Barley derived spent grains: conversion to bioethanol

Stuart James Wilkinson

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Abstract

Brewers spent grains (BSG) are an abundant co-product from the beer brewing industry. The current predominant use for BSG is as cattle feed. However, this is a low value use. As such, higher value uses for BSG are being sought. One such option is the production of bioethanol from the polysaccharides (cellulose and hemicellulose in the lignocellulosic matrix) found in BSG, and this thesis aimed to develop this process. This was achieved through investigating and attempting to optimise a range of different pre-treatments in order to enhance the subsequent cellulolytic enzyme saccharification yields to produce a high glucose concentration feedstock which could then be fermented to produce bioethanol. For the pre-treatment step of the process, a wide range of protocols were investigated and optimised (at high solids loading; ≥25% w/v). These included dilute acid and alkali hydrothermal, alkaline peroxide, caustic (NaOH) and microwave based autohydrolytical protocols which were all capable (under optimal conditions) of achieving close to 90% theoretical glucose yields when using an excess of cellulolytic enzyme. Optimisation of the enzymatic saccharification step was attempted and involved using a batch-fed protocol, with supplementary enzymes, and a high-torque mixing system were still only able to achieve ca. 43% theoretical glucose yields when operating at 15% w/v solids loading. Limited saccharification yields appear to be the rate limiting step for bioethanol production from BSG as it limited the concentration of glucose in the feedstocks produced. In addition, entirely biological routes to generate bioethanol were investigated using consolidated bioprocessing (CBP), which entailed using a consortium of fungal microorganisms. A primary filamentous fungal species was used as a cellulolytic enzyme factory whilst a yeast strain was used to ferment any liberated sugars to ethanol. From all of the fungal based CBP systems tested the traditional saké fermentation system (*A.oryzae* and *S.cerevisiae* NCYC479) was shown to achieve the highest ethanol yields (ca. 37 g/L within 10 days).

1 Introduction

The global consumption of finite crude oil based fuels and the subsequent climatic change due to the emission of greenhouse gases has led to production of biofuels as potential alternatives (Chundawat et al., 2011; Mussatto et al., 2006). Biofuels would ideally be second generation (advanced generation), being sustainable and renewable, using either low market value or waste products as the initial substrate instead of non-renewable crude oil or potential food sources. This is in contrast to first generation biofuels that are in conflict with food production due to their use of substrates such as sugarcane or maize (Rosegrant & Msangi, 2014; Youngs & Somerville, 2014). The central concept of biofuels is the production of an organic based industrial fuel molecule such as ethanol via a microbiological species in some form of bioreactor, using a renewable material such as a derivative of plant biomass, in an attempt to gain oil-independence and minimise the detrimental effects oil based products have on the environment. Bioethanol is an ideal transportation fuel with its 98 octane rating and high heat of vaporisation that results in superior performance in specifically engineered engines compared to gasoline (Shindo & Tachibana, 2006).

The biofuel production process brings with it a huge range of new scientific and technological difficulties which must be overcome to generate a truly renewable and sustainable biofuel (Xiros & Christakopoulos, 2009). The use of agricultural or industrial waste streams (such as brewers spent grains or BSG) has considerable potential for conversion of the otherwise unused polysaccharide content from the cell walls to ethanol utilising a fermentative microbial species. The difficulties arise due to the recalcitrant nature of the biomass, which is highly resistant to degradation or depolymerisation to utilisable sugars for fermentation. Overcoming the recalcitrance of the cell wall polymers requires a significant amount of industrial or biological processing of some form.

Another consideration is that the polysaccharide and aromatic biopolymers found in plant biomass based substrates such as BSG have considerably higher oxygen to carbon content than that of crude oil (Chundawat et al., 2011). This creates difficulty in reducing them to higher energy density molecules that are compatible with the current industrial infrastructure likely to utilise any such biofuel.

This introductory chapter will attempt to explore all the significant factors involved in successful bioconversion of barley derived spent grains to ethanol. Much of the information gained from research into other lignocellulosic substrates such as wheat straw, switch grass and corn stover can potentially be applied to BSG. Therefore various parts will be directed to general lignocellulosic biofuel considerations whilst other parts will deal specifically with respect to BSG.

1.1 Biofuel considerations

There are many important factors that need to be considered when evaluating a potential biofuel. Crucially the process must be as efficient as possible, generating the maximum amount of energy from a minimal chemical and energy input. The fuel versus food debate must also be considered in that the substrate used initially should ideally not take anything away from a potential food source (Chundawat et al., 2011; Pimentel & Patzek, 2005). Also the substrate availability should be high. Another likely significant cost that needs to be considered is the transport and storage costs of the substrate and ultimate product. Local acquisition of the substrate and also local use of the final product would significantly enhance the overall efficiency of the process.

The production of a biofuel such as ethanol from a lignocellulosic material such as BSG is usually a four-stage affair when using a conventional chemical engineering or biotechnological approach: with an initial diminution step (particle size reduction), then a

pre-treatment step to deconstruct the material, then a secondary enzymatic hydrolysis step to liberate fermentable sugars and finally the actual fermentation to ethanol itself (Aliyu & Bala, 2011; Carvalheiro et al., 2008; Chundawat et al., 2011; Mussatto et al., 2006; White et al., 2008).

1.2 Origins of BSG: co-product from the brewing industry

BSG is a co-product generated in large quantities from the mashing process (the solubilisation of the malt or cereal grains to facilitate suitable wort extraction) associated with the beer production industry (Mussatto et al., 2006; Robertson et al., 2010; Xiros & Christakopoulos, 2009). After coffee, tea, milk and carbonated soft drinks, beer is the most frequently consumed beverage in the world, with over 1.34 billion hectolitres produced annually (Aliyu & Bala, 2011). Therefore huge quantities of BSG are generated annually with approximately 3.4 billion tonnes (wet weight) from the European Union alone. BSG represents approximately 85% of the total by-products generated from the brewing process. BSG accounts for 30-60% of the biological oxygen demand (BOD) and suspended solids generated in a typical brewery (Aliyu & Bala, 2011). Although the price of BSG fluctuates, its cost is generally low with prices as low as approximately $14 per tonne (wet weight) in US dollars (Mussatto et al., 2008). It is therefore abundant, readily available, low cost (as categorised as a co-product) with great industrial exploitation potential. The abundance of BSG satisfactorily answers the substrate availability question.

1.3 BSG production

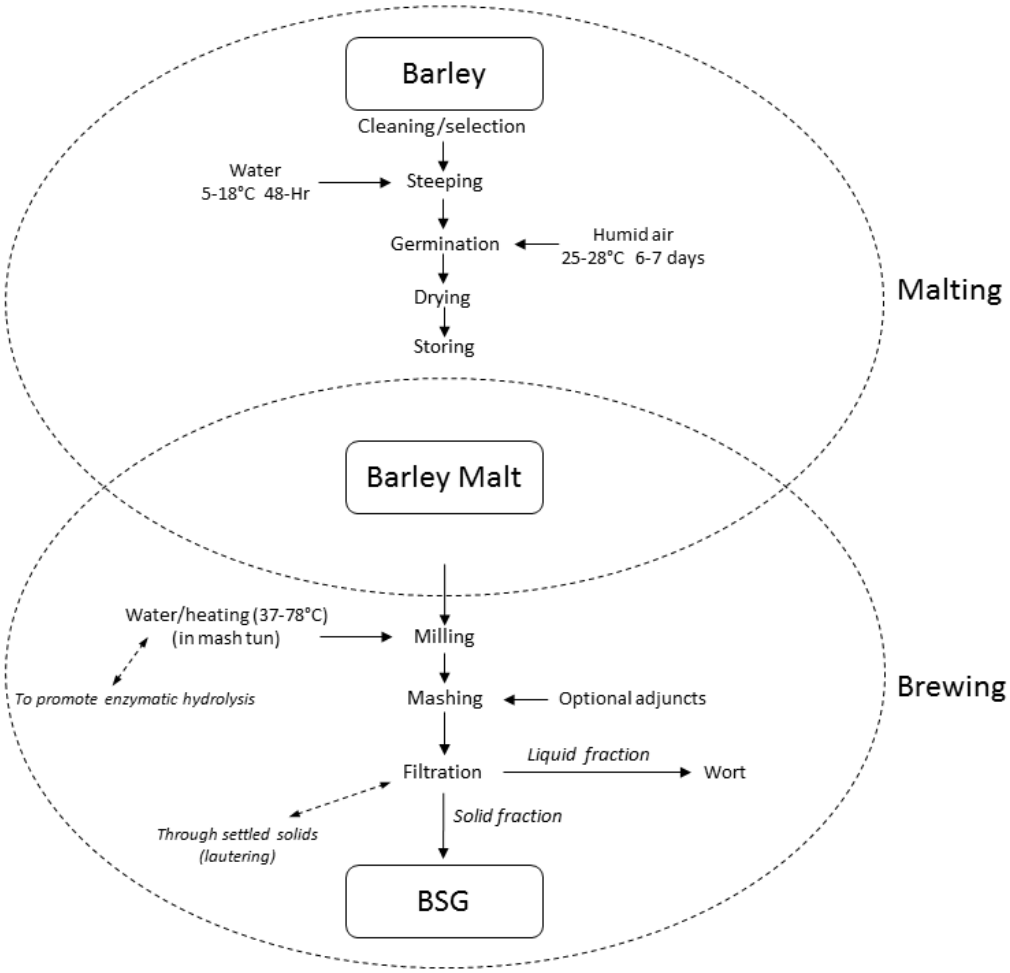
In basic terms BSG is produced after the barley grains are steeped or hydrated (to create the optimum moisture content) to initiate germination then dried (kilning), milled and then the mashing process completes the endosperm modification (Figure 1.1) and release of enzymes to liberate the stored carbohydrate and protein for use in beer fermentations (Aliyu & Bala,

2011; Mussatto et al., 2006; Xiros & Christakopoulos, 2009). In more detail, germination is a carefully controlled small amount of growth of the shoot and root (plumule and radicle respectively) that triggers enzymatic release of carbohydrases and proteases (Mussatto et al., 2006). The kilning stage then stops any further growth. The mashing stage then promotes the enzymatic hydrolysis of the prolamins (main storage proteins) and stored starch in the endosperm (main storage carbohydrate). The starch must be gelatinised prior to enzymatic hydrolysis from its more crystalline form. It is then further hydrolysed to liquid called wort containing various subunits including dextrins, maltotriose, maltose and ultimately the constitutional monomer of all these compounds, glucose. The collective ability of the several starch degrading enzymes that accumulate within the barley grain (during malting) to effectively hydrolyse the starch component after soaking in hot water is termed its diastatic power (Arends et al., 1995; Palmer et al., 1989). This is measured in degrees Lintner in America and Windisch-Kolbach units (°WK) in Europe and a grain with the power to completely self-hydrolyse is thought to have a diastatic power of around 35°Lintner or 94 Windisch-Kolbach units.

1.4 BSG composition

BSG usually originates from barley (*Hordeum vulgare* L.) which is the most economically important cereal grain after wheat, maize and rice (Gupta et al., 2010; Palmer et al., 1989). BSG is a heterogeneous, complex matrix composed of mainly the barley husk, pericarp tissue and remaining endosperm fragments (Forssell et al., 2008; Palmer et al., 1989). Figure 1.2 illustrates the morphology of a barley kernel.

Figure 1.1 Schematic overview of BSG production from a typical brewery. (Mussatto et al, 2006)

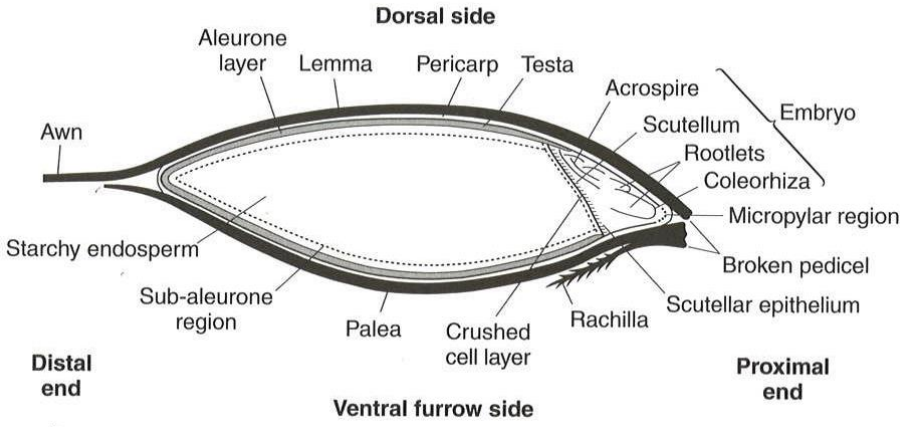


There are a large numbers of factors affecting the chemical composition of BSG and these include: the particular variety of barley (cultivar), geographical location of the crop growth (environmental parameters: temperature, soil type and composition, climate), annual time of harvest, malting and mashing conditions (the degree of endosperm modification) and also the presence and quality of any adjuncts used in the brewing process (Gupta et al., 2010; Mussatto et al., 2006).

1.4.1 Gross morphology

The barley husk is a multi-layered dead tissue region that is effectively the protective seed coat of which most of the lignin is located here along with high concentrations of silicates and inorganic material (ash) and some small quantities of resins and tannins (Mussatto et al., 2006). The non-parenchymatic tissue nature of BSG means that pectin is less prevalent and therefore less of an issue for deconstruction, removing the need for specialised pectinase enzymes (Beldman et al., 1987). Approximately 80% of BSG (dry weight) is plant cell wall material which is the source of the lignocellulosic material that could be processed to liberate fermentable sugars (White et al., 2008).

Figure 1.2 Shows a schematic diagram of barley kernel morphology (Briggs et al., 2004)**.**



1.4.2 Chemical composition

There has been a wide variety of compositional analyses conducted on BSG from numerous published sources (Table 1.1). Approximately 50% of the dry weight of BSG is polysaccharide (Chundawat et al., 2011). The carbohydrate content is mostly in the form of arabinoxylans (hemicellulosic origin) and β-glucans (cellulosic origin) with the possibility of some residual starch from the endosperm fragments that may be present (Aliyu & Bala, 2011; Carvalheiro, 2004; Chundawat et al., 2011). Morphologically the origin of the β-glucans is from the endosperm cell walls of the barley, whilst the aleurone layer (which separates the

starchy endosperm from the pericarp and husk) is mostly arabinoxylan rich. The outer layers of the barley kernels are more complex: here cellulose microfibrills are combined with arabinoxylans and lignin in a more complex matrix. There may also be some pectin present in the cell walls.

Table 1.1 Shows the compositional ranges of various components of BSG (Mussatto et al., 2006)

Component	Range (%)
Cellulose	17-25
Hemicellulose	22-28
Protein	15-24
Lignin	12-28
Lipid	9-11
Ash	2-5

Other various compounds in BSG are (all in ppm): biotin (0.1), choline (1800), folic acid (0.2), niacin (44), pantothenic acid (8.5), riboflavin (1.5), thiamine (0.7), pyridoxine (0.7), calcium (1040), magnesium (688), silicon (242), phosphorus (1977) (Aliyu & Bala, 2011; Mussatto et al., 2006). There are also trace amounts of cobalt, copper, iron, manganese, potassium, selenium, sodium and sulphur (Gupta et al., 2010).

The polysaccharide and lignin components will be discussed in more detail in the lignocellulosic material section (1.6).

1.4.2.1 Protein content

The protein found in BSG is mostly hordein based (barley prolamins) but with some glutelins, globulins, albumins (Celus et al., 2009; Forssell et al., 2008). Hordeins are low-lysine, high-proline, and high-glutamine alcohol-soluble proteins. The bulk of the protein fraction is located inside the aleuronic cells and any remaining starchy endosperm fragments. Disulphide linked aggregates can often be found in BSG and are artefacts from the denatured

proteins that are generated during the mashing process. Up to 65% of the malt proteins are retained in BSG making this a considerable fraction that warrants the consideration of another use for this material. *Bacillus licheniformis* alcalase® (a commercial enzyme cocktail produced by Novozymes A/S, Denmark) has a high peptidase and limited α-amylase function and capable of hydrolysis of the BSG protein fraction (Celus et al., 2009). Subtilisin A is the main enzyme responsible. Subtilisin is an exclusively prokaryotic serine endopeptidase, with the serine amino acids forming the active site (Jacobs et al., 1985). Overall, the relatively high protein content also means that BSG should provide a good nitrogen source for any fermentations conducted downstream of any processing steps.

The ash component is mainly the remaining non-combustible, non-organic material such as metal salts (Ulery et al., 1993; Vassilev et al., 2010).

1.4.2.2 Moisture content

The moisture content of BSG is a crucial point for consideration. After the mashing process in brewing, the moisture content is high usually at 71-81% (Mussatto et al., 2006; Robertson et al., 2010). This has two major draw-backs: firstly, weight and volume of the product are both increased considerably due to the water content. This has numerous practical implications such as the volume required for mass storage is therefore increased and the cost of transport is also increased considerably. This has cost and efficiency implications for any processing or potential industrial use unless done at the original site of BSG production (i.e. the brewery). Secondly, the high moisture content results in very rapid microbial spoilage if left untreated (Robertson et al, 2010, Mussatto et al, 2006). Essentially its positive properties for potential bioconversion to ethanol are also potential negative properties as it is such a good microbial growth substrate. Sodhi et al (1985) found that after storage for 30 days, untreated BSG had eight different fungal isolates growing on it including *Aspergillus*,

Fusarium, *Mucor* and *Penicillium* species. Additionally lactic acid bacteria have been able to grow on BSG (Novik et al., 2007). Therefore either a costly drying process must be carried out to drop the moisture content to below 10% or pH adjustment must be conducted which would inhibit the bulk of any potential microbial growth.

1.4.2.3 Resident microflora on BSG

The inherent physico-chemical properties of the BSG, in particular its high moisture and nutritional content mean that it is highly likely that some resident micro-flora will be present on BSG direct from the brewery in the absence of some form of sterilisation or intensive drying (Robertson et al., 2010). Mainly thermophillic aerobic bacterial spores ($<10^7$ cells per g BSG) are usually present in BSG from a typical brewery but this can rapidly change depending on the specific storage conditions. The relatively high temperatures used in the mashing process would likely kill off (or at least inactivate) everything except thermophillic bacterial spores hence their predominance.

1.5 Current uses of BSG

1.5.1 Animal feed

The actual final use for BSG is usually dependent on the local economic and market considerations but it is primarily used as animal feed due to its composition, having a considerable protein fraction and considerable carbohydrate fraction although in a format that only ruminants can utilise due to the lignocellulosic recalcitrant nature (Schwencke, 2006). BSG is often sold at a very low price to farmers willing to collect it (Jay et al., 2008; Mussatto et al., 2006; Xiros & Christakopoulos, 2009). Cow's diets supplemented with BSG result in a higher milk yield compared to controls via the addition of ruminant indigestible protein or RUP (Gupta et al., 2010; Mussatto et al., 2006). The crude fibre content is what makes BSG unsuitable for non-ruminants as they are not able to digest it (Schwencke, 2006).

This reliance on cattle farmers renders the use of BSG as animal feed heavily dependent on the presence of cattle farmers in the local area otherwise transport costs (of the suitable quantities of BSG needed to be considered as a viable animal feed alternative) would outweigh the low market value. Therefore in areas with no cattle farming or areas where the market value for animal feed is very low, an alternative use for BSG is sought from an economic perspective. In addition the high ecological cost of producing beef (from any feed source etc.) has called into question this practice for the use of BSG as not the most environmental friendly and sustainable option (Subak, 1999).

1.5.2 Incorporation into human food products

BSG can also be used in human food production, often in the form of baked goods (Gupta et al., 2010). It has numerous health benefits including acting as dietary fibre (gastrointestinal health) and preventing coronary disorders (as dietary BSG has the ability to drop serum cholesterol concentrations; Gupta et al., 2010, Aliyu and Bala, 2011). BSG is too granular for direct incorporation into foods and must therefore be converted into high protein flour. This can then be used to manufacture biscuits, whole wheat bread, cakes and other assorted foodstuffs. However, sensory testing of products that incorporated flour made from BSG (such as bread) imparted an unusual taste which limited the quantities of BSG that could be incorporated or blended in (Hassona, 1993). Overall this limits how much BSG could feasibly be used in this role. Additionally animal food based (for animals other than ruminants etc.) uses of BSG fractions or flours have been tested through incorporation into products such as fish food (McCarthy et al., 2013) or dog biscuits (http://brewscuit.com/).

1.5.3 Building materials

Due to the low ash content and high fibrous material content (from the cellulose and hemicellulose components) in BSG, it can be used in the production of building materials (Aliyu & Bala, 2011; Mussatto et al., 2006). Bricks manufactured from BSG are higher

strength, higher porosity (higher water absorption capacity) and lower density (better thermal insulation properties) than clay bricks. Therefore with the constant endeavour for new sustainable building materials this represents an area for future development of potentially great interest to the construction industry.

1.5.4 Bio-adsorbent applications

There is a potential use for BSG as a bio-adsorbent for industrial applications. This is because BSG has the ability to adsorb metal ions due to its reactive functional groups such as hydroxyl, carboxyl and amine groups, allowing it to be used to remove heavy metals from waste water streams (Aliyu & Bala, 2011). This functionality also works with various organic compounds such as industrial dyes. Untreated BSG can remove in excess of 90% of the colour of acid orange 7 dye (2-naphthol orange; used in the fabric industry for colouring materials) from waste water at 30°C (Aliyu & Bala, 2011). However, with all adsorption functions of BSG, the solution pH greatly affects the ability of BSG to immobilise compounds as an excessively low pH would result in the saturation of the BSG binding sites with hydrogen ions. The adsorptive properties also enable its use in reducing any organic material from wastewater streams not just heavy metals. Overall this represents an interesting potential application for BSG for the future.

1.5.5 Pulp/paper production

BSG also has commercial potential in the pulp/paper production industry (Mussatto et al., 2008). Using hydrogen peroxide as a more environmentally friendly bleaching agent than chlorine, writing paper quality material has been generated exclusively using BSG as the substrate. Alkaline media is used which shifts the hydrogen peroxide equilibrium to the formation of hydroperoxide ions (-OOH) and this is the principle bleaching agent (Mussatto, 2009). This particular anion is a strong nucleophile and converts electron rich chromophores such as unsaturated aldehydes, ketones and phenolic rings to their non chromophoric variants

hence the observed colour change. Prior to this oxidative bleaching the BSG undergoes a deconstructive pre-treatment specifically designed to hydrolyse and solubilise lignin and hemicellulose whilst attempting to leave the cellulose intended for pulp production relatively intact and undamaged. This could present an interesting opportunity for on-site processing within a brewery that may then allow for the production of packaging or labels for beer bottles or similar.

1.5.6 Microbial metabolic products

Most importantly due to its compositional nature (but also due to the high moisture content) BSG can be used for the production of commercially valuable products via microbial means (Aliyu & Bala, 2011). Examples of this are the production of lactic acid or xylitol, with BSG as the predominant, primary growth substrate for the micro-organisms (Aliyu & Bala, 2011; Gupta et al., 2010; Mussatto & Roberto, 2006; White et al., 2008). Residual microflora are normally already present on the surface of BSG (if the BSG is not significantly dried) to metabolically kick-start the deconstructive processes. Lactic acid (2-hydroxypropanoic acid) is an important industrial chemical used in food manufacture, fermentations, pharmaceutical and chemical industry. Large scale, cost effective, commercial production using *Lactobacillus delbruckii* could be used to produce lactic acid as a precursor to the polymer polylactic acid (PLA) from BSG as the growth substrate (Mussatto et al., 2007). Additionally genetically modified yeast could also be used to produce lactic acid (Porro et al., 1999). Improved process economics could then result in more commercial use of PLA as this polymer is actually biodegradable under the right conditions (in the presence of a suitable amount of oxygen). However PLA is difficult to recycle and requires almost complete depolymerisation in order to do so.

Xylitol is a sugar alcohol used in the food industry as a sweetener without the negative dietary and dental concerns of sugars (Mussatto & Roberto, 2006). Various yeasts have been

shown to produce high xylitol yields when grown on BSG feedstocks (Aliyu & Bala, 2011; Mussatto & Roberto, 2005).

Another industrially valuable class of biopolymer that can be produced microbially using BSG are pullulans (Aliyu & Bala, 2011). These are extracellular water soluble polysaccharides that are polymers of α-glucans, mainly maltotriose (α-1, 6 glycosidic linked). Pullulans have adhesive properties and form fibres, compression mouldings and strong oxygen impermeable films that can be used in industrial applications such as adhesives (Oguzhan & Yangilar, 2013). Making pullulan production cheaper could increase their use commercially.

Along the same lines as xylitol, lactic acid and pullulan production, BSG could be used as the substrate for the microbial production of various commercially valuable enzymes (Aliyu & Bala, 2011; Mussatto et al., 2006). These could include the cellulases and xylanases used in the biofuel sector.

1.5.7 Direct application as a fuel

BSG can also be directly burnt for energy in certain power stations, as a whole material, co-combustion with wood chips or the lignin component exclusively, generating steam for other industrial processes (Schwencke, 2006; Touš et al., 2011). Unfractionated BSG has a heat of combustion of approximately 21 MJ/kg which is similar to that of dry wood or lignite coal. But this value heavily depends on the moisture content of BSG as the vaporisation energy cost of the water content can be high. Also direct combustion of BSG is not favoured as the protein fraction is lost and significant quantities of nitrogen oxides (NO_x) and sulphur dioxide (SO_2) are generated in the process, therefore overall this would not be generally considered an environmentally friendly process (Touš et al., 2011).

1.5.8 Biogas

BSG has been investigated as a potential Biogas substrate with some success (Mussatto et al., 2006). The primary biogas fuel methane is produced by acetotrophic methanogenesis using a group of microbes in a single bioreactor (along with some carbon dioxide and hydrogen gas co-products). However some of the phenolic lignin breakdown products are significantly toxic and inhibit the biogas microbes, indicating further optimisation of this process would be required to maximise the process efficiency (Mussatto et al., 2006).

1.5.9 Growth of edible fungi

The naturally high moisture content and high protein content of BSG has led to development of high growth rates of fungal species such as *Pleurotus ostreaus*, an edible mushroom (Gregori et al., 2008). *P. ostreaus* uses the lignocellulosic substrate as an activational trigger for production of lignolytic and cellulolytic enzymes that deconstruct the material and facilitate growth.

1.5.10 Basic principles involved in the conversion of BSG to ethanol

The combination of the considerable polysaccharide composition of BSG and its low market value makes bioconversion of the storage sugars to ethanol for incorporation into biofuels an attractive idea. This would be done via fermentation of BSG derived sugars utilising a microbial species. However there are a number of technical hurdles which must first be overcome before this can be achieved. The initial difficulty arises from the format of the potential sugars, as the recalcitrant nature of lignocellulosic material found in BSG does not allow it to be utilised in its native form (Chundawat et al., 2011). Without some form of initial physico-chemical processing (or thermo-chemical pre-treatment) and subsequent enzymatic hydrolysis the material simply cannot be fermented to ethanol. This essential pre-treatment process is likely to be energy demanding therefore very costly (Mussatto et al., 2006). Also the complex nature of the material requires an equally complex cocktail of

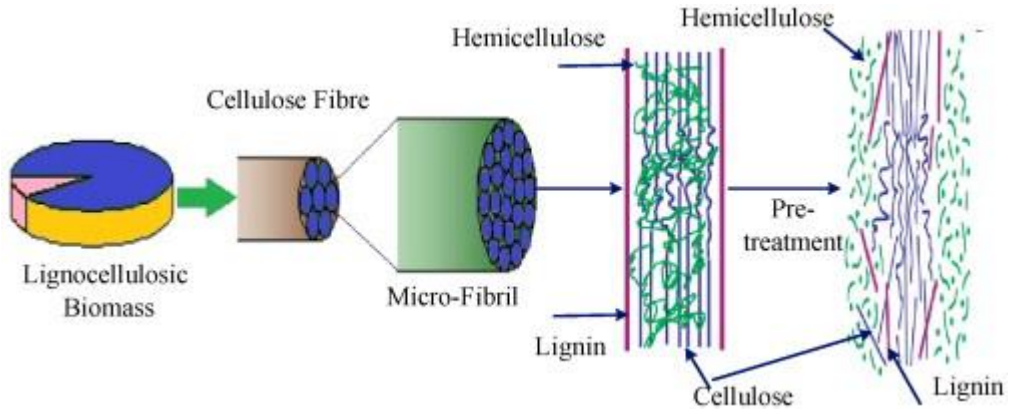
specific enzymes to liberate any stored sugars. Use of numerous enzymes on an industrial scale is likely to be prohibitively expensive. Also the specific type of feedstock generated must be of a fermentable nature for the microbial species used. Therefore all processes must be optimised for minimal energy or chemical input to ensure the genuine green credentials of the biofuel are preserved and the process does not consume more energy than it creates. Optimisation of the pre-treatment process is also essential in order to minimise the excessive degradation of any liberated sugars to products that function as fermentation inhibitors for any downstream processes. Following the fermentation step the ethanol must be recovered, usually by distillation. This is a costly but necessary process. However distillation only becomes economically viable when the ethanol concentration in the media exceeds 4-5% v/v (Gírio et al., 2010). This would indicate the minimum ethanol titre that would need to be achieved. Following fully optimised traditional fermentation conversion rates this would indicate that an absolute minimum of 10% fermentable sugar (ca. 100 g/L) must be present initially in any BSG derived feedstock to justify any fermentation to ensure an ethanol concentration economically worthy of distillation is achieved.

1.6 Lignocellulosic material: the natural recalcitrant barrier

The cell wall polysaccharides found in BSG are present in the form of a complex matrix of lignocellulosic material (Forssell et al, 2008, Russ et al, 2005, White et al, 2008, Chundawat et al, 2011, Aliyu & Bala, 2011, Carvalheiro et al, 2004). This is a recalcitrant material, highly resistant to degradation by physical, chemical or biological attack (by abiotic or biotic means) that is part of the reason for the evolutionary success of plant species (Lagaert et al., 2009; Obel et al., 2009). Lignocellulosic material is considered to be produced as a direct response to the coevolution of plants with animals and other potential predators such as various microbial species that may attempt to consume the plant material as a food source. The cell wall polysaccharides alone are very stable with a half-live (at room temperature) in

the region of 20 million years (Dodd & Cann, 2009). The lignocellulosic material is found in cell walls, which are normally composed of three distinct layers: middle lamella, primary cell wall and the secondary cell wall (Chundawat et al., 2011).

Figure 1.3 Schematic overview of lignocellulosic material (Chandra et al., 2007).



The three main components; cellulose, hemicellulose and lignin, exhibit structural characteristics such as a high degree of polymerisation, high crystallinity, and an apparent random construction. In addition, all three are inter-linked together by various means (Figure 1.3) which overall creates such a complex structure that makes biodegradation or digestion by enzymatic means highly difficult (Chundawat et al., 2011). Due to the complexity of the composition of lignocellulosic material, each of the three main components will be discussed individually.

1.6.1 Cellulose

This is a homo-polymer composed of 3nm thick microfibrills of 36 crystalline parallel linked, linear β-1, 4 D-glucose units (Chundawat et al., 2011; Foyle et al., 2007; Taherzadeh & Karimi, 2008). Cellulose is largely crystalline in structure (50-90%), with the remaining fraction in the amorphous form (Chundawat et al., 2011). Cellulose exhibits extensive inter and intramolecular hydrogen bonding and van der Waals forces (Figure 1.4) which are

largely responsible for the observed high degree of crystallinity. Hydrolytic deconstruction of cellulose generates various glucose based fragments but mostly in the form of β-D-glucans. These are mixed linkage (1, 3 & 1, 4) linear homopolymers of β-D glucopyranosyl residues and originate from cellulose hydrolysis, and these are major fibre components. Various cellodextrins may also be produced from further hydrolysis of β-D-glucans. Cellodextrins are smaller, low molecular weight oligosaccharides produced by various cellulase class enzymes. They are typically anything greater than two degrees of polymerisation (>2 Dp). There are different crystalline polymorphs of cellulose that exist but the most abundant in higher plant derived lignocellulosic material is cellulose I_{β} (Chundawat et al., 2011). Various thermo-chemical pre-treatments can change one polymorph of cellulose into another which can possibly assist in the subsequent deconstruction.

1.6.1 Hemicellulose

This is a low molecular weight, branched heteropolymer with a xylose backbone (Figure 1.5) with various monosaccharide side chains that are plant dependant in their composition but usually pentose based as either arabinose, mannose, rhamnose or galactose (Gírio et al., 2010; Gupta et al., 2010; Yarbrough et al., 2009).

Figure 1.4 Basic structure of crystalline cellulose showing extensive nature of hydrogen bonding network (adapted from Chundawat et al, 2011).

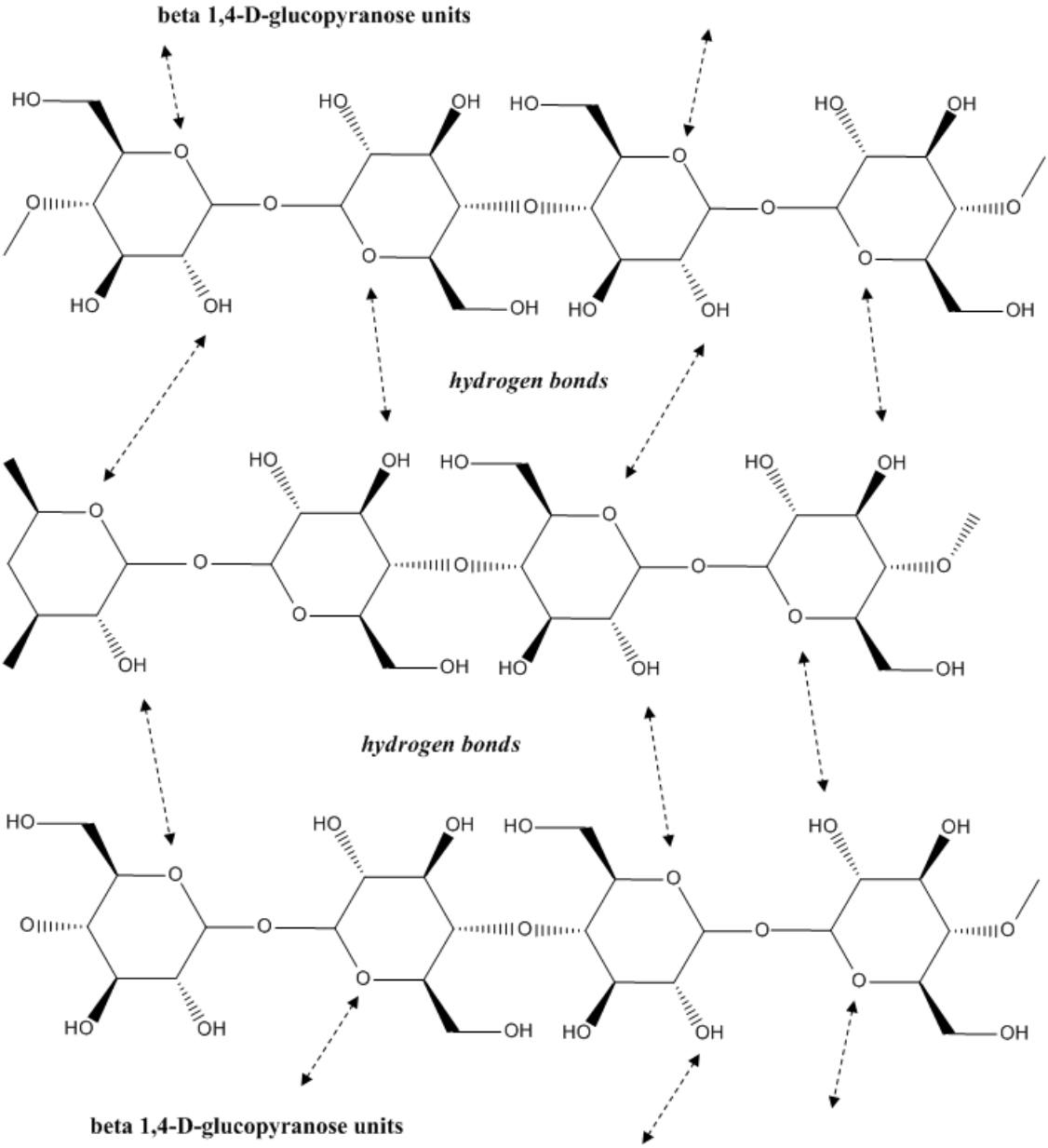
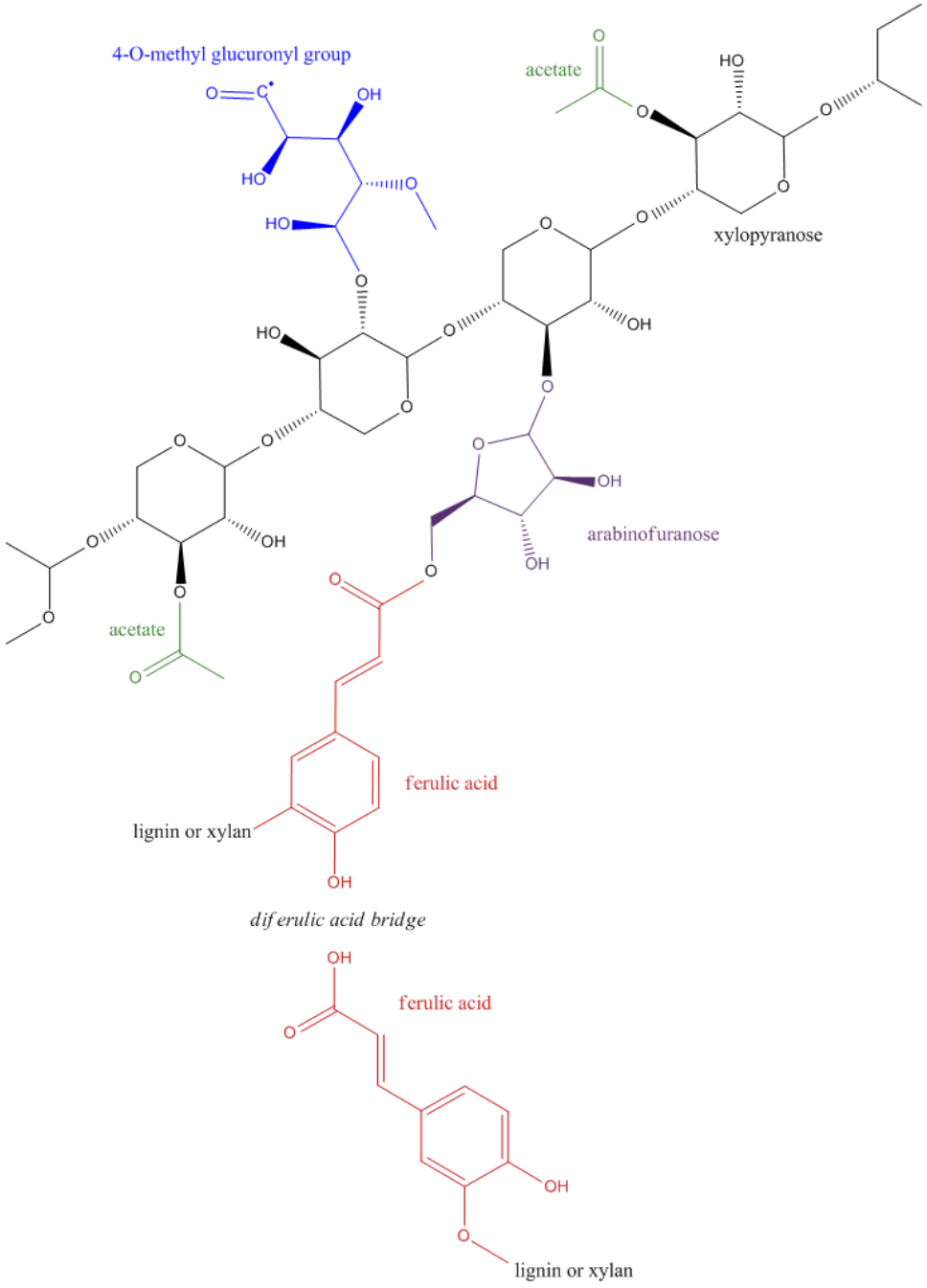


Figure 1.5 Basic structure of various linkages found in BSG derived hemicellulosic xylans (adapted from Dodd & Cann, 2009, Gupta et al, 2010).



Hemicellulose is generally considered amorphous in structure. It forms both covalent and non-covalent bonds with cellulose and lignin and is hydrogen bonded to cellulose (Gírio et

al., 2010). The side chains can have ferulic acid residues which can form ester linkages with lignin. It is the acetylation of the side chains in hemicellulose that is responsible for the water based autohydrolytical pre-treatments that can be employed in the deconstruction of the biomass to generate feedstocks, as the cleaving of these generates free acetic acid (Gupta et al., 2010; Taherzadeh & Karimi, 2008). The acetylation is thought to stabilise the glycosidic links in hemicellulose, increasing the structural strength. The gross hemicellulose structure can be subdivided into various forms of a polymer generally termed xylan. Xylans are composed of xylose, arabinose, glucuronic acid and possibly glucuronyl, acetyl, feruloyl groups (Dodd & Cann, 2009, Forssell et al, 2008, Girio et al, 2010). Fundamentally the composition of xylans is a repeating β-1,4- xylose backbone with either acetyl, arabinofuranosyl or 4-O-methylglucuronyl groups as side chains (Dodd & Cann, 2009). Depending on the side chains xylans can either be classified as one of four main groups:

1: homoxylans: no side groups (just β-1,3- xylose & β-1,4- xylose)

2: arabinoxylans: are constructed from a 1,4-β D-xylopyranose backbone substituted with arabinofuranosyl (often arabinose) residues at the O2 or O3 atom in xylose. In BSG the arabinose: xylose ratio is approximately 0.5.

3: glucuronoxylans: 4-O-methyl-α-D-glucuronic acid is substituted at the O-2 of xylose.

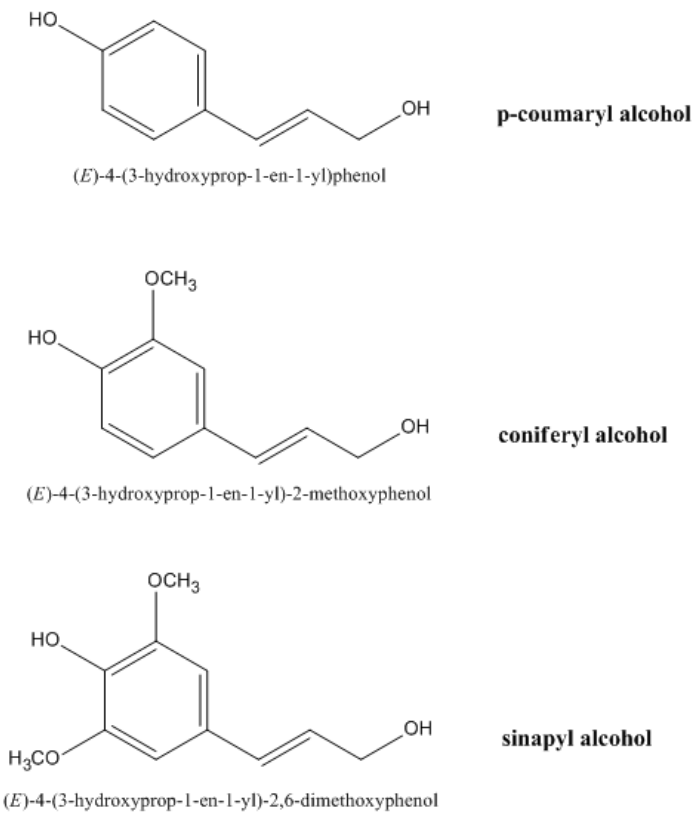
4: arabinoglucuronoxylans: arabinofuranosyl, glucuronic acid and acetyl side chains linked to the xylose backbone.

In addition to xylans present in hemicellulose, mannans may also be present. These are structurally similar polysaccharides composed of repeating backbone of β-1,4 mannose monosaccharide monomers (Girio et al, 2010). These can also have substituted side chains at various points as xylans can.

1.6.2 Lignin

Lignin is a random, amorphous, high molecular weight, hetero-polyphenolic macromolecule which is covalently linked to hemicellulose (Carvalheiro et al., 2008; Kajikawa et al., 2000; Taherzadeh & Karimi, 2008; Yarbrough et al., 2009). The basic composition of lignin involves the polymerisation of three monolignol monomers (Figure 1.6), each methoxylated to varying degrees (they are glycosylated monolignols that actually originate from the amino acid phenylalanine).

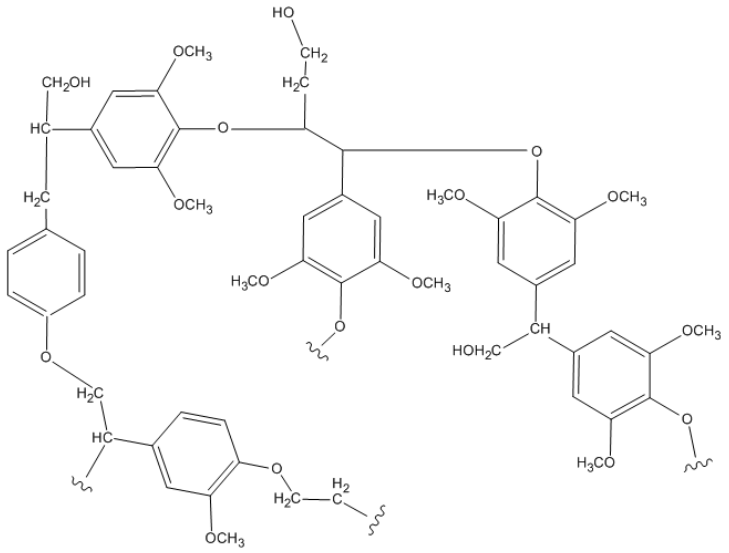
Figure 1.6 Monolignols of lignin construction (Morreel et al., 2004; Wang et al., 2010).



The three monomers that co-polymerise are: p-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol, with all three in the form of various phenylpropanoids: *p*-hydroxyphenol, guaiacyl and syringal. These three sub-units randomly polymerise resulting in huge structural

variation in lignin, with different plant material having different ratios of these, hence the difficulty for enzymatic based deconstruction (Figure 1.7).

Figure 1.7 Suggested possible structure for core lignin (adapted from Chundawat et al, 2011).



The lignified material often exists within the middle lamella, cell corners and secondary cell walls of cells. Its main function is to withstand attack via external compressive forces (Donohoe et al., 2008; Taherzadeh & Karimi, 2008). In addition to the three fundamental monomers, lignin can have additional randomly inserted molecules such as catechol, hydroquinone, coniferyl, 4-methyl catechol, vanillyl alcohol, syringic, vanillic, palmitic, ferulic, coumaric acids which again increases the variability (and therefore further increases its resistance to specific enzymatic based deconstruction) of the structure. Lignin associates with the cell wall polysaccharides (cellulose and hemicellulose) via the hydroxycinnamic acids ferulic acid and *p*-coumaric acid, which form ester linkages to arabinose side chain residues in xylans and hydroxyl groups in lignin (Dodd & Cann, 2009). Ferulic acid may also form di-ferulic acid linkages between neighbouring ferulic acid residues attached to arabinose

molecules on adjacent xylans, which (in combination with the plethora of covalent bonds, hydrogen bonds and van der Waals interactions) starts to explain the complex multi-interactions which give rise to matrix responsible for the recalcitrant nature of lignocellulosic material. As a result, the main monomeric products released during the hydrolysis of lignin are the phenolic hydroxycinnamic acids ferulic acid and *p*-coumaric acid (Forssell et al., 2008). There is also the possibility of ferulic acid dimers (and even trimers) being released (from the di-ferulic acid bridges). As these acid residues are mostly esterified into the lignocellulosic matrix they are only detected after an alkaline based reagent is used to disrupt the structure or the enzyme ferulolyesterase.

1.6.3 Value added co-products

Independent of the theoretically available fermentable sugars that are present in the lignocellulosic material in BSG, there are numerous other compounds that may be industrially or commercially valuable (Forssell et al., 2008; Yang & Wyman, 2007). There are also alternative uses to some of the fractions which may become recoverable after some form of lignocellulosic deconstruction. The xylan derivatives can be used in the food industry as hydrocolloids and some xylo-oligosaccharides can be used as prebiotics (digestive aids due to their function as substrates for species such as *Bifidobacteria*), antioxidants in the form of ferulic acid from lignin or as a precursor for the food favour compound vanillin, and the protein fraction or protein hydrolysates can be used as emulsifying agents (Forssell et al, 2008) or aquaculture feeds (McCarthy et al., 2013). The pyrolytic thermal decomposition (to effectively burn without the presence of oxygen) of lignocellulosic material releases the lignin based methoxy phenols, guaicol and syringol (2-methoxyphenol and 2,6 dimethoxyphenol respectively). These hydroxycinnamic acids (and the derivative guaicol in particular) can be used as precursors for the valuable food flavour molecule, vanillin

(Figure 1.11) and also coumaric acid can be converted into ferulic acid and then vanillin (Lesage-Meessen et al., 1996).

1.7 Pre-treatment strategies

1.7.1 Deconstruction overview:

Due to the complexity of lignocellulosic material a pre-treatment process is usually essential as the efficiency of enzyme hydrolytic saccharification alone is usually poor, liberating approximately only 20% of the theoretical yield of fermentable sugar available (Sanchez & Cardona, 2008). There are various forms of pre-treatment involving physical disruption of material such as milling for example, or chemical disruption of the material such as acid hydrolysis (or a combination of the two). Pre-treatment can raise the sugar yield to approximately 90% of the theoretical amount available rendering it essential usually (as sugar yields are only ca. 20% theoretical without it), but this affects the economy of the overall process (due to the chemical and thermal energy costs of pre-treatment). Hemicellulose solubilisation (or hydrolysis of some form) is often essential for effective saccharification as it is the primary store of pentose sugars but also as it (in combination with lignin) significantly limits the access of cellulases to cellulose. Whilst some degree of lignin disruption or modification is suggested to be required for achieving high sugar yields from lignocellulosic biomass, there does not always appear to be a direct correlation between lignin removal and the digestibility of cellulose, which reinforces the complex nature of the material. The moisture content of the substrate also heavily influences the effectiveness of the pre-treatment (Keshwani & Cheng, 2009). The pre-treatment process usually attempts to increase the overall substrate surface area, as this results in more sites for enzymatic adsorption and subsequent attack. It also attempts to reduce the crystallinity of cellulose, as a low crystallinity index results in more exposed glycosidic linkages for enzymatic attack

(Yang & Wyman, 2007). The enzymatic hydrolysis rate of cellulose is proportional to the specific surface area that is accessible to the cellulase enzyme and inversely proportional to the crystallinity index of cellulose (Sinitsyn et al., 1989). A high degree of polymerisation of cellulose limits the number of free reducing ends available for enzymatic attack (thus requiring a more complex enzyme mixture of both endo and exo acting forms of the enzymes). The pre-treatment process as a whole is generally pH dependant in that a low pH protocol (such as acid hydrolysis) primarily targets hemicellulose removal, whilst a high pH protocol (such as ammonia treatment) generally targets lignin exclusively and the polysaccharides remain virtually intact (Chundawat et al, 2011).

The severity of the reaction conditions heavily influence the products formed (Carvalheiro et al., 2008). Higher temperatures (and/or pressures) and higher concentrations of any liquid catalyst used (with the exception of using water) can increase sugar yield but only up to a point. Exceeding the optimal reaction conditions can then result in excessive sugar degradation to downstream fermentation inhibitors giving an overall lower sugar yield. However, more extreme reaction conditions obviously result in a faster overall processing time (reduced process cycle times). Carvalheiro et al (2008) found that the maximum yield of solubilised xylan obtained from agricultural residues using an autohydrolytical pre-treatment was after 120 – 180 min at 150°C, compared to just 20 and 5 min at 170 and 190°C (respectively). Clearly a compromise must be found between processing time (process efficiency) and energy input. The majority of lignocellulosic pre-treatments using some variant of a liquid catalyst (high temperature water, acid, alkali or organic solvent etc.) result in a solid phase of mostly cellulosic composition (and possibly condensed, insoluble lignin) and a hydrolysate phase (the liquid fraction) consisting of the solubilised hemicellulose and any solubilised phenolics of lignin origin (Chundawat et al, 2011, Carvalheiro et al, 2008, Girio et al, 2010). High initial solids loadings are often required to generate a sugar

concentration worthy of fermenting as a sugar concentration step can be conducted but the economic impact of this would be unfavourable and also fermentation inhibitor generation could be increased due to increased thermally induced sugar degradation. There are a number of important factors to consider when evaluating a pre-treatment such as attempting to use the minimal chemical input (Yang and Wyman, 2008), the minimal energy input (for heating or particle size reduction), use of the least environmentally damaging chemicals where possible and using non specialist materials for any hardware were possible (Petersen et al, 2009). Value added co-product recovery costs (non-fermentative co-products etc.) are important should that be a target for the process. Where chemical addition is unavoidable, green chemistry should be sought, with reagents that are the least harmful to the environment, either non-volatile or low volatile, non-toxic and if possible chemicals and/or processes where the reagents can be recycled (El Seoud et al., 2007). Overall choosing the pre-treatment that gives the highest enzymatic digestibility (highest enzymatic saccharification yields) whilst allowing the lowest enzyme loading (lowest dose) is desirable, as this is a costly but essential step. There is not a current single pre-treatment that manages to satisfy all the desired criteria for a true advanced generation biofuel hence the large number of techniques discussed. Some pre-treatments use no chemical reagents but require high energy inputs (often high temperatures for instance) whilst others avoid high energy inputs but require environmentally unfriendly chemical reagents which require would need to be recycled to achieve favourable life cycle assessment. To discuss the current pre-treatments available for deconstruction of BSG, lignocellulosic material will be considered in general and the pre-treatments will be divided into categories based upon their mode of disruption. The main categories are: 1) high temperature mechanical based disruptive techniques (termed thermo-mechanical), 2) water based autohydrolytical, 3) acid based hydrolytical, 4) alkali

based caustic and 5) non-pH function chemical reagents (solvent fractionation including ionic liquids).

1.7.2 Thermo-mechanical deconstruction

This relies on high temperature reaction conditions and usually a large physical energy input often in the form of shearing, extruding, and milling/grinding (Chundawat et al, 2011, Carvalheiro et al, 2008, Girio et al, 2010). Thermo-mechanical disruption can also be coupled with a chemical reagent to enhance the process further. Whilst thermo-mechanical protocols can be effective, overall the considerable energy input required is problematic as it reduces life cycle assessment (LCA) favourability.

1.7.2.1 Milling

There are two major variants of milling that could theoretically be applied to BSG; wet disk milling and dry ball milling (Hideno et al., 2009). Milling is fairly high energy intensive but offers a somewhat environmentally friendly option due to the lack of chemical input. Also unlike other chemical and high temperature pre-treatments no sugar degradation occurs therefore no downstream inhibitors are generated. Wet disk milling allows a slurry of BSG and water to be processed and has been used effectively on other lignocellulosic biomass. Wet disk milling is also a lower energy technique rendering it more economically viable on a commercial scale. When using other lignocellulosic biomass types such as rice straw, dry ball milling resulted in ca. 12% higher sugar yields than wet disk milling (Hideno et al., 2009). However, wet disk milling requires only 5% of the energy input of dry ball milling. Milling is likely to enhance any enzymatic saccharification simply through increased surface area allowing better enzyme access (White et al, 2008). Milling also aids storage and handling through better compacting. Jay et al. (2008) found that BSG could be split into 5 fractions through combined milling and vibratory sieving. Material retained on 500, 250 and 150 µm

sieves was composed of mostly arabinoxylan based material, whilst the material that passed through a 106 and 53 µm sieve was mostly very fine particles of protein and starch fragments. Differences in the specific types of lignin based phenolic acids were observed in different fractions, but each fraction still had some variety present. This suggests that some degree of chemical fractionation could be achieved by purely physical means. Fractionation of BSG to yield a protein enriched fraction and a crude fibre enriched fraction through size exclusion sieving has been developed up to pilot plant scale by Heineken Breweries (Schwencke, 2006). In this case the protein fraction is often sold as cattle feed (depending on local economic factors) whilst the fibrous fraction is burnt to generate steam. This process replaces long cycle CO_2 (such as fossil fuel burning) with short cycle CO_2 (balanced CO_2 output with the amount initially absorbed by the growing biomass) and therefore contributes to emissions reduction targets such as those set at the Kyoto summit agreement (Schwencke, 2006).

1.7.2.2 Thermo-mechanical extrusion

Used in the food industry, the effectiveness of thermo-mechanical extrusion is heavily dependent on the operating temperature and pressure (Lamsal et al., 2010). Frictional and shear force are responsible for the results of this technique (Lamsal et al., 2010; Lee et al., 2010). There are many variables associated with extruder operation such as the screw type and the chosen dye and these could be specifically optimised for BSG pre-treatment. Extrusion cooking at a high moisture level is an energy efficient variant of this process (Ben-Gera et al., 1983). However thermo-mechanical extrusion often needs the addition of some form of chemical reagent to improve its overall effectiveness as sugar yields with this alone are often sub-optimal (Lamsal et al., 2010).

1.7.2.3 Steam explosion

Steam explosion is a synergistic combination of both thermo-mechanical and water based autohydrolytical pre-treatment at high temperature and pressure in a sealed reactor (Girio et al, 2010, Chundawat et al, 2011, Carvalheiro et al, 2008, Sanchez and Cardona, 2008). This technique typically utilises steam at ca. 200°C and 15-22 bar pressure and partially hydrolyses hemicellulose and partially depolymerises lignin. The rapid depressurisation of the generated steam effectively explodes the biomass, causing fibrillation. The subsequently generated hydrolysate contains pentose sugar derivatives and phenolics from the lignin. The removal of hemicellulose is effectively by autohydrolysis (as with most of the water based pre-treatments) which involves the cleaving of acetyl groups in the hemicellulose that form acetic acid which then hydrolyses the glycosidic linkages and the β-o-4-ester linkages found in hemicellulose and lignin (Carvalheiro et al., 2008; Gírio et al., 2010; Petersen et al., 2009). The procedure does not completely remove lignin, instead it redistributes it to condensation sites usually found on the surface of the cellulose in proximity to the lignin. These condensed lignin droplets operate as competitive inhibitors (through competitive adsorption at active sites) with any subsequently added cellulose enzymes attempting digestion and as such can reduce enzymatic saccharification yields. This redistribution of lignin following solubilisation is common to many alkaline reagent based pre-treatments. The effect of particle size of the initial substrate is more crucial with steam explosion (or any steam based pre-treatment) as larger particle sizes may limit the uniform transfer of heat of the steam (Carvalheiro et al., 2008).

1.7.3 Autohydrolytical water based pre-treatments

This can be considered the central hub of current lignocellulosic pre-treatments and involves the use of water in an autocatalytic process (Carvalheiro et al., 2004; Gírio et al., 2010; Gupta et al., 2010; Sun et al., 2005; Taherzadeh & Karimi, 2008). Generally these techniques

specifically target the hemicellulose. Under suitable conditions of temperature and pressure, the water molecules cleave the acetylated side chains from hemicellulose and the resulting formation of acetic acid then catalyses the hydrolysis of glycosidic links, as described previously with steam explosion (section 1.7.2.3). Autohydrolysis has a similar mechanism to dilute acid hydrolysis (section 1.7.4.2). Water molecules undergo auto-ionisation producing hydronium ions (H_3O^+) that start the reaction off, cleaving acetyl substitutions on the hemicellulose to produce acetic acid which then takes over as the main reactive species in further hydrolysis reactions (Carvalheiro et al., 2008). A relatively high hemicellulose recovery (55-84% w/w) is possible using autohydrolytical pre-treatments (Girio et al., 2010). The liquid to solid ratio (of water to BSG) may be between 2 and 90 (w/w) but is normally closer to 10. Autohydrolytical water based pre-treatments look to be a promising option as they do not require chemical reagents and can be effective with relatively small quantities of water and moderate temperatures (and therefore moderate energy input).

1.7.3.1 Hydrothermal pre-treatment

This process is also autocatalytic as with steam explosion but operates at a more gentle, lower temperature in the range of 120-220°C (Carvalheiro et al., 2008, Girio et al., 2010, Chundawat et al., 2011). Hydrothermal pre-treatment is a relatively simple technique with low generation of degradation products. Suitable for hemicellulose hydrolysis and subsequent generation of xylan based oligosaccharides (Girio et al., 2010). At temperatures below 100°C, there is no hydrolytic effect on the lignocellulosic material (Carvalheiro et al., 2008). At temperatures above 220°C, cellulose begins to depolymerise. At temperatures above 250°C pyrolysis reactions begin to occur.

1.7.3.2 Hot compressed water

This involves using water almost at the supercritical level, again in an autohydrolytical fashion of disruption as described with steam explosion and hydrothermal pre-treatment (Brunner, 2009).

1.7.3.3 Supercritical processing

This often uses either supercritical CO_2 or water (Gírio et al., 2010; Zhao et al., 2009c). Supercritical fluids are compounds above their critical temperature and critical pressure but below the pressure that would condense them into a solid. This is effectively a form of extreme hot compressed water treatment but with extra lignocellulosic pore penetrating power. In the case of water, at supercritical point it is also able to act as a solvent for non-ionic species (as opposed to ionic only at ambient temperature and pressure) which is very useful for biopolymer hydrolysis. Supercritical CO_2 offers gas-like mass transfer properties and liquid-like solvation power (Taherzadeh & Karimi., 2008). Typically water becomes supercritical at 374°C and 22MPa which results in good lignin removal and cellulose hydrolysis but the severity of the conditions results in considerable pentose and hexose degradation to non-fermentable fragments and fermentation inhibitors. One strategy would be to use supercritical water or CO_2 in an optimised, shorter initial pre-treatment for lignin removal then switch to subcritical conditions for a less severe hydrolysis step for sugar hydrolysis, attempting to minimise degradation product (inhibitor) generation (Taherzadeh & Karimi, 2008). Supercritical CO_2 treatment could involve the use of CO_2 generated from the fermentation stage of the process and recycled (Yang and Wyman., 2008). This would increase the process efficiency considerably as industrial scale use would likely be very expensive (Taherzadeh & Karimi., 2008).

1.7.3.4 Steam and chemical

This may involve the use of steam and an additional chemical reagent such as an acid or an alkali such as alkaline peroxide (Sun et al., 2005, Carvalheiro et al., 2008). The steam is targeted towards hemicellulose removal or hydrolysis and the alkaline peroxide targets the lignin for removal. The alkaline peroxide may function by cleaving the α-benzyl ether linkages between lignin and hemicellulose. Alkaline peroxide based pre-treatment is discussed further in alkaline pre-treatment reagents (section 1.7.5).

1.7.4 Acid based pre-treatments

Acid based pre-treatments often use sulphuric acid but can also use hydrochloric or nitric acid (Sanchez & Cardona., 2008, White et al., 2008). The use of acid based pre-treatments needs to be carefully optimised for monosaccharide generation for subsequent fermentation as it can generate excessive degradation products (fermentation inhibitors). Major effects of acid pre-treatment are a reduction in cellulose crystallinity, separation of the polysaccharides from the lignin fraction, and depending on the strength of the acid used, solubilisation of lignin and then subsequent condensation/coalescence onto cellulose in the form of droplets. Overall acid based pre-treatments can be very effective but the need to minimise the acid input or recycle the acid in some way is a technical hurdle (Sanchez & Cardona., 2008).

1.7.4.1 Concentrated acid hydrolysis

This can involve using concentrations such as 72% H_2SO_4 or 41% HCl (Girio et al., 2010). Such conditions are very effective at total hydrolysis: down to monomeric sugars and without the use of enzymes but overall the protocol has many shortfalls. This concentrated acid is often excessively severe therefore results in considerable degradation of sugars to inhibitory products such as 5-hydroxymethylfurfural (HMF) and furfural. It can also be expensive and environmentally unfavourable. The highly corrosive nature of the concentrated acid therefore

requires specialist reaction equipment such as austenitic steel alloys (chromium and nickel based) as corroded metals can release highly toxic ions (Gírio et al., 2010). Finally the process requires a neutralisation step prior to fermentation of any feedstock (and the neutralisation step itself may generate unwanted compounds such as a large quantity of metal salts). Overall life cycle assessment (LCA) discourages strong acid use in a theoretical large scale model of a bioethanol plant (Uihlein & Schebek, 2009). The current availability of aggressive, highly effective enzyme mixtures now permits the use of weaker, less severe acid pre-treatments (Chundawat et al, 2011). However concentrated acid reactions proceed effectively at much lower temperatures which reduce the energy input required (Girio et al, 2010). Acid recovery would be essential though for achieving suitable process economy.

1.7.4.2 Dilute acid hydrolysis

The process works through the released protons cleaving heterocyclic ether bonds between sugars in the cellulose and hemicellulose biopolymers (White et al., 2008, Girio et al., 2010). The use of nitric or hydrochloric acid may also enhance the deconstruction process through the presence of nucleophilic chloride ions from HCl or the oxidative action of HNO_3 (White et al., 2008). The process is often heated and acts as a combination of both autohydrolytical degradation and acid catalysed hydrolysis (Carvalheiro et al., 2008). Typically up to 0.5N sulphuric acid concentration is used at 90-130°C for up to 4 h: this results in good digestibility (good sugar yields from enzymatic saccharification) without excessive degradation product formation (Yang & Wyman., 2008). Sugar yields following cellulose digestion increased from 22% to 40% (of the initial dry weight BSG) after dilute acid treatment (White et al., 2008). Sugar yields were mostly composed of xylose and arabinose from hemicellulose degradation as the acid is not strong enough for degradation of crystalline cellulose hence little glucose was formed. However, cellulose crystallinity is reduced to some extent and there is less linkage of the polysaccharides to the lignin present. A flow through

system removes more lignin in comparison to batch treatment using dilute acid as there is less condensation of the solubilised lignin as droplets on the cellulose (Yang & Wyman., 2008). Dilute acid hydrolysis may still require a neutralisation step prior to fermentation but the volume of neutralisation reagent (and volume of deleterious neutralisation product formation) would be considerably less than that with concentrated acid hydrolysis (Girio et al., 2010). Dissolution of CO_2 into the liquor of lignocellulosic material and water could form carbonic acid which could act as the catalyst as an alternative to traditional acid addition (Carvalheiro et al., 2008). Carbonic acid is particularly suitable for BSG hydrolysis under mild pressure and pH conditions. Should the pre-treatment stage of BSG processing be conducted at an already existing brewery then CO_2 generated from various other industrial processes such as beer fermentations could be recycled back into use, thus reducing the carbon emissions and accrediting the green credentials of the overall process. Experimental data from van Walsum and Shi (2004) suggested that the presence of carbonic acid during hot water pre-treatment (180-220°C) of aspen wood significantly increased the concentration of xylose and xylan oligomers in the hydrolysate compared to water alone. This would suggest hemicellulose solubilisation and would suggest that better access of cellulase enzymes to cellulose would then be achieved.

1.7.4.3 Electrolysed water

This technique uses a water electrolyser which creates acid and alkali species to disrupt the hydrogen bonding network that is associated with cellulose (Wang et al., 2010). This technique is advantageous as it does not require strong acid or alkali generation but the final sugar yields are low compared to 1% sulphuric acid treatment at 200°C or ammonia fibre expansion (AFEX described later in section 1.7.5; Weiss et al., 2010). However electrolysed water is much faster than the latter treatments requiring only 24 min at 210°C. The reactive species generated result in fractures and pore formation in the cellulose structure, with low

pH electrolysed water exhibiting an oxidative mode of action whilst at high pH electrolysed water functioning as a reducing agent (Wang et al., 2010). Further options to enhance electrolysed water pre-treatment to achieve higher sugar yields closer to techniques such as AFEX would be to operate it in a 2-stage process combining it with an initial pre-treatment with 3% hydrogen peroxide, with the first stage designed to remove lignin and hemicellulose (if the treatment was severe enough) and the second stage specifically designed to improve cellulose digestibility (Wang et al., 2010).

1.7.4.4 Microwave heating technology

This uses electromagnetic waves and is a very efficient, fast heating technique but does require the calculation of various factors such as the dielectric constant of BSG (how polarisable a material is in an electric field) and the dielectric loss, the production of heat/energy that is lost in the presence of a varying electrical field (Orozco et al., 2007). Whereas conventional ovens and heating techniques use relatively inefficient direct superficial heat transfer, microwaves oscillate polarised species and the resulting friction between molecules generates the heat (Hu & Wen, 2008). As with many lignocellulosic pre-treatments microwave heating can be augmented with the addition of some form of chemical pre-treatment such as an acid or an alkali. This combination with an alkali proves highly effective as the alkali targets the lignin for dissolution or removal whilst the microwave heating targets the hemicellulose. Whilst approximately 99% of the theoretical sugar yield can be achieved using this combination, it requires a high liquid: solid ratio (low solids loading) as the microwave radiation needs a high water quantity for effective heat transfer to the biomass. Also as with any heating technique, excessive reaction conditions can generate degradation products (Zhang & Zhao, 2009). In addition to microwave based heating strategies, other forms of irradiation have been investigated with regards to their effectiveness with lignocellulosic deconstruction (Taherzadeh & Karimi., 2008). These include gamma

rays and electron beams. Additionally ultrasonic assisted pre-treatments using acoustic energy have also been shown to function effectively on lignocellulosic biomass (Bussemaker & Zhang, 2013; Luo et al., 2014). These techniques can be very useful in enhancing enzymatic saccharification of lignocellulosic material. However due to their very high energy nature the process must be highly optimised as excessive degradation of the hydrolysed sugars to fermentation inhibitors is very likely. Microwave heating can also be combined with non-pH dependant chemical reagents such ionic liquids for increased effectiveness due to their rapid heating profile (Zhang & Zhao., 2009). The problem with microwave technology as a viable pre-treatment is the difficulty encountered when attempting to scale up the process to commercial industrial scale from laboratory small scale reactions.

1.7.5 Alkali based pre-treatments

These pre-treatment systems often use aqueous sodium hydroxide or lime (calcium hydroxide) but could potentially use the hydroxide of any alkaline earth metal (Hu & Wen, 2008., Girio et al., 2010, Carvalheiro et al., 2008). The major effects of alkaline based pre-treatments are the complete lignin removal with high enough alkali strength (typically 0.2-0.3 g/g biomass). There are generally fewer degradation products than acid pre-treatments but use of alkali based reagents can result in hemicellulose degradation which reduces the final sugar yield. These alkali based pre-treatments are thought to act via a swelling mechanism which increases the substrate porosity and allows enhanced enzymatic access to the polysaccharides (mainly enhances access to cellulose). In essence it is saponification of the ester bonds that link hemicellulose to lignin that occurs. (Carvalheiro et al., 2008). The generation of excessive concentrations of carboxylic acids from alkaline based pre-treatments can be a greater fermentation inhibitive factor than that of furan based inhibitors from sugar degradation (Girio et al., 2010). Typical reaction conditions for alkaline pre-treatment are: 30-130°C temperature and 10 min to 18 h reaction times. Alkali based pre-treatment is

especially suitable where the recovery of hydroxycinnamic acids (ferulic and *p*-coumaric acids) is desired. The combination of an alkali based liquid catalyst and ultrasonic assisted extraction improves the yield of solubilised hemicellulose (xylans) and lignin than the use of just the alkali reagent alone (Carvalheiro et al., 2008). The main advantages of alkali based pre-treatments are that generally lower reaction temperatures and pressures can be used which impacts the economics of the process (lower energy input and lack of requirement for high pressure resistant bioreactors). The downside to alkaline based pre-treatments is similar to that of any chemical based pre-treatment systems; the difficulty and cost associated with recycling the alkaline reagent if high concentrations are used.

1.7.5.1 Cold caustic ammonia

Aqueous ammonia solutions are used at low temperatures rendering it highly efficient in terms of energy input (Carvalheiro et al., 2008). Ammonia only significantly reacts with lignin and can completely remove all lignin if the concentration is high enough. Aqueous ammonia solutions leave both hemicellulose and cellulose intact which increases the theoretical sugar yield considerably compared to using sodium hydroxide. This is especially significant for BSG as hemicellulose concentrations can be up to 30% - 35% (dry weight). Ammonia recovery would need to be attempted though in order to increase the 'green credentials' of the process and recycle it. However ammonia based pre-treatment alone still results in relatively lower final sugar yields (lower degree of enhancement of enzymatic saccharification) than some other pre-treatments (such as steam explosive based autohydrolytical pre-treatment for example) as NH_4 resistant regions of lignin can still impede cellulase access to cellulose if the process is not severe enough (not severe enough reaction conditions).

1.7.5.2 Ammonia recycled percolation (ARP)

ARP uses a continuous flow through system targeted at delignification, through a reactor column at ca. 170°C that prevents lignin deposition (the condensation or coalescence that can occur after the initial dissolution of lignin) in an attempt to minimise the inhibition it can potentially physically cause cellulase enzymes, impeding them from access to the cellulose substrate (Gupta et al., 2009, Girio et al., 2010, Carvalheiro et al., 2008). The typical ammonia concentration is 10-15% with a reaction time of no more than 90 min and a percolation rate of 5 mL/min (Carvalheiro et al., 2008). However as with the standard ammonia pre-treatment systems it still results in relatively lower final sugar yields if not optimised and the cellulose remains intact. However, ARP can still prove effective at hemicellulose solubilisation, achieving between 40-60% removal (Girio et al., 2010). The remaining cellulosic biomass is then highly enzymatically digestible due to the almost complete removal of lignin and a significant degree of hemicellulose removal (Carvalheiro et al., 2008). ARP also works via a similar swelling mechanism that cleaves ether and ester linkages between lignin and the polysaccharides as traditional ammonia (and other alkaline based) pre-treatments work. However there are cost implications associated with the recovery of the ammonia and health and safety issues related to the use of large industrial quantities of ammonia.

1.7.5.3 Ammonia fibre expansion (AFEX)

AFEX combines ammonia treatment and extruder based mechanical deconstructive action to achieve a high sugar yield after enzymatic saccharification (Chundawat et al., 2011, Carvalheiro et al., 2008, Girio et al., 2010). It can be seen as a combination of an alkaline pre-treatment but also with the mechanical effects similar to those of steam explosion (Carvalheiro et al., 2008). AFEX typically uses anhydrous ammonia at concentration of 1-2 kg ammonia per kg biomass at temperatures of 40-140°C and high pressures of 250-300 psi

for periods of only a few minutes. The pressure is then rapidly released. Therefore it could be interpreted as ammonia explosion pre-treatment. It uses ammonia to predominantly specifically attack the lignin, whilst leaving cellulose and hemicellulose intact (Keshwani & Cheng, 2009). With optimisation and severe enough reaction conditions the technique can also be used for solubilisation of cellulose and hemicellulose, with yields over 85% theoretical achievable (Girio et al., 2010). Also the ammonia can be almost completely recovered (approximately 99% of the original quantity used) improving the process economics and LCA favourability considerably.

1.7.5.4 FIBEX

This is a further development of the AFEX procedure with the whole process transferred to an extruder for greater effectiveness (Gupta et al., 2009). It uses less ammonia than AFEX making it a 'greener' option. The lignocellulosic material is soaked in aqueous ammonia at a relatively low temperature and the hemicellulose is virtually intact afterwards. Subsequent treatment with cellulase and xylanase enzymes then proves very effective in achieving a high degree of saccharification. FIBEX (like many other lignocellulosic pre-treatments) can be enhanced via the addition of hydrogen peroxide as another additional reactive species, without detrimentally increasing the severity of the process to the point were excessive sugar degradation products are formed.

1.7.5.5 Hydrogen peroxide (alkaline peroxide)

This utilises oxidative cracking by a hydro-peroxyl reactive species generated from hydrogen peroxide (Banerjee et al., 2011; Wang et al., 2010). These reactive species can cleave lignin rings, aryl-ether bonds and react with chromophoric groups, changing colours. Typical reaction conditions are 2% H_2O_2 extraction at 90°C for 2 h at pH 11.5 but can use lower temperatures and longer reaction times such as 50°C for 16 h at pH 12.5 (Fang et al., 1999).

Whilst the hydroperoxyl reactive species are not technically alkaline based reagents, the requirement of pH 11.5 renders the addition of a significant quantity of NaOH usually mandatory to achieve this. Therefore some degree of caustic based reactivity is highly likely as an additional pre-treatment effect (Carvalheiro et al., 2008; Li et al., 2012). Whilst alkaline peroxide is suggested to specifically target lignin, Wang et al (2010) suggest it can also solubilise hemicellulose. Reactions conditions are suggested to be 30-80°C for 45 min to 5 h using 1-3% H_2O_2 (Carvalheiro et al., 2008). In addition to the hydro-peroxyl radicals formed from the hydrogen peroxide, hydroxyl radicals are also generated and these produce water soluble degradation products. Both the hydroxyl and hydro-peroxyl radicals attack the α-benzyl ether linkages between lignin and hemicellulose, resulting in improved enzymatic access and therefore better saccharification. Alkaline peroxide can also be combined with steam explosion (200-220°C, 15-22 bar, 3-30 min) for very effective deconstruction (Sun et al., 2005). The steam is more specifically targeted towards hemicellulose removal and the alkaline peroxide targets the lignin for removal. However the longer more extreme reaction conditions often result in very dark coloured hydrolysates indicating significant sugar degradation product formation and therefore lower overall final sugar yields. Alkaline peroxide pre-treatments are suggested to have faster kinetics at lower temperatures than standard NaOH based treatments indicating some potential economic benefits, and when combined with steam explosion provides a highly effective lignocellulosic deconstruction protocol (Carvalheiro et al., 2008).

1.7.6 Non-pH dependant functionality chemical pre-treatments

1.7.6.1 Sonochemical attack: Supersonic/shockwave steam

This process works via sonolysis in that wave energy is targeted at the biopolymer (the lignocellulosic matrix) and can potentially deconstruct if the wave energy is high enough and

enough exposure is given (Bennett, 2013; Kardos & Luche, 2001; Montalbo-Lomboy et al., 2011). Sonochemical attack has the ability to cleave large macromolecules like those associated with lignocellulosic material found in BSG but needs a high liquid to solid ratio (low solids loading) to facilitate effective transmission of the mechanical wave energy to the target molecule (e.g. lignin or hemicellulose). This requirement of very low solids loading would make the technique inefficient on a large industrial scale and also with a potentially unacceptable water requirement.

1.7.6.2 Organic solvents

These techniques are often simply referred to as organosolv and use polar organic solvents such as ethanol or methanol which have proved to be very effective at removal of lignin (Taherzadeh & Karimi., 2008, Carvalheiro et al., 2008, Girio et al., 2010, Chundawat et al., 2011). Some degree of hemicellulose hydrolysis can also occur. The organic solvents impregnate the lignocellulosic biomass and solubilise the lignin. This technique can be good at facilitating the recovery of valuable co-products such as phenol derivatives, antioxidants such as ferulic acid and other vanillin precursors potentially used in the food industry (Yang & Wyman., 2008). An operational example is the Lignol organosolv biorefinery: water and ethanol is used in a ratio of 1:1 at 200°C and at 400 psi pressure. The lignin is in a soluble form and recovered in a flash tank. Furfural and hemicellulose derivatives are recovered from the hydrolysate (liquid fraction) as with many other pre-treatments. When higher temperatures are used acetic acid is generated in a similar fashion to that seen with water based autohydrolytical pre-treatments. However, not all organosolv based pre-treatments need high temperatures, with some operating at room temperature (Girio et al., 2010). The use of copious quantities of organic solvents is expensive and without an expensive separation step for recovery, the disposal of large amounts of organic solvents is highly environmentally unfriendly (Girio et al., 2010, Carvalheiro et al., 2008). One option is the use

of ethanol generated downstream of the process (in the fermentation step of a bioethanol process) and recycling some quantity of this back for use in the organosolv pre-treatment step.

1.7.6.3 Cellulose and Organic Solvent Lignocellulosic Fraction (COSLIF)

Cellulose and Organic Solvent Lignocellulosic Fraction often utilises either a phosphoric acid-acetone-water mixture, or alternatively ionic liquids (Chundawat et al., 2011; Zhu et al., 2009). COSLIF uses a lower reaction temperature and lower pressure than many other pre-treatment systems in order to minimise sugar degradation product formation. Reaction conditions are typically 50°C for 30-60 min at 1 atm pressure. Scanning electron microscopy (SEM) has shown that COSLIF results in different structural changes to lignocellulosic material than that caused by acid based pre-treatments (Zhu et al., 2009). Compared to dilute acid pre-treatment it removes less hemicellulose but more lignin. It also increases the digestibility (degree of enzymatic saccharification) of the larger glucose based oligosaccharides (glucans) compared to dilute acid treatment, and also decreased the cellulase enzyme loading (decreased doses) required to do so. The practical difficulty of the technique is that it requires a separation step for the solvent used which is likely to be expensive. The presence of any residual organic solvent or ionic liquid would inhibit cellulase enzymes leading to reduced final sugar yields (Chundawat et al., 2011).

1.7.6.4 Ionic liquids

Ionic liquids are low melting point salts in the liquid state (with small lattice enthalpies) that contain only anions and cations (El Seoud et al., 2007; Gírio et al., 2010; H Clark et al., 2009; Zhao et al., 2009a). Ionic liquids are liquid electrolytes that conduct electricity but more importantly their ionic composition gives them the ability to compete for hydrogen bonding in macromolecules such as those found in lignocellulosic material. The overall effect is that

the structure is significantly disrupted which can often result in depolymerisation as a result of swelling of the molecule (Gírio et al., 2010; Zhang & Zhao, 2009). In addition the process requires a minimal energy input as it can function at room temperature for highly effective cellulose digestion (Zavrel et al., 2009). Minimal degradation products or fermentation inhibitors are formed with this technique but the ionic liquids themselves (if present in higher concentrations) are likely to be unfavourably toxic to the fermentation species used downstream (El Seoud et al., 2007). The solution is again a costly separation step. Combining ionic liquids with microwave heating technology has been shown to considerably improve the effectiveness as the ionic liquids themselves usually exhibit a rapid heating profile therefore minimising energy input (Zhang & Zhao., 2009).

1.7.6.5 Surfactants

As re-condensed lignin (after its initial solubilisation) has been shown to reduce enzyme activity (especially cellulase activity) through adsorptive, non-competitive inhibition, surfactants can be used to decrease this deleterious adsorption (Gupta et al., 2009). Surfactants lower the surface tension between the re-condensed lignin and the substrate therefore allowing greater adsorptive surface area for the enzymes to function. Qing, Yang et al. (2010) found that when various surfactants were used in addition to weak acid hydrolysis they increased lignin removal and therefore reduced the subsequent amount of non-productive enzyme binding on the surface of corn stover (when compared to weak acid hydrolysis alone). The solvent based surfactant Tween-80 in particular increased the effectiveness of the subsequent enzymatic saccharification by approximately 10% and therefore enhanced the overall sugar recovery from the lignocellulosic biomass.

Overall there are many pre-treatments available for lignocellulosic biomass. Table 1.2 summarises the main advantages and disadvantages of a range of key pre-treatment technologies.

1.8 Generation of degradation products (inhibitors): overview

An unfortunate consequence of the likely need to pre-treat the lignocellulosic biomass in BSG is that the process can be difficult to control. Therefore reactions can easily exceed the desired point of polysaccharide hydrolysis and enter the realm of downstream process inhibitor generation (Allen et al., 2010; Heer & Sauer, 2008; Liu, 2006; Palmqvist & Hahn-Hagerdal, 2000a; Palmqvist & Hahn-Hägerdal, 2000b). These undesirable inhibitory compounds originate from the three main lignocellulosic components; lignin, cellulose and hemicellulose, usually from degradation or chemical modification of the monomer subunits. Such degradation products (inhibitors) affect downstream processes, often having detrimental effects on both the hydrolytic enzymes used for saccharification and also the microbes responsible for ethanol production.

Acid and alkali based pre-treatments give rise to many different furan derivatives, weak acids and phenolics which all have inhibitory actions against the most commonly used commercial fermentative species, *Saccharomyces cerevisiae* (Palmqvist & Hahn-Hagerdal, 2000a; Sanchez & Cardona, 2008).

1.8.1 Sugar degradation products (inhibitors)

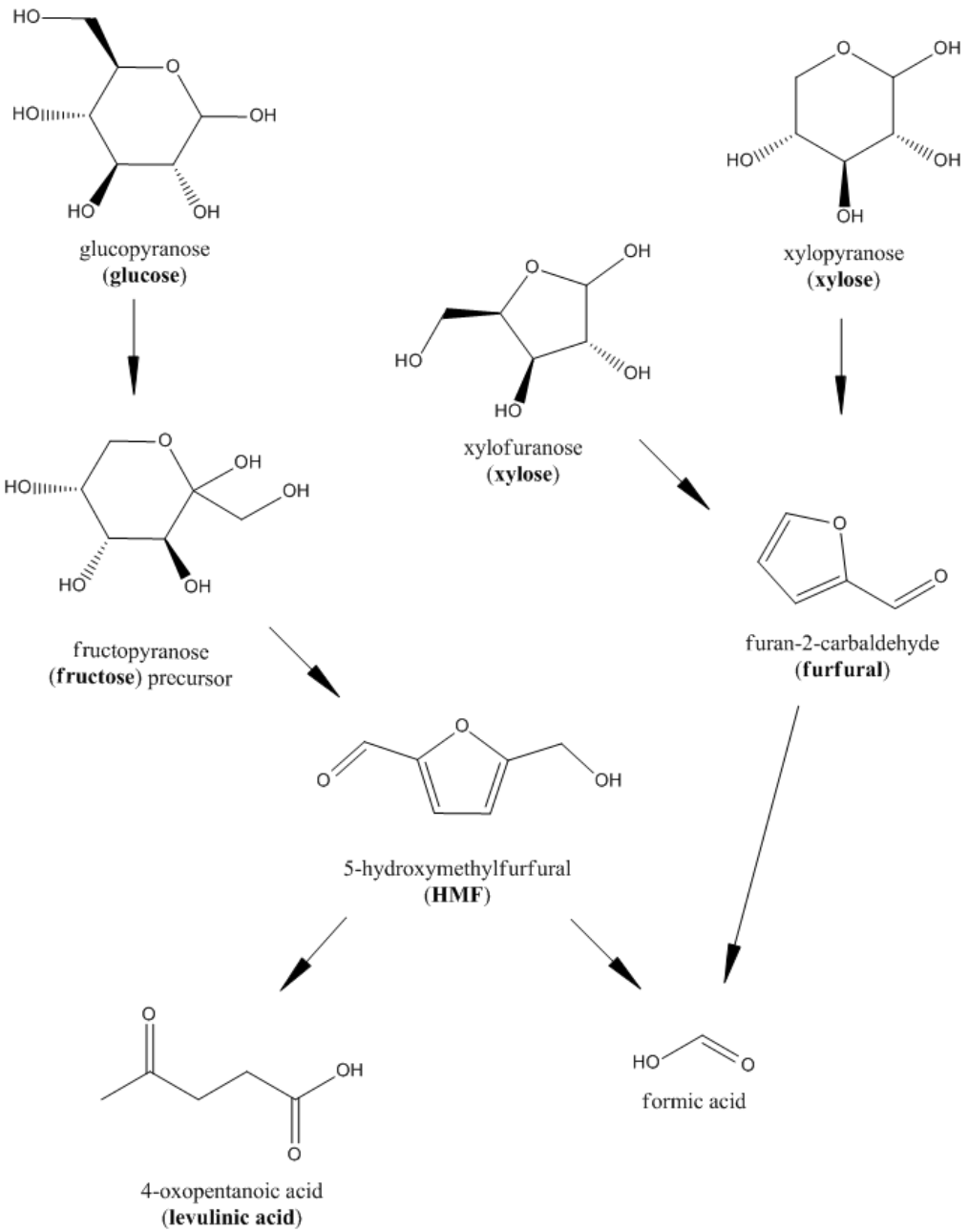
The major sugar derived fermentation inhibitors are furfural, 5-hydroxymethylfurfural, levulinic acid and formic acid (Heer and Sauer, 2008, Palmqvist and Hahn-Hagerdal, 2000a). For an overview of sugar derived inhibitor formation see Figure 1.9.

Table 1.2 Summary of advantages and disadvantages of different pre-treatments

Pre-treatment	Advantages	Disadvantages
Thermo-mechanical extrusion[1-3]	Uses existing food production technology	Only moderate effectiveness; often requires additional chemical reagents
Dilute acid hydrothermal[2, 4] (1% H2SO4)	Highly effective hemicellulose removal at moderate (120-160°C) low cost reagent	Potentially high degree of furan inhibitors generated
Low temperature alkaline[2, 4] (NaOH<100°C)	Highly effective lignin removal, low energy	High viscosity product and potential for high degree of phenolic inhibitors
Autohydrolytical[1-4] (steam explosion)	Rapid and highly effective hemicellulose removal, no chemical reagent use	High energy and requires specialised equipment
Autohydrolytical[5, 7] (microwave)	Rapid and highly effective hemicellulose removal, no chemical reagent use	High energy and requires specialised equipment
Ammonia recycled percolation[2, 4] (ARP)	Fast processing, highly effective	High cost of recovery of ammonia, high reaction temperatures
Ammonia fibre expansion[1, 2, 4] (AFEX)	Highly effective, high recovery of ammonia is achievable	High energy, requires high pressure equipment, ammonia must be recycled
Alkaline peroxide[2, 8]	Effective lignin modification, faster kinetics at lower temperatures than other alkaline reagents	Hazardous to handle and store reagent
Supercritical H_20/CO_2[4-6]	Highly effective lignin removal and partial cellulose hydrolysis	High energy, requires specialist equipment and high degree of inhibitors generated
Ionic liquids[4, 9]	Complete structure disruption, very low energy input, minimal inhibitor generation,	Very high cost reagents, complexity of recovery of recovery of reagents
Organosolv[1, 2, 4, 5] (ethanol/methanol)	Effective and good for recovery of phenolic based co-products	High energy and High cost of recovery of solvents used (or disposal of)

([1]Chundawat et al., 2011, [2]Carvalheiro et al., 2008, [3]Lamsal., et al, 2010, [4]Girio et al., 2010, [5]Taherzadeh & Karimi., 2008, , [6]Yang & Wyman., 2007, [7]Orozco et al., 2007, [8]Wang et al., 2010, [9]El Seoud et al., 2007).

Figure 1.8 Production of fermentation inhibitors derived from sugars (Palmqvist & Hahn-Hagerdal, 2000a; Palmqvist & Hahn-Hägerdal, 2000b).



2-fural-aldehyde or furfural originates from pentose sugar degradation, mostly xylose or arabinose (Allen et al, 2010, Heer and Sauer, 2008, Palmqvist and Hahn-Hagerdal, 2000a). Furfural is usually present in higher concentrations following pre-treatments such as acid

hydrolysis as the pentose sugars that hemicellulose is composed of are more susceptible to thermal and chemical degradation than hexose sugars. 5-hydroxy-methyl-2-fural aldehyde or HMF is generated from the degradation of mainly glucose, but actually any hexose sugar including galactose and mannose which may also be present (Liu, 2009, Heer and Sauer, 2008, Palmqvist and Hahn-Hagerdal, 2000a). HMF is produced via glucose dehydration to fructose, as a precursor to HMF. Levulinic acid is another compound with inhibitory action that can originate from glucose degradation via HMF as an intermediate, often also with formic acid as a co-product of the reaction (Palmqvist and Hahn-Hagerdal, 2000a). Formic acid can also originate from glucose as it is a secondary degradation product via either furfural or HMF as an intermediate (Palmqvist and Hahn-Hagerdal, 2000a). Acetic acid also originates mainly from the breakdown of hemicellulose based pentose sugars: xylose and arabinose (Liu 2006, Larsson et al, 1999, Palmqvist and Hahn-Hagerdal, 2000a). However, a minor source can be acid soluble lignin when hydrolysed (Nicholls et al, 2010).

1.8.2 Lignin degradation products (inhibitors)

These are generally considered to be more toxic to fermentative microbial species than low molecular weight organic acids such as acetic acid (Palmqvist and Hahn-Hagerdal, 2000a). Production of lignin degradation products (LDPs) increases when the liquid: solid ratio of the pre-treatment decreases (higher solids loading during pre-treatment; more viscous) and surprisingly also when acid concentration is lower and reaction times are shorter. This is because when the acid concentration is higher a large concentration of the native lignin is solubilised and the 'saturated' hydrolysate then results in a large number of condensation/coalescence reactions occurring, depositing lignin back out of solution as droplets (often onto the surface of any cellulose or other suitable surface present). The deposited lignin droplets have a much more rigid structure than the original native lignin format. This makes it much more difficult to remove the lignin in any subsequent attempts

downstream. Reaction times also affect lignin solubilisation, with longer reaction times again leading to lower LDPs due to a prolonged initial solubilisation period that subsequently results in more condensation reactions. The solution to this would be an optimised lignin solubilisation reaction time and acid concentration and/or a continuous flow protocol that allowed any solubilised lignin to be fractionated from the processing stream. This would drop the lignin concentration below the critical point that initiated the re-polymerisation (condensation) reactions. The most common lignin based degradation products are the hydroxycinnamic acids *p*-coumaric and ferulic acid (Figure 1.10). These are often also the released products when lignin and hemicellulose linkages are hydrolysed (Palmqvist and Hahn-Hagerdal, 2000a). However there are various other phenolic inhibiters of lignin origin including: vanillin, vanillic acid, syringaldehyde, 4-hydroxybenzoic acid, and less significantly acetovanillione, 4-hydroxybenzaldehyde, 4-hydroxyacetophenone and 3,5-dimethoxy-4-hydroxyacetophenone (Luo et al., 2002).

Both *p*-coumaric acid and ferulic acid can be converted into vanillin which is a value added product used extensively in the food production industry (Lesage-Meessen et al., 1996). Industrial synthesis of vanillin (either by chemical engineering or biological engineering) is significantly more cost effective than using natural vanillin. Lesage-Meessen et al (1996) describe a two-stage biological approach to vanillin synthesis using two strains of filamentous fungi, *Aspergillus niger* and *Pycnoporus cinnabarinus*. The *A. niger* initially converts ferulic acid into vanillic acid and then the *P. cinnabarinus* converts the vanillic acid intermediate into vanillin. There are also various chemical reagents that can modify similar compounds such as guiacol into vanillin (Figure 1.11).

Table 1.3 provides a summary of the main inhibitors generated from lignocellulosic biomass.

Figure 1.9 Lignin-based degradation products, including the hydroxycinnamic acids: *p*-coumaric and ferulic acid.

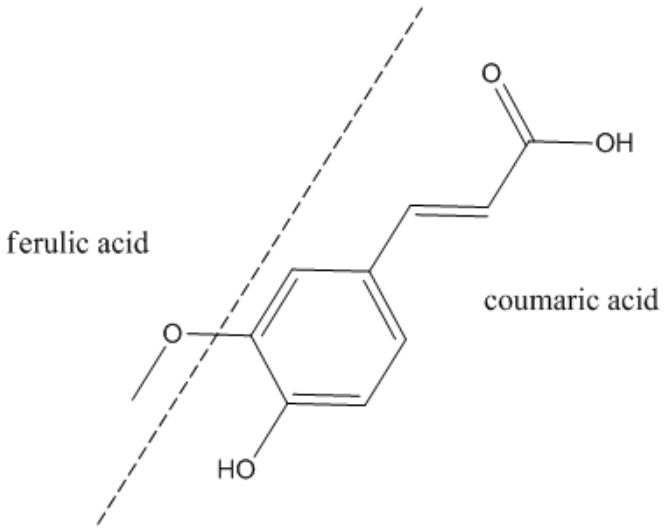
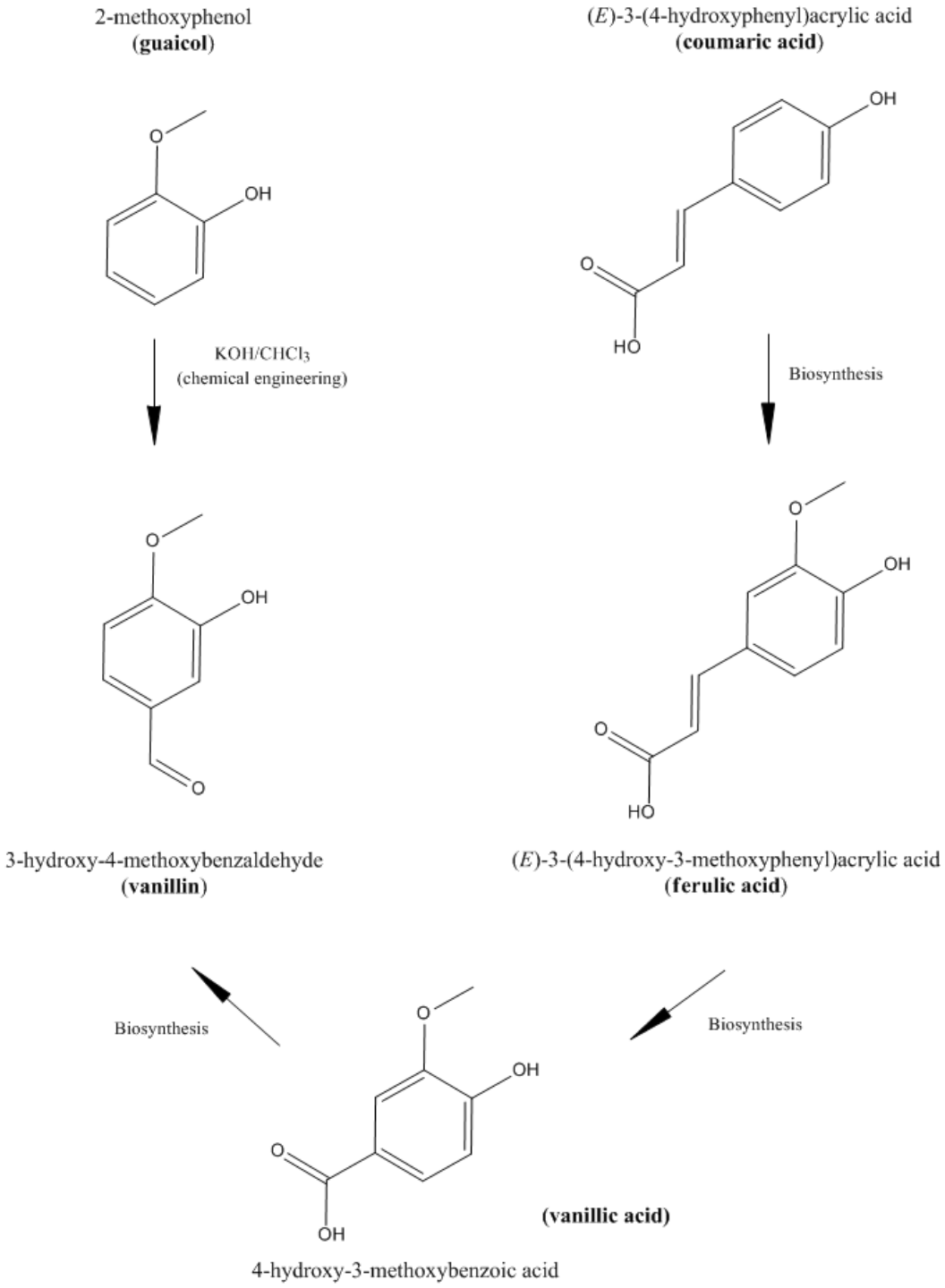


Table 1.2 Summary of main lignocellulosic inhibitors

Inhibitor	Origin	Reference
Small organic acids		
Acetic acid	Hemicellulose/Cellulose	Luo et al., 2002, Palmqvist &Hahn-Hagerdal., 2000b
Formic acid	Hemicellulose/Cellulose	Palmqvist &Hahn-Hagerdal., 2000b
Levulinic acid	Hemicellulose/Cellulose	Palmqvist &Hahn-Hagerdal., 2000b
Furans		
5-HMF	Cellulose/Hemicellulose	Luo et al., 2002, Palmqvist &Hahn-Hagerdal., 2000b
Furfural	Hemicellulose	Luo et al., 2002, Palmqvist &Hahn-Hagerdal., 2000b
Phenolic based		
p-Coumaric acid	Lignin	Klinke et al., 2004
Ferulic acid	Lignin	Luo et al., 2002, Klinke et al., 2004
Vanillic acid	Lignin	Luo et al., 2002, Klinke et al., 2004,
Vanillin	Lignin	Luo et al., 2002, Palmqvist &Hahn-Hagerdal., 2000b
Syringaldehyde	Lignin	Luo et al., 2002, Palmqvist &Hahn-Hagerdal., 2000b

Figure 1.10 Vanillin synthesis from hydroxycinnamic acids (Hansen et al., 2009; Lesage-Meessen et al., 1996; Negishi et al., 2009).



1.9 Enzymatic saccharification

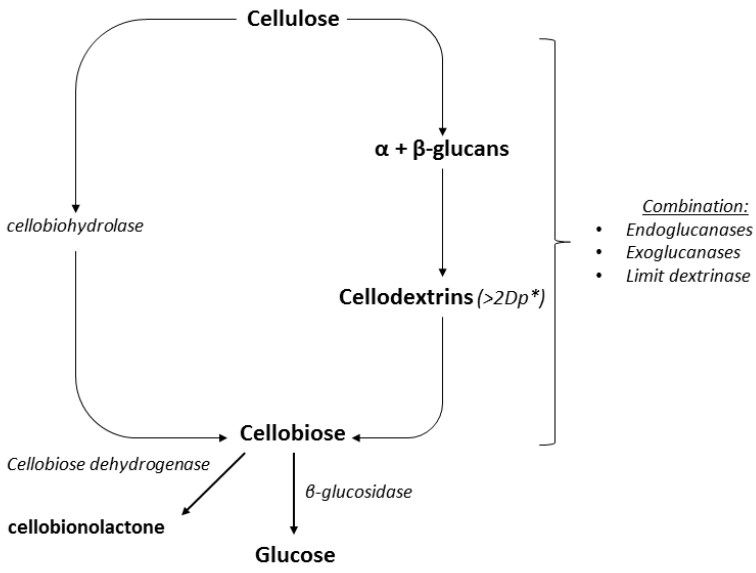
The complex nature of the polysaccharides found in BSG result in efficient biocatalytic saccharification requiring either a secondary acid based hydrolytic treatment (after the initial pre-treatment step etc.) or a complex mixture of expensive exogenous enzymes is usually required (Chundawat et al, 2011, Carvalheiro et al, 2008). Secondary acid based hydrolysis (post-hydrolysis) can be conducted using H_2SO_4 at a concentration of 0.5-6.5% (w/w) at a temperature of 100-135°C with a reaction time of up to 10 h (Carvalheiro et al, 2008). This can be very effective, achieving close to 100% theoretical sugar recovery. The process is also relatively cheap and rapid. The downside is that the acidic pH requires neutralisation and may corrode any equipment such as bioreactors (prior to the neutralisation step). The neutralisation products (likely to be metallic salts) will also need to be removed as they can be problematic for any downstream fermentation or further enzyme based steps. Therefore an entirely biological option is sought in the form of various enzymes. These would not require any neutralisation or high operating temperatures and would not lead to any further formation of degradation products from the sugars due to specificity of the enzymes, unlike any secondary acid based protocol. Due to the wide range of specificity of their action, the potential enzymes that could be used will be discussed individually and grouped together depending on their target substrate. For the purpose of this introductory thesis chapter, purely free, secreted non-complexed enzymes mostly of fungal origin will be discussed as these have been investigated the most. Bacterial cellusomic enzyme protein complexes will not be discussed. All the polysaccharide depolymerising enzymes effectively work by very similar mechanisms: hydrolysis of the glycosidic linkages found between the monomer subunits by either inversion or retention of the stereochemical configuration at the anomeric carbon (one of the carbons bonded to the oxygen on the ring of a monosaccharide (Dodd & Cann., 2009). There are two alternative mechanisms by which this can occur but both utilise a pair of

carboxylic acid molecules at the active site of the enzyme. Both systems use a double displacement mechanism which involves two successive molecular substitution reactions. Firstly the carboxylic acid nucleophile assembles a covalent enzyme-substrate intermediate complex and secondly another intermediate complex occurs but this time using a water molecule. Alternatively, inverting glycoside hydrolases use a single displacement reaction where the carboxylic acid acts as a base to activate a water molecule for attack at the anomeric carbon atom. The enzymatic deconstructive requirements for each of the major components of the BSG based lignocellulosic material (cellulose, hemicellulose and lignin) will each be discussed individually.

1.9.1 Enzymatic based deconstruction of cellulose

There are a wide variety of cellulose acting (or cellulose fragment acting) enzymes which can be sub-divided into many different categories. Figure 1.12 illustrates a schematic overview of enzyme based cellulose deconstruction.

Figure 1.11 Cellulose deconstruction (Chundawat et al., 2011; Langston et al., 2011).



*Dp = degree of polymerisation

1.9.1.1 Cellulases

The term cellulase encompasses a wide category of enzymes with very different activities. They adsorb onto the surface of cellulose and require as much surface area as possible to be effective. As such a higher available cellulose surface area results in greater adsorption of these enzymes (Chundawat et al, 2011). Essentially the cellulose accessibility to cellulose is one of the major rate limiting steps of enzymatic hydrolytic saccharification. Cellulose with a low crystallinity index has more exposed glycosidic links therefore the resulting enzymatic hydrolysis (saccharification) is much more effective than highly crystalline cellulose as a substrate. A higher degree of polymerisation also limits the number of free reducing ends, and as a result efficient hydrolysis requires a mixture of both endo and exo acting glucanases (cellulase derivatives).

1.9.1.2 Cellobiose dehydrogenase

This enzyme oxidises soluble cellodextrins (glucose oligosaccharides of greater than 2 units), mannodextrins (mannose oligosaccharides of greater than 2 units) and lactose (glucose and galactose) to their corresponding lactones or cyclic esters (Langston et al, 2011, Higham et al, 1994). The reason for this is currently undecided. It may be to inhibit the repolymerisation of cellulose after hydrolysis or to limit the competitive feedback product inhibitive effect of high concentrations of cellobiose that may be present when cellulose is deconstructed.

1.9.1.3 Cellobiohydrolases

This category of enzymes release cellobiose from the terminal end of crystalline cellulose, or any β-1,4-D-glycosidic linked oligosaccharide (Chundawat et al, 2011).

1.9.1.4 Endo- β-1,4-glucanases

These attack internal β-1,4-D-glycosidic bonds in cellulose and create smaller oligosaccharide fragments therefore more free terminal ends become available for Cellobiohydrolases to act upon (Gupta et al, 2010) .

1.9.1.5 β-glucosidase

This hydrolyses soluble cellodextrins such as cellobiose, cellotriose and cellotetraose to glucose monomers (Gupta et al, 2010, Chundawat et al, 2011).

1.9.2 Enzymatic based deconstruction of hemicellulose

To effectively and efficiently depolymerise xylan based polysaccharides to produce a fermentable feedstock would require endo 1,4-β xylanases, β-D-xylosidases, α-L-arabinofuranosidases, α-glucuronidases, acetyl-xylan esterases and also ferulic/coumaric acid esterases (Dodd and Cann, 2009, Girio et al, 2010). Figure 1.13 presents a schematic overview of hemicellulose deconstruction.

1.9.2.1 β-D-xylosidases

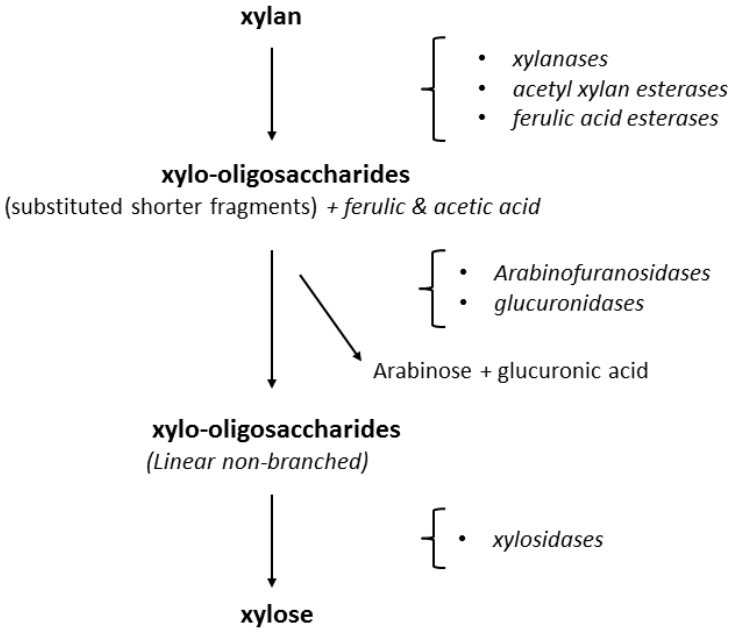
β-D-xylosidases release xylose from the non-reducing ends of xylo-oligosaccharides (Dodd and Cann, 2009, Girio et al, 2010). These typically work after xylanases have cleaved larger xylan fragments into smaller oligosaccharides giving rise to more non-reducing ends for the β-xylosidases to act upon.

1.9.2.2 α-glucuronidases

These liberate the glucuronic acid from the terminal, non-reducing xylose molecule in arabinoglucuronoxylans (Dodd and Cann, 2009, Girio et al, 2010). Large amounts of acetylation in the locality of the glucuronic acid residue on the arabinoglucuronoxylan can reduce the activity of the α-glucuronidase enzyme. These (like β-xylosidases) also normally

function more downstream of xylanases, due to the requirement of smaller xylan fragments to work with.

Figure 1.12 Xylan (hemicellulosic) deconstruction overview (adapted from Dodd and Cann, 2009, Gupta et al, 2008, Yarborough et al, 2009, Girio et al, 2010).



1.9.2.3 Acetyl-xylan esterases

These are xylanolytic esterases (Dodd & Cann., 2009, Girio et al., 2010). Arabinoglucuronoxylans tend to be acetylated at the O-2 and/or O-3 positions of the xylose chain, and also often have esterified ferulic or coumaric acid groups at the 5'-OH of the arabinofuranosyl groups. Acetylated groups such as these often reduce the catalytic capability of many xylanolytic enzymes (most significantly β-xylanases and α-glucuronidases) by impeding successful binding and therefore inhibit hydrolysis. Acetyl-xylan esterases function to liberate any acetate functional group that may be present to form free acetic acid.

1.9.2.4 Endo 1,4-β xylanases

These internally acting enzymes cleave the β-1,4 glycosidic links between xylose molecules (Dodd & Cann., 2009, Girio et al., 2010). The activity of these enzymes can be checked to some extent using non-decorated, commercially available xylo-oligosaccharides (with no side chain substitutions) but this is limited as they are not 'native' naturally occurring xylans. As the xylanases are highly specific and recognise the side chain 'decorations' the final hydrolysis products will be highly influenced by this.

1.9.2.5 α-L-arabinofuranosidases

These enzymes remove the arabinose residues from the xylose backbone of arabinoglucuronoxylans (Dodd and Cann., 2009, Girio et al., 2010). These enzymes can be subdivided into 3 types depending on the substrates on which they work:

1) Only active on short arabino-oligosaccharides and *p*-nitrophenyl-α-L-arabinofuranoside.
2) Active on both short oligosaccharides and longer polysaccharides like arabinan and arabinoxylan.
3) Active on arabinoxylan only.

1.9.2.6 Mannases

Mannanases are involved in the hydrolysis of β-1,4 mannans (Girio et al., 2010). As mannans may be present the inclusion of endo and exo-acting mannanses could be crucial for maximising the monosaccharide concentration of any potential BSG derived feedstock. These can act to cleave the β-1,4 glycosidic links liberating free mannose monomers and manno-oligosaccharides.

1.9.3 Enzymatic based deconstruction of starch

There is the possibility of the presence of some residual starch (starchy endosperm fragments) in BSG present due to sub-optimal mashing conditions during the brewing process. As such this warrants the inclusion of a brief overview of the hydrolytic enzymes involved in starch depolymerisation (Gupta et al., 2010, Forssell et al., 2008, Mussatto et al., 2006). Starch is a mixture of amylose (a linear polymer of α-1,4-D glucopyranose units) and amylopectin (a branched heteropolymer consisting of α-1,4 & α-1,6-D-glucopyranose units). The main starch degrading enzymes can be sub-divided into various categories. Figure 1.14 depicts a schematic overview of the depolymerisation of starch.

1.9.3.1 α-amylase

Functions via endo hydrolysis of random glycosidic linkages in amylo based polymers of greater than 3 subunits (Gupta et al, 2010).

1.9.3.2 β-amylase

Catalyses the hydrolysis of maltose disaccharides from the non-reducing end of amylo oligosaccharides (Gupta et al., 2010). The main sources for β-amylase enzymes are from the seeds of higher plants (including barley) but the enzyme can also be produced by certain fungi and bacteria (Atia et al., 2003; Kunamneni et al., 2005).

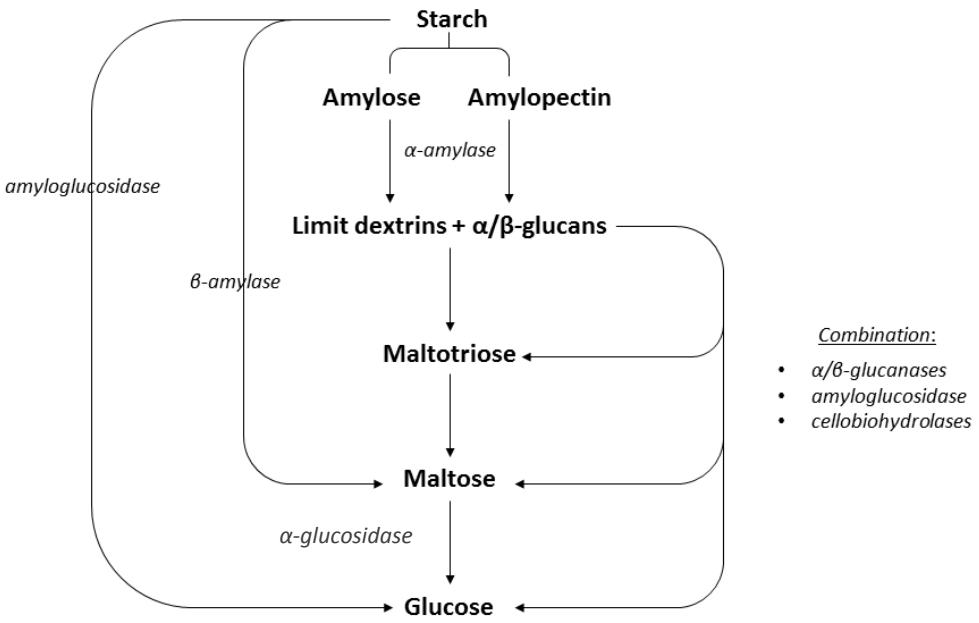
1.9.3.3 Amyloglucosidase

This functions via an exo-hydrolysis based mechanism, releasing glucose monosaccharides from the terminal end of malto-oligosaccharides and amylo-polysaccharides (Gupta et al, 2010).

1.9.3.4 α-glucosidase

Essentially an amyloglucosidase but more limited to activity on smaller oligosaccharides and polysaccharide fragments (Gupta et al., 2010).

Figure 1.13 Starch degradation (adapted from Gupta et al, 2010, Forssell et al, 2008, Mussatto et al, 2006, Briggs et al, 2004).



Addition of α & β amylase enzymes could be included in the enzymatic cocktail used to hydrolyse any starch present to generate maltotriose, maltose and glucose monomers. Limit dextrinase could also be added to cleave any dextrins (at the 1,6-α glycosidic links) produced by the α-amylase (Evans et al., 1994; Evans et al., 2003). This would create more free terminal ends for the β-amylase and amyloglucosidases to function more effectively. However the marginal improvement in liberated sugar concentrations due to the likely very small amount of starch found in BSG and the cost of the inclusion of such enzymes would make their commercial use unlikely.

1.9.4 Enzymatic based deconstruction of lignin

1.9.4.1 Ligninases

Ligninases act specifically on the lignin and are often made by higher fungi that grow in the wild on lignocellulosic substrates such as decaying plant biomass (Linko & Haapala, 1993).

These may use free radicals to attack the lignin structure, in forms such as manganese peroxidase, lignin peroxidase, laccase and other variants that use hydrogen peroxide as the reactive species. In the case of lignin peroxidase, the production relies on vetratryl alcohol (a secondary fungal metabolite) as an enzyme co-factor for production of the enzyme.

1.9.4.2 Ferulic & coumaric acid esterases

Esterases of this variety catalyse the release of free ferulic acid from feruloylated arabinoxylan-oligosaccharides (Forssell et al, 2008., Dodd & Cann., 2009). These enzymes employ a trio of amino acids (Serine-Histidine-Asparagine) via a method similar to that used by serine proteases (where peptide bonds in proteins are hydrolysed using a serine active site) such as the endopeptidase subtilisin. The 2 stages of this hydrolysis method are: initial acylation of the nucleophilic serine residue (donation of an electron pair) and then finally deacylation with water acting as the nucleophile. The breakage of these ferulic and di-ferulic bridges also then results in the presence of free arabinoxylo-oligosaccharides as the linkages are separated, rendering the inclusion of a FAE enzyme in any BSG hydrolysis enzyme cocktail highly desirable (Forssell et al., 2008).

1.9.5 Commercial enzyme mixtures currently available

Various commercial enzyme preparations are available for use on lignocellulosic biomass such as BSG. Such companies include; Novozymes, Genencor (DuPont), DSM and Verenium. Many of these have been microbially produced using fungal species such as *Aspergillus niger* and *Trichoderma reesei* effectively as bio-factories (Chundawat et al., 2011; Gírio et al., 2010; Nieves et al., 1997). Both these species are naturally occurring biodegrading fungal organisms that may have been genetically modified to enhance the transcription and translation of lignocellulosic deconstructive enzymes. *A.niger* is suggested to only secrete β-glucosidase whilst *T.reesei* secretes many different cellulases and has an

overall much more complex catalytic arsenal. These include cellobiohydrolases, β-glucosidases and endoglucanases. *T.reesei* also produces hydrolytic hemicellulose degrading enzymes including endo-1,4 β xylanases, mannanases, α-galactosidases, acetyl xylan esterases and arabinofuranosidases.

1.9.5.1 Enzymatic saccharification efficiency factors

Most enzyme systems are subject to various factors that affect the kinetics of the process of saccharification. These include reaction parameters such as vessel temperature, pH, and subjectivity to inhibition by various means (Chundawat et al, 2011, Dodd & Cann, 2009). Enzymatic inhibition has two main variants: competitive and non-competitive (Dodd & Cann., 2009). Competitive inhibition is described as when a molecule binds to the active site or an allosteric site on the enzyme that prevents the target substrate successfully binding with the enzyme (usually through modification of the active site itself). Alternatively non-competitive inhibition is the reduction of the activity of the enzyme (even with successful binding of the enzyme and the target substrate) caused by the interaction of another molecule (Dodd & Cann., 2009). Endo and exo-glucanases are susceptible to non-competitive inhibition by soluble cello-oligosaccharides, such as cellobiose and glucose. β-glucosidase appears to be exclusively inhibited through competitive inhibition, but this observation is based on when using a purely cellulose substrate (with no hemicellulose or lignin present) so this may not accurately reflect its activity on lignocellulosic substrates.

The solids loading (the solid to liquid ratio) at which the enzyme step is conducted is very likely to play a key role in the final yields achieved (Chundawat et al., 2011). The desire to conduct this stage at high solids loading (>10% w/v) in order to achieve high sugar yields and increase the quantity of biomass that can be processed per unit time must be balanced with the likely decreased effectiveness of the enzyme that is likely due to mass transfer limitations

and enzyme mobility issues (excessively viscous slurries will limit the ability of the enzyme to 'find' all the substrate). Improved mixing regimes and new technology (such as ultrasound assisted methodologies; Bennett, 2013) may be needed to assist in boosting the efficiency of this crucial step. Due to the rate limiting factors that affect enzymatic hydrolysis of the BSG polysaccharides (such as inhibition of some type) a sequential batch addition process (batch-fed biomass addition) may give the most efficient results. Optimisation of the addition rate of new substrate and product removal could be conducted to maximise efficiency and minimise any product inhibition.

Overall the enzymatic saccharification stage is highly complex with many factors affecting the efficiency of conversion (sugar release) of cellulose (Chundawat et al., 2011). It is likely that a significant amount of optimisation based experimentation would be required to tailor each specific process and achieve acceptable sugar yields.

1.10 Fermentation of any liberated sugars present in feedstocks

1.10.1 Yeast candidates: *Saccharomyces cerevisiae*:

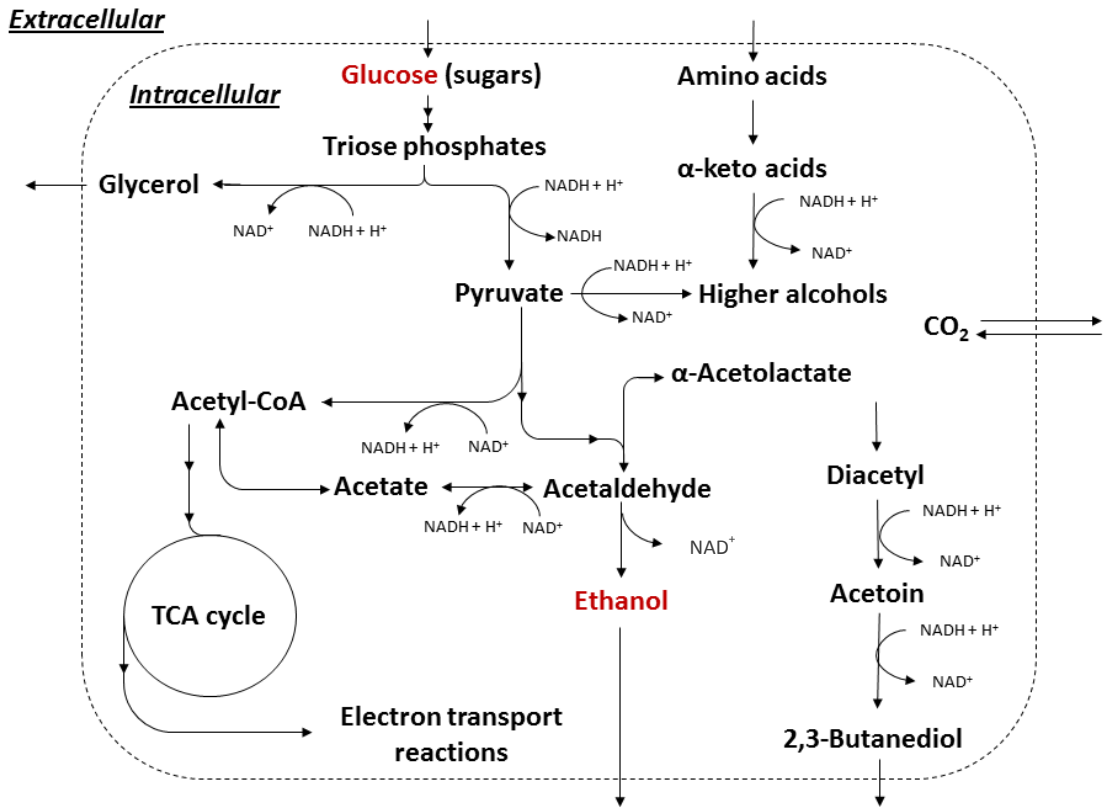
Saccharomyces cerevisiae is most likely the preferred fermentative species due to its high ethanol tolerance and excellent fermentation rate of C6 hexose sugars (Jeffries, 2006; Palmqvist & Hahn-Hagerdal, 2000a; Palmqvist & Hahn-Hägerdal, 2000b). Whilst it is not exclusively the only fermentation option, it will be discussed here as a benchmark species most likely to be used for bioethanol production.

As with many organisms metabolic energy in yeasts such as *S.cerevisiae* is conserved via high energy phosphate bonds in molecules such as adenosine triphosphate or ATP (Briggs et al, 2004). The reducing power of these systems is transferred via pyridine dinucleotide coenzymes such as nicotinamide adenine dinucleotide (NAD^+) and nicotinamide adenine dinucleotide phosphate ($NADP^+$). These reactions involve the balance of redox potential

within the cell which must be maintained at all times. The coenzyme reactions involve the transfer of two hydrogen ions from the substrate, one of which is transferred to the coenzyme to produce NADH which is then free to do any reduction reaction with the substrate (e.g. sugar). During fermentative metabolism the oxidative metabolic pathway is not operational and NAD^+ is regenerated by the reduction of acetaldehyde to ethanol (the target product in BSG bioethanol production; Figure 1.15). The key to successful biofuel production is in keeping the fermentative species producing ethanol and not reverting to oxidative biomass generative metabolism or diauxic shift that may oxidise any ethanol produced (in the absence of another carbon source).

The carbon end points (products) during fermentative growth of yeasts include ethanol, glycerol, lactic acid, acetic acid and carbon dioxide, indicating that 100% total efficiency of conversion to ethanol cannot ever be realistically achieved (Briggs et al, 2004). A proportion of the produced carbon dioxide is fixed in various carboxylation reactions and therefore 'recycled'. Glycerol is produced in response to osmotic stress and is essential for protecting the yeast cells. Glycerol production (via reduction by the glycerol-3-phosphate dehydrogenase enzyme) also regenerates excess NAD^+ (produced from biomass generation metabolism) to maintain redox balance. But other carbon end points are not recycled or essential and therefore in the interest of maximising the efficiency of the BSG derived biofuel, fermentation optimisation would need to be conducted to maximise the formation of ethanol as the carbon end point and not to other metabolites. Figure 1.15 depicts a schematic overview of a yeast based anaerobic fermentation.

Figure 1.14 Simplified overview of *S.cerevisiae* anaerobic fermentation of ethanol and some associated redox-balancing reactions using nicotinamide adenine dinucleotide (NAD) (adapted from Briggs et al, 2004, Palmqvist & Hahn-Hagerdal, 2000a).



1.10.2 Fermentative substrate utilisation capability of *S.cerevisiae*

Saccharomyces cerevisiae has the necessary genes to produce the required transport and metabolism proteins (enzymes) for anaerobic fermentation (to ethanol) of many starch based mono, di or tri-saccharides (Jeffries, 2006, Girio et al, 2010, Briggs et al, 2004). It has 19 separate genes encoding hexose sugar transport proteins (Jeffries, 2006). This clearly favours hexose sugar utilisation with fermentative yeasts. Sugar (such as glucose) uptake can occur freely by diffusion into the cell but mostly occurs using an active process of pumping against a concentration gradient but this is dependent on the specific sugar (Jeffries, 2006). Biofuel feedstocks with a complex mixture of sugars can result in a limitation of the rate of ethanol production as organisms need to activate different metabolic pathways for each different

substrate, hence a short lag phase may be observed at each transition point (Briggs et al., 2004; Jeffries, 2006). This would suggest that efficient bioethanol production would suit a feedstock in which the BSG polysaccharides had been almost completely hydrolysed to as small a number of different sugar fragments, (ideally monosaccharides) as possible (Jeffries, 2006). Yeasts have the ability to select which sugar to use first in a complex feedstock (Briggs et al., 2004; Jeffries, 2006). In traditional brewers wort glucose is utilised first then sucrose. In the specific case of sucrose, it is hydrolysed via an invertase enzyme that is produced in the periplasm and results in the subsequent glucose and fructose liberation (Briggs et al., 2004). The next sugar used is maltose (Briggs et al., 2004). When maltose levels drop to the undetectable level maltotriose is used (Briggs et al., 2004). Most *S.cerevisiae* yeast strains cannot metabolise amylo-oligosaccharides of greater than three subunits (Dp 3) except for a few strains that can utilise Dp4 oligosaccharides (Briggs et al., 2004). Alternatively it can only metabolise glucose (Dp 1) from all the potential saccharide fragments from cellulosic origin, not even cellobiose (Dp 2) or cellotriose and cellotetraose (Dp3, Dp4 respectively; Chundawat et al., 2011). This is due to the β-1,4 glycosidic bond that joins cello oligosaccharides compared to the α-1,4 glycosidic linkage that joins malto-oligosaccharides (of starch origin). This suggests any BSG pre-treatment and enzymatic strategy must completely hydrolyse the cellulose fraction down to its constituent smallest subunits of glucose. This requirement for total hydrolysis will increase the probability of generation of glucose based degradation products (inhibitors) such as HMF. Some lager producing *Saccharomyces* strains can utilise melibiose as they have the α-D-galactosidase enzyme that cleaves it into galactose and glucose (Briggs et al., 2004; Hilkka et al., 1991; Ostergaard et al., 2000). *S.cerevisiae diastaticus* can utilise various dextrins (higher Dp glucose based oligosaccharides) but in side reactions it simultaneously produces styrene and

4-vinyl guaicol which could decrease the ethanol yield should it be used for biofuel fermentations (Dequin, 2001; Laluce & Mattoon, 1984).

1.10.3 Bacterial alternatives to *S.cerevisiae*

Bacterial species such as *E.coli* can metabolise xylose and arabinose to ethanol, however their use has various shortfalls when compared to using yeast (Girio et al, 2010). These include a neutral pH operating range of pH 6-8., which renders any fermentation susceptible to contamination. Also *E.coli* has a relatively lower tolerance to any potential fermentation inhibitors (sugar and lignin degradation products) compared to *S.cerevisiae.* Also the end products are mixed (including acetic acid and lactic acid) and not exclusively ethanol (Gírio et al., 2010). *Zygomonas mobilis* is another bacterial option. This has a high ethanol yield and ethanol as the exclusive end product. As its glucose metabolism uses the Entner-Doudoroff (ED) pathway instead of the Embden-Meyerhoff-Parnas (EMP) pathway that *S.cerevisiae* and *E.coli* use, it only produces 1 ATP molecule per glucose molecule instead of the 2 ATP molecules from the EMP pathway. The consequence of this is that less cell mass (biomass) is produced which results in a higher specific ethanol productivity. But again *Z.mobilis* suffers from the same bacterial shortfalls as *E.coli,* with a narrow neutral pH range and low inhibitor tolerance in comparison with *S.cerevisiae*. Additionally *Z.mobilis* will only utilise glucose, therefore some degree of strain improvement is needed to increase substrate capacity. Pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) are the essential enzymes for conversion of EMP pathway derived pyruvate to ethanol (Girio et al, 2010). The genes for these enzymes could be taken from *Z.mobilis* and integrated into a single plasmid for insertion into *E.coli,* and then mutants selected that showed high ADH activity in order to enhance the potential ethanol production. However, this still does not overcome the difficulty of the low ethanol tolerance of *E.coli* which would limit the final ethanol yields achievable.

1.10.4 Nitrogen assimilation and biofuel feedstocks

The presence of some assimilable nitrogen in the biofuel fermentation feedstock is essential as it can limit the fermentation rate if low (Aliyu and Bala, 2010, Briggs et al, 2004). *S.cerevisiae* can utilise amino acids, small peptides, ammonium salts, urea, purines and pyrimidines (Briggs et al, 2004). Therefore hydrolysed BSG should provide any required nitrogen in theory. However as yeast do not produce proteases that are secreted (rather they remain intracellular) un-hydrolysed BSG protein would not suffice as it would likely be physically too large to enter the cell in its original format (Celus et al., 2009). This would require some sort of hydrolytic pre-treatment of the BSG or the inclusion of proteases in the enzymatic digestion cocktail used for saccharification (Forssell et al, 2008, Celus et al, 2009).

1.10.5 Fermentative metabolism of *S.cerevisiae*: C6 sugar utilisation but not C5

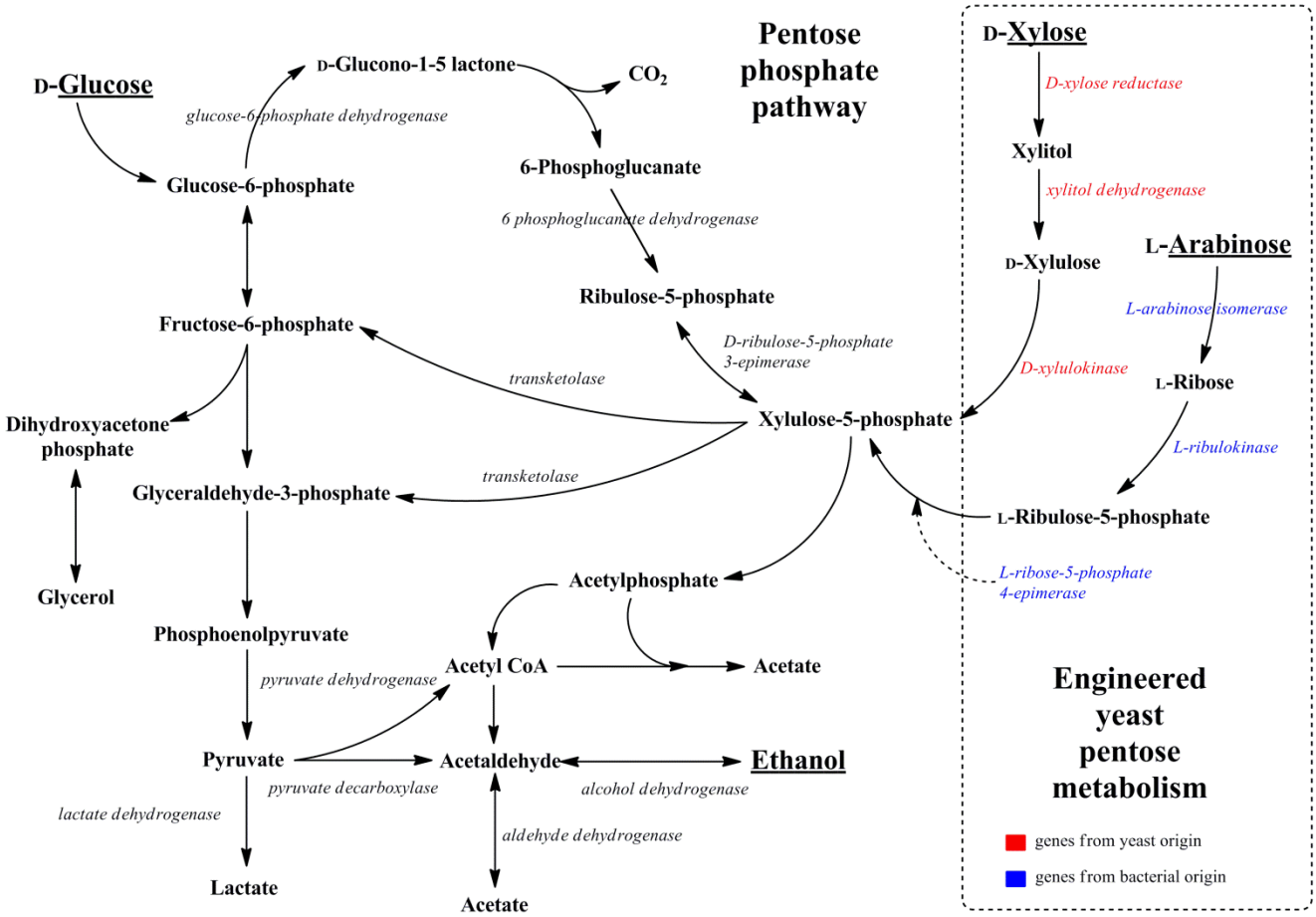
S.cerevisiae has all the necessary C5 pentose sugar transport and metabolism genes but does not choose to express them for an unknown reason, even in the presence of such sugars (Jeffries, 2006, Girio et al, 2010). It can transport pentose sugars such as xylose into the cell using its hexose transporters. However, these have a 2-fold lower affinity for pentose sugars than hexose sugars (Girio et al, 2010). It will only transport pentose sugars in the absence of glucose due to its high affinity for glucose. Gene cloning could be used to insert a higher affinity pentose transporter into *S.cerevisiae* (Jeffries, 2006). Ideally this would be a facilitator protein that worked by facilitated diffusion rather than proton symport as the latter requires more energy to function, rendering it less effective under anaerobic conditions where ATP production would be restricted.

C5 pentose sugar metabolism requires activity of the pentose phosphate pathway or PPP (Carvalheiro et al, 2008, Jeffries, 2006). The PPP has two basic phases: an oxidative phase and a non-oxidative phase.

Recombinant DNA technology has been used to insert into *S.cerevisiae* the genes that encode two essential enzymes in the PPP; xylose reductase and xylitol dehydrogenase from a pentose metabolising yeast such as *Scheffersomyces stipitis* (*Pichia stipitis*) in an attempt to encourage it to utilise xylose (Jeffries, 2006). However, this approach still results in poor yeast growth on media containing exclusively xylose. This is likely to be due to a disproportion of metabolites or cofactors. These result in an excess of NADH that cannot be recycled through respiration under the oxygen limited fermentation conditions that are present. Normally *S.cerevisiae* would respond by producing glycerol but in this case it cannot do so (Jeffries, 2006). This would result in a redox imbalance and adversely affect cellular metabolism. In the absence of some oxygen the yeast would not produce ethanol but rather xylitol in an attempt to restore the redox balance. This confirms the requirement of respiration for xylose metabolism in *S.cerevisiae*. Figure 1.16 illustrates an overview of the pentose phosphate pathway and also some optional exogenous genes that could be inserted to aid pentose sugar conversion to ethanol.

Both the yeasts *Scheffersomyces stipitis* and its anamorph, *Candida shehatae* can metabolise C5 pentose sugars but produce a limiting, inefficient amount of ethanol, typically less than 0.5 g ethanol per g sugar (Jeffries, 2006, Girio et al, 2010). Both species are also more sensitive to pH and the potential fermentation inhibitors (sugar and lignin degradation products) than *S.cerevisiae*.

Figure 1.15 Simplified pentose phosphate pathway with optional cloned genes from both bacterial and yeast origin to aid C5 sugar utilisation (adapted from Jeffries, 2006, Jeppsson et al, 2002, Walfridsson et al, 1995).



Unlike the anaerobically fermenting *S.cerevisiae*, both *C. shehatae* and *S. stipitis* species require the presence of some oxygen for C5 pentose utilisation which can promote respiration and oxidation of any ethanol produced (Gírio et al., 2010; Jeffries, 2006). This is a real issue for successful bioconversion of BSG to ethanol as the hemicellulose content is greater than the cellulose content therefore C5 pentose sugars will be more predominant than C6 hexoses. Successfully finding or producing a yeast strain that will both utilise C5 pentose sugars but also not incorporate them into biomass (rather anaerobically ferment to ethanol) is a major hurdle. Various techniques including random mutagenesis, accelerated evolution technology and recombinant DNA technology for gene cloning have all been used in various ways to

either overexpress certain C5 metabolism genes or block undesirable pathways through gene knock-outs (Girio et al, 2010). An example of a beneficial gene knock out would be to inhibit the production of xylitol, a common pathway especially in bacterial fermentative species and instead shift the metabolic flux towards ethanol production instead, therefore maximising conversion efficiency (Girio et al., 2010). However this can lead to problems as many genes encode multifunctional proteins that may have hidden fundamental roles in cellular metabolism and their absence may result in unstable mutants with traits such as very slow growth rates, rendering them commercially unusable (Girio et al., 2010).

Variations of genetically modified (GM) *S.cerevisiae* have been created with xylose reductase and xylitol dehydrogenase enzymes from *Pichia* species in an attempt to overcome the pentose sugar utilisation hurdle and enhance C5 sugar based metabolism. However these strains still only offer limited fermentation to ethanol capacity. Strain improvements that show overexpression of the pentose phosphate pathway genes (those responsible for xylose catabolism in *S.cerevisiae*) have also been attempted (Girio et al., 2010). Additionally some GM yeast strains have been produced with xylose isomerase genes of fungal origin (de Figueiredo Vilela et al., 2013; Zhou et al., 2012).

An option of fractionating the C5 pentose and C6 hexose sugars (with the C5 sugars in the hydrolysate from pre-treatment and the C6 sugars in the remaining solid residue) and then using separate fermentation vessels could be considered should a single species not present itself capable of metabolising both substrates. Alternatively a multi-strain, single bioreactor model could be developed and potentially optimised for both C5 and C6 co-fermentation. The holy grail in 'green' efficient processing would be the incorporation of more species, possibly fungal, such as *A.niger* or *T.reesei* into the same single C5/C6 bioreactor, to function as the lignocellulosic deconstructive instruments, effectively pre-treating the BSG with lignocellulosic and carbohydrate depolymerising enzymes (and not harsh industrial physico-

chemical pre-treatments) generating the feedstock for the fermentations, similar almost to a saké fermentation (Steinkraus, 1997). The bioreactor would function like an ecosystem in a continuous flow operation with continuous waste product removal and new substrate addition to limit any feedback inhibition issues. Xiros and Christakopoulos (2009) investigated a single step, consolidated system of ethanol production using the mesophilic fungus *Fusarium oxysporum*. The fungus attempts both lignocellulosic deconstruction and also the fermentation step to produce the ethanol through what is termed consolidated bioprocessing (CBP). *F.oxysporum* was able to ferment both glucose and xylose over 96 h and produce ethanol but only to 44% of the theoretical maximum yield based upon the sugar concentration present. Also no arabinose utilisation was observed at all. Glucose was utilised first and then subsequently xylose. Effective xylose utilisation required a small amount of aeration to address the redox imbalance associated with xylo-conversion to ethanol, and xylose conversion only occurs when glucose levels are sufficiently low as it may interfere with xylose transport into the cell. However, BSG was not the exclusive substrate as the feedstock was a combination of corn cob and BSG, to encourage lignocellulolytic enzyme production. Further work would need to be conducted to configure the process to operate using purely BSG. Also the fungus was not able to produce any greater ethanol concentration when the sugar concentration was higher than 6% w/v. This is very low compared to other fermentative species such as *S.cerevisiae* that can utilise sugar concentrations in excess of 20% w/v, and reaching ethanol concentrations over 20% v/v (Girio et al., 2010). Also the use of NaOH based alkali pre-treatment of the BSG increased the subsequent maximum ethanol yield to 60% of theoretical maximum, suggesting that initial BSG hydrolysis is a significant rate limiting step in the process. This further confirms the highly complex and recalcitrant nature of the lignocellulosic material found in BSG. Other than the reported activity of certain GM variants of a *Geobacillus* strain of bacteria developed by TMO renewables (discussed in more

details in section 1.12.3) it also suggests that currently no one single organism has all the capabilities for commercially efficient CBP based ethanol production (Girio et al., 2010).

Shindo and Tachibana (2006) describe a simultaneous co-fermentation using the two yeasts *S.cerevisiae* (fermenting the C6 sugars) and *Yamadazyma stipitis* (fermenting both C5 and C6 sugars but predominantly C5). Carbohydrase enzymes were simultaneously added to the feedstock with the yeast and produced an initial sugar concentration of 10% (w/v) that was fermented to a final ethanol concentration of 16 g/L after 4 days at 30°C. The co-fermentation reached a higher final ethanol concentration than either species did alone on the same feedstock. Also catabolite repression did not occur during the co-fermentation as it did with the *Y. stipitis* exclusive fermentation. Repeated batch fermentations were conducted using both yeast species immobilised on glass beads and then washed prior to the start of each batch.

Continuing on this theme the use of recombinant DNA technology could also be used to attempt the expression of non-native lignocellulolytic enzymes (such as xylanases and cellulases from *A.niger* or *T.reesei*) in *S.cerevisiae*, with it functioning as a single complete system (again a consolidated bioprocessing approach) degrading the lignocellulosic material in addition to fermenting it (Girio et al., 2010). However this could result in an excessive metabolic burden and decrease the overall production of ethanol. Therefore it could be a more sensible strategy to simply utilise *S.cerevisiae* as the specialist fermenter that it is and use another species for the hydrolysis of BSG (for the saccharification step).

1.11 Fermentation inhibitors: modes of action

Each of the fermentation inhibitors (sugar and lignin degradation products) that can be potentially generated during excessive pre-treatment reactions involving BSG function in

slightly different ways and as such each will be discussed individually (Palmqvist & Hahn-Hagerdal, 2000a).

The small organic acids such as formic and acetic acid, exhibit toxicity through freely entering the cytoplasm of the cell due to their physically small size (Palmqvist & Hahn-Hagerdal, 2000a; Palmqvist & Hahn-Hägerdal, 2000b). The toxicity is a function of the drop in cytosolic pH (known as the anion accumulation theory) where the dissociated acid is caught within the cell which results in more influx of un-dissociated acid. This further lowers the pH which decreases metabolic activity. In the case of acetic acid alone, at low concentrations (<100 mM) there appears to be a fermentative advantage to its presence (Linde et al., 2008; Palmqvist & Hahn-Hagerdal, 2000a; Palmqvist & Hahn-Hägerdal, 2000b). Ethanol yields are higher in the presence of small amounts of acetic acid, and the final ethanol titre is also reached sooner, increasing fermentation efficiency (Palmqvist & Hahn-Hagerdal, 2000a; Palmqvist & Hahn-Hägerdal, 2000b; Sanchez & Cardona, 2008). This could be due to efforts being made to actively pump the hydrogen ions out of the cell via a plasma membrane ATPase. This function requires energy (ATP) to operate. Therefore the increased energy demand encourages the fermentative metabolism even more to generate the required ATP, therefore generating more ethanol in the process as a by-product. However, excessive ATP expenditure (to expel H^+ ions) will eventually inhibit yeast growth. Attempts to buffer the pH of the hydrolysate could be used to overcome the inhibitory effect of these small organic acids. However hydrolysates are often a complex mixture of different inhibitors.

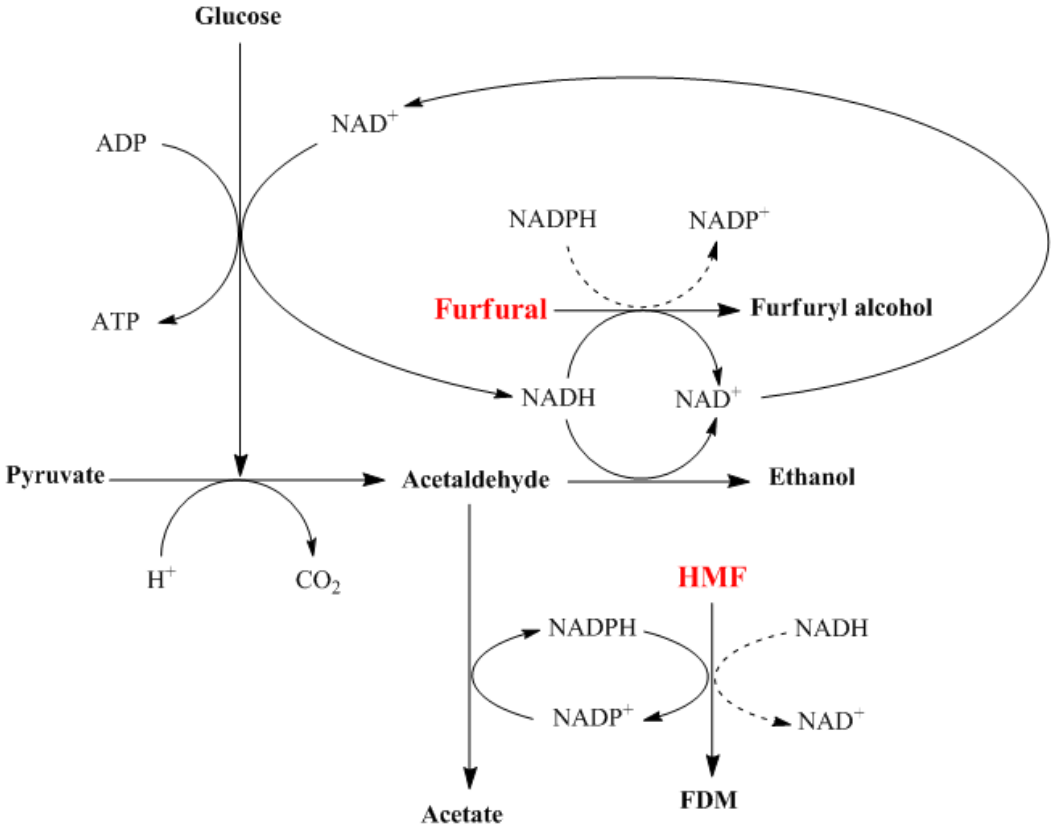
1.11.1 Furan derivatives

Furfural interferes with electron transport and disrupts fundamental glycolytic pathway activity in fermentative species such as *S.cerevisiae* (Palmqvist & Hahn-Hagerdahl, 2000a, Palmqvist & Hahn-Hagerdahl, 2000b, Heer & Sauer, 2008, Liu, 2006, Allen et al, 2010). The

presence of furfural affects the NADH dependant enzyme alcohol dehydrogenase (ADH) which reduces furfural to furfural alcohol (Liu, 2006). The reduction affects the redox balance of the cell and inhibits the production of glycerol (Liu, 2006; Wang et al., 2001). This is also why the presence of low levels of furfural may increase overall ethanol production as less carbon is diverted into by-product formation (glycerol). However, the cell cannot survive indefinitely without glycerol therefore confirming high furfural concentrations are likely to be toxic (Liu, 2006). In the presence of furfural, a state of competition for reduction then exists between the ADH enzyme and the glycerol-3-phosphate enzyme (reducing furfural to furfural alcohol and dihydroxy-acetone-phosphate to glycerol respectively). This leads to overall lower intracellular NADH levels (and low ATP levels). Higher furfural concentrations are likely to decrease the final ethanol yield as there is also substrate competition for the ADH enzyme. This is because in addition to reducing furfural to furfural alcohol, ADH also reduces acetaldehyde to ethanol, the primary fermentative mechanism for ethanol production (Briggs et al, 2004, Liu, 2006). Therefore the presence of significant furfural concentrations inhibits the ability of ADH to produce ethanol. The result is significant amounts of acetaldehyde need to be excreted (as acetaldehyde is also metabolically toxic to the yeast cell at higher concentrations). Furfural may also be oxidised by the enzyme aldehyde dehydrogenase (ALDH) to another fermentative inhibitor, furoic acid (Liu, 2006). This enzyme is also responsible for oxidising acetaldehyde to acetate (acetic acid) therefore further inhibiting the cellular ability to detoxify acetaldehyde. Furfural also affects mitochondrial membranes in some way which results in the accumulation of highly reactive oxygen species that cause cellular damage (Allen et al, 2010). Lag phase cells are more sensitive to furfural as they cannot repair any resulting damage through the normal macromolecule synthesis mechanism (Heer & Sauer, 2008). Pyruvate dehydrogenase may also be inhibited by both furfural and HMF (Liu, 2006).

There is very little experimental data on the mechanism of fermentative inhibition by HMF (Liu, 2006). Due to the similarity of HMF in structure to furfural, with a furan ring and aldehyde group, it is likely that the inhibitory mechanisms could be similar, essentially upsetting the redox balance of the cell. HMF is 'detoxified' within the cell by conversion to 2,5-bis-hydroxymethyl furan (Liu, 2006). However, in contrast to furfural reduction (that uses NADH) HMF requires a different coenzyme for reduction, NADPH (Figure 1.17). NADPH is required for biomass production (amino acid and nucleotide synthesis) and the presence of high concentrations of HMF could out compete for the reducing capacity of NADPH (Palmqvist & Hahn-Hagerdal, 2000a; Palmqvist & Hahn-Hägerdal, 2000b). This substrate dominance at higher concentrations could explain the observed metabolic inhibition (Liu, 2006). Figure 1.17 illustrates a schematic overview of the simplified conversion pathways for furan based compounds.

Figure 1.16 Conversion pathways for furan inhibitors (adapted from Liu, 2006, Palmqvist & Hahn-Hagerdahl, 2000b).



1.11.2 Lignin derived phenolic inhibitors

These phenolic compounds tend to be lipo-soluble acids which can freely enter the cell when undissociated (Heer & Sauer, 2008, Palmqvist et al, 1999). Upon entering the neutral pH cytoplasm they subsequently dissociate and their toxicity can be a function of the resulting pH crash which likely inactivates various essential cellular processes (Palmqvist & Hahn-Hagerdahl, 2000a, Palmqvist & Hahn-Hagerdahl, 2000b). The aromatic nature of these phenolic inhibitors also causes pH related toxicity similar to that of furan based inhibitors but through a different mechanism (Palmqvist & Hahn-Hagerdahl, 2000a). They are effectively membrane toxic, causing membrane swelling which leads to increased membrane fluidity and allowing an uncontrolled proton influx (and subsequent further pH crash). Their amphipathic molecular nature is very similar to that of membrane phospholipids hence their ability to easily infiltrate cell membranes. In comparison with non-phenolic inhibitors they are more toxic to most fermentative species.

As the majority of fermentation inhibitors produced from the pre-treatment stage are often confined to the liquid hydrolysate (such as the furan derivatives and a significant proportion of the phenolics) it is possible that this fraction could be discarded and the cellulosic fraction alone enzymatically hydrolysed and fermented. This would also overcome the difficulty of C5 pentose fermentative metabolism using *S.cerevisiae*. However this would reduce the overall process efficiency as a significant proportion of the total theoretical sugar yield would not be utilised. Therefore a viable alternative end use of the C5 pentose sugar fraction needs to be sought in order to improve the life cycle assessment and increase the viability of this option.

1.12 Optimising fermentations

1.12.1 Overview

Many pre-treatment techniques (with the exception of AFEX) strip potential fermentation nutrients (such as minerals and proteins) away from lignocellulosic material (Chundawat et al, 2011). This could result in the need for supplemental nutrient addition to ensure adequate microbial growth but would likely impact the overall process efficiency and economics. The pH and buffering capacity of any BSG derived feedstock would need to be investigated to ensure suitability for the chosen fermenting species. At the usual pH for yeast fermentations (pH 4-5) the presence of any acetic acid in the feedstock generated from the pre-treatment process will be un-dissociated and therefore free to diffuse into the cell and impart its inhibitory effect (section 1.11; Palmqvist & Hahn-Hagerdahl., 2000a, Palmqvist & Hahn-Hagerdahl., 2000b White et al., 2008). Therefore a higher pH feedstock (>pH 5.5) would minimise any acetic acid based inhibitory effect.

One optimisation strategy could be the investigation of simultaneous saccharification and fermentation (SSF). This involves a continuous flow mechanism instead of a batch process system where sugar is continually removed as it is produced by the enzymes acting upon the pre-treated lignocellulosic material by the fermenting species thus reducing any end product inhibition of the enzymes (Girio et al., 2010). This decrease in inhibition could lead to the use of lower enzyme loading and the use of a single bioreactor would reduce production costs and reduce the space required (reduce the required footprint). Overall this would improve the process economics considerably. The disadvantages of SSF are that re-use of the microbes is almost impossible as the separation from lignin is highly difficult. Also any enzymatic hydrolysis would need to be conducted at a lower temperature than may be optimal due to the presence of the microbe. This could decrease the enzymatic hydrolysis (saccharification)

efficiency. Also it assumes a species can be found that can utilise both C6 and C5 sugars and tolerate any inhibitors that may be present.

1.12.2 Microbial strain selection and development for biofuel fermentations

Genetically modifying fermentative species such as *S.cerevisiae* to achieve greater tolerance by overexpression of various genes is one optimisation strategy. Overexpression of alcohol dehydrogenases or aldo-keto-reductases in an attempt to enhance the chemical reduction therefore detoxification of any furan based fermentation inhibitors that may be present in the feedstock to their corresponding less toxic alcohols (furfural alcohol and 2,5-bis-hydroxymethyl furan respectively) would be one strategy (Heer & Sauer., 2008, Liu., 2006). However due to both the main furan based inhibitors (furfural and HMF) potentially having different cellular mechanisms, any strategy for increased tolerance would need to be specifically targeted at one or the other compound depending on which was deemed most problematic. The same is true for the lignin based phenolic inhibitors. With exposure to furfural or HMF at up to 30mM, ethanologenic yeast will display an extended initial lag phase, with no glucose utilisation or ethanol production during this period (Liu., 2006). This is thought to represent a change in gene expression and the subsequent physiological shift in reaction to the chemical stress the yeast is exposed to. After the initial extended lag phase has been overcome the yeast then cease to be inhibited even when re-dosed with a similar concentration of either furan. This suggests further tolerance could be developed by accelerated evolution technology or via genetic modification. Depending on the specific pre-treatment strategy used for BSG, even if using an optimised protocol, the feedstock could still have numerous fermentative inhibitors present. Even at sub-lethal concentrations of each compound individually, the inhibitors may have synergistic toxic effects when present together. This would require a multi-resistant strain to be developed. Integrated gene microarray technology could be used to track changes in the transcriptome in profile response

to complex mixtures of different inhibitors. Liu (2006) found that within 10 min of exposure to HMF, several hundred *S.cerevisiae* genes had significantly different expression levels compared to controls, confirming the dynamic nature of chemical stress tolerance in fermentative yeasts.

1.12.3 TMO Renewables: current state of the art technology and processing

TMO claim to have a fully operational lignocellulosic conversion to ethanol demonstration facility utilising a number of different feedstock's including BSG (Atkinson et al., 2006). The current bacterial species used (codenamed TMO 242) can allegedly metabolise C3, C5, C6 sugars and various oligosaccharides including cellobiose, xylobiose and starch. It has been tested on generic xylan material obtained from Sigma-Aldrich which had been treated with a cocktail of hemicellulases to liberate monosaccharides, disaccharides, trisaccharides and oligosaccharides. The sugar utilisation was tracked and confirmed usage of both C5 and C6 monosaccharides and also some ethanol was produced. The lignocellulosic feedstock's are initially pre-treated using high temperature steam and then subjected to a cocktail of exogenous enzymes (in combination with some enzymes actually secreted by the organism itself) to liberate the feedstock sugars. The species they began modifying was a gram positive thermophillic soil bacteria *Geobacillus thermoglucosidius* (Atkinson et al., 2013a). The thermophillic nature of the bacteria allows fermentations to be run at temperatures in excess of 50°C (and often up to 70°C) allowing very fast fermentation rates (and therefore greater overall efficiency as the final ethanol titre can be reached much sooner than a conventional yeast based fermentation). Another advantage is that at temperatures above 50°C ethanol can be removed as vapour (therefore reducing the inhibitory effect on fermentation of high ethanol concentrations and facilitating an easier end product separation). The high temperatures and rapid fermentation rates also allow very high sugar concentrations to be used and results in a bioreactor design that can be considerably smaller in footprint (and

therefore less expensive to produce) than a more traditional temperature fermenter and still achieve the same productivity. The use of very high fermentation temperatures also has another major advantage. This is due to TMO not operating under sterile conditions (and also with the lignocellulosic feedstock's entering the facility potentially contaminated with their own native microorganisms) which would normally present a very high risk of fermentation contamination. However the high fermentation temperatures do not allow many species other than TMO 242 to thrive or even survive. As previously mentioned TMO 242 also secretes some of its own native hydrolytic saccharification enzymes, producing oligomeric sugars (often Dp 2-4) of which it can intake and ferment to ethanol. The precise details of exactly which specific enzymes TMO 242 secretes and on what specific substrates they function on are currently commercial secrets.

1.12.3.1 Bacterial metabolic summary:

TMO had summarised that essentially bacterial metabolism converted glucose to pyruvate (and in doing so generated ATP; Atkinson et al, 2006). What happens to the pyruvate afterwards depended on the species and the specific environmental conditions but there were three main pathway options:

- Under aerobic conditions: use of the citric acid cycle (pyruvate converted to acetyl coenzyme A) via the enzyme pyruvate dehydrogenase (PDH)
- Under anaerobic conditions: pyruvate converted to acetaldehyde via pyruvate decarboxylase (PDC) and then onto form ethanol via alcohol dehydrogenase (ADH)
- Pyruvate converted to lactate via lactate dehydrogenase (LDH)

Many strategies have been employed by TMO to enhance the wild type strain of *G. thermoglucosidius* including recombination with heterologous PDC and ADH genes to unlock the metabolic pathway of ethanol production (Atkinson et al, 2006). Up-regulation of

PDH genes to produce more acetyl coenzyme A was another strategy as acetyl coenzyme A can be subsequently converted to ethanol (Atkinson et al, 2012). Other novel approaches that have been attempted are the production of sporulation deficient strains (Atkinson et al., 2013b) and insertion of extra genes for improved amylase function and over expression of native amylase encoding genes to enhance starch based metabolism (Atkinson et al, 2012).

1.12.3.2 ***Sporulation deficient thermophillic fermentation species****:*

Sporulation is when growing cells are triggered to become metabolically dormant in order to enhance their dispersal and/or for survival for extended periods in unfavourable environmental conditions (Atkinson et al., 2013b). Sporulation pauses active metabolism. Therefore should the initiation of sporulation occur during fermentation (in response to rising ethanol concentrations for instance) then no more ethanol would be produced. The primary regulator for sporulation is a DNA binding protein *spoOA* (stage O sporulation protein A). This is a response regulator derivative of a transcription factor. Therefore the inactivation of the gene for *spoOA* via insertion of plasmid DNA into chromosomal DNA produces sporulation deficient mutants.

1.12.3.3 *LDH gene knockouts:*

The lactate dehydrogenase gene (LDH) knock out prevents lactate production from pyruvate and under the right environmental conditions actually encourages ethanol production if PDC and ADH genes are also present (Atkinson et al, 2006, 2012).

1.12.3.4 *Up-regulation of PDH gene:*

The up-regulation of the PDH gene promotes the conversion of pyruvate to acetyl coenzyme A (Atkinson et al., 2012). Under suitable conditions acetyl coenzyme A can be used to produce acetaldehyde and subsequently ethanol. Up-regulating PDH levels also aids ethanol production indirectly as it reduces pyruvate levels as higher concentrations can inhibit

glucose uptake and inhibit glycolysis. Up-regulation of PDH appears to enhance the activity of PDC and therefore more acetaldehyde is produced than acetyl coenzyme A.

1.12.3.5 *Inactivation of the PFL gene:*

Pyruvate formate lyase (PFL) converts pyruvate to acetyl coenzyme A and formate under anaerobic conditions (Atkinson et al., 2012). Although acetyl coenzyme A can be converted to ethanol, the formate is an unwanted by-product as it inhibits fermentative metabolism. Therefore inactivation of the PFL gene would be highly desirable as it would leave only the PDH complex metabolic route to process any pyruvate resulting in a more favourable set of carbon end products.

1.12.3.6 *Enhanced Amylase activity:*

TMO have also developed two strategies of increased expression of native amylase genes and also insertion of additional heterologous amylase encoding genes (Atkinson et al, 2012). The increased expression was achieved by incorporation of strong upstream promoters into the genome. Both the promoters and additional amylase encoding genes were all obtained from *G.stearophilus* and again constructed plasmids were used as vectors for the transformation.

1.13 Life Cycle Assessment (LCA)

Any bioethanol production protocol or biorefinery (the process of fractionation of biomass to its constituent components for use as value added products or energy production) using BSG could use life cycle assessment (LCA) to determine the efficiency of the whole process. LCA is essentially 'cradle to the grave' analysis that incorporates every input and output and considers the effect that the existence of the product has on the environment (Cherubini, 2010; Ou et al., 2009). This includes energy input, material input (e.g. initial raw material or any chemical pre-treatment), distribution and transport costs of all components and any outputs that may be environmentally damaging or have an associated high energy cost of

disposal (Cherubini, 2010). Generally speaking biofuels are considered carbon neutral as their combustion releases the same amount of CO_2 as that was captured by the growing plant initially. However greenhouse gas (GHC) emissions can be higher than ideal during the production of the biofuel due to the use of fossil fuels for pre-treatment energy requirements. As BSG is an industrial biomass co-product none of the concerns about dedicated energy crops apply. There is no likely significant risk of reduction of carbon storage in carbon pools (resulting in soil nutrient depletion) with BSG as with the use of some agricultural crops or waste residues. The incorporation of LCA into biofuel generation such as conversion of BSG to bioethanol would assist in optimisation of all processes and give a true representative overview of exactly how green the whole process was. This would ensure the production of the biofuel does not consume more energy than it creates or generates excessive pollutant outputs negating any theoretical environmental benefits compared to traditional fossil fuels. In essence LCA could ensure the green credentials of biofuel are maintained. Overall preliminary studies indicate lignocellulosic biorefineries such as those using BSG could save up to 80% GHC emissions compared to traditional fossil fuels.

1.14 Conclusion

The chemical composition, low market value and abundance of BSG render biocatalytic conversion to a biofuel such as ethanol a highly attractive processing option. There are numerous options for pre-treatment of BSG to enhance enzymatic saccharification to liberate fermentable sugars. Currently no single pre-treatment ticks all the boxes concerning ‘green’ energy efficient technology and chemistry. Further optimisation and work is needed to incorporate energy and financial factors into the BSG deconstruction and fermentation model through LCA. The decision on which pre-treatment would likely be based around the scale of the processing plant desired as some technology would be harder to scale up economically than others. Advances in enzyme technology and production could increase the efficiency

and cost effectiveness of the fermentable sugar liberation step with ever aggressive products arriving on the market. Should the technology and science be developed to an economically viable point, the future of BSG could involve on-site processing and fermentation to biofuel ethanol at sites on or near to breweries solving the storage and transport cost issues associated with biofuel substrate acquisition. Breweries could recycle their own co-products to power other industrial applications within the traditional brewing process and therefore significantly reduce their CO_2 emissions whilst simultaneously reducing energy costs.

1.15 Aim of this research

For the purpose of this thesis a comprehensive and systematic evaluation of a wide range of different processing options for BSG were investigated. This was in an attempt to create a higher value use for the BSG than simply feeding it to cattle or burning it directly for a small quantity of energy. This research furthers the scientific understanding of the future options of what BSG can be used for in order to increase its value and to minimise any wastage of this often overlooked lignocellulosic biomass type. This research has also elucidated how much of the current lignocellulosic biomass science (such as that related to wheat straw, miscanthus and corn stover etc.) can be directly applied to BSG.

The primary focus of this research was to evaluate the feasibility of producing bioethanol from the lignocellulosic component found within BSG. This would require the deconstruction of the lignocellulosic matrix to allow for the depolymerisation of the complex carbohydrates (primarily cellulose) to allow the liberation of simple sugars (predominantly glucose) which could then be used as a feedstock for fermentation to ethanol.

As such the scope of this research project was extremely wide and involved working in many different fundamental areas and scientific disciplines due to the sheer complexity of the task of bioethanol production from lignocellulosic biomass.

Preliminary investigational work (Chapter 3) looked at the chemical composition of BSG as a starting point for tracking any changes that may have occurred as a result of any subsequent processing. Ultrastructural imaging was also conducted using scanning electron microscopy (SEM) to gain an insight into the heterogeneity of the material. Rudimentary dry sieving based attempts at fractionation of the protein component were also conducted. Finally direct attempts at enzymatic saccharification were conducted in an effort to avoid the usage of any energy or chemically intensive pre-treatment step beforehand.

The project then moved on to the evaluation and screening of thermo-chemical pre-treatment systems (the primary stage for the processing of lignocellulosic biomass when attempting to produce bioethanol). The large body of research in this particular area was conducted in order to establish which of the many available pre-treatment options were most suited to BSG in terms of enhancing the subsequent enzymatic saccharification yields (Chapter 4 –6). Efficacy of pre-treatment effectiveness was measured using glucose yields achieved as this was likely to be the primary carbon source used for ethanol production via fermentation using a yeast strain. All experimental work was conducted with the aim of minimising the amount of chemical reagent or thermal energy input that was required to still achieve high cellulose to glucose conversion rates. In addition attempts were made to push the functional solids loading (increase the solid to liquid ratio that the pre-treatment step could successfully operate at) in order to boost both the efficiency and the productivity of the process. Maximising the solids loading at each stage of the process would ultimately increase the amount of biomass (BSG) which could be processed per unit time and also aid the achievement of suitably high glucose concentration feedstocks. Producing high glucose concentration feedstocks would then allow suitably high ethanol concentrations to be produced from fermentations that may make distillation of the product economically viable. Dilute acid and alkali based hydrothermal protocols were investigated which screened a wide

range of potential pH dependant reagents at 121°C (Chapter 4). Following on from this further evaluation of the best performing caustic (alkaline) reagents which included NaOH and alkaline peroxidase (H_2O_2) at various temperatures were also investigated (Chapter 5). Finally autohydrolytical based protocols were evaluated which used a high temperature aqueous (water) based deconstruction mechanism in an attempt to avoid the usage of environmentally unfriendly chemical reagents (Chapter 6).

Subsequently experiments were conducted looking exclusively at the second major stage of bioethanol production; the enzymatic saccharification step (Chapter 7). Efforts were based around maximising the achieved glucose yields whilst attempting to minimise the dosage of these cellulolytic enzyme preparations as a cost control measure. Again effort was made to push the functional solids loading at which this step could be conducted to boost biomass processing and achieve suitably high glucose concentrations for subsequent fermentations. The effect of different mixing protocols and batch-fed biomass addition along with the inclusion of potentially beneficial supplementary enzymes and cofactors was also investigated in an effort to further boost glucose yields.

In contrast to the predominantly chemical engineered approach to production of bioethanol from BSG (conventional advanced generation biofuel production) various entirely biological approaches were investigated. These strategies employed a consortia of microorganisms in an attempt to produce ethanol without the need for any expensive and energy intensive pre-treatments or exogenous enzyme usage (Chapter 8). This consolidated bioprocessing (CBP) approach could yield a low cost albeit slower alternative strategy for deriving increased value uses for BSG. Various filamentous fungi species were partnered with various yeast strains and the subsequent ethanol production yields were quantified. Further investigation of the best performing permutation was also conducted in an attempt to further understand what was occurring in terms of both the carbon source utilisation (which components if any in BSG

were being biologically deconstructed to ultimately generate any ethanol produced) and also to attempt to decipher any symbiosis between the filamentous fungi and the yeast strain that may have been occurring.

Finally attempts were made to try and fractionate the considerable protein content of BSG to generate an additional value added product using both biocatalytic (enzyme based) systems and also using thermo-chemical (caustic reagent based) systems (Chapter 9). Attempts were then made to combine both the protein fractionation and the bioethanol production systems to create a biorefining system to maximise the usage of all the components in BSG.

Overall the wide range of research presented here provides a comprehensive dissection of processing options for BSG. This research represents a resource that can provide a platform for future, higher value uses for BSG when a possible paradigm shift in economics may render some of the options presented here more economically viable as the cost of the finite fossil fuel based energy sources (such as crude oil and shale gas) eventually increases as reserves diminish.

2 Materials and methods

2.1 Brewers Spent Grains (BSG)

Unless otherwise stated, samples of BSG were sourced from the SABMiller 10 hl Research Brewery (Sutton Bonington, UK) and originated from a high gravity brewing process (ca. 1060 OG) using 100% barley malt grist and lauter-tun based brewhouse technology (with a continuous sparging regime using 80°C hot water until a bright, clear wort had been observed in the sight glass). Additionally, unless otherwise stated all experiments were conducted using BSG that was dried overnight in an oven at 105°C to achieve a moisture content of less than 10% (w/w) and then ground to a particle size of less than 212 µm (ground using a conventional coffee grinder and then sieved to determine the maximum size of particles; discussed further in section 3.2.4) to ensure adequate homogeneity prior to all experimentation. Due to the logistical difficulties of obtaining, transporting and adequately storing (in order to avoid the rapid microbial spoilage and degradation etc.) the large quantities of BSG that would be needed for the research presented here within this thesis all subsequent experiments were conducted using BSG obtained in-house from the SABMiller pilot plant at Sutton Bonington. The initial grist used was 100% malt and from lauter-tun brewhouse technology.

2.2 Reagents

All reagents were of AnalaR grade and obtained from Sigma-Aldrich (UK) and Fisher Scientific (UK). All water used was deionised reverse osmosis and of ≥18 mega-ohm purity.

2.3 Compositional analysis techniques

2.3.1 Cellulose and hemicellulose quantification in BSG and other insoluble residues using a two-stage complete acid hydrolysis method

The total monosaccharide contents of materials were quantified following complete acid hydrolysis of samples using a modified and updated version of the assay described by Dubois et al (1956). Dried biomass (30 mg) was weighed into a heat resistant Pyrex reaction vessel (50 mL). To each tube was added 1 mL of 12 M $H_2SO_4$and the contents incubated at 37°C in a water bath for1 h. Water (11 mL) was added to dilute the acid concentration to 1 M and the contents further incubated at 100°C for 2 h. The resulting solutions were then syringe-filtered (GF/C 25 mm filter diameter/1.2 μm pore size, Whatman, USA) and the concentration of monosaccharides quantified by High-Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD; see section 2.9.1). The monosaccharide concentrations in the pre-treated BSG solid residues were used as a basis to calculate the percentage of theoretical sugar yields achieved following enzymatic saccharification. Cellulose and hemicellulose concentrations were calculated from glucose and xylose concentrations (respectively) after stoichiometric correction for dehydration using the following equations obtained from Sluiter & Sluiter, (2010):

$$0.9 = \text{cellulose anhydrous correction factor} = 1 - \frac{glucose\ MW\ (180) - water\ MW\ (18)}{glucose\ MW\ (180)}$$

$$0.89 = \text{hemicellulose anhydrous correction factor} = 1 - \frac{xylose\ MW\ (150) - water\ MW\ (18)}{xylose\ MW\ (150)}$$

Whilst the hemicellulose was not composed of purely xylose but rather a complex mixture of xylose, arabinose, glucose, galactose (and other sugars) this two stage acid hydrolysis and the associated calculations were shown to reliably quantify hemicellulose and provide a similar figure to gravimetric based assays for hemicellulose yet much faster and higher throughput.

2.3.2 Alternative gravimetric hemicellulose quantification method for comparison

A modified version of the protocol described by Sun and Tomkinson (2002) was used for gravimetric determination of hemicellulose concentration to confirm the validity of using the two stage complete acid hydrolysis method described in section 2.3.1. For this assay 1000 mg of BSG was weighed into a 40 mL centrifuge tube and 20 mL 4M KOH added. The sample was then gently agitated on a roller bed for 2 hours. Centrifugation was then performed at 5000 rpm for 5 min (Heraeus Megafuge 16, Thermo Scientific, UK). The supernatant was filtered using Buchner filtration apparatus and GMF filter paper (Whatman, USA). The filtrate was then collected and the pH adjusted to 5.5 using glacial acetic acid and then transferred into a centrifuge tube of known weight. 80% acetone solution (in water) was added at a ratio of 2:1 (80% acetone: filtrate) and centrifuged at 3500 rpm for 5 min. The supernatant was then discarded and the remaining pellet broken up with a spatula and subjected to two rounds of ethanol washes. The ethanol washes consisted of addition of 95% ethanol (ratio of 2 : 1, 95% ethanol: pellet volume), centrifugation at 3500 rpm for 10 min then the supernatant discarded. The pellet was then dried overnight in an oven (OV/100/F/DIG oven; Genlab, UK) at 45°C and the remaining residue considered to be hemicellulose and calculated gravimetrically (accurately weighed to 3 decimal places using an analytical balance; model AG204, Mettler–Toledo, Switzerland). All analyses were conducted in triplicate.

2.3.3 Acetyl bromide soluble lignin quantification in BSG and various insoluble residues using a spectrophotometric assay

Acetyl bromide soluble lignin (ABSL) was analysed in the starting BSG and the solid residues derived from various alkaline pre-treatments using a variant of the method described by Fukushima and Hatfield (2004). Biomass samples (100 mg) in individual tubes were mixed with 4 mL of 25% acetyl bromide in glacial acetic acid and incubated at 50°C for 2 h in a water bath. After allowing the solution to cool to approximately room temperature the volume was adjusted to 16 mL with glacial acetic acid and left to settle for 30 min. An aliquot (0.5 mL) of this solution was further diluted with 2.5 mL of glacial acetic acid, and 1.5 mL of 0.3 M NaOH and the contents mixed by hand agitation. Aliquots of 0.5 M hydroxylamine hydrochloride (0.5 mL) and glacial acetic acid (5 mL) were then added and the absorbance of the resulting mixture measured at 280 nm (7315spectrophotometer, Jenway, UK). ABSL concentrations were determined based on an extinction coefficient measured at 280 nm using a range of authentic lignin standards (alkali lignin, low sulfonate content, Sigma–Aldrich, UK).

2.3.4 Protein quantification in BSG and various insoluble residues using a flash nitrogen (Dumas) analyser

A Thermo Flash Nitrogen Analyser (ThermoFisher Scientific, Waltham, Massachusetts, USA) was used to determine protein content. 50 mg of dried sample was sealed in a tin capsule and inserted into a combustion chamber (heated to 900°C and included an oxidation catalyst). Oxygen was added to the helium carrier, which facilitated combustion of the sample at ca. 1800°C. The combustion gases were then passed into the reduction reactor (heated to 680°C and containing copper) where nitrogen oxides were converted to elemental nitrogen. Carbon dioxide, sulphur dioxide and water were removed via filters of soda lime, magnesium perchlorate and a molecular sieve. Finally the helium stream passed the nitrogen

through a nitrogen separation column (50°C) and into a thermal conductivity detector. Analysis was achieved using the Eager 300 software with an L-aspartic acid sample for calibration. Protein was determined using the N x 6.25 conversion factor. All analyses were conducted in triplicate.

2.3.5 Starch quantification in BSG using a glucose oxidase/peroxidase kit

Starch was quantified using a glucose oxidase/peroxidase kit (GAGO-20, Sigma-Aldrich) according to the manufacturer's instructions. The kit is for the quantitative, enzymatic based determination of glucose in biomass. After enzymatic hydrolysis the liberated glucose is first oxidized to gluconic acid and then hydrogen peroxide by glucose oxidase. The hydrogen peroxide then reacts with *o*-dianisidine in the presence of the peroxidase enzyme to form a coloured product. The oxidized *o*-dianisidine then reacts with sulfuric acid to form a more stable coloured product. The intensity of the colour is then measured at 540 nm using a spectrophotometer and is proportional to the original glucose concentration. All analyses were conducted in triplicate.

2.3.6 Ash quantification of BSG using a muffle furnace

Ash content was determined according the method proposed by Santos (2003) however due to constraints imposed by the available equipment a lower ashing temperature was used However this was not considered to have any impact on the validity of the results. 1.0 g of BSG was weighed into crucibles of known weight which were then placed into a muffle furnace at 580°C for 24 hours. After ashing the samples were placed directly into a desiccator for 30 minutes to prevent the hygroscopic sample and crucible drawing moisture from the environment. The sample was then accurately weighed to 3 decimal places using an analytical balance (model AG204, Mettler–Toledo, Switzerland). All analyses were conducted in triplicate.

2.3.7 Total solvent extractable lipid quantification

Total lipids were quantified using a modified method based upon Cequier-Sanchez et al (2008). 400 mg of BSG was weighed into a Pyrex reaction vessel (50 mL). 12 mL of a dichloromethane: methanol mixture (2:1, v/v ratio) was added and then gently agitated for 2 hours using a roller bed. The sample was then syringe filtered (GF/C 25 mm filter diameter/1.2 µm pore size, Whatman, USA) into a centrifuge tube of known weight to remove the unwanted solids. 2.5 mL KCl solution (0.88% w/v; chilled to approx. 4°C) was then added to the filtrate which was then vigorously agitated by hand shaking for 5 min. Centrifugation (Heraeus Megafuge 16, Thermo Scientific, UK) was then performed at 1500 rpm for 5 min. The aqueous upper phase was then removed and the lower organic phase was then dried overnight at 40°C (OV/100/F/DIG oven Genlab, UK). The total solvent extractable lipid was then quantified by gravimetric analysis. All analyses were conducted in triplicate.

2.3.8 Determination of the moisture content of BSG

The moisture content of 'dried' BSG was determined (after drying in the oven overnight at 105°C) using a HR83 Moisture Analyzer (Mettler Toledo, UK) using a temperature of 105°C until a constant weight was achieved. All analyses were conducted in triplicate.

2.3.9 Multi element analysis of biomass by Inductively Coupled Plasma Mass Spectrometry (ICPMS).

Multi-element analysis was conducted on a range of recovered residues. The analysis was conducted using ICP-MS (Thermo-Fisher iCAP-Q) with a 'Flatopole collision cell' (charged with helium gas) upstream of the analytical quadrupole to reduce polyatomic interferences. Internal standards were introduced to the sample stream via a T-piece and included Sc (50 µg L-1), Ge (20 µg L-1) Rh (10 µg L-1) and Ir (5 µg L-1) in the preferred matrix of 2% HNO3. External calibration standards were all in the range 0 – 100 µg L-1 (ppb). Samples were introduced via a covered autosampler (Cetac ASX-520) through a concentric glass venturi

nebuliser (Thermo-Fisher Scientific). Sample processing was undertaken using 'Qtegra software' (Thermo-Fisher Scientific). The quantitative multi-element analysis included (alphabetically): Ag, Al, As, B, Ba, Be, Cd, Ca, Co, Cr, Cs, Cu, Fe, K, Li, Mg, Mn, Mo, Na, Ni, P, Pb, Rb, S, Se, Sr, Ti and (semi-quantitatively) Tl, U, V and Zn.

2.4 Recovery and processing of insoluble residues after all pre-treatments

Unless otherwise stated, after each different pre-treatment of BSG was conducted (and the specific pre-treatment protocols are described separately within each relevant, subsequent chapter) all the experimental runs were adjusted to pH 5.0 (±0.2) with either glacial acetic acid or 40% NaOH (w/v). The biomass was then centrifuged at 5000 rpm for 10 min and the supernatant removed for the analysis of sugars and known yeast-inhibitory compounds (sugar and lignin degradation products). The remaining residue was then exhaustively washed with water (by repeated re-suspension and centrifugation at 5000 rpm for 10 min, discarding the supernatant each time). The remaining residues were then oven-dried overnight at 60°C prior to any compositional analysis or attempted subsequent enzymatic saccharification.

2.5 Cellulolytic enzyme hydrolysis protocols

2.5.1 Preparation of cellulolytic enzyme buffer

For all cellulolytic enzyme hydrolysis attempts 50 mM sodium citrate buffer (pH 5.0 for Cellic® CTec2 and pH 4.8 for Celluclast®) was added to the correct quantity of biomass to achieve the required solids-loading.

All enzymatic saccharification protocols were conducted at 50°C with agitation at 150 rpm (MaxQ 4358 shaking incubator, Thermo Scientific, UK). Various different incubation periods were investigated from 12-72 h, as discussed in individual results sections.

2.5.2 Celluclast® protocol

The initial assessments of the efficacy of different pre-treatment processes were conducted using a low solids-loading protocol (0.5% w/v) with lyophilised Celluclast® cellulase from *Trichoderma reesei* (ATTC 26921, Sigma-Aldrich, UK) using an excess of enzyme to achieve, as closely as possible, the maximum sugar concentration obtainable. Pre-treatment residue (200 mg) was mixed with 40 mL of a 1 g/L Celluclast® solution in 50mM sodium citrate buffer and incubated as above (section 2.5). This gave an enzyme dosing of ca. 50 FPU/g biomass. Following saccharification, samples were centrifuged at 5000 rpm for 10 min and the supernatant sampled for quantification of sugars via HPLC.

2.5.3 Cellic® CTec2 protocols

To investigate high solids loading based enzyme saccharification of pre-treated BSG and for the preparation of feedstocks for trial fermentations the decision was taken to use commercially applicable enzymes and dose rates, so that a more representative evaluation of overall process viability was attained. Hence, lyophilised Celluclast® cellulase was replaced with Novozymes Cellic® CTec2 (kindly supplied by Novozymes A/S, Demark) and enzymatic saccharification was conducted at more realistic solids loadings (5% - 25% w/v). Various dosages were investigated from 10 – 160 FPU/g biomass (filter paper units per gram biomass) and dispersed in the required volume of 50 mM sodium citrate buffer to give the desired solids loading. The Cellic® CTec2 had a total cellulase activity of 204 -102 FPU/mL in undiluted format which was determined according to Ghose (1989) and Adney and Baker (1996) and described in section 2.8. After the required incubation period samples were centrifuged at 5000 rpm for 10 min and the supernatant removed for HPLC quantification of sugars and use in the subsequent fermentation trials. The glucose concentration present in the Cellic® CTec2 enzyme preparation was analysed and subtracted from each enzymatic

saccharification sugar yield to allow accurate calculation of achieved % of theoretical glucose yields.

2.6 Determination of filter paper (cellulase) activity of enzyme preparations used for subsequent saccharification

This was done in order to standardise the dosing of the cellulolytic enzyme preparations and provide an internationally recognised dosing unit. Filter paper discs (Whatman No 1; 90 mm diameter; Whatman Ltd., Kent, UK) were cut into 1 × 10 cm rectangular strips of 50 mg weight. Rectangular filter paper strips were rolled up and placed into test tubes (13 × 100 mm) containing 1.0 mL of 50 mM sodium citrate buffer (pH 4.8). An aliquot (0.5 mL) of various diluted enzyme mixtures were then each added separately to the tubes. Initial experiments used a wide range of dilutions (1:10, 100, and 1000) to determine the appropriate dilution that would be needed to obtain the target 2.0 mg of glucose goal as the protocol suggests. Glucose standards were prepared by adding 0.5 mL solution (concentration range: 1 – 3.35 mg/0.5 mL) to 1.0 mL of 50 mM sodium citrate buffer. Reaction vessels were incubated for 1 h at 50°C in a water bath (Grant Instruments, UK). After the required incubation period, 3 mL of 3, 5-dinitrosalicylic acid (DNS) reagent was added to each test tube (prepared as described in Adney and Baker; 1996). Reaction vessels were then put in a water bath at 100°C for 5 min to facilitate the colour development of the reaction. The DNS reagent is reduced to 3-amino-5-nitrosalicylic acid if reducing sugars are present which involves the oxidation of the carbonyl group on the sugar to a carboxyl group. After the boiling step to develop the colour the absorbance at 540 nm measured using a Jenway 7315 UV–Vis spectrophotometer (Bibby Scientific Limited, Staffordshire, UK). Glucose standards were used to plot a standard calibration curve. Glucose concentrations in the unknown samples were then calculated from a standard curve prepared using known glucose concentrations.

The following equation was then used to calculate FPU:

$$\frac{(2.0\ mg\ glucose\ \ /\ 0.180\ mg\ glucose\ /\ \ \mu mol)}{0.5\ ml\ diluted\ enzyme\ x\ 60\ ml} = 0.37\ \mu mol\ /\ minute\ ml$$

$$= \frac{filter\ paper\ activity}{\frac{0.37}{[enzyme\ dilutiion]\ \ releasing\ \ 2.0\ mg\ glucose}}\ \ units\ \ ml$$

2.7 High performance liquid chromatography (HPLC)

2.7.1 Quantification of monosaccharides using High-Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD)

The monosaccharide concentrations resulting from the 2-step total acid hydrolysis of various insoluble residues (carbohydrate based compositional analysis of these materials etc.) were analysed using an ICS 3000 system from Dionex fitted with a CarboPac PA20 column (150 mm × 3.0 mm; Dionex, USA) and a high performance anion exchange pulsed amperometric electrochemical detector (HPAE-PAD; Dionex, USA) as the sensitivity of detection of this system was suitable for the small quantity of biomass that the compositional assay used. The chromatographic conditions were as follows: isocratic elution with 10 mM NaOH at 0.5 mL/min flow rate with a column regeneration step using 200 mM NaOH at 0.5 mL/min. Analysis was completed within 18 min and data were recorded using Chromeleon Chromatography software (Version 6.80, Dionex softron, Germering, Germany). The linearity of detection response was confirmed and quantification of sugars was based on peak

areas, relative to those for authentic external standard series of monosaccharides (at a concentration range of 1–100 g/L) using mannitol as an internal standard.

2.7.2 Quantification of glucose, ethanol, acetic and formic acid concentrations using reverse phase high performance liquid chromatography (RP-HPLC) with refractive index (RI) detection

The glucose and ethanol concentrations attained from enzymatic saccharification experiments and also from fermentations was analysed by HPLC. Additionally the small organic acids potentially generated from the pre-treatment process (acetic and formic acid) were also quantified by HPLC. The HPLC system consisted of an AS-2055 Intelligent Auto-sampler and a PU-1580 Intelligent HPLC Pump (Jasco, Japan) fitted to a Rezex ROA Organic Acid H+ organic acid column (5μm, 7.8 mm × 300 mm; Phenomenex, UK) operated at ambient temperature. Elution was isocratic, with a mobile phase of 0.005 N H_2SO_4 (0.5 mL/min) and a Refractive Index cell (RI-2031 Intelligent Refractive Index detector, Jasco, Japan) was used for detection. The injection volume was 10 μL. Data were acquired using the Azur software package v. 4.6.0.0 (Datalys, France). Quantification was performed against authentic external standards (5, 10, 20 and 40 g/L for ethanol, 6.25, 12.5, 25, 50 and 100 g/L for glucose and 0.3125, 0.625, 1.25, 2.5 and 5 g/L for acetic and formic acid).

2.7.3 Quantification of sugar and lignin based degradation products (downstream process inhibitors) concentrations using reverse phase high performance liquid chromatography (RP-HPLC) with photodiode array (PDA) detection

Metabolic process inhibitors were quantified by HPLC using UV detection at 280 nm (2695 HPLC system and 996 Photodiode Array Detector, Waters, USA) with UV spectra for secondary confirmation. The chromatographic conditions were as follows: Techsphere ODS C18 column (5 μm, 4.6 mm × 250 mm; HPLC Technologies, UK) at ambient temperature.

The mobile phase was a binary mixture of 1% acetic acid (solvent A) and methanol (solvent B) with an overall flow rate of 1.0 mL/minute. The system was operated in gradient mode, ramping from 20% to 50% methanol over 30 minutes with a 100% methanol column cleaning phase and a 9-minute re-equilibration period. The sample injection volume was 10 µL. Data were recorded using Millennium Chromatography software (Waters, USA). Quantification was performed by comparison of peak areas of a range of authentic standards (0.1-1.0 g/L concentration range) including furoic acid (furan-2-carboxylic acid), furfural (furan-2-carbaldehyde), HMF (5-hydroxymethylfurfural), vanillic acid (4-hydroxy-3,4-dimethoxybenzoic acid), vanillin (4-hydroxy-3-methoxy-benzaldehyde), ferulic acid (3-(4-hydroxy-3-methoxyphenyl)-2-propenoic acid), *p*-coumaric acid (β-(4-hydroxyphenyl) acrylic acid), syringaldehyde (4-hydroxy-3,4-dimethoxy-benzaldehyde), acetovanillone (4-hydroxy-3-methoxyacetophenone), sinapic acid (3,5-dimethoxy-4-hydroxycinnamic acid), acetosyringone (4-hydroxy-3,4-dimethoxyacetophenone), *p*-hydroxybenzaldehyde (4-hydroxybenzaldehyde), and *p*-hydroxybenzoic acid (4-hydroxybenzoic acid).

2.8 Yeast based fermentations of feedstocks produced from BSG (after pre-treatment and enzymatic saccharification)

2.8.1 Preparation of yeast cultures prior to fermentations

S.cerevisiae Strain NCYC479 was chosen as the primary yeast species to assess the fermentability of any generated feedstock from BSG due to its high ethanol tolerance phenotype. For various other experiments a comparison was also made using *S.cerevisiae* Strain NCYC2592 (a distillers strain also exhibiting a high ethanol tolerance phenotype). The alternative yeast strains *K.marxianus* NCYC1426 and NCYC179 and *K.wickerhamii* strain NCYC546 were also tested in certain experiments due to their potential pentose sugar utilisation capabilities (both for cellular metabolism and possibly for some limited

fermentation to ethanol). All strains were obtained from NCYC (National Collection of Yeast Cultures, UK). Sequential 3-stage propagation was conducted for each yeast strain in order to culture a sufficient cell density for the required number of fermentation vessels. All propagation was conducted using 4% YPD media containing 4% glucose, 2% peptone and 1% yeast extract (Oxoid, UK) in the required amount of water. The first propagation step involved incubation of the 10 mL inoculated yeast culture in a sterilec30 mL plastic vessel (Sterillin, UK) at 25°C with agitation at 150 rpm, for 48 hours (CERTOMAT BS-1 incubator, Sartorius, Germany). The second stage was dispersion of the 10 mL culture into a further 100 mL of sterile 4% YPD (in a 250 mL glass conical flask) and a further incubation period of 48 hours (150 rpm agitation). The third and final stage of propagation was the dispersion of the 100 mL yeast culture into 1000 mL of sterile 4% YPD (in a 2000 mL glass conical flask) and a final incubation period of 48 hours (150 rpm agitation). After the final incubation period the cultures were subjected to three cycles of washes prior to use. The wash cycles consisted of addition of 100 mL water (into a sterile 500 mL Nalgene centrifuge tube) and centrifugation at 5000 rpm for 5 min (Heraeus Megafuge 16, Thermo Scientific, UK) and the supernatant discarded. The remaining yeast pellet was re-suspended in 100 mL sterile water via vortex mixing (Labnet, USA) and centrifuged (again in the 500 mL Nalgene tube) at 5000 rpm for 5 min. This wash step was repeated 3 times to ensure all the residual glucose and YPD nutrients had been removed prior to inoculation. After the final centrifugation step of the final wash the supernatant was discarded and the yeast pellets re-suspended in 50 mL sterile water. Cell counts were then conducted using a haemocytometer to determine the concentration of cells present (cells/mL). Methylene blue was used to indicate cell viability numbers. The yeast propagation was conducted in triplicate for each strain in order to produce a sufficient quantity of cells for use in all the experimental runs. All the experimental vessels were inoculated with the required yeast strain at a concentration of 1×10^6 cells/mL

unless otherwise stated (a small sub-section of experiments that used a higher pitching rate are described in sections 4.2.4 and 4.3.8). Specific experiments in which higher pitching rates were used (in order to attempt to overcome the inhibitory effects of a range of degradation products) are described separately in section 4.2.7.1.

2.8.2 Fermentation protocols

2.8.2.1 *S.cerevisiae* based fermentations

Unless otherwise stated the *S.cerevisiae* based fermentations were conducted (using 100 mL of each specific feedstock) in glass serum bottles (125 mL; Wheaton, USA) fitted with air-tight rubber septa, in order to maintain as anaerobic an environment as possible. Custom made non-return valves were used to allow the escape of fermentation gases (primarily CO_2) whilst minimising air input using the method described in Quain et al., (1985). Fermentations were conducted over a range of temperatures (MIR-253 incubator, Sanyo Electric Co., Japan) but predominantly at 15°C with magnetic stirring at 120 rpm. The 15°C temperature that the majority of bioethanol fermentations were conducted at was used due to a technical oversight. This was later concluded not to be the most efficient temperature for rapid bioethanol production but was used simply as it was similar to the saké fermentation temperature from which the *S.cerevisiae* NCYC479 yeast strain originated. Additionally a full optimisation of the fermentation stage was considered beyond the scope of this thesis as the fermentations were only included purely as proof of principle of ethanol production.

Progression of the *S.cerevisiae* fermentations was tracked by the weight loss of vessels as a result of the liberation of CO_2 associated with the conversion of sugars to ethanol (Quain et al., 1985). The fermentation vessels were weighed at frequent intervals until a constant weight was achieved which was considered to indicate the end of the fermentation. Upon completion of the fermentation samples were taken for HPLC analysis of ethanol (section

2.7.2). All fermentations were conducted in triplicate. Achieved % theoretical ethanol yields were calculated using the starting glucose concentration of the feedstock and the x 0.51 ethanol conversion factor (Badger, 2002). Whilst this weight loss based protocol may have not been the most ideal method of tracking fermentation progression it did provide some degree of insight and should highlight any major fermentation issue such as a long lag phase or stuck fermentation (delayed attenuation) possibly due to a deficiency of some nutrient in the produced feedstock or the presence of an inhibitory compound. Due to the very small volume and sealed nature of the fermentation vessels it was not possible to sample at various intervals throughout the fermentation for subsequent ethanol production and sugar utilisation quantification by HPLC. Production of a suitably large quantity of each feedstock for the fermentations was also a technical hurdle given the small volume of the reaction vessels used for many experiments (small volume pre-treatment and enzyme saccharification reactions etc.) and this made the use of a larger fermentation volume (which may have then enabled regular sampling for time course based analysis) unfortunately not a practically viable option.

2.8.2.2 *Kluyveromyces Spp*. based fermentations

The *Kluyveromyces Spp* based fermentations of hydrolysates generated directly from the acid-based pre-treatment (Section 4.3.8; looking at the potential use of the pentose sugars present, especially xylose) were conducted using 100 mL (of the required hydrolysate) in glass serum bottles (125 mL; Wheaton, USA) with foam bungs. All the experimental vessels were inoculated with the required yeast strain at a concentration of 1 x 10^6 cells/mL unless otherwise stated. Fermentations were conducted over a range of temperatures (MIR-253 incubator, Sanyo Electric Co., Japan) but at 25°C with magnetic stirring at 120 rpm. Any Xylose utilisation and any ethanol production were then quantified using HPLC. The yeast strains were propagated as described in section 2.8.1.

2.8.2.3 Consolidated bioprocessing (CBP) fermentations

A range of CBP based fermentations (Chapter 8) were conducted at either 15°C or 30°C (MIR-253 incubator, Sanyo Electric Co., Japan) in 500 mL conical flasks with foam bungs. The vessels were inoculated with the filamentous fungi and required yeast strains on day 0 and left for ca. 20 days (with no additional agitation or mixing). Sugar liberation (by the filamentous fungi) and ethanol production (by the yeast strain/s) were also quantified using HPLC. The yeast strains (either *S.cerevisiae* or *Kluyveromyces Spp* strains or both) were inoculated at 1 x 10^6 cells/mL. The yeast strains were propagated as described in section 2.9.1. Specific details of the exact protocols used are given in Chapter 8.

2.9 Statistical analysis and experimental design:

Experimental design, response surface modeling and ANOVA were all performed using Design Expert v 7.0 (Stat-Ease Inc., Minneapolis, USA). Additional ANOVA (followed by the Tukey HSD test) and t-test statistical analyses were performed using the software package SPSS 16.0 for Windows (SPSS, Germany).

The experimental designs and response surface modelling (RSM) used a D-optimal format from which the D-optimal algorithm chose the treatment combinations (the exact parameter settings; e.g. reaction times and temperatures or enzyme doses) to include in the design from a specific range for that particular parameter set by the user. This reduced the total number of experiments needed to be conducted from that of a full factorial experimental design yet still provided statistically valid data.

Unless otherwise stated all experiments (pre-treatments, enzymatic saccharification reactions and fermentations) were conducted in triplicate (biological replication).

3 Preliminary analysis and experiments investigating BSG

3.1 Introduction

In order to objectively evaluate alternative uses for BSG there was a requirement to know the starting composition of the material to allow tracking of compositional changes in relation to processing strategies. To achieve this, a full suite of compositional analyses were conducted. In addition, ultrastructural imaging was performed using scanning electron microscopy (SEM) to gain an insight into the physical details and morphology of the material that makes up BSG. The minimal amount of high quality ultrastructural information and imaging of BSG available from published data at the current time justified this. Ultrastructural imaging was performed to enable visual tracking of changes to the structure of the material in response to different processing techniques such as pre-treatments or enzyme digestions (saccharification).

The protocol described in Schwenke (2006) involved the use of vibratory wet sieving of a slurry of BSG (and hot water) to achieve some degree of fractionation of the protein (into the resulting liquid fraction) from the structural carbohydrates. This achieved the separation of the cellulose and hemicellulose (which remain in the solid residue) through particle size differences. As such, an experiment was designed and conducted to investigate the possibility that dry sieving technology could achieve similar fractionation. Fractionation of the protein component of BSG could prove highly lucrative enabling its use for a commercial application such as animal feed or used in high gravity fermentations as a nitrogen source whilst leaving the structural carbohydrate enriched residue free for possible use in bioethanol production. Dry sieving is technically less demanding, requires less expensive equipment and uses no water, therefore should it prove to be effective it would render its use on a larger scale much more commercially viable and therefore more life cycle assessment (LCA) friendly.

Finally direct cellulolytic enzymatic saccharification attempts were investigated using Novozymes Cellic® CTec2. This involved no prior thermo-chemical pre-treatment and would establish if the prior processing stages of the beer brewing process (malting, mashing and lautering etc.) had sufficiently modified the structure of the lignocellulosic matrix in the BSG to reduce the recalcitrance to a point where an energy intensive pre-treatment was not actually needed. In addition to purely cellulolytic enzyme based direct saccharification attempts, Novozymes Ondea® Pro was also tested as it was a mixture of various enzymes designed specifically for hydrolysing various components of un-malted barley and therefore may have been effective in achieving direct saccharification of BSG.

3.2 Materials and methods

3.2.1 Brewers spent grains (BSG)

In addition to the 100% malt based, lauter tun derived BSG from the SABMiller pilot plant (Sutton Bonington, UK) used for all major experiments an additional sample of BSG was obtained from Molson-Coors brewery (Derbyshire, UK) which used mash filter based brewery technology and an initial grist composition of 90% malt and 10% wheat.

3.2.2 Compositional analysis of BSG

A full range of compositional assays were conducted in order to try and achieve mass balance and account for every component present in BSG samples from both lauter tun and mash filter based brewing technology. The comparison of two different BSG samples would help to evaluate the compositional differences between the different BSG samples that may arise due to use of different brewhouse technology and the addition of non-barley based cereal adjuncts in the initial grist. All assays and techniques were performed as described in sections 2.3.1 – 2.4.1. All analyses were conducted in triplicate.

3.2.3 Scanning electron microscopy (SEM) of BSG

Unground samples of native BSG and samples of BSG following direct cellulolytic enzyme hydrolysis attempts using Cellic® CTec2 (Novozymes, Bagsvaerd, Denmark) were dried overnight at 105°C (OV/100/F/DIG oven, Genlab, UK) and imaged using an FEI Quanta 3D 200 scanning electron microscope (FEI company, Oregon, USA). It was operated at 1 – 5 kV. In order to improve the image quality the samples were coated with carbon. The carbon coating was done for 20 seconds using an Agar Turbo Carbon Coater (Agar Scientific Ltd, Stansted, UK).

3.2.4 Particle size analysis and Sieving based fractionation attempts

Particles within the BSG were sized using a sieve stack (AS200 Digital, Retsch, UK). Dry BSG was gently broken up by hand to separate the aggregated particles. For the sizing analysis BSG was placed at the top of the sieve stack and shaken using an amplitude of 0.5 *g* for 10 minutes. In the initial experiment pre-weighed sieve trays with pore sizes of 2 mm, 1.70 mm, 1.18 mm, 710 μm, 425 μm, 212 μm, 106 μm and a base tray (<106 μm) were used. It was possible to separate the sample into fractions ranging from larger grains with almost complete husks (>2 mm) to fine flours (<106 μm). These were then re-weighed after separation had been completed to ascertain fraction proportions within the sample.

In an initial experiment for the sieving based fractionation attempts, particles within the unground BSG material were sized using a sieve stack (AS200 Digital, Retsch, UK) and sieve trays with pore sizes as described above (2 mm - <106 μm). Each size fraction then had cellulose, hemicellulose and total nitrogen (protein) quantified (specific protocols as previously described in section 2.3.1). All particle size analyses and sieving based fractionation attempts were conducted in triplicate.

The success of the preliminary sieving experiment warranted the expansion of the sieve size fractionation range to include smaller sizes in an attempt to further improve the degree of fractionation. In the secondary experiment particles within the BSG material were again sized using a sieve stack (AS200 Digital, Retsch, UK) but this time with sieve trays with pore sizes of 106 μm, 75 μm, 63 μm, 45 μm and a base tray (<45 μm). Prior to sieving the BSG was ground to a fine powder (using a mortar and pestle) to enhance the recovery of smaller size fractions to ensure enough material for the compositional assays. Each size fraction then had cellulose, hemicellulose and total nitrogen (protein) quantified as in the initial experiment using the methods described in section 2.3.1 – 2.4.1.

3.2.5 Enzyme saccharification of BSG without thermo-chemical pre-treatment

3.2.5.1 Cellulolytic enzyme hydrolysis using Novozymes Cellic°CTec2

Enzyme hydrolysis was conducted using the Cellic°CTec2 protocol at 50°C for 24 h as described in Section 2.7. An enzyme dose range of 10 - 160 FPU/g BSG at a very low solids loading of 0.5% (w/v) was used in order to ensure dose and solids loading were not rate limiting factors and to give a best case scenario (in terms of liberating maximal sugar yields). After incubation samples were centrifuged and liberated sugar concentrations quantified as described in section 2.7.

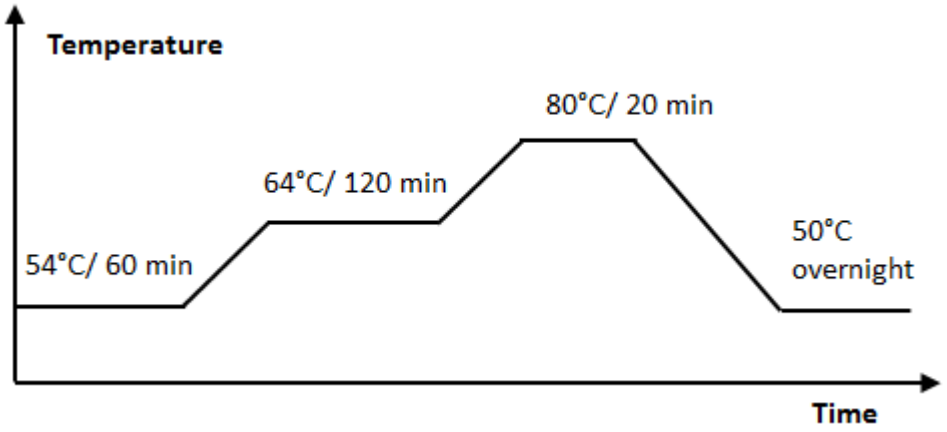
3.2.5.2 Enzymatic saccharification using Novozymes Ondea® Pro

The commercial enzyme preparation Ondea® Pro (Novozymes A/S, Demark) is designed to facilitate beer brewing from un-malted barley (Novozymes Ondea® Pro MSDS, 2011). It is reported to offer a substantial energy saving over the use of malted barley for beer brewing (Novozymes Ondea® Pro MSDS, 2011). It is a complex mixture of various enzymes including a protease (EC 232-991-2), pullulanase (EC 232-983-9), α-amylase (EC 232-565-6), cellulase (EC 232-734-4), lipase (EC 232-619-9) and an endo 1,4- xylanase (EC 232-800-

2). This combination of different barley specific enzymes may have worked better (in terms of liberating maximal sugar yields) than when compared to cellulolytic enzyme preparations such as Cellic® CTec2 as the enzymes may have been better suited to BSG. In addition the Ondea® Pro may have been sufficient to modify and deconstruct the BSG component matrix to allow a high degree of fermentable sugar liberation without any pre-treatment.

A series of 8 enzyme loading rates of 4, 8, 12, 16, 20, 40, 80 and 160 mg enzyme per g BSG were prepared using 50 mM sodium citrate buffer (pH 5.8 which was within the suggested operating range of the product of pH 5.7-5.9, adjusted via addition of 10 M NaOH). Many of these loading rates were in excess of the dosing suggested by Novozymes for saccharification of un-malted barley (1.5–2.5 kg enzyme per tonne of raw barley or ca. 2 mg enzyme per g substrate) in order to provide a best case scenario. Larger volume stock solutions of the enzyme preparations at the required concentrations were produced due to the very small amounts of enzyme to be added. Stock solutions were prepared by adding 20, 40, 60, 80, 100, 200, 400 and 800 mg of the Ondea® Pro enzyme preparation each separately dispersed in a final volume of 1000 mL 50mM sodium citrate buffer (pH 5.8, adjusted via 10 M NaOH). The pre-dosed enzyme buffer mix was then thoroughly mixed via magnetic stirring for 5 min (Variomag compact, Thermo Scientific, UK). 40 mL of each enzyme pre-dosed buffer was then added to 200 mg BSG in a pre-weighed 50 mL centrifuge tube. A modified variation of the mashing schedule for Ondea® Pro (the specific stand times used etc.) advised by Novozymes was used as outlined in Figure 3.1. The same temperatures were used but with double the stand times advised by Novozymes to give each enzyme additional time to function. A temperature controllable water bath was used for the incubation. In addition a final overnight stand at 50°C was also used to maximize the chances of the enzymes working effectively.

Figure 3.1 Temperature and time profile of the incubation stands used for the saccharification attempts using Novozymes Ondea®Pro directly on BSG.



After the final incubation the liquid fraction was removed using centrifugation at 3500 rpm for 10 min (Heraeus Megafuge 16, Thermo Scientific, UK). The supernatant was then analyzed for monosaccharides (ICS 3000 HPAE-PAD; pa20 column, Dionex, USA, section 2.7.1). The residue was then subjected to 3 rounds of water washes (20 mL water added and centrifugation at 3500 rpm for 5 min then supernatant discarded) to remove any components of the Ondea® Pro preparation and dried overnight at 40°C (OV/100/F/DIG oven, Genlab, UK). The dried remaining insoluble residue was analyzed for any compositional changes (protein, cellulose, hemicellulose, lignin and total lipid) and any mass loss calculated to determine any effects of the enzyme mixture using the compositional assay techniques described previously in section 2.3.1 – 2.4.1. BSG controls (without any digestion by Ondea® Pro) were run alongside each compositional assay as controls for comparison. All analyses were conducted in triplicate.

3.3 Results and discussion

3.3.1 Compositional analysis of BSG originating from both lauter tun and mash filter based brewing technology

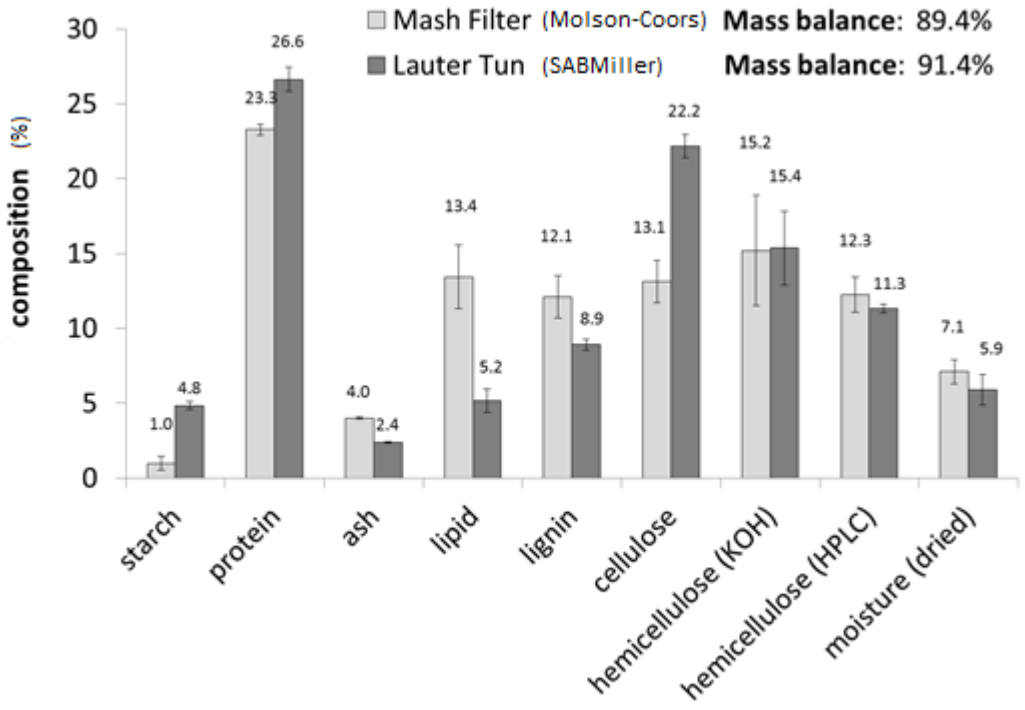
A degree of compositional variability for some of the components was observed between the two BSG types that originated from the different brewery technology used (Figure 3.2). The largest variability was seen in starch and total lipid with the lauter tun derived BSG containing only ca. 38% of the total lipid content found in the mash filter derived BSG but also the mash filter derived BSG sample containing only ca. 20% of the starch contained in the lauter tun derived BSG. The mash filter derived BSG contained only ca. 59% of the cellulose concentration that the lauter tun derived BSG contained. Other noticeable differences were seen in the lignin content with the lauter tun derived BSG containing only ca. 73% as much lignin as the mash filter derived BSG. Additionally the mash filter derived BSG sample contained only 87% of the protein present in the lauter tun derived. Other components such as hemicellulose were present in more similar proportions between the two different BSG samples.

The variation in composition seen in some component proportions was considered to be due to both the type (of brewhouse technology used) and degree of optimization of the brewing process that formed the two different BSG samples and also the variation in the grist composition from which each different sample of BSG arose. The mash filter derived BSG was produced at the Molson Coors brewery (Burton-upon-Trent, Derbyshire, UK) and was brewed using mash filter technology to separate the wort. This resulted in a finer milling process being used initially (via a hammer mill) which then alters the rate and degree of extraction and solubilisation of material from in the grist. In addition exogenous enzymes were also used during the mashing stage to further enhance the recovery of fermentable

extract. Alternatively the lauter tun derived BSG used for these experiments was produced at the SABMiller pilot plant brewery research facility here at University of Nottingham, Sutton Bonington. The process involved lauter tun based filtration of the wort (for separation) which requires a more course, larger particle size of the grist (therefore a less intensive milling protocol is used initially) in order to serve as an effective filtration bed. The larger particle size affects the rate and degree of extractive removal of components from the malt due to a reduced surface area. Also the SABMiller pilot plant was considered to be a research facility in its relative infancy and therefore the brewing process was not as optimized as a fully operational, large scale commercial brewery such as that at Molson-Coors. This would have likely resulted in a less exhaustive, lower extraction efficiency of soluble material from the grist compared to the large scale commercial brewery source of the mash filter derived BSG. This could explain the observed differences in protein, cellulose and particularly starch. The variation in lipid and lignin was considered to be due to the variation in the malted barley used in both grist's from different breweries (different cultivar, harvest time and storage conditions etc.) and also the use of the wheat adjunct (as part of the grist) for the mash filter derived BSG from Molson-Coors. In addition exogenous mash enzymes were not used at the SABMiller brew whereas the brew at Molson-Coors did. The SABMiller pilot plant grist (used for the lauter tun derived BSG) was composed of 100% barley whilst the Coors grist (used for the mash filter derived BSG) was a mixture of 90% malt (barley) and 10% wheat. Whilst information on the exact type, cultivar, harvest time and storage conditions of the barley that composed both the different grist types was not available (due to commercial confidentiality issues with the two different breweries), it was likely there were differences due to the use of a different source for each malt.

For the lauter tun BSG sample a mass balance of 91.4% was achieved. For the mash filter BSG sample a mass balance of 89.4% was achieved.

Figure 3.2 Compositional analytical overview and mass balance for BSG originating from both mash filter and lauter tun brewhouse technology. Data are the mean ± SD of five replicate measurements. Mass balance calculated using hemicellulose determination from KOH digestion gravimetric assay.



Given the analytical variability of each technique and assay that was used to perform each of the compositional analyses these values were considered adequate to account for the majority of components present in each different BSG sample. In addition to the compositional analysis that was conducted particle size analysis of un-ground lauter tun BSG was conducted and concluded that the majority of material found within the sample was between the 0.425 mm and 0.71 mm size (Table 3.1).

Table 3.1 Size distribution of the particles of unground lauter tun derived BSG. Sieving conducted using an amplitude of 0.5 *g* for 10 minutes. Data are the mean of three replicate measurements.

Sieve size (mm)	Mass (%)
2	7.86
1.7	2.08
1.18	7.07
0.71	12.9
0.425	65.5
0.212	4.23
0.166	0.29
Base (<0.166)	0.08

3.3.2 Scanning electron microscopy (SEM) based ultrastructural imaging of BSG

3.3.2.1 BSG originating from lauter tun based brewing

3.3.2.1.1 Ultrastructure of grist (100% malted barley)

The initial grist was imaged in an attempt to try and establish a 'baseline' for the material (that ultimately ends up as BSG) as it may have assisted in identification of the ultrastructure of the subsequent BSG. Figure 3.3 (a-f) all show internal endosperm tissue fragments at various magnifications that are covered in granules that are likely of starch origin due to their characteristic, somewhat spherical shape and size of approximately 20 μm in diameter (Wilson et al., 2006).

3.3.2.1.2 Ultrastructure of BSG:

The BSG ultrastructural imaging showed that all the granules of suspected starch origin seen in the previous grist SEM images had been stripped away and in addition the surface topography of the inner endosperm was now more craterous with visible cavities where material had clearly been extracted through the mashing conditions associated with the

brewing process (Figure 3.4; f). Overall the endosperm surface appears more rugged and Figure 3.4 (a) shows a small residual piece of clearly smooth outer husk material in the center of a larger piece of inner endosperm tissue. Figure 3.4 (e) shows a larger piece of the smooth waxy outer husk with a smaller piece of rugged endosperm tissue resting on the surface.

3.3.2.1.3 Ultrastructure of lauter tun derived BSG after enzyme saccharification:

The immediately noticeable effect of the cellulolytic enzyme treatment is a reduction in the ruggedness of the surface topography of the inner endosperm tissue fragments (Figure 3.5 a-d). They appear less craterous and almost eroded with less distinct cavities where the enzyme had digested the surface tissue. However the typical size of the endosperm tissue fragments was still similar to non-enzyme treated BSG fragments. This may assist in the explanation of why only moderate amounts of liberated monosaccharides were detected (relative to the theoretical concentration that was potentially available) after this enzyme hydrolysis (i.e. a direct saccharification attempt without pre-treatment; see section 3.2.6 for experimental details). It may suggest that cellulolytic enzymes can only function on the exposed surface layers of tissue and cannot penetrate into the inner more recalcitrant lignocellulosic material. This hypothesis could be supported by the fact that in part many pre-treatment techniques function as swelling agents to increase the porosity of the material (amongst other effects) which enables better enzyme access to the internal regions of the material being processed. Figure 3.5 (a) shows what is suspected to be stress fractures occurring in vertical lines down the outside of the fragment in the center of the image. This could be as a result of attempted external action of the hydrolytic enzymes present in the Novozymes Cellic® CTec2 preparation used in the direct saccharification attempts.

Figure 3.3 SEM analysis of SABMiller brewery grist (100% malt) at various magnifications a-f: endosperm tissue fragments.

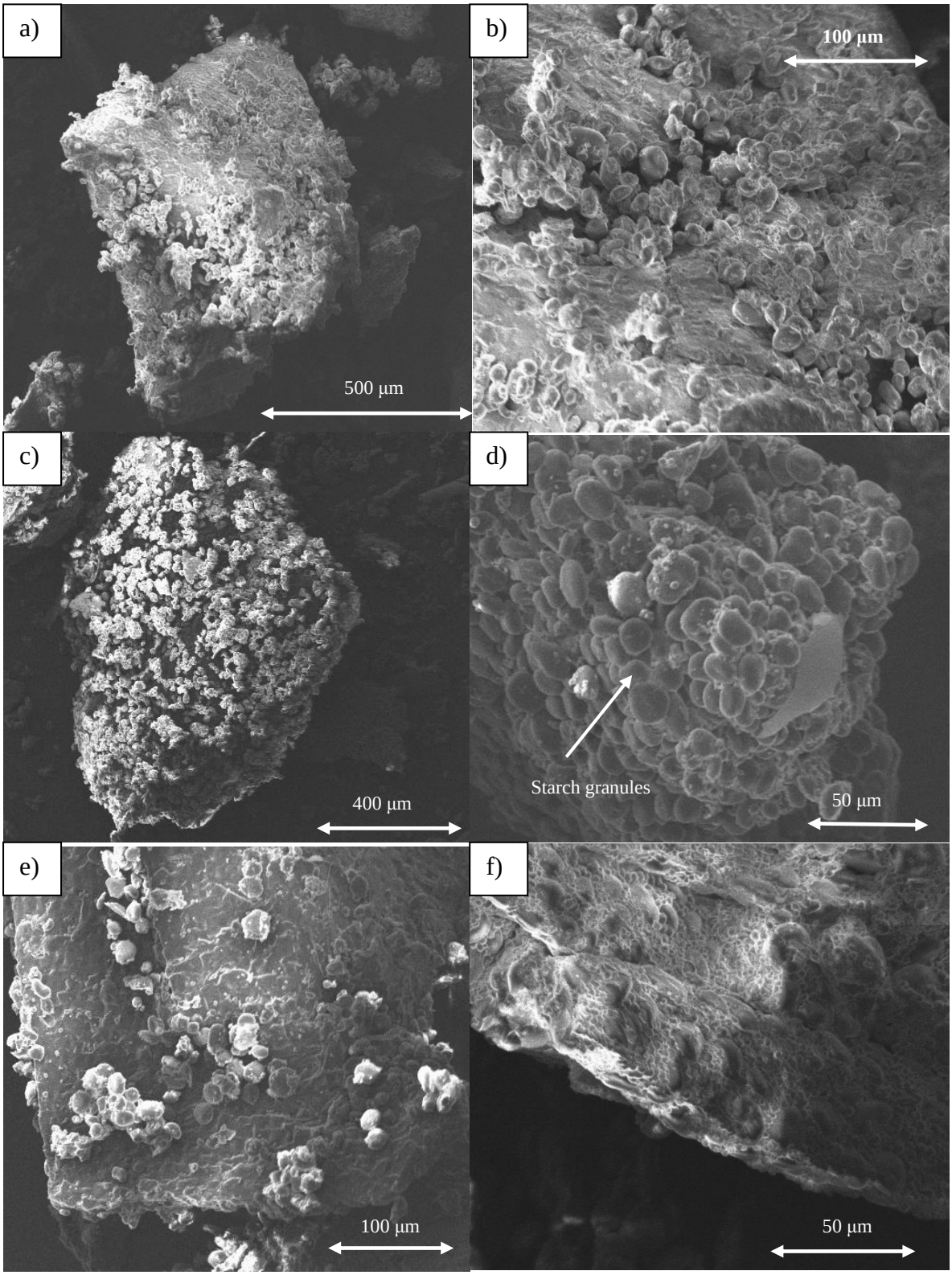


Figure 3.4 SEM analysis of SABMiller brewery grist BSG (100% malt derived) at various magnil a-f: endosperm and outer husk tissue.

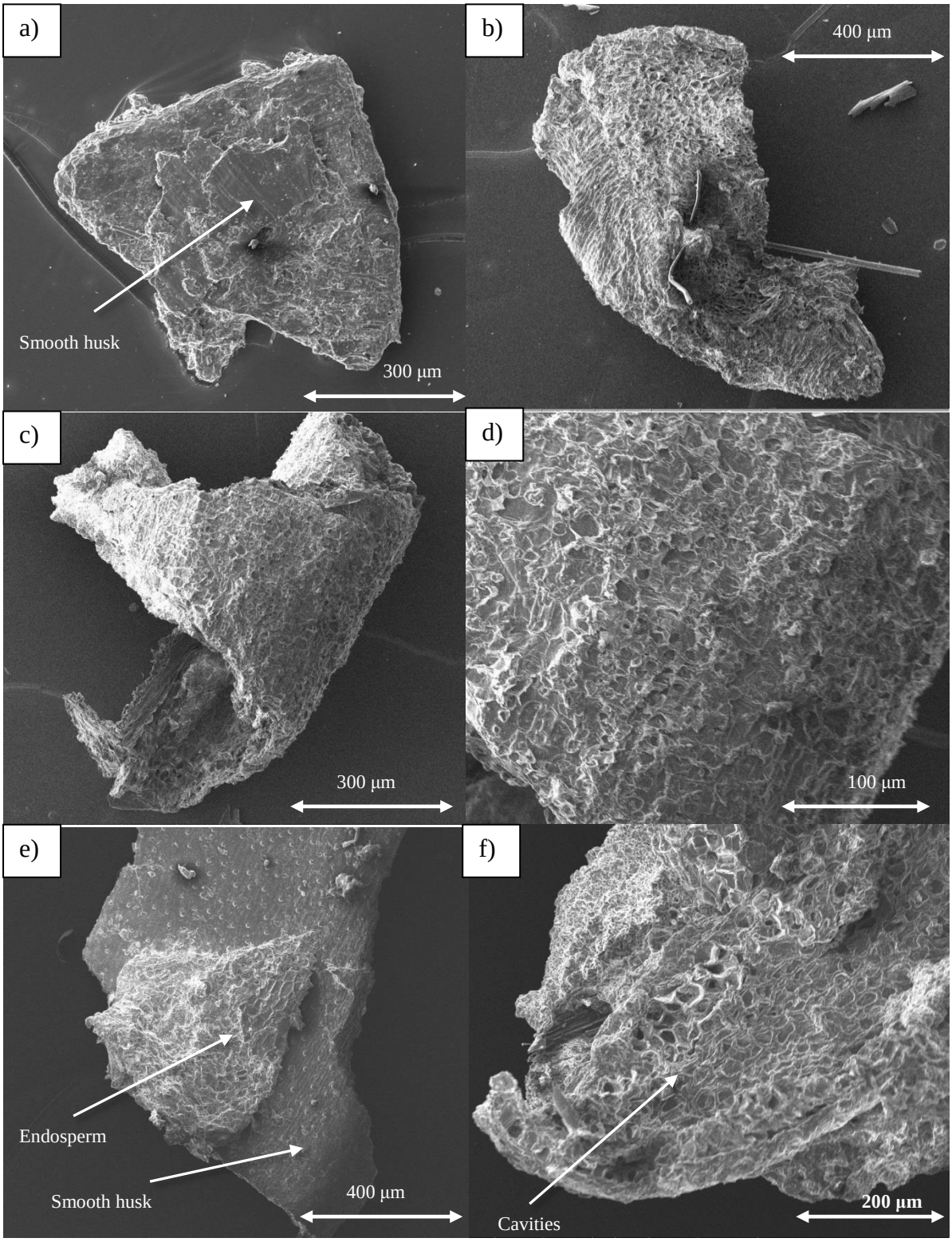
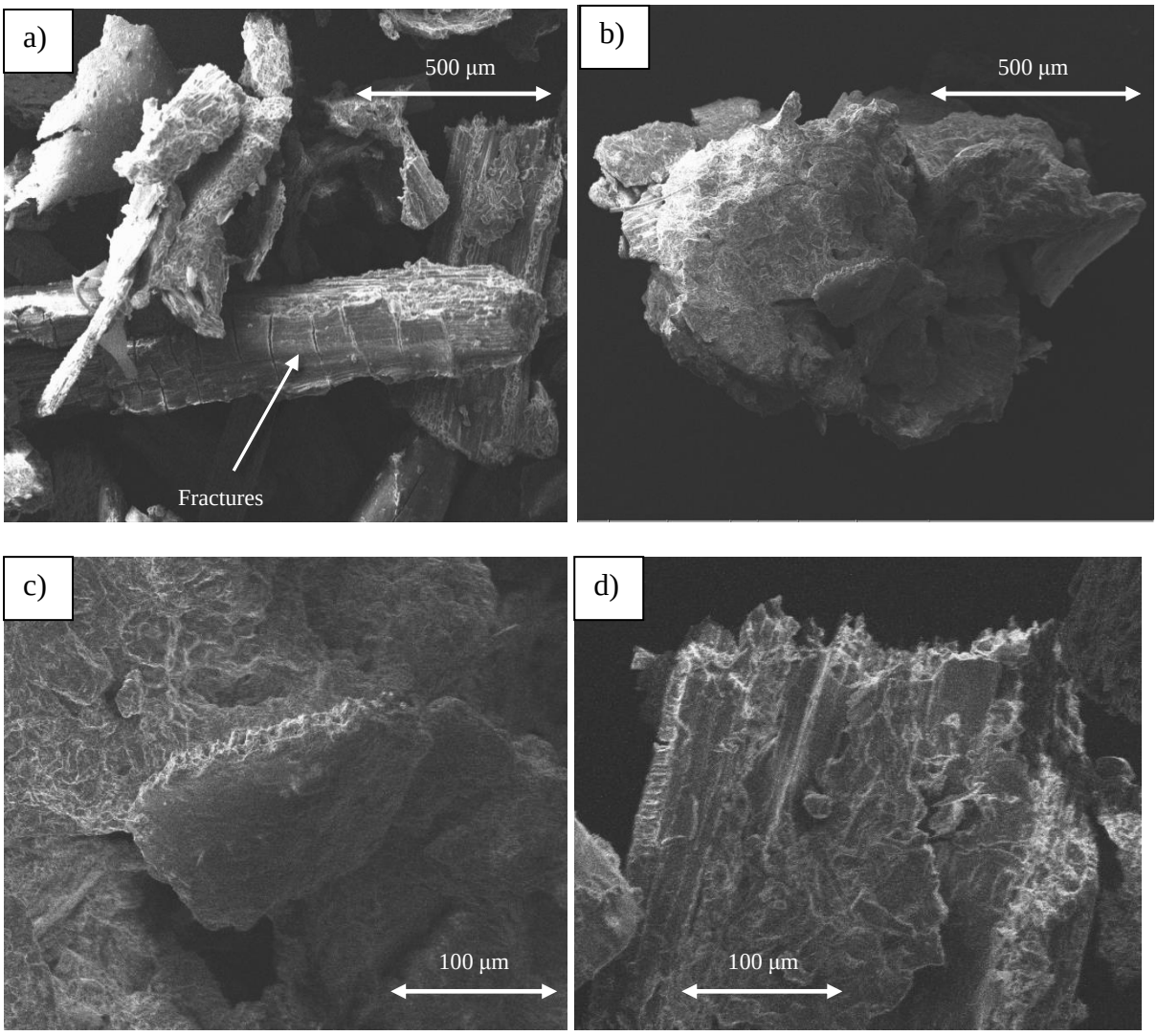


Figure 3.5 SEM analysis of BSG post-treatment with cellulolytic enzymes at various magnifications a-f: endosperm and outer husk tissue.



3.3.2.2 Ultrastructure of BSG originating from mash filter based brewing

The BSG which originated from a brewery using mash filter technology (and which used additional exogenous enzymes during mashing) and a wheat adjunct (10%) showed generally similar ultrastructural characteristics to that of the BSG which originated from using lauter tun brewhouse technology (and 100% malt grist). Figure 3.6 (a) shows long strands of fibers which are considered to belong to that of the wheat adjunct that was present. Figure 3.6 (b) shows a particularly clear image of the cavities left behind after the extraction of material

during mashing processes associated with brewing. These endosperm fragments may however appear to be more craterous due to the highly optimized brewing process extracting more material as described previously in the compositional analysis discussion in section 3.3.1. Figure 3.6 (e-f) all show in clear detail the smooth waxy outer husk layer with its consistent characteristic dimples on the surface, whilst Figure 3.6 (d) shows a fragment of endosperm tissue resting on top of a dimpled, smooth surfaced piece of outer husk tissue.

3.3.2.2.1 Ultrastructure of mash filter derived BSG after attempts at direct cellulolytic enzymatic saccharification

Figure 3.7 (c) indicates the highly heterogeneous nature of the BSG as even after enzymatic hydrolysis the sample is still comprised of many different types of material; smaller inner endosperm fragments and longer, flatter pieces of outer husk tissue (with both outer and inner orientations visible). The characteristic largely smooth but dimpled waxy outer husk layer remains largely intact (as seen in Figure 3.7 b). As also seen in the SABMiller pilot plant (lauter tun based) enzyme digested BSG, the inner endosperm tissue fragments of the mash filter derived BSG appear considerably eroded and overall smoother in appearance (Figure 3.7 d-f). Figure 3.7 (a) illustrates a transverse, cross-sectional fragment of tissue that appears to possibly show internal hollow tubes (cavities) remaining after enzymatic based extraction of hydrolyzed material from within the core. The fracture of the material fragment that exposed this cross-section may have enabled better enzymatic access to the inner material within the tube-like structures seen here. In the absence of any such fracture the enzyme is unlikely to have been able to penetrate into the inner tube- like structures hence the majority of tissue fragments appear only externally eroded through the action of the cellulolytic enzymes.

Figure 3.6 SEM analysis of BSG (derived using mash filter brewhouse technology) at various magnifications. a-f: endosperm and outer husk tissue.

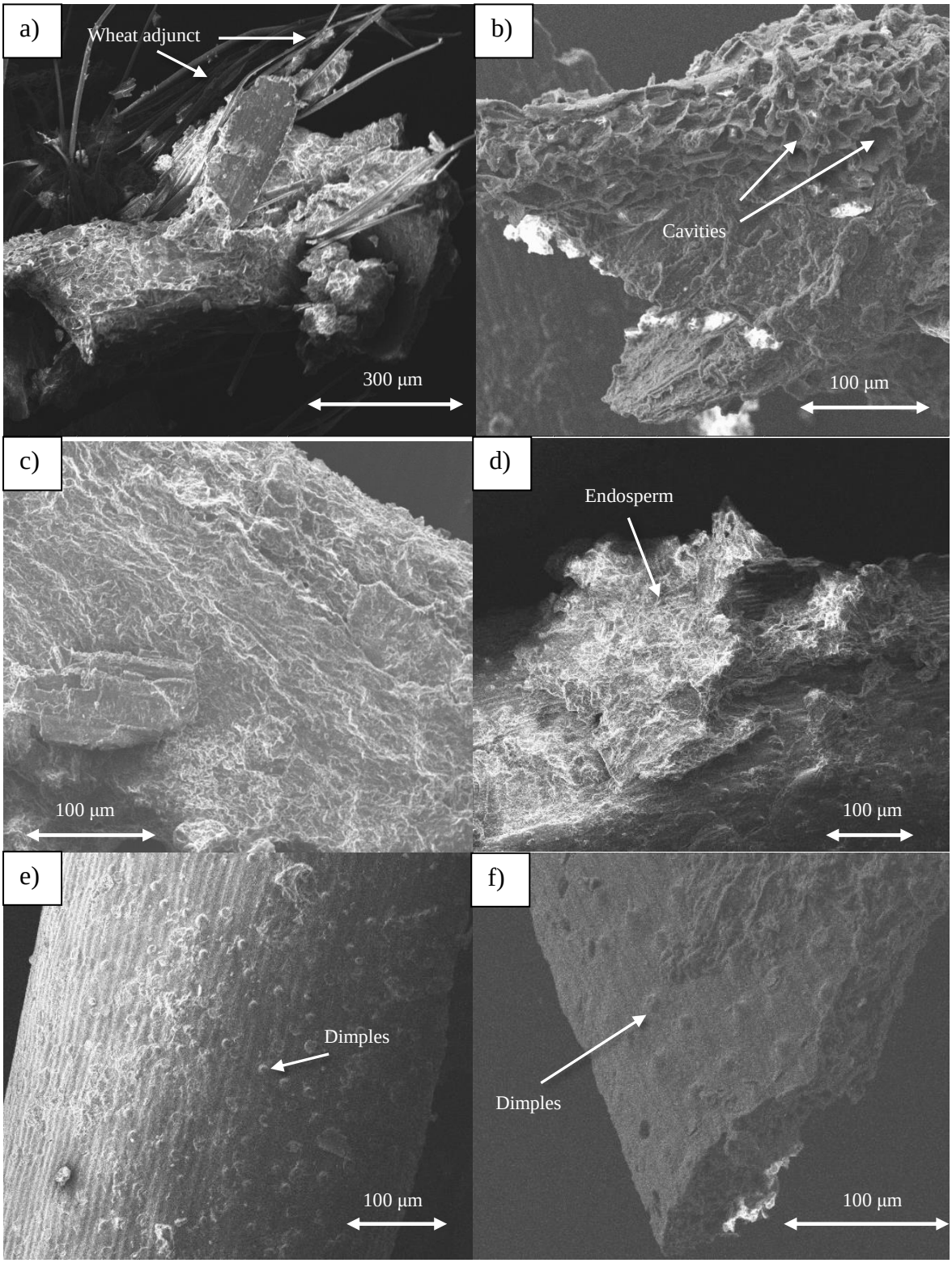
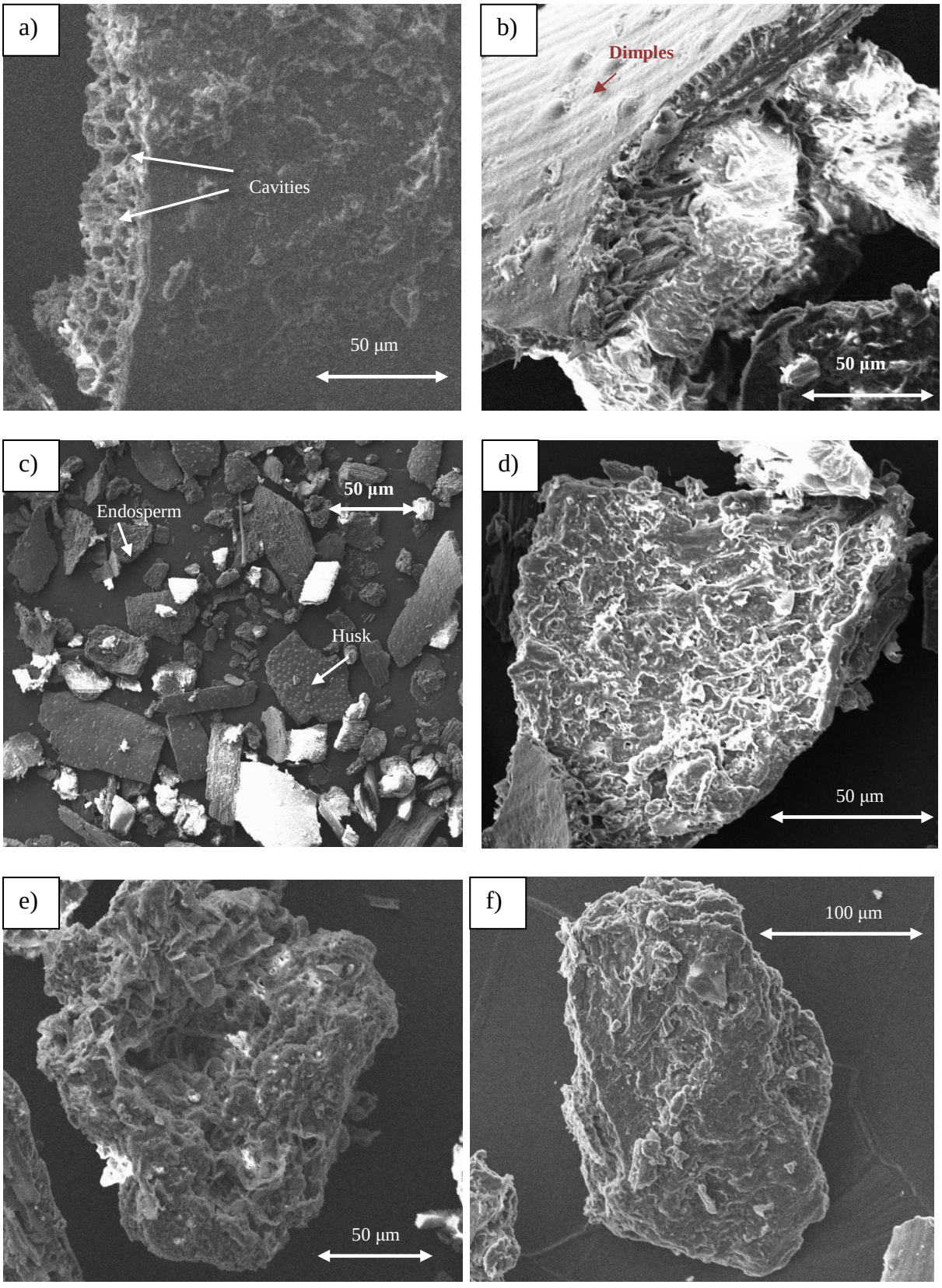


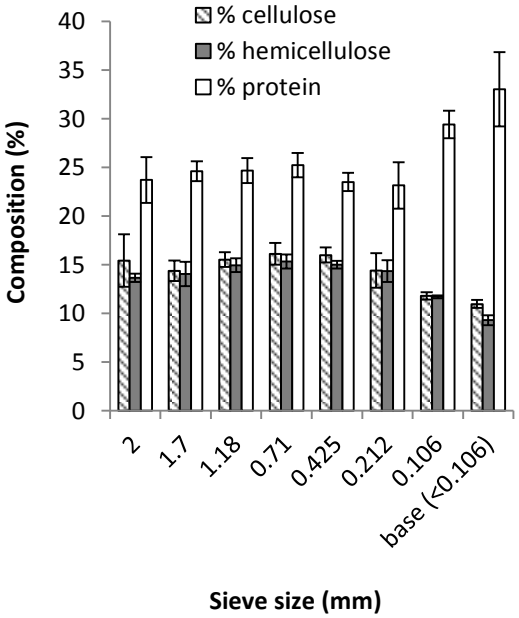
Figure 3.7 SEM analysis of BSG (from mash filter based brewing) post-treatment with cellulolytic enzymes at various magnifications. a-f: endosperm and outer husk tissue.



3.3.3 Sieving based protein fractionation attempts

Each size fraction of BSG that was isolated in section 3.2.4 was isolated and cellulose, hemicellulose and total nitrogen (protein) quantified. The compositional analysis showed that once the particle size dropped below 212 μm (i.e. recovered by sieve size 106 μm and below) there was a significant difference ($p<0.05$) in the concentration of protein and structural carbohydrates (cellulose and hemicellulose) compared to larger size fractions (Figure 3.8). In the 106 μm size fraction and base tray (<106 μm) the protein concentration was higher (ca. 31 % protein compared to only ca. 24% for the larger sieve sizes). The protein was enriched in certain fractions as the concentration of structural carbohydrates diminished. Likewise the concentration of structural carbohydrates (cellulose and hemicellulose) in the 106 μm size fraction and base tray (<106 μm) were lower (ca. 11 % and 11% respectively compared to ca. 15% and 15% respectively for the larger sieve sizes).

Figure 3.8 Compositional analysis differences of the different sized particles of BSG. Sieving conducted using an amplitude of 0.5 *g* for 10 minutes. Data are the mean ± SD of three replicate measurements.



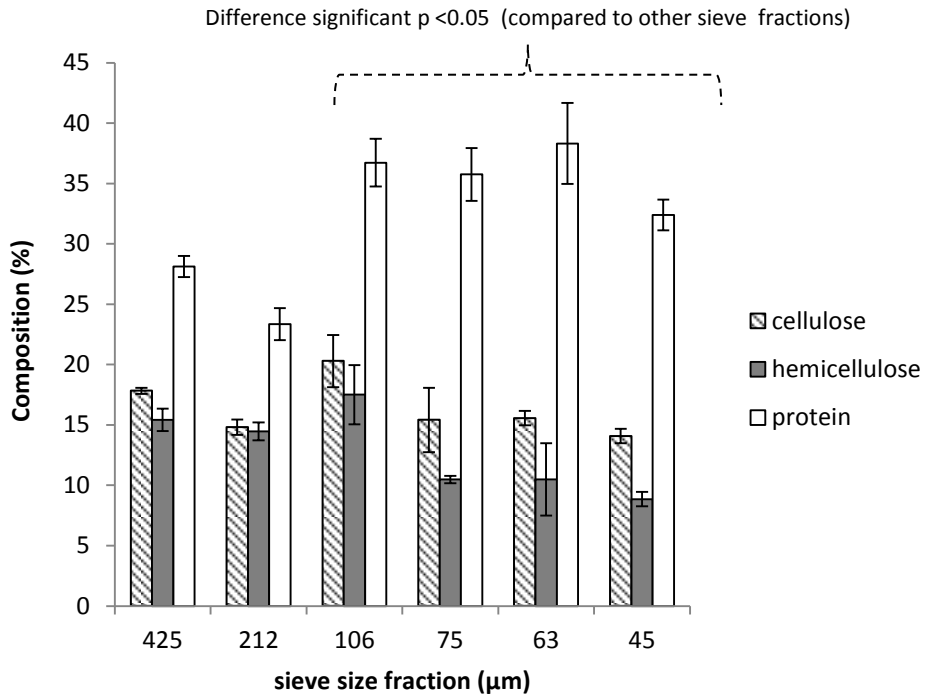
The data obtained via the preliminary sieving experiment warranted the expansion of the sieve size range to include smaller sizes (106 μm, 75 μm, 63 μm, 45 μm and a smaller base tray; <45 μm) in an attempt to further improve the degree of fractionation. Consequently a small but significant ($p<0.05$) decrease in the hemicellulose component of all the smaller fractions together as a group (the 75, 63 and 45 μm sieve sizes) was observed compared to the larger 106 μm sieve size (ca. 10 % hemicellulose for the smaller sieve sizes compared to 20% for the 106 μm sieve size respectively). However, no significant difference in protein between each individual smaller sieve size of 75, 63 and 45 μm (Figure 3.9) was seen. This difference was observed as a cut-off point indicating that somewhere in the region of 106-75 μm (i.e. anything smaller than 212 μm) was the threshold for recovering increased protein content particles. However, the variation in protein was the most interesting observation. The 106 μm sieve size and below showed a significant ($p<0.05$) increase in protein compared to the larger size sizes (ca.28% for the 106 μm and smaller fractions compared to the 425 and 212 μm sizes). However there was no significant difference between the individual smaller, 106 – 45 μm sieve sizes, indicating that 106 μm was the cut-off point regarding protein enrichment.

3.3.4 Enzymatic saccharification of BSG without pre-treatment

3.3.4.1 Cellulolytic enzyme saccharification attempts using Novozymes Cellic® CTec2

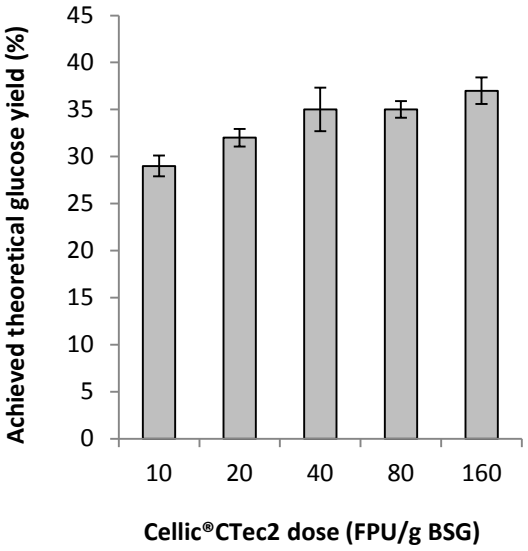
As the BSG had already undergone a degree of processing (through its production via the beer brewing process; specifically the milling and mashing stages in particular) it was considered that direct enzymatic saccharification alone may be sufficient to achieve high liberated glucose concentrations. However, % theoretical glucose yields (calculated from the total glucose content of the BSG as described in section 2.3.1) of ca. 30-35% were obtained regardless of the dose of Cellic® CTec2 used (Figure 3.10).

Figure 3.9 Compositional analysis of smaller sized particles of BSG. Sieving conducted using an amplitude of 0.5 *g* for 10 minutes. Data are the mean ± SD of three replicate measurements.



Although these yields were lower than expected, increasing the enzyme dose by x 16 from 10 FPU/g BSG to 160 FPU/g BSG (with doses of 40 FPU/g biomass already considered in excess) only liberated an extra 8% theoretical glucose, illustrating that enzyme dose was not rate limiting. In addition to the low cellulose conversion yields it must be noted that all reactions were conducted at extremely low solids loading (0.5% w/v) in order to give a 'best case' scenario. Clearly these very dilute reaction conditions would not be economically viable for any large scale process. Overall it can be concluded that some form of thermo-chemical pre-treatment would still be needed to enhance the enzymatic saccharification yields and achieve suitably high glucose yields for production of any feedstocks for bioethanol fermentations.

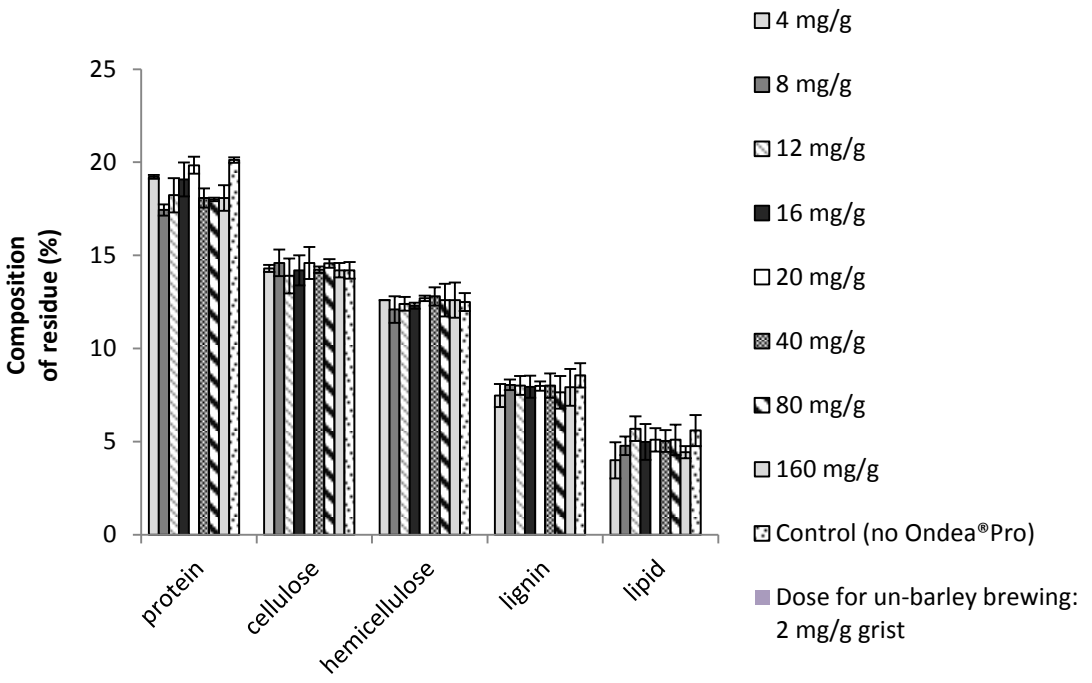
Figure 3.10 % theoretical glucose yields achieved using different cellulolytic enzyme doses on BSG with no prior thermo-chemical pre-treatment. Digestion was conducted using Novozymes Cellic® CTec2 at 50°C for 24 h using 50 mM sodium citrate buffer at pH 5.0 to achieve a 0.5% (w/v) solids loading. Data are the mean ± SD of three replicate measurements.



3.3.4.2 Direct enzymatic saccharification attempts using Novozymes Ondea® Pro

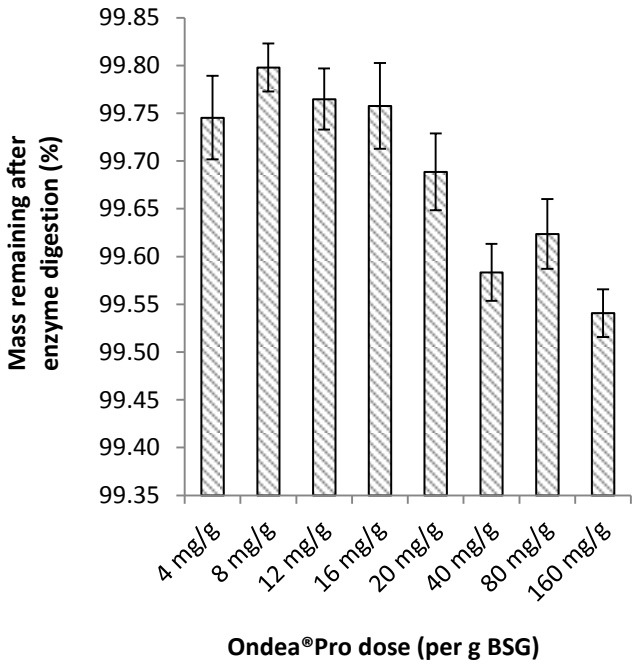
Compositional analysis of the various insoluble residues that remained after BSG was incubated with various doses of Novozymes Ondea®Pro indicated no significant effects on any of the five major components found in BSG (protein, cellulose, hemicellulose, lignin or total lipid) were observed when compared to non-enzyme treated BSG controls (Figure 3.11). This was unexpected, especially considering the maximum dose used was almost a factor of 100 higher than the suggested dose of Ondea® Pro used for barley based brewing (2 mg/g grist). The lack of any significant compositional change was also confirmed by analysis of mass loss following the enzymatic digestion which indicated an almost negligible mass loss following treatment with Ondea® Pro, even at the highest concentration of 160 mg/g used (Figure 3.12).

Figure 3.11 Compositional changes in response to using a range of different doses of the brewing specific commercial enzyme preparation Novozymes Ondea® Pro directly applied to BSG. Data are the mean ± SD of three replicate measurements.



Literature obtained from Novozymes suggests that there is a required synergy between the enzymes present in the Ondea® Pro preparation and the naturally occurring proteases and β-amylases found in barley in order for the enzyme based 'malting' to be successful. This may explain the lack of any observed effect on the BSG following enzymatic digestion with Ondea® Pro as there would not have been any significant quantities (if any at all) of active forms of the endogenous enzymes left in the BSG. However an important consideration is also that most if not all the extractable material of which the Ondea® Pro had been designed to liberate had already been removed (by the previous mashing and brewing processes that derived the BSG). Overall this confirmed that Ondea®Pro would be of no use for attempted direct saccharification of the lignocellulosic material found in BSG.

Figure 3.12 Percentage mass loss achieved using a range of different doses of the brewing specific commercial enzyme preparation Novozymes Ondea® Pro directly on BSG. Data are the mean ± SD of three replicate measurements.



3.4 Conclusions

Initial preliminary visual inspection of BSG indicated it was a heterogeneous mixture of a multitude of differing sized fragments. Particle size analysis using a sieve stack concluded the majority of fragments were approximately 425 μm in size. Compositional analysis utilising a wide range of assays pinpointed several compositional differences between two different samples of BSG that were derived from both lauter tun and mash filter based brewhouse technology. The largest differences in composition observed between the two different samples were total lipid and starch, with the lauter tun derived BSG having ca. 62% less lipid and 80% more starch remaining than in the mash filter derived BSG. In addition the lauter tun derived BSG had ca. 41% more cellulose and 13% more protein than the mash filter derived BSG. Overall a general trend of more extraction of almost every component was seen with the mash filter derived BSG. This was considered to be partly due to the presence of some wheat adjunct along with the malted barley that comprised the grist from the mash filter

brewery sample but more importantly due to the more effective extraction technology associated with brewing using a mash filter (Cooper et al., 1998). The finer (smaller) particle size of the grist that was used with the mash filter would have enhanced the degree of extraction of material from the grist compared to the larger particle sizes of the grist (after milling) used in the lauter tun based brewery. Overall an acceptable degree of mass closure (mass balance of ca. 89-91% w/w) was achieved for each different sample of BSG (both lauter tun and mash filter derived).

Ultrastructural analysis of various BSG based samples using scanning electron microscopy (SEM) confirmed the heterogeneous mixture of different sizes and types of fragments that BSG is composed of. Comparison of BSG to the starting malt grist (before brewing) confirmed the removal of the starch granules present in the initial malt. Imaging (of the residues that remained after direct enzymatic saccharification attempts using the cellulolytic enzyme mixture Cellic®CTec2) revealed a noticeable degree of erosion of the superficial edges of many of the BSG fragments. However, the general size of the fragments was similar to before any enzyme digestion which suggested only very minimal saccharification was likely occurring as a result of the enzyme activity. HPLC based glucose quantification after saccharification also confirmed this. Dry sieving based fractionation attempts suggested that some degree of protein fractionation could be achieved through specific size separation of fragments that compose BSG. Smaller particle sizes appeared to have a higher protein concentration with ca. 28% more protein for the 106 μm and smaller sized fractions compared to the larger 425 μm and 212 μm sized fractions. However the explanation for this presently remains unclear.

Direct attempts at enzymatic saccharification using the brewing industry specific product Ondea®Pro from Novozymes were unsuccessful with only minimal mass loss and minimal changes in the concentration of any major components found within the biomass. Similarly,

generally poor overall glucose yields (<35% theoretical yields) were attained from direct attempts at enzymatic saccharification using the lignocellulosic targeted product Cellic®CTec2 from Novozymes. Overall this confirmed the ultrastructural imaging based hypothesis that suggested minimal saccharification was occurring due to minimal physical changes observed after enzyme digestion. In conclusion it confirmed the requirement of some form of thermo-chemical pre-treatment would be needed. This would be in order to enhance the enzymatic saccharification yields sufficiently in order to achieve the desired glucose concentrations needed for subsequent biofuel fermentations but also in order to achieve a suitable good conversion efficiency of cellulose to glucose to validate the 'green credentials' of any proposed process of bioethanol production from BSG.

This would subsequently lead the research towards the direction of comprehensive evaluation of various different pre-treatment strategies.

4 Dilute acid and alkali catalysed hydrothermal pre-treatments for bioethanol production from Brewers Spent Grains

4.1 Introduction

Dilute acid or dilute alkali-catalysed hydrothermal (121°C 30 min) pre-treatment processes for BSG were investigated with a view to maximising the sugar yields achieved from the subsequent enzymatic saccharification of the remaining insoluble residues. A wide range of different acid and alkali catalytic reagents were screened to evaluate their effectiveness as pre-treatment reagents. Quantification (by HPLC) of downstream process inhibitors (that may have been generated from excessive thermal reactions involving the degradation of any liberated sugars or lignin based phenolics) was then conducted to determine any significant detrimental effects of the pre-treatments.

The impacts of solids-loading (solid to liquid ratio) both during pre-treatment and enzymatic saccharification were investigated in an attempt to maximise the amount of BSG that could be processed per unit time (per batch) and in an effort to liberate sufficiently high glucose concentrations to manufacture feedstocks for bioethanol production. A selection of analytical techniques was then used to evaluate the effect of the various pre-treatments on the composition of the remaining insoluble residues.

Subsequently, laboratory-scale fermentations (using *S.cerevisiae* NCYC479) were conducted using the feedstocks from enzyme hydrolysates resulting from optimised acid and alkali pre-treatment processes. This would evaluate the feasibility of bioethanol production using both these pre-treatment systems and highlight any fermentability issues that may have been attributed to either type of feedstock. In addition fermentations were also conducted using the hydrolysates that were generated directly from the pre-treatment stage when using a 1% HCl

acid reagent in an attempt to utilise this potentially valuable C5 pentose sugar laden process stream.

4.2 Materials and methods

4.2.1 Hydrothermal pre-treatment protocol

Both acid and alkali based hydrothermal pre-treatments were conducted in screw-capped glass bottles (100-1000 mL) heated at 121°C for 30 min using a 40L bench top autoclave (Priorclave, Tactrol 2, RSC/E, UK). Pre-treatment reagent solutions were prepared at the required concentrations (1% HCl, 1-5% NaOH w/v) and had the appropriate quantity of BSG added (10 – 125 g) to give the required solids-loading (5-50% w/v). After pre-treatment all experimental runs were adjusted to pH 5.0 (± 0.2) with either glacial acetic acid or 40% NaOH (w/v). The biomass was then centrifuged at 5000 rpm for 10 min and the supernatant removed for the analysis of sugars and known yeast-inhibitory compounds (including quantification of acetic acid concentrations to ensure there would be no downstream inhibitory effects from the glacial acetic acid used for the neutralization step).

The remaining residues were then washed and dried using the standardized protocol for recovery and processing of pre-treated biomass as described in section 2.5.

4.2.2 Quantification of the total arabinose, glucose and xylose contents of BSG and the insoluble residues remaining after various pre-treatments

Monosaccharide concentrations in the starting BSG and the remaining insoluble residues after the various pre-treatments were quantified using the two-stage complete acid hydrolysis protocol described in section 2.3.1.

4.2.3 Enzymatic saccharification of the insoluble residues remaining after pre-treatment

All enzymatic saccharification attempts using the hydrothermal pre-treated residues were conducted over 24 h incubation periods at 50°C with agitation at 150 rpm (MaxQ 4358 shaking incubator, Thermo Scientific, UK). 50 mM sodium citrate buffer (pH 5.0) was added to the biomass to achieve the required solids-loading.

The initial assessments of the efficacy of different pre-treatment processes were conducted using very low solids-loading with lyophilised Celluclast® cellulase from *Trichoderma reesei* (ATTC 26921, Sigma-Aldrich, UK) using the protocol described in section 2.7.1.

High solids-loading, low enzyme dose enzymatic saccharification reactions were conducted using Novozymes Cellic® CTec2 (Novozymes A/S, Demark) as described in section 2.7.2. The Cellic® CTec2 was dosed at 10 per 200 mg biomass (equivalent to 10 FPU/g biomass) and at 15% (w/v) solids loading for 24 h. After saccharification all samples were then centrifuged (5000 rpm, 10 min) prior to sampling for HPLC based glucose quantification as described in section 2.9.2.

4.2.4 Optimisation of the dilute acid-catalysed hydrothermal pre-treatment for BSG

Preliminary experiments investigated the effectiveness of a 1% HCl hydrothermal pre-treatment on BSG at solids-loadings of 5-25% (w/v). The main measurements used to compare the pre-treatment efficacy were the % mass loss during pre-treatment and the sugar yields following enzymatic saccharification of pre-treatment solid residue using Celluclast® cellulase. Following this, experiments at 25% (w/v) solids-loading were then conducted using alternative acid catalysts (either acetic, boric, formic, lactic, phosphoric, nitric or sulphuric acids) each at a concentration of 1% (w/v) to determine which was most effective.

The individual and combined impacts of solids-loadings during pre-treatment (1% HCl at 25-50% w/v solids loading) and during enzymatic saccharification with Novozymes Cellic® CTec2 (at 15-30% w/v solids loading), on final sugar yields attained were investigated using a D-optimal designed experiment (19 experimental runs; see Appendix 2 for full details). The D-optimal algorithm chose the treatment combinations to be conducted. This reduced the total number of experiments needed to be conducted from that of a full factorial experimental design yet still provided a statistically valid data set.

4.2.5 Optimisation of an alkali-catalysed hydrothermal pre-treatment for BSG

Preliminary experiments tested a range of alkaline earth metal hydroxides (NaOH, KOH or $Ca(OH)_2$) as pre-treatment reagents at 1% (w/v) and at a solids-loading of 25% (w/v). Subsequently a D-optimal designed experiment was conducted (14 experiments, 1 block) to investigate the individual and combined effects of varying NaOH concentration (1-5% w/v) and % solids-loadings (15-25% w/v) during pre-treatment. (see Appendix 2 for full details).

4.2.6 Direct comparison of 1% HCl and 3% NaOH hydrothermal pre-treatment

For a direct comparison of the overall suitability of 'optimised' 1% HCl and 3% NaOH hydrothermal pre-treatments for bioethanol production, the subsequent fermentation performance of feedstocks after subsequent enzymatic saccharification was investigated utilising *S.cerevisiae* NCYC479. To produce the fermentation media, 25% (w/v) pre-treatment solids-loading was used, since it was within the effective range for both the 1% HCl and 3% NaOH systems and 15% (w/v) solids-loading was used for enzymatic saccharification with Novozymes Cellic® CTec2.

4.2.6.1 Fermentations of two optimised feedstocks

Trial fermentations of the hydrolysate generated after enzymatic saccharification were conducted. In addition trial fermentations were also conducted using the hydrolysate directly

from the acid catalysed pre-treatment step and also the hydrolysates generated. No suitable feedstock was produced from the alkali catalysed pre-treatment (the hydrolysate did not have any significant quantity of low molecular weight sugars etc.) therefore no fermentations could be conducted for this process stream. The different chemical mechanism of deconstruction (through which the alkali reagent functioned compared to the acid reagent) was suggested to result in only larger molecular weight oligosaccharides liberated into the pre-treatment hydrolysate. This led to an extremely high viscosity hydrolysate that was almost gel-like in appearance.

All fermentations were conducted using *S.cerevisiae* NCYC479 (pitched at 1 x 10^6 cells/mL) using the fermentation and propagation protocols described in section 2.10.1-2. Achieved % theoretical ethanol yields were then calculated using the starting glucose concentration of the feedstock and the x 0.51 ethanol conversion factor (Badger, 2002).

The attempted utilisation of the C5 pentose sugar component that was present in the acid based pre-treatment generated hydrolysate involved trial fermentations using various *Kluyveromyces* strains including *K.marxianus* strain NCYC1426, *K.wickerhamii* strain NCYC546 and *K. dobzhanskii* strain NCYC538. The yeast propagation for these *Kluyveromyces* strains (prior to fermentations) was exactly the same as with *S.cerevisiae* propagation as described in section 2.10.1. The *Kluyveromyces* fermentations were conducted identically to those described in section 2.10.2 (pitched at 1 x 10^6 cells/mL as for the *S.cerevisiae* fermentations) with the exception of use of non-sealed vessels (conical flasks with sterile foam bungs) to allow air ingress to encourage any potential aerobic pentose phosphate metabolism. This was considered to offer both aerobic and anaerobic fermentative metabolic pathways which may have increased the probability of successful utilisation of pentose sugars for ethanol production.

Additional *S.cerevisiae* fermentation experiments were conducted using the acid-derived pre-treatment generated hydrolysate (in an attempt to utilise the glucose present) but this time using a higher pitching rate of 1 x 10^8 cells/mL. This was in order to determine if a higher pitching rate could overcome the inhibitory effect of the furan based inhibitors that were present in the hydrolysate.

All trial fermentations were not optimised in anyway and were simply proof of principle that ethanol could be produced from the generated feedstocks. The trial fermentations would highlight any issues regarding fermentability such as an excessively long initial lag phase.

4.2.7 Viscosity measurement

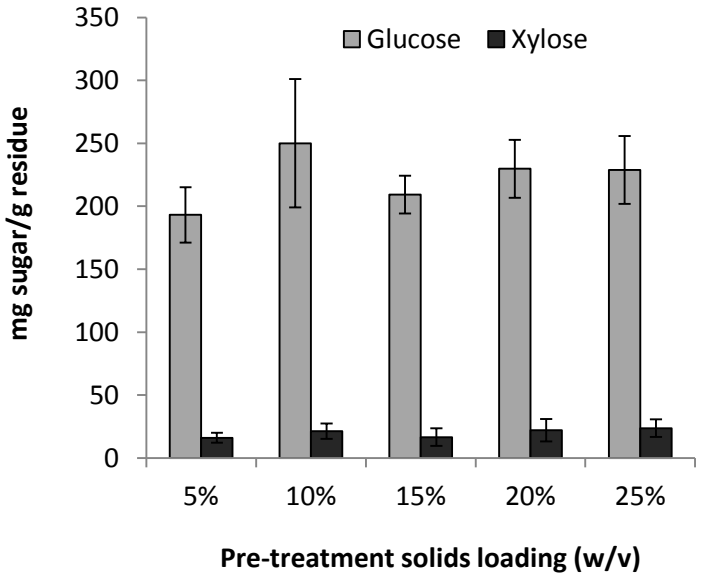
Visual inspection of the slurries that remained after both optimised hydrothermal pre-treatments suggested a significant increase in viscosity of the variant from NaOH based pre-treatment compared to that of the acid based pre-treatment. Therefore attempts were made to measure the relative viscosity of both different slurries. A rotational rheometer (CVO-Rheometer, Bohlin Instruments, UK) fitted with 40 mm parallel plates (150 µm gap height) was used to characterise the rheology (at 25 °C) of selected samples of pre-treated BSG material. Samples were the crude contents of the pre-treatment vessels resulting from 25% w/v solids-loading hydrothermal processes using either 1% HCl or 3% NaOH (each treated at 121°C for 30 min). The shear stress was increased stepwise from 0.01 to 100 Pa. Due to the stick slip phenomenon (particles temporarily arrange themselves in the direction of the compression force being applied and then rapidly slip over each other; Stickel et al, 2009) which often occurs during the increasing stress ramp only the viscosity data acquired during the decreasing ramp was used.

4.3 Results and discussion

4.3.1 The impact of pre-treatment solids-loading

Ability to operate at a high solids-loading is important to the viability of any large scale biofuel production process, due to lower water requirements (and the associated heat input to achieve treatment temperatures) and higher final sugar concentrations (Galbe & Zacchi, 2012; Lu et al., 2009; Petersen et al., 2009). A preliminary investigation was conducted into the impact of solids-loading (5-25% w/v) during the pre-treatment of BSG with 1% (w/v) HCl using the standard hydrothermal conditions (121°C/ 30 min). Using an excess of Celluclast® cellulase enzyme, a similar degree of enzymatic saccharification (200-250 mg glucose/ g pre-treated residue) was achieved regardless of the pre-treatment solids-loading used, confirming that this pre-treatment was effective at solids-loadings up to 25% w/v (Figure 4.1).

Figure 4.1 The impact of pre-treatment % solids-loading (5-25%) on sugar yields following enzymatic saccharification of the BSG pre-treatment solid residue. Hydrothermal pre-treatment: 1% HCl at 121°C for 30 min. Digestion of pre-treated residue: 24 h incubation (50°C) using 200 mg residue to 40 mL of a 1 g/L Celluclast® cellulase solution buffered to pH 5.0. Data are the mean ± SD of three replicate measurements.



4.3.2 Evaluation of different acid and alkali catalysts (each at 1% w/v) during hydrothermal pre-treatment (25% w/v solids-loading).

4.3.2.1 Acid reagents

Based on the sugar yields following saccharification of pre-treatment residues with excess Celluclast® cellulase, 1% HCl performed better in the hydrothermal pre-treatment than all other acid reagents tested (Table 4.1). This treatment liberated 242 mg of glucose per gram of pre-treated residue (which corresponded to 86% of maximum, based on its composition).

There was a clear division in efficacy between the weak acids (acetic, boric, formic and lactic; 24.4-29.9% of theoretical glucose yield on saccharification of the pre-treated residue), and the strong acids (phosphoric, nitric, sulphuric and hydrochloric; 44.2-86.3% glucose yield). Comparing the acids at equivalent percent (w/v) meant that the reagent pH varied both according to the strength of the acid and its molar mass (Table 4.1). Thus, glucose yield on saccharification increased substantially when using an acid pre-treatment reagent with a pH of less than 2. Since there was variation in efficacy between acids of similar reagent pH (e.g. nitric and sulphuric), a role for the counter-ion in the deconstruction of BSG, supplementary to that of the protons, is possible. In particular, there is a suggested role of chloride ions in deconstructing lignocellulosic materials, which may partly account for the enhanced efficacy of HCl relative to other strong acids. Published studies on the use of ionic liquids as lignocellulosic biomass pre-reagents have utilised chlorides such as ammonium chloride and methylimidazolium chloride (Fort et al., 2007; Zhu et al., 2006). It has been proposed that the chloride ions may be key components that are responsible for disrupting hydrogen bonding within the lignocellulosic matrix and thus allowing better subsequent enzymatic access to the cellulose (White et al., 2008). White et al. (2008) also reported that nitric or hydrochloric acids were more effective pre-treatment reagents for BSG than sulphuric acid used at the same molarity. Higher sugar yields on saccharification correlated with higher mass losses

during pre-treatment (linear R^2 = 0.87), confirming the generic relationship between the removal of material and improved enzyme digestibility (improved saccharification) of the residue. Furthermore, there was a trend of increasing production of furan based yeast-inhibitory compounds (sugar degradation products) with increasing sugar yields (Table 4.2). Thus, the most effective acid pre-treatment reagents generated furfural and 5-hydroxymethylfurfural as a consequence of their action. However, neither was released at concentrations thought to be inhibitory for *S.cerevisiae* according to published data (Allen et al., 2010; Liu, 2006). The most effective acid reagents (Table 4.1) and in particular HCl, required a greater quantity of 40% w/v NaOH to adjust to pH 5 prior to enzymatic saccharification (Table 4.2). Over and above differences caused by the varying pH values of the 1% acid solutions, this was probably due to the presence of acetic acid residues hydrolysed from the hemicellulose.

4.3.2.2 Alkali reagents

Using liberated glucose and xylose concentrations after enzymatic saccharification as an indicator of enzyme digestibility, at 1% w/v concentrations NaOH yielded ca. 14% -17% more glucose than KOH or $Ca(OH)_2$ respectively (Table 4.1). This was probably due to the higher molarity of NaOH present (0.25 M) compared to both KOH and $Ca(OH)_2$ (0.18 and 0.14 M respectively). However, at the same w/v concentrations, subsequent sugar yields were poor in comparison with the strong acid reagents (Table 4.1), achieving less than 40% of theoretical maxima. As with the acid-catalysed pre-treatments, lower sugar yields corresponded with lower percentage mass loss during pre-treatment.

HPLC analysis of liquor generated directly from the 1% w/v alkaline reagent hydrothermal pre-treatment of BSG revealed the absence of any mono, di or trisaccharides, suggesting that only a limited breakdown of hemicellulose had happened under these conditions. All 3 alkaline pre-treatment processes required an identical concentration of glacial acetic to adjust the pH of the resulting material to 5.0 (Table 4.2). The concentration of hydroxycinnamic acid (lignin derived) yeast-inhibitory compounds detected in the pre-treatment liquors increased with increasing effectiveness of the alkaline reagent. This is in agreement with the observations of Mussatto et al. (2008), who reported that increased lignin removal in BSG enhanced subsequent enzyme digestibility. However the dilute 1% (w/v) concentrations of alkaline reagent were clearly not capable of enhancing subsequent enzyme digestibility to the same degree as strong acid reagents at 1% (v/v), suggesting a greater proportion of lignin (or possibly hemicellulose) may need to be solubilised to achieve this.

4.3.3 HCl (1% w/v) catalysed pre-treatment: impacts of solids-loadings during both pre-treatment and enzymatic saccharification stages on achieved sugar yields.

19 pre-treatment (1% HCl hydrothermal) experiments were conducted using a D-optimal design space with two factors: pre-treatment solids-loading (25-50% w/v) and the solids-loading during enzyme digestion (15-30% w/v) of the pre-treatment residue. Response data were modelled against these factors using Design Expert software v 7.0 (Stat-Ease, MN, USA).

4.3.3.1 Sugar liberation into the hydrolysate generated directly from pre-treatment

The derived models for sugar release into the pre-treatment hydrolysate (liquid fraction) indicated a non-linear relationship was observed for glucose liberation in response to increasing solids-loading during pre-treatment (Figure 4.2A).

Table 4.1 Effectiveness of different acid or alkaline reagents in the hydrothermal pre-treatment (121°C/ 30 min; 25% w/v solids-loading) of Brewers Spent Grains. Treatments are compared in terms of % mass loss of solids across the pre-treatment, and the glucose and xylose yields when the pre-treatment solid residue was digested with an excess of Celluclast® cellulase from *Trichoderma reesei*. Data are the mean ±SD of 3 replicate measurements.

			mg sugar/g residue		% of theoretical (from pre-treated residue)	
	Calculated reagent pH at 25°C	mass loss (%)	Glucose	Xylose	Glucose	Xylose
1% acetic acid (0.17 M)	2.77	11.0 ±1.98	53.0 ±4.6	21.0 ±1.8	29.9 ±2.7	19.4 ±1.0
1% boric acid (0.16 M)	5.01	11.1 ±0.9	43.5 ±2.0	19.6 ±3.9	24.4 ±3.4	18.3 ±3.6
1% formic acid (0.22 M)	2.21	11.4 ±1.3	46.3 ±4.9	20.8 ±2.8	25.7 ±2.1	19.1 ±2.5
1% lactic acid (0.11 M)	2.42	12.3 ±1.1	53.3 ±5.5	17.5 ±3.3	28.1 ±2.8	16.7 ±2.3
1% phosphoric acid (0.10 M)	1.37	42.7 ±2.5	126.4 ±2.8	18.6 ±1.5	50.6 ±6.9	40.7 ±4.2
1% nitric acid (0.16 M)	0.80	46.7 ±3.1	113.4 ±6.5	14.2 ±2.0	44.2 ±4.2	29.6 ±4.6
1% sulphuric acid (0.10 M)	0.89	54.1 ±2.1	206.8 ±4.6	25.3 ±4.9	77.0 ±2.5	63.4 ±3.5
1% hydrochloric acid (0.27 M)	0.47	61.5 ±4.1	242.7 ±5.2	21.8 ±2.2	86.3 ±3.2	98.0 ±4.2
1% NaOH (0.25 M)		28.3 ±0.5	104 ±6.1	37 ±0.08	37.7 ±1.3	18.1 ±0.08
1% KOH (0.18 M)		25.1 ±1.9	85 ±3.1	31 ±1.6	32.3 ±2.5	16.1 ±1.4
1% $Ca(OH)_2$ (0.14 M)		26.0 ±0.9	82 ±2.5	29 ±2.0	31.2 ±1.7	15.0 ±2.2

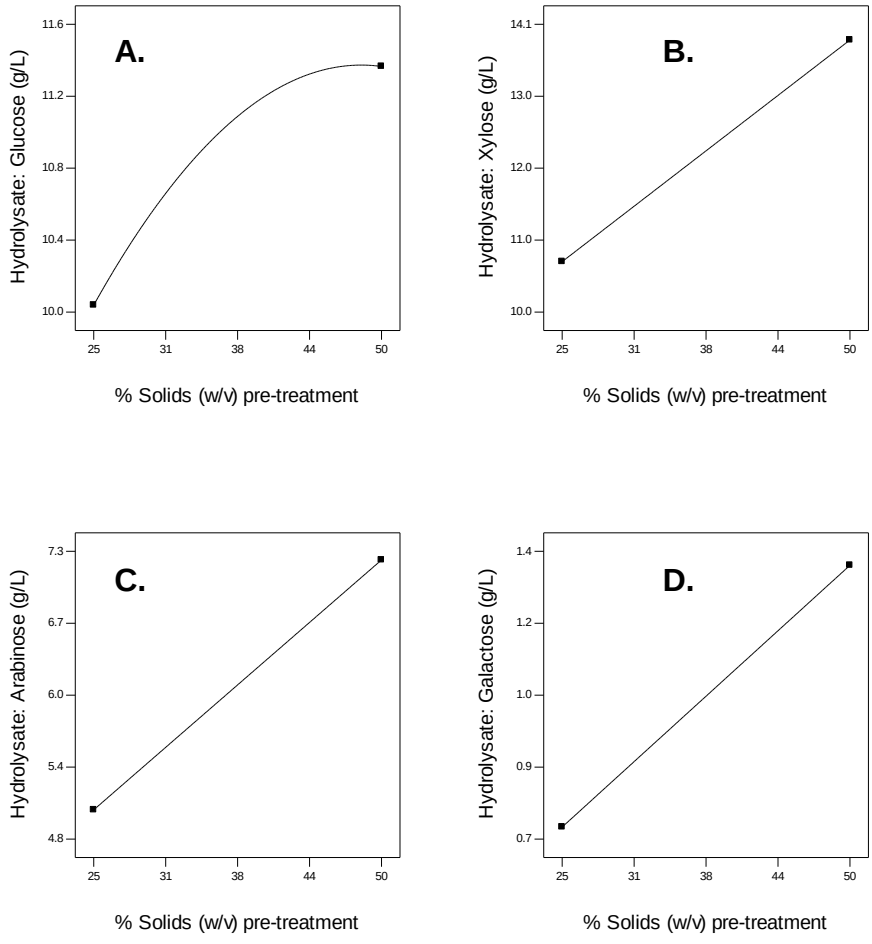
Table 4.2 Quantification of the generated inhibitory compound concentrations from acid and alkali reagents (1%) for the hydrothermal pre-treatment (121°C, 30 min) of BSG. Factors: (i) the generation of compounds with potential yeast-inhibitory activity and (ii) the volume of neutralisation reagent required following pre-treatment and prior to enzymatic saccharification.

	Concentrations of potential yeast-inhibitory compounds (g/L)				Volume (mL) of neutralisation reagent[1] required to adjust to pH 5.0
	HMF	Furfural	Coumaric acid	Ferulic acid	
1% acetic acid	0.061 ±0.003	0.011 ±0.006	-	-	0.38 ±0.02
1% boric acid	0.022 ±0.004	0.0022 ±0.001	-	-	0.38 ±0.1
1% formic acid	0.12 ±0.06	0.040 ± 0.007	-	-	0.40 ±0.1
1% lactic acid	0.088 ±0.04	0.020 ±0.005	-	-	0.38 ±0.07
1% phosphoric acid	0.19 ±0.2	0.25 ±0.1	-	-	0.73 ±0.04
1% nitric acid	0.091 ±0.01	0.091 ±0.01	-	-	0.75 ±0.1
1% sulphuric acid	0.23 ±0.01	0.46 ±0.1	-	-	0.75 ±0.05
1% hydrochloric acid	0.44 ±0.06	0.41 ±0.1	-	-	2.0 ±0.1
1% NaOH	-	-	0.022 ±0.007	0.24 ±0.01	1.0 ±0.2
1% KOH	-	-	0.0096 ±0.001	0.20 ±0.02	1.0 ±0.1
1% $Ca(OH)_2$	-	-	0.011 ±0.003	0.12 ±0.02	1.0 ±0.2

[1] Acid reagent pre-treatment residues were adjusted to pH 5 with 40% (w/v) NaOH whilst alkaline reagent pre-treatment residues were adjusted to pH 5 using glacial acetic acid. Data are the mean ±SD of 3 replicate measurements.

Liberated glucose in the pre-treatment hydrolysate most likely originated from the hydrolysis of amorphous regions of cellulose present within BSG. The fact that glucose concentrations in the pre-treatment hydrolysate 'flattened off' above 44% solids suggests a limit at very high solids levels to the hydrolysing power of the 1% w/v HCl system at 121°C and in the presence of increasing concentrations of amorphous cellulose. In contrast, a linear increase in the concentrations of xylose, arabinose and galactose with increasing pre-treatment solids-loading over the range 25-50% w/v (Figure 4.2 B-D).

Figure 4.2 Design Expert single factor plots for the analysis of the effect of solids-loading (w/v) on the concentrations of monosaccharides liberated directly into the hydrolysate generated during the 1% HCl catalyzed hydrothermal pre-treatment of BSG. A: Glucose (model R^2: 0.85), B: xylose (model R^2: 0.91), C: arabinose model (R^2: 0.96) and D: galactose (model R^2: 0.81).

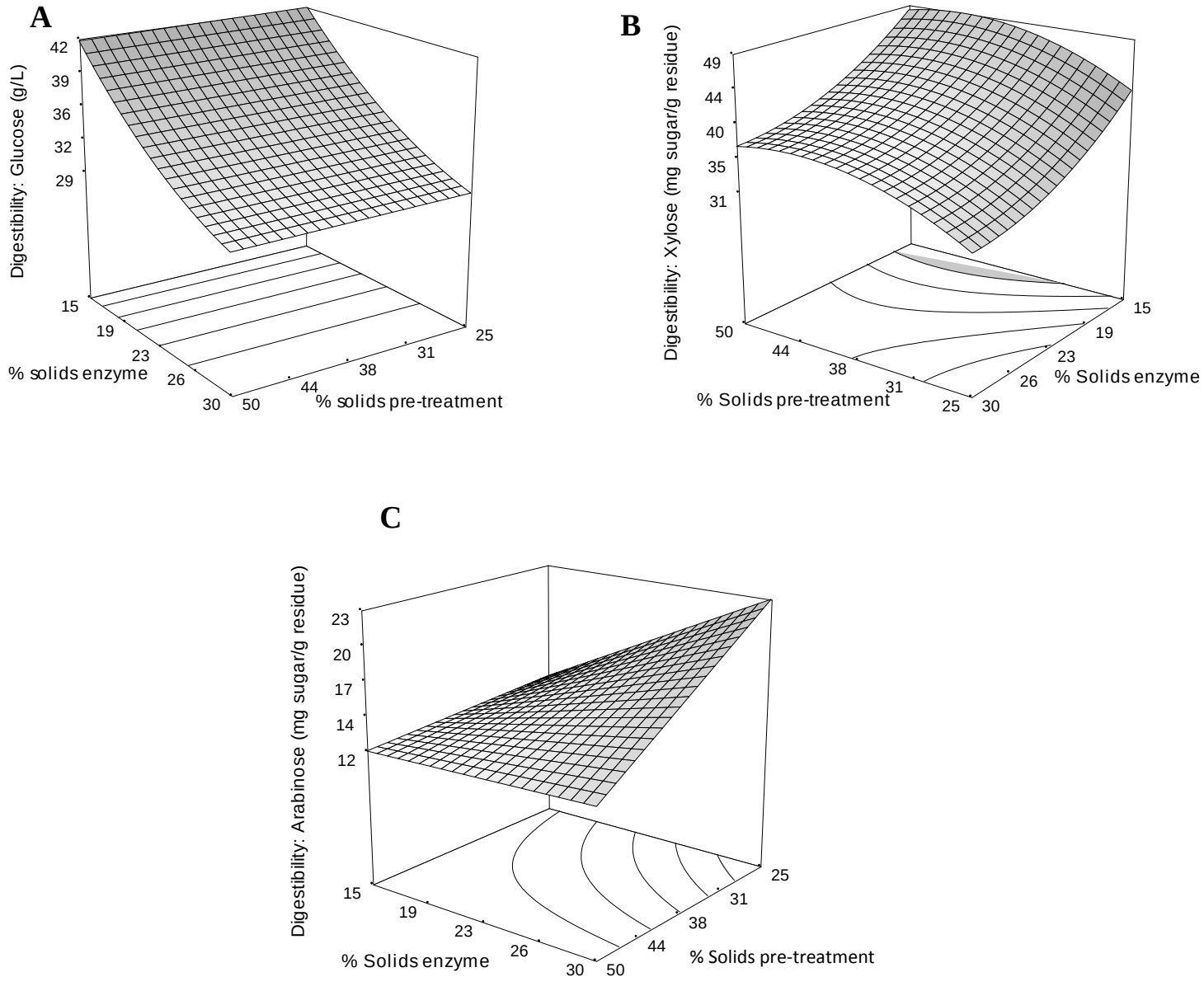


4.3.3.2 Sugar yields from enzymatic saccharification of the pre-treatment generated insoluble residues

Models were also developed for the sugar concentrations resulting when each of the 19 pre-treatment solid residues were subsequently digested with Novozymes Cellic® CTec2 (10 FPU/g biomass, 24 h incubation at 50°C; Figure 4.3). The derived model for glucose (Figure 4.3A) indicated that solids-loading during the enzymatic saccharification step was the main determinant of yield, whilst that during pre-treatment had only a minor influence. Thus, pre-treatment efficacy was retained up to 50% solids-loading using the 1% HCl hydrothermal system (Figure 4.3A) and glucose yield was maximized (ca. 42 g/L) at the lowest (15% w/v) solids-loading during enzymatic saccharification. For xylose (Figure 4.3B), an enhancement in yield was observed when increasing pre-treatment solids-loading from 25% (w/v) to 50%, at which point this trend flattened off, possibly indicating the upper limits of the effectiveness of the pre-treatment had been reached (Figure 4.3B). At these very high pre-treatment solids-loadings it can be concluded that either less of the total hemicellulose present was being solubilized by the pre-treatment (therefore rendering the associated benefit of better enzymatic access to cellulose less pronounced) or that larger molecular weight xylan or arabinoxylan fragments were produced rather than complete hydrolysis to produce monomeric xylose and arabinose. Both scenarios would represent an overall reduction in enzymatic saccharification efficiency and therefore lower fermentable sugar yields, indicating an optimal solids-loading, using the current protocol, of less than 50% (w/v). As noted with glucose, increasing solids-loading during enzymatic saccharification lowered xylose yields (Figure 4.3B). Arabinose yield exhibited a different trend across the design space as compared with glucose or xylose. At higher pre-treatment solids-loadings (ca. 50% w/v) the solids-loading used during the enzyme step did not impact on arabinose yield (Figure 4.3C).

However, at lower solids-loading during pre-treatment (25% w/v) arabinose yield increased linearly in response to increasing solids-loading during enzymatic digestion.

Figure 4.3 Design Expert 3D response surface analysis of the effects of (i) % solids-loading (w/v) during pre-treatment of BSG and (ii) % solids-loading (w/v) during the enzymatic saccharification step on the resultant sugar concentrations (g/L). A: Glucose (model R^2: 0.98) B: Xylose (model R^2: 0.94) C: Arabinose (model R^2: 0.93). Pre-treatment: 1% HCl catalysed hydrothermal (121°C 30 min). Enzyme digestion: Novozymes Cellic® CTec2 (10 µl enzyme per 200 mg residue, 10 FPU/g biomass, 24 h incubation at 50°C).



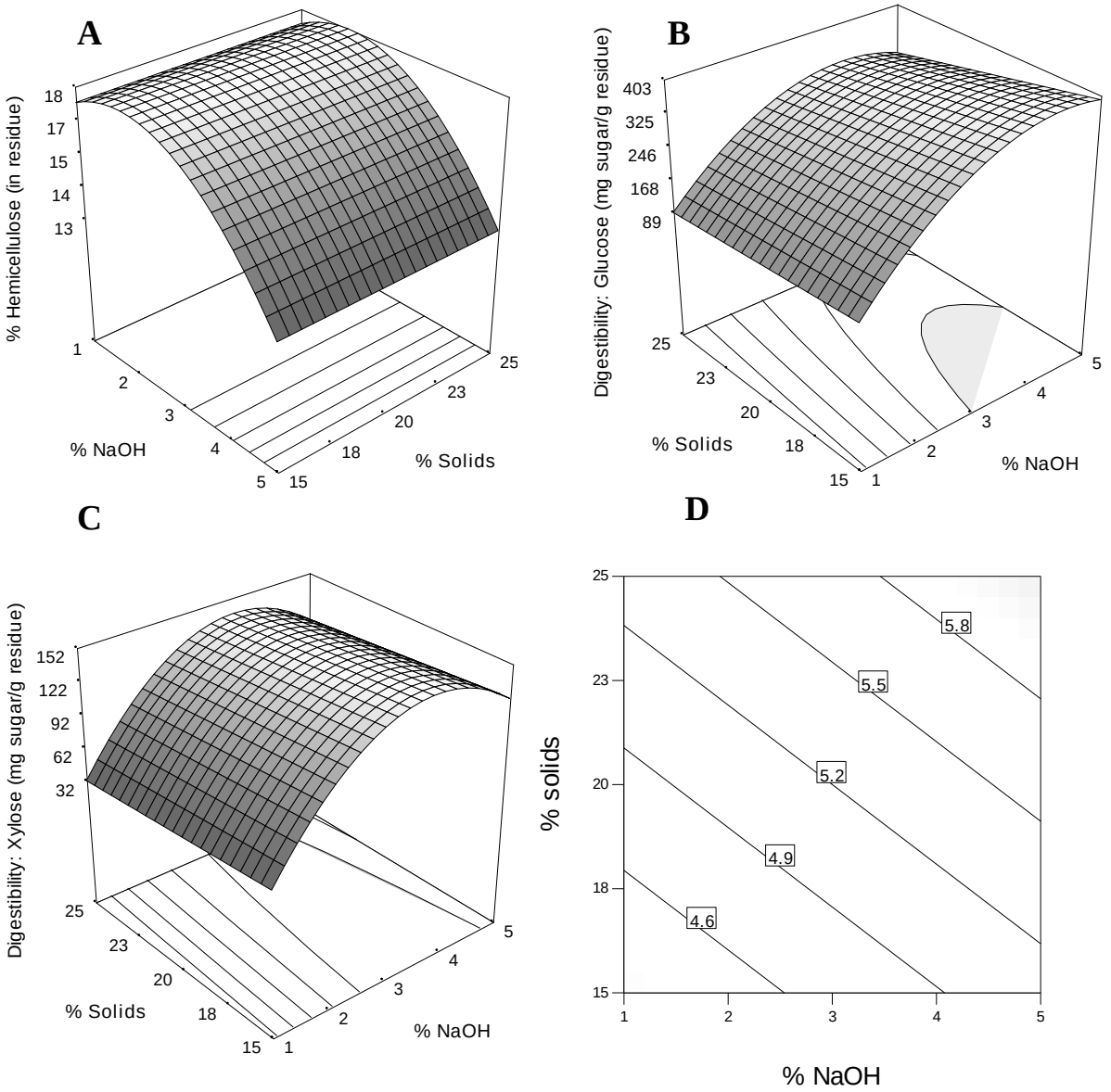
4.3.4 Alkaline pre-treatment: impacts of solids-loading and NaOH concentration.

14 pre-treatment experiments were conducted using a D-optimal design space with two factors: pre-treatment solids-loading (15-25% w/v) and NaOH concentration (1-5% w/v). Response data were modelled against these factors using Design Expert software v 7.0 (Stat-Ease, MN, USA).

4.3.4.1 Solubilisation of hemicellulose

The hemicellulose content (%) of the solid residue recovered following pre-treatment decreased as the concentration of NaOH was increased from 1% to 5% w/v (Figure 4.4A). Thus, under the present hydrothermal process conditions (121°C / 30 min), dilute NaOH only solubilised a small amount of hemicellulose (13% of that present in the feedstock using 5% NaOH), to leave behind a residue which still contained 13-18% hemicellulose on a dry weight basis. This contrasts with the action of higher concentrations of NaOH at higher temperatures or for longer reaction times (>100 h), where, in experiments conducted with wheat straw, it has been shown to readily solubilise up to 80% of hemicellulose present (Janker-Obermeier et al., 2012; Sun et al., 1995). Sun et al. (1995) reported xylan loss of 76.4% from wheat straw treated at room temperature for 144 h with 1.5% (w/v) NaOH; however, the work was conducted at very low solids loading (2.5%). Janker-Obermeier et al. (2012) reported 73% xylan solubilisation using 5% (w/v) NaOH treatment of wheat straw at 140°C for 10 min in a microwave reactor; once again at low solids loading (10%).

Figure 4.4 Modeled data for the hydrothermal pre-treatment (121°C for 30 min) of BSG at varying NaOH concentrations (1-5% w/v) and solids contents (15-25 % w/v). A: hemicellulose content (% w/w) of the resulting solid residue (model R^2: 0.64); and B: Glucose (mg/g biomass model R^2: 0.99), C: Xylose (mg/g biomass model R^2: 0.96) and D: arabinose yields (mg/g biomass; model R^2: 0.50) following enzymatic saccharification of the pre-treatment residue. Digestion of pre-treated residue: 24 h incubation (50°C) using 200 mg residue to 40 mL of a 1 g/L Celluclast® cellulase solution buffered to pH 5.0



There were negligible quantities of low molecular weight sugars in the liquor generated directly from the pre-treatment step in the current experiments. Hence solubilised hemicellulose was present mainly as high molecular weight fragments and this caused the alkaline-pre-treated samples to have high viscosities, particularly at high solids-loadings (≥25% w/v). This could cause processing difficulties if such a high solids process were scaled up. Furthermore, if the aim was to maximise the conversion of substrate sugars to biofuel, this fraction would need to be treated with a xylanase specifically targeting the relevant sizes of xylan fragments, which would represent an additional cost.

4.3.4.2 Saccharification yields from the NaOH-catalysed hydrothermal pre-treatment residues

Pre-treatment residues were digested using 1 g/L Celluclast® cellulase at 50°C, pH 5, for 24 h. The resultant concentrations of glucose, xylose and arabinose (Figures 4.4 B-D) were modelled across the experimental design space using Design Expert software v 7.0 (Stat-Ease, MN, USA). High glucose yields could be attained using this system, reaching >300 mg glucose per gram of pre-treatment residue and with greater yields favoured by higher NaOH concentrations and lower pre-treatment solids-loadings (Figure 4.4 B). The major effect was due to the concentration of caustic used, whilst at any given NaOH concentration increasing solids-loading caused a minor reduction in glucose yield. Bearing in mind the requirement to minimise reagent usage, at any given solids-loading a concentration of around 3-4% w/v NaOH was sufficient to maximise glucose release in this system. This coincided with the concentration range where NaOH was observed to solubilise greater amounts of hemicellulose (Figure 4.4 A), a finding in agreement with prior observations of enhanced sugar yield as hemicellulose solubilisation increases (Lee et al., 2008; Mussatto et al., 2008). This suggests that the primary mode of action of the dilute NaOH hydrothermal pre-treatment of BSG is a physical effect, allowing greater enzyme access to the β-1,4 glycosidic links of

cellulose during subsequent saccharification, but that it did not directly liberate low molecular weight sugars from direct breakage of glycosidic linkages.

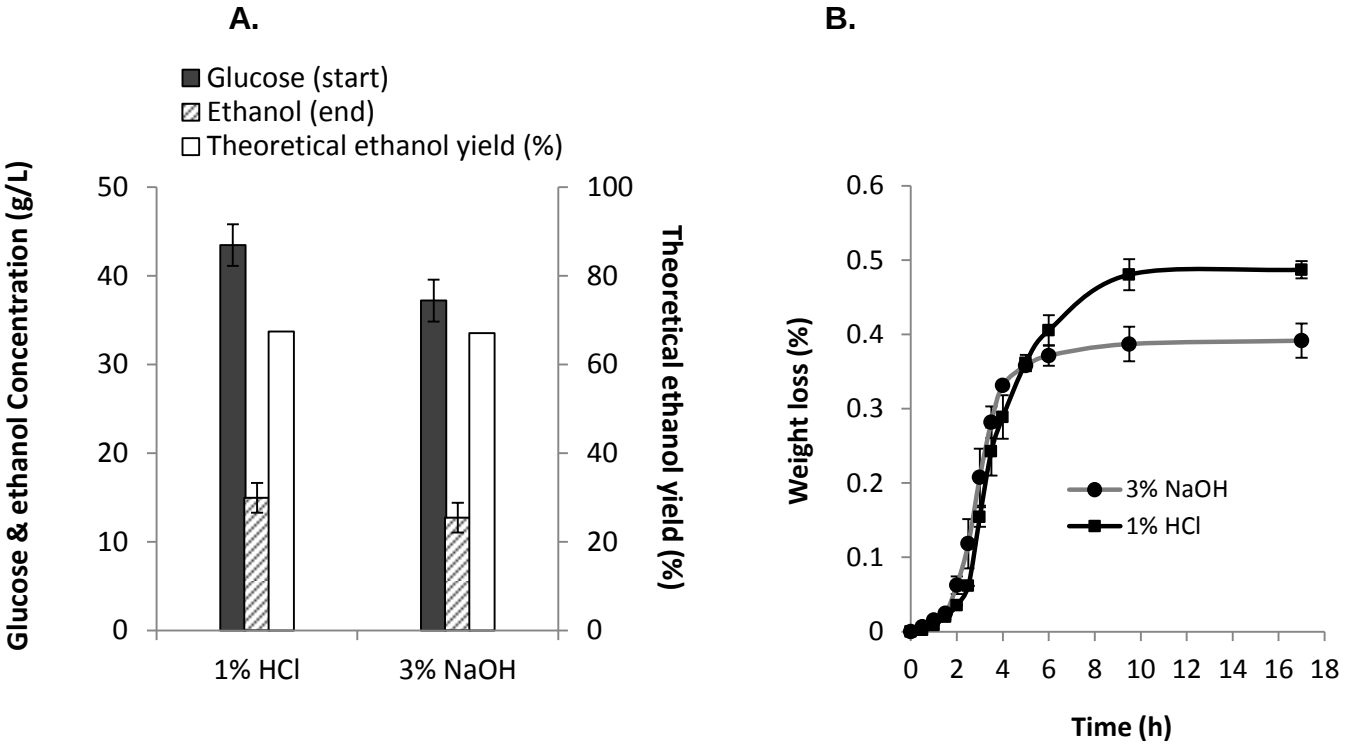
Xylose yield (Figure 4.4 C) exhibited a somewhat similar trend across the design space to that observed for glucose. However as the concentration of NaOH exceeded 4% (w/v) the concentration of xylose recovered after the enzymatic saccharification step decreased. This may have been due to the greater proportion of hemicellulose which was solubilized (into the pre-treatment liquor) at these higher NaOH concentrations. Hydrothermal degradation of xylose (xylans) into furan derivatives may also have contributed to lower xylose yields at higher NaOH concentrations. Furfural was found to be present in the hydrolysates at 0.045 and 0.21 g/L when using 4% and 5% (w/v) NaOH respectively. In comparison only trace concentrations of furfural were detected when using 1-3% (w/v) NaOH. The liberation of arabinose after enzymatic saccharification exhibited a linear trend of increasing arabinose with both increasing solids-loading and increasing NaOH concentration (Figure 4.4 D). However the overall concentrations of arabinose present were minor, relative to those of glucose and xylose as would be expected due to its lower proportion by mass in hemicellulose.

4.3.5 Fermentation of BSG derived feedstocks (pre-treated and saccharified) using *S. cerevisiae* NCYC479

From prior experiments, it was noted that hydrothermal pre-treatments utilizing 1% w/v HCl or 3% w/v NaOH were approximately equivalent in terms of their glucose yield following saccharification (ca. 40 g/L at a solids-loading of 25% w/v). In order to ascertain whether there were specific advantages or disadvantages of the two techniques pertaining to the subsequent fermentation step, trial fermentations were conducted with *S.cerevisiae* NCYC479 using BSG hydrolysates resulting from the two pre-treatment systems. Similar final ethanol concentrations were attained in each system (13-15 g/L) which equated to

approximately 67% of theoretical ethanol yield from glucose (Figure 4.5A). Achieved % theoretical ethanol yields were calculated using the starting glucose concentration of the feedstock and the x 0.51 ethanol conversion factor (Badger, 2002).

Figure 4.5 A comparison of fermentation progression and yield data for the fermentation of enzymatic digested hydrolysates (feedstocks) resulting from acid versus alkaline hydrothermal pre-treatment processes. A: Theoretical ethanol yield based on the initial glucose concentration in the feedstock. B: Fermentation progression monitored by weight-loss of vessels, due to CO_2 evolution. Pre-treatment: 1% HCl or 3% NaOH catalysed hydrothermal (121°C 30 min). Enzyme saccharification: Novozymes Cellic® CTec2 (10 FPU/g biomass, 24 h incubation at 50°C). Fermentations conducted at 15°C with stirring (120 rpm) and using *S.cerevisiae* strain 479 at a pitching rate of $1x10^6$ cells/mL in 100 mL of hydrolysate. Data are the mean ± SD of three replicate experiments.



Overall, glucose utilization was high, with both 1% HCl and 3% NaOH hydrolysates being completely depleted of glucose by the end of the fermentation (data not shown). The available glucose which was not converted to ethanol could have possibly been used to support yeast growth and a short phase of aerobic respiration at the onset of fermentations. However no yeast growth analysis or viability assays were conducted therefore this cannot be confirmed. Additionally if the Crabtree effect was in effect (the inhibition of respiration by

glycolysis due to the presence of the glucose) then the production of biomass would have been unlikely. Therefore the glucose not converted to ethanol may have been used for an alternative carbon end product that was required by the yeast. Regarding fermentation progression (Figure 4.5B), the 3% NaOH hydrothermal pre-treatment generated feedstock performed very similarly to the 1% HCl hydrothermal pre-treatment in terms of the lag phase at the start of fermentation (Figure 4.5B). The NaOH system attenuated more rapidly and at a lower % weight loss, which suggests a lower overall evolution of CO_2. However, this would have likely been simply due to the difference in starting glucose concentration of the two feedstocks. However both systems attenuated rapidly (by ca. 10 h) considering the low fermentation temperature (15°C) which was likely due to the relatively low glucose concentrations present in the feedstocks initially.

4.3.6 Final waste residue following fermentation

Following the fermentation of samples as described in Section 4.3.5, a considerable mass of insoluble co-product was generated. Greater than 25% (dry weight basis) of this remaining residue was found to be protein, when using 1% w/v HCl hydrothermal (121°C 30 min) pre-treatment. This co-product is thus of potential high value and could, for example, be incorporated into animal feed products or be used as a nitrogenous supplement for high gravity brewing if deemed to be assimilable by yeast. Further processes could be developed to enrich the protein component of the final waste residue through fractionation, then leaving a final insoluble, highly recalcitrant fibrous waste product (likely to be highly enriched in lignin and ash) that could be directly burnt for energy either through pyrolysis (Becidan et al., 2007) or plasma gasification (Zhang et al., 2012) making almost complete use of the material.

4.3.7 Comparison of 1% HCl and 3% NaOH hydrothermal pre-treatments of BSG (121°C for 30 min at 25% w/v solids-loading)

Mass losses during the 1% w/v HCl and 3% w/v NaOH hydrothermal pre-treatments were comparable with one another (ca. 50-60%; Table 4.3). As noted, there was a slightly greater glucose yield following saccharification for the 1% HCl pre-treatment process. At 3% NaOH, the improved sugar yields (Table 4.3) relative to a 1% NaOH hydrothermal pre-treatment (Table 4.1) were accompanied by greater yields of lignin degradation products (61% and 25% more coumaric and ferulic acid, respectively; Table 4.2 and Table 4.3).

Table 4.3 Sugar yields and the generation of yeast inhibitory compounds (sugar and lignin degradation products) from acid and alkali hydrothermal pre-treatment of BSG.

	BSG hydrothermal Pre-treatment conditions (25% w/v solids-loading)	
	1% HCl (121°C/30 min)	3% NaOH (121°C/30 min)
Sugars in Liquor following pre-treatment		
Arabinose (g/L)	8.5 ±1.6	0.0084 ±0.2
Galactose (g/L)	1.6 ±0.05	-
Glucose (g/L)	15.3 ±2.1	0.02 ±0.01
Xylose (g/L)	16.0 ±1.1	0.0079 ±0.002
Yeast inhibitory compounds in liquor		
HMF (g/L)	0.44 ±0.07	-
Furfural (g/L)	0.41 ±0.02	-
Coumaric acid (g/L)	-	0.057 ±0.01
Ferulic acid (g/L)	-	0.32 ±0.01
Mass loss on pre-treatment	61.5 ±2.6	51.2 ±1.6
Sugars in hydrolysate after enzymatic digestion of pre-treated residue[1]		
Glucose (g/L)	43.5 ±0.8	37.2 ±0.5
Xylose (g/L)	9.9 ±0.2	4.6 ±0.01
Sugars (% of theoretical)		
Glucose	55.4 ±0.9	31.6 ±2.9
Xylose	98.4 ±1.8	7.4 ±0.51

[1] Digestion with Novozymes Cellic® CTec2 (10 FPU/g biomass); 15% solids-loading, 24 h at 50°C.

However, the concentrations of potential yeast-inhibitory compounds (degradation products) detected in the hydrolysates from both the 1% HCl and 3% NaOH pre-treatments were all

within published limits of tolerability for *S.cerevisiae* (Allen et al, 2010, Liu, 2009, Palmqvist and Hahn-Hagerdal, 2000).

The higher concentration of NaOH, relative to HCl, required for pre-treatment to achieve a similar process yield, meant that more 'neutralisation' reagent was required for the NaOH system in order to adjust the pH to 5.0 prior to enzyme addition. As a consequence higher concentrations of metal salts (i.e. sodium chloride and sodium acetate) are formed through neutralisation reactions when using strong alkaline reagents. This required extensive washing of the biomass in order to minimise any detrimental effects of the metal salts on the enzymes and fermentative organisms used downstream. Whilst this was not problematic on a laboratory scale, there would be implications for process water usage on a large scale.

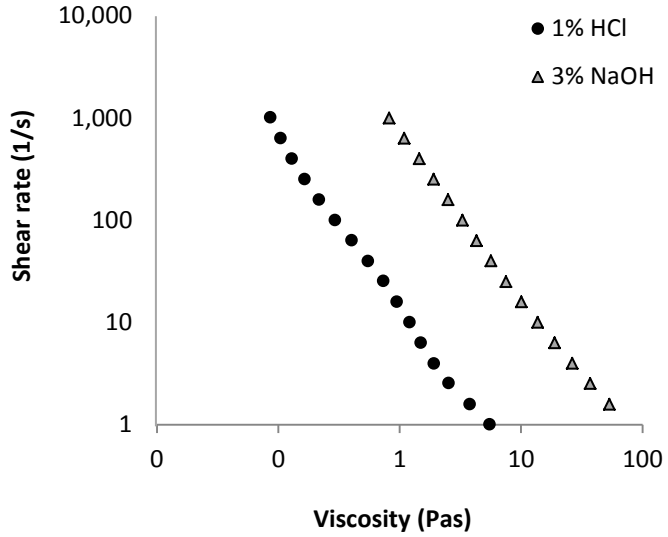
Evaluation of the total quantity of fermentable sugars liberated from each pre-treatment must include the glucose and xylose released into the hydrolysate during the pre-treatment stage. The release of monomeric sugars into this liquor from the 3% NaOH pre-treatment was negligible (Table 4.3). However, for the 1% HCl pre-treatment at 25% w/v solids-loading the liquor contained an additional 569 mg glucose and 595 mg xylose, derived from treatment of 12.5 g of BSG. When this source of fermentable sugars within the process was included in the yield calculations, overall yields of glucose and xylose were found to be enhanced by 35% and 85% respectively when using 1%HCl, relative to the 3% NaOH hydrothermal treatment. Sugar yield targets for commercialization of any bioethanol process would likely be in the region of a minimum of 100 g/L glucose whilst still achieving greater than 80% of theoretical yield. The high glucose (86%) and xylose (98%) yields attained following hydrothermal pre-treatment with 1% w/v HCl at 25% solids (Table 4.1) and using an excess of lyophilized Celluclast® cellulase at 0.5% w/v solids, indicated the effectiveness of the pre-treatment. Using a more commercially relevant dose of Novozymes Cellic®CTec2 and operating at 15% w/v solids-loading during saccharification (Table 4.3) glucose yields were

reduced by around 45% compared to the more dilute assay, indicating sub-optimal saccharification. The viscosity of the mixtures when using high solids-loadings during enzymatic saccharification is thought to limit the mobility of the enzymes, thus resulting in the lower observed yields. To overcome this and achieve better sugar yields within a practical timescale most likely requires a greater enzyme dosage in combination with more effective mixing of the enzyme and substrate. Solids-loading during both the pre-treatment and enzymatic saccharification stages could be increased to try and achieve the 100 g/L glucose target. However, efficiency of conversion (the percentage of theoretical sugar yield achieved) would likely be reduced below the 80% target.

Rheological analysis of the materials resulting from both 1% w/v HCl and 3% w/v NaOH hydrothermal pre-treatments of BSG at 25% (w/v) solids-loading indicated that the 1% w/v HCl pre-treated material had a significantly lower viscosity than the 3% w/v NaOH pre-treated material (e.g. at a shear rate of 10 s-1, the respective viscosities were approximately 1.21 Pas and 13.7 Pas; Figure 4.7).

The use of alkali catalysed hydrothermal pre-treatment at very high solids-loading would thus present considerable practical difficulties in pumping or transporting the material in a large scale biofuel plant, requiring more energy relative to that required if using an acid reagent at an equal solids-loading.

Figure 4.6 Viscosity analysis for a decreasing shear stress ramp of resulting material from hydrothermal pre-treatment (121°C 30 min) of BSG at 25% (w/v) solids loading using 1% HCl or 3% NaOH reagents. Rheometer parameters: 40 mm parallel plates (150 µm gap height), shear stress 0.01-100 Pa at 25°C.

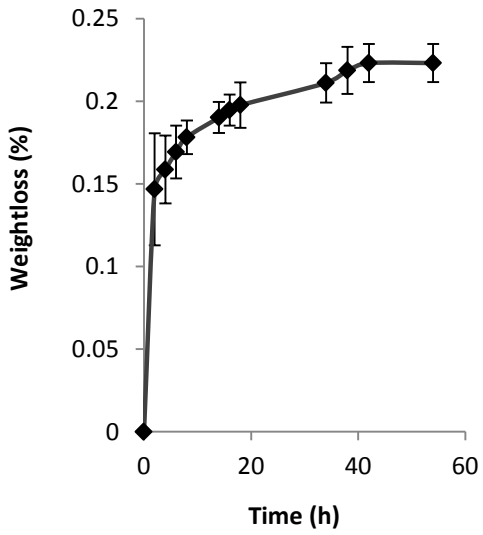


4.3.8 Fermentation of the hydrolysate generated directly during 1% HCl hydrothermal pre-treatment

An efficient bioethanol production process based on BSG would need to utilize the sugars released into the pre-treatment hydrolysate, as well as those remaining in the solid residue. In this experiment, *S.cerevisiae* NCYC479 inoculated at an increased pitching rate of 1 x 10^8 cells/mL was used to ferment 100 mL of this liquor directly, at 15°C. This strain rapidly assimilated >99% of the available glucose with a minimal lag phase, even in the presence of a significant quantity of known metabolic inhibitors (0.44 and 0.41 g/L HMF and furfural respectively). The fermentation vessel weight loss data indicated that the majority of the fermentable sugar (i.e. glucose) was utilized within the first 4 h (Figure 4.7). A final ethanol concentration of 5.4 g/L was produced which equated to 69% of theoretical maximum. This yield was of a similar order to that achieved following saccharification of the pre-treatment solid residue (Section 4.3.5). The success of the fermentations indicated that acetic acid concentrations in this pre-treatment hydrolysate (released from hemicellulose during pre-treatment, but were not quantifiable via HPLC due to co-chromatography with other analytes)

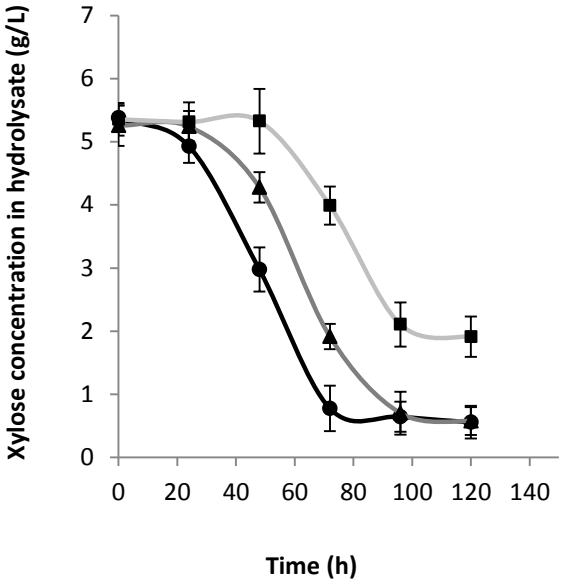
were within tolerable limits for *S.cerevisiae* NCYC479. Overall it showed that whilst the 1 x 10^8 cells/mL pitching rate was very high, a sufficient yeast cell density could overcome even significantly inhibitory concentrations of furan based inhibitors. The quality of the yeast used for a fermentation of this type would not necessarily be a factor which could allow the use of expired 'waste' yeast from the brewing industry to be used. However this high pitching rate would not likely be feasible for very large scale commercial bioethanol production (other than within a brewery etc.). As such inhibitor tolerant yeast strain development would still be required. However whilst this *S.cerevisiae* based fermentation of the pre-treatment hydrolysate was shown to effectively utilise the C6 (hexose) glucose sugars it did not utilise the considerable quantity of C5 pentose sugars. Therefore, some limited attempts were made to find an alternative yeast strain that could potentially utilise C5 sugars. This involved testing various *Kluyveromyces* yeast strains that could possibly not only metabolise C5 pentose sugars but also possibly produce some additional ethanol.

Figure 4.7 Percentage weight loss of fermentation vessels containing C5 pentose sugar rich hydrolysate generated directly from the pre-treatment of BSG using 1% HCl at 121°C for 30 min. Fermentation conducted in sealed 125mL serum bottles using *S.cerevisiae* NCYC479 (at an increased pitching rate of 1x10^8 cells per mL) at 15°C with 100 mL hydrolysate. Data is expressed as mean ± SD of three replicates.



Using xylose uptake as a crude indicator of fermentation progression, three *Kluyveromyces* strains were tested and each exhibited a considerable length in the lag phase before they began to either metabolize (or simply uptake) the xylose (Figure 4.8). However by 48 hours strains *K.marxianus* NCYC1426 and *K.wickerhamii* NCYC546 began using xylose with NCYC1426 performing better (more xylose uptake). By 72 hours strain NCYC1426 had reached its maximal xylose usage point and strain NCYC546 by 96 hours.

Figure 4.8 Xylose uptake from fermentations of a hydrolysate generated directly from pre-treatment of BSG using 3 different strains of *Kluyveromyces.* (●)NCYC1426: *K. marxianus,* (▲) NCYC546:*K. wikerhamii*, (■) NCYC538: *K. dobzhanskii.* Fermentation carried out in 100 mL conical flasks at 25°C with 50 mL hydrolysate and agitated at 120 rpm using magnetic stirring. Pre-treatment; 1% HCl at 121°C at 25% (w/v) solids loading. Data is expressed as mean ± SD of three replicates.



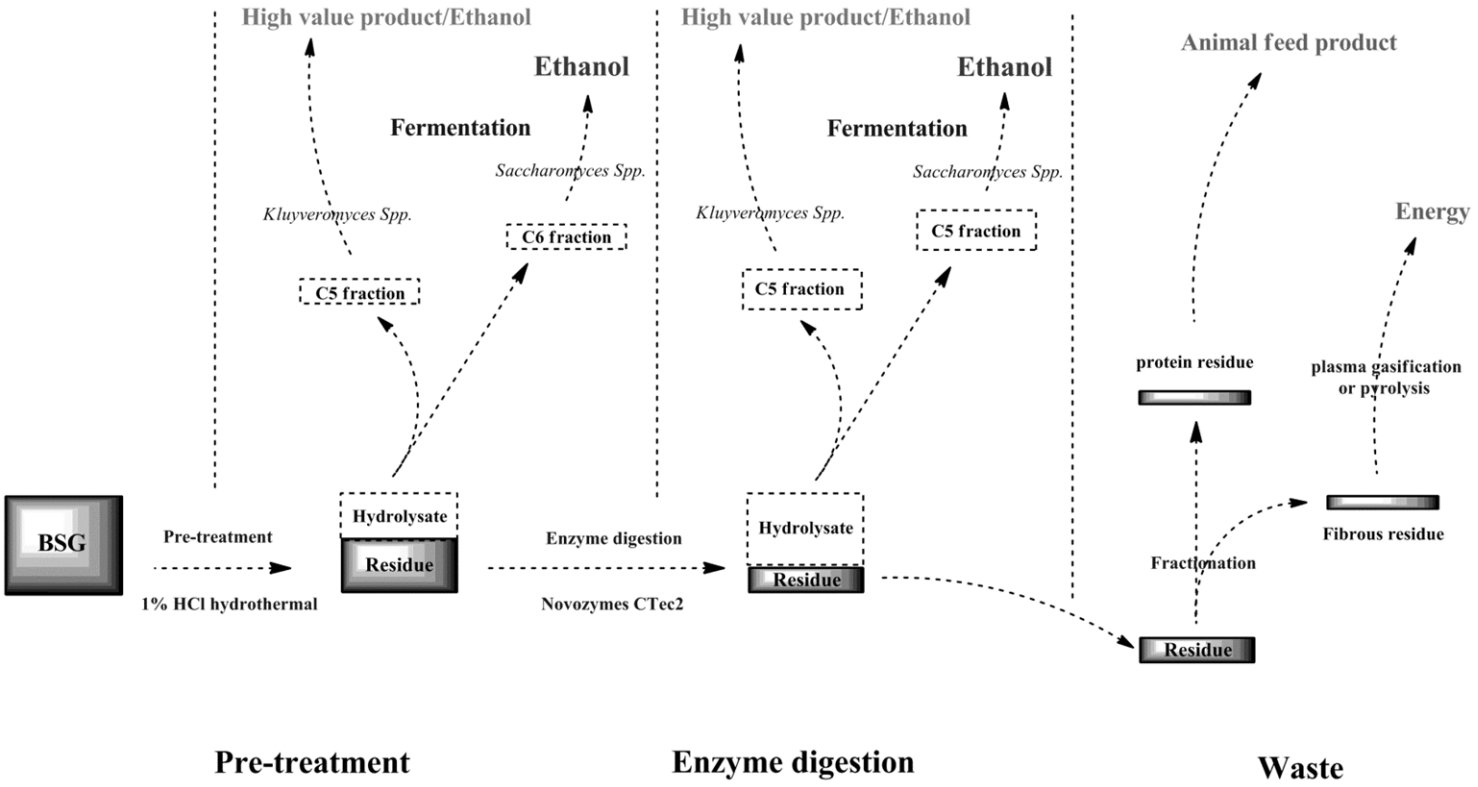
Both NCYC1426 and NCYC546 strains ultimately utilized a similar level of xylose (ca. 90%), with both strains out-performing the *K.dobzhanskii* NCYC538 strain in terms of both the final degree of and rate of usage of xylose. Exclusively in terms of xylose utilization the NCYC1426 strain appeared to be the best candidate for a potential C5 based fermentation. Arabinose utilization over the course of the fermentation exhibited a similar trend (in terms of the performance of the different strains was similar) was observed with strain 1426 appearing to utilize the arabinose most rapidly (data not shown). However negligible final ethanol concentrations were detected in all of the samples from all the different strains. There was also an absence of any xylitol or other likely alternative carbon flux indicator (alternative carbon end products to ethanol). This suggested that all the *Kluyveromyces* strains were either using the xylose for biomass generation or an alternative unknown by-product. Alternative process options would include using the C5 sugar processing stream as a microbiological substrate for the growth of an organism (such as a *Kluyveromyces* strain) but for an alternative high value metabolic product (or platform chemical) rather than ethanol as the target end product. This could be achieved using a GM approach. Another option would be the conversion of the xylose found within the pre-treatment generated hydrolysate into a more suitable format for potential fermentation to ethanol. One option to achieve this would be to convert the xylose into its isomer xylulose via the enzyme xylose isomerase which various *Kluyveromyces* strains have been shown to be able to ferment to ethanol (Chiang et al., 1981; Johansson & Hahn-Hägerdal, 2002; Kötter & Ciriacy, 1993). This could be achieved via the use of exogenous xylose isomerase enzyme to catalyze the conversion. In order to enhance the recovery and enable re-use the enzyme could be immobilized on inorganic beads of some variety such as silica.

4.4 Conclusions

Dilute acid and alkali-catalysed hydrothermal pre-treatments of BSG were investigated as potential cost effective treatments, not requiring the expensive recycling of reagents associated with the use of pre-treatment reagents such as ionic liquids for instance. In terms of sugar yield following saccharification of the pre-treatment residue, 1% w/v HCl and 3% w/v NaOH hydrothermal pre-treatments were found to be effective at a solids-loading of 25%. Although the feedstocks resulting from enzymatic saccharification of the remaining insoluble residues after either pre-treatment could be fermented to yield ethanol concentrations of the order of 12-15 g/L within less than 20 h, the 1% HCl pre-treatment system was preferred due to the lower quantity of reagent used, lower quantities of neutralisation salts formed and the lower viscosity of the system at equivalent pre-treatment solids-loadings.

The total amount of fermentable sugars generated was also higher in the acid system, when sugars released into the pre-treatment hydrolysate were taken into account. With regard to developing a cost efficient process to derive added value from this brewery co-product, it was identified that the liquid fraction (hydrolysate) generated during this pre-treatment could equally be fermented by *S. cerevisiae* to generate additional ethanol. Furthermore, the final solid residue from pre-treatment which remained after enzymatic saccharification was a protein-enriched (ca. 25%) stream of potential value. This could allow for the development of a more efficient processing strategy for BSG that utilised more than just the cellulosic component for biofuel production and a crude proposal of this potential process is shown in Figure 4.9.

Figure 4.9 Proposed processing strategy for BSG and associated potential fractions generated and their possible uses when using 1% HCl 121°C pre-treatment, subsequent enzymatic saccharification using Novozymes Cellic® CTec2 and yeast-based fermentations.



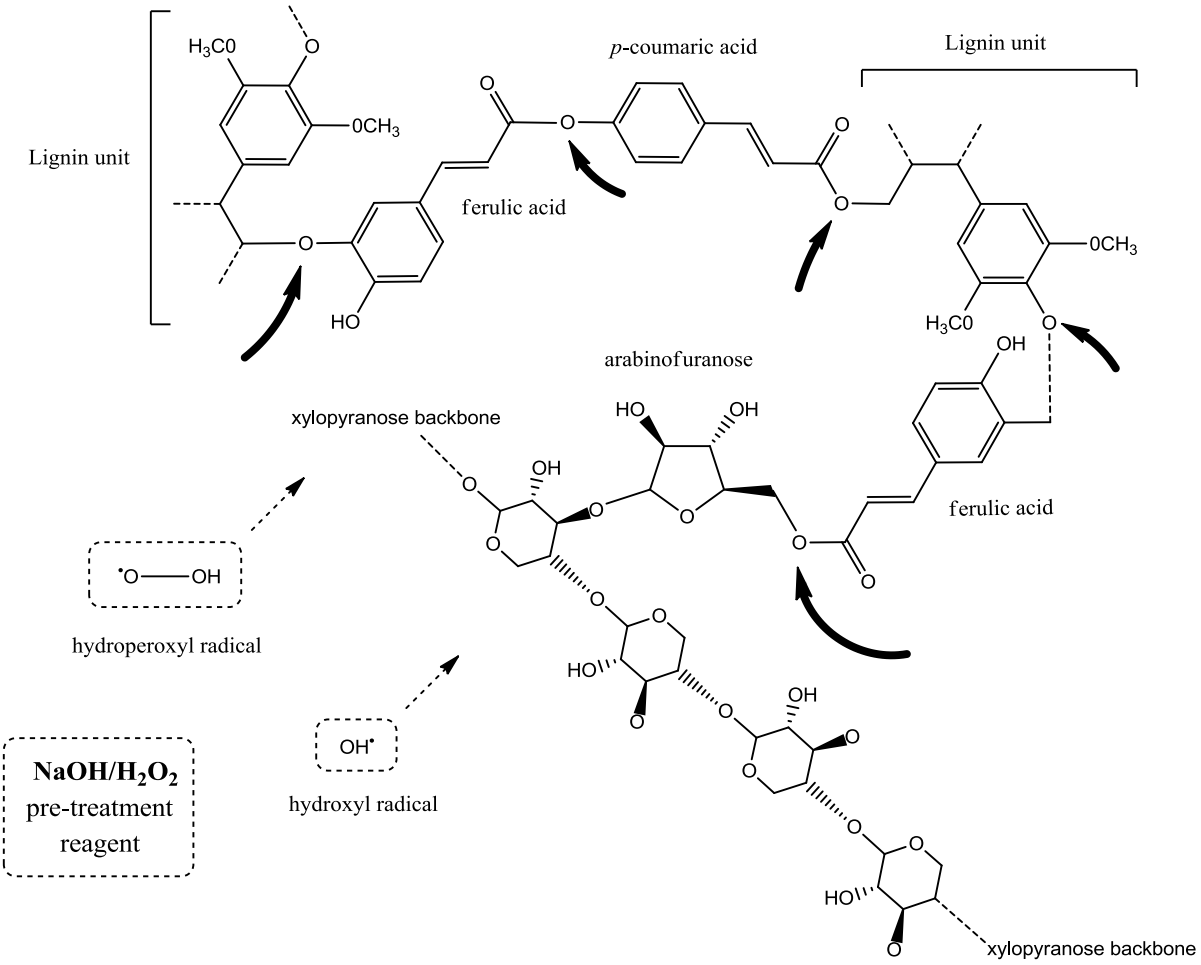
5 Optimisation of alkaline reagent based chemical pre-treatment of Brewers Spent Grains for bioethanol production

5.1 Introduction

Alkaline reagents (such as NaOH) are considered to work primarily by removing lignin (and, to a lesser extent, hemicellulose) which facilitates better enzymatic access to the cellulose and hemicellulose components (Dehnavi et al., 2011). They are also proposed to act via a swelling mechanism which increases the porosity of the biomass (Zhu et al., 2008). The mechanisms which are active during alkaline pre-treatment involve saponification of the ester bonds that link hemicellulose to lignin (Carvalheiro et al., 2008). Removal of lignin may be a key objective in order to improve enzymatic saccharification yields, both because it improves the physical access of cellulolytic enzymes to cellulose and because it lowers the potential for non-productive enzyme binding to lignin. Lignin hydrolysis can result in free ferulic acid and *p*-coumaric acid liberation (Figure 5.1). In addition to NaOH at various concentrations and process temperatures, the use of alkaline peroxide pre-treatments has been investigated for various lignocellulosic biomass types (Yamashita et al., 2010). This pre-treatment method reagent is involves a combination of hydrogen peroxide and sodium hydroxide at pH 11.5 (Cara et al., 2006; Carvalheiro et al., 2008; Saha & Cotta, 2008; Yang et al., 2002). Alkaline peroxide pre-treatments are also considered to target the breakdown of lignin and under the correct reaction conditions can target hemicellulose (Wang et al., 2010). The hydroxyl and hydro-peroxyl radicals (and, to a lesser extent, superoxide radicals) formed in the reagent mixture attack the α-benzyl ether linkages between lignin and hemicellulose through oxidative cracking (Figure 5.1). This initiates the breakdown of the lignocellulose complex, leading to enhanced sugar yields during subsequent enzymatic saccharification (Yamashita et al., 2010). To some extent this mimics the mode of action of native lignin peroxidase and

Mn-dependent peroxidase enzymes used by various fungal species to depolymerise lignin in nature (Ahmad et al., 2010; Bonugli-Santos et al., 2010).

Figure 5.1 Proposed points of attack of hydroxyl and hydroperoxyl radical species in breaking down the lignocellulosic structures present in BSG during alkaline peroxide pre-treatment (Buranov & Mazza, 2008; Crestini & Argyropoulos, 1997).



If on-site production of bioethanol from BSG could be achieved within the brewery it could then be used for steam generation and recycled into the brewing process. On-site utilisation would also negate the requirement and associated costs of storage (or disposal) and transport

of the large quantities of BSG produced. For the successful large scale, economically viable, operation of second generation biofuel production any pre-treatment employed must be highly optimised in terms of minimising chemical and energy inputs (to assist and ensure in achieving a net energy gain) and effective operation at high solids loadings (>25% w/v) would likely be a prerequisite to achieve this. Due to the already widely known effectiveness of alkaline reagents for pre-treatment of lignocellulosic biomass (Carvalheiro et al., 2008; Yamashita et al., 2010; Zhu et al., 2008), this chapter aimed to investigate the effectiveness of NaOH based caustic and alkaline peroxide based pre-treatment of BSG at commercially applicable very high solids loadings and their respective enhancement of subsequent enzymatic saccharification for sugar liberation and fermentation to bioethanol. The research also aimed to examine whether reduction of lignin in pre-treated residues correlated with their enhanced enzymatic digestibility.

5.2 Materials and methods

5.2.1 Alkaline reagent-based pre-treatment of BSG

All pre-treatments were conducted using 500 mL borosilicate glass bottles, in which the appropriate amount of BSG (12.5-125 g) and alkaline reagent (either 1-5% NaOH or 1-5% H_2O_2) were blended to achieve the required solids loading [25-50% (w/v)]. The pH of H_2O_2 solutions were adjusted to 11.5 using 40% (w/v) NaOH solution. Following mixing of the contents, the pre-treatments were conducted by incubating sample bottles in a water bath at the required temperature (20°C, 50°C or 100°C) and for the required duration (1-12 h). After pre-treatment all pre-treated slurries were neutralized to pH 7.0 (±0.5) using glacial acetic acid. The entire biomass slurry was then transferred into 500 mL Nalgene® polypropylene centrifuge tubes and centrifuged at 5000 rpm for 10 min (Heraeus Megafuge 16; Thermo Scientific, UK) and the supernatant removed. The remaining residues were then washed and

dried using the standardized protocol for recovery and processing of pre-treated biomass as described in section 2.5.

5.2.2 Quantification of the total arabinose, glucose and xylose contents of BSG and the insoluble residues remaining after alkaline based pre-treatments

Monosaccharide concentrations in the starting BSG and the remaining insoluble residues after the alkaline pre-treatments were quantified using the two-stage complete acid hydrolysis protocol described in section 2.3.1.

5.2.3 Analysis of the lignin contents of native and pre-treated BSG samples

The acetyl bromide soluble lignin (ABSL) concentrations in BSG and alkaline pre-treated insoluble residues were quantified using the protocol described in section 2.3.3.

5.2.4 Enzymatic saccharification of alkaline pre-treated BSG samples

All enzymatic saccharification attempts using the pre-treated residues were conducted over 24 h incubation periods at 50°C with agitation at 150 rpm (MaxQ 4358 shaking incubator, Thermo Scientific, UK). 50mM sodium citrate buffer (pH 5.0) was added to the biomass to achieve the required solids-loading.

The initial assessments of the efficacy of different pre-treatment processes were conducted using very low solids-loading with lyophilised Celluclast® cellulase from *Trichoderma reesei* (ATTC 26921, Sigma-Aldrich, UK) using the protocol described in section 2.7.1.

High solids-loading, low enzyme dose enzymatic saccharification reactions were conducted using Novozymes Cellic® CTec2 (Novozymes A/S, Demark) as described in section 2.7.2. The Cellic® CTec2 was dosed at 50 FPU/g biomass and at 15 % (w/v) solids loading and using a 24 h reaction time. After saccharification all samples were then centrifuged (5000

rpm, 10 min) prior to sampling for HPLC based glucose quantification as described in section 2.9.2.

5.2.5 Fermentations of two optimised feedstocks

Two alkaline pre-treatments of BSG were selected, on the basis of high sugar yields following enzymatic saccharification, and used to generate sufficient feedstocks to conduct laboratory scale fermentations. These pre-treatments were (each at 25% w/v solids loading): i) 5% alkaline peroxide at 100°C for 5 h (i.e. the 20°C variant of the system was not used due to its poor yields) and ii) 5% NaOH at 50°C for 12 h. Enzymatic saccharification of the pre-treatment residues was conducted using Cellic® CTec2 as described in Section 5.2.4. Fermentations of the feedstocks produced after the alkaline pre-treatments and enzymatic saccharification of the remaining insoluble residue were conducted using *S.cerevisiae* NCYC479 using the fermentation and propagation protocols described in section 2.9.1-2. Achieved % theoretical ethanol yields were then calculated using the starting glucose concentration of the feedstock and the x 0.51 ethanol conversion factor (Badger, 2002). These trial fermentations were not optimised in any way and were simply used as proof of principle that ethanol could be produced from the generated feedstocks. It was anticipated that the trial fermentations would highlight any issues regarding fermentability such as an excessively long initial lag phase.

5.2.6 Optimisation of the fermentation performance of NaOH pre-treatment derived feedstock through nutrient supplementation

The base (control) alkaline pre-treatment derived feedstock was produced using the optimised process described in section 5.2.1. This used 5% NaOH at 50°C for 12 h at 25% (w/v) solids loading. After neutralisation of the pH (using glacial acetic acid) the remaining residues were then washed and dried using the standardized protocol for recovery and processing of pre-treated biomass as described in section 2.5. Enzymatic saccharification of the insoluble

residue that remained after alkaline pre-treatment was conducted using Novozymes Cellic CTec2 using an identical protocol as described in section 5.2.4.

Supplementation of the base alkaline derived feedstock was then conducted using $ZnSO_4$, $MgCl_2$, $CaCl_2$ (at 0.75 mM – 1.5 mM concentrations) or bacteriological peptone (Oxoid, UK and dosed at 10 g/L – 20 g/L) or a combination of both the metal salts and peptone together. Fermentations of the base and supplemented feedstocks were then conducted using *S.cerevisiae* NCYC479 using the fermentation and propagation protocols described in section 2.8.

5.3 Results and discussion

The results reported in Sections 5.3.1 to 5.3.4 are from a series of experiments targeted at maximising the release of sugars during enzymatic saccharification following pre-treatments with either NaOH or alkaline peroxide. An excess of Celluclast® enzyme was used for saccharification in these experiments using the protocol described in section 2.7.1. Whilst the cost of enzyme usage at such levels would not be viable at commercial scale, excess enzyme was used in order to indicate the maximum potential sugar release resulting from each of the pre-treatments.

5.3.1 5% Alkaline peroxide pre-treatment of BSG at room temperature (20°C)

Following pre-treatments with alkaline reagents at varying solids contents, the solid residue was separated from the liquid phase (hydrolysate) containing solubilised material, washed and dried as described in section 2.4. In order to determine the impacts of each treatment on BSG breakdown, compositional analysis of both the raw material and of the residue following pre-treatment was performed (Table 5.1). Components which were enriched in the solid residue were those most resistant to the pre-treatment conditions, whereas components which were readily solubilised into the pre-treatment hydrolysate were reduced

proportionately in the solid residue. Primary targets in this regard were the enrichment of cellulose and the removal of some lignin.

With regard to the degree of cellulose enrichment (as indicated by glucose concentration; Table 5.1A) alkaline peroxide at room temperature (20°C) was only moderately effective, although the efficacy was broadly similar at all solids loadings tested (25% - 45% w/v) with a slight drop off above 30% w/v solids. The concentration of xylose in pre-treated residues was also relatively consistent (ca. 250 mg/g residue) with little change noted as solids loading increased, suggesting there was not a significant quantity of hemicellulose being directly solubilised into the pre-treatment liquor at this low reaction temperature (20°C). Following saccharification with excess Celluclast® cellulase enzyme, the yields of glucose, arabinose and xylose were measured and expressed as a percentage of theoretical maximum (Figure 5.2A), based on the mass of residue hydrolysed and its analysed composition (Table 5.1). At room temperature 5% alkaline peroxide liberated ca. 45% and 35% of theoretical glucose and xylose respectively (Figure 5.2A). A decrease in achieved glucose yield (from 55% down to 40%) was observed as pre-treatment solids loading increased from 25% to 45% w/v which showed that the pre-treatment still worked somewhat (albeit modestly) even at very high solids loadings. Overall very low % theoretical arabinose concentrations were liberated with all solids loadings evaluated. Other than the possibility of their release and subsequent chemical breakdown to unknown degradation products, this was considered to be due to an unknown reason.

A small reduction in acetyl bromide soluble lignin (ABSL) was noted in pre-treated solid residues (Table 5.1A) but there was no obvious relationship between the amounts of lignin removed and the observed variations in sugar yields.

Table 5.1 Composition of BSG and of the solid residues remaining after the specified alkaline pre-treatments. Part A) Alkaline peroxide pre-treatments. Part B) NaOH pre-treatments.

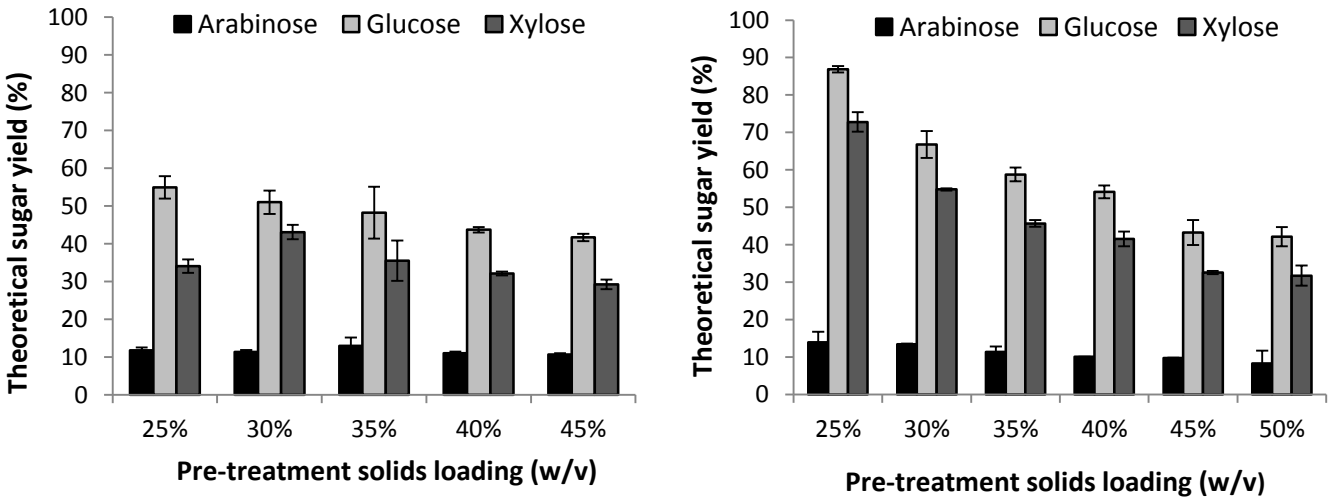
A)				*mg/g (sugar/biomass)*			
Pre-treatment	*Temperature (°C)*	*Solids loading (% w/v)*	*Reaction time (h)*	*Arabinose*	*Glucose*	*Xylose*	*Lignin:ABSL % (w/w)*
BSG				104 ± 4.1	202.3 ± 13.4	276.2 ± 14.7	6.01 ± 0.50
Alkaline peroxide	*20*	25	5	60.1 ± 4.2	363.2 ± 7.8	247.5 ± 3.1	5.56 ± 0.97
(5% H_2O_2 pH 11.5)	*20*	30	5	60.1 ± 2.8	365.0 ± 7.9	246.5 ± 1.0	5.47 ± 0.15
	20	35	5	59.5 ± 5.6	337.7 ± 8.9	234.0 ± 1.0	5.84 ± 0.70
	20	40	5	64.5 ± 2.3	336.4 ± 6.3	249.2 ± 12.3	5.50 ± 0.29
	20	45	5	64.5 ± 1.1	324.2 ± 3.7	232.3 ± 1.4	5.41 ± 0.50
Alkaline peroxide	*100*	25	5	48.9 ± 7.6	499.6 ± 4.1	219.2 ± 6.4	5.41 ± 0.07
(5% H_2O_2 pH 11.5)	*100*	30	5	50.0 ± 6.4	495.0 ± 9.0	222.2 ± 7.7	5.87 ± 0.08
	100	35	5	50.9 ± 3.6	428.0 ± 7.7	240.1 ± 6.7	5.45 ± 1.15
	100	40	5	47.5 ± 5.8	351.5 ± 0.5	249.3 ± 16.0	5.26 ± 0.75
	100	45	5	58.7 ± 2.2	344.9 ± 3.8	267.6 ± 0.1	5.43 ± 1.08
	100	50	5	55.5 ± 1.6	295.8 ± 12.5	271.3 ± 2.3	5.44 ± 1.20
Alkaline peroxide	*100*	30	1	40.5 ± 1.9	340.2 ± 12.6	249.0 ± 2.3	5.67 ± 0.24
(5% H_2O_2 pH 11.5)	*100*	30	3	46.5 ± 2.4	387.2 ± 4.2	233.1 ± 2.8	5.94 ± 0.65
	100	30	5	50.0 ± 9.0	491.0 ± 17.6	221.8 ± 1.5	5.87 ± 0.08

B)					*mg/g (sugar/biomass)*			
pre-treatment	*Temperature (°C)*	*NaOH (%)*	*Solids loading (% w/v)*	*Reaction time (h)*	*Arabinose*	*Glucose*	*Xylose*	*Lignin:ABSL % (w/w)*
NaOH	*50*	5	25	12	54.6 ± 2.4	567.2 ± 4.1	200.2 ± 3.4	4.06 ± 0.0020
	50	5	30	12	52.8 ± 2.5	550.2 ± 3.0	207.0 ± 1.1	4.24 ± 0.27
	50	5	35	12	53.3 ± 4.6	530.9 ± 9.1	220.7 ± 10.6	4.76 ± 0.67
	50	5	40	12	55.8 ± 4.6	453.9 ± 4.4	232.5 ± 7.9	4.84 ± 0.13
	50	5	45	12	60.7 ± 6.0	401.9 ± 2.5	244.3 ± 2.4	5.24 ± 0.092
	50	5	50	12	61.2 ± 2.3	400.5 ± 12.8	245.1 ± 7.5	5.45 ± 0.52
NaOH	*50*	1	25	12	73.4 ± 6.1	314.2 ± 7.1	259.6 ± 4.7	5.59 ± 0.15
	50	3	25	12	58.2 ± 6.2	389.2 ± 9.2	239.3 ± 10.5	5.23 ± 0.51
	50	5	25	12	69.7 ± 6.3	565.2 ± 7.6	204.2 ± 5.5	4.02 ± 0.010
NaOH	*50*	5	30	2	54.9 ± 3.3	500.2 ± 11.3	233.1 ± 5.3	4.42 ± 0.050
	50	5	30	4	47.2 ± 1.8	503.2 ± 10.6	226.9 ± 3.3	4.35 ± 0.046
	50	5	30	6	49.7 ± 0.3	516.2 ± 2.7	215.6 ± 6.1	4.36 ± 0.27
	50	5	30	12	52.8 ± 1.1	547.2 ± 1.7	209.3 ± 1.3	4.14 ± 0.27

The lack of any correlation between enhanced enzymatic digestibility and lignin concentrations in the pre-treated residues could be caused by the re-condensation (post pre-treatment coalescence reactions) of any depolymerised or solubilised lignin back onto the surface of the lignocellulosic material (Fullerton, 1987; Gellerstedt et al., 2004; Li et al., 2012). Therefore changes in net lignin concentrations may not be detectable albeit that the alkaline peroxide reagent evidently performed some degree of chemical modification of the lignocellulose structure, which resulted in greater enzymatic access to the cellulose. Whilst significant concentrations of lignin-derived inhibitors were not detected (via HPLC) there may have been an increase in the release of other, currently unknown lignin-degradation products. Consequently this may have explained the enhancement of saccharification seen with the alkaline peroxide pre-treatment system.

Figure 5.2 Impact of pre-treatment solids loading on sugar yields following enzymatic saccharification of pre-treated BSG residues generated using 5% alkaline peroxide (5% H_2O_2 at pH 11.5; achieved via 40% NaOH) for 5 h at **A**) 20°C and **B**) 100°C.

A. **B**



5.3.2 5% Alkaline peroxide pre-treatment of BSG at 100°C

The extent of cellulose enrichment (as indicated by glucose enrichment) in the solid residues after pre-treatment with 5% alkaline peroxide at 100°C decreased as solids loading increased (Table 5.1A). However at the lower solids loadings tested (25% & 30% w/v) the degree of glucose enrichment was much greater (ca. 500 mg/g residue) than that seen at similar solids loadings with the 20°C pre-treatment (ca. 360 mg/g residue). The higher temperatures used in these experiments were evidently required for effective action of the reagent on BSG. At 100°C some hydrothermal (autohydrolytical) deconstruction mechanisms may also be operative in these systems. A significant degree of enrichment of cellulose within pre-treated residues is desirable as it indicates fractionation of less desired components and presents a greater concentration of substrate (i.e. cellulose) for the cellulases during enzymatic saccharification. In addition, further value streams could be derived from components solubilised into the pre-treatment hydrolysate. Xylose concentrations in the pre-treated residues displayed the opposite trend to glucose concentrations, whereby the degree of hemicellulose solubilisation decreased with increasing solids loading (Table 5.1A) and this was due to an unknown reason. In terms of sugar release following enzymatic saccharification there was a decrease in the efficacy of alkaline peroxide pre-treatments at 100°C when using higher solids loadings (compare Figures 5.2A and B at 25% and 50% solids loadings). Glucose yields approaching 90% of theoretical were attained at 25% (w/v) solids loading. However, sugar yields diminished as solids loadings increased (Figure 5.2B), so that at 45% w/v, for example, there was very little difference between sugar yields resulting from pre-treatment temperatures of 20°C and 100°C. As with the 20°C variant of the system, overall very low % theoretical arabinose concentrations were liberated with all solids loadings evaluated. Other than the possibility of their release and subsequent chemical breakdown to unknown degradation products, this was considered to be due to an unknown

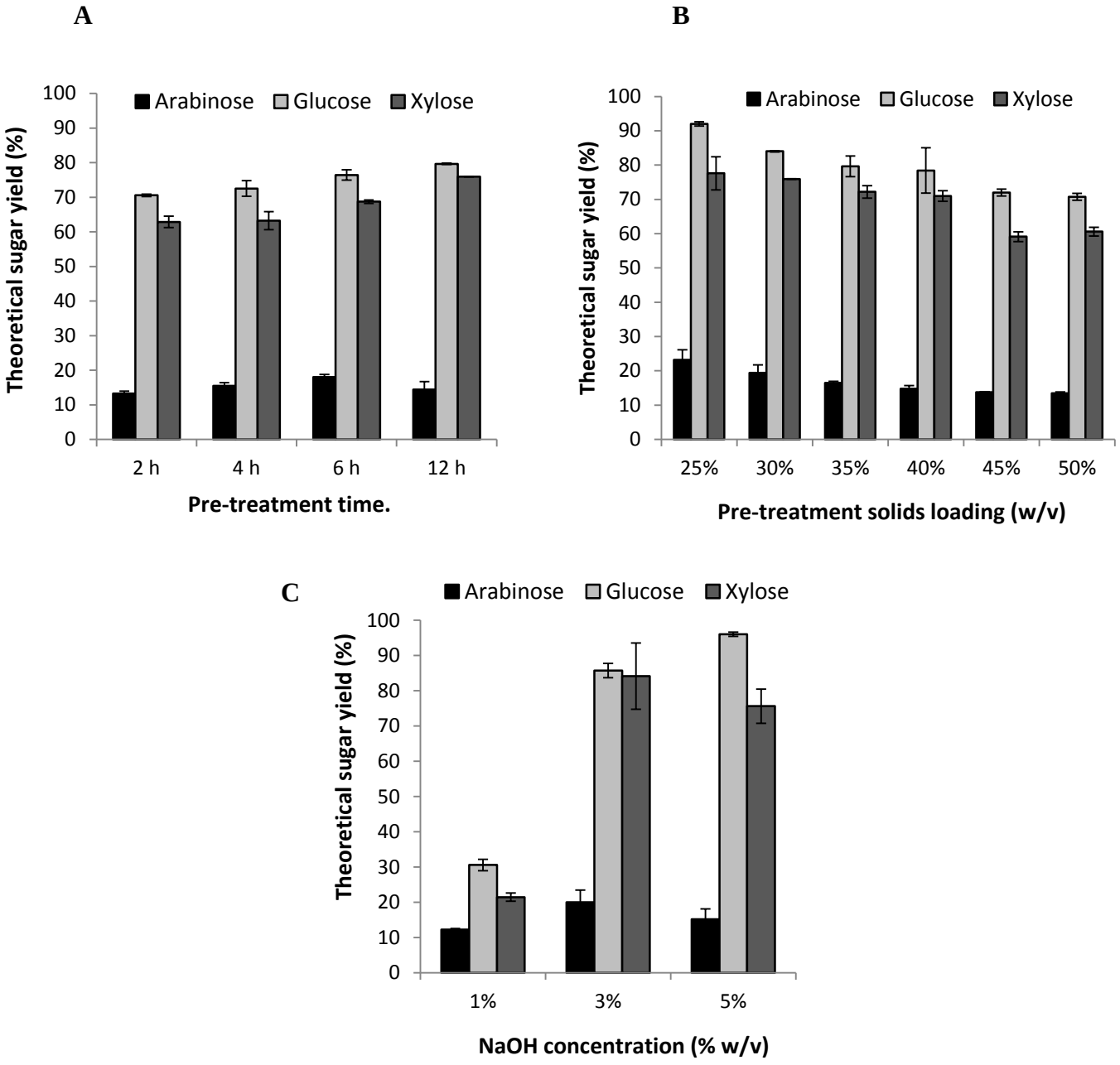
reason. There was no obvious correlation between the lignin contents of pre-treated residues (all ca. 5.4% w/w) and the observed increases in cellulose enrichment and % theoretical sugar yields with decreasing solids loading (Table 5.1A). As with the 20°C variant of this system, significant concentrations of lignin-derived inhibitors were not detected but there may have been an increase in the release of other, currently unknown lignin-degradation products. Consequently this may have explained the enhancement of saccharification seen with the alkaline peroxide pre-treatment system. The effects of shortening pre-treatment time at 30% w/v solids loading were investigated (Figure 5.3A). Whilst shorter holding times at 100°C would be desirable from the perspective of process energy balance, the degree of enrichment of cellulose in the pre-treated residues was significantly reduced at shorter reaction times (Table 5.1A). A small but significant (P<0.01: one way ANOVA) decrease in xylose concentrations of the residues was also seen with decreasing reaction time.

5.3.3 5% NaOH pre-treatment of BSG at 50°C

Effective pre-treatment processes were developed using 5% NaOH at moderate temperature (50°C) and a range of solids contents (Table 5.1B and Figure 5.3B). Xylose concentrations of the pre-treated solid residues increased with increasing solids loading, which suggests that increased solids loading had a negative impact on hemicellulose solubilisation (Table 5.1B). Furthermore, cellulose enrichment in the solid residues declined significantly (P<0.01: one-way ANOVA) with increasing solids loading when using a 12 h reaction time (Table 5.1B). At 25% (w/v) solids glucose concentrations in excess of 565 mg/g were attained in pre-treatment residues. However, even at very high solids loadings [50% (w/v)] glucose concentrations in excess of 400 mg/g residue were still achieved. Following saccharification with an excess of Celluclast® cellulase enzyme, theoretical sugar yields were observed to be maintained, even at very high solids loadings (Figure 5.3B). Pre-treatment with 5% NaOH at 50°C for 12 h at 25% (w/v) solids resulted in glucose yields from the pre-treated residues in

excess of 90% theoretical (Figures 5.3B & C). Whilst sugar yields displayed a downwards trend at higher pre-treatment solids loadings, yields of around 70% theoretical glucose were retained even at 50% (w/v).

Figure 5.3 Sugar yields following enzymatic saccharification of pre-treated BSG residues using NaOH. A) 5% NaOH at 30% solids (w/v) and 50°C for 2-12 h. B) 5% NaOH for 12 h at 50°C with 25-50% w/v solids loading, C) 1-5% NaOH at 25% solids (w/v) and 50°C for 12 h. Enzymatic hydrolysis conducted using 1 g/L Celluclast® cellulase with 24 h incubation at 50°C and 150 rpm agitation. Data are the mean ± SD of 3 replicate measurements.



The ultimate choice of solids loading for a process based around this pre-treatment would be a balance between the percentage of theoretical sugars yielded and the need to derive concentrated sugar solutions for fermentation through working at high solids. Lignin concentrations in the 5% NaOH, 50°C pre-treated residues decreased with decreasing solids loading (Table 5.1B). As the extent of lignin removal increased, so did the cellulose enrichment and subsequently the percentage of theoretical glucose yields attained. The use of NaOH concentrations less than 5% w/v lowered the degree of cellulose enrichment in the pre-treated residues from ca. 565 mg/g with 5% NaOH down to ca. 315 mg/g with 1% NaOH (Table 5.1B). Also there was a significant increase ($P<0.01$: one-way ANOVA) in hemicellulose solubilisation using 5% w/v NaOH, as opposed to 1 or 3 % w/v NaOH, which was evident in the lower xylose content of the pre-treated residue (Table 5.1B). Achieved theoretical sugar yields following treatment with an excess of Celluclast® cellulase enzyme confirmed the superiority, in terms of glucose yields of using 5% NaOH (Figure 5.3B). However, pre-treatment at 25% w/v solids using 3% NaOH for 12 h still resulted in glucose yields as high as 85% of theoretical. Once again there was a trend towards improved cellulose enrichment and higher theoretical sugar yields as more lignin was removed at higher concentrations of NaOH (Table 5.1B). This supports the hypothesis that some degree of lignin solubilisation or depolymerisation was occurring to enhance sugar yields from BSG pre-treated with NaOH. In addition it supports the results described in Dehnavi et al., (2011) which describe the effectiveness of NaOH to fractionate the lignin component found in BSG. The effect of shortening pre-treatment time was investigated at 30% w/v solids loading (Table 5.1B; Figure 5.3C). It was notable that theoretical glucose yields in excess of 75% were still attained following pre-treatment with 5% NaOH for durations as short as 2 h at 50°C (Figure 5.3C). This pre-treatment thus has the potential to operate efficiently at very high solids loadings using moderate temperatures and relatively short contact times. The

technology could potentially be integrated within conventional brewery processes as the treatment time of 2 h is within most brewery turnaround times for mash separation; i.e. each batch of spent grains generated could be discharged and pre-treated in time for the following brew. Spent grains are discharged from mash separation at temperatures in excess of the required 50°C and this excess heat could in part be recovered to heat the required volume of caustic. Modern breweries are equipped to routinely handle NaOH at these concentrations as a part of their cleaning in place (CIP) regimes. Whilst this pre-treatment strategy may not currently be realistic for wide-scale roll-out of the technology within all breweries, it does represent an interesting area of future work that could be further optimised.

5.3.4 Fermentation of BSG derived sugars: a comparison of feedstocks generated following i) alkaline peroxide and ii) NaOH pre-treatments

5.3.4.1 Fermentation feedstocks

In this experiment feedstocks with comparable glucose contents were prepared from BSG using optimised processes for each of the two alkaline pre-treatments investigated in sections 5.3.2 and 5.3.3. The pre-treatments which were compared (both at 25% w/v solids loading) were: 5% NaOH (at 50°C for 12 h) and 5% alkaline peroxide (at 100°C for 5 h). Table 5.2 provides a comparison of the composition of these feedstocks. In contrast to the data reported for pre-treatment optimisation, lower, but commercially relevant, concentrations of cellulolytic enzymes were used to prepare fermentation media from BSG, in order to provide a more realistic appraisal of the overall process feasibility.

Thus, enzymatic saccharification used more commercially relevant doses of Novozymes Cellic® CTec2 (51 FPU/g biomass) at 15% w/v solids loading – a much higher percentage solids than the dilute enzymatic saccharification conducted to appraise maximum sugar release in earlier experiments (0.5% w/v solids loading).

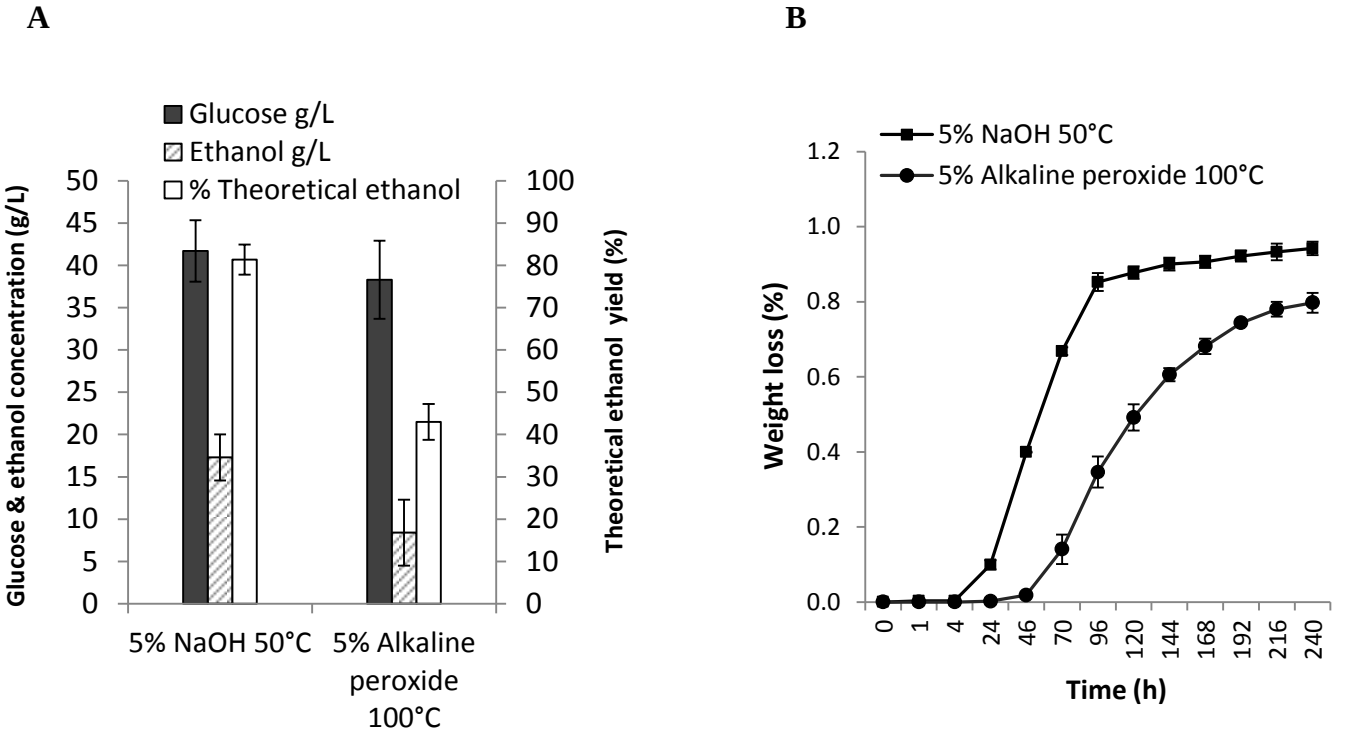
Table 5.2 Sugar and inhibitor (degradation product) analysis of feedstocks produced by alkaline pre-treatments of BSG

	Sugar g/L		% of theoretical		Inhibitors (g/L)	
	glucose	xylose	glucose	xylose	Ferulic acid	*p*-coumaric acid
5% alkaline peroxide 100°C 5 h 25% (w/v) solids	41.7	14.6	49.2	40.2	0.091	0.043
5% NaOH 50°C 12 h 25% (w/v) solids	38.3	10.1	51.2	41.3	0.27	0.054

N.B. These data relate to feedstocks generated from saccharification using commercially relevant enzyme loadings and at high solids content. The sugar yields are thus lower than those attained by the same pre-treatments during optimisation experiments featuring excess amounts of enzyme in dilute solution. Enzymatic hydrolysis used Novozymes Cellic® CTec2 (50 µl per 200 mg biomass, 51 FPU/g biomass , at 15% w/v solids loading using 50 mM Sodium citrate solution buffered at pH 5.0) for 24 h at 50°C. Data are the mean of 3 replicate measurements.

This resulted in glucose concentrations of 41.7 g/L and 38.3 g/L from the NaOH and alkaline peroxide pre-treated residues respectively (Figure 5.4A).

Figure 5.4 A comparison of fermentation progression and yield data for the fermentations (*S.cerevisiae* NCYC479) of enzymatic hydrolysates (feedstocks) resulting from NaOH versus alkaline peroxide based pre-treatment processes. **A**) Initial glucose concentration, final ethanol concentration and % theoretical ethanol concentration at start and end of fermentation for each pre-treatment. Data are the mean ± SD of three replicate measurements. **B**) Fermentation progression (monitored by vessel weight loss) of feedstocks derived from pre-treated and saccharified BSG pitched with approximately 10^6 cells/mL of *S. cerevisiae* strain NCYC479 and incubated at 15°C: (■) pre-treatment with 5% NaOH at 25% w/v solids, 50°C for 12 h , (●) pre-treatment with 5% alkaline peroxide at 25% w/v solids, 100°C for 5 h.



Results for the analysis of the yeast inhibitory compounds (lignin degradation products) ferulic acid and *p*-coumaric acid in each feedstock are also presented (Table 5.2). It should be noted that a wider range of yeast inhibitory substances (e.g. organic acids, furan sugar degradation products) were screened for, but were not found to be present in alkaline pre-treated BSG. This would make alkaline based pre-treatments attractive in terms of the low concentrations of inhibitors present in the feedstocks produced. The presence of ferulic and *p*-coumaric acids is a result of lignin depolymerisation and to some degree benzyl ether bond

cleavage. The 5% NaOH pre-treatment yielded slightly higher concentrations of each inhibitory phenolic compound as compared with the alkaline peroxide method (Table 5.2). The concentrations of *p*-coumaric and ferulic acids in both the NaOH and alkaline peroxide pre-treated feedstocks (0.043 – 0.27 g/L or 0.3 – 1.4 mM) were below those which have been shown to be inhibitory for *S. cerevisiae* (Adeboye et al., 2015) and as such should not have significantly affected fermentation performance. Saccharified feedstocks were fermented as prepared (Section 5.2.4) and without further processing to remove inhibitory compounds.

5.3.4.2 Fermentation performance and ethanol yields

The derived feedstocks were fermented with *S. cerevisiae* strain NCYC 479 at 15°C and fermentation progression monitored by periodically measuring the weight loss due to CO_2 evolution (Figure 5.4B). Fermentation of the NaOH generated feedstock progressed better than for the alkaline peroxide feedstock, as indicated by a shorter lag phase and faster attenuation (Figure 5.4B). Even after 46 h, the alkaline peroxide generated feedstock had not begun to ferment to any significant extent (as indicated by negligible weight loss of the fermentation vessels) and fermentations took significantly longer to attenuate than for the NaOH treated BSG feedstocks. The final mean ethanol yields achieved from the NaOH and alkaline peroxide fermentations were 17.3 g/L and 8.4 g/L respectively (Figure 5.4B). This corresponded to ca. 81% and 43% of theoretical ethanol yields respectively. Achieved % theoretical ethanol yields were calculated using the starting glucose concentration of the feedstock and the x 0.51 ethanol conversion factor (Badger, 2002). The relatively poor fermentation performance of the alkaline peroxide derived feedstock could have been caused by residual hydroperoxyl radicals (HOO^*) or more likely by peroxide mediated reaction products retained in the pre-treated BSG. Various hydroperoxyl compounds have been shown to disrupt yeast metabolism (Dröge, 2002). Adjusting the system to pH 5 for optimal saccharification using Novozymes Cellic® CTec2 might have encouraged the formation of

hydroperoxyl radicals from any superoxide precursors (O_2^-) present (Boulton, 2013; Briggs et al., 2004; Kumar et al., 2009). Future studies could be conducted to quantify these radicals using chemical probes and Fenton oxidation chemistry as outlined in Lindsey & Tarr., (2000). A shift in carbon flux away from ethanol production, due to a stress response caused by the presence of superoxide radicals, could also be a factor in the low ethanol yield achieved using the alkaline peroxide generated feedstock. Increased production of trehalose (a glucose based disaccharide) is one possibility as this often occurs in yeast exposed to environmental stress conditions as it maintains the structural integrity of the cytoplasm (Wiemken, 1990). Another possibility is the increased production of glycerol in order to balance redox potential (Nevoigt & Stahl, 1997) which may possibly be affected by the presence of increased free radicals. Alternatively, the lack of correlation between lignin removal and enzyme digestibility observed when using alkaline peroxide pre-treatments may have indicated the perseverance of hydrophobic lignin breakdown products (which aqueous washing failed to remove) that exhibited yeast inhibitory activity in some capacity.

5.3.4.3 Optimisation of the fermentability of the NaOH pre-treatment derived feedstock

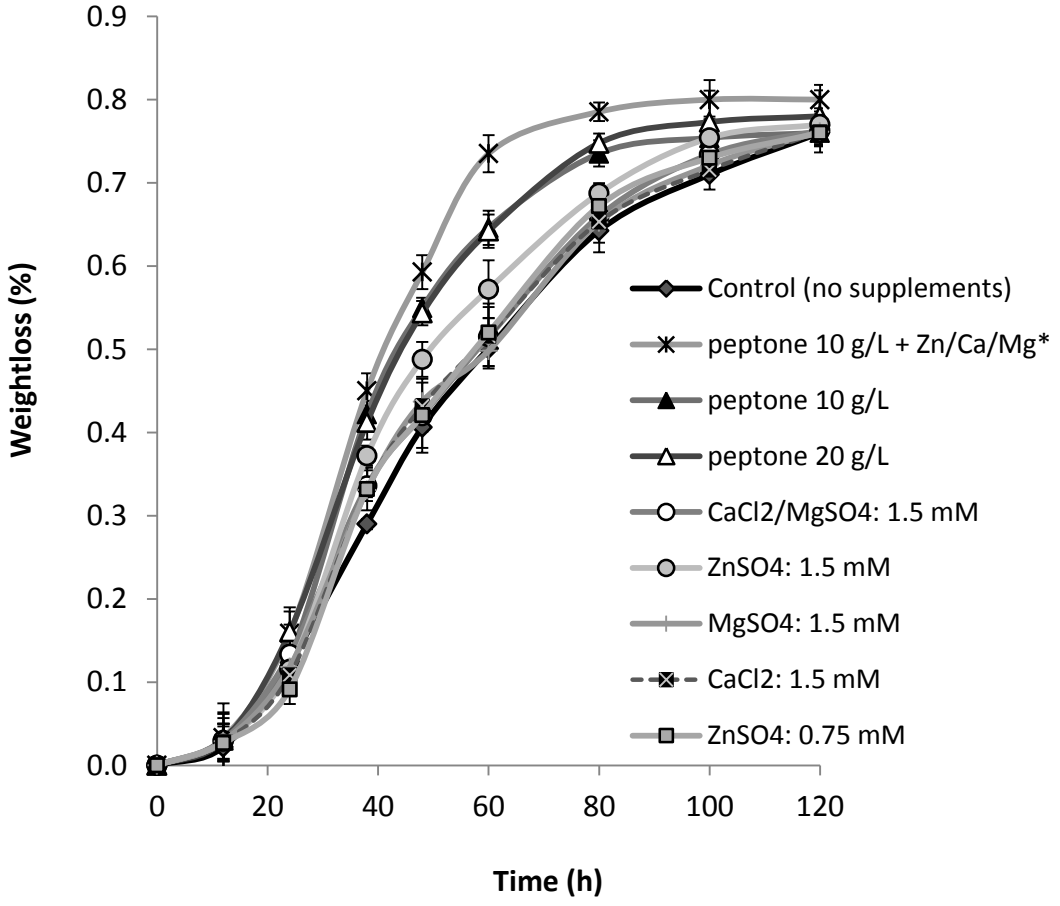
The high sugar yields that were achieved using the optimised low temperature caustic pre-treatment (5% NaOH at 50°C for 12 h) made this pre-treatment strategy a very attractive, relatively energy efficient (without requiring hydrothermal temperatures etc.) processing option. However, the downside to this pre-treatment protocol was the relative slow fermentation performance of the feedstock that was produced (after enzymatic saccharification) with it requiring ca. 70 h before beginning to attenuate (as discussed in section 5.3.4.2). Therefore attempts were made to improve the fermentation performance of this feedstock through the addition of supplementary nutrients that included peptone and a selection of micronutrient metal salts. Whilst the low fermentation temperature used (15°C)

may have contributed to the slow fermentation progression, this was not considered at the time due to a technical oversight.

Peptone, although a complex nutrient mixture, was included primarily as an additional nitrogen source to establish whether the feedstock may have been nitrogen deficient possibly through the action of the caustic pre-treatment removing or irreversibly damaging the native protein content present in the BSG. Whilst peptone may have not been the most cost effective nitrogenous adjunct for large-scale, commercial use, it was used purely for proof of principle to evaluate nitrogenous supplementation. Preliminary success with peptone may have then warranted further investigation of lower cost nitrogenous supplements, even possibly attempting to solubilise or fractionate part of the protein component found in BSG as the source. Similarly micronutrient associated metal salts ($ZnSO_4$, $MgSO_4$ and $CaCl_2$ at 1.5 mM) were also tested to establish if a severe deficiency may have existed in the feedstock as various published work had suggested their supplementation could potentially improve fermentation performance (Nabais et al., 1988; Zhao et al., 2009b).

From all the individual supplements tested peptone addition was shown to offer the biggest improvement in rate of attenuation. Peptone addition (although supplemented at a very high concentration to ensure not rate limiting; up to 20 g/L) reduced the apparent time from which the weight loss of the fermentation vessel began to cease (which was used as a crude marker for fermentation completion) from ca. 120 h (for the control vessels using the NaOH derived feedstock without supplements) to ca. 80 h when dosed at either 10 g/L or 20 g/L (Figure 5.5). The next best performing individual supplement was the inclusion of $ZnSO_4$ at 1.5 mM. Whilst this was considered a huge dose it did reduce the fermentation time (again from ca. 120 h) to ca. 100 h. The use of a lower $ZnSO_4$ concentration (0.75 mM) also offered some improvement but less so than when dosed at 1.5 mM.

Figure 5.5 Effect of metal salts and peptone additions on the fermentation progression of enzymatic hydrolysates (feedstocks) resulting from an optimised low temperature caustic pre-treatment process.



*metal salt concentrations all dosed at 1.5 mM.

Fermentation progression (monitored by vessel weight loss) of feedstocks derived from pre-treated and saccharified BSG. Pitched with approximately 10^6 cells/mL of *S. cerevisiae* strain NCYC479 and incubated at 15°C. Pre-treatment: 5% NaOH at 25% w/v solids, 50°C for 12 h. Enzymatic saccharification: Novozymes Cellic® CTec2 (20 FPU/g biomass, 24 h incubation at 50°C, pH 5.0 ± 0.1) at 15% (w/v) solids loading. Starting glucose concentration ca. 40 g/L. Data are the mean ± SD of three replicate measurements.

In contrast the use of $MgSO_4$ and $CaCl_2$ either individually or dosed simultaneously together offered very little improvement over the control feedstock suggesting the initial feedstock was not deficient in these particular metal salts. Overall the largest improvement in fermentation performance was seen with the simultaneous use of peptone (dosed at 10 g/L) and all the metal salts ($ZnSO_4$, $MgSO_4$ and $CaCl_2$; dosed at 1.5 mM) together. This combination was shown to reduce the fermentation time to ca. 60 h which represented a 50%

time saving over the control vessels. This improvement would suggest the poor fermentation issue of the base feedstock was more complex than just the deficiency of one single mineral or nutrient. Alternatively, the slow fermentation issue may have been related to the presence of an inhibitory compound (i.e. a lignin-degradation product such as a lipo-soluble phenolic acid but of unknown identity as the HPLC-based method was not exhaustive) of which the supplements possibly assisted in the metabolic detoxification of.

As all the fermentation permutations (with each of the different supplements etc.) started at the same initial gravity (a glucose concentration of ca. 39 g/L) any observed differences in rate of attenuation were considered to be due to the effect the supplements alone. Also the final ethanol concentrations achieved from each fermentation permutation were all similar achieving ca. 16 g/L and ca. 80% theoretical yield. Only the rate of attenuation was affected (improved) by the addition of the best performing additives. It should be noted that the control fermentations (with no supplementary additives) within this batch of optimisation experiments took slightly longer than fermentations of a similar produced feedstock presented in Figure 5.4A (section 5.3.4). This was considered to be due to the larger scale of the pre-treatment and enzyme saccharification steps that were conducted in order to ensure enough base feedstock was produced to conduct all the optimisation experiments. As a consequence of the scaling up (with the associated variation in efficiency of each of the process steps) it was suggested that a higher concentration of an unknown inhibitory compound may possibly have been present. HPLC based analysis of the base feedstock using the sugar and lignin derived inhibitor screening method (described in section 2.8.3) did not identify any significant differences in the spectrum or quantity of any known inhibitors when compared to the smaller scale feedstocks that were produced for the previous fermentations (section 5.3.4.2) However, this HPLC based inhibitor screening method was not exhaustive and there may have been as of yet unidentified degradation products generated from the pre-

treatment step. This may have put the yeast under some additional metabolic stress that resulted in a slightly longer attenuation time. Alternatively, whilst all reasonable steps were taken during the propagation of the yeast (prior to all the fermentations) to ensure a similar culture of cells was generated for controlled experimental conditions, there may have been slight differences in the health of the yeast cells. Whilst cell counts and cell viability assessments were made, no other assays or assessments regarding yeast cell health were conducted. As such there may have been slight differences in the health of the yeast culture (such as intracellular trehalose or ergosterol concentrations) which then resulted in the slower fermentation rate.

5.4 Conclusions

Here we have developed two alternative strategies for the alkaline reagent based pre-treatment of BSG to maximise the release of sugars for bioethanol production. Alkaline peroxide treatment using 5% H_2O_2 at pH 11.5 was optimal at 100°C for 5 h with a solids loading of 25% and resulted in theoretical glucose yields, using excess enzyme levels, of between 85-90%. Several effective pre-treatments were developed utilising NaOH at a moderate process temperature (50°C) and at concentrations of 3 or 5% w/v. Maximum theoretical glucose yields (>90%) were obtained using 5% NaOH for 12 h at 25% w/v solids loading. However, this treatment still performed well at even higher solids loadings (up to 50% w/v) or when treatment time was reduced as low as just 2 h at 30 % w/v solids loading. For NaOH pre-treatments the extent of lignin removal from pre-treated residues broadly correlated with the efficacy of the pre-treatment in terms of final saccharification yields. However, for the alkaline peroxide systems no such correlation was observed.

Feedstocks prepared using an optimised 5% NaOH pre-treatment gave superior fermentation performance and resulted in higher ethanol yields than resulted from the use of alkaline

peroxide derived feedstocks. Thus, when specifically considering alkaline based pre-treatments, the 5% NaOH pre-treatment is preferred for future development due to its superior flexibility regarding process times and solids loadings, as well as the noted superior fermentation performance and ethanol yield (when compared to the alkaline peroxide system). The latter remains modest (17 g/L ethanol) and will only be significantly improved if enzymatic saccharification is developed to work optimally at higher solids loadings than is currently the case. In addition, even the NaOH derived feedstock exhibited a somewhat slower fermentation progression when compared to feedstocks produced using the dilute acid or alkali catalysed hydrothermal pre-treatments (121°C) described in the previous chapter (section 4.3.5). However various supplements, namely peptone and zinc were shown to significantly improve the fermentation performance (rate of attenuation) of the NaOH derived feedstock. Overall, large scale trials and economic calculations would need to be conducted to establish whether the cost of these fermentation additives would be financially justified.

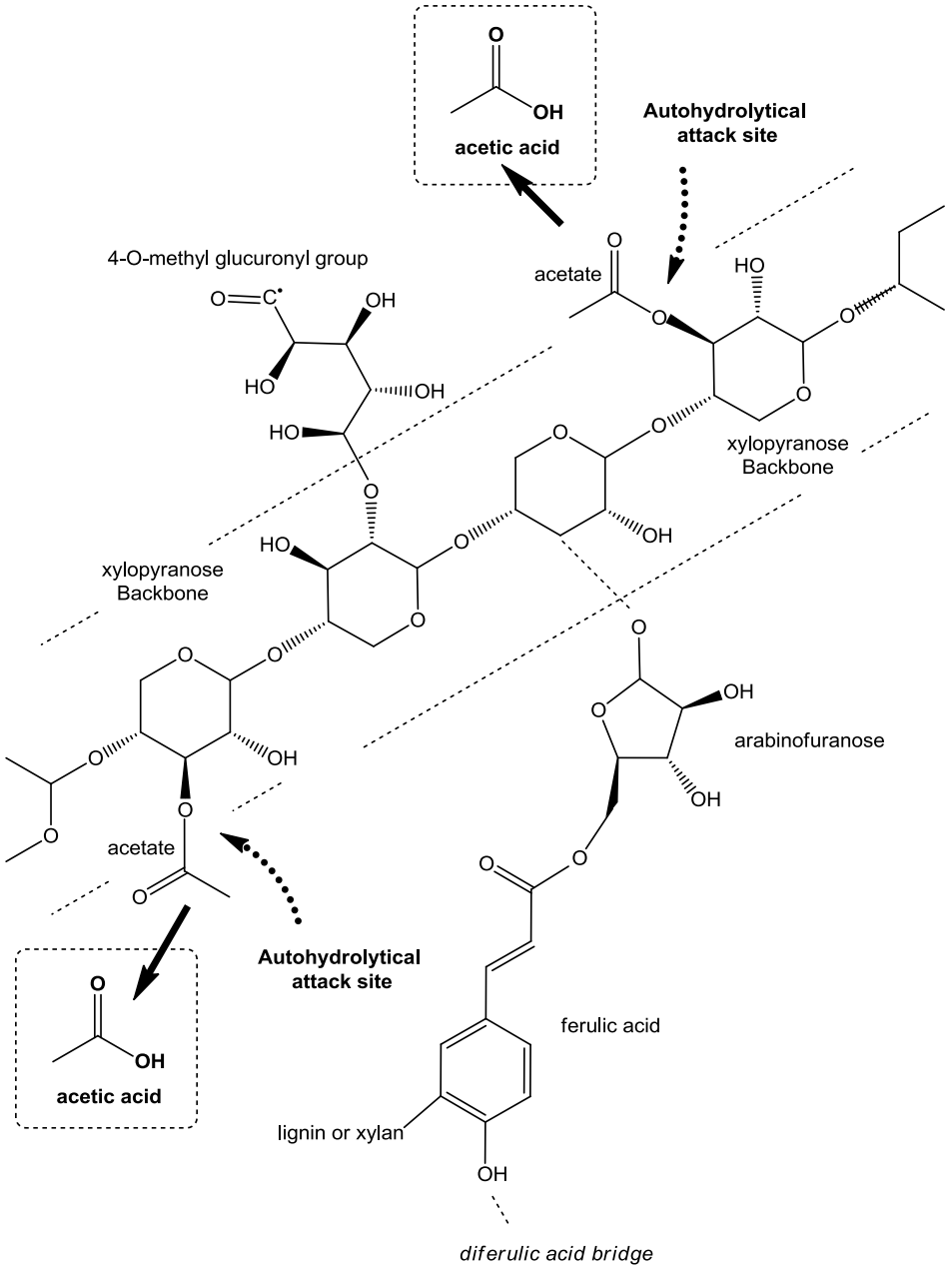
6 Optimisation of hydrothermal pre-treatment methods for degradation of BSG

6.1 Introduction

The primary mechanism of both the autohydrolytical and acid-catalysed hydrothermal pre-treatment of lignocellulosic biomass involves removal of hemicellulose, which exposes the remaining cellulose fraction thus making it more amenable to enzymatic saccharification (Chundawat et al., 2011; Lee et al., 2008; Sanchez & Cardona, 2008). The specific mechanism of autohydrolytical pre-treatment involves cleavage of acetylated xylose units from hemicellulose, yielding free acetic acid residues (Figure 6.1). The acetic acid thus released then acts to further hydrolyse the remaining hemicellulose present. Use of an additional acid catalyst in theory re-enforces this acid-catalysed hydrolysis through the supply of additional protons.

The research described in this chapter addresses the need for viable processes to operate at high solids content (up to 50% w/v); otherwise the cost of heating process water likely becomes prohibitive, even though yield and material handling considerations might suggest working at lower solids-loadings. The prevalence of hydrothermal and steam explosion pre-treatments in existing pilot plant and lignocellulosic bioethanol demonstration facilities indicates their favourability (both practically and economically) when deconstructing lignocellulosic biomass on a commercial scale (Balat, 2011; Chen & Qiu, 2010; Coppola et al., 2009; Ibrahim, 2012; Talebnia et al., 2010). As such it was chosen to evaluate their application to BSG, due to their relative simplicity, cost-effectiveness and in particular as a comparison of autohydrolytical (purely aqueous) pre-treatments with the acid and alkali based pre-treatments evaluated in chapters 4-5.

Figure 6.1 Suggested autohydrolytical cleavage of acetate groups to liberate free acetic acid for further hydrolytic breakdown of hemicellulose (adapted from Dodd & Cann, 2009 and Gupta et al, 2010).



In contrast to more conventional heating methods microwave irradiation (dielectric heating) offers an alternative method for the rapid and controlled generation of heat within materials (Janker-Obermeier et al., 2012; Nüchter et al., 2003), to stimulate hydrothermal reaction conditions. It has also been speculated that rapid internal heating of water distributed

throughout a lignocellulosic material could help to disrupt that structure effectively. Microwave based pre-treatment has been shown to work effectively with many lignocellulosic feedstocks such as rape straw, liberating ca. 54% of theoretical glucose (Lu et al., 2011), sugarcane bagasse (liberating a reducing sugar yield of 0.83 g/g dry biomass (Binod et al., 2012), wheat straw (Jackowiak et al., 2011; Xu et al., 2011) and even BSG, liberating 62% of all arabinoxylans and feruloylated arabinoxylo-oligosaccharides (Coelho et al., 2014).

The present research within this chapter aimed to evaluate the feasibility of a bioethanol production process from BSG, with a focus on optimising the microwave based pre-treatment step as has been done with the previous pre-treatment based chapters 4-5. For the reasons outlined above, we elected to evaluate autohydrolytical and acid-catalysed hydrothermal pre-treatment of BSG at commercially relevant solids loadings (>25% w/v) and using a microwave reactor. It should be noted that the microwave reactor used in these studies was a piece of laboratory apparatus, designed for rapid and accurately controlled heating. Following enzymatic saccharification we conducted trial fermentations again using *Saccharomyces cerevisiae* to assess the fermentability of the feedstocks produced from BSG and the resultant ethanol yields.

6.2 Materials and methods

6.2.1 Microwave based pre-treatment of BSG

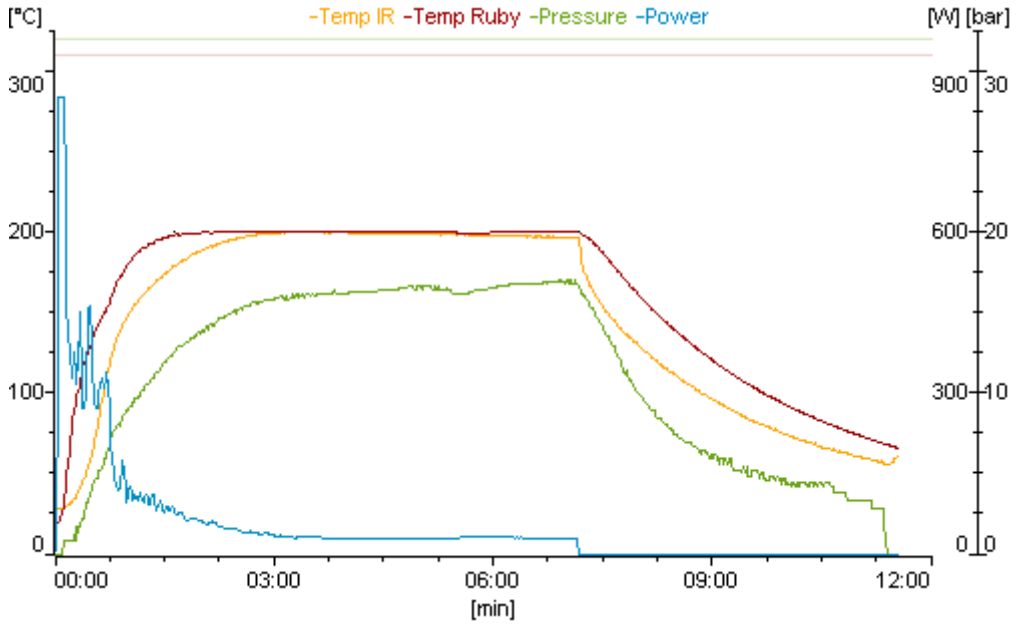
Both autohydrolytical and dilute acid catalyzed microwave based pre-treatment of BSG were conducted using a laboratory scale Monowave 300 microwave synthesis reactor (Anton Paar Gmbh, Gratz, Austria) using both infra-red and ruby based fibre optic thermometers for accurate temperature control. Figure 6.2 depicts an example output chart displaying the parameters from a typical 200°C pre-treatment run using the microwave reactor.

Autohydrolytical pre-treatment was investigated using a 120-200°C temperature range, a reaction time range of 1-15 min and over a 2.5 - 35% (w/v) range of solids loadings (Tables 6.1-6.2). The experimental design software DesignExpert (Stat-Ease Inc., Minneapolis, MN, USA) was employed to create experimental designs that would reduce the total number of experimental runs whilst still accurately statistically modeling the data to determine any variance (see Appendix 2 for full details).

Acid catalysed pre-treatment was conducted using 1% HCl (v/v) using a temperature range of 120-200°C, a reaction time range of 1-15 min and a 30-50% (w/v) range of solids loadings. Although acid catalysed pre-treatments had been previously tested in chapter 4, this additional research was conducted to evaluate the effect of an additional acid based catalyst at higher temperatures than had been previously tested (in excess of 121°C autoclave temperature).

BSG (0.7 - 5.0 g) was accurately weighed into the specified glass G30 (30 mL) microwave reaction vessels (Anton Paar Gmbh, Gratz, Austria) with 8.0 - 25 mL water to achieve the correct solids loading. The experimental runs were then heated to the correct temperature and for the desired time period and then cooled initially through the microwaves own air based cooling system to 70°C and then finally through submersion into cold water to stop any further reactions. Any remaining hydrolysate (the liquid fraction) after pre-treatment was then sampled and analyzed for sugar concentration and metabolic inhibitor (degradation products) concentrations via HPLC. Pre-treated slurries generated when using very high solids loadings did not produce any hydrolysate for analysis as the biomass absorbed all the free liquid. The remaining residues were then washed and dried using the standardized protocol for recovery and processing of pre-treated biomass as described in section 2.5.

Figure 6.2 Parameters associated with microwave treatment of BSG The displayed parameters are power consumption, pressure, temperature as detected by infra-red and temperature as detected by fluorescence of the fiber optic ruby thermometer. Running conditions: 200°C 5 min 30 sec .



6.2.2 Quantification of total glucose and xylose concentrations in BSG and the insoluble residues remaining after microwave based pre-treatments

Monosaccharide concentrations in the starting BSG and the remaining insoluble residues after the microwave based pre-treatments were quantified using the two-stage complete acid hydrolysis protocol described in section 2.3.1. This allows for the calculation of % theoretical sugar yields after subsequent enzymatic saccharification.

6.2.3 Enzymatic saccharification of microwave pre-treated BSG

All enzymatic hydrolysis attempts using the hydrothermal pre-treated residues were conducted over 24 h incubation periods at 50°C with agitation at 150 rpm (MaxQ 4358 shaking incubator, Thermo Scientific, UK). 50mM sodium citrate buffer (pH 5.0) was added to the biomass to achieve the required solids-loading.

As with all previous pre-treatment based work (Chapters 4-5) the initial assessments of the efficacy of different pre-treatment processes were conducted using very low solids-loading with lyophilised Celluclast® cellulase from *Trichoderma reesei* (ATTC 26921, Sigma-Aldrich, UK) using the protocol described in section 2.7.1.

After the initial assessments had been made using Celluclast®, high solids-loading, low enzyme dose enzymatic saccharification reactions were conducted using Novozymes Cellic® CTec2 (Novozymes A/S, Demark) as described in section 2.7.2. The Cellic® CTec2 was dosed at 20 FPU/g biomass and at 15 % (w/v) solids loading for 24 h.

After saccharification all samples were then centrifuged (5000 rpm, 10 min) prior to sampling for HPLC based glucose quantification as described in section 2.9.2.

6.2.4 Fermentations of two optimised feedstocks

Trial fermentations were conducted using two feedstocks (selected due to their high sugar yields) from both an optimised autohydrolytical pre-treatment (200°C for 10 min) and an optimised acid catalysed (1% HCl) hydrothermal pre-treatment (160°C for 5 min) of BSG with the pre-treatments both conducted at 30% w/v solids loading. The subsequent enzyme saccharification step was conducted using the Novozymes Cellic® CTec2 protocol described in section 6.2.2. Fermentations of the feedstocks produced after the microwave based pre-treatments and enzymatic saccharification of the remaining insoluble residue were conducted using *S.cerevisiae* NCYC479 using the fermentation and propagation protocols described in section 2.10.1-2. Achieved % theoretical ethanol yields were then calculated using the starting glucose concentration of the feedstock and the x 0.51 ethanol conversion factor (Badger, 2002). These trial fermentations were not optimised and were simply conducted as proof of principle that ethanol could be produced from the generated feedstocks and to highlight any significant fermentability issues.

6.2.5 Optimisation of a phosphoric acid based hydrothermal pre-treatment system

Based upon the somewhat effective enhancement of enzymatic saccharification that a phosphoric acid based pre-treatment system (1% H_3PO_4 at 121°C) had previously shown in chapter 4 (achieving ca. 51% theoretical glucose yield; section 4.3.2) an additional investigation was conducted to establish whether glucose yields could be boosted further. This optimisation would look at higher phosphoric acid concentrations and higher reaction temperatures in an attempt to match the yields achieved using the benchmark 1% HCl pre-treatment protocol at 121°C (>80% theoretical glucose yields) but also the successful use of phosphoric acid could potentially provide a food grade pre-treatment option. Whilst food grade processing was not relevant to bioethanol (fuel ethanol) production, it could be relevant should on-site processing of BSG occur within a brewery as any food grade ethanol could be re-used through incorporation into alcoholic beverages. A range of phosphoric acid concentrations (1% -5% v/v) and reaction temperatures (120°C - 200°C) were used at 25% w/v solids loading for each pre-treatment. The experimental design software DesignExpert (Stat-Ease Inc., Minneapolis, MN, USA) was employed to create experimental designs that would reduce the total number of experimental runs whilst still accurately modeling the data to determine any variance (Table 6.3). All microwave based pre-treatments were conducted as described in section 6.2.1. The remaining residues were then washed and dried using the standardized protocol for recovery and processing of pre-treated biomass as described in section 2.5. Quantification of the concentration of any sugar and lignin degradation products (downstream metabolic process inhibitors) in the pre-treatment generated hydrolysate was then conducted using HPLC as described in section 2.9.3. Enzymatic saccharification of the remaining insoluble residues was then conducted using an excess of Celluclast® cellulase as

described in section 2.7.1. Liberated glucose concentrations after enzymatic saccharification were then quantified using HPLC as described in section 2.9.2.

6.3 Results and discussion

Sections 6.3.1 and 6.3.3 report results from a series of optimisation experiments aimed at maximising the sugar concentrations liberated from BSG (post enzymatic saccharification) using a range of either autohydrolytical or acid catalysed hydrothermal pre-treatments. An excess of Celluclast® cellulase was used in each case for saccharification, in order to establish the efficacy of each specific pre-treatment, and did not reflect a commercially viable enzyme dosage. However section 6.3.4 describes the fermentation of feedstocks produced under more commercially relevant saccharification conditions, using a low dose of Novozymes Cellic® CTec2 at high solids loading.

6.3.1 Autohydrolytical pre-treatment of BSG using a microwave reactor

6.3.1.1 Hydrothermal pre-treatment (autohydrolysis based) at low solids loading

Preliminary work investigated the impact of temperature (110-200°C) and time (1-10 min) on the efficacy of pre-treatments at low solids-loading (2.5% w/v). As the BSG had already been through an industrial process (the malting and mashing of the beer brewing process) the experiment was designed to evaluate whether lower temperatures could be used to pre-treat BSG compared to other lignocellulosic biomass types such as wheat straw in an effort to minimise energy usage. DesignExpert was used to create a design space that would rapidly screen a wide range of pre-treatment temperatures and reaction times (whilst minimising the total number of experiments needed) in order to find a suitable set of conditions for further optimisation work. It did not test every temperature within the specified range but rather a representative selection. A total of 20 pre-treatments were conducted using a D-optimal designed experiment (see Appendix 2 for full details). The efficacy of the microwave-based

pre-treatments was evaluated using mg of sugar liberated per g of (pre-treated) biomass after enzymatic saccharification. Whilst this unit (mg/g) did not provide any process efficiency measure (as calculation of % theoretical yields would) it avoided the need for comprehensive compositional assays (such as quantification of the total sugar concentrations of each differently pre-treated biomass sample). Therefore it was purely used as a rapid method for preliminary screening of the different reaction temperatures. Quantification of the sugars liberated following enzymatic saccharification (using an excess of Celluclast® cellulase) indicated that temperatures approaching 200°C and reaction times of at least 5 min were required in order to achieve glucose yields of 300 mg/g pre-treated BSG (Figure 6.3). Whereas at lower process temperatures of 110-150°C, glucose yields of only 50 mg/g were achieved. Thus, autohydrolysis of BSG under the hydrothermal conditions used here required temperatures of ca. 200°C to attain sugar yields approaching theoretical maximum following enzymatic saccharification of the pre-treated BSG solid residue. Further optimisation could be conducted in the future to fine tune the exact minimum effective pre-treatment temperature (e.g. whether ca. 195°C would still function equally effectively as 200°C) but for all our subsequent work a 200°C temperature was used.

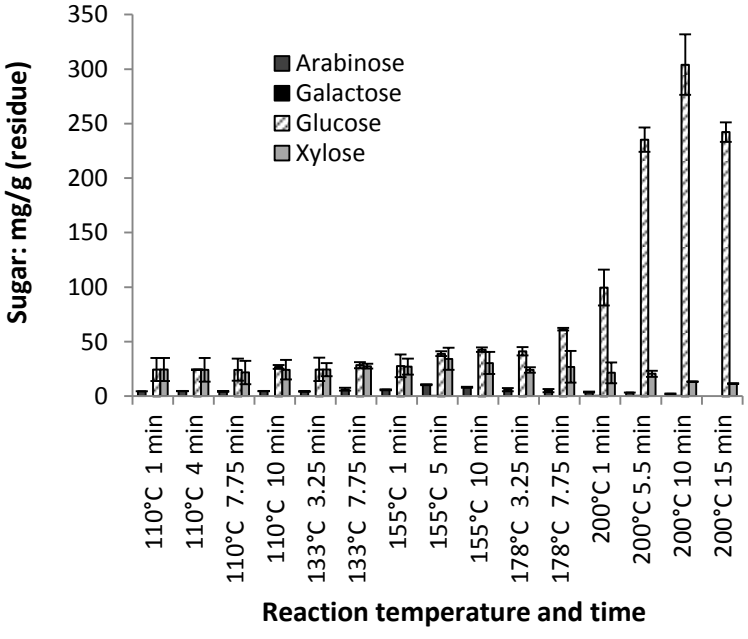
6.3.2 Hydrothermal pre-treatment (autohydrolysis) at high solids loading

A total of 19 pre-treatments at 200°C were conducted using a D-optimal designed experiment (see Appendix 2, Table 6.3 for full details) with two factors varied: pre-treatment solids loading (10-35% w/v) and reaction time (5-15 min) and the response data were then modelled against these factors. DesignExpert created a design space that evaluated these parameter ranges but did not test every permutation possible in order to reduce the total number of experiments required yet still provide statistically valid data.

6.3.2.1 Analysis of the hydrolysate (liquid fraction) generated directly from the pre-treatment step

Because the solid residue following hydrothermal pre-treatment of lignocellulosic materials is enriched in cellulose and therefore contains the majority of available sugars, the hydrolysate generated during pre-treatment is often overlooked as a potential source of fermentable sugars. Nevertheless, it represents a pool of additional sugars and may also be a source of high value chemicals (e.g. 5-hydroxymethylfurfural or HMF); therefore its compositional characterisation is desirable as a part of process optimisation.

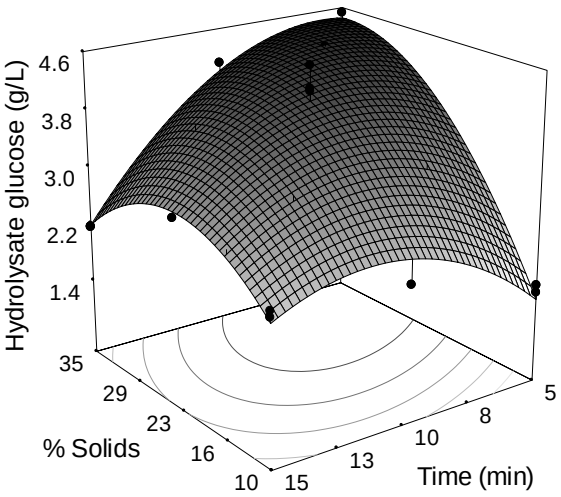
Figure 6.3 The impact of microwave-based pre-treatment reaction temperature and reaction time on sugar yields following enzymatic saccharification of the BSG pre-treatment solid residue. D-optimal algorithm chosen treatment combinations (reaction temperatures and times: see Appendix2, Table 6.3 for specific experimental design conditions). Preliminary screening of autohydrolytical pre-treatment of BSG at 2.5% (w/v) solids loading. 24 h enzyme incubation (50°C) using 200 mg residue to 40 mL of a 1 g/L Celluclast® solution buffered to pH 5.0 ± 0.1. Data are the mean ± SD of a minimum of two replicate measurements. Additional experimental runs were conducted to ensure a minimum of at least two replicates of each reaction condition permutation.



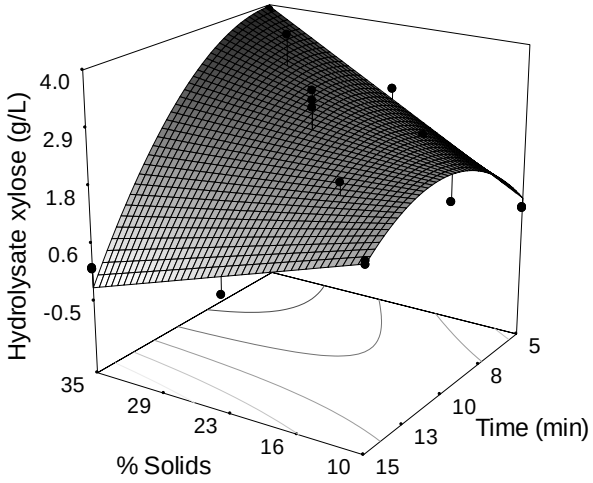
Modelling of glucose released into the hydrolysate during hydrothermal pre-treatment at 200°C showed that a maximum concentration of around 4.5 g/L resulted from short holding times (ca. 5 min) at high solids loadings (35% w/v; Figure 6.4A).

Figure 6.4 Design Expert 3D response surface models of the effects of (i) % solids loading (w/v) and (ii) reaction time (min) during 200°C microwave-based pre-treatment of BSG. Temperature previously optimised therefore not varied as an experimental parameter here. Effects described as on the resultant sugar concentrations liberated directly into the pre-treatment hydrolysate (g/L). A: Glucose (R^2: 0.84) B: Xylose (R^2: 0.96) C: 5-Hydroxymethylfurfural (R^2: 0.98). D: Furfural (R^2: 0.81). Autohydrolytical pre-treatment utilized a Monowave 300 microwave synthesis reactor with G30 vessels (Anton Parr).

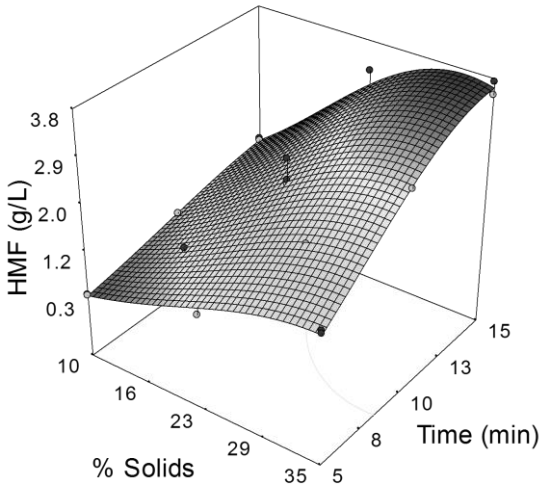
A) Glucose



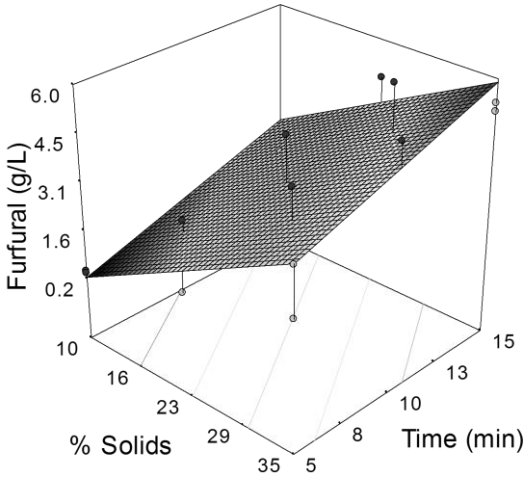
B) Xylose



C) 5-Hydroxymethylfurfural



D) Furfural



At low solids-loadings an increase of pre-treatment holding time over the range 5 – 15 min first increased and then decreased the glucose concentration in the pre-treatment hydrolysate. This is thought to represent a balance between the extent to which the more thermally labile amorphous regions of cellulose were broken down to release glucose and the subsequent degradation of glucose to HMF with prolonged holding at 200°C. At high solids-loadings the predominant effect of increasing holding time was to reduce glucose concentrations released into the hydrolysate, presumably once again due to degradation to generate HMF. The model of HMF concentration in the pre-treatment hydrolysate (Figure 6.4C) supports this hypothesis, since HMF concentrations were maximal at the highest solids content (35% w/v) and longest treatment time.

Xylose in the pre-treatment hydrolysate (liquid phase) originates from the hydrothermal breakdown of hemicellulose. The trends in xylose concentrations across the design space exhibited some similarity to the glucose results, with peak concentrations of 4.0 g/L released at the highest solids content and shortest treatment time (Figure 6.4B). Once again, albeit with a slightly modified response surface, the analytical concentration of xylose is hypothesized to be a balance between its release through breakdown of hemicellulose polymers and its thermal degradation to products such as furfural (Figure 6.4D). In contrast to glucose, xylose hydrolysate concentrations exhibited a small linear decline with increasing solids loading at 15 min reaction time (from 1.8 g/L to 0.55 g/L). Clearly at 15 min treatment time, the onwards degradation of the released xylose outweighed the concentration effect of working at higher solids which was evident at a 5 min treatment time (Figure 6.4B). Furfural concentrations were maximal under these conditions (Figure 6.4D). Evaluation of any pre-treatment must also consider the degree of generation of downstream processing inhibitors, such as HMF and furfural which are generated from thermal degradation of hexose and pentose monosaccharides (respectively). These furan compounds can reduce the efficiency of

the subsequent enzymatic saccharification and fermentation steps conducted downstream (Allen et al., 2010; Carvalheiro et al., 2008; Liu, 2006; Palmqvist & Hahn-Hagerdal, 2000a). They are liberated into the hydrolysate (liquid fraction) generated during pre-treatment, particularly when exposing materials to high temperatures for prolonged periods. Both HMF and furfural have well established inhibitory actions on many fermentative species such as *Saccharomyces cerevisiae*, hence their production should be minimised wherever possible (Allen et al., 2010; Palmqvist & Hahn-Hagerdal, 2000a). They primarily affect the redox balance of the cell, with furfural affecting the function of alcohol dehydrogenase (ADH) and HMF affecting nicotinamide adenine dinucleotide phosphate (NADPH) function. Quantification of potential yeast-inhibitory compounds in the pre-treatment hydrolysate found that HMF and furfural were the only significant inhibitors detected, with furfural being present in greater concentrations than HMF (maximally 6.0 g/L for furfural compared to 3.8 g/L for HMF: Figure 6.4C-D respectively). This was as expected, bearing in mind the known function of hydrothermal pre-treatments in solubilising hemicellulose (Garrote et al., 1999). A general correlation was seen with increasing concentrations of both furfural and HMF as solids loading and reaction time increased. Whilst prolonged treatment time favoured furfural production across the design space, the solid-loading during pre-treatment had the greater impact on furfural concentration. HMF concentrations exhibited a different trend with a linear increase in response to increasing reaction time (at any given solids loading) but with a more significant effect (steeper linear ramp) at higher solids loadings compared to at lower solids loadings (Figure 6.4C). In contrast to the linear furfural concentration increase at all reaction times as solids loading increased, at longer reaction times (ca. 15 min) HMF concentrations only increased until ca. 29% solids loading, beyond which they began to plateau. However, with shorter reaction times (ca. 5 min) an almost linear increase in HMF concentrations was observed with increasing solids loadings beyond ca. 25% (w/v). The

furfural concentrations of ca. 5 g/L generated from autohydrolytical pre-treatment of BSG at 200°C when using solids loadings greater than 29% w/v would be metabolically inhibitory for many fermentative species such as *S.cerevisiae* (Allen et al., 2010; Liu, 2006) rendering some form of biomass washing or dilution necessary. However fractionation of both furfural and HMF from a waste stream after such a biomass washing step could generate additional revenue due to the high value of both compounds as chemical intermediates for various industrially relevant compounds such as the solvent tetrahydrofuran (THF) and various plastics including polyesters (Chheda et al., 2007; Yemiş & Mazza, 2012).

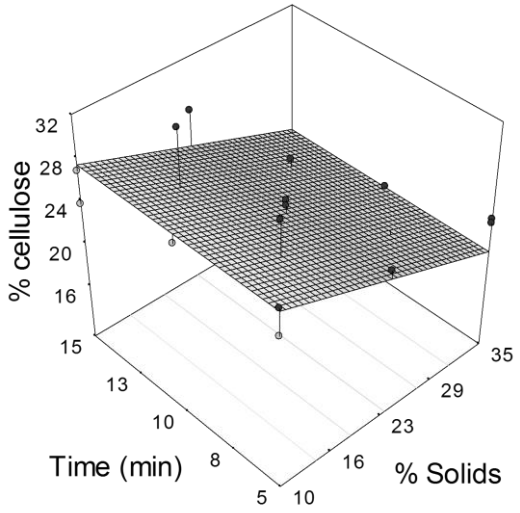
6.3.2.2 Sugar yields achieved after the enzymatic saccharification step

Following each pre-treatment the remaining solid residue was separated from the liquid fraction in order to remove potentially detrimental metabolic process inhibitors that would likely interfere with the subsequent enzymatic saccharification and fermentation steps. All pre-treated biomass was then washed and dried. Compositional analysis of these solid residues was undertaken both to enable accurate calculation of theoretical sugar yields after enzymatic saccharification and to assess, to some extent, the physical impact of each pre-treatment on the composition of BSG derived material. Hydrothermal pre-treatments primarily target hemicellulose solubilisation and the subsequent liberation of its monomeric constituents into the liquid fraction. Consequently this results in enrichment of the more recalcitrant components, such as crystalline cellulose. Producing a material enriched in cellulose could be deemed favourable if the ultimate intention is to ferment glucose to bioethanol using C-6 hexose fermenting yeast strains. The proportion of cellulose in solid residues of pre-treatments across the design space (for specific details see Appendix2, Table 6.4) is illustrated as a response surface in Figure 6.5A. There was a linear trend of decreasing cellulose enrichment as solids-loading was increased (Figure 6.5A) as a consequence of a smaller degree of hemicellulose solubilisation (Figure 6.5B). Reaction time appeared to be a

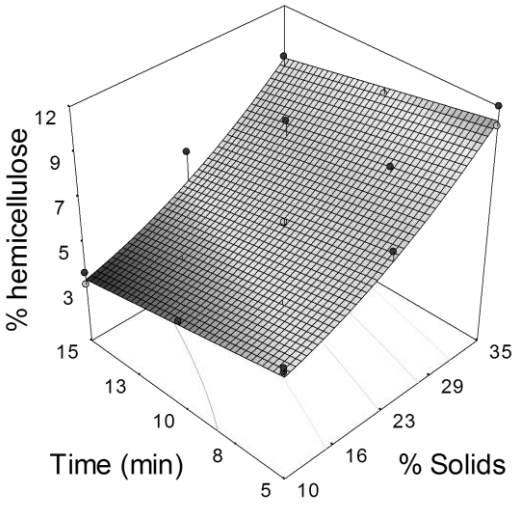
less influential parameter in this model, with a small decrease in cellulose enrichment occurring as reaction time was increased at each solids-loading.

Figure 6.5 Design Expert 3D response surface models the effects of (i) % solids loading (w/v) and (ii) reaction time (min) during 200°C pre-treatment of BSG. Temperature was previously optimised therefore not varied as an experimental parameter here. A: % cellulose content of the solid residue following pre-treatment of BSG (R^2: 0.56). B: % hemicellulose content of the solid residue following pre-treatment of BSG (R^2: 0.91). C: % theoretical glucose yield after enzymatic saccharification (R^2: 0.78). D: % theoretical xylose yield after enzymatic hydrolysis (R^2: 0.75). Autohydrolytical pre-treatment utilized a Monowave 300 microwave synthesis reactor with G30 vessels Anton Parr). Digestion of pre-treated residue: 24 h incubation (50°C) using 200 mg residue to 40 mL of a 1 g/L Celluclast® solution buffered to pH 5.0 ± 0.1.

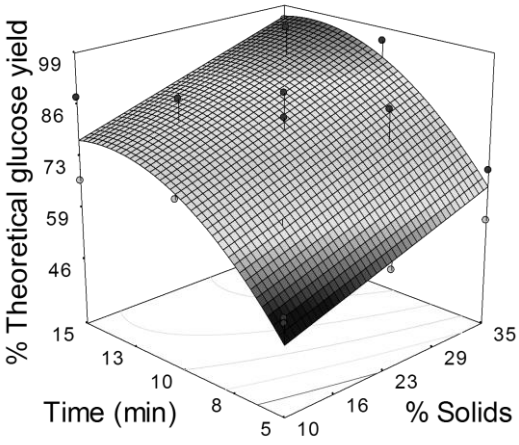
A) Cellulose



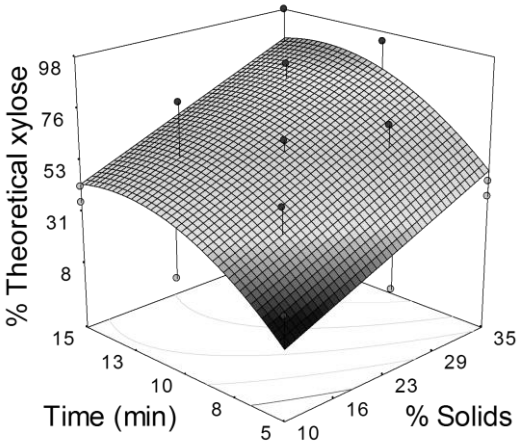
B) Hemicellulose



C) Glucose



D) Xylose



The solid residues of pre-treated BSG were then treated with an excess of Celluclast® cellulase enzyme and the sugars released were determined by HPLC. Response surface models were fitted to this data to show the impacts of treatment time (5-15 min) at solids-loadings between 10-35% w/v on the concentrations of glucose (Figure 6.5C) and xylose (Figure 6.5D). At any given pre-treatment reaction time increasing solids-loading (during the pre-treatment step) resulted in higher glucose yields (expressed as a percentage of theoretical maximum) following enzymatic saccharification. At 35% w/v solids-loading, pre-treatment at 200°C for 15 min gave glucose yields approaching 100% of theoretical (Figure 6.5C). The increase in theoretical glucose yields with increasing solids-loading was unexpected and was possibly due to a smaller proportion of cellulose being present within these solid residues (Figure 6.5A). This would have subsequently resulted in a greater ratio of enzyme to cellulose when saccharifying samples pre-treated at higher solids-loadings, thus increasing the probability of complete enzymatic saccharification through more successful enzyme-substrate binding. This hypothesis was supported by the model derived to predict hemicellulose content of the remaining solid residues after pre-treatment (Figure 6.5B). This confirmed that hemicellulose content of the remaining residue increased (in an almost linear fashion) with increasing solids loading during the pre-treatment step. This pointed to a drop in effectiveness of the pre-treatment (reduced hemicellulose solubilisation) as the solids loading was increased.

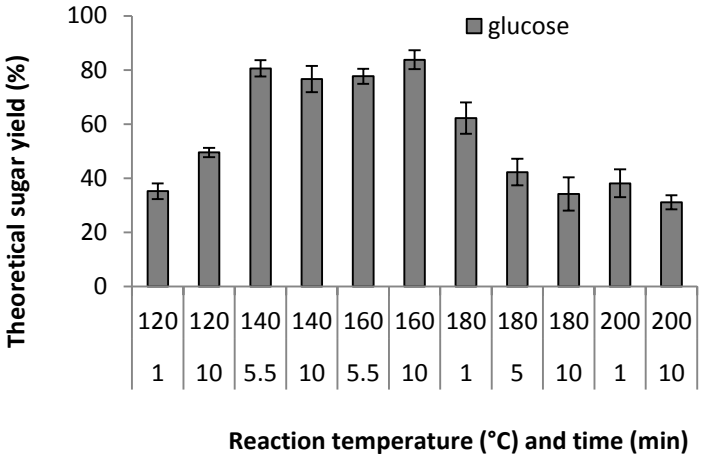
The trend in glucose yields with process time at all solids-loadings investigated (Figure 6.5B) indicated that extension of reaction times beyond 15 min was unlikely to result in any further increase in glucose yield. A similar model was derived to predict xylose yields across the design space (Figure 6.5D). Once again, sugar yield was maximised at the highest pre-treatment solids-loading and with treatment times between 13-15 min.

6.3.3 Microwave-based dilute acid hydrothermal pre-treatment of BSG

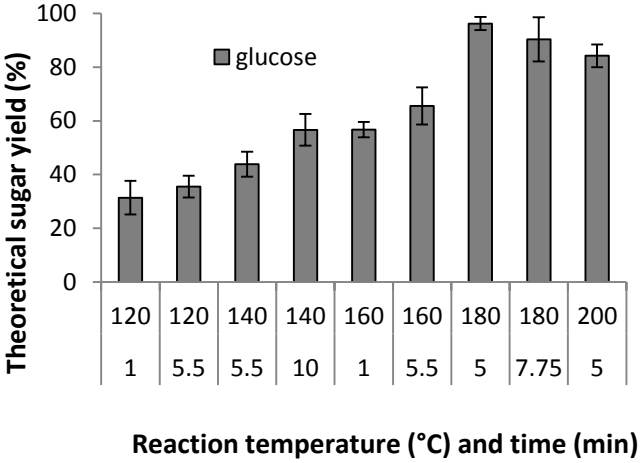
The impact of adding an acid catalyst (1% HCl v/v) on the hydrothermal pre-treatment of BSG was investigated over a range of treatment times (1-10 min), temperatures (120-200°C) and solids loadings (30% - 50% w/v). At such high solids loadings it was not physically possible to recover the liquid fraction after pre-treatment. However the subsequent biomass was washed extensively prior to enzymatic saccharification to remove potential metabolic process inhibitors. The percentage of theoretical sugars yielded following saccharification is shown as a function of process temperature and time at 30%, 40% and 50 % w/v solids loadings (Figure 6.6). At 30% w/v solids-loading it was apparent that incorporation of the acid catalyst enabled high sugar yields (close to 80% of theoretical) to be attained at a lower temperature range (140-160°C) than was required in the absence of HCl (Figure 6.6A). The acid catalyst acts to enhance the autohydrolysis mechanism through provision of additional protons. However, use of 1% HCl at temperatures of 180-200 °C decreased theoretical glucose yields as low as ca. 40% of theoretical, even at relatively short process times (e.g. 1 min). At these temperatures the acid catalyst promotes not only the release of glucose and xylose, but also their subsequent thermal degradation. In contrast to these findings, when pre-treatment solids-loading was increased to 40% w/v, a temperature of 180°C was required in order to achieve greater than 80% theoretical glucose and xylose yields (Figure 6.6B). Treatment at 140-160°C with 40% solids loading only achieved approximately 40-60% of theoretical glucose and xylose yields. Furthermore, increasing pre-treatment solids loading to 50% w/v required temperatures of 200°C to approach 80% theoretical sugar yields (Figure 6.6C). Thus, at very high solids loadings (40 & 50% w/v) pre-treatment temperature was a more significant parameter than reaction time when using the 1% HCl catalyst. However, at 30% w/v the impact of process time on % theoretical sugar yields was apparent at high temperatures, presumably due to the impact of accelerated sugar degradation reactions.

Figure 6.6 The impact of reaction temperature (120-200°C) and reaction time (1-10 min) at various solids loading during acid catalysed hydrothermal pre-treatment of BSG on % theoretical sugar yields following enzymatic saccharification of the pre-treated solid residue. A: 30% w/v solids loading, B: 40% w/v solids loading, C: 50% w/v solids loading. Data are the mean ± SD of three replicate measurements. 1% HCl hydrothermal pre-treatment utilized a Monowave 300 microwave synthesis reactor with G30 vessels (Anton Parr). Enzymatic saccharification of pre-treated residue: 24 h incubation (50°C) using 200 mg residue to 40 mL of a 1 g/L Celluclast® cellulase solution buffered to pH 5.0 ± 0.1.

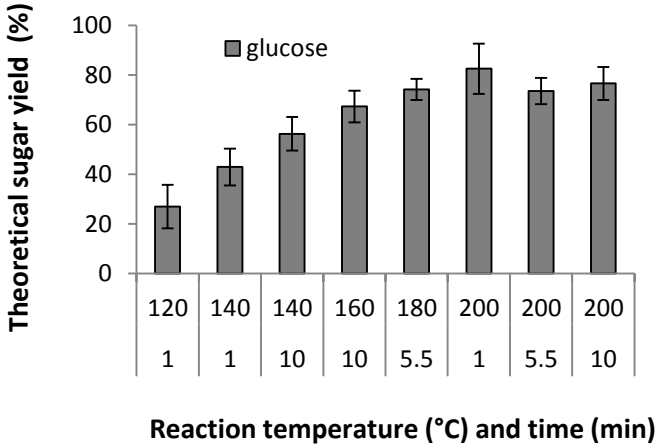
A) 30% w/v solids loading



B) 40% w/v solids loading



C) 50% w/v solids loading

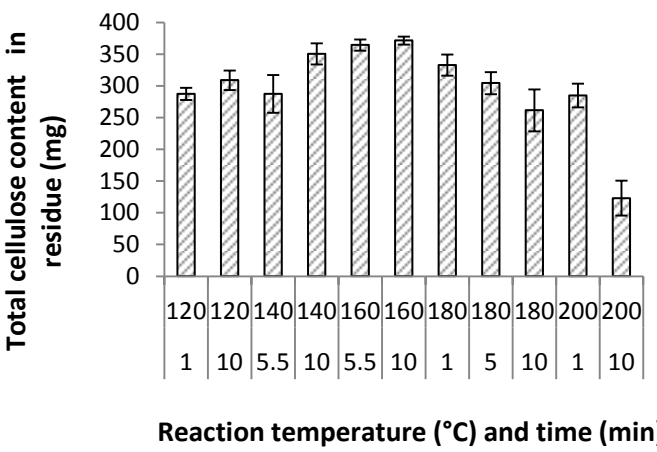


6.3.3.1 Total glucose concentrations in the solid residues resulting from 1% HCl catalysed hydrothermal pre-treatment of BSG

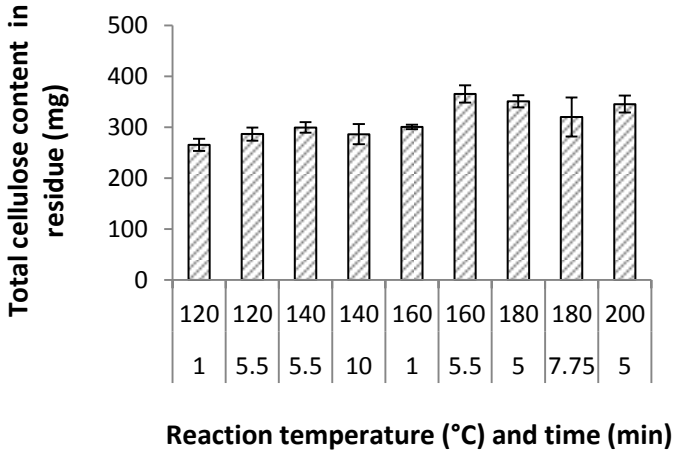
In contrast to the results for direct hydrothermal (autohydrolytical) pre-treatment, in experiments with addition of 1% HCl, a positive correlation was observed between cellulose concentrations in the pre-treated solid residues and % theoretical sugar yields achieved following saccharification, at all solids loadings tested (Figure 6.7A-C).

Figure 6.7 The impact of reaction temperature (120-200°C) and reaction time (1-10 min) at various solids loading during 1% HCl acid catalysed hydrothermal pre-treatment of BSG on cellulose concentration of the remaining pre-treated solid residue after complete acid hydrolysis. A: 30% solids loading, B: 40% solids loading, C: 50% solids loading. Data are the mean ± SD of three replicate measurements. 1% HCl hydrothermal pre-treatment utilized a Monowave 300 microwave synthesis reactor with G30 vessels (Anton Parr). Cellulose concentration expressed as glucose; mg sugar per g solid residue.

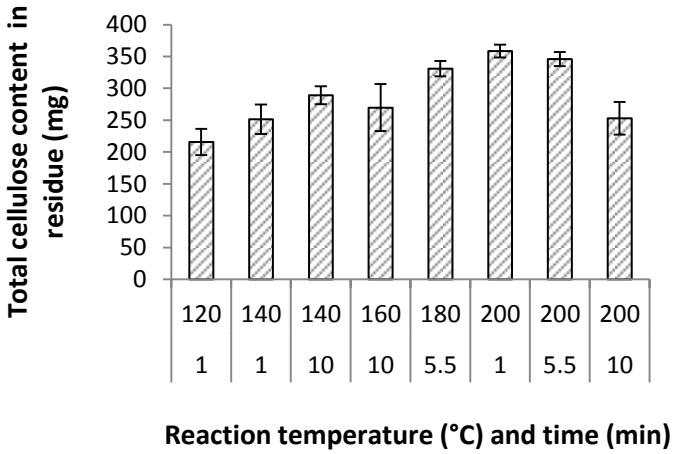
A) 30% w/v solids loading



B) 40% w/v solids loading



C) 50% w/v solids loading

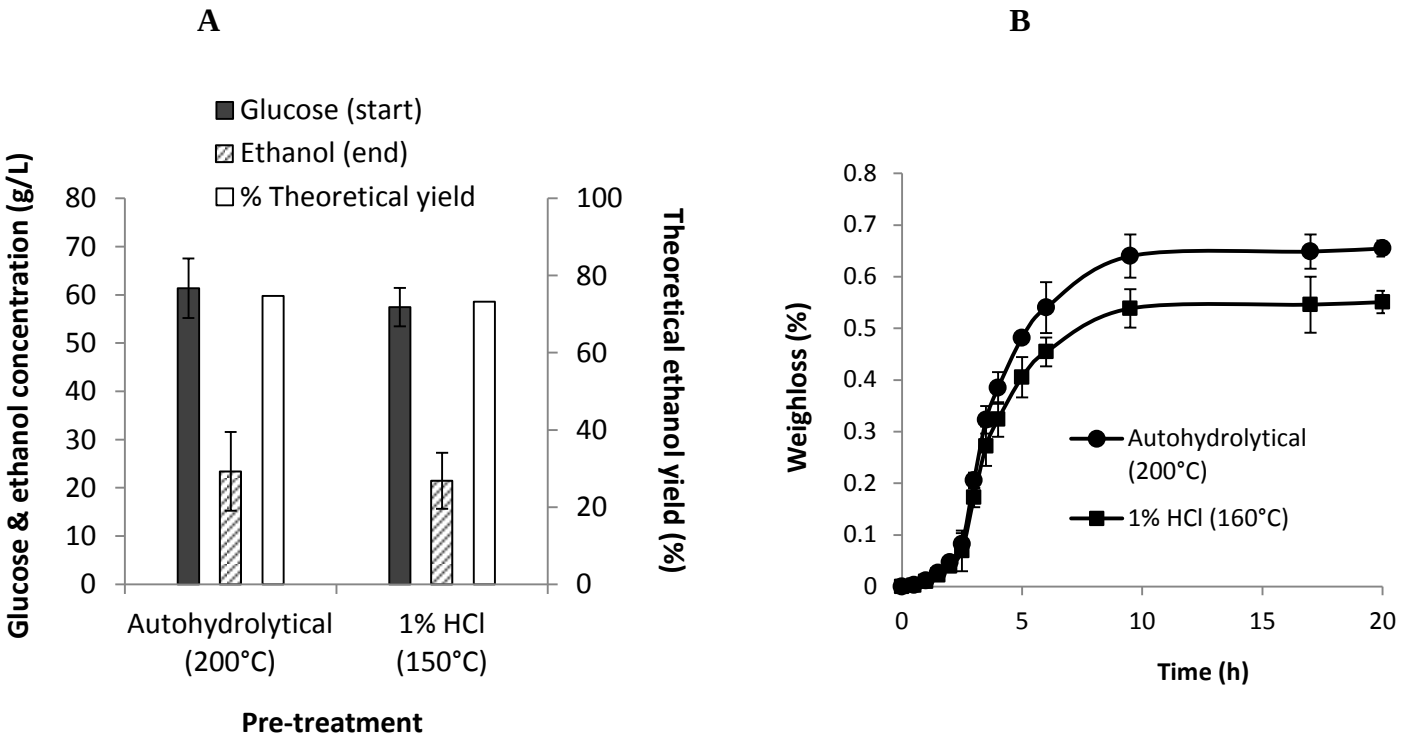


Thus, enriching the cellulose fraction of BSG by effectively removing hemicellulose also enhanced enzymatic digestibility of the material and the proportion of cellulose which could then be released as glucose. Whilst the mechanisms of autohydrolysis and acid catalysed hydrothermal pre-treatment are considered to be similar in nature (Garrote et al., 1999; Hendriks & Zeeman, 2009) it appears that the lower pH attained with HCl addition enhanced subsequent digestibility of cellulose in this system. Alternatively, other researchers have proposed that the chloride ions added in HCl exert a catalytic effect, either through nucleophile activity (White et al., 2008) or possibly through an ionic liquid based mechanism, disrupting hydrogen bonding within cellulose microfibrills and further enhancing enzymatic access and thus enzymatic sugar yields (Liu et al., 2012; Su et al., 2011).

6.3.4 Fermentations of two optimized feedstocks

Two optimized pre-treatment processes (200°C, 10 min autohydrolytical and 160°C, 5 min 1% HCl dilute acid hydrothermal pre-treatments; both at 30% w/v solids loading) were chosen for the production of feedstocks to conduct trial fermentations. These treatments were approximately matched in terms of the glucose concentrations attained following saccharification (61 g/L and 57 g/L respectively; Figure 6.8A). In this experiment realistic constraints were placed upon enzymatic digestion, as opposed to the vast excess of cellulases used previously to compare and contrast the efficacy of pre-treatments. Thus saccharification was conducted at 15% (w/v) solids loading and with a commercially applicable dose of Novozymes Cellic® CTec2. The trial fermentations were conducted using *S. cerevisiae* NCYC479.

Figure 6.8 Yield data (A) and fermentation progression (B) from fermentations of enzymatic hydrolysates (feedstocks) resulting from either optimized autohydrolytical or dilute acid hydrothermal pre-treatment processes. A: Theoretical ethanol yield based on the initial glucose concentration in the feedstock. B: Fermentation progression monitored by weight-loss of vessels, due to CO_2 evolution. Pre-treatment: Autohydrolytical (200°C 10 min) or 1% HCl catalysed hydrothermal (160°C 5 min) both at 30% solids loading (w/v). Enzyme saccharification: Novozymes Cellic® CTec2 (20 FPU/g biomass, 24 h incubation at 50°C, pH 5.0 ± 0.1) at 15% (w/v) solids loading. Fermentations conducted at 15°C with stirring (120 rpm) and using *S. cerevisiae* NCYC479. Data are the mean ± SD of three replicate experiments.



Similar final ethanol concentrations (ca. 22 g/L) resulted from fermentation of either of the two feedstocks and this equated to approximately 75% and 73% of theoretical ethanol yield (Achieved % theoretical ethanol yields were calculated using the starting glucose concentration of the feedstock and the x 0.51 ethanol conversion factor). For the autohydrolysis and 1% HCl catalyzed pre-treatment feedstocks respectively (Figure 6.8A).

A small amount cellular growth at the start of fermentations is thought to explain the proportion of glucose not ultimately fermented to ethanol. This is because after the biomass was pre-treated the washing steps would have removed any inhibitors that were generated from the thermal breakdown of sugars. As we did not conduct any enzymatic saccharification

optimization or fermentation optimization these ultimate ethanol yields could be improved with further work. Fermentation progression (followed by vessel weight loss resulting from CO_2 evolution) was also similar when comparing the two feedstocks. Each exhibited similar and relatively short lag phases and both attenuated after ca. 10 hours (Figure 6.8B). On average, the autohydrolytical pre-treatment generated feedstock resulted in a slightly greater percentage weight loss over the course of fermentation, which likely indicated a greater evolution of CO_2; however this was considered to be due to the very slightly higher glucose concentration present in the initial feedstock. Overall two highly effective hydrothermal microwave pre-treatments were developed for the optimal release of fermentable sugars from BSG.

6.3.5 Optimisation of a Phosphoric acid based hydrothermal pre-treatment system

As with all previous pre-treatment efficacy assessments conducted, % theoretical glucose yields (after enzymatic saccharification) were used as a response factor for the derived models used for the attempted optimisation.

The 3D response surface models suggested that, as hypothesised, either higher reaction temperatures (than the 121°C used for the initial screening of the phosphoric acid; Chapter 4) or higher acid concentrations than the previously used 1% v/v were required in order to achieve >75% theoretical glucose yields (Figure 6.9). The best compromise between reaction temperature and acid concentration was shown to be 160°C and 3% H_3PO_4 which achieved >90% theoretical glucose yield which was comparable to the target efficacy of the benchmark 1% HCl 121°C hydrothermal pre-treatment that achieved ca. 86% theoretical glucose yield (as discussed in section 4.3.2). Alternative reaction parameter optimisations that also achieved >90% theoretical glucose yields were found to be either 150°C and 5% H_3PO_4 or 190°C and 1% H_3PO_4. Should any large scale use of this phosphoric acid based pre-treatment

system be conducted the choice of specifically which optimisation permutation was chosen would largely depend on which was most economical. This would relate to the energy costs of attaining the required reaction temperature and the cost and availability of food-grade phosphoric acid.

However, whilst very high glucose yields were possible using this phosphoric acid catalysed pre-treatment system the degree of generation of the sugar degradation products Furfural and HMF (2-furancarbaldehyde and 5-hydroxymethylfurfural) in response to the most effective pre-treatment permutations (e.g. >180°C and >2% H_3PO_4) was severe. Furfural and HMF concentrations of ca. 20 g/L and 5 g/L (respectively) were present in the liquid fraction (pre-treatment generated hydrolysate) which would likely present a significantly inhibitory effect on downstream processes such as any fermentations conducted (Figure 6.10). This would require significant aqueous based washing of the biomass essential in order to reduce the concentration of these inhibitory compounds to tolerable levels.

However, the option would exist to separate the degradation product laden pre-treatment hydrolysate from the remaining insoluble biomass (via filtration or centrifugation). This would then allow successful feedstock production from the insoluble residue after an enzymatic saccharification step. The degradation product laden hydrolysate could then be used for an alternative process or detoxified possibly through stripping of the volatile compounds (such as furfural) through a boiling step similar to that achieved with wort in the brewery in the kettle boil.

Figure 6.9 Design Expert 3D response surface model of the effects of (i) reaction temperature (°C) and (ii) phosphoric acid concentration (% v/v) during hydrothermal pre-treatment of BSG. Pre-treatment conducted at 25% w/v solids loading using a Monowave 300 microwave synthesis reactor with G30 vessels (Anton Parr). D-optimal algorithm chosen treatment combinations (120°C - 200°C reaction temperatures and 1% - 5% phosphoric acid concentrations: see Table 6.3 for specific experimental design conditions). Response factor: Glucose liberation (%theoretical glucose yield) after enzymatic saccharification of remaining insoluble residue (R^2: 0.74). Enzymatic saccharification conducted using 24 h enzyme incubation (50°C) using 200 mg residue to 40 mL of a 1 g/L Celluclast® cellulase solution buffered to pH 5.0 ± 0.1.

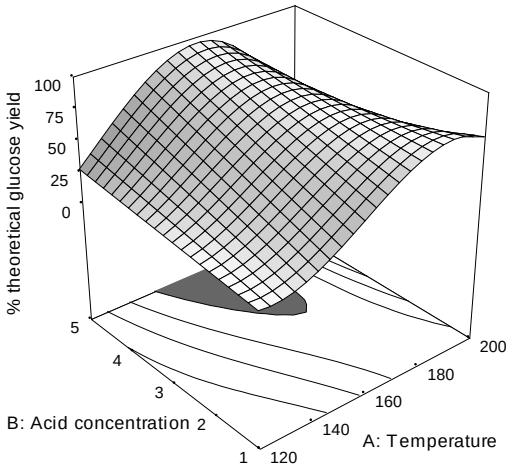
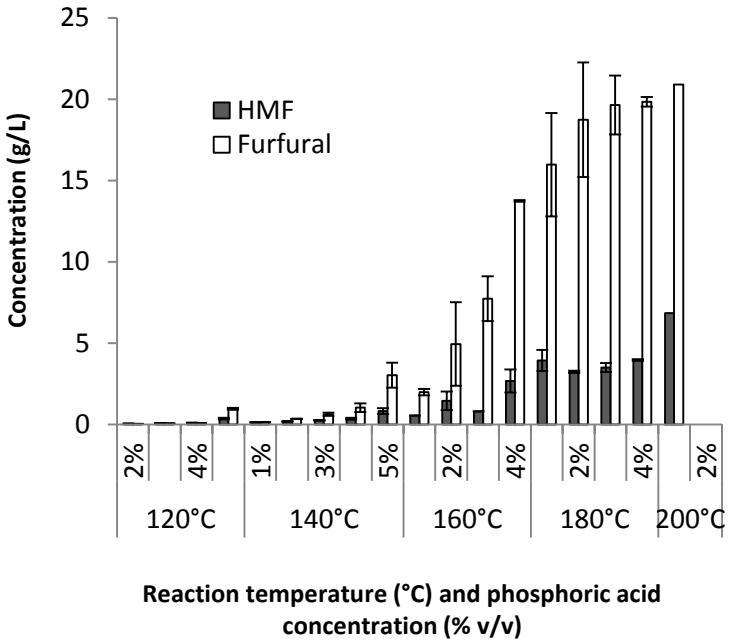


Figure 6.10 Sugar degradation product concentrations generated from phosphoric acid catalysed hydrothermal pre-treatment of BSG. Pre-treatment conducted at 25% w/v solids loading using a Monowave 300 microwave synthesis reactor with G30 vessels (Anton Parr). D-optimal algorithm chosen treatment combinations (120°C - 200°C reaction temperatures and 1% - 5% phosphoric acid concentrations: see Table 6.3 for specific experimental design conditions).



6.4 Conclusions

Overall, microwave-based autohydrolytical and dilute acid catalysed hydrothermal pre-treatment of BSG were found to be efficient in terms of subsequent enzymatic saccharification yields (>90% theoretical glucose yields) at high solids loading rates (25% w/v). Also the feedstocks generated using these pre-treatments produced good final ethanol titres (>75% theoretical yield) and rapid fermentability. This research has also proved invaluable for the evaluation of dilute acid based hydrothermal pre-treatment at higher temperatures than previously tested. Further work defining the exact dielectric properties of BSG could be conducted which may then allow for specific adjustment of the operational parameters of the microwave to further improve the dielectric heating efficiency and thus further reduce the energy input for the pre-treatment step whilst still maintaining its efficacy. Additionally an optimised phosphoric acid based hydrothermal system was developed which was capable of liberating >90% theoretical glucose yields whilst also offering a food grade pre-treatment option.

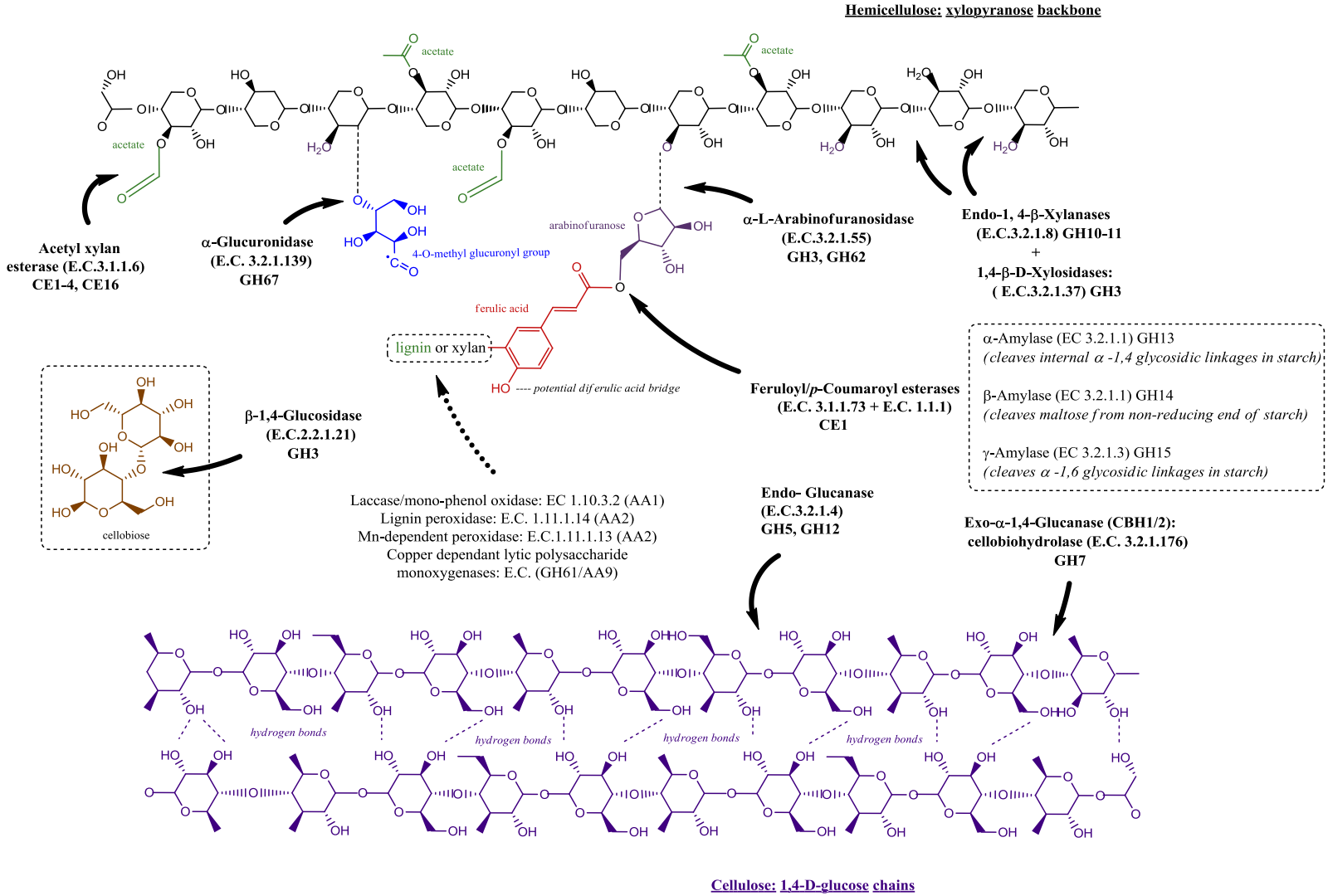
7 Maximising the enzymatic saccharification yields (at high solids loading) from acid catalysed hydrothermal pre-treated BSG for bioethanol production

7.1 Introduction

Numerous effective chemical and thermal pre-treatments for brewers spent grains (BSG) have been developed and have been shown to enhance the subsequent enzymatic saccharification yields achieved from lignocellulosic biomass (White et al., 2008). However, large doses of expensive commercial enzymes are still usually required to achieve high conversion efficiencies of cellulose to glucose (Chundawat et al., 2008; Leathers, 2003; Qing et al., 2010b). Therefore optimisation of the enzymatic saccharification stage is a key objective in the cost-effective production of lignocellulosic biofuels. Maximising the operational solids loading used (a high solid to liquid ratio) during the enzymatic saccharification step, whilst minimising the required enzyme dose are likely to be key factors which need to be addressed in order to produce high glucose concentrations cost effectively and minimise water usage (Hodge et al., 2008; Kristensen et al., 2009). Commercial scale bioethanol production would likely require a minimum glucose concentration of ca. 100 g/L (ca. 10% w/v) in order to realistically produce the benchmark minimum ethanol concentration of ca. 50 g/L (ca. 5% v/v) which may then facilitate economically viable distillation (Lau & Dale, 2009). Operation at high solids loading (>10% w/v) during the enzymatic saccharification step would likely be essential in order to achieve this through limiting the dilutional effect of surplus liquid water (Kristensen et al., 2009). However, operation at high solids loading would likely drop the conversion efficiency of cellulose to glucose resulting in % theoretical glucose yields well below acceptable limits of process efficiency. This is possibly due to rheology related mass transfer limitations because of the high viscosity of the media impeding enzymatic access to all of the available substrate

(cellulose) and impeding dispersal of the hydrolysis products which may feedback inhibit the cellulases (Chundawat et al., 2008; Gan et al., 2003). Mixing technology may play some part in solving this through ensuring better enzyme substrate interactions (especially at very high solids loading; >20% w/v). However, some research has indicated there may only be minimal improvements in achieved glucose yields through additional mixing (Kristensen et al., 2009). Achieving effective mixing at very high solids loadings may also prove prohibitively energy intensive due to the amount of torque likely required to agitate highly viscous slurries of the biomass. There is also a minimum requirement of some available water for the successful catalytic function of many lignocellulolytic enzymes due to their hydrolytic mechanism of glycosidic bond fracturing through addition of a water molecule (Horn et al., 2012). This would suggest that an upper limit exists with regards to the maximum functional solids loading that can be used during the enzyme saccharification step as going beyond this would detrimentally limit the available free water present. Various enzymes from the CAZy database (http://www.cazy.org/) of carbohydrate degradation specific enzymes are likely to be involved in the successful degradation of lignocellulosic substrates such as BSG, many of which come from fungal enzymatic systems such as brown and white rot fungi (Delmas et al., 2012; Evans et al., 1994). These enzymes include members of the GH (glycoside hydrolase) family, CE (carbohydrate esterase) family and PL (polysaccharide lyase) family (Figure 7.1). Most commercial enzyme preparations designed for advanced generation biofuel processes are tailored to be as broad spectrum as possible so as to be effective on the wide variety of lignocellulosic substrates of interest such as woody biomass, energy crops/grasses and industrial waste streams and co-products. (Harris et al., 2010).

Figure 7.1 Schematic representation of the mode of action of enzymes involved in deconstruction and saccharification of lignocellulosic material (adapted from Beg et al., 2001; Dodd & Cann, 2009; Faulds et al., 2004; Faulds & Williamson, 1995; Gupta et al., 2010; Kuhad et al., 2011; Rai, 2009 and http://www.cazy.org/).



However the highly substrate specific nature of the format of the lignocellulose (such as the specific side chain decorations of the xylan backbone which composes hemicellulose) may render generic enzyme preparations sub-optimal in terms of the specific content of their constituent enzymes or the dose of any one particularly crucial enzyme for any particular substrate (Dodd & Cann, 2009; Gilbert et al., 2008). Therefore addition of specific ancillary enzymes (used in conjunction with a broad spectrum commercial enzyme mixture) could prove effective in maximising glucose yields from a specific lignocellulosic substrate such as BSG through the cleaving of a particular linkage or bond that is critical to the recalcitrance of a particular substrate (Chundawat et al., 2008).

Additional enzymes could include acetyl xylan esterases (CE1 family) which hydrolyse the ester linkages of acetate side groups on the xylose backbone of hemicellulose (Shallom & Shoham, 2003; Zhang et al., 2011). Other options are arabinofuranosidases (GH62) which hydrolyse the terminal non-reducing α-L-arabinofuranoside residues in α-L-arabinosides present in varying intervals as side chain decorations in arabinoxylans and hemicellulose (Poutanen, 1988; Schwarz et al., 1995). The successful de-branching of these residues may be crucial for complete and effective hydrolysis of branched formats of hemicellulose.

Other non CAZy classified candidates could include ferulic acid esterases (furuloyl esterases) which have been shown to release ferulic acid (and other hydroxycinnamic acids) from substrates such as brewers spent grains (Bartolome & Gómez-Cordovés, 1999; Faulds et al., 2002). In addition proteases may be able to hydrolyse some of the considerable protein content within the BSG which may indirectly improve physical access of any cellulase enzymes to the crystalline cellulose present but also solubilise vital free amino nitrogen (FAN) into the generated feedstock that would be essential for yeast nutrition. The several kinds of endopeptidase and exopeptidase found in the commercially available proteinase preparation pronase (from *Streptomyces griseus*) has been used to digest various cattle feeds

such as grasses in an attempt to produce low nitrogen feeds (Abe et al., 1979). Pronase may target dextrans (glucan) and has been shown to be more effective in doing this than even mild acid hydrolysis (Kato et al., 1991). There are also various brewing specific commercial protease mixtures used for protein hydrolysis of malted barley that may still be effective in fractionation of some of the protein content of BSG. Various starch targeted amylases may also assist in boosting final glucose concentrations as there may be some residual starch present in the BSG (dependant on the brewing technology used upstream, such as whether lauter tun or mash filter brewhouse apparatus was used and whether or not exogenous enzymes were used during the mashing stage thus enhancing the degree of starch extraction).

The CAZy GH61 family of glycoside hydrolases have been indicated to play an important role in the oxidative cleavage of certain linkages in lignocellulolytic substrates in nature with many GH61 genes being indicated to be present in the genome of various fungal species including the brown rot fungi *Postia placenta* (Quinlan et al., 2011). As a result additional GH61 enzymes have been included in certain commercial enzyme products such as Novozymes Cellic® CTec2 (Cannella et al., 2012). Various published work has suggested they may be able to boost the effectiveness of other cellulase class enzymes such as endo and exo-1,4-β-glucanases and in particular cellobiohydrolases (Beeson et al., 2011; Harris et al., 2010; Horn et al., 2012; Quinlan et al., 2011). In addition various redox active cofactors such as gallate and ascorbate have been suggested to function as possible enhancers of GH61 enzyme activity as they may act as electron donors or chemical reductants (Horn et al., 2012; Quinlan et al., 2011). Also various metal ions (in particular copper) have been suggested to bind to the active site of GH61 class enzymes and play a crucial role in their activity (Quinlan et al., 2011). The oxidative based activity of the GH61 family may also suggest that the provision of supplementary oxygen or aeration may be a low cost option which could boost their catalytic activity (Beeson et al., 2011; Horn et al., 2012).

This chapter presents an investigation looking at a range of parameters associated with the efficacy of enzymatic saccharification of pre-treated BSG (in respect of their significance with regards to achieved glucose yields).

For the pre-treatment of the BSG prior to all enzymatic saccharification experiments the optimised dilute acid catalysed hydrothermal protocol (1% HCl at 121°C; discussed in Chapter 4) was chosen as a benchmark due to its proven effectiveness (ca. 86% theoretical glucose yields achievable) and its practicality, as large quantities of BSG could be relatively rapidly pre-treated (within ca. 30 min) compared to the >12 h pre-treatment time for the 5% NaOH alkali-based system. Also after pre-treatment the product from the 1% HCl system had a much lower viscosity (than that derived from the 5% NaOH system) allowing for much easier processing and recovery of all the pre-treated biomass. Additional factors for choosing the 1% HCl pre-treatment system included the lower reaction temperature (121°C; and therefore lower energy input) compared to that of autohydrolytical pre-treatment (200°C) and phosphoric acid catalysed hydrothermal pre-treatment (160°C).

For the actual enzymatic saccharification experiments the factors investigated were: simultaneous effect of variation of the solids loadings at several different cellulolytic enzyme doses, the effect of extension of hydrolysis incubation time, a range of different mixing protocols, the effect of supplementation of the cellulolytic enzymes with various additional enzymes and enzymatic cofactors and also the effectiveness of batch-fed protocols. Finally the development of a novel, high solids loading enzymatic saccharification protocol is demonstrated. This was a consolidated approach of a combination of a selection of the previously optimised parameters in an attempt to further maximise achieved glucose yields. If successful it is anticipated that this could then possibly provide the basis for a commercial industrial scale saccharification step as part of the process of biofuel production from BSG.

7.2 Materials and methods

7.2.1 Pre-treatment of BSG prior to saccharification experiments

All enzymatic saccharification experiments were conducted using dilute acid catalysed hydrothermal pre-treated BSG. The pre-treatment was conducted according to the optimised protocol described in section 4.2.2, using 121°C hydrothermal conditions with a 1% HCl acid catalyst at 25% w/v solids loading. The remaining insoluble residue was then used for all the saccharification experiments after recovery and processing as described in section 2.4.

Whilst the pre-treatment step did liberate a significant quantity of sugars (ca. 15 g/L glucose and xylose concentrations) directly into the supernatant or hydrolysate (particularly xylose from hemicellulose depolymerisation) this was set aside as the focus of this research was more specifically on optimising the subsequent enzymatic saccharification of the remaining insoluble residue that remained after pre-treatment.

7.2.2 Quantification of the total glucose contents of BSG and the insoluble residue remaining after pre-treatment

Glucose concentrations in the starting BSG and the remaining insoluble residue after the acid catalysed hydrothermal pre-treatment were quantified using the two-stage complete acid hydrolysis protocol described in section 2.3.1. This would then allow for the calculation of accurate % theoretical sugar yields after any subsequent enzymatic saccharification.

7.2.3 Enzymatic saccharification of pre-treated BSG.

All enzyme hydrolysis reactions (the saccharification of the insoluble residues after pre-treatment of the BSG as described in section 7.2.1) were conducted using the commercial enzyme preparation Novozymes Cellic® CTec2 with a 50°C incubation temperature used for all saccharification reactions following the general outline of the protocol described in section

2.7.1. The efficacy of all enzymatic saccharifications was evaluated via the achieved glucose yields (both g/L and % theoretical yields). All analyses were conducted in triplicate.

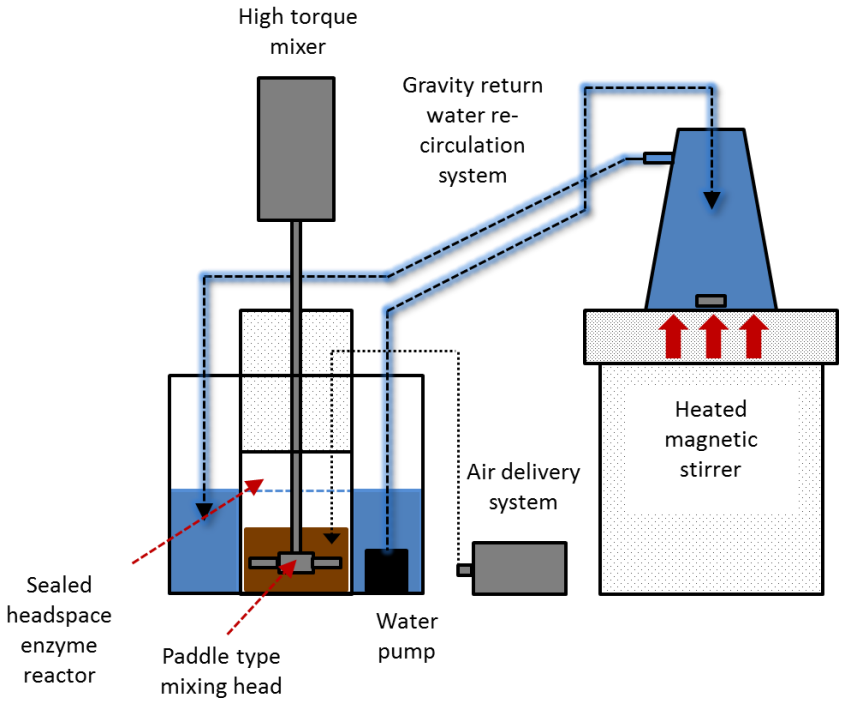
The Cellic® CTec2 used for saccharification had a total cellulase activity of 200 FPU/mL in undiluted format and was determined according to the protocol described in section 2.8. An enzyme dosing range of 10 - 160 FPU/g biomass was used. The correct quantity of pre-treated BSG (0.5 – 15 g) 50 mM sodium citrate buffer (pH 5.0 ± 0.1) were combined to produce a slurry and achieve the various desired solids loadings (5% - 50% w/v). After the correct incubation period (10-72 h) all enzyme saccharification preparations were centrifuged at 5000 rpm for 10 min and the supernatant removed and sampled for HPLC based quantification of glucose concentrations (as described in section 2.9.2). Prior to all enzymatic saccharification experiments the glucose concentration present in the Cellic® CTec2 enzyme preparation was determined by HPLC and subtracted from final enzymatic saccharification glucose yields to allow calculation of accurate % of theoretical yields (which were expressed as a percentage of the total glucose content of the pre-treatment generated residue prior to saccharification; as described in section 7.2.2).

7.2.3.1 Evaluation of the effect of various mixing protocols during enzymatic saccharification

A standard shaking incubator method using agitation at 150 rpm (MaxQ 4358 shaking incubator, Thermo Scientific, UK) was used as a benchmark with various alternative mixing protocols then compared against it. Efficacy of each different mixing protocol was determined by the liberated glucose yields achieved. Roller bed mixing protocols were conducted at 50 rpm using an SRT6D roller bed (Stuart Scientific, UK) whilst magnetic stirring protocols were conducted at 150 rpm using a multi-plate magnetic stirrer (Variomag poly 15, Thermo Scientific, UK). Both the roller bed and multi-plate magnetic stirrers were

housed within an MIR-253 incubator (Panasonic, Japan) to achieve the 50°C temperature optima for the Cellic® CTec2. A high torque mixing (HTM) protocol was conducted using a custom made, 2-piece, sealed headspace reaction vessel housed within a water bath which acted as an incubator (Figure 7.2). A 6-bladed paddle type mixer (at 150 rpm) was used for the actual agitation. The effect of supplemental aeration on enzymatic saccharification (as aeration was suggested to aid the function of GH61 carbohydrate-degrading enzymes present in the Cellic® CTec2) was investigated using the custom HTM system with an additional 2 mm air delivery line which supplied air from an external air pump (TetraTec APS100 Air Pump: 100 L/h, 2.5w). All analyses were conducted in triplicate.

Figure 7.2 Schematic diagram of high-torque mixing system and optional additional aeration.



7.2.3.2 Effect of supplementation of the Cellic® CTec2 with additional enzymes and cofactors on achieved glucose yields

The effectiveness of additional hydrolytic enzymes or various cofactors (in conjunction with Novozymes Cellic® CTec2) was investigated using the aerated HTM protocol as described in section 7.2.3.1. In particular the aeration was supplied to enhance any activity of the redox-dependant cofactors (the ascorbic acid and $Cu(NO_3)_2$) that were suggested to potentially assist in the functioning of the GH61 enzymes (Horn et al., 2012; Quinlan et al., 2011).

All analyses were conducted in triplicate. For preliminary proof of principle experiments all enzymes and cofactors were dosed at levels which were considered to be in excess (100 µl aliquot of each enzyme preparation as supplied) in order to ascertain any additional improvement in achieved glucose yields compared to the use of Novozymes Cellic® CTec2 alone. Additional xylanases were tested using Novozymes Cellic® HTec2 (100 µl/10 g biomass). Ferulic acid esterase was tested using Prozomix Feruloyl esterase (CAZy CE1 carbohydrate esterase family, from *Clostridium thermocellum*: 100 µl/10 g biomass; activity: 112.5 Units/mL). One unit was defined as the amount of enzyme required to release 1 µM of ferulic acid per minute from 32 µM methyl ferulate in 50 mM sodium phosphate buffer, pH 6.0, at 37°C, and at 335 nm. Acetyl xylan esterase was tested using Prozomix Acetyl xylan esterase (CAZy CE3, carbohydrate esterase family 3, from *Clostridium thermocellum ATCC 2740,* 100 µl/10 g biomass; activity: 112.5 Units/mL). One unit was defined as the amount of enzyme required to release 1 µM of p-nitrophenol per minute from p-nitrophenyl acetate (1 mM in the assay) in 50 mM phosphate buffer, pH 7, at 50°C, containing 1 mg/mL of BSA. Arabinofuranosidase was tested using Prozomix α-L-Arabinofuranosidase (CAZy GH51, glycoside hydrolase family 51, from *Streptomyces coelicolor A3,* 100 µl/10 g biomass; activity: 125 Units/mL). One unit was defined as the amount of enzyme required to release 1

μM of p-nitrophenol per minute from p-nitrophenyl α-L-arabinofuranoside (1 mM in the assay) in 50 mM phosphate buffer, pH 7, at 60°C, containing 1 mg/mL of BSA.

The addition of starch degradation enzymes was tested using α-amylase and glucoamylase (Megazyme, Ireland) with each enzyme tested individually (100 μl/10 g biomass) and also both enzymes simultaneously (100 μl of each/10 g biomass). The specific activity of α-amylase was 3000 Units/mL. One Unit was defined as the amount of enzyme required to release one μM of p-nitrophenol from blocked p-nitrophenyl-maltoheptaoside per minute (in the presence of excess α-glucosidase) at pH 6.0 and 40°C. The specific activity of glucoamylase was 160 Units/mg (60°C, pH 4.5 on soluble starch). One Unit of glucoamylase activity is defined as the amount of enzyme required to release one μg of β-D-glucose reducing-sugar equivalents per minute from soluble starch (10 mg/mL) in sodium acetate buffer (100 mM) at pH 4.5.

The effect of addition of proteolytic enzymes was investigated using the commercial preparations Alphalase™ NP (Danisco) and Pronase E (Sigma-Aldrich, UK). Alphalase™ NP is a commercial enzyme mixture (derived from *Bacillus amyloliquefaciens*) used within the beer brewing industry in order to assist in the hydrolysis of the protein component found within malted barley (the pre-cursor of BSG) in order to boost free amino nitrogen (FAN) levels to assist in high gravity fermentations thus rendering it potentially effective on the native protein fraction found in BSG.

Alphalase™ NP was dosed at 100 μl/10 g biomass. Alphalase™ NP has a suggested dosing of 0.1-0.3 kg/MT grist when used during the mashing stage of commercial beer brewing therefore the dose used here was also considered to be in excess. The Alphalase™ NP was prepared in 50 mM sodium citrate buffer at pH 6.5.

Pronase E is a commercial mixture of ca. 10 proteases from *Streptomyces griseus* K-1 and includes five serine-type proteases, two zinc endopeptidases, two zinc leucine aminopeptidases and one zinc carboxypeptidase (with an overall very broad spectrum of activity). Pronase E was prepared as a 1% w/w solution; 1 g/100 mL of 50 mM sodium citrate buffer (pH 7.5). The specific activity of Pronase E was 4 Units/mg. One unit of Pronase E was defined as the amount of enzyme required to hydrolyse casein to produce colour equivalent to 1.0 μM (181 μg) of tyrosine per min at pH 7.5 at 37°C (colour by Folin-Ciocalteu reagent).

For the evaluation of the effect of the supplementary protease enzyme preparations, both BSG and pre-treated BSG were subjected to a 5 h pre-incubation with each of the protease solutions (150 rpm agitation; MaxQ 4358 shaking incubator, Thermo Scientific, UK) using 15% w/v solids loading (at 60°C for the Alphalase™ NP and 37°C for the Pronase E) and prior to the saccharification stage using Novozymes Cellic® CTec2 (as described in section 2.5). The effect of a protein denaturation step after the protease pre-incubation was also investigated. The denaturation was conducted by incubation at 90°C for 10 min in a water bath. This was designed to denature and inactivate the proteases prior to dosing with Novozymes Cellic® CTec2 (to minimise any possible degradation of the lignocellulolytic enzymes found within the Cellic® CTec2 by the proteases). After denaturation samples were cooled to 50°C prior to dosing with Cellic® CTec2 and then further incubated at 50°C (150 rpm agitation; MaxQ 4358 shaking incubator, Thermo Scientific, UK) for the required time period.

In addition the supplementation with the potential GH61 enzyme family cofactors, copper and ascorbate ($Cu(NO_3)_2$ and ascorbic acid were tested (in conjunction with Cellic® CTec2 that supplied the GH61 enzymes) using a concentration range of 2.5 mM – 10 mM

incorporated into the standard buffer used for saccharification (50 mM sodium citrate; prepared as described in section 2.5).

7.2.3.3 Effect of variation of solids loading and enzyme dose during saccharification on achieved glucose yields

The individual and combined impacts of solids-loadings (5-25% w/v) and enzyme dose (Novozymes Cellic® CTec2; 10-160 FPU/g biomass) during enzymatic saccharification of pre-treated BSG were investigated using a D-optimal designed experiment (30 experiments, see Appendix 2 for experimental details). Response data (glucose yields) were then modelled against these factors using Design Expert software v 7.0 (Stat-Ease, Mn, USA). For this preliminary investigation a simplified shaking incubator (SI) protocol was utilised due to its high-throughput nature in comparison to the optimised aerated HTM system which consisted of a 'single-shot' bioreactor.

7.2.3.4 Effect of variation of solids loading and reaction time during saccharification on achieved sugar yields

The individual and combined impacts of solids-loadings (5-25% w/v) and reaction time (10-72 h) during enzymatic saccharification of pre-treated BSG (as described in section 2.3) were also investigated using a D-optimal designed experiment (30 experiments, see Appendix 2 for experimental details). Novozymes Cellic® CTec2 was used as the enzyme preparation (20 FPU/g biomass). Response data (glucose yields) were then modelled against these factors using Design Expert software v 7.0 (Stat-Ease, Mn, USA).

7.2.3.5 Batch-fed enzymatic saccharification experiments

These were conducted to evaluate their effectiveness against non-batch-fed saccharification protocols to determine whether the staggered addition of biomass achieved higher glucose yields. This experiment was an attempt to take advantage of the observed liquefaction that

occurred with all previous enzymatic saccharification trials (of pre-treated BSG) after ca. 12 h of hydrolysis. The increased liquefaction may have allowed for increased biomass addition without the requirement of any additional enzyme dosing. This staggered addition of biomass was considered to be more likely to result in high glucose concentration feedstocks than the staggered addition of more enzymes.

Preliminary experiments utilised the standard shaking incubator (SI) protocol (48 h incubation at 50°C with agitation at 150 rpm, MaxQ 4358 shaking incubator, Thermo Scientific, UK) due to its high throughput nature, using the correct amount of biomass (0.5 - 5 g of pre-treated BSG) to achieve the desired solids loading rates (5-50% w/v) but with the biomass split into two aliquots with the second aliquot added after 24 h incubation. All analyses were conducted in triplicate.

7.2.3.6 Combined enzymatic saccharification protocol

A combined enzymatic saccharification protocol was formulated from a combination of all of the previous individual experimental optimisations in an attempt to further maximise the achieved glucose yields when operating at high solids loading. This protocol was based on all the parameters that were previously concluded to play a significant role in enhancing the glucose yields achieved. The combined protocol involved using the aerated HTM system (Figure 7.2) operating at 50°C for 72 h incubation (considered an excess reaction time and thus non rate limiting) with a batch-fed approach (tested over a final solids loading range of 5-25% w/v). The system used a low dose of Novozymes Cellic® CTec2 (10 FPU/g biomass; to simulate a commercially viable large scale industrial process) and with the additional enzymes; ferulic acid esterase, acetyl xylan esterase, arabinofuranosidase, Cellic® HTec2.

All additional enzymes were dosed at 100 µl and prepared as described previously in section 2.5.4) and also with the additional enzymatic cofactors ($Cu(NO_3)_2$ and ascorbic acid (both dosed at 10 mM concentrations). All analyses were conducted in triplicate.

7.2.4 Quantification of free amino nitrogen (FAN) levels

FAN was determined by the European Brewery Convention ninhydrin colorimetric assay using glycine as the standard (Analytica-EBC, 1998).

7.2.5 Measurement of the protein content of the insoluble residue following pre-treatment of the BSG

A Thermo Flash Nitrogen Analyser (ThermoFisher Scientific, Waltham, Massachusetts, USA) was used to determine protein content using the protocol described in section 2.3.4. All analyses were conducted in triplicate.

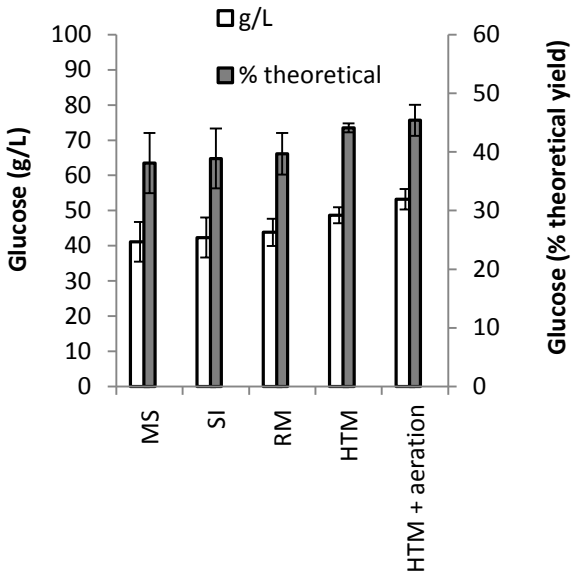
7.3 Results and discussion

7.3.1 Effect of variation of the mixing protocol on enzymatic saccharification yields from pre-treated BSG

Attempts were made to boost achieved sugar (glucose) yields from the enzymatic saccharification of pre-treated BSG through use of different mixing protocols. Higher energy mixing protocols may have reduced mass transfer limitations that may have limited enzyme activity. These experiments found that magnetic stirring (MS), shaking incubation (SI) and roller mixing (RM) producing similar results with ca. 40 g/L final glucose concentrations and achieved ca. 39% theoretical glucose yields after 72 h incubation when using an excess dose (160 FPU/g biomass) of Novozymes Cellic® CTec2 (Figure 7.3). However the use of a high torque mixing system (HTM) was shown to boost glucose yields very slightly (to ca. 49 g/L and 44% of theoretical yield). This showed the other protocols were sub-optimal in terms of their agitation and the enhanced mixing of the enzyme and substrate that occurred with the

HTM system was beneficial. Supplementation of the HTM system with additional aeration further increased the glucose yields (to ca. 53 g/L and 45% of theoretical yield) indicating the additional oxygen present in the air supplied may have enhanced the activity of some saccharification component, possibly the GH61 oxidative enzymes found within the Novozymes Cellic® CTec2. The precise mechanism of the beneficial effect of the additional aeration is currently unknown.

Figure 7.3 Impact of using shaking incubator, magnetic stirring, rollerbed mixing, high-torque mixing and aerated high-torque mixing protocols during enzymatic saccharification of pre-treated BSG on achieved glucose concentrations. Enzymatic saccharification conducted using Novozymes Cellic® CTec2 (excess enzyme dose: 160 FPU/g biomass with 72 h incubation at 50°C, 15% w/v solids loading) on pre-treated BSG (1% HCl 121°C 30 min pre-treatment, 25% w/v solids loading). Key: MS: magnetic stirring, SI: shaking incubator, RM: roller bed mixing, HTM: high torque mixing, HTM+ aeration: high torque mixing with additional aeration supplied from an external air pump.

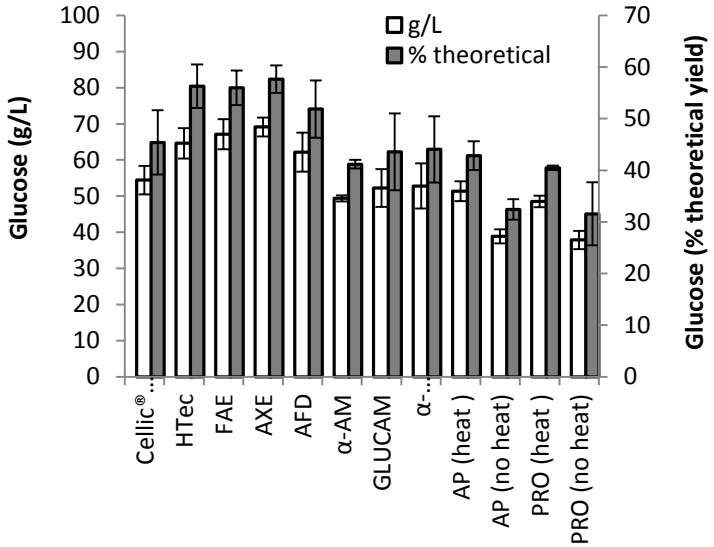


7.3.2 Effect of supplementation of the Cellic® CTec2 with additional enzymes and enzymatic cofactors on saccharification yields from pre-treated BSG

Enzymatic saccharification experiments utilising the aerated HTM mixing protocol (using Novozymes Cellic® CTec2) indicated that supplementation with the commercial protease mixtures (Alphalase NP and Pronase E with a 5 h pre-incubation prior to the saccharification

step with Novozymes Cellic® CTec2) did not result in any subsequent enhancement of the liberation of glucose from the pre-treated BSG compared to when using Novozymes Cellic® CTec2 alone (yields were actually slightly lower; ca. 50 g/L and 41% of theoretical; Figure 7.4).

Figure 7.4 Impact of using a range of supplementary enzymes in conjunction with Cellic® CTec2 during enzymatic saccharification of pre-treated BSG on achieved glucose concentrations. Enzymatic saccharification conducted using Novozymes Cellic® CTec2 (excess enzyme dose: 160 FPU/g biomass with 72 h incubation at 50°C, 15% w/v solids loading) using aerated high-torque mixing (HTM) on pre-treated BSG (1% HCl 121°C 30 min pre-treatment, 25% w/v solids loading) with all supplementary enzymes dosed at 100 µl (considered in excess dosages).



Key:	
HTec:	Xylanases
FAE:	Ferulic acid esterases
AXE:	Acetyl xylan esterases
AFD:	α-L-Arabinofuranosidase
α-AM:	Amylase
GLUCAM:	Glucoamylase
α-AM/GLUCAM:	Amylase + Glucoamylase
AP (heat):	Alphalase protease with heat inactivation
AP (no heat):	Alphalase protease without heat inactivation
PRO (heat):	Pronase protease with heat inactivation
PRO (no heat):	Pronase protease without heat inactivation

The use of either of the protease mixtures without a heat mediated inactivation stage (denaturation of the proteases) prior to the subsequent saccharification step with Novozymes Cellic® CTec2 significantly decreased the glucose yields achieved (ca. 38 g/L and 32% of theoretical yield) compared to when using Cellic® CTec2 alone. This suggested that some proteolytic attack of the carbohydrate degrading enzymes within the Cellic® CTec2 occurs as a direct result of the presence of the proteases. Overall the lack of any enhancement in glucose yields indicates that the considerable protein fraction which is still present within the BSG after hydrothermal pre-treatment (ca. 22% w/w crude protein) does not impede the activity of lignocellulolytic enzymes contained within Novozymes Cellic® CTec2. This would confirm as expected that proteinaceous tissues within the BSG are generally morphologically discreet from the lignocellulosic matrix. However, whilst glucose yields may not have been boosted by the addition of the proteases their inclusion could still be considered as it may also be desirable to liberate free amino acids (or low molecular weight peptides) into the hydrolysate which is generated from the saccharification step (the actual feedstock subsequently fermented to bioethanol) thus increasing the free amino nitrogen (FAN) content. This could potentially improve the subsequent fermentation performance which could be particularly useful for very high gravity fermentation systems (using very high initial sugar concentrations) as it may assist in reducing the osmotic stress on the yeast (or bacterial species) used and allow for production of high ethanol titres. Therefore, the lack of any observed improvement of the enzymatic saccharification yields with the use of additional proteases may not definitively exclude their incorporation into enzymatic cocktails used (for the saccharification step which would be required for biofuel production) due to the considerable, currently under-utilised protein fraction found within pre-treated BSG. Both protease mixtures were indicated to be effective at increasing FAN liberation from pre-treated BSG as levels were indicated to rise from ca. 23 mg/L to >100 mg/L when using the 5 h pre-

incubation. Due to this success of FAN liberation from pre-treated BSG an identical protocol was conducted but testing the protease mixtures on the starting BSG biomass (before any pre-treatment). This resulted in an even greater increase in FAN from 51 mg/L to >180 mg/L. This data would suggest that hydrothermal pre-treatment does either liberate or modify a significant proportion of the protein present in the BSG thus explaining the lower FAN concentrations liberated by the proteases on pre-treated BSG. Overall this could allow the generation of a FAN enriched solution before the saccharification step using Cellic® CTec2 and is the subject of current on-going research (Chapter 9). This could either be used for incorporation into any subsequent feedstock after saccharification or form an additional high value product stream which could be fractionated.

In a similar fashion supplementation with either (or both) of the starch degrading enzymes, glucoamylase (amyloglucosidase) and α-amylase did not significantly increase enzymatic saccharification yields (glucose yields) beyond that achieved by Novozymes Cellic® CTec2 alone. Some starch does usually remain present in BSG (typically <5% w/w) unless a very intense and effective mashing protocol has been used during the initial beer brewing process such as use of mash filter brewing technology and the associated brewhouse apparatus such as a hammer mill (e.g. extra finely milled grist used initially and also possibly with additional saccharification enzymes added during mashing; both of which aid extraction efficiency of starch removal from the malted barley). However the hydrothermal pre-treatment temperatures (121°C) that were used initially would have very likely already gelatinised and solubilised any residual starch that may have been present (in the BSG) into the pre-treatment generated hydrolysate. Therefore starch degradative enzymes did not directly boost glucose yields through starch hydrolysis or indirectly through enhanced access of the cellulases (present in the Novozymes Cellic® CTec2) to cellulose through removal of any starch grains that may have physically impeded the enzymes.

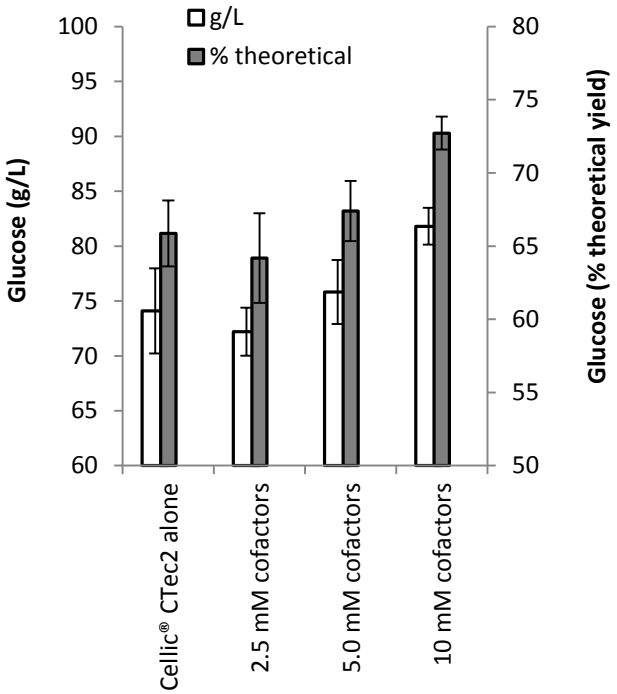
In contrast to this the supplementation with additional xylanases (Novozymes Cellic® HTec2), ferulic acid esterases, acetyl xylan esterases and α-L-arabinofuranosidases all resulted in a significant increase ($p<0.05$) in enzymatic saccharification glucose yields compared to Cellic® CTec2 alone. The ferulic acid esterase, acetyl xylan esterase and xylanases (Cellic® HTec2) were indicated to achieve the greatest enhancement in glucose yields (ca. 64 g/L and 58% of theoretical yields) whilst the α-L-arabinofuranosidase liberated ca. 62 g/L glucose and 52% theoretical yield. This suggested the pre-treated BSG still contained various lignocellulosic linkages that to a small extent impeded the function of the cellulases found within Novozymes Cellic® CTec2. It further supports the idea that specific lignocellulosic biomass such as BSG requires a tailor made enzyme cocktail in order to maximise the glucose yields achieved. Whilst Novozymes Cellic® CTec2 is a highly effective commercial enzyme preparation on its own (and a complex mixture of different lignocellulolytic enzymes) specific substrates may require larger quantities of specific enzymes e.g. ferulic acid (feruloyl) esterases, due to the prevalence of particular linkages found within it.

Alternatively specific additional enzymes may be needed due to the format of the specific components present that may be chemically modified as a result of the specific pre-treatment step which had been employed upstream of the enzymatic hydrolysis step.

Overall it indicates a requirement for comprehensive optimisation of the enzymatic saccharification step for each specific lignocellulosic substrate. In the specific case of BSG as the feedstock it may also indicate that each different batch may also require some degree of optimisation in order to maximise the saccharification yields due to compositional differences that arise as a result of the use of differing barley cultivars used initially in the grist or different subsequent mashing conditions used during brewing (wort production).

Additionally the supplementation of the aerated HTM protocol (using Novozymes Cellic® CTec2) with the GH61 indicated enzymatic cofactors copper and ascorbate showed that a small but significant increase ($p<0.05$) in glucose yields was achieved when using a 10 mM concentration of both cofactors simultaneously (liberating ca. 58 g/L glucose and ca. 56% of theoretical yield; Figure 7.3).

Figure 7.5 Impact of the simultaneous addition of both the enzymatic cofactors $Cu(NO_3)_2$ and ascorbate in conjunction with Cellic® CTec2 during enzymatic saccharification of pre-treated BSG on achieved glucose concentrations. Enzymatic saccharification conducted using Novozymes Cellic® CTec2 (excess enzyme dose: 160 FPU/g biomass with 72 h incubation at 50°C, 15% w/v solids loading) using aerated high-torque mixing (HTM) on pre-treated BSG (1% HCl 121°C 30 min pre-treatment, 25% w/v solids loading) with a range of $Cu(NO_3)_2$ and ascorbic acid concentrations (2.5-10 mM with equal concentrations of both reagents).



This suggested that GH61 enzymes found within Novozymes Cellic® CTec2 may be responsive to increased concentrations of redox active cofactors and function sub-optimally without this additional supplementation and confirmed the work of Quinlan et al (2011) and Beeson et al (2011). Further work would need to be conducted in order to ascertain whether

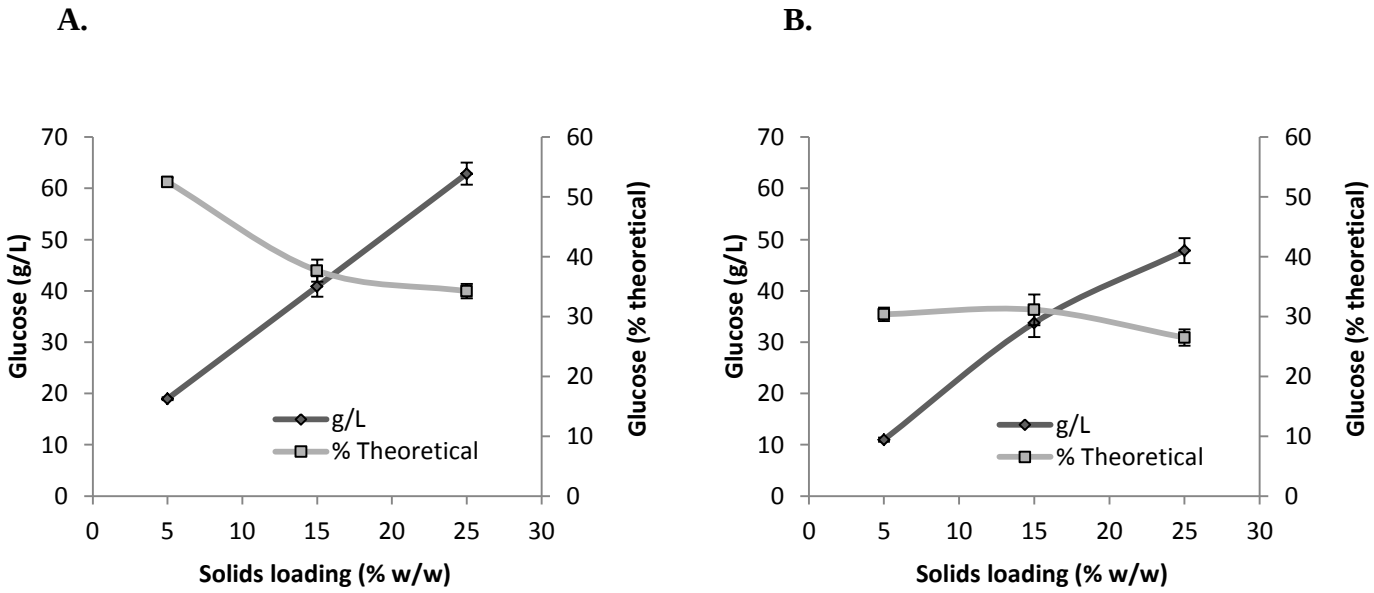
the presence of copper ions at concentrations of around 10 mM (should those concentrations be retained in the feedstock generated) would be detrimental to any downstream fermentations conducted as various yeast species have been indicated to be sensitive to it at high concentrations of copper ions resulting in sub-optimal fermentation performance (Dönmez & Aksu, 1999). However these suggested GH61 enzymatic cofactors may offer a more cost effective alternative to augmentation of generic lignocellulosic enzyme cocktails with additional expensive exotic enzymes. Copper is suggested to bind to a classical type II site that is located at the N terminus of the enzyme (Quinlan et al., 2011). Oxygen can then bind to the Copper ion and provides an oxidising effect that aids the function of the enzymes active site. Additionally the small molecule redox-active cofactor ascorbic acid further enhances the oxidising power of the GH61 enzyme complex.

Whilst some members of the GH61 family exhibit weak β-1,4 endoglucanase activity it is unlikely they act upon single cello-oligomeric chains as it has been indicated that no soluble reaction products such as cellobiose or glucose are detected when they are used alone on lignocellulosic substrates (Harris et al., 2010; Horn et al., 2012). In addition they do not appear to enhance the activity of cellulases on pure cellulose (Harris et al., 2010). In conclusion it would then suggest that GH61 class enzymes may act upon a rare bond found only in lignocellulosic materials (which are obviously not found in pure cellulose) that may possibly obstruct the normal function of other primary cellulases in some way. Alternatively they may function via a non-hydrolytical based mechanism possibly similar to the mechanism of carbohydrate binding modules (CBMs) in that they may penetrate micro-cracks in crystalline cellulose and cause localised swelling that enables free water to penetrate, thus further increasing the porosity allowing better access for additional enzymes to function (Arantes & Saddler, 2010).

7.3.3 Effect of variation of the solids loading and enzyme dose used during enzymatic saccharification on liberated glucose yields

Two-dimensional variants of the derived models for glucose release (using only a selection of the data set) found that when using an excess of Novozymes Cellic® CTec2 (160 FPU/g biomass) increasing solids loading during enzymatic saccharification from 5% w/v to 25% w/v resulted in a linear increase in g/L glucose concentrations liberated (Figure 7.6A).

Figure 7.6 Effect on achieved glucose concentrations of variation of the solids loading used during enzymatic saccharification of pre-treated BSG. Enzymatic saccharification conducted using Novozymes Cellic® CTec2 with 72 h incubation at 50°C, 5-25% w/v solids loading) using standard shaking incubator protocol (SI) on pre-treated BSG (1% HCl 121°C 30 min pre-treatment, 25% w/v solids loading). Figure A shows the theoretical maximum yield achieved using an excess enzyme dose (160 FPU/g biomass) whereas Figure B shows the more realistic achieved yield when constraints of enzyme pricing are considered and a lower enzyme dose used (10 FPU/g biomass).



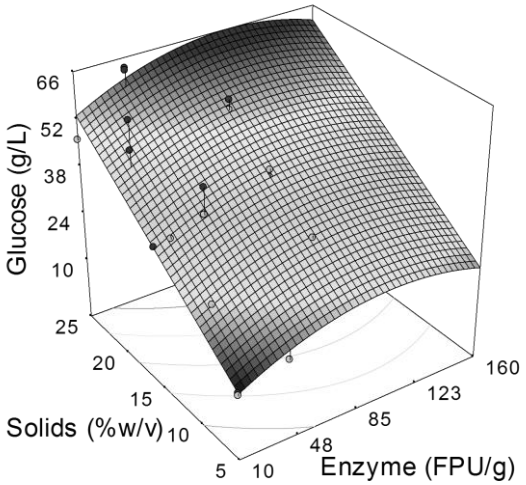
However, % theoretical glucose yields dropped as solids loading was increased, particularly sharply with the increase from 5% w/v to approximately 15% w/v. Increasing solids loading beyond 15% w/v resulted in only a minor drop in % theoretical glucose yields. In comparison when shifting to using a low dose of Novozymes Cellic® CTec2 (10 FPU/g biomass) increasing solids loading from 5% w/v to 25% w/v also resulted in an almost linear increase

(albeit lower ramp than when using an excess enzyme dose) in g/L glucose concentrations liberated (Figure 7.6B). However, in contrast to when using an excess enzyme dose there was only a very small drop in % theoretical yields (from ca. 30% to 26%) as solids loading was increased from 5% to 25% (w/v) suggesting that higher solids loading is optimal when using low enzyme doses . A direct comparison of the % theoretical glucose yields at 25% solids loading with both a low and an excess enzyme dose found that x16 enzyme dose increase only resulted in an increase from 26% to 34% of theoretical glucose yield. This may indicate that low enzyme doses at high solids loading may be optimal in terms of maximising efficiency of processing large quantities of biomass (i.e. pre-treated BSG). If achieving the highest g/L glucose concentration was the primary target for rendering large scale bioethanol fermentation economically viable and % theoretical yield a secondary objective (which could be sacrificed somewhat) then the use of very high solids loading could prove very effective as high g/L glucose concentrations could be achieved. Three-dimensional response surface models (3D RSM) of the glucose release data looking at the simultaneous effect of variation of solids loading and enzyme dose (using Novozymes Cellic® CTec2) showed that increasing enzyme dose from 10 to 160 FPU/g biomass at any given solids loading between 5% and 25% w/v did not significantly increase the g/L glucose concentration liberated (Figure 7.5A). For achieving optimal g/L glucose concentrations a low enzyme dose, high solids loading combination was confirmed to be optimal as was previously apparent as with the 2D models (Figure 7.6B).

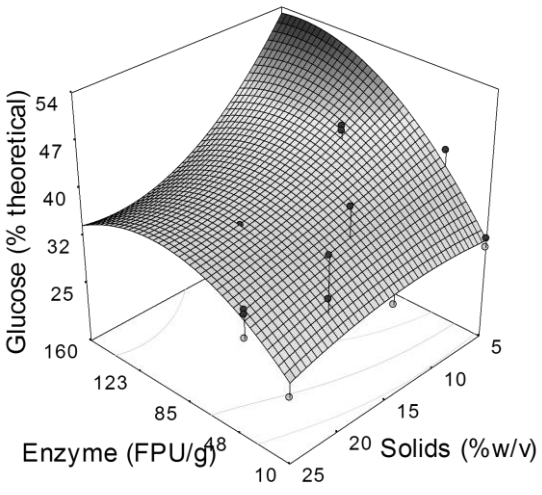
However, in contrast to this, increasing solids loading at any given enzyme dose was indicated to be a significant factor affecting glucose yields. 3D RSM of % theoretical glucose yield data indicated that at low enzyme doses (10 FPU/g biomass) the increase in solids loading was not a significant parameter.

Figure 7.7 3D response surface models showing the simultaneous effects of the factors (i) % solids-loading (w/v) and (ii) enzyme dose for saccharification. A: Glucose: g/L (model R^2: 0.94) B: Glucose: % theoretical yield (model R^2: 0.86). Enzymatic saccharification conducted using Novozymes Cellic® CTec2 (10-160 FPU/g biomass) with 72 h incubation at 50°C, 5-25% w/v solids loading) using standard shaking incubator protocol (SI) on pre-treated BSG (1% HCl 121°C 30 min pre-treatment, 25% w/v solids loading).

A.



B.



In contrast to this at high enzyme doses (160 FPU/g biomass) increasing solids loading was a significant parameter, resulting in a considerable reduction in % theoretical glucose yields (Figure 7.5B). Increasing enzyme dose at low loadings loading (5% w/v) was shown to have a much more significant effect on achieved glucose yield than at high solids loading (25% w/v). For optimal conversion efficiency (optimal % theoretical yields achieved) a low solids loading, high enzyme dose combination was suggested to be most suitable. Overall the enzyme reaction kinetics seen here did not appear to follow the traditional Michaelis–Menten model. This is likely due to the (relatively) highly insoluble slurries (with significant quantities of hydrophobic components) being used as opposed to highly soluble, dilute enzyme-substrate mixes.

7.3.4 Effect of variation of solids loading and reaction time of enzymatic saccharification on achieved glucose yields

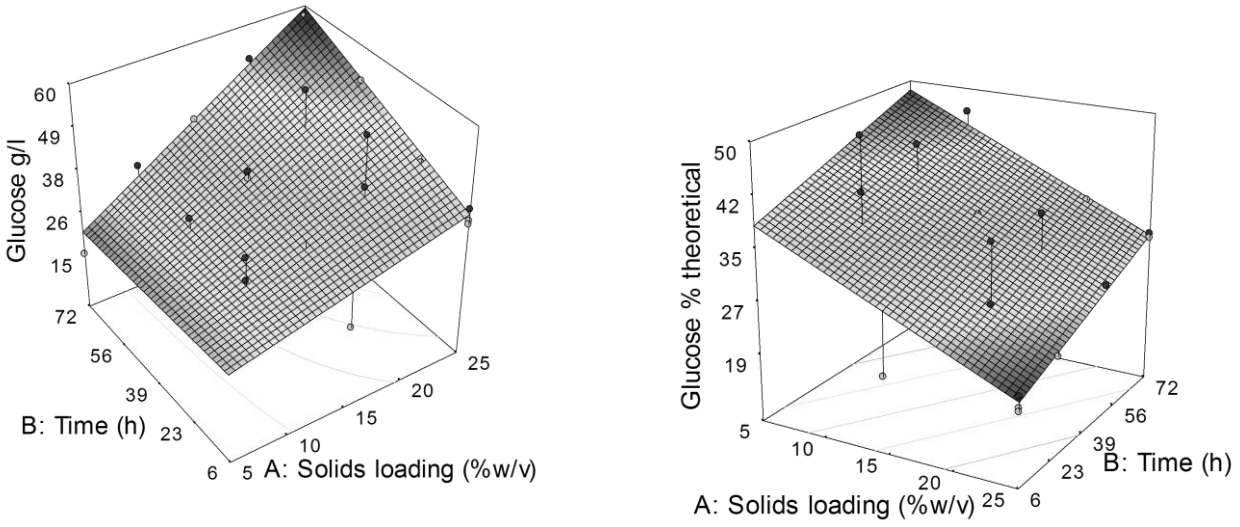
As described in section 3.3, a higher throughput system for enzymatic saccharification was utilised for the investigation of the optimal hydrolysis (reaction) time varied in response to variation of the solids loading used during enzymatic saccharification.

Evaluation of the 3D RSM data found that liberated glucose concentrations (g/L) peaked (achieving 60 g/L) at the maximal solids loading tested (25% w/v) and when the longest reaction time (72 h) was used (Figure 7.6A). At low solids loading (5% w/v) an extension of the reaction time beyond 6 h did not result in any significant increase in glucose liberation. However at high solids loading (25% w/v) extension of the reaction time beyond 10 h resulted in a linear increase in glucose liberation. This data showed that in terms of g/L glucose liberation the optimal reaction time depends heavily on the actual solids loading used for the enzymatic saccharification. It was shown that increasing the solids loading from 5% to 25% w/v when using a shorter reaction time (10 h) did result in a small but significant increase in liberated glucose concentrations (g/L). However, this effect was much more pronounced when using a longer reaction time (>24 h).

In direct contrast to g/L glucose liberation, the % theoretical glucose yields showed that a linear drop in conversion efficiency was observed (regardless of reaction time used) as solids loading was increased (Figure 7.6B). As expected the highest conversion efficiencies achieved (ca. 50% of theoretical yield) were observed when the lowest solids loading was used (5% w/v). At any given solids loading used the extension of reaction time from 10 h to 72 h was shown to offer only a relatively small increase in % theoretical glucose yield (from ca. 40% to 50% theoretical yield at 5% w/v solids loading).

Figure 7.8 3D response surface models showing the simultaneous effects of the factors (i) % solids loading (5-25% w/v) and (ii) reaction time (6-72 h) during enzymatic saccharification of pre-treated BSG on the resultant glucose concentrations liberated. A: Glucose; g/L (R^2: 0.78), B: Glucose; % theoretical yield (R^2: 0.65). Enzymatic saccharification conducted using Novozymes Cellic® CTec2 (20 FPU/g biomass) with 6-72 h incubation at 50°C, 5-25% w/v solids loading) using standard shaking incubator protocol (SI) on pre-treated BSG (1% HCl 121°C 30 min pre-treatment, 25% w/v solids loading).

A) **B)**



This would indicate that extension of the reaction time from ca. 24 h to 72 h would not be economically viable when conducting an enzyme saccharification step at less than 25% (w/v) solids loading.

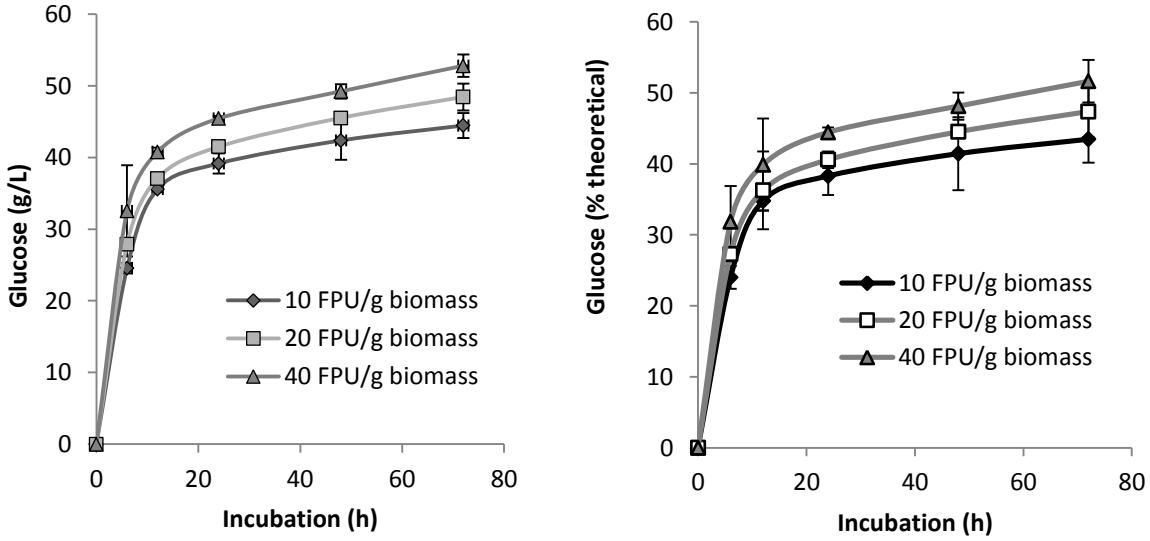
7.3.5 Effect of variation of reaction time of enzymatic saccharification at various low enzyme doses on achieved glucose yields

Using a 6-72 h time course (again with a standard SI based protocol for higher throughput than the aerated HTM system) a very similar trend in liberated g/L glucose concentrations was observed across a low range of enzyme doses (10-40 FPU/g biomass; Novozymes Cellic® CTec2) when operating at a static 15% w/v solids loading (Figure 7.7A). The predominant bulk of the total final saccharification yields were achieved by ca. 24 h (ca. 86% of the final glucose yields that were achieved when using a 72 h reaction time). Regardless of

the enzyme dose used, any extension of the reaction time beyond ca. 24 h resulted in only a small increase in achieved glucose yields. A very similar trend was observed with % theoretical yields (Figure 7.7B). Overall the data found that at 15% (w/v) solids loading and when using low enzyme doses, reaction times of ca. 24 h would be optimal as doubling the reaction time to 48 h only liberated ca. 9% more glucose (g/L).

Figure 7.9 Effect of simultaneous variation of the enzyme dose and solids loading used during enzymatic saccharification on achieved glucose concentrations. A: Glucose (g/L) and B: Glucose (% theoretical yield). Enzymatic saccharification conducted using Novozymes Cellic® CTec2 (10-40 FPU/g biomass) with 6-72 h incubation at 50°C, 15% w/v solids loading) using standard shaking incubator protocol (SI) on pre-treated BSG (1% HCl 121°C 30 min pre-treatment, 25% w/v solids loading).

A) **B)**



7.3.6 Investigation of batch-fed protocols on enzymatic saccharification glucose yields

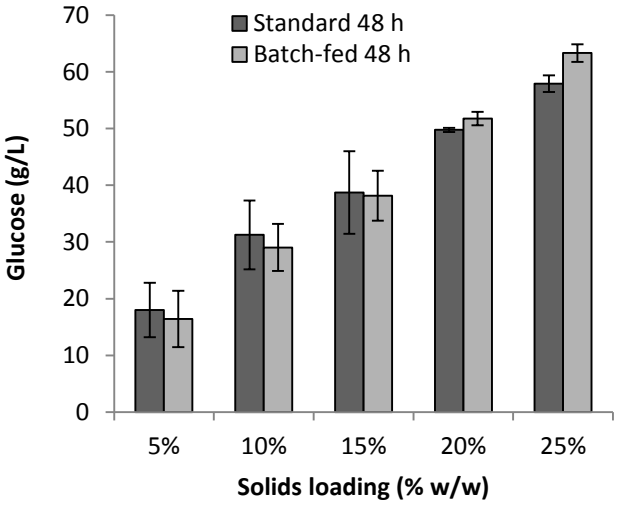
The preliminary screening of batch-fed protocols (in which the required amount of pre-treated BSG to achieve the desired solids loading was added in two aliquots with the second

aliquot added after 24 h incubation) was also initially conducted using a high-throughput SI protocol as opposed to the optimised aerated HTM system.

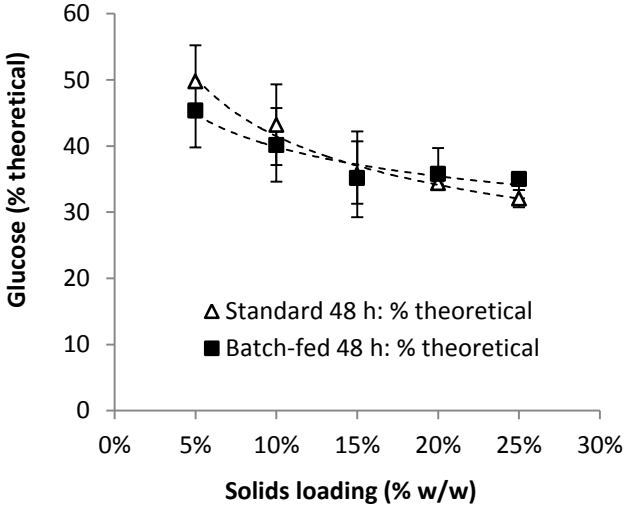
The use of the batch-fed protocol resulted in small but significant (p<0.05) increases in final g/L glucose concentrations (ca. 12% more glucose) when solids loading exceeded 20% (w/v) in comparison to a standard, non-batch-fed protocol using both an identical enzyme dose and identical incubation period but with all the biomass added entirely at the start of the incubation period (Figure 7.8A). Whilst the degree of relative enhancement of the batch fed protocol at both 20% and 25% w/v solids loadings was similar (ca. 12% increases in % theoretical yield compared to the standard non-batch-fed protocol at similar final solids loading) the effect on final g/L glucose concentrations was more pronounced as solids loading increased due to the presence of ever greater quantities of biomass. The % theoretical glucose yields followed a similar trend with batch-fed protocols only functioning slightly more effectively (than non-batch-fed protocols) at very high solids loading (≥20% w/v; Figure 7.8B). Overall this indicated that when operating at ≥20% w/v solids loading, splitting the biomass into two equal aliquots whilst using an identical enzyme dose boosted both final g/L glucose concentrations and % theoretical yields through effectively halving the operational solids loading for the first 24 h of a 48 h saccharification period. The initial 24 h saccharification period facilitated a considerable degree of liquefaction (and reduced viscosity) of the slurry which likely assisted in some reduction of any mass transfer limitations possibly enabling better enzyme mobility and thus better enzyme-substrate linkages.

Figure 7.10 Efficacy of batch-fed enzymatic saccharification protocols compared to non-batch-fed protocols. A: Glucose (g/L) and B: Glucose (% theoretical yield). Enzymatic saccharification conducted using Novozymes Cellic® CTec2 (50 FPU/g biomass) with 48 h incubation at 50°C, 5-25% w/v final solids loading) using standard shaking incubator protocol (SI) on pre-treated BSG (1% HCl 121°C 30 min pre-treatment, 25% w/v solids loading). Batch-fed protocol had the required biomass quantity split into two equal portions with the second aliquot added after 24 h.

A.



B.



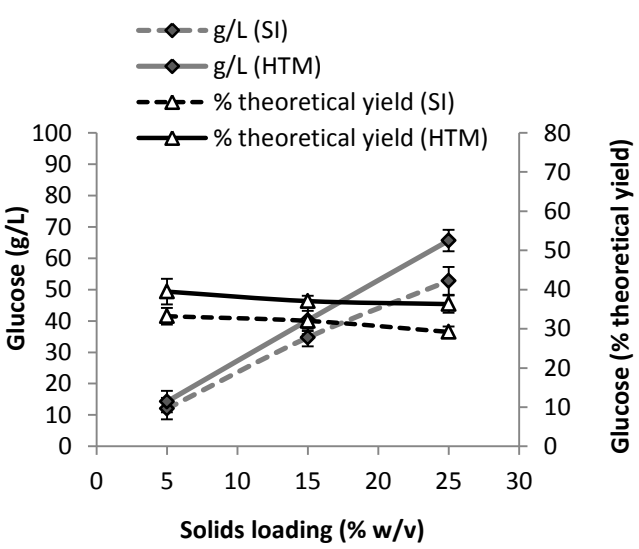
7.3.7 Comparison of the effect of increasing solids loading during enzymatic saccharification using both an optimised aerated HTM protocol and a standard SI protocol

Both g/L glucose concentrations and % theoretical glucose yields were indicated to be improved through the use of the aerated HTM protocol compared to the standard SI protocol with both responses ranging from ca.10% to 25% higher across all solids loadings tested (Figure 7.9A). However, the degree of improvement (achieved using the aerated HTM protocol) for both response parameters increased as solids loading increased with the greatest improvement observed at the highest solids loading tested (ca. 25% improvement in glucose yields at 25% w/v solids loading). This would indicate the HTM protocol overcame some of

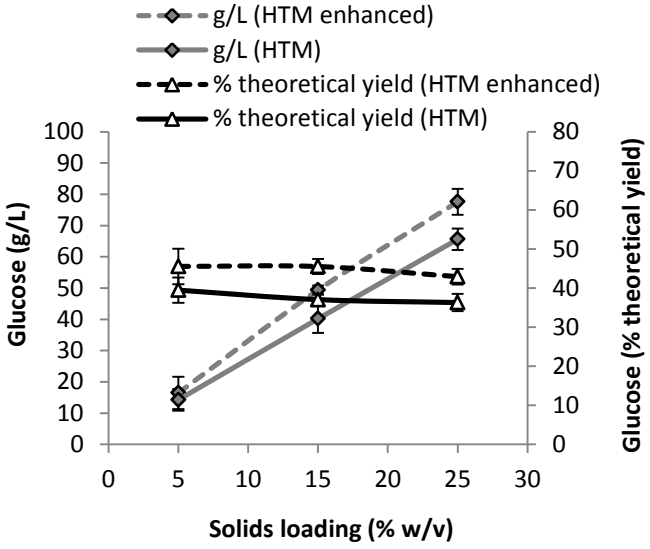
the substrate insolubility issues which limited mass transfer. Overall it found that higher energy mixing regimes may still play some part in facilitating effective operation of high solids loading enzymatic saccharification of pre-treated lignocellulosic biomass such as BSG.

Figure 7.11 Comparison of the effect of variation of solids loading on post enzymatic saccharification glucose concentrations achieved using different protocols. A) shaking incubator (SI) protocol compared to the aerated high-torque mixing (HTM) protocol. B) aerated HTM protocol alone compared to the consolidated protocol (aerated HTM with additional enzymes and cofactors). Enzymatic saccharification conducted using Novozymes Cellic® CTec2 (10 FPU/g biomass and incubated at 50°C for 72 h) on pre-treated BSG (1% HCl 121°C 30 min, 25% w/w solids loading) over a 5%-25% solids loading range. A) Standard shaking incubator (SI) system (150 rpm) compared to aerated high-torque mixing (HTM) system (150 rpm). B): HTM aerated protocol (non-batch-fed with no additives) compared to a HTM aerated protocol batch fed (biomass split into 2 x aliquots; second added after 24 h) with additional enzymes: FAE; ferulic acid esterase, AXE; acetyl xylan esterase, AFD; α-L-arabinofuranosidase, HTec2; Cellic® HTec2 xylanases (all at 100 µl doses) and Cu(NO3)2 and ascorbic acid (both at 10 mM).

A.



B.



7.3.8 Combined enzymatic saccharification protocol

The combination of the aerated HTM system but also simultaneously incorporating a batch-fed approach when using a low enzyme dose (Novozymes Cellic® CTec2: dosed at 10 FPU/g biomass) and a synergistic combination of various additional enzymes and cofactors previously indicated to enhance the saccharification yields (ferulic acid esterase, acetyl xylan esterase, Novozymes Cellic® HTec2, α-L-arabinofuranosidase, $Cu(NO_3)_2$ and ascorbic acid) resulted in an improvement of ca.13% to 19% more glucose (both g/L and % theoretical yields, compared to the use of a non-batch-fed variant of the aerated HTM protocol without any supplements) across all solids loadings tested (Figure 7.9B).

As previously observed with the aerated HTM system (in comparison to the standard SI protocol) the degree of improvement for both response parameters (g/L and % theoretical glucose yields) increased as solids loading increased but in this case with the greatest improvement observed at 15% w/v solids loading (ca. 19% improvement in glucose yields). Increasing solids loading to 25% w/v then reduced the relative degree of improvement in achieved glucose yields to only ca. 15% more compared to the aerated HTM system alone. This may have indicated that 15% solids loading was a ‘sweet spot’ in terms of optimal conversion efficiency (optimal % theoretical glucose yields).

This ever smaller relative increase in glucose yields even with the inclusion of all the optimisations suggested the point of diminishing returns had likely been reached. It indicated that simultaneous inclusion of all the additives (supplementary enzymes and enzymatic cofactors) alongside the main cellulolytic enzyme cocktail (Cellic® CTec2) would not be economically viable in terms of their incorporation into any large scale enzymatic saccharification process as use of any one supplementary additive alone would likely do the job effectively and facilitate a good degree of enhancement of saccharification. The choice of

which particular additive would likely depend solely on what was the most economically viable and readily available.

Overall, even with all the indicated optimisation strategies employed simultaneously, only relatively low % theoretical glucose yields were achieved (ca. 43% when using 25% w/v solids loading). This could be due to a number of possible factors. Some degree of product inhibition is a possibility (catabolite repression) as cellobiose has been shown to feedback inhibit various endocellulases (Xiao et al., 2004). However the inclusion of high concentrations of β-glucosidase enzymes within the Novozymes Cellic® CTec2 preparation should in theory combat this issue to a significant degree through cleaving the cellobiose molecules into their constituent monomeric glucose sub-units. This was therefore considered unlikely to be the cause of the low yields. In addition HPLC analysis of all post enzymatic saccharification supernatants (the hydrolysates produced from all experiments etc.) did not show any significant cellobiose concentrations to be present that would have possibly been an indicator of feedback inhibition. Alternatively inhibition of the enzymes from deleterious product formation that may have occurred during the upstream pre-treatment process (such as liberation of lignin based hydroxycinnamic acids or furan based hemicellulose or cellulose degradation products) can be discounted in this case as all experiments were conducted using thoroughly washed biomass prior to the enzymatic hydrolysis reactions.

Another possible factor is that adsorption of various cellulases to the cellulose substrate has been experimentally shown to decrease with increasing solids loading (Kristensen et al., 2009). This could possibly be due to mass transfer limitations due to the insoluble nature of the slurries used when attempting high solids loading enzyme saccharification (the low free water content and thus low ‘mobile phase’ in which the enzymes can freely move throughout the media). This is particularly applicable to pre-treated lignocellulosic substrates such as BSG as the upstream processing (the thermo-chemical pre-treatment step in particular)

generates a wide range of sizes of fragments of the material (some of which are very small; < 106 μm) that saturates the liquid or 'mobile phase'. Additionally the build-up of hydrolysis end products such as high concentrations of glucose and cellobiose have been shown to physically impede the adsorption of endocellulases to cellulose (Stutzenberger & Lintz, 1986). The general observed trend of a linear increase in liberated glucose concentrations (g/L) as solids' loading was increased (for much of the experimental data presented here) would suggest that when very high solids loadings were used (>25% w/v) sufficient glucose concentrations may be present to impede any further successful adsorption (or mobility of) the carbohydrate binding domains (CBMs). This could assist in explaining the sub-optimal % theoretical glucose yields. It is the CBMs that facilitate the adsorption and subsequent activity and mobility of the critical endocellulases that initially cleave internal β-1,4 glycosidic linkages thus generating more free reducing ends that other cellulase class enzymes can attack (Arantes & Saddler, 2010). The modification of endocellulase CBMs (including their substitution for higher binding affinity variants) in order to enhance both their activity and their resistance to high solid to liquid ratio environments thus improving their function at high solids loading has also been considered (Taylor et al., 2012). The issue of this pseudo form of non-competitive inhibition by enzyme reaction products such as glucose could possibly be solved through investigation of simultaneous saccharification and fermentation (SSF) approaches. This would enable the selective uptake and metabolism of reaction products such as glucose thus reducing their deleterious feedback inhibitive effects. However, the system would need to be carefully designed as some provision for fermentation product removal would be required, especially in the case of bioethanol production as ethanol is a known inhibitor of various cellulases although less so than the degree of feedback inhibition that occurs as a result of cellobiose build-up (Ooshima et al., 1985). The use of a thermophillic fermentative organism in conjunction with the high temperature optima (50°C)

of Novozymes Cellic® CTec2 may enable some degree of evaporative recovery of any ethanol produced as an extremophile may tolerate short periods of temperature spikes which could be used to enhance recovery. Alternatively, a sealed system that artificially reduced the atmospheric pressure within the bioreactor to below the vapour pressure of ethanol could then enable lower evaporative temperatures. However, this would not solve the issue of the build-up of any other non-volatile metabolic waste product which may still be equally inhibitory towards various enzymes.

7.4 Conclusions

Here we have evaluated the significance of numerous different parameters associated with enzymatic saccharification (at high solids loading) of pre-treated BSG in an attempt to maximise the achieved glucose yields. We conclude that supplementation of the cellulolytic enzymes with certain additional enzymes and cofactors can significantly boost achieved glucose yields. Furthermore batch-fed protocols and improved slurry agitation through aerated high-torque mixing can also offer significant enhancements in glucose yields. From our individual experimental investigations and optimisations we have developed a novel, combined enzymatic saccharification protocol which functioned effectively at high solids loadings in an attempt to further maximise achieved glucose yields. Glucose yields of ca. 78 g/L and ca. 43% of theoretical yield were achieved when operating at 25% w/v solids loading and using a commercially applicable low dose of Cellic® CTec2 (10 FPU/g biomass). However, our investigations reveal that additional development is required in order to either increase these glucose yields further or maintain them when increasing the operational solids loading beyond 25% w/v. Additionally the % theoretical glucose yields dropped as solids loading (during saccharification) was increased which indicated that one or the other could be sacrificed depending on whether the priority was either maximal biomass processing or high saccharification efficiency.

8 Evaluation of a fungal based consolidated bioprocessing approach to bioethanol production from brewers spent grain

8.1 Introduction

As a consequence of the technical difficulties and the economic implications of using a conventional approach to advanced generation biofuel production (thermo-chemical pre-treatment, enzyme saccharification and then fermentation to ethanol) various research has looked at entirely biological alternatives. These include simultaneous saccharification and fermentation (SSF) and consolidated bioprocessing or CBP (Lynd et al., 2005; Olson et al., 2012; Xu et al., 2009). Most attempts at CBP involve the use of just one solitary organism, such as yeast strains (Hasunuma & Kondo, 2012; van Zyl et al., 2007) or bacteria such as various *Clostridia* species (Jin et al., 2011; Lynd et al., 2005; Salimi et al., 2010), often genetically modified (GM) for expression or over expression of lignocellulolytic enzymes . However the use of obligate anaerobic species such as *Clostridia* has numerous technical difficulties associated with it not least due to the pathogenicity of many strains to humans. Also the widespread use of GM organisms has additional implications and restrictions for their usage in many parts of the world. Additionally, there have been attempts at achieving the production of ethanol from filamentous fungi already capable of secreting lignocellulolytic enzymes (Srinivasan, 2009).

In the absence of any thermo-chemical pre-treatment the purely biological deconstruction and saccharification of any lignocellulosic feedstock would likely require a wide variety of enzymes to achieve similar ultimate ethanol productivity as conventional advanced generation biofuel production. This is because lignocellulosic materials in the wild are usually deconstructed by a consortium of different microorganisms (often many fungal species) working symbiotically and would likely include a large number of carbohydrate

degrading (CAZy or Carbohydrate-Active enZYmes) and associated enzymes (Delmas et al., 2012; Martinez et al., 2009; Znameroski & Glass, 2013).

With this inherent natural propensity of many fungal species to deconstruct lignocellulosic material in the wild this research evaluated the potential production of ethanol via CBP utilising entirely fungal based consortia. A primary organism such as a filamentous fungus could be used to biologically deconstruct the lignocellulosic material. This could be achieved through secretion of its native lignocellulolytic enzymes. This could be as a substitute for the use of expensive exogenous commercial enzyme preparations and possibly also even negate the requirement for the use of chemically or energetically intensive pre-treatment steps. Subsequently, a secondary fungal species such as a particular yeast strain could then ferment any liberated monomeric sugars into ethanol. The use of a consortium of organisms in this way could enable a non-GM based CBP system utilising both the secretion of high enzyme titres from various filamentous fungal species and the rapid fermentation rates and ethanol tolerance of various yeast strains.

Various primary fungal candidates exist with considerable known arsenals of secreted lignocellulolytic enzymes, including *Aspergillus niger, Aspergillus oryzae, Trichoderma reesei* and *Humicola insolens*. However *A.oryzae* is of particular interest even though this species is more widely recognised as a specialist in starch hydrolysis (α-glycosidic linkages instead of the β-glycosidic linkages found in cellulose). The production of the Japanese alcoholic beverage saké utilises a consortium of *A.oryzae* and a yeast strain *Saccharomyces cerevisiae* (often NCYC479, although not solely) to produce high concentrations of ethanol (ca. 20% ABV) from the starch component found within rice (Takagi et al., 2005). The *A.oryzae* organism is responsible for the secretion of the enzymes, primarily α-amylases; endo-1,4-α-D-glucan glucohydrolase (EC 3.2.1.1) that hydrolyse the starch into glucose that the *S.cerevisiae* then utilises for ethanol production (Francis et al., 2003). However *A.oryzae*

has also been shown to secrete lignocellulolytic enzymes (endocellulases and various xylanases) in significant quantities when cultured in media containing lignocellulosic biomass (Acharya et al., 2010). The saké fermentation system could therefore represent the basis for the development of a CBP approach for commercial bioethanol production from lignocellulosic materials, as both the *A.oryzae* and *S.cerevisiae* are known to exhibit suitably high ethanol tolerance phenotypes (Brown et al., 1981; Watanabe et al., 2011). This would enable potentially high ethanol titres to be produced if sufficient sugar concentration could be liberated from the biomass.

BSG typically contains between 15% and 27% protein. This, in contrast to many other potential lignocellulosic biofuel substrates such as wheat straw or switch grass, renders BSG an ideal substrate for microbial growth due to the significant nitrogenous component (i.e. to facilitate the production and secretion of various enzymes). BSG has been used as a growth substrate for the production of various cellulase enzymes (endo and exoglucanases) using the fungus *Trichoderma reesei* (Sim & Oh, 1990). In addition, BSG has also been used as a substrate for cultivation of α-amylases using *A.oryzae* (Francis et al., 2003). *A.oryzae* has been reported to secrete a number of proteases when grown on wheat straw (Melikoglu, 2012) indicating the potential ability to utilise the significant nitrogenous component found within BSG.

This research aimed to evaluate various novel combinations of organisms in CBP based ethanol production from BSG. Comparison was also made to a SSF based approach using only an exogenous enzyme preparation (Novozymes Cellic® CTec 2) with various yeast strains. In addition the gene expression of a selection of key carbohydrate degrading (CAZy) enzymes and associated enzymes by the *A.oryzae* within the saké based CBP system (utilising BSG as the growth substrate) were investigated using qRT-PCR in an attempt to

identify what specific substrates were utilised within the BSG and the order of their deconstruction (substrate preference) by the *A.oryzae* primary fungal species.

8.2 Materials and methods

8.2.1 Quantification of the total glucose contents of BSG

Glucose concentrations in BSG were quantified using a two-stage complete acid hydrolysis protocol as described in section 2.3.1. The quantification of total glucose concentrations in the starting BSG would allow a crude approximation of the % theoretical ethanol yields to be estimated should any have been produced from the CBP combinations tested. The achieved % theoretical ethanol yields were then calculated using the total glucose concentration found within the BSG (and multiplied by the quantity of BSG present) and the x 0.51 ethanol conversion factor (Badger, 2002).

8.2.2 CBP attempts and initial culture of organisms

All CBP based experiments were conducted using 50 g of dried and ground BSG unless otherwise stated (section 8.2.3.4.1) with 200 mL of water in 500 mL conical flasks with foam bungs and then inoculated with both the required primary filamentous fungal and secondary yeast species (both on day 0) and then incubated at the required temperature for approximately 20 days. Samples of the supernatant were then taken for various analyses (monosaccharide liberation, ethanol production and assessment of lignocellulolytic enzyme activity) at various intervals. The efficacy of each CBP system was then evaluated in terms of maximal ethanol concentrations generated and volumetric productivity (ethanol yield per unit time). All experiments were conducted in triplicate.

8.2.2.1 Propagation of *Saccharomyces* and *Kluyveromyces Spp*.

S.cerevisiae NCYC479 (the saké strain) and *S.cerevisiae* NCYC2592 (the distillers strain) were both chosen to partner the primary fungal candidates due to their high ethanol tolerance

phenotypes that should enable the production of ethanol should sufficient sugars be liberated. *K.marxianus* strains NCYC1426 and NCYC179 and *K.wickerhamii* strain NCYC546 were also tested due to their potential pentose sugar utilisation capabilities (both for cellular metabolism and possibly for some additional fermentation of these C5 sugars to ethanol) in the instance that there was sufficient pentose sugar liberation from the hemicellulose degradation. Propagation of all yeast strains was conducted using the protocol described in section 2.8.1. All the experimental vessels (both CBP and SSF attempts) were inoculated with the required yeast strain (or strains where applicable) at a concentration of approximately 1 x 10^6 cells/mL.

8.2.2.2 CBP system utilising *A.niger* with either *S.cerevisiae* NCYC2592 or NCYC479

Spores of *A.niger* (N402) were grown on PDA slopes (potato dextrose agar; Oxoid) supplemented with 10 mM uridine (Sigma-Aldrich) in vials at 28°C with agitation at 120 rpm (MaxQ 4358 shaking incubator, Thermo Scientific, UK) until they had conidiated. The spores were harvested prior to inoculation by harvesting into 0.1% (v/v) tween 20. An aliquot (1.0 mL) of tween 20 was pipetted into the PDA slope to wash the spores into the solution. The concentration of spores in the solution was then determined using a microscope and haemocytometer. All the experimental vessels contained 50 g ground BSG and 200 mL water and were inoculated with *A.niger* spores to a concentration of approximately 1 x 10^7 spores per mL (considered to be an excess of spores) and then inoculated with either *S.cerevisiae* NCYC2592 or NCYC479 also at concentration of approximately 10^6 cells per mL. The vessels were then incubated at 25°C for 17 days.

8.2.2.3 CBP system utilising *Humicola insolens* with either *S.cerevisiae* NCYC2592 or NCYC479

Spores of *Humicola insolens* were grown up in YpSs media containing 1.5% bacto-soluble starch, 0.4% yeast extract, 0.1% dipotassium phosphate and 0.05% magnesium sulphate (Oxoid, UK) in vials at 40°C in the dark until they had germinated. All experimental vessels contained 50 g dried, ground BSG and 200 mL water and were inoculated with 1.0 g ± 0.1 of *H.insolens* filamentous tissue and then inoculated with either *S.cerevisiae* NCYC2592 or NCYC479 at concentration of approximately 10^6 cells/mL and incubated at 30°C for 20 days. This temperature was considered a compromise between the 40-45°C degree optima for the *H.insolens* but also to ensure viability of the *S.cerevisiae* strains (as they were not thermo-tolerant yeast strains).

8.2.2.4 Saké based CBP permutations utilising *A.oryzae* as the primary filamentous fungal species.

Due to the relative high performance in terms of ethanol yields seen in preliminary experiments looking at the saké based CBP system utilizing *A.oryzae* and *S.cerevisiae* NCYC479 (when cultured using dried and ground BSG), a range of additional experiments were conducted looking at the use of a multitude of different BSG derived biomass types in an attempt to further increase the ethanol yields generated. These experiments used either 50 g dried BSG, 50 g dried and ground BSG, 50 g BSG which had been subjected to 1% v/v HCl 121°C hydrothermal pre-treatment or 50 g ‘wet’ BSG direct from the lauter tun (dry weight, corrected for moisture content which was determined at 105°C until a constant weight was achieved using a HR83.P moisture analyzer, Mettler Toledo, UK).

8.2.2.4.1 Standard saké based protocol

The 50 g of BSG derived biomass was combined with 200 mL water and all vessels were then inoculated with 10 g of rice-based Koji mold containing *A.oryzae* (Akita Konno Co., Ltd., Japan) and inoculated with either *S.cerevisiae* NCYC479 (as with a standard saké based fermentation) or a modified version of the saké system in which the original saké yeast strain was substituted for the distillers strain (*S.cerevisiae* NCYC2592*)* with both inoculated at concentration of approximately 10^6 cells/mL and incubated for 20 days at either 15°C or 30°C.

8.2.2.4.2 pH adjusted variant of the saké based system

In addition a pH adjusted variant of the *A.oryzae/S.cerevisiae* NCYC2592 CBP system was conducted identically to the standard method described in section 8.2.2.4.1 with the exception of daily adjustment of the culture media to maintain pH 5.0 ± 0.1 (via 40% w/v NaOH) to match that of the previously determined pH of the *A.oryzae/S.cerevisiae* NCYC479 CBP system in order to establish whether variation in pH may have been a factor in the differences seen in ethanol yields.

8.2.2.4.3 Enzyme supplemented variants of the saké based system

Enzyme supplemented saké based CBP experimental variants were conducted using an identical approach as section 8.2.2.4.1 except with the addition of 1 mL Novozymes Cellic® CTec2 (kindly supplied by Novozymes A/S, Demark) into each vessel on day 10. Whilst cellulolytic enzyme supplementation would appear to defeat the purpose of CBP it was evaluated purely to establish if enzyme secretion (from the primary fungal species; *A.oryzae*) was a limiting factor in the production of ethanol. The Cellic® CTec2 was dosed at 10 FPU/g biomass which was calculated based upon the activity of the undiluted enzyme preparation determined according to Ghose (1987) using the protocol outlined in section 2.8. Day 10 was

chosen for the addition of the enzyme preparation as preliminary work concluded that maximal ethanol yields had been achieved by this point. The concentration of glucose initially present in the Novozymes Cellic® CTec2 was quantified by HPLC and its contribution (in terms of potential fermentative conversion to ethanol) subtracted from the subsequent ethanol yields achieved (for all enzyme supplemented experimental variants) in order to accurately determine the boosted effect of the addition of the exogenous enzymes.

8.2.2.4.4 Hybrid saké based system with additional *H.insolens*

An additional hybrid saké based system was also investigated and conducted similar to the standard saké protocol as described in section 2.3.4.1, but with the addition of 1.0 ± 0.1 g of pre-cultured *H.insolens* filamentous fungal biomass tissue and then incubated for 20 days at either 15°C or 30°C. This was in order to establish whether the incorporation of an additional primary fungal species with a somewhat differing (to *A.oryzae,* but also potentially complementary) lignocellulolytic enzyme arsenal could ultimately lead to the production of higher maximal ethanol yields than the use of either fungal species alone.

8.2.2.4.5 Alternative secondary yeast partners used with the saké based CBP system

To test the effectiveness of alternative yeast partners (other than or in conjunction with *S.cerevisiae* NCYC479) when paired with *A.oryzae* as the primary fungal species for CBP based ethanol production, *Kluyveromyces Spp*. based experimental variants were conducted identically to the purely *S.cerevisiae* based permutations except using either *K.marxianus* NCYC1426, *K.marxianus* NCYC179 or *K.wickerhamii* NCYC546 (all inoculated at 1 x 10^6 cells/mL). In addition, a consortium of both the various *Kluyveromyces* strains and *S.cerevisiae* NCYC479 were tested (with *A.oryzae*). This was to determine whether the addition of a *Kluyveromyces Spp* yeast resulted in higher maximal ethanol concentrations possibly through fermentation of some of the pentose sugars present in the supernatant (in

particular as a result of the additional xylanases added as part of the Cellic® CTec2 enzyme boosting) which the *S.cerevisiae* was unable to utilise. These experimental variants were initially inoculated with only *S.cerevisiae* NCYC479 on day 0 and incubated at 15°C until day 10 (which primarily targeted the fermentation of the hexose sugars liberated). On day 10 the vessels were then inoculated with the desired *Kluyveromyces Spp* (at 1 x 10^6 cells/mL). Additional sparged aeration was also provided from day 10 onwards in order to encourage the aerobic based pentose metabolism of the *Kluyveromyces Sp.* Enzyme boosted variants of these *Kluyveromyces Spp.* based CBP systems were also conducted using Novozymes Cellic® CTec2 (10 FPU/g biomass) dosed on day 10 as described in section 8.2.2.4.3.

8.2.2.4.6 Saké based system using 1% HCl catalysed 121°C hydrothermal pre-treated BSG

In addition to the investigation of CBP based ethanol production from native, unmodified BSG, the effect of using thermo-chemically pre-treated BSG was accessed in order to establish whether additional deconstruction of the lignocellulosic matrix prior to inoculation of the fungal species resulted in any increase in the maximal ethanol yields generated. Pre-treatment of BSG was conducted using the optimised dilute acid catalysed hydrothermal protocol described in Chapter 4, at 121°C for 30 min using a 40L benchtop autoclave (Priorclave, Tactrol 2, RSC/E, UK) and all reaction vessels were screw capped glass bottles (1000 mL). Pre-treatment reagent solutions at the required concentration (1% HCl v/v) had the appropriate quantity of BSG added (125 g) to give the required solids loading (25% w/v). After pre-treatment all experimental runs were neutralized to pH 5.0 (±0.2) via 40% NaOH (w/v). The biomass was then centrifuged at 5000 rpm for 10 min (Heraeus Megafuge 16; Thermo Scientific, UK) and the supernatant removed. The remaining residue was then subjected to five cycles of washes with water, conducted by re-suspension and centrifugation at 5000 rpm for 10 min, discarding the supernatant each time. The remaining residues were

then dried overnight at 60°C prior to use in the CBP experiments which were conducted as described in section 8.2.2.4.1.

8.2.2.4.7 Koji mold experimental controls

Experimental controls were prepared identically as the standard saké-based protocol described in section 8.2.3.4.1 (i.e. with koji mold; *A.oryzae* and also *S.cerevisiae*) except without the addition of BSG. Any ethanol subsequently produced in these controls was then subtracted from the ethanol yields achieved when actually using the BSG in order to compensate for the contribution of the fermentation of starch based glucose which was present in the rice-based koji. This would then allow for accurate determination of achieved ethanol yields from the BSG based biomass alone.

8.2.2.5 Comparative SSF system utilising Novozymes Cellic® CTec2 with either *S.cerevisiae* NCYC2592 or NCYC479

As an experimental comparison to the purely biological based CBP systems of bioethanol production, an SSF system which substituted the primary fungal species used with the CBP systems for the use of exogenous lignocellulolytic enzymes (Novozymes Cellic® CTec2) was tested. This was in order to establish whether more of a chemical engineering based approach (i.e. utilizing separate pre-treatment, enzyme saccharification and fermentation steps) could be developed in order to produce similar ethanol yields or improved volumetric productivity (and thus less reliance on the relatively slower propagation and culturing of fungal species). This was also to ascertain whether the role of the primary fungal species within the CBP systems were simply as lignocellulolytic enzyme factories with their secreted enzymes solely responsible for only conducting the saccharification or whether there were additional positive effects of the presence of the fungi. This could involve the physically disruptive effects of their growing hyphae (as similar final ethanol yields as with the CBP

system would be expected with an SSF system if only the presence of the enzymes was key). Experimental vessels contained 50 g dried and ground BSG and 200 mL water and were dosed with 2.5 mL Novozymes Cellic® CTec2 (10 FPU/g BSG on day 0). The Cellic® CTec2 had a total cellulase activity of 201 FPU/mL in undiluted format which was determined according to Ghose (1989). Vessels were then inoculated with either *S.cerevisiae* NCYC2592 or NCYC479 at concentration of 1 x 10^6 cells per mL and incubated for 20 days at 30°C. As with all the CBP experiments the flasks were sampled on various days for ethanol and sugar quantification by HPLC. The 30°C inoculation temperature was used as a compromise between the 50°C optimal temperature of the Novozymes Cellic® CTec2 enzyme preparation and the functional temperature range of the *S.cerevisiae* strains in order to attempt to achieve some degree of enzymatic saccharification of the BSG but maintain yeast cell viability and thus encourage ethanol production. The concentration of glucose initially present in the Novozymes Cellic® CTec2 was quantified by HPLC and its theoretical contribution to the final ethanol yields subtracted from the final achieved yields to allow accurate determination of the effectiveness of the SSF protocol.

8.2.3 Effect of the presence of ethanol on fermentation performance and final cell densities and cell viabilities of *S.cerevisiae* strains NCYC479 and NCYC2592

A range of ethanol concentrations (1-16% v/v) were incorporated into 4% YPD media and their effect on both *S.cerevisiae* NCYC479 and NCYC2592 was evaluated over the course of a (predominantly anaerobic) 96 h fermentation. This was in order to determine whether the consistently observed differences in ethanol production of the two yeast strains during CBP based fermentations could be attributed to differences in the ethanol tolerance of the two yeast strains. The fermentations were conducted using 100 mL of ethanol augmented 4% YPD media in 125 mL glass serum bottles (Wheaton, USA) using the method described in

Quain et al (1985) with air-tight rubber septa used in order to maintain an anaerobic environment to mimic the physic-chemical conditions of the saké based CBP systems. Custom made non-return valves were also used to allow the escape of fermentation gases (primarily CO_2) whilst minimising any air input. Each vessel was inoculated by transfer of an aliquot of a propagated cell suspension of *S.cerevisiae* NCYC479 or NCYC2592 (propagation as previously described in section 2.9.1) in order to achieve a pitching rate of 1 x 10^6 cells/mL. Fermentations were conducted using the protocol described in section 2.9.2. Upon attenuation cell counts and cell viability analysis was conducted microscopically using a haemocytometer and utilising methylene blue staining. Samples were also taken for HPLC analysis of glucose concentrations to quantify usage. All fermentations were conducted in triplicate.

8.2.4 Semi-quantitative assay for evaluation of the secreted cellulase and xylanase enzyme activity in the supernatants of the CBP variants

The detection of any cellulase or xylanase activity in the supernatants of each of the CBP experiments was achieved using the Congo red staining method (Béguin, 1983; Lamed et al., 1987). The Congo red assay involved a 10 µl volume of each supernatant deposited into wells in the agar plate which contained either carboxymethylcellulose (CMC) or xylan. The preparations were then incubated for 2 h at 37°C after Congo red staining of the CMC or xylan. Endoglucanase or endo xylanase activity was then detected by the appearance of clear spots on the plate with the size of clearing approximately proportional to the degree of cellulose or xylan hydrolysis.

8.2.5 Carbon source utilisation analysis of *S.cerevisiae* strains NCYC479 and NCYC2592 using a phenotypic microarray

The Biolog Omnilog phenotypic microarray system (Biolog, USA), utilising the YT MicroPlateTM yeast identification system (Biolog, USA) was used according to the

manufacturer's instructions and the method of Praphailong et al (1996) to investigate whether the significant differences in maximal ethanol concentrations that were observed between the *S.cerevisiae* NCYC479 and NCYC2592 yeast strains when partnered with *A.oryzae* (in the saké based CBP system) were due to the ability of one strain to use additional carbon sources that could have possibly been liberated from the hydrolytic breakdown of the BSG. The YT MicroPlateTM identification system was employed as a rapid screening tool which utilised 31 different carbon sources (Figure 8.1) in conjunction with a tetrazolium redox dye which changed colour in response to a chromophoric conformational change should any metabolic activity occur (facilitated by NADH). The system was also coupled with a detector that could accurately distinguish small changes in colour and thus quantify any variation in the ability of a particular yeast strain to utilise a specific carbon source. The *S.cerevisiae* yeast strains NCYC479 (the saké strain) and *S.cerevisiae* NCYC2592 (the distillers strain) were propagated using the standard protocol described in section 2.8.1. The yeast cells were then inoculated into sterile RO water in 20 × 100-mm glass test tubes and adjusted (diluted) to a transmittance of 62 % (~5 × 106 cells/ml) using a Biolog turbidimeter (Biolog, USA). An aliquot (100 μL) of each of the diluted cell cultures was then inoculated into each of the 31 different wells on the YT MicroPlate. The plates were the incubated at 25°C for 96 h with a reading of redox signal intensity taken every 15 min. A positive result from the Biolog YT microplate system for the utilisation of a potentially novel carbon source would then warrant the conduction of larger scale 'macrolog' fermentations (and thus enabling sampling for HPLC analysis) to confirm the substrate was actually being converted to ethanol and not an alternative carbon end point and thus potentially explaining the ethanol yield differences observed between the two yeast strains.

Figure 8.1 Biolog YT microplate system used for investigation of carbon source utilisation differences between *S.cerevisiae* strains NCYC479 and NCYC2592.

BIOLOG

Yeast Identification Test Panel

YT MicroPlate™

A1 Water	A2 Acetic Acid	A3 Formic Acid	A4 Propionic Acid	A5 Succinic Acid	A6 Methyl Succinic	A7 L-Aspartic Acid	A8 L-Glutamic Acid	A9 L- Proline	A10 D-Gluconic Acid	A11 Dextrin	A12 Inulin
B1 Cellobiose	B2 Mentiobiose	B3 Maltose	B4 Maltotriose	B5 D-Melezitose	B6 D-Melibiose	B7 Palatinose	B8 D-Raffinose	B9 Stachyose	B10 Sucrose	B11 Trehalose	B12 Turanose
C1 N-Acetyl-D-Glucosamie	C2 α-D-Glucose	C3 D-Galactose	C4 D-Psicose	C5 L-Sorbose	C6 Salicin	C7 D-Mannitol	C8 D-Sorbitol	C9 D-Arabitol	C10 Xylitol	C11 Glycerol	C12 Tween 80
D1 Water	D2 Fumaric Acid	D3 L-Malic Acid	D4 Methyl Succinate	D5 Bromosuccinc Acid	D6 L-Gluamic Acid	D7 γ-Aminobutyric Acid	D8 α-Keto Glutaric Acid	D9 2- Keto-D-Gluconic Acid	D10 D-Gluconic Acid	D11 Dextrin	D12 Inulin
E1 Cellobiose	E2 Gentiobiose	E3 Maltose	E4 Maltotriose	E5 D-Melexitose	E6 D-Melibiose	E7 Palatinose	E8 D-Raffinose	E9 Stachyose	E10 Sucrose	E11 Trehalose	E12 Turanose
F1 N-Acetyl D-Glucosamie	F2 D-Glucosamine	F3 α-D-Glucose	F4 D-Galactose	F5 D-Psicose	F6 L-Rhamnose	F7 L-Sorbose	F8 α-Methyl-D-Glucoside	F9 β- Methyl-D-Glucoside	F10 Amygdalin	F11 Arbutin	F12 Salicin
G1 Maltitol	G2 D-Mannitol	G3 D-Sorbitol	G4 Adonitol	G5 D-Arabito	G6 Xylitol	G7 i-Erythritol	G8 Glycerol	G9 Tween 80	G10 L-Arabinose	G11 D-Arabinose	G12 D-Ribose
H1 D-Xylose	H2 Methyl Succinate +D-Xylose	H3 N-Acetyl-L-Glutamic Acid +D-Xylose	H4 Guinic Acid +D-Xylose	H5 D-Glucuronic Acid+D-Xylose	H6 Dextrin +D-Xylose	H7 α-D-Lactose +D-Xylose	H8 D-Melibiose +D-Xylose	H9 D-Galactose +D-Xylose	H10 m-Inositol +D-Xylose	H11 1,2-Propanediol + D-Xylose	H12 Acetoin +D-Xylose

FIGURE 1. Carbon Sources in YT MicroPlate

Metabolism Tests Turbidity Tests

8.2.6 Headspace analysis screening the supernatants generated from various CBP system permutations for volatile compounds using Gas Chromatography-Mass Spectrometry (GC-MS)

GC-MS headspace analysis was employed as a rapid and efficient screening tool that facilitated preliminary exploratory analysis looking for the presence of any unique volatile organic chemical candidates (VOCs). These are metabolic products generated from either the primary fungal species or the particular accompanying yeast species that can play important

interspecies signalling roles in natural environments (Morath et al., 2012; Shank & Kolter, 2009). These may have potentially exhibited either symbiotic signalling (metabolic boosting effects etc.) or conversely inhibitory properties that may have elucidated the observed significant differences in maximal ethanol yields that were indicated to be dependent on which specific secondary yeast partner was used with the *A.oryzae*. The use of hard ionisation MS detection was chosen as it enabled any generated ion fragments to be cross-referenced against mass spectral libraries to assist in the identification of any candidates.

The protocol involved using a Trace GC Ultra gas chromatograph with a dual stage quadrupole MS (both from Thermo, USA). Solid-phase microextraction (SPME) was conducted using headspace vials containing 10 mL supernatant (filtered, GF/C 25 mm filter diameter,1.2 μm pore size, Whatman, USA) with a 50/30 μm DVB-CAR-PDMS (divinylbenzene-Carboxen-polydimethylsiloxane, Carbowax-divinylbenzene and polydimethylsiloxane) stableflex 24 GA fibre used in conjunction with a Combi PAL autosampler (CTC Analytics, Switzerland). Operating parameters were as follows: 22 mm vial penetration, a 10 min extraction time, a 54 mm injection penetration and a 10 min desorption time. The column used was a ZB-WAX (30 m × 0.25 μm I.D, 1 μm film thickness; Phenomenex, UK). The GC had a split-splitless injector which was held at 230°C. Analysis was conducted following 10 min desorption and splitless injection. The GC program started at 37°C for 3 min and then increased at 3°C/min from 37°C to 250°C where it was held at for a further 3 min. During the GC run, a constant pressure (18 psi) of the carrier gas (Helium) was maintained. The mass spectra were obtained by electron impact (EI) ionisation and the MS transfer line was held at 250°C. The detector was set in TIC mode (full scan) from *m*/*z* 50 to 650. The results were analysed using Xcalibur software (Thermo, USA).

8.2.7 Measurement of the physico-chemical environmental parameters of the saké based (*A.oryzae*) CBP system permutations using either *S.cerevisiae* NCYC479 or NCYC2592

Various environmental parameters were measured at various intervals over a 20 day fermentation using the saké based (*A.oryzae*) CBP system with two *S.cerevisiae* strains (either NCYC479 or NCYC2592). This was to investigate if the observed ethanol yield differences could be attributed to a metabolic effect of a particular yeast strain resulting in the significant variation of a key environmental parameter which may have affected the ultimate ethanol productivity in some way. Dissolved oxygen concentrations were measured using a VISIFERM dissolved oxygen ARC sensor probe with the corresponding ARC handheld wireless receiver (Hamilton Bonaduz AG, Switzerland). In addition pH was measured using a FG2-Fieldkit FiveGo™ pH Meter (Mettler Toledo, UK). Free amino nitrogen (FAN) was measured using glycine as a standard according to the European brewing convention method (Analytica – EBC, 2000).

8.2.8 Quantitative assessment of cellulolytic enzyme activity of the supernatants from both the *S.cerevisiae* NCYC479 and NCYC2592 variants of the saké based (*A.oryzae*) CBP system

As an additional effort to investigate possible explanations for the differences in ethanol yields when using the two different yeast strains the cellulase activity of the two supernatants was externally tested in order to establish whether the presence of a particular yeast strain induced a greater secretion of cellulase enzymes from the *A.oryzae*, thus liberating greater glucose yields which may then be converted into additional ethanol.

Samples of the supernatant (1.0 mL) were taken on day 8 from both the *S.cerevisiae* NCYC479 and NCYC2592 variants of the saké based (*A.oryzae*) CBP system and externally assessed for cellulolytic enzyme activity using 50 mg Whatman No.1 filter paper (VWR, UK)

using a 24 h incubation at 15°C. There was no external adjustment of the pH and the degree of glucose liberation from the filter paper was used as an indicator of cellulolytic activity. No pH adjustment was conducted in order to establish whether the small pH difference observed between the two different yeast strain supernatants was responsible for a greater liberation of glucose (either through greater cellulase secretion or through enhanced activity of any cellulases present). This was as glucose was the likely primary carbon source used by the *S.cerevisiae* for fermentation of any ethanol produced and thus explained the differences in maximal ethanol yields. After incubation the samples were centrifuged at 5000 rpm for 10 min and the supernatant analysed via HPLC for glucose liberation. Initial HPLC analysis of the CBP system supernatants (right at the start; time point zero) was also conducted and indicated that negligible glucose concentrations were present initially (at the start of the assay) therefore no subtractions were necessary for accurate calculation of liberated glucose yields. In addition enzyme blanks were also included (using only 50 mg filter paper and 1 mL water in place of the supernatant) and used to adjust the final glucose yield calculations.

8.2.9 Quantitative real time polymerase chain reaction (qRT-PCR) based analysis of CAZy gene expression in *A.oryzae* and ADH2 alcohol dehydrogenase gene expression in *S.cerevisiae* when used in the saké based CBP system

qRT-PCR was undertaken in order to attempt to determine which specific components found within the BSG the *A.oryzae* were deconstructing for use as a carbon source and any obvious sequence of usage, possibly related to the degree of recalcitrance of each component. In addition qRT-PCR was also conducted to investigate the expression levels of the ADH2 alcohol dehydrogenase gene in both the NCYC479 and NCYC2592 strains of *S.cerevisiae* (when combined with the *A.oryzae* using the saké based CBP system) to determine if

differential expression could possibly explain the observed differences in maximal ethanol yields attained when using these strains in the saké based CBP system.

For both the CAZy and ADH2 gene expression analysis saké based CBP protocols were conducted with the required yeast strain (as previously described in section 8.2.2.4.1) with sacrificial replicates for each required sample point (days 1, 5, 10, and 15; each in triplicate). Sampling involved the separation of the liquid phase (supernatant) from the solid phase via filtration using miracloth (Merck KGaA, Darmstadt, Germany). The solid and liquid samples were each separately flash-frozen in liquid nitrogen and then stored at -80°C until required. All PCR primers were designed using the Eurofins Primer Design Tool and synthesised by Eurofins (MWG Operon, Germany) from target candidate genes identified from the *Aspergillus oryzae* genome database (http://www.aspgd.org/) and *Saccharomyces* genome database (http://www.yeastgenome.org/). Table 8.1 displays the genes which were chosen as target carbon source utilisation candidates (for the subsequent primer design). These were considered likely to represent key enzymes that would be required somewhere within the reaction sequence of the hydrolytic breakdown of a particular polymeric component (e.g. cellulose) found within the BSG and thus infer its utilisation. Three suitable housekeeping genes (which were considered to be consistently stably expressed etc.) were chosen as system controls for expression levels of the target CAZy genes to be compared against (for normalisation against etc.). These consisted of the genes TUB1; which encoded tubulin (Tominaga et al., 2006), 18S rRNA (Schüller, 2003) and ACT1; which encoded actin (Shen et al., 2004). The ACT1 gene was also used as the housekeeping gene for normalisation of the *S.cerevisiae* ADH2 data set.

The specificity of the designed primers for the target CAZy genes was tested using PCR against the extracted DNA of both the *A.oryzae* and *S.cerevisiae* strains NCYC479 and NCYC2592 to ensure they only worked for the desired organism. Reference DNA was

extracted from propagated cultures of both *A.oryzae* and both *S.cerevisiae* strains (both cultured in 4% YPD media at 25°C for 72 h) using a Fungi Genomic DNA Isolation Kit for the *A.oryzae* (Norgen Biotek, Canada) and a yeast DNA extraction kit (ArchivePure Yeast; 5 prime, USA) both according to the manufacturer's instructions. The PCR conditions were as follows; 35 cycles using 94°C for denaturation, 60°C for annealing and 72°C for elongation, using a TC-512 PCR thermocycler (Techne, UK). Confirmation of the PCR products was conducted via electrophoresis using TAE (0.04 M Tris- acetate, 0.001 M EDTA) 1% agarose gels (run at 100v for 30 min) with ethidium bromide staining for visualisation utilising a UVP gel documentation system (UVP Ltd., UK) with comparison against a 100 bp DNA ladder (New England Biolabs, USA). After successful confirmation of the activity of the CAZy gene designed primers specifically only with *A.oryzae* (and ADH2 primers with only the *S.cerevisiae strains*), total RNA was extracted from the frozen samples (from the 20 day fermentations using the saké based CBP system) and purified using a fungal RNA purification kit for the *A.oryzae* (Norgen Biotek Plant/Fungi Total RNA Purification Kit, Canada) and a yeast RNA extraction kit (RiboPure Yeast; Ambion Life Technologies, USA) with both used according to the manufacturer's instructions. All extracted RNA concentrations were then quantified using a NanoVue™ microvolume spectrophotometer at both 230 and 260 nm (GE healthcare, USA). First strand cDNA synthesis from the extracted RNA was then performed using a first strand cDNA synthesis kit (GE healthcare, USA) according to the manufacturer's instructions.

Table 8.1 Target CAZy and associated carbon source utilisation indicator genes from *A.oryzae,* alcohol dehydrogenase gene from *S.cerevisiae* and all housekeeping genes used for all qRT-PCR conducted.

Gene	Activity (and Orthologous genes in *Aspergillus* species)		Primer sequence (5'-3')
CAZy and associated genes (A.oryzae)			
AO090011000745	*axeA*, acetyl xylan esterase activity (ORF, Verified)	Fw:	CCGCAGAACTACGAGGAAAC (20)
		Rv:	ACACCGCCAGCCAAAATAC (19)
AO090001000066	*faeB*, ferulic acid esterase activity (ORF, Verified)	Fw:	CCCTCAATGTAACGACCTCC (20)
		Rv:	AAGCAAAATCCTCAACCACC (20)
AO090005001210	predicted xylanase activity	Fw:	ATCCCATCCCACCTACAAC (19)
	(hydrolase: An01g13340 *A.niger*)	Rv:	TCCTCATTTTCACGAATCCAC (21)
AO090113000148	β- glucosidase	Fw:	TCAGATGACGAAGGAGCAG (19)
	(An03g03740 *A. niger*)	Rv:	CATACCGAGGACCATATCCAG (21)
AO090010000746	*glaA*, glucoamylase (ORF, Verified)	Fw:	GTGTCTTTCTCCTCTTGTCTCC (22)
		Rv:	TTTGCTTCTGTGCTCAGCC (19)
AO090005001553	Ortholog(s) have cellulase activity	Fw:	CTCAATGGCTCAAGGACAAC (20)
	(cellulose binding activity: AO090011000715 *A.oryzae*)	Rv:	GATAATCCAGCATTCCCTTCAC (22)
AO090038000175	predicted cellulase activity, hydrolase activity	Fw:	TTCCACCTCTTCTTCCTCCC (20)
	(Putative endo-glucanase: An03g05380 *A.niger*)	Rv:	GCTTCCCTCAAATTGTGTCTC (21)
(S.cerevisiae)			
YMR303C	*ADH2*; alcohol dehydrogenase II;	Fw:	ATGCTAAGGCGATGGGTTAC (20)
		Rv:	ACGACTGCGCTAACAATGTC (20)
housekeeping genes (A.oryzae)			
AO090701000065	*actA*: one of two actin genes in *A. oryzae*	Fw:	AGCCCCTATCAACCCTAAGTCC (22)
		Rv:	ACACCGTCACCAGAATCCAG (20)
AO090206r00001	18S ribosomal RNA (Verified)	Fw:	TCAACACGGGGAAACTCAC (19)
		Rv:	CGCAATTAAGCAGACAAATCAC (22)
AO090005000840	Ortholog(s) are structural cytoskeleton activity	Fw:	CCAAGAACATGATGGCTGCT (20)
	(tubulin: An01g05650 *A.niger*)	Rv:	CTTGAAGAGCTCCTGGATGG (20)
(S.cerevisiae)			
YFL039C	*ACT1*; Actin; structural protein, cell cytoskeletal functions	Fw:	AGCCCTATCAACCCTAAGTCC (22)
		Rv:	ACACCGTCACCAGAATCCAG (20)

Quantitative RT-PCR was then performed with a StepOnePlus real-time PCR system (Applied Biosystems, USA) using Fast SYBR® green mastermix (Applied Biosystems, USA) according to the manufacturer's guidelines. Negative controls (without cDNA) were also run. Running conditions were as follows; 40 cycles of 94°C for 1 min (denaturation), 60°C for 30 sec (primer annealing), 72°C for 30 sec (polymerisation). Melt curve analysis was also conducted subsequently for the evaluation of the degree of formation of primer dimers. Technical replicates were conducted in triplicate for the qRT-PCR in addition to the triplicate biological replication.

8.2.10 Measurement of the protein content of solid residues remaining after saké based CBP ethanol production from BSG

A Thermo Flash Nitrogen Analyser (ThermoFisher Scientific, Waltham, Massachusetts, USA) was used to determine protein content using the protocol described in section 2.3.4. All analyses were conducted in triplicate.

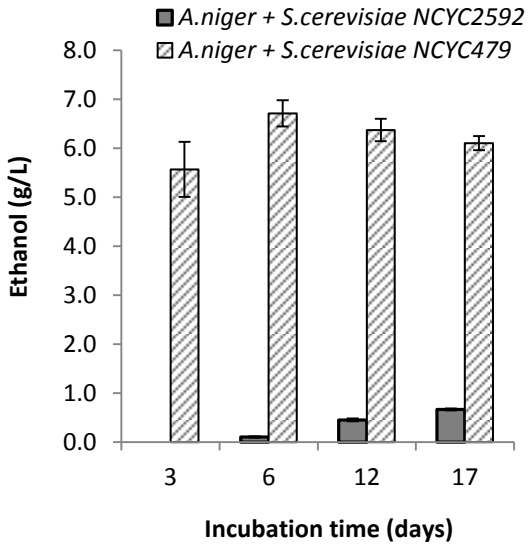
8.3 Results and discussion

8.3.1 CBP using *A.niger* with either *S.cerevisiae* NCYC2592 or NCYC479

A.niger was in theory an excellent CBP primary fungal candidate with a known large arsenal of lignocellulosic degrading enzymes (Chandra et al., 2007; Faulds & Williamson, 1995; Kang et al., 2004). It is used in commercial biotechnology as an enzyme factory due to its high capacity secretory system (Guillemette et al., 2007; Pel et al., 2007). It was therefore expected to perform well in terms of the production of relatively high concentrations of ethanol when partnered with a suitable *S.cerevisiae* strain, such as either NCYC2592 or NCYC479. However, the difference in ethanol yields (and also rate of production) achieved between the two *Saccharomyces* strains when paired with *A.niger* was significant with the maximal ethanol yield achieved when using *S.cerevisiae* NCYC479 being 6.7 g/L after just 6

days compared to only 0.67 g/L after 17 days when using *S.cerevisiae* NCYC2592 (Figure 8.2). This ca. 90% improvement in final ethanol yield achieved when using *S.cerevisiae* NCYC479 was both unexpected and unexplained as both strains were expected to perform similarly given their high ethanol tolerance phenotypes. The attenuation or attainment of maximal ethanol yields after just 6 days indicated that the *A.niger* had probably saccharified all the lignocellulosic material available by this time point. HPLC analysis of the supernatants did not reveal any sugars present (any monosaccharides or oligosaccharides less than Dp5) at any of the sample time points (when using either yeast strain partner) indicating that any sugar liberation from the lignocellulosic material was likely occurring in very small trace amounts and was also being utilised immediately by either the yeast species or the filamentous fungus present.

Figure 8.2 Ethanol concentrations generated using a consolidated bioprocessing process at 25°C with a consortium of *Aspergillus niger* and two separate strains of *Saccharomyces cerevisiae.* Strains NCYC2592 or NCYC479 used. CBP conducted using 50 g (dried and ground) BSG with 200 mL water.

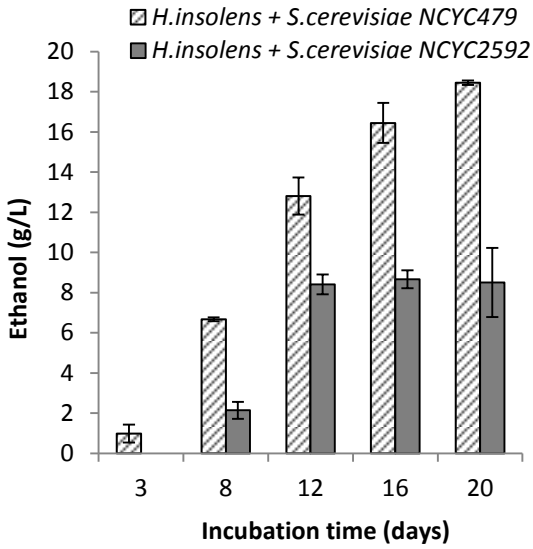


The rapid utilisation of any liberated sugars by the yeast species (the *S.cerevisiae*) could limit catabolite repression or product inhibition of any enzymes secreted by the primary deconstructive organism (the fungus *A.niger* in this case) and may also further induce enzyme secretion from the fungus due to the lack of immediately available carbon sources. This is similar to the phenomenon described in Suto & Tomita (2001) in which it was reported that cellulose induced expression of cellulase encoding genes in fungi but glucose repressed cellulase expression. Semi-quantitative lignocellulolytic enzyme analysis (utilising Congo red staining of CMC and xylan based agar plates described in section 8.2.4) indicated that a relatively moderate activity of both cellulases and xylanases was present in the supernatant from the NCYC479 experimental variants. However, negligible activity for both enzyme classes was detected in the NCYC2592 variant supernatants, which possibly explains the observed differences in final ethanol productivity, perhaps due to limited saccharification of the polymeric, complex carbohydrates.

8.3.2 CBP using *H.insolens* with either *S.cerevisiae* NCYC2592 or NCYC479

The thermophillic, saprophytic fungus *H.insolens* was another potential CBP candidate as it was known to have a high capacity secretory system that included various cellulases and xylanases (Schülein, 1997). Both yeast variants (*S.cerevisiae* NCYC2592 & NCYC479) of the *H.insolens* CBP system successfully produced ethanol directly from BSG but with strain NCYC479 again significantly out-performing NCYC2592, producing a maximal ethanol concentration of ca. 20 g/L by day 12, compared to only ca. 8.5 g/L by day 12 for the NCYC2592 variant which corresponded to ca. 57% more ethanol (Figure 8.3).

Figure 8.3 Ethanol concentrations generated at various time points from consolidated bioprocessing at 30°C using a consortium of *Humicola insolens* and two separate strains of *Saccharomyces cerevisiae.* Strains NCYC2592 or NCYC479 used. CBP conducted using 50 g (dried and ground) BSG with 200 mL water.



Semi-quantitative analysis of enzyme activity indicated a greater activity of both cellulase but in particularly xylanase activity in the supernatant produced using yeast strain NCYC479 compared to NCYC2592. Increased xylanase activity may have increased hemicellulose solubilisation thus improving the physical access of any secreted cellulases that were present to the cellulose substrate. This may have therefore liberated higher glucose concentrations. The supernatant from the NCYC2592 variant only indicated a negligible degree of cellulase activity suggesting minimal secretion of these enzymes from *H.insolens* in this CBP system. This was again a possible explanation for the higher ethanol concentrations produced (with the NCYC479 variant) due to a greater degree of saccharification of the lignocellulosic material. HPLC analysis of the supernatants from both yeast strain variants did not detect any significant concentrations of any yeast friendly, directly metabolisable mono, di or trisaccharides at any sample points. This indicated that these specific sugars were being solubilised in very small amounts from the lignocellulosic material and were likely being

utilised immediately by the yeast species. In addition to the superior ethanol yields which were achieved with the NCYC479 variant, a significant difference in the degree of filamentous fungal biomass production was observed with the NCYC479 variant, producing a mean quantity of 5.6 g ± 1.2 g of *H.insolens* fungal biomass (dry weight) compared to only 1.3 g ± 0.4 g biomass for the NCYC2592 variants. This large increase in fungal biomass purely in response to the inclusion of a different yeast partner was due to an unknown reason. Possible explanations may include that unlike when using *S.cerevisiae* NCYC2592, the NCYC479 strain did not fully deplete and therefore did not rate limit a particular micronutrient that was crucial for fungal biomass generation. This could possibly be due to defective uptake mechanisms from the media or possibly more optimised metabolism resulting in a lower net requirement of a particular component. In direct comparison to the *A.niger* CBP system, the *H.insolens* CBP system produced significantly higher maximal ethanol yields, especially when using *S.cerevisiae* NCYC479. It was suggested that a greater degree of cellulolytic enzyme secretion was achieved when using *H.insolens* (compared to *A.niger*) which liberated greater sugar concentrations and therefore in turn greater ethanol yields. Again, the increased ethanol yields when using the *S.cerevisiae* NCYC479 variant were due to an unknown reason.

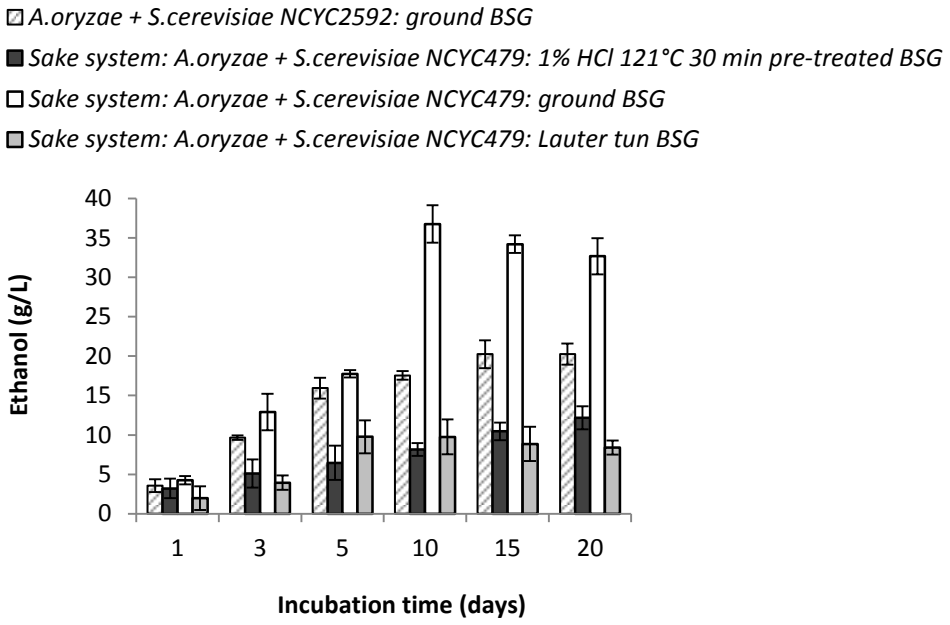
8.3.3 CBP using *A.oryzae* with *S.cerevisiae* NCYC479 (Saké based CBP system)

8.3.3.1 Using non pre-treated BSG

The *A.oryzae* or saké based CBP system indicated that relatively high ethanol concentrations of ca. 37 g/L could be attained with 10 days incubation at the low temperature of 15°C when using dried and ground BSG (Figure 8.4). These ethanol concentrations were significantly higher than those achieved using any other CBP system tested. Maximal ethanol yields achieved when using ‘wet’ (and unground) BSG direct from the lauter tun were significantly

lower (optimally ca. 9.8 g/L after 5 days) compared to when using dried, ground BSG (ca. 74% less etc.).

Figure 8.4 Ethanol concentrations generated at various time points from consolidated bioprocessing of BSG at 15°C using a saké based consortium of *Aspergillus oryzae* and *Saccharomyces cerevisiae* NCYC479. CBP conducted using either 50 g BSG (dried and ground), 50 g BSG direct from the brewery lauter tun (corrected for moisture content) or 50 g hydrothermal pre-treated BSG (1% HCl 121°C 30 min). Inoculated with *A.oryzae* and *S.cerevisiae* NCYC2592 and incubated at 15°C.

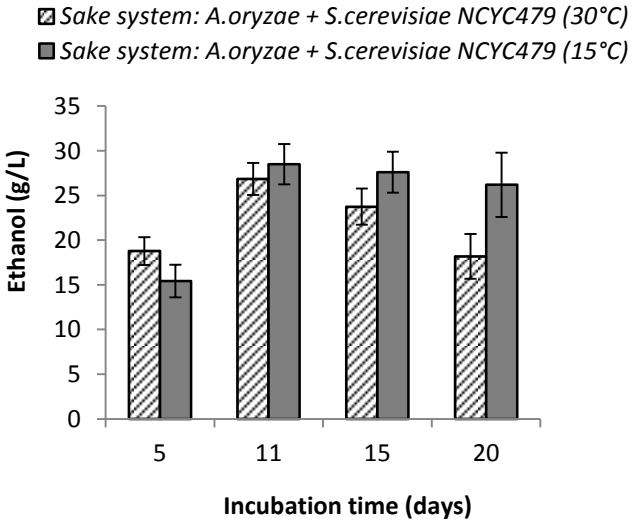


This revealed the requirement for some form of particle size reduction of the BSG in order to enhance the biocatalytic saccharification of the lignocellulosic material by the enzymes secreted from the *A.oryzae*. A reduction in particle size may also then allow greater penetration of the substrate by the fungal hyphae as described by Lee (1997) and therefore facilitate more successful interaction of the secreted enzymes and the substrate. The apparent superiority of the saké based CBP system (over the other permutations of fungal species tested) in terms of the high ethanol yields achieved was also somewhat unexpected as *A.oryzae* is not specifically considered a cellulolytic fungal species (although does have some cellulolytic enzyme capacity), instead rather more of a starch hydrolysis specialist (Francis et al., 2003). However, significant cellulase and xylanase enzyme activity was detected in the

supernatants generated after 5 days incubation and onwards. As with all the other CBP systems tested here, negligible low molecular weight sugar concentrations (<Dp5) were detected in all the supernatants at all sampled time points. Overall this suggested that all the primary fungi species where not producing cello-oligosaccharides (cellobiose, triose, tertraose etc.) from the deconstruction of the BSG unless they were larger than Dp5; i.e. cellodextrin sized carbohydrate molecules. It is widely known that *S.cerevisiae* cannot ferment cello-oligosaccharides including cellobiose or any higher molecular weight β-glycosidic bond linked oligosaccharides due to both the lack of a functional membrane transporter and a suitable β-glucosidase (Ha et al., 2011). Although the HPLC based analytical system employed could not detect oligosaccharides greater than Dp5 it was also considered unlikely that any of the primary fungi species used in these CBP systems would have been able to directly utilise these larger molecular weight sugars in the supernatant either. Further hydrolysis of these cellodextrins to lower molecular weight sugars such as glucose that would have been more easily transported into the fungal cells would be a more probable mechanism of carbon source utilisation for both the filamentous fungi and yeast. A very small quantity of ethanol was produced in the koji controls (i.e. vessels containing no BSG; with just the koji *A.oryzae* inoculum and *S.cerevisiae*) which could be attributed to hydrolysis of the very small quantity of starch component that was present in the koji inoculum. However this was subtracted from the final ethanol yields generated so as to allow for accurate evaluation of the degree of usage of the BSG as the carbon source. Therefore, the presence of a small quantity starch initially in the media (from the koji) could have possibly facilitated the initial growth of the *A.oryzae* (as a rapidly utilisable carbon source favoured by the *A.oryzae* with its strong arsenal of amylase degrading enzymes) which then subsequently encouraged greater lignocellulolytic enzyme secretion due to a fungal culture with greater viability once the media was carbon source depleted (in terms of its favoured amylose based

carbon sources). As the *A.oryzae* searched for an alternative, replacement carbon source and ultimately finding only lignocellulosic material remaining, this would likely trigger a shift in gene expression towards the production of lignocellulolytic enzymes. Subsequent experiments running the saké based CBP system (*A.oryzae* and *S.cerevisiae* NCYC479) at both 15°C and 30°C indicated that at both temperatures similar ethanol concentrations were achieved at each time point sampled. Operating at 30°C was found to be optimal at the 5 day time point (ca. 19 g/L ethanol compared to ca. 15 g/L for 15°C: Figure 8.5). However, from day 11 onwards the 15°C temperature appeared to be slightly more optimal (generating ca. 6% to 30% more ethanol compared to those achieved at 30°C). Overall it indicated the process could function effectively over a wide temperature range without the requirement of careful temperature control (and therefore possibly reducing heating or cooling operational costs). This may enable the process to function in a wide geographical or climatic range, at ambient temperature but most specifically without precise temperature control.

Figure 8.5 Ethanol concentrations generated at various time points from consolidated bioprocessing at 15°C and 30°C using the saké based consortium of *A.oryzae* and *S.cerevisiae* NCYC479 with dried and ground BSG. CBP conducted using 50 g BSG with 200 mL water. Data are the mean ± SD of three replicate experiments.



A key factor associated with the use of *A.oryzae* is that unlike many other related species it does not produce aflatoxin (Machida et al., 2005). Although the genes are present for the aflatoxin synthesis pathways, very few expressed sequence tags (ESTs) are present. This has the added advantage in that any ethanol produced through an *A.oryzae* based CBP approach with BSG would be theoretically food grade. Food grade ethanol could have a range of uses including potentially being used in the production of alternative brewery products such as the formulated ready to drink (RTD) alcoholic beverages should on-site processing (CBP based 'recycling' of the BSG via the saké based system) be conducted within a brewery and would represent a considerably higher value product than the current market price of fuel grade ethanol. The concept of incorporation of any food grade ethanol produced into beverages may not be immediately realistic but does open up a range of new possibilities. However great care would need to be taken to avoid *A.oryzae* based contamination of brewery malt or cereal adjunct stocks.

8.3.3.2 Saké based CBP system (*A.oryzae* with *S.cerevisiae* NCYC479) using 1% HCl catalysed 121°C hydrothermal pre-treated BSG

The investigation of whether thermo-chemical pre-treatment of the lignocellulosic material in BSG prior to its usage in the saké based CBP system indicated that maximal ethanol concentrations of ca. 12 g/L were achieved after 20 days incubation (Figure 8.4).

From this data it can be concluded that usage of pre-treated BSG was sub-optimal compared to using non-pre-treated BSG (when dried and ground prior to use) as ethanol yields were ca. 68% less. This system also appeared to attenuate less rapidly than the non-pre-treated BSG experimental variant (requiring 20 days for maximal ethanol concentrations to be attained instead of 10 days for the non-pre-treated variant). Relatively moderate cellulase activity was the only enzyme activity detected in the supernatants when using pre-treated BSG. However,

the absence of any xylanase activity was not surprising as the acid catalysed hydrothermal pre-treatment would have likely solubilised the majority of hemicellulose present in the starting BSG material leaving cellulose as the only significant structural polysaccharide present in any significant amount as suggested in Chapter 4. However the degree of cellulase activity observed in the supernatants when using pre-treated BSG was also less than that observed during the non-pre-treated BSG saké based CBP system indicating that non-modified lignocellulosic material appeared to be a better activator of cellulase enzyme secretion from the *A.oryzae*. This may have been due to a more native, non-modified structure of the lignocellulose (and subsequently also the more native format of the cellulose present) being successfully recognised better or alternatively possibly resulting in more successful enzyme-substrate interactions with the non-pre-treated BSG. In addition, the recognition of other lignocellulosic components such as hemicellulose could possibly be crucial in triggering optimal secretion of cellulases through some form of signalling. This could be similar to the importance of the XLR-1 xylan degradation regulator (found in an alternative fungal species *Neurospora crassa*) with regards to its requirement of induction of other cellulolytic encoding genes (Sun et al., 2012). Alternatively a starvation response may have been triggered which resulted in the secretion of a broad spectrum of exploratory CAZy enzymes as has been shown to occur with *A.niger* grown on lignocellulosic substrates such as wheat straw (Delmas et al., 2012). Again HPLC analysis of the supernatants did not detect any fermentable low molecular weight sugars to be present at any sample point again further confirming that any sugar liberation was at best small and likely utilised immediately by either of the organisms.

8.3.3.3 *A.oryzae* based CBP system substituted with *S.cerevisiae* NCYC2592

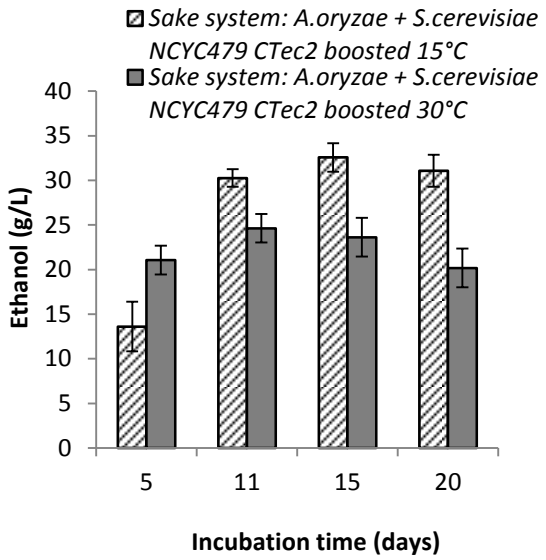
Retaining the *A.oryzae* fungal species but substituting the original *S.cerevisiae* NCYC479 yeast strain partner for the NCYC2592 strain resulted in maximal ethanol concentrations of

only ca. 20 g/L after 15 days (Figure 8.4). This equated to ca. 45% lower maximal ethanol yields achieved than when using the NCYC479 yeast strain which further confirmed its apparent superiority compared to the NCYC2592 strain. Whilst moderate cellulase and xylanase enzyme activity was detected in the supernatants at various time points it was determined to be less than that observed with the *S.cerevisiae* NCYC479 variant (although using the same primary filamentous fungus; *A.oryzae*) Which was a surprising observation. As such the likely lower sugar yields (lower through reduced saccharification due to lower enzyme activity) may have possibly explained the lower observed maximal ethanol productivity. It suggested the specific co-culture of *S.cerevisiae* NCYC479 with *A.oryzae* was in some way optimal regarding the degree of saccharification achieved.

8.3.3.4 CBP using an enzyme boosted variant of the saké based system (*A.oryzae* and *S.cerevisiae* NCYC479 with additional Novozymes Cellic® CTec2)

In an attempt to increase the maximum ethanol concentrations generated from the saké based CBP system and establish whether the secretion of cellulolytic enzymes (primarily cellulases and xylanases) were a rate limiting factor in ethanol production, enzyme based 'boosting' with Novozymes Cellic® CTec2 was investigated (with the enzyme addition occurring on day 10; once previously determined maximum ethanol concentrations had already been reached). However very similar ethanol concentrations were attained (ca. 30 g/L on days 11-15) to those achieved with the non-enzyme boosted approach (Figure 8.6).

Figure 8.6 Ethanol concentrations generated at various time points from consolidated bioprocessing of BSG at both 15°C and 30°C using a consortium of *Aspergillus oryzae* and *Saccharomyces cerevisiae* NCYC479 with the addition of Novozymes Cellic CTec2 on day 10. CBP conducted using 50 g BSG with 200 mL water. Cellic® CTec2 dosed at 10 FPU/g biomass. Data are the mean ± SD of three replicate experiments.

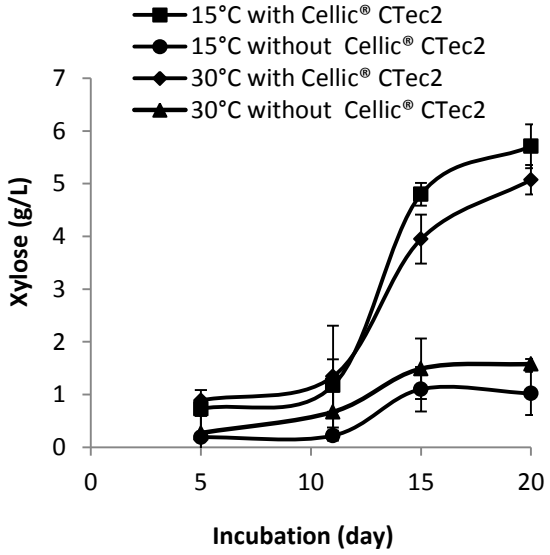


This suggested that cellulolytic enzyme secretion by the *A.oryzae* was not a rate limiting factor in terms of ethanol productivity from the *S.cerevisiae*. However, the actual activity of any endogenous natively secreted enzymes could be a factor with mass transfer limitations reducing yields somewhat through limited physical access to the cellulose component due to the relative high viscosity of the media. Operation of the saké based CBP system at a lower solids loading (lower solid to liquid ratio of the culture) or the use of stirred or mixed fermentation vessels could possibly result in higher ethanol yields through increasing successful enzyme-substrate interactions. Paradoxically, however, enzyme secretion has been shown to be greater on solid state cultures as opposed to highly dilute liquid cultures (Machida et al., 2008). Genetic analysis of the profile and expression levels of various genes (or mRNA) within the *A.oryzae* could also possibly indicate specifically what components within the BSG the fungus was saccharifying to enable the *S.cerevisiae* to produce the ethanol. Some degree of utilisation of the tiny fraction of residual starch present in the BSG is

a possibility. This starch would be present due to the slightly sub-optimal starch extraction efficiency during non-enzyme augmented mashing of lauter tun based brewing technology (from which the BSG was obtained) compared to the better extraction achieved utilising a mash filter with the associated smaller milled particle size of the grist used initially. However, when using 50 g BSG which had a total starch content determined to be less than 1.5% (w/w) and assuming 100% theoretical conversion efficiency of that starch to glucose via the amylose based enzymes secreted by the *A.oryzae* (and subsequently 100% theoretical conversion of that liberated glucose to ethanol via fermentation by the *S.cerevisiae*) the maximum ethanol concentration attained from starch (also assuming zero usage by the *A.oryzae* which is also highly unlikely) would be 0.51 g (ca. 2.5 g/L). Therefore, the ca. 37 g/L ethanol concentrations attained using the saké based CBP system indicated that starch alone could not be the only carbon source being utilised. The consistent observation of again generally lower ethanol yields achieved when using 30°C compared to 15°C (even with the additional Cellic® CTec2 enzyme boosting etc.) was unexpected (Figure 8.6). With the optimal functional temperature of the Cellic® CTec2 being 50°C, the higher temperature would have been considered to facilitate improved enzyme activity and thus liberated higher sugar concentrations (consequentially likely resulting in higher maximal ethanol concentrations). This mirrored the results from the standard saké based CBP system (*A.oryzae* and *S.cerevisiae* NCYC479; with no additional Cellic® CTec2 boosting etc.) in which the 15°C variant performed at least as good as if not better than the 30°C variant in terms of maximal ethanol productivity (as seen in Figure 8.5). However temperature reduction has been indicated to possibly play a crucial role in maintaining continued expression of extracellular hydrolases when using *A.oryzae* in a solid-state cultivation system for the traditional manufacture of products such as soy sauce (Machida et al., 2008). The reduced mobility of the secreted enzymes (and thus reduced activity somewhat) due to the

slightly higher viscosity of solid state cultures at lower temperatures may significantly reduce product feedback based inhibition of the expression of hydrolases therefore resulting in higher secreted enzyme concentrations. This may assist in explaining the superior (or at least equal) ethanol yields achieved at lower temperatures as additional enzyme secretion may have compensated for the generally lower rate of enzyme activity compared to the 30°C system. In addition, this viscosity related enzyme mobility issue may explain the relatively slow rate of ethanol production due to the slow rate of saccharification. However, it does not explain the slightly lower ethanol yields achieved at 30°C when using additional exogenous enzyme boosting (Cellic® CTec2) as this should in theory combat this issue. This is currently due to an unknown reason. Analysis of liberated xylose concentrations in the supernatants from both the 15°C and 30°C enzyme boosted variants of the saké CBP system indicated a significantly higher concentration was present in both (ca. 5 g/L maximally) compared to the non-enzyme boosted variants (ca. 1 g/L maximally; Figure 8.7).

Figure 8.7 Detected xylose concentrations liberated at various time points from a saké based system of consolidated bioprocessing. CBP conducted using 50 g dried ground BSG (with 200 mL water) at both 15°C and 30°C using a consortium of *A.oryzae* and *S.cerevisiae* NCYC479 with and without the addition of Novozymes Cellic® CTec2 (10 FPU/g biomass) on day 10. Data are the mean ± SD of three replicate experiments.



This suggested the xylanases present in the Novozymes Cellic® CTec2 hydrolysed an additional proportion of the hemicellulose present in the BSG resulting in significantly higher free xylose concentrations. However, this somewhat increased hemicellulose hydrolysis did not significantly improve the final ethanol yields achieved (as would be expected through better cellulase access to the cellulose through removal of more of the hemicellulose) possibly indicating some proportion of the cellulose component was still restricted from the cellulases present in the Cellic® CTec2. This may have proposed that the exogenous, broad spectrum activity xylanases from the Cellic® CTec2 were not as effective as the specific native xylanases secreted from the primary fungi partners. The underutilised xylose concentrations in the supernatant offer an additional carbon source which could also be fermented to ethanol using an additional, specialised C5 pentose fermenting species which likely had a more broad potential substrate spectrum, such as various strains of the yeast *Kluyveromyces* (Fonseca et al., 2008; Wilkins et al., 2008). This would likely require (at least in part) the generation of aerobic environmental conditions somewhat in order for the pentose phosphate pathway to be utilised. This could render the use of *Kluyveromyces Spp*. difficult under traditional anaerobic fermentation conditions (for example if attempting to use *Kluyveromyces Spp*. simultaneously with *S.cerevisiae* etc.).

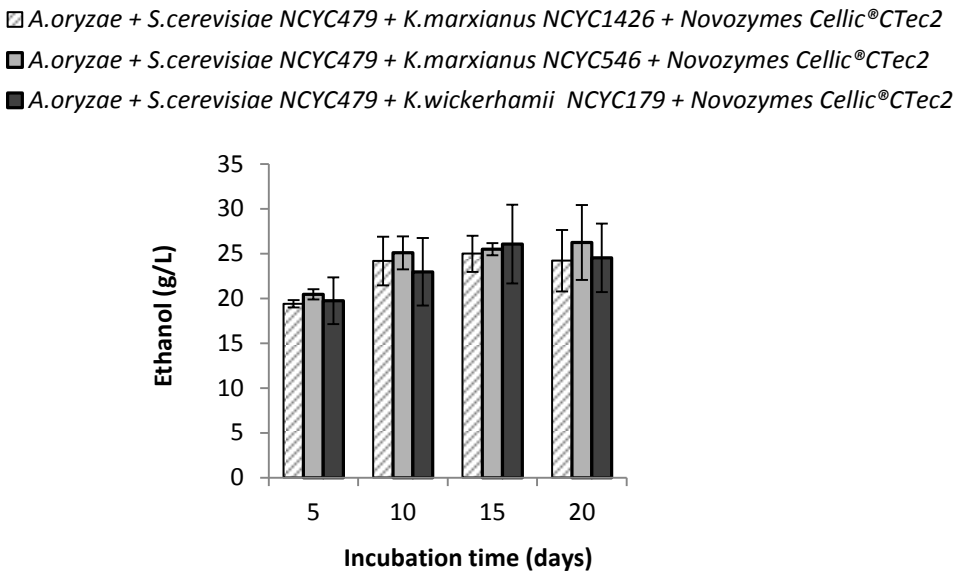
8.3.3.5 Use of *Kluyveromyces* yeast strains with the *A.oryzae* based CBP system

As previously discussed the increased xylose concentrations detected in the supernatant from the saké based CBP system (*A.oryzae* with *S.cerevisiae* NCYC479) when the additional enzyme supplementation was conducted suggested that the inclusion of an additional fermentative organism potentially capable of utilising pentose sugars could possibly enable the production of higher maximum ethanol concentrations. However, none of the permutations tested (utilising any of the *Kluyveromyces Spp*. used either alone with *A.oryzae* or even any *Kluyveromyces Spp*. in a triple consortium with both the *A.oryzae* and

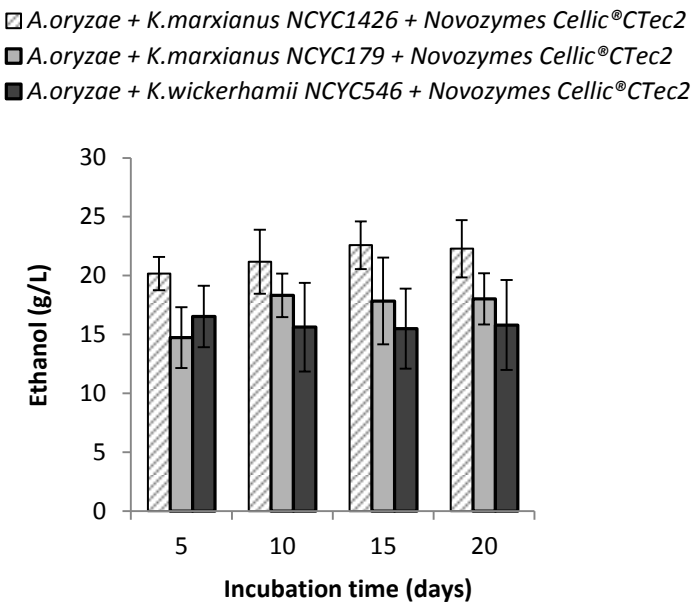
S.cerevisiae NCYC479) produced higher maximum ethanol concentrations than the standard saké based system of *A.oryzae* with *S.cerevisiae* NCYC479 alone. All three *Kluyveromyces* species (*K.marxianus* NCYC1426, *K.marxianus* NCYC179 and *K.wickerhamii* NCYC546) were indicated to perform similarly in terms of their generated ethanol yields (Figures 8.8A-B and 8.8). The Cellic® CTec2 enzyme-boosted variant of the standard saké based system (*A.oryzae* and *S.cerevisiae* NCYC479) when also inoculated with any of the *Kluyveromyces* species (on day 12) with the corresponding commencement of external aeration to create aerobic conditions (i.e. after an initial preliminary anaerobic fermentation period using only *S.cerevisiae* NCYC479 to efficiently utilise the C6 sugars) produced less than 25 g/L ethanol (Figure 8.8B).

Figure 8.8 Ethanol concentrations generated at various time points from consolidated bioprocessing of BSG at 25°C using *A.oryzae* and 3 different strains of *Kluyveromyces*; *marxianus* NCYC1426, *marxianus* NCYC179 and *wickerhamii* NCYC546. CBP conducted using 50 g BSG with 200 mL water. A) inoculated initially with *S.cerevisiae* NCYC479 on day 0 and then subsequently inoculated with *Kluyveromyces spp*. and boosted with Novozymes Cellic® CTec2 (10 FPU/g biomass) both on day 10. B) Inoculated with only *Kluyveromyces spp*. and boosted with Novozymes Cellic® CTec2 (10 FPU/g biomass) on day 10 (with no initial *S. cerevisiae* fermentation). Data are the mean ± SD of three replicate experiments.

A)



B)



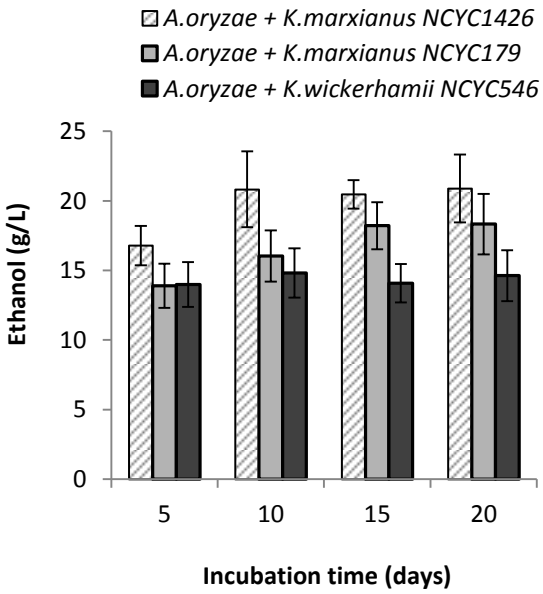
This was again revealed to be inferior to the standard saké based CBP system in terms of maximal ethanol yields achieved. Investigation of the three *Kluyveromyces* species independently when combined with the *A.oryzae* alone (i.e. without the initial *S.cerevisiae* fermentation) also indicated that lower maximal ethanol yields (ca. 14-21 g/L) were produced than when using the standard saké based system (*S.cerevisiae* NCYC479). The purely *Kluyveromyces* based CBP combinations with *A.oryzae* both with and without additional enzyme boosting (addition of Novozymes Cellic® CTec2) produced ca. 15-23 g/L maximal ethanol concentrations (Figures 8.8B and 8.9).

Overall this indicated that all the *Kluyveromyces* species tested were inferior to *S.cerevisiae* NCYC479 in terms of maximal ethanol productivity and also that either the *Kluyveromyces* species were not utilising a significant amount of the liberated xylose from the enzyme boosting for the production of ethanol (instead possibly for biomass production or an alternative metabolic carbon end product than ethanol). Alternatively, it may have suggested the *S.cerevisiae* was possibly metabolising some of the ethanol that had already been

produced in the absence of any other readily utilisable carbon source being present. However for this to occur there would need to be some oxygen present.

Quantification of the expression levels of the ADH2 gene that encodes an alcohol dehydrogenase (in *S.cerevisiae* NCYC479) responsible for the key primary stage of the conversion of ethanol to acetaldehyde (Figure 8.16) could be used to indicate whether this could be a rate limiting factor in terms of the upper limit on ethanol production that is achievable from all the CBP system combinations that involve the use of this specific yeast.

Figure 8.9 Ethanol concentrations generated at various time points from consolidated bioprocessing of BSG at 25°C using *A.oryzae* and 3 different strains of *Kluyveromyces; marxianus* NCYC1426, *marxianus* NCYC179 and *wickerhamii* NCYC546. CBP conducted using 50 g BSG with 200 mL water with no additional enzyme boosting and no initial *S.cerevisiae* NCYC479 inoculation and pre-fermentation stage. Data are the mean ± SD of three replicate experiments.



This is the subject of current research and could indicate possible modification routes (possibly through a knock out of the ADH2 gene or artificial down regulation of its expression) in order to further increase maximal ethanol yields from this CBP system through closure of this metabolic pathway thus somewhat disabling ethanol degradation. The superiority of the *S.cerevisiae* NCYC479 (in terms of ethanol production) if proven to be

related to reduced expression of the ADH2 gene could possibly be due to some degree of artificial selection pressure exerted by human activity, brewing saké over many hundreds of years that has favoured the symbiotic paring of (and subsequent co-evolution of) *S.cerevisiae* strains that produce higher final ethanol titres. The improved ethanol production phenotype of the *S.cerevisiae* NCYC479 strain could possibly be due to the continued use of cell lines with defective *ADH2* genes (or defective transcriptional expression factors) resulting in a reduced capacity to utilise ethanol as a carbon source. Alternatively it may simply utilise glucose faster than its competitors. Due to the complexity and dynamic environments of the CBP systems the actual answer could likely be a combination of various factors.

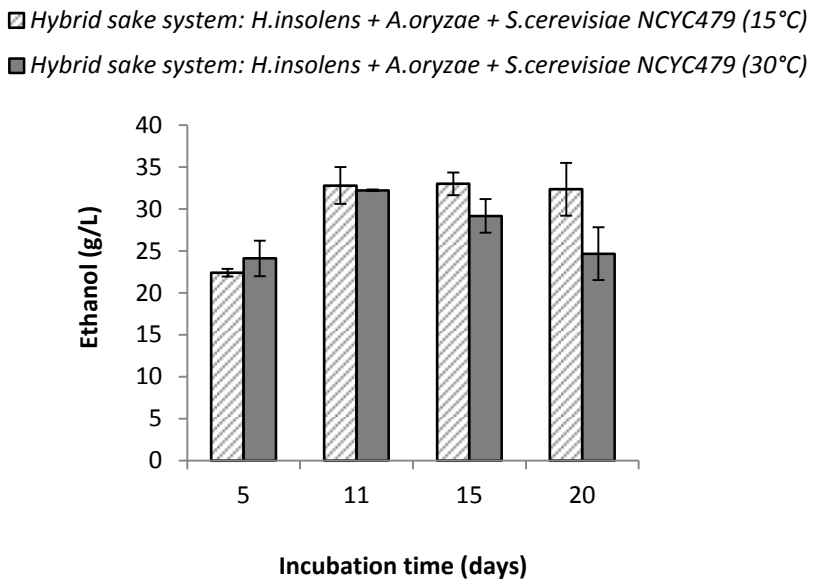
8.3.4 CBP using a consortia of *H.insolens*, *A.oryzae* and *S.cerevisiae* NCYC479 (hybrid Saké based system)

The success of both the saké based (*A.oryzae*) and *H.insolens* based CBP systems (specifically when both systems used the *S.cerevisiae* NCYC479 yeast strain) warranted the investigation of a combination of the three species used as a hybrid saké based consortia to determine whether the variation in the lignocellulolytic enzyme arsenal that each different primary filamentous fungal species would provide resulted in any significant increase in ethanol production (through improved saccharification) when compared to the use of either species alone. However, similar ethanol yields were achieved at most sample time points (Ca. 25-35 g/L ethanol for all sample points with both of the 15°C and 30°C temperature variants used; Figure 8.10) as with the standard saké system (using purely *A.oryzae* and *S.cerevisiae* NCYC479) indicating the presence of the *H.insolens* did not appear to significantly improve ethanol productivity.

However, higher ethanol concentrations were initially achieved after just 5 days (16% and 36% increases in ethanol yields for the 15°C and 30°C hybrid variants respectively) compared to the standard saké system signifying a short term efficiency enhancement

especially at the higher 30°C temperature, likely due to the thermophillic nature of the *H.insolens* with its 45°C optima and suggesting some early additional deconstructive enzyme contribution.

Figure 8.10 Ethanol concentrations generated at various time points from consolidated bioprocessing of BSG at both 15°C and 30°C using a consortium of *Humicola insolens, Aspergillus oryzae* and *Saccharomyces cerevisiae* NCYC479. CBP conducted using 50 g BSG with 200 mL water. Data are the mean ± SD of three replicate experiments.



For any potential commercial application of the technology an evaluation would need to be made to establish whether the day 5 ethanol yield improvement (specifically the volumetric productivity) was sufficient to justify the inclusion of the *H.insolens*. Semi quantitative enzyme assays showed a slightly higher cellulase and xylanase concentration (or associated activity) in this hybrid trio CBP system supernatant compared to either the standard saké system or the purely *H.insolens* CBP system alone. This suggested either a cumulative net increase in the total cellulolytic enzyme concentration (due to both fungal species) or that the presence of the additional organism triggered additional secretion from one species, possibility due to additional environmental competition for limited carbon sources. However the overall lack of a significant increase in the maximal ethanol yield compared to the standard saké based CBP system (even with higher cellulose and xylanase activity detected in

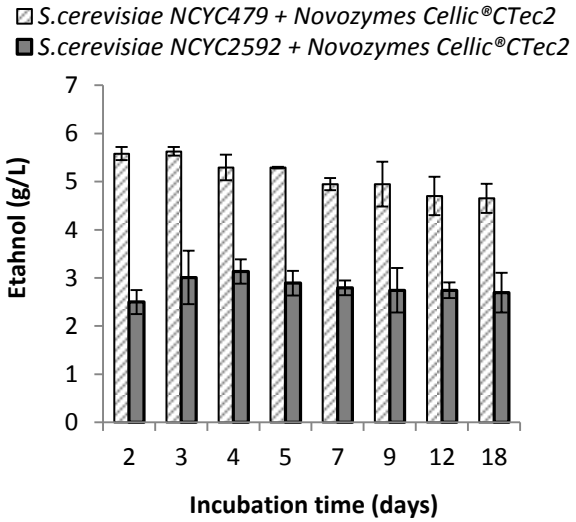
the supernatant) again indicated that lignocellulolytic enzyme secretion was possibly not a significant rate limiting factor or that it was not the only factor involved. The cumulative, increased filamentous fungal biomass that would have been present when using this triple consortium may have simply utilised more of the liberated sugars (for biomass production) hence no detectable increase in ethanol output from the partnering yeast species. This could lead to GM approaches to knock out (or reduce through down regulation of the expression of a key gene in the pathway) the ability of the primary fungal species to utilise glucose subsequently forcing it to utilise the pentose sugars present that *S.cerevisiae* could not use. This could be done through knock-out of the genes encoding hexose sugar transporters (removing the ability of the fungi to uptake hexose sugars) or a range of key enzymes including those involved in the TCA cycle, the predominant system used by filamentous fungi for metabolism of hexose sugars (Chambergo et al., 2002). This would leave the glucose exclusively for the yeast to rapidly ferment to ethanol.

8.3.5 SSF system using Novozymes Cellic® CTec2 with either *S.cerevisiae* NCYC2592 or NCYC479

The substitution of the primary filamentous fungal species for an exogenous enzyme cocktail (Cellic® CTec2; that definitively contained a significant concentration of xylanases and in particular aggressive cellulases) was used to evaluate both the *S.cerevisiae* strains directly and also to establish whether the primary fungal species used in the CBP systems were simply functioning as lignocellulolytic enzyme factories. As observed with all the previous CBP experimental data the use of *S.cerevisiae* strain NCYC479 produced significantly higher ethanol yields than strain NCYC2592, achieving a maximal ethanol yield of over 5.6 g/L after just 3 days (Figure 8.11). In comparison the maximal ethanol yield attained when using strain NCYC2592 was approximately 45% less (3.1 g/L after 4 days). This confirmed that

S.cerevisiae strain NCYC479 appears to be a better fermentative candidate for bioethanol production when using a fungal based CBP or SSF approach.

Figure 8.11 Ethanol concentrations generated at various time points from a simultaneous saccharification and fermentation (SSF) protocol using BSG at 30°C using Novozymes Cellic® CTec2 and two strains of *S.cerevisiae*. SSF conducted using 50 g BSG with 200 mL water. Cellic® CTec2 dosed at 10 FPU/g biomass. Strains NCYC2592 and NCYC479 used. Data are the mean ± SD of three replicate experiments.



These results also indicated that the superiority of NCYC479 yeast strain was not exclusive to when used in CBP systems. Although the relative difference in ethanol yields between the two yeast strains when used in the SSF system was less than compared to when using the CBP systems, this would suggest that the observed superiority of strain NCYC479 was due to more than just symbiotic signalling between the yeast and the primary filamentous fungi species used. A combination of factors is likely. Overall the SSF system was indicated to achieve significantly lower final ethanol yields per unit of BSG than when using the best CBP systems. However, this system could attain maximal yields in just 3 days. Further optimisation could potentially be conducted to attempt to optimise the enzymatic conversion efficiency through a more effective mixing regime allowing better enzyme substrate

interaction or a short pre-incubation phase (prior to the yeast inoculation) at a higher temperature closer to the 50°C optima for Cellic® CTec2.

8.3.6 Ethanol tolerance of *S.cerevisiae* NCYC479 and NCYC2592 yeast strains

The superiority of *S.cerevisiae* NCYC479 in terms of ethanol productivity was not indicated to be due to ethanol tolerance. This was as both NCYC479 and NCYC2592 performed similarly (in terms of attenuation, final cell counts and final cell viabilities) across a 1% to 16% v/v ethanol concentration range when initially incorporated into 4% YPD media for a 96 h fermentation (Table 8.2). In addition both strains are widely renowned as exhibiting high ethanol tolerance phenotypes (Ogawa et al., 2000; Watanabe et al., 2009) and the ethanol concentrations generated from all CBP trials were all well below prescribed inhibitory levels (concentrations did not exceed 4% ABV with any CBP system). This initially suggested that *S.cerevisiae* strain NCYC479 may have had the ability to either utilise additional carbon sources that strain NCYC2592 could not (which may have been present from the deconstruction of the BSG) or alternatively that the synergistic or symbiotic combination of using strain NCYC479 with the primary fungal species possibly induces greater lignocellulolytic enzyme secretion. This could possibly be through some form of chemical signalling. Alternatively the differences could be due to presence of an inhibitory molecule (or greater concentrations of any such compound) present or generated exclusively with the use of strain NCYC2592. These could be similar to the K toxins produced by certain 'killer' yeast strains including various *S.cerevisiae* strains (Gulbiniene et al., 2004; Palpacelli et al., 1991). Another possibility was that the presence of NCYC479 either inhibited the primary fungal species from utilising the available nutrients somewhat or that NCYC479 simply utilised the available glucose significantly faster than the primary fungal species. This could be possibly through the presence of very high affinity glucose transporters such as improved variants of the HXT2 gene (Kruckeberg & Bisson, 1990).

Table 8.2 Effect ethanol concentrations (0%-16% v/v) on attenuation rate, cell density, cell viability and final glucose concentration after fermentation at 15°C using *S.cerevisiae* NCYC479 and *S.cerevisiae* NCYC2592. Inoculated at an initial cell density of 1.0E+06 cells/mL. 4% YPD media components: 4% glucose, 2% peptone, 1% yeast extract. Attenuation determined at point at which fermentation vessels reach a constant weight. Data are the mean ± SD of three replicate experiments.

	S.cerevisiae NCYC479				*S.cerevisiae* NCYC2592			
Ethanol concentration (v/v)[1]	Attenuation (h)[2]	cells/mL[3]	% dead[3]	glucose: g/L[3]	Attenuation (h)[2]	cells/mL[3]	% dead[3]	glucose: g/L[3]
0%	7	9.7E+0.8 (± 5.6E+0.6)	3.5 ± 1.4	0 ± 0	4	1.3E+0.9 (± 4.5E+0.6)	1.3 ± 0.6	0 ± 0
1%	7	9.6E+0.8 (± 9.5E+0.6)	3.9 ± 0.5	0 ± 0	7	1.3E+0.9 (± 8.5E+0.6)	1.5 ± 1.0	0 ± 0
2%	18	9.3E+0.8 (± 5.1E+0.6)	4.1 ± 1.1	0 ± 0	22	1.1E+0.9 (± 3.5E+0.7)	2.4 ± 0.1	0 ± 0
4%	23	6.7E+0.8 (± 5.6E+0.6)	5.1 ± 2.1	0 ± 0	27	5.6E+0.8 (± 3.2E+0.6)	3.7 ± 2.5	0 ± 0
8%	28	3.3E+0.8 (± 5.3E+0.6)	6.5 ± 1.6	0 ± 0	32	2.0E+0.8 (± 3.3E+0.7)	9.1 ± 0.2	0 ± 0
16%	n/a	5.6E+0.7 (± 2.8E+0.6)	30.5 ± 6.3	44.4 ± 0.5	n/a	5.5E+0.7 (± 2.8E+0.6)	22.3 ± 8.9	43.9 ± 0.2

[1]*Incorporated into initial 4% YPD media*

[2]*Attenuation based upon a constant weight achieved*

[3]*At end of 96 h fermentation*

Data are the mean ± SD of three replicate experiments

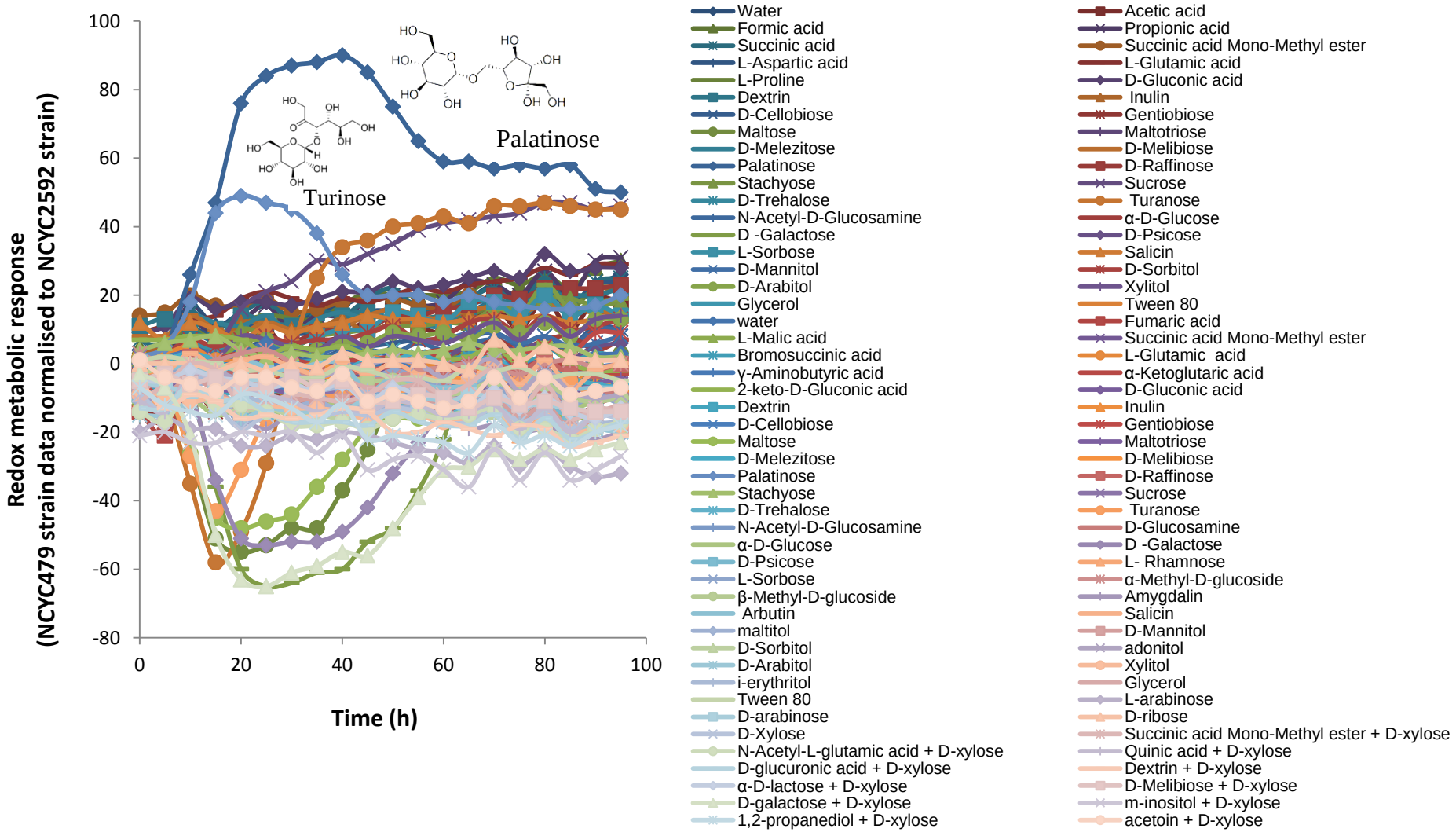
8.3.7 Investigation of carbon source utilisation by *S.cerevisiae* strains NCYC479 and NCYC2592 via phenotypic microarray.

Use of the YT microplate yeast identification system in conjunction with the Biolog Omnilog phenotypic microarray system indicated that strain NCYC479 had the capability of utilising the disaccharides palatinose (isomaltulose) and turanose whereas strain NCYC2592 did not (Figure 8.12). Although in *S.cerevisiae* the gene *AGT1* encodes a permease that can actively transport various α-glucosides including turanose into the cell (Machida et al., 2008) thus potentially supporting the concept that NCYC479 may have a wider spectrum of potential carbon sources than NCYC2592, the presence of significant quantities of either of these specific disaccharides was not likely to arise from the deconstruction and hydrolysis of the structural polysaccharides within the BSG. As a consequence this was therefore highly unlikely to explain the significant differences in ethanol yields. No other differences in carbon source utilisation were detected between the two strains which then shifted the focus instead towards the a different cause for the consistent differences in ethanol yields observed between the two different yeast strain variants

8.3.8 Screening of various CBP supernatant samples for potential symbiotic signalling or inhibitory compounds via GC-MS headspace analysis

GC-MS based headspace analysis of supernatant samples from both the standard saké based system (*A.oryzae*) and *H.insolens* CBP system (from both CBP system yeast variants; using either of the *S.cerevisiae* strains NCYC479 or NCYC2592) found several potential candidates for either some form of symbiotic signalling or inhibitory molecules (that were exclusively present when using specifically one or the other yeast strain) which could have possibly played a role in the significant differences in ethanol yields.

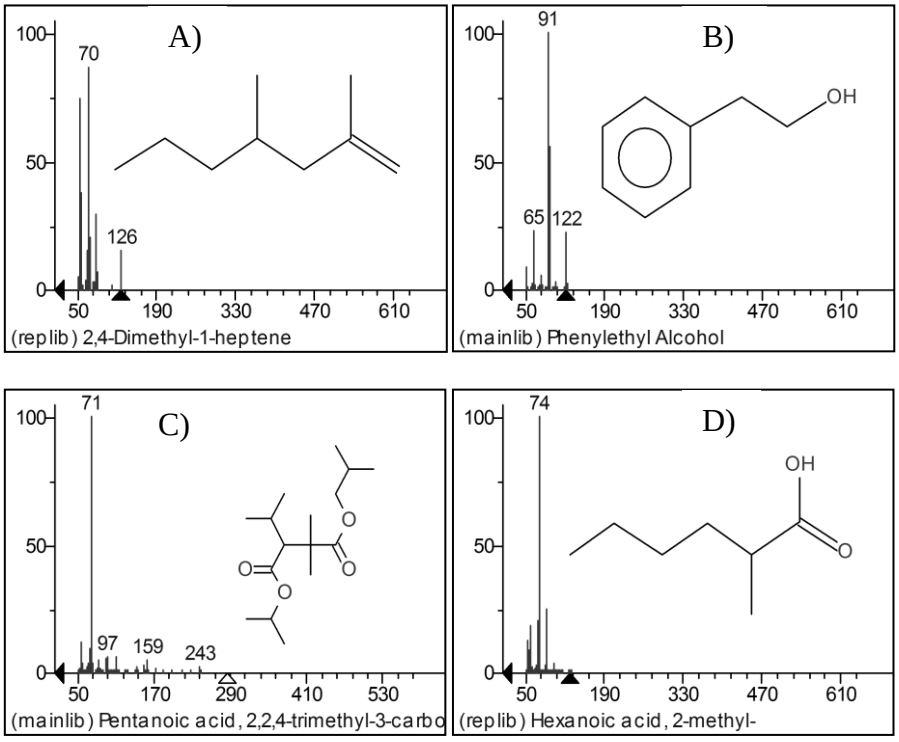
Figure 8.12 Carbon source utilisation analysis using a phenotypic microarray for yeast strains; *S.cerevisiae* NCYC479 and NCYC2592.



The idea was that the presence (or absence) of a unique compound may have had an effect on either the saccharification step (increased fermentable sugar liberation by the primary fungus through triggering increased enzyme secretion) or an effect on the fermentation step (improved fermentation efficiency or improved sugar uptake by the yeast, therefore more carbon flux towards ethanol production). Conversely a unique compound present may have had an inhibitory effect on either enzyme secretion (or affected enzyme activity; possibly through active site modification or competitive inhibition).

Hexadecanoic acid and pyrazole were only detected in the samples originating from when *S.cerevisiae* NCYC2592 was used as the yeast partner in both the *A.oryzae* (saké) and *H.insolens* CBP systems (Figure 8.13).

Figure 8.13 Mass spectral library (GC-MS) based compound identification examples from headspace analysis (SPME) of supernatants from two yeast strain variants of the saké-CBP system. A) 2,4-Dimethyl-1-heptene. B) Phenyl ethyl alcohol. C) Pentanoic acid. D) Hexanoic acid.



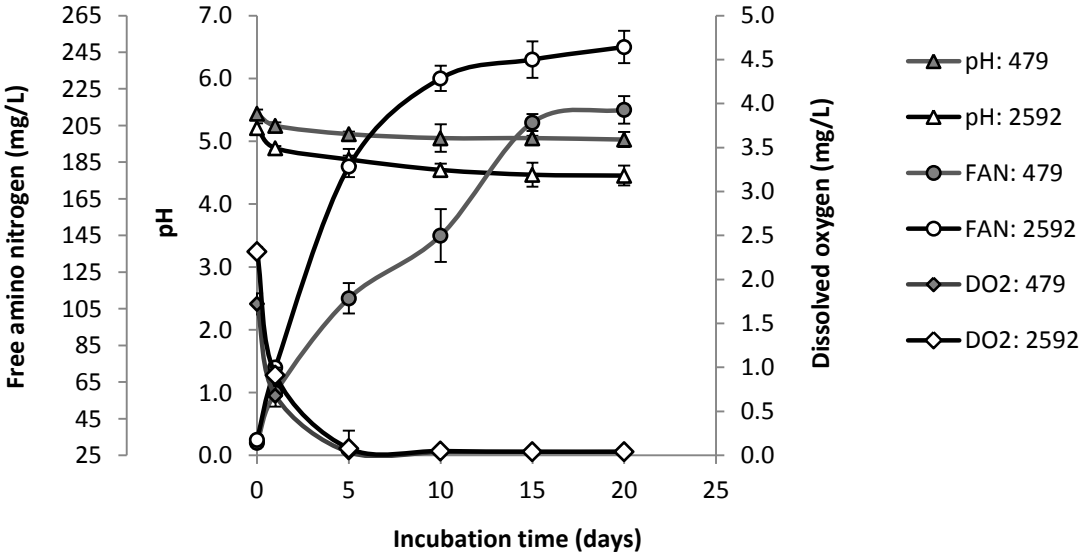
Hexadecanoic acid may have antifungal properties (along with certain other saturated fatty acids) through membrane disruptive effects (Morath et al., 2012) and pyrazole is capable of inducing inhibition of various yeast alcohol dehydrogenases (Shank & Kolter, 2009; Weinhandl et al., 2014) which may indicate a possible role in the observed sub-optimal ethanol yields through disruption of the ethanolic fermentative pathway. It is highly unlikely the *S.cerevisiae* NCYC2592 strain would produce a compound such as pyrazole that would self-inhibit its own ADH enzymes (as it would impede its own ability to produce energy via a fermentative pathway). Therefore it would be more likely it would originate from the *A.oryzae*, possibly as an adaptive strategy to survive, in an attempt out-compete the yeast for the limited available nutrients present. Whereas the *S.cerevisiae* NCYC479 yeast stain was more suited to co-culture (with the *A.oryzae*) and as such may not have triggered such an inhibitory response from the *A.oryzae*. Alternatively pentanoic acid, phenyl-ethyl alcohol (benzyl alcohol), octanoic acid (ethyl ester) were only found when using *S.cerevisiae* NCYC479 as the yeast partner in both the *A.oryzae* and *H.insolens* CBP systems. This redundancy (the consistent detection of these molecules with both primary filamentous fungal species variants of the CBP systems) was considered to increase the probability that one or more of these molecules could potentially function in enhancing (or reducing) the ultimate ethanol productivity in some way. Current research is looking at the supplementation of these chemical candidates into test systems as to whether they exert any fermentation enhancement or disruption effects.

8.3.9 Evaluation of any physico-chemical environmental parameters differences between the saké based (*A.oryzae*) CBP system variants using either *S.cerevisiae* NCYC479 or NCYC2592

Dissolved oxygen concentrations were determined to be virtually identical for both yeast strain CBP variants (starting at ca. 2 g/L and dropping to 0 g/L by day 5; Figure 8.14)

indicating this was not a significant factor regarding the explanation of the differing maximal ethanol yields. In addition this indicated that use of either yeast strain quickly resulted in the generation of anaerobic fermentation conditions. This relatively rapid depletion of dissolved oxygen levels could have possibly had a negative effect on the growth or more specifically the enzyme secretion from the *A.oryzae*.

Figure 8.14 Physico-chemical environmental data from saké based (*A.oryzae*) CBP systems of bioethanol production using BSG with both *S.cerevisiae* NCYC479 and NCYC2592 at 15°C for 20 days. CBP conducted using 50 g BSG with 200 mL water. Data are the mean ± SD of three replicate experiments.



Measurement of the free amino nitrogen (FAN) at various time points indicated that unlike a standard *Saccharomyces Spp.* based brewing fermentation, the FAN concentration actually increased (when using either yeast strain) over the duration of the fermentation (Figure 8.14). Initially both of the CBP systems (both *S.cerevisiae* NCYC479 and NCYC2592 variants of the saké based system) started with FAN levels of ca. 25 mg/L, rising to greater than 200 mg/L by day 20. Incidentally FAN levels were consistently measured to be ca. 10-30% higher in the *S.cerevisiae* NCYC2592 variant compared to the NCYC479 variant. This would initially suggest that FAN was not a limiting factor in the maximal ethanol yields achieved in

either yeast strain variant and also not responsible for the observed differences between the two yeast strain variants. FAN has been shown to be crucial for rapid attenuation and high ethanol yields in many fermentations using S.*cerevisiae*, especially high gravity fermentations (Dekoninck et al., 2012; Dragone et al., 2004; Thomas & Ingledew, 1990). Thus its limitation may somewhat contribute to the lower yields seen when using NCYC2592 if it had been rate limiting. Free amino nitrogen derived in-situ through some degree of hydrolysis of hordein protein fraction present within BSG by a secreted protease from the filamentous fungus would likely be an excellent substitute for a supplied exogenous nitrogen source when conducting high gravity fermentations. However, the specific amino acid profile liberated from the BSG protein fraction could affect the degree of enhancement of the fermentation. For example glycine (even though yeast cells are readily able to uptake it) has been indicated to actually increase the attenuation time and decrease the total yeast cell count for various fermentations suggesting that not all amino acids are created equal in terms of FAN (Thomas & Ingledew, 1990). Therefore, should the specific yeast partner (either *S.cerevisiae* NCYC479 or NCYC2592) which is paired with the *A.oryzae* somehow affect the amino acid profile of the FAN within the supernatant such as through an effect on the transcription of specific proteases secreted by the *A.oryzae* (thus possibly affecting the specific amino acids cleaved from the hordein polypeptides present) it may ultimately limit the maximal ethanol yield.

qRT-PCR could be employed in the future to look at gene expression of protease encoding genes within the *A.oryzae* (such as aminopeptidases which could cleave individual amino acids from terminal ends of proteins or polypeptides). These proteases may be responsible for the observed boost in measured FAN levels or possibly responsible for providing additional substrates for gluconeogenesis thus possibly somewhat explaining the differences in ethanol productivity.

The lack of detectable low molecular weight sugars detected in the supernatant at all the time points tested (for both yeast variants of the *A.oryzae* based CBP system) would definitively put the fermentation into the low gravity category and thus differences in specific FAN composition would be considerably less likely to be a limiting factor in the observed maximal ethanol differences, especially given the overall increase in FAN over the time course.

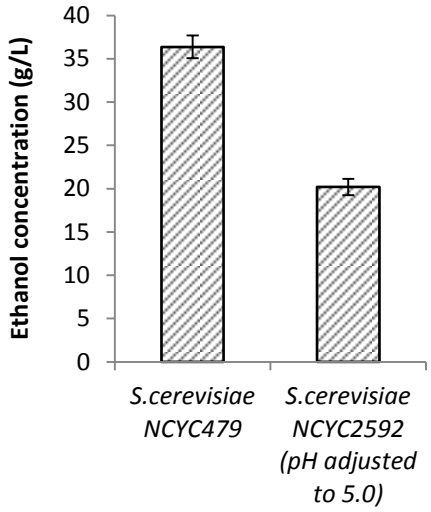
Measurement of the pH (over the course of the 20 day CBP fermentation) highlighted a small consistent (observed with both yeast strain variants) drop occurred as the fermentation progressed. This was expected to be somewhat similar to what occurs with beer fermentations where a proportion of the CO_2 generated by the yeast (through fermentative metabolism) would form carbonic acid (H_2CO_3) and reduce the pH.

Additionally, the measurement of pH levels indicated a small variation with the NCYC2592 variant maintaining a consistently slightly lower pH (ca. pH 4.5) compared to the NCYC479 variant (ca. pH 5.0; Figure 8.14). This may have had a small effect on the ultimate ethanol productivity of the yeast, however, the difference was not considered significant enough to have resulted in the ca. 43% higher maximal ethanol yields seen when using NCYC479. This is because the pH of both supernatants was considered to be close to optimal for both of the yeast strains and would not have caused any major metabolic issues. An alternative possibility could be the pH difference of the supernatants affected the activity of one or more of the key CAZy based saccharification enzymes secreted by the *A.oryzae*. The overall result may have been that less directly fermentable sugar (glucose) was liberated from the cellulose thus a lower overall ethanol productivity from the yeast. To investigate this hypothesis the cellulolytic enzyme activity of the supernatants of both yeast strain variants (NCYC479 and NCYC2592) of the saké based CBP system (sampled on days 4 and 8; and considered to reflect phases of enzyme secretion most likely to indicate any differences) was externally tested ex vivo of the CBP system. The native pH and the 15°C operating temperature were

used for the assay in order to accurately model the in-vivo environmental parameters of the saké based CBP system and utilised Whatman® No 1 filter paper (cellulose based) as the substrate. Ultimately a mean glucose liberation difference of only ca. 9% was observed between the two different yeast strain supernatants and thus indicated that cellulolytic enzyme activity was not significantly affected by the 0.5 unit pH difference and consequently did not significantly affect the saccharification yields or maximal ethanol yields.

The traditional cultivation of *A.oryzae* conidiospores for subsequent commercial usage has been shown to be enhanced by using a slightly alkaline pH through the addition of ash leaves (Machida et al., 2008). The effect is attributed to prevention of contamination by other microorganisms (that are less able to tolerate alkaline conditions etc.). However as the preparation for all the CBP based experiments were conducted entirely under sterile, aseptic conditions the probability of contamination was considered negligible. This in conjunction with the fact that both *A.oryzae* based CBP variants (with either *S.cerevisiae* NCYC479 or NCYC2592) had a relatively similar, acidic supernatant pH it was considered unlikely to be the cause of the ethanol productivity differences. Confirmation of the lack of a pH based effect was then achieved when a modified variant of the *A.oryzae* and *S.cerevisiae* NCYC2592 CBP system with the pH adjusted and maintained at 5.0 ± 0.1 (and thus identical to the NCYC479 variant) still produced ca. 45% less maximal ethanol than the *A.oryzae* and *S.cerevisiae* NCYC479 CBP system under otherwise identical conditions (Figure 8.15).

Figure 8.15 Maximal ethanol concentrations (achieved on day 10) from a variant of the *A.oryzae* and *S.cerevisiae* NCYC2592 CBP system in which the pH was adjusted to 5.0 ± 0.1 to match the native pH of the *A.oryzae* and *S.cerevisiae* NCYC479 system. CBP conducted using 50 g BSG with 200 mL water. Data are the mean ± SD of three replicate experiments.

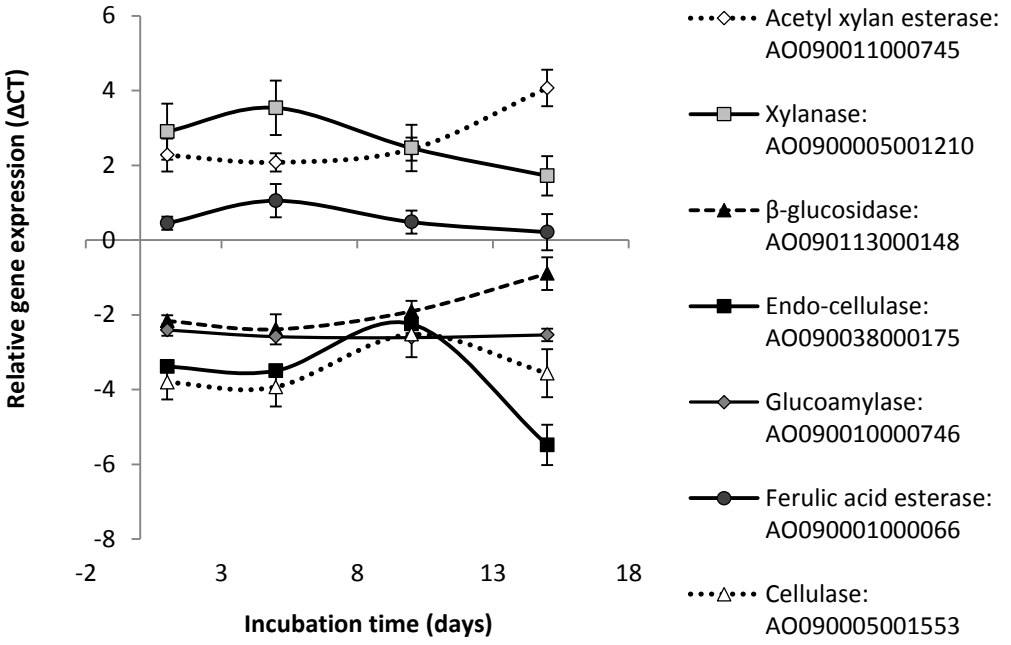


8.3.10 qRT-PCR based gene expression analysis

8.3.10.1 CAZy and other associated genes in *A.oryzae* when paired with *S.cerevisiae* NCYC479 for CBP based ethanol production from BSG

Looking at quantitative gene expression of the representative CAZy targets (as a rapid and fairly crude initial screen for carbon source usage indications) relative to the ACT housekeeping gene (which itself showed consistently stable, steady state expression at all time-points tested thus validating its choice for this role) an interesting sequence of gene expression up-regulation was observed (Figure 8.16).

Figure 8.16 qRT-PCR tracking expression levels of 7 *A.oryzae* target CAZy and associated genes throughout the time course of a saké based CBP system. All data normalised to ACT housekeeping gene expression levels. CBP conducted using 50 g BSG and 200 mL water with *A.oryzae* and *S.cerevisiae* NCYC479. Data are the mean ± SD of three replicate experiments.



The first gene indicated to be up-regulated was the xylanase (AO090005001210) which then peaked at day 5, beyond which its expression began to decline. This appeared to show the *A.oryzae* targeted the hemicellulose (xylan) fraction for depolymerisation first, possibly due to its relatively lower recalcitrance compared to that of crystalline cellulose or lignin. This was also somewhat similar to the pattern (expression sequence) seen by Delmas et al (2011), using next generation RNA sequencing technology (RNA-seq) looking at *A.niger* when cultured purely on another lignocellulosic material wheat straw which suggested some similarity between *A.oryza*e and *A.niger* in terms of their preferential carbon source in the absence of their preferred choices of glucose or starch etc. However, the transcriptional responses (specifically the detection of any up-regulation of expression of the CAZy and associated lignocellulolytic enzymes) of the *A.oryzae* grown on BSG was considerably slower than the response seen in *A.niger* cultured on wheat straw (days for the *A.oryzae* as

opposed to only hours as seen with *A.niger*) suggesting the possibility that a true starvation response was not being seen initially in the *A.oryzae* cultured on BSG. This would suggest that (at least initially) the *A.oryzae* may have been utilising a non-lignocellulosic carbon source such as the trace amounts of residual starch present or possibly gluconeogenesis through metabolism of hordein prolamin glycoproteins or their constituent glucogenic amino acids such as proline (Shewry & Tatham, 1990). Alternatively, the apparent transcriptional delay (seen here with the *A.oryzae*) in the up-regulation of the xylanase gene expression in comparison to that of *A.niger* cultured on wheat straw as described in Delmas et al (2011), could be due to the increased viscosity of the saké based CBP culture media used here due to the use of a higher solid to liquid ratio (higher solids loading) which consequently affected the mass transfer and may have affected the rate of confirmation of the presence of hemicellulose (xylan). This delay in 'sensing' hemicellulose may have somewhat hindered any significant secretion of xylanases. In conjunction with the observed decline in expression of the target xylanase gene (AO090005001210) beyond day 5, a simultaneous increase in the expression of the endo-cellulase gene (AO090038000175) was observed, peaking at ca. day 10. This showed the *A.oryzae* subsequently moved from primarily hemicellulose as the carbon source to that of cellulose as an endo-cellulase would likely be required initially to cleave internal sites along the cellulose fibres in order to expose free reducing ends for any additional exo-cellulases to attack and release lower molecular weight cello-oligomers (such as cellobiose which in turn could be further depolymerised to glucose ultimately). This hypothesis was further supported by a similar degree of up-regulation of another gene (AO090005001553) with predicted cellulase activity from day 5 onwards as was seen with the endo-cellulase. Beyond day 10 a sharp decline was then seen in expression levels of both the genes (AO090005001553 and AO090038000175) with predicted cellulose activity. In the absence of any detectable free monosaccharide concentrations (especially the lack of detected

hexose sugars; likely due to their rapid utilisation by either the primary fungus or the yeast once liberated) it could suggest that catabolite repression was not the causal factor for the subsequent down-regulation of the endo-cellulase encoding genes beyond day 10. An alternative explanation could be that the endo-cellulases where targeting primarily amorphous, non-crystalline cellulose present within the lignocellulosic matrix due to its reduced recalcitrance in comparison to crystalline cellulose and thus more readily hydrolysed to liberate metabolisable sugars. Once this more amorphous cellulose had been hydrolysed by the cellulases the fungus may have mistakenly detected the cellulose component had been fully depleted and thus triggered the down regulation of the endo-cellulases. This hypothesis would suggest that a preliminary thermo-chemical pre-treatment of the BSG such as the 1% HCl acid catalysed hydrothermal protocol previously tested (Chapter 4) could facilitate a higher maximal ethanol yield through modification of the lignocellulosic matrix. However, as this was not the case it may propose that there may be other thermo-chemical pre-treatments that are more suitable for enhancing BSG based CBP using the standard saké based system such as the use of alkaline or caustic reagents. These may include NaOH or alkaline peroxide reagents which have been indicated to work very effectively in the pre-treatment of BSG (Chapter 5). These are suggested to alter the crystallinity of cellulose (possibly increasing the conversion of crystalline cellulose to a more amorphous cellulose format to some degree) and act as swelling agents on the fibrils (Zhu et al., 2008). This may facilitate a modified transcriptional response from the *A.oryzae* (either alternative gene expression or increased up-regulation) and thus possibly achieve a higher maximal ethanol yield. From approximately day 5 onwards and similar to as seen with both the endo-cellulases, a simultaneous steady increase in the expression of a β-glucosidase gene (AO090113000148) was observed which continued steadily increasing as the time course progressed. This would suggest it was being expressed in response to the preliminary activity of the endo-cellulases liberating cello-

oligosaccharides whose non-reducing ends would then be prime substrate candidates for the β-glucosidase to cleave into its constituent glucose monomers. With a similar magnitude to the β-glucosidase up-regulated expression pattern the acetyl xylan esterase gene (*axeA*: AO090011000745) expression levels were also indicated to very steadily increase from day 5 onwards. This could indicate the increased expression of 'scoping' enzymes that may have some activity on an additional substrate present within the BSG (such as on the side chain decorations on one of lignocellulosic polymers present) and thus potentially able to liberate an additional metabolic carbon source which the *A.oryzae* could utilise. This could have been an indication the *A.oryzae* was beginning to deplete the all of the easily accessible (more easily hydrolysed) carbon sources and indicating the preliminary stages of some format of a true starvation response. Some degree of up-regulation of the ferulic acid esterase gene (*faeB*: AO090001000066) was indicated at an early stage of the time course, peaking at ca. day 5. This could suggest that either liberated hydroxycinnamic acids (predominantly ferulic acid) could be used by the *A.oryzae* as a carbon source or alternatively that cleavage of the di and tri-ferulic acid esterified cross linkages between the various lignocellulosic polymers was a key step in reducing the overall recalcitrance and thus improving either physical access or the performance of other secreted CAZy enzymes. In contrast expression levels of the glucoamylase gene (*glaA*: AO090010000746) remained constant throughout the duration of the experiment indicating that the *A.oryzae* was either not utilising the marginal starch component present within the BSG (as increased α-glycosidic bond hydrolysis would have indicated) or that it was occurring at a continually low, steady state. This may indicate the *A.oryzae* could simultaneously utilise more than one carbon source and that it does not necessarily discreetly shift discreetly from one particular carbon source to another.

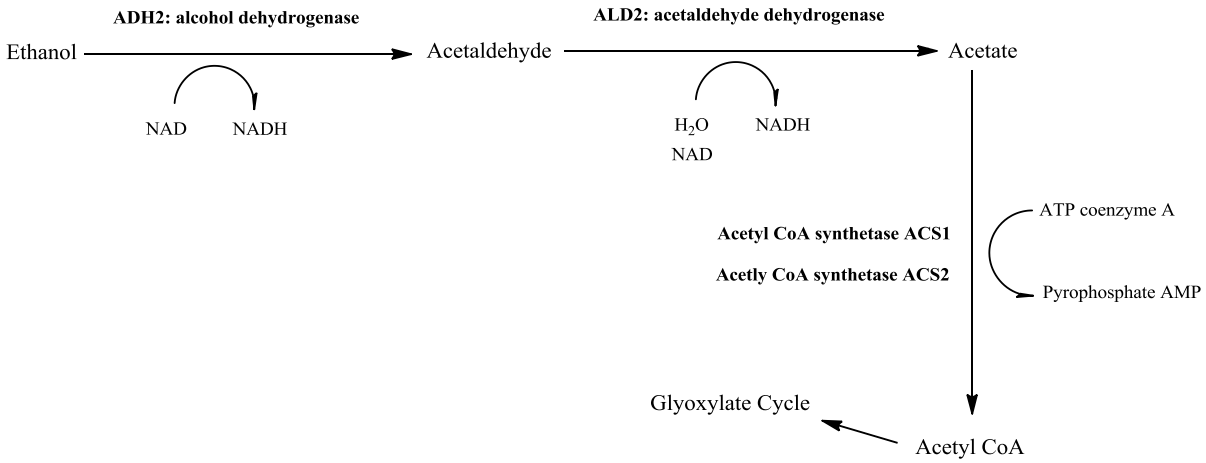
If secretion of an additional enzyme such as a ferulic acid esterase (by the filamentous fungi) is possibly a rate limiting factor then optimisation could be conducted to attempt to enhance

this through encouraging the over-expression either through genetic engineering or accelerated evolution technology such as MAGE; *multiplex automated genome engineering* (Wang et al., 2009). In addition, as all experimental runs were conducted using static, semi-solid state bioreactors (with high initial viscosity of the media), additional mixing could also be employed to attempt to enhance fungal growth (and thus possibly achieve greater enzyme secretion) which could in turn facilitate better enzyme and substrate interactions potentially generating greater fermentable sugar yields and therefore possibly greater ultimate ethanol yields. The low solubility of oxygen (in water) relative to other dissolved solutes can rate limit aerobic fungal growth through limited oxygen mass transfer (especially in high dissolved solids bioreactors such as those employed here) thus likely limit the secretion of various lignocellulolytic enzymes (Weber & Agblevor, 2005). In addition, the mycelial growth of any filamentous fungal species within a solid state or high solids CBP reactor further affects the rheological properties of the supernatant (potentially leading to further increases in viscosity) which may further limit oxygen mass transfer. Thus, the anaerobic environmental conditions generated by the saké based *A.oryzae* system (with either of the yeast strain variants tested) could play a small part in the explanation of the limit of maximal ethanol yields achieved, through limitations of fungal growth (and therefore possibly limitations on activity of saccharification enzymes). Additionally the traditional brewing of the saké beverage is capable of achieving ca. 20% ABV under very similar conditions to those used for the CBP fermentations. Therefore the co-culture is clearly much better suited to starch-based saccharification and fermentation rather than the recalcitrant lignocellulosic biomass. Overall, the issue is likely to be considerably more complex and dynamic than due to one single factor.

To compound the difficulties faced with the potential use of some form of mixing or agitation of the slurry, both fungal hyphae and cellulases are shear stress sensitive rendering agitation

based improvements in oxygen mass transfer challenging (Weber & Agblevor, 2005). Micro-bubble based dispersion systems could possibly be investigated as opposed to conventional mixing protocols. Ideally a dissolved oxygen concentration (DO_2) of ≥20% air saturation would provide sufficient fungal growth parameters (Schell et al., 2001). However this could possibly reduce the ethanol yield from the *Saccharomyces Spp.* and shift the carbon flux away from ethanol production via fermentation and rather to biomass generation somewhat. Additionally, the creation of an aerobic environment could increase the possibility of the utilisation of any ethanol previously produced via fermentation as a carbon source by the yeast in the absence of any other easily utilisable carbon source (with the ethanol converted initially to acetaldehyde via the *ADH2* gene encoded alcohol dehydrogenase; Figure 8.17).

Figure 8.17 Ethanol utilisation via the ADH2 pathway (glyoxylate cycle) (adapted from www.yeastgenome.org, Weinhandl et al, 2014, Schüller 2003).



Therefore, further investigation would be required in order to optimise any such strategy employed, as a compromise between boosting enzyme secretion (and activity) and maintaining optimum ethanol fermentation conditions.

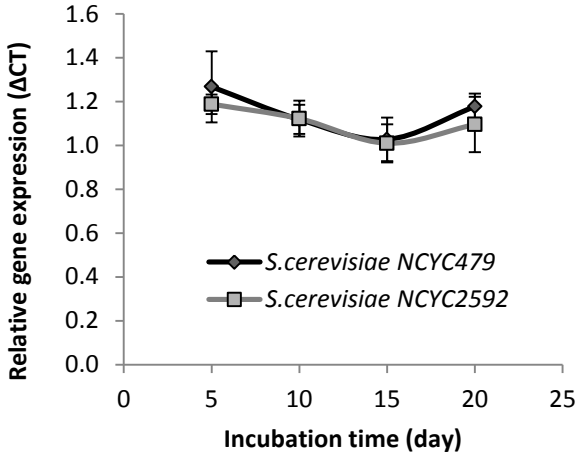
Whilst somewhat off-track, yet still an interesting idea is that in addition to the potential application of the saké based CBP biofuel production system utilising and effectively 'recycling' BSG, another avenue of potentially simultaneous development which could also

be conducted on-site within a brewery (or in an associated bottling plant nearby) would be the use of *A.oryzae* to decompose biodegradable plastic such as poly-butylene succinate or PBS (Maeda et al., 2005). *A.oryzae* has both cutinase (*CutL1*) and hydrophobin genes (such as *rolA*) present within its genome which have been shown to be responsible for the deconstructive mechanism with cutinase facilitating the actual decomposition of the plastic and hydrophobin acting as 'scaffolding' for the specific site recruitment of the cutinase onto the hydrophobic surfaces of biodegradable plastics (Machida et al., 2008; Maeda et al., 2005). The successful use of biodegradable plastics for commercial soft drinks distribution could enable research and development of a biodegradable plastic beer bottle (designed and optimised for flavour stability and minimal oxygen pick up) which could then be recycled on site at the brewery or bottling plant by the *A.oryzae*.

8.3.10.2 ADH2 gene expression in *S.cerevisiae* NCYC479 and NCYC2592 when paired with *A.oryzae* (saké system) for CBP based ethanol production from BSG

qRT-PCR looking at relative gene expression levels of the ADH2 alcohol dehydrogenase gene in the two yeast strain variants (*S.cerevisiae* NCYC479 and NCYC2592) when used in the saké based CBP system showed little or no difference in their expression profiles over the course of the 20 day fermentations (Figure 8.18). Both strains exhibited a steady decline in expression from ca. day 5 to ca. day 15 suggesting the presence of glucose within the media was causing repression of gene expression. However, the expression increased in both strains from day 15 onwards which corresponded to the peak of ethanol production after ca. 15 days. This suggested a starvation response may now be occurring as some degree of increased 'recycling' of the produced ethanol was likely occurring. This did not, however, explain the differences in observed maximum ethanol concentrations seen between the two strains and this is still due to an unknown reason and is the subject of current research.

Figure 8.18 qRT-PCR tracking relative expression levels of the alcohol dehydrogenase gene (ADH2) in two different yeast strains throughout the time course of a saké based CBP system. Strains *S.cerevisiae* NCYC479 or NCYC2592 used with *A.oryzae* using 50 g BSG and 200 mL water. qRT-PCR for ADH2 expressed as relative to ACT housekeeping gene. Data are the mean ± SD of three replicate experiments.



8.4 Conclusions

This work is one of the first major investigations that utilises an entirely fungal co-culture (consortium of two different fungi) for ethanol production from BSG using a CBP approach. From the multitude of different permutations of primary fungi species and yeast strains tested, the saké brewing combination of *A.oryzae* and *S.cerevisiae* NCYC479 was the most effective combination. Maximum ethanol yields of ca. 37 g/L could be produced within 10 days and also with the best volumetric ethanol productivity (3.5 g/L/day). This equated to ca. 79% of the theoretical ethanol yield (assuming it derived from the cellulose component of BSG alone). 94 kg of pure ethanol could be produced from 1 metric tonne of BSG using 36 hL water. Whilst this system is arguably too slow for commercial viability at scale it represents a very 'green', low cost and energy efficient method of producing bioethanol. The results of this work open up further avenues for exploration and optimisation of entirely fungal based bioethanol production systems from lignocellulosic biomass such as BSG. In

particular the work highlights the versatility of the saké based fermentation system as a valuable bioprocessing tool that warrants further investigation.

9 Biorefining BSG: Enzymatic and thermo-chemical based protein fractionation

9.1 Introduction

In addition to the considerable amount of complex, structural carbohydrate present in BSG (in the form of the lignocellulosic matrix) there is also a considerable quantity of protein that still remains in the biomass after the brewing process has been completed. Typically values range from 10% to 30% protein (Xiros & Christakopoulos, 2012). In present research we have found that values are ca. 25% protein for BSG originating from 100% malt brewing (Chapter 3). Therefore, for every metric tonne of BSG (1000 kg dry weight) there could be ca. 250 kg of pure protein. The fractionation of this considerable yet under-utilised proteinaceous component found within BSG could represent a high value product stream.

The bulk of this thesis specifically relates to bioethanol production from BSG (i.e. from the carbohydrate components). However, due to the low market value of fuel ethanol the intention was to attempt to develop a duel-phased process that also created alternative high value products (such as a high protein extract) in addition to the bioethanol. The production of two high value products may then increase the life cycle assessment (LCA) favourability of the process sufficiently to consider the roll-out of the technology on a commercial scale. However, the successful linkage of two separate processes (such as protein fraction and bioethanol production) would require careful investigation in order to ensure that either process did not detrimentally affect the other.

Various research groups have reported protocols that can achieve some degree of protein fractionation using a wide range of different reagents and techniques (Celus et al., 2007; Celus et al., 2009; Crowe et al., 1985; Ishiwaki et al., 2000; Tang et al., 2009). In particular, caustic-based (NaOH) protein fractionation reagents as described in Celus (2007) were of particular interest. As caustic-based compounds had been previously shown to be highly effective pre-treatment reagents for BSG (Chapter 5) there was the possibility that a caustic-based protein fractionation protocol may also provide some degree of pre-treatment effect. As such there may then have been no need for a separate pre-treatment step which could potentially save a considerable quantity of energy or chemical usage yet still produce two high value products.

Should an effective protein fractionation protocol be developed and shown to be sufficiently economically viable to process the spent grains on a commercial scale, then it could be used for a multitude of goods including incorporation into human or animal food products (Ainsworth et al., 2007; Denstadli et al., 2010; Hassona, 1993; McBurney & Rasco, 1989; Stojceska & Ainsworth, 2008). Alternatively, as the BSG obviously originates from within breweries, a high protein process stream could be recycled back into the beer brewing process. This could be through its use as a nitrogenous adjunct (a supplement) for high gravity fermentations. High gravity fermentations require a higher concentration of bioavailable, low molecular weight proteinaceous compounds or free amino nitrogen (FAN) than lower gravity fermentations. This is because the yeast are exposed to a greater degree of osmotic stress due to the high sugar concentrations used initially and high ethanol concentrations produced ultimately (Piddocke et al., 2011; Puligundla et al., 2011). The additional FAN is used to avoid nutrient depletion issues that arise with high gravity brewing. However, the specific amino acid profile has been shown to influence the degree of enhancement of fermentation performance, with high glycine levels actually reducing the

fermentability of a feedstock (Thomas & Ingledew, 1990). However, Essien and Udotong (2008) report that at least 17 of the major amino acids were present in the proteinaceous material from BSG which could confirm its suitability as a balanced nutritional additive or fermentation additive.

Little if any published literature exists to the author's knowledge that has investigated or developed an effective protein fractionation process from BSG that has been confirmed to have no detrimental effects on the complex carbohydrates that constitute the lignocellulosic material found within BSG. These complex carbohydrates can represent a significant source of potentially fermentable and therefore highly valuable sugars. In particular no published work evaluates the effect of protein fractionation protocols on the subsequent enzymatic saccharification yields achieved using cellulolytic enzyme cocktails (e.g. for the production of biofuel feedstocks). The protein fractionation protocols may have provided a sufficient 'pre-treatment' effect, thus enabling enzymatic saccharification to be conducted immediately afterwards.

Here we have developed a fractionation process (the solubilisation of part of the protein component found in BSG) that produces a high value protein enriched product. In addition the protein fractionation process did not detrimentally affect the carbohydrate component (cellulose and hemicellulose) found in BSG. This would then still enable the production of a biofuel from the carbohydrate component, offering two high value products.

9.2 Materials and methods

9.2.1 Enzyme based attempts at fractionation of the protein component in BSG

To investigate a biocatalytic protein solubilisation option, two commercial protease mixtures; Pronase (Sigma-Aldrich) and Alphalase™ NP (Danisco) were chosen for evaluation of their protein fractionation abilities on BSG.

9.2.1.1 Preliminary testing of Pronase and Danisco Alphalase NP protease mixtures

In terms of attempting to develop an effective fractionation protocol of the protein component found within BSG whilst also attempting to utilise the lignocellulosic component for biofuel production a number of potential stages for the protein fractionation step to be conducted were identified. Previous work had optimised a dilute acid based pre-treatment protocol (1% HCl, 121°C; Chapter 4) that achieved a high degree of enhancement of the subsequent enzymatic saccharification yields. As such a high glucose, highly fermentable feedstock was produced. However, we also previously concluded from % nitrogen analysis of the insoluble residues that remained after each processing step (the pre-treated BSG biomass and the final insoluble residue that remained after the enzymatic saccharification step) that the total protein content had not significantly decreased from that of the initial BSG (ca. 20% - 25% w/w). Therefore the 3 potential stages for attempting protein fractionation were: the initial BSG, the residue that remained after pre-treatment and the residue that remained after enzymatic saccharification.

Alphalase™ NP is a commercial preparation which is produced by *Bacillus amyloliquefaciens* and used within the brewing industry to aid in the hydrolysis of the protein present within malted barley thus boosting free amino nitrogen (FAN) levels to support high gravity fermentations. The Alphalase™ NP was dosed at 100 µl/10 g biomass. The

Alphalase™ NP has a suggested dosing of 0.1- 0.3 kg/MT grist when used during the mashing stage of beer brewing. Therefore the dose used here for this preliminary work was considered to be greatly in excess. The Alphalase™ NP was prepared in 50 mM sodium citrate buffer at pH 6.5.

Pronase is a commercial mixture of many proteases from *Streptomyces griseus* K-1 with an overall very broad spectrum of activity. Pronase was prepared at 0.5 g/L using 50 mM sodium citrate buffer (pH 7.5). The specific activity of Pronase was 4 Units/mg with one unit of Pronase was defined as the amount of enzyme required to hydrolyse casein to produce colour equivalent to 1.0 μM (181 μg) of tyrosine per min at pH 7.5 at 37°C (colour by Folin-Ciocalteu reagent).

Both the Pronase and Alphalase™ NP protein fractionation experiments were conducted using 24 h incubation periods (at 37°C and 60°C respectively) at 15% (w/v) solids loading which was achieved using the correct amount of biomass and 50 mM sodium citrate buffer at pH 7.5 and pH 6.5 respectively. The samples were then centrifuged at 5000 rpm for 5 min and the supernatant was analysed for FAN and total protein using the protocols outlined in sections 7.2.4 and 7.2.5 respectively. Substrate controls (blanks) were run alongside with only enzyme and buffer for subtraction of protein and FAN levels from the enzyme preparations for accurate calculation of achieved protein solubilisation concentrations.

9.2.1.2 Optimisation of Pronase based protein fractionation using BSG

As preliminary experimental work had concluded that using the Pronase protease mix outperformed the Alphalase™ and when used directly on BSG (as opposed to using it on pre-treated BSG or after enzymatic saccharification) liberated the highest concentration of protein and highest free amino nitrogen (FAN) concentrations into the generated hydrolysate, further work was conducted in order to attempt to optimise this protein solubilisation protocol as the

dosage used for the preliminary investigation (0.5 g/L) was considered largely ineffective. Therefore factors optimised included; increased Pronase dose (0.625 – 10 g/L), the solids loading used (5% - 25% w/v) and the reaction time (4 - 64 h). Efficacy of the protein solubilisation was determined again by the protein (Bradford assay based) and FAN concentrations liberated into the hydrolysate.

The Pronase optimisation was conducted using a D-optimal designed experiment (see Appendix 2 for full details). The D-optimal algorithm chose the treatment combinations to be conducted. This reduced the total number of experiments needed to be conducted from that of a full factorial experimental design yet still provided a statistically valid data set.

9.2.2 Caustic based thermo-chemical attempts at fractionation of the protein component found within BSG

9.2.2.1 Preliminary investigation of the caustic protocol

A very slightly modified variant of the caustic protein fractionation protocol described in Celus et al (2007) was used initially as a starting point to attempt to evaluate the techniques effectiveness. This protein fractionation step involved a caustic digestion of the BSG using 0.3M NaOH (at 15% w/v solids loading) at 60°C for 1 h. The samples were then centrifuged at 5000 rpm for 5 min. The supernatant was then removed and analysed for FAN and protein concentration. The separated hydrolysates were then pH adjusted to 4.0 ± 0.1 (using 2M citric acid) to facilitate protein precipitation and then centrifuged (10000 RPM for 15 min) and the supernatant discarded. The protein enriched pellet was then oven dried at 70°C overnight to aid storage stability. Efficacy of the protein fractionation protocols was determined through quantification of total protein and/or free amino nitrogen (FAN) concentration in the hydrolysate (i.e. the liquid fraction or supernatant that was produced). In addition the % nitrogen content (which could then allow calculation of the % protein content) of the final

remaining insoluble residue was quantified to confirm a diminishment in protein content was accompanying the increased liberation into the hydrolysate. The dried protein enriched pellets also had % nitrogen determined to assist in tracking the degree of protein solubilisation and exactly how concentrated in protein the pellets actually where. FAN was measured using glycine as a standard according to the European brewing convention method (Analytica – EBC, 2000) as described in section 7.2.4. Total Protein quantification was conducted using the Coumassie (Bradford) reagent protein assay kit as described by in section 7.2.5. The % nitrogen quantification (and subsequent % protein calculation) of the starting BSG and the remaining insoluble residues was conducted using a Thermo Flash Nitrogen Analyzer using the method described in section 2.3.4.

9.2.2.2 Optimisation of the caustic thermo-chemical protein fractionation protocol:

Following the largely unsuccessful attempts at recreating the protein fractionation results from BSG achieved using the exact protocol described in Celus et al (2007) a subsequent optimisation experiment was conducted. This was designed to modify the reaction conditions of the original protocol in order to attempt to improve its efficacy and liberate a greater protein concentration. This involved using a range of caustic concentrations and a range of reaction temperatures (at 15% w/v solids loading using 0.3 M – 0.9 M NaOH and incubated at 60°C - 100°C for 1 h). After incubation samples were centrifuged (5000 RPM for 5 min) and the hydrolysate decanted and sampled for protein quantification using the Coumassie (Bradford) reagent protein assay kit (Thermo-Fisher, UK) as previously described. The separated hydrolysates were then pH adjusted to 4.0 ± 0.1 (2M citric acid) and an additional chilling stage (to ca. 1°C for 1 h) was added in order to attempt to aid protein precipitation. The chilled hydrolysates were then centrifuged (10000 RPM for 15 min) and supernatant discarded after confirmation of minimal protein concentration (via the Bradford assay as previously described). The remaining protein enriched pellet was then oven dried at 70°C

overnight to aid storage stability. Quantification of the % protein concentration of the pellets was again conducted using the method outlined in section 2.3.4.

9.2.2.3 Direct attempts at cellulolytic enzymatic saccharification of the remaining insoluble residue after protein fractionation

This was conducted in order to ascertain whether the caustic protein fractionation protocols achieved any degree of enhancement of subsequent enzymatic digestion akin to that of conventional thermo-chemical pre-treatments which can also involve the use of caustic reagents as previously described in Chapter 5. It would also establish whether any deleterious effects on the subsequent enzymatic saccharification yields were observed in response to running the caustic based protein fractionation protocol prior to any pre-treatment or enzymatic saccharification step. Enzymatic saccharification was conducted using the insoluble residues that remained after the caustic based protocols and with native (unmodified) BSG as a control to compare any potential 'pre-treatment' effect of the caustic protein fractionation protocol against.

High solids-loading (10% w/v), low enzyme dose (20 FPU/g biomass) enzymatic saccharification reactions were conducted using Novozymes Cellic® CTec2 (Novozymes A/S, Demark) at 50°C for 24 h using the general protocol described in section 2.7.2. Samples were then centrifuged (5000 rpm, 10 min) prior to sampling for HPLC based glucose quantification as described in section 2.9.2.

9.2.2.4 Quantification of the total glucose contents of BSG and the insoluble residues remaining after caustic based protein fraction

Total glucose concentrations in the BSG and each of the remaining insoluble residues after the caustic based protein fractionation attempts were quantified using the two-stage complete

acid hydrolysis protocol described in section 2.3.1. This allowed the calculation of accurate % theoretical glucose yields after enzymatic saccharification.

9.3 Results and discussion

9.3.1 Enzyme based attempts at fractionation of the protein component in BSG

9.3.1.1 Preliminary testing of Pronase and Danisco Alphalase NP

Initial studies concluded that the Pronase commercial mixture of proteases performed better in terms of protein fractionation than did the brewing industry specific Alphalase NP, regardless of which of the 3 stages of the process (the starting BSG, pre-treated BSG or the remaining insoluble residue after enzymatic saccharification) the protease mixtures were used (Table 9.1). Slightly higher total protein (Bradford) and FAN concentrations were liberated into the hydrolysate (liquid fraction) when using the Pronase mixture as compared to the Alphalase NP. In addition the preliminary work concluded that in terms of maximal protein and FAN liberation, the initial BSG co-product was probably the best biomass on which to use the protease enzymes as it resulted in significantly higher yields than when using the insoluble residues remaining after either pre-treatment (before Cellic® CTec2 based enzymatic saccharification) or after enzymatic saccharification (Table 9.1). The data showed that only minimal differences in protein yields were seen when the enzymes were applied to pre-treated BSG, regardless of the exact pre-treatment protocol (1% HCl or 5% NaOH). The actual liberated protein yields were very low (typically ca. 100 μg/mL in the hydrolysate; Table 9.1). This indicated that optimisation work may be required (dose, reaction time and solids loading optimisation etc.) to attempt to increase the degree of protein solubilisation achieved using this enzyme based method.

Table 9.1 Preliminary testing of the effectiveness of two commercial protease mixtures at solubilising the protein content of BSG, as well as the insoluble residues that remained after pre-treatment and after cellulolytic enzyme saccharification.

	Liquid Fraction (hydrolysate)	
	FAN (mg/L)	Protein (μg/mL)
BSG Pronase E	126 ± 9	160 ± 35
BSG Alphalase NP	102 ± 11	24 ± 13
BSG both protease mixes	115 ± 16	131 ± 23
1% HCl 121°C pre-treated (Before CTec2) Pronase E	89 ± 4	89 ± 23
1% HCl 121°C pre-treated (Before CTec2) Alphalase NP	60 ± 8	82 ± 41
1% HCl 121°C pre-treated (Before CTec2) both protease mixes	57 ± 5	118 ± 19
1% HCl 121°C pre-treated (After CTec2) Pronase E	44 ± 7	124 ± 26
1% HCl 121°C pre-treated (After CTec2) Alphalase NP	25 ± 4	106 ± 31
1% HCl 121°C pre-treated (After CTec2) both protease mixes	34 ± 8	142 ± 45
5% NaOH 50°C pre-treated (Before CTec2) Pronase E	76 ± 2	109 ± 21
5% NaOH 50°C pre-treated (Before CTec2) Alphalase NP	12 ± 5	103 ± 16
5% NaOH 50°C pre-treated (Before CTec2) both protease mixes	43 ± 9	152 ± 9
5% NaOH 50°C pre-treated (After CTec2) Pronase E	63 ± 7	107 ± 24
5% NaOH 50°C pre-treated (After CTec2) Alphalase NP	5 ± 1	105 ± 32
5% NaOH 50°C pre-treated (After CTec2) both protease mixes	22 ± 3	126 ± 13

Pronase hydrolysis protocol: dosed at 0.5 g/L and prepared using 50 mM sodium citrate buffer (pH 7.5) at 10% (w/v) solids loading with 24 h incubation at 37°C. Danisco Alphalase NP hydrolysis protocol as with Pronase except dosed at 10 μL/g biomass (preparation used as supplied) and at pH 6.5 with incubation at 60°C. All samples then centrifuged at 5000 rpm for 10 min and supernatant sampled for protein and FAN analysis. Pre-treatments all conducted at 25% solids loading (w/v). Protocols: 1% HCl 121°C pre-treatment for 30 min or 5% NaOH at 50°C for 12 h. Residues then adjusted to pH 5.0 (via 40% NaOH or glacial acetic acid) and exhaustively washed with water. Centrifugation at 5000 rpm for 10 min and supernatant discarded. Insoluble residue dried overnight at 70°C. Enzyme saccharification protocol: Novozymes Cellic® CTec2 dosed at 20 FPU/g biomass at 15% (w/v) solids loading and achieved using 50 mM sodium citrate buffer (pH 5.0). Incubation at 50°C for 48 h at 150 rpm agitation. Data are the mean ± SD of three replicate experiments.

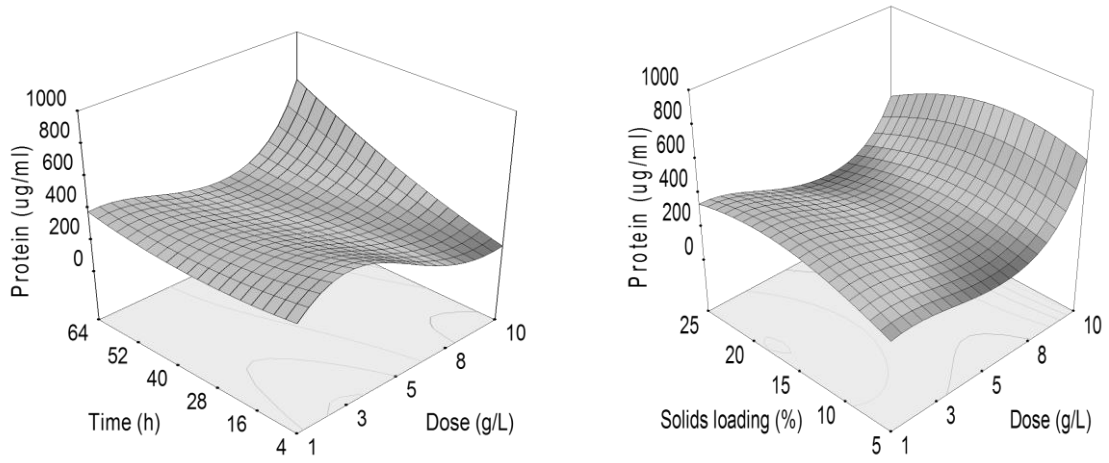
9.3.1.2 Optimisation of the Pronase based protein fractionation protocol

Looking initially at the total protein concentration (liberated into the hydrolysate) as a response factor in a 3D response surface model (with the simultaneous variation of enzyme dose from 0.625 g/L – 10 g/L and reaction time from 4-64 h) it was concluded that variation of the dose had little effect except at very high addition rates (10 g/L) with very long (64 h) reaction times (Figure 9.5A). The use of a 10 g/L Pronase dose for 64 h liberated ca. 800 μg/mL (0.8 mg/mL) protein compared to ca. 200 μg/mL (0.2 mg/mL) when using a 1 g/L dose for 64 h. This unfortunately showed that even with very high enzyme doses no substantial protein solubilisation from the BSG could be achieved. Such doses would not be economically viable as the cost of 1.0 g of Pronase is Ca. $120. This showed that utilising a caustic based protein fractionation protocol (as described in section 9.3.1) may not only be much cheaper but could also be better; but also the caustic reagent (NaOH) would be already present within the brewery (as part of the CIP systems) which could make on-site processing within a brewery a very attractive option. Further evaluation of the 3D RSM, evaluating the interaction between reaction time and enzyme dose found that only at very high doses (10 g/L) does reaction time become significant, with longer reaction times liberating higher protein concentrations (Figure 9.5A).

The assessment of an alternative 3D RSM again using protein liberation as the response but this time plotted against solids loading (5% - 25% w/v) and Pronase dose (0.625 g/L – 10 g/L) found that solids loading did limit the liberated protein concentration (Figure 9.1B). Increasing the solids loading (increasing the amount of biomass present) at any dose did not result in a significant increase in protein liberation. This suggested the amount of enzyme present could not cope with the amount of biomass that was present as it would be expected that increased protein would be liberated with the increased biomass present. At doses of 0.625 g/L to ca. 8 g/L the increase in solids loading from 5% to 25% w/v did not result in any

significant increase in protein liberation. Incidentally, at very high Pronase doses (10 g/L) the increase in solids loading resulted in a slight decline in liberated protein as solids loading was increased beyond ca. 15% w/v. This was an unexpected observation and could possibly be due to mass transfer limitations at such high solids loadings. The increased viscosity of the slurry (enzyme and biomass mixture) may have limited the mobility of the protease enzymes therefore reducing the efficacy of enzyme-substrate binding.

Figure 9.1 3D response surface models (RSM) showing the simultaneous effects of various factors including; Pronase dose (g/L), reaction time (h) and operational solids loading (% w/v) on protein liberation into the generated hydrolysate. Chart A; Pronase hydrolysis of BSG conducted at 15% solids achieved using 50 mM sodium citrate buffer (pH 7.5), using a Pronase dose range of 0.625 – 10 g/L and incubation over a range of 4 – 64 h at 37°C. Protein quantification achieved using the Coumassie (Bradford) reagent protein assay. (Model R^2: 0.84). Chart B: Pronase hydrolysis of BSG conducted at 5% - 25% w/v range of solids loadings achieved using 50 mM sodium citrate buffer (pH 7.5), using a Pronase dose range of 0.625 – 10 g/L and incubation at a temperature of 37°C for 64 h. Protein quantification achieved using the Coumassie (Bradford) reagent protein assay. (Model R^2: 0.84).



A **B**

9.3.2 Caustic based thermo-chemical attempts at solubilising of the protein component found within BSG

9.3.2.1 Preliminary investigation of the caustic protocol

The initial work which followed the protocol outlined by Celus et al (2007) was shown to be ineffective in fractionating a substantial quantity of the protein present in the BSG as values of only ca. 3 mg/mL total protein and ca. 23 mg/L free amino nitrogen (FAN) were achieved (Table 9.1). A total mass loss of only ca. 7% was achieved using this protocol, which confirmed the poor protein solubilisation seen. In contrast, Celus et al (2007) reported that ca. 17% mass loss was purported to be achievable. Either the NaOH concentration (0.3 M) or the reaction temperature (60°C) was therefore considered to be insufficient with this particular batch of BSG. This considerable reduction in effectiveness of the protocol (only achieving ca. 41% of the mass loss compared with that seen by Celus et al, 2007) was possibly attributed to differences in chemical composition of the BSG, due to differences in the original barley cultivar used and/or differences in the preceding brewing process. This would suggest that each different batch of BSG may need specific optimisation of this technique in order to maximise the quantity of protein solubilised.

9.3.2.2 Optimisation of the caustic protein fractionation protocol

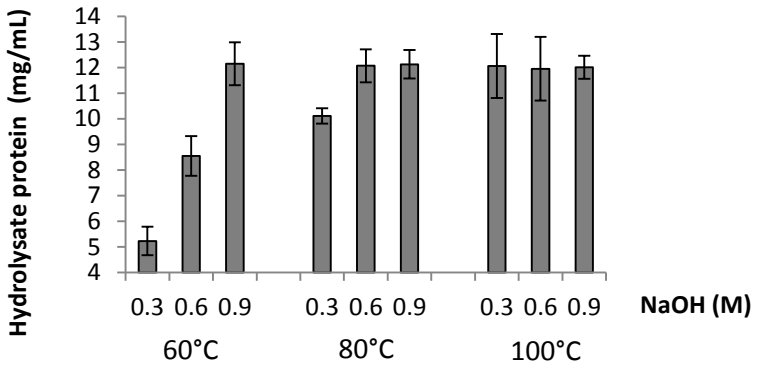
Modification of the original protocol evaluating a range of different NaOH concentrations (0.3M – 0.9M) and a range of different temperatures (60°C – 100°C) found that either a higher temperature or stronger caustic concentration was required to liberate a significant quantity of protein from the BSG (Figure 9.2).

Table 9.2 Preliminary attempts at caustic based thermo-chemical solubilisation of the protein component of BSG

	Liquid Fraction[2] (hydrolysate)		Protein in biomass	Mass loss
	FAN (mg/L)	Protein (mg/mL)	%	%
BSG	N/A	N/A	24.8 ± 1.1	N/A
Remaining insoluble residue after attempted caustic extraction[1]	23.1± 3.1	3.1± 1.2	21.1 ± 0.8	7.2 ± 0.4

[1]caustic extraction of BSG protein conducted using 15% (w/v) solids loading achieved using 0.1 M NaOH at 60°C for 60 min using the protocol described in Celus et al (2007).
Samples then centrifuged at 5000 rpm for 5 min and protein enriched supernatant removed.
Protein precipitation step then conducted via adjusting supernatant pH to 4.0 ± 0.1 (using 2.0 M citric acid) and subsequent centrifugation at 10 000 rpm for 10 min.
Residue then dried at 70°C overnight before protein quantification.
[2]Liquid fraction (hydrolysate) refers to the protein enriched supernatant remaining after attempted fractionation.
Data are the mean ± SD of three replicate experiments. N/A = not applicable.

Figure 9.2 Quantification of total protein liberated into the hydrolysates generated from using a range of different temperatures and NaOH concentrations to solubilise the protein component of BSG.



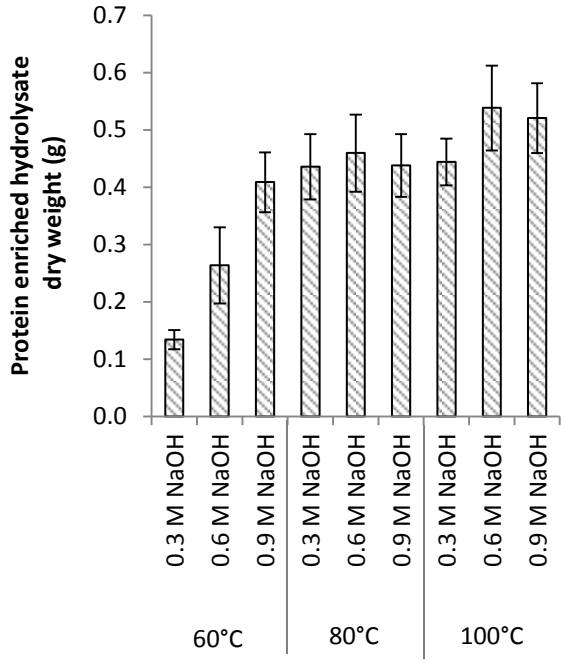
Protein quantification conducted using the Coumassie (Bradford) reagent protein assay. Protein solubilisation of BSG conducted using a range of caustic concentrations and a range of reaction temperatures (0.3 M – 0.9 M NaOH and incubated at 60°C - 100°C for 1 h, using an initial solids loading of 15% w/v). After incubation samples were centrifuged (5000 rpm; 5 min) and hydrolysate decanted and sampled for protein quantification as described above. Hydrolysates then had pH adjusted to 4.0 ± 0.1 (2M citric acid) and chilled to 1°C for 1 h. Then centrifuged (10000 rpm for 15 min) and supernatant discarded. Protein enriched pellet then oven dried overnight at 70°C to aid storage stability. Data are the mean ± SD of three replicate experiments.

The optimal parameters (those achieving the best protein yields with the minimum amount of reagent or minimal temperature requirement etc.) were found to be either 60°C using 0.9M NaOH or 80°C using 0.6M NaOH with both permutations liberating ca. 12 mg/mL protein into the hydrolysate. Evaluation of the dry weights of the recovered protein enriched pellets that were ultimately obtained after the different caustic protocols also confirmed that these two permutations of reaction conditions gave similar results (Figure 9.3).

The 60°C, 0.9 M NaOH and 80°C 0.6 M NaOH treatments produced protein enriched pellets weighing between 0.41 - 0.46 g from the original 1.5 g of BSG used. Considering the ca. 25% (w/w) protein content of the starting BSG this would correspond to greater than 100% theoretical protein yield if the pellet was assumed to be purely protein. As this was impossible it would indicate the protocol did not just solubilise protein alone and likely fractionated some quantity of other components such as a small amount of lignin and/or hemicellulose as caustic reagents have been shown to do when used on lignocellulosic biomass such as BSG (Chapter 5). The data also showed that treatments at 100°C (especially when using 0.6 M or 0.9 M NaOH concentrations) did produce heavier recovered protein pellets. However it is suggested that at this temperature the protocol may be losing its selectivity for protein fractionation and could be solubilising a significant quantity of other components, such as lignin and/or hemicellulose as previously described. Quantification of the protein content of these recovered pellets would therefore be needed in order to establish whether this was the case as it may show an overall lower protein concentration was present with the use of the 100°C temperatures. Analysis of the % protein content of the recovered pellets found that the 80°C 0.6 M NaOH variant produced a pellet with the highest protein content of ca. 53% w/w (Table 9.3). It also confirmed that the pellets that were derived from the use of the 100°C temperatures had a significantly lower protein concentration (ca. 30% w/w) relative to the best performing lower temperature variants (the 80°C and 0.6 M NaOH

derived protein pellets containing >50% protein). This confirmed that at 100°C a significant quantity of components other than just protein were being solubilised (fractionated) and that the greater recovered pellet weight did not reflect a greater degree of protein solubilisation.

Figure 9.3 Dry weight of the recovered BSG derived protein fractions (protein pellets) obtained using a range of different caustic (NaOH) concentrations and temperatures.



Initial protein solubilisation conducted using a range of caustic concentrations and a range of reaction temperatures (0.3 M – 0.9 M NaOH and incubated at 60°C - 100°C for 1 h, using an initial solids loading of 15% w/v). Precipitated protein enriched hydrolysates were centrifuged (10,000 rpm, 10 min) and supernatant discarded. Proteinaceous pellet then oven dried overnight (70°C) prior to weighing. Values are based upon an initial starting weight of 1.5 g BSG. Data are the mean ± SD of three replicate experiments.

Corresponding analysis of the final 'waste' insoluble residues that remained after all of the caustic protocol variants had been conducted (that should have then had significantly reduced protein contents if any solubilisation had occurred) found that the 80°C 0.6 M NaOH variant ultimately had the lowest final protein content (7% w/w; Table 9.3). However, there was very little difference in the remaining protein concentrations between the different samples (with the exception of the lower NaOH concentration, lower temperature treatments; 0.3 M - 0.6 M

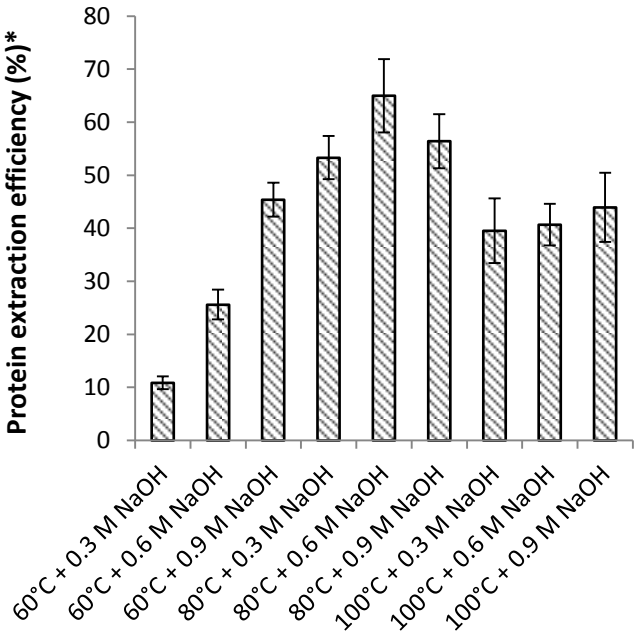
at 60°C) suggesting that a similar total amount of protein was being solubilised by each of the different protocols. The only differences where in the selectivity (or purity) of solubilisation and the data suggested that the 0.6 M NaOH at 80°C treatment achieved both the greatest purity of and also the greatest degree of solubilisation of the protein content. This was confirmed when calculating the achieved protein extraction efficiency which again showed the 80°C 0.6 M NaOH treatment achieved a ca. 65% extraction compared to only ca. 40% extraction efficiency for the 100°C treatments, since their protein selectivity was lower (Figure 9.3). ICP-M S (Inductively Coupled Plasma Mass Spectrometry) was used (primarily for quantification of iron levels) to analyse the elemental composition of the protein extract that was produced using the optimised fractionation protocol (0.6 M NaOH at 80°C). This analysis concluded the alkali and alkali earth metals (K, Na, Ca and Mg) were generally the most abundant elements present in the extract with concentrations in the range of 1000 – 10000 mg/kg (Figure 9.5). High levels of sulphur and phosphorus were also shown to be present (ca. 400 and ca. 1800 mg/kg respectively). However, as previously stated the primary function of the ICP-MS analysis was to quantify the iron (Fe) concentration that was present in the protein extract. This was to evaluate the suitability of the protein extract as a potential nitrogenous supplement (adjunct) for use in high gravity brewing, since high levels of Fe would present a problem with regards to flavour stability in the finished beer (through Fenton based oxidative chemical reactions). However the ICP-MS analysis concluded that iron levels would unlikely be a significant issue as they were only ca. 20 mg/kg. In addition the limited use of a small quantity of the protein extract in a large fermentation vessel would also likely dilute the iron concentration substantially.

Table 9.3 Quantification of total nitrogen (% protein) content of various insoluble residues generated from a range of different temperatures and NaOH concentrations used to solubilise the protein component of BSG.

	Protein in biomass[1]
	%
BSG	24.8 ±1.1
Insoluble residues after caustic extractions	
60°C + 0.3 M NaOH	14.3 ± 1.9
60°C + 0.6 M NaOH	10.8 ± 0.7
60°C + 0.9 M NaOH	8.5 ± 1.1
80°C + 0.3 M NaOH	9.5 ± 2.0
80°C + 0.6 M NaOH	7.0 ± 0.9
80°C + 0.9 M NaOH	7.5 ± 1.4
100°C + 0.3 M NaOH	7.7 ± 1.6
100°C + 0.6 M NaOH	7.5 ± 0.9
100°C + 0.9 M NaOH	7.1 ± 1.7
Recovered protein pellets	
60°C + 0.3 M NaOH	30.1 ± 2.4
60°C + 0.6 M NaOH	36.1 ± 2.9
60°C + 0.9 M NaOH	41.3 ± 1.6
80°C + 0.3 M NaOH	45.5 ± 3.2
80°C + 0.6 M NaOH	52.6 ± 3.6
80°C + 0.9 M NaOH	47.9 ± 4.1
100°C + 0.3 M NaOH	33.1 ± 3.7
100°C + 0.6 M NaOH	29.2 ± 2.8
100°C + 0.9 M NaOH	31.4 ± 3.4

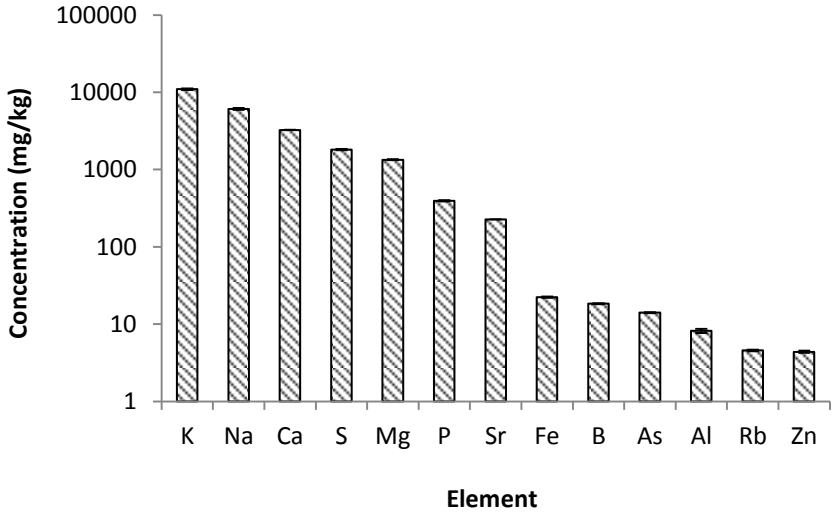
[1]Total protein content of insoluble residues remaining after various caustic protein fractionation protocols and also of the dried protein enriched fractions recovered. Data are the mean ± SD of three replicate experiments.

Figure 9.4 Protein extraction efficiency of various caustic based solubilisation protocols



*calculated from the recovered pellet weights and their corresponding % protein concentration after using 1.5 g BSG initially with a 24.8% protein content. Data are the mean ± SD of three replicate experiments.

Figure 9.5 Multi element analysis of the protein extract by Inductively Coupled Plasma Mass Spectrometry (ICP-MS)



Multi-element analysis of the protein extract produced using the optimised caustic fractionation protocol (0.6 M NaOH at 80°C). Analysis conducted using ICPMS (Thermo-Fisher iCAP-Q) with a 'Flatopole collision cell' (typically charged with helium gas). Data are the mean ± SD of two replicate experiments and only major elements found to be present are included.

9.3.2.3 Effect of the caustic protein fractionation on the efficacy of subsequent cellulolytic enzymatic saccharification yields

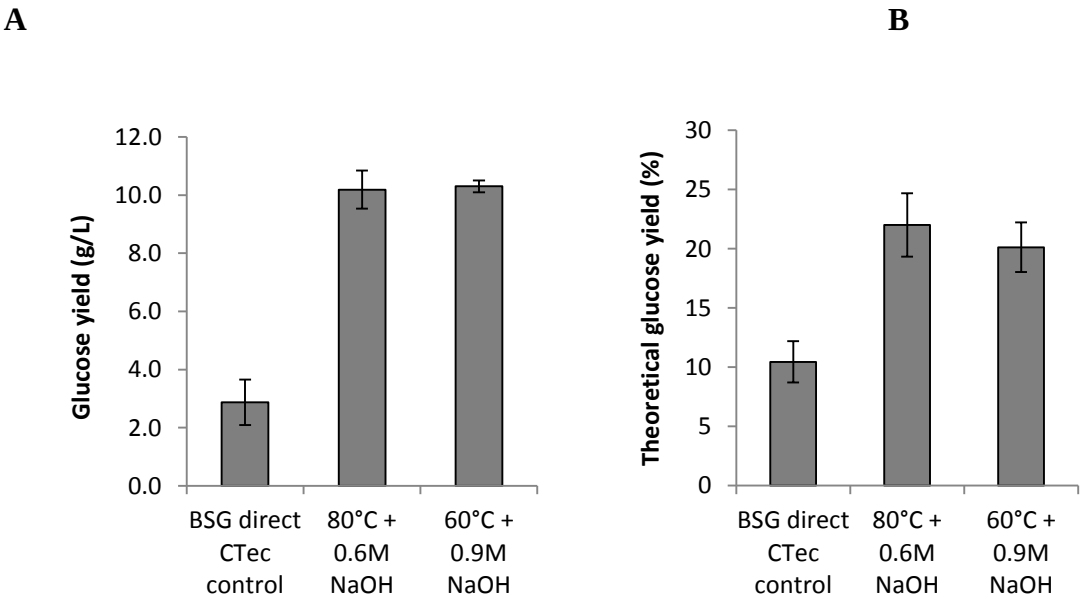
The optimised protein solubilisation protocols (either 0.6 M NaOH at 80°C or 0.9 M at 60°C) were shown to function somewhat as a very mild pre-treatment as glucose yields were slightly higher than when conducting enzymatic saccharification directly on BSG without the prior protein solubilisation (ca. 20% theoretical yields with the prior protein fractionation step compared to just 10% theoretical yield for direct saccharification on BSG; Figure 9.6A-B). This was somewhat as suspected as previous work reported that usage of NaOH as a pre-treatment reagent (albeit when using higher 5% w/v or ca. 1.3 M NaOH concentration at 50°C for 12 h) is highly effective in enhancing the subsequent enzymatic saccharification yields achieved afterwards (Chapter 5).

However overall the achieved glucose yields were far lower than those attained when conducting a fully optimised pre-treatment step prior to enzymatic saccharification. This was likely due to the lower NaOH concentration and the significantly shorter reaction time used as it was only ca. 8% of the reaction time that the optimised 5% NaOH at 50°C pre-treatment protocol described in Chapter 5.

Overall this validated the consideration of the inclusion of the caustic fractionation protocol into an overall biorefining process that could also produce a valuable biofuel process stream as well as a high value, protein enriched product from the same co-product. However, for the successful production of bioethanol from the remaining biomass (after the protein fractionation etc.) a subsequent thermo-chemical pre-treatment would still be required. This would be in order to reduce the recalcitrance of the lignocellulosic matrix and achieve a suitable high degree of saccharification from the depolymerisation of the complex carbohydrates to produce a high glucose content feedstock (as the protein fractionation process alone did not function effectively enough as a pre-treatment). However, the only mild

'pre-treatment' effect was considered a positive aspect because of the desired selectivity of the protein fractionation step. However, it would likely mean that an entirely new optimised pre-treatment protocol would need to be developed as previously optimised protocols (such as the 1% HCl 121°C and 5% NaOH 50°C techniques described in Chapters 4-5) would unlikely still be optimal.

Figure 9.6 Glucose yields (g/L and % theoretical) obtained from subsequent cellulolytic enzymatic saccharification of the insoluble residue remaining after subjecting the BSG to two different optimised caustic protein solubilisation protocols.



Enzymatic saccharification protocol: Novozymes Cellic® CTec2 dosed at 20 FPU/g biomass using a 10% (w/v) solids loading. 50°C incubation for 48 h with 150 rpm agitation. Samples then centrifuged (5000 rpm, 10 min) prior to sampling for HPLC based glucose quantification. Direct BSG controls conducted using identical protocol except without the caustic protein fractionation protocol. Data are the mean ± SD of three replicate experiments. **A**) Glucose yield (g/L). **B**) Glucose yield (% theoretical).

9.4 Conclusions

Both thermo-chemical (caustic) and enzymatic protocols for attempted fractionation of the protein component in BSG were evaluated. An optimised caustic protocol (using 0.6 M

NaOH at 80°C) shown to perform much better of the two and also at a much lower cost. The optimised caustic protocol demonstrated a viable option for achieving a good degree of solubilisation of the protein component (>65% theoretical yields) found within BSG.

The optimised caustic protein solubilisation protocol was also shown to exhibit a small 'pre-treatment' effect in that achieved glucose yields (after enzymatic saccharification) were higher than those achieved directly on just BSG. However the degree of the enhancement was not considered sufficient alone to allow for the successful linkage of the protein fractionation step and biofuel production systems. As such the formulation of a novel biorefining process from BSG that effectively produces two high value process streams (a high protein product and a biofuel such as ethanol) would require further work in order to optimise a suitable pre-treatment step after the protein fractionation step.

10 Conclusions

10.1 Overview

In order to successfully process BSG and find higher value uses for it than the current predominant low value use as cattle feed, then a detailed understanding of its structure and chemistry is required. This would then allow for targeted approaches towards utilisation of the various components found within BSG. Preliminary work was conducted which determined the chemical composition and ultrastructural characteristics of malted barley-based BSG (Chapter 3). Fractionation of the considerable protein content found within BSG was attempted using dry sieving technology (based upon fragment size differences) as a more simplified, crude effort to investigate the vibrational wet sieving based protocol described in Schwencke (2006). Direct enzymatic saccharification of BSG was also attempted in order to evaluate whether there was definitely the requirement of a thermo-chemical pre-treatment step prior to the enzymatic saccharification step, as this could potentially save a large quantity of energy if it could have been avoided.

With regards to advanced (second) generation bioethanol production, many different variants of effective pre-treatments have been proposed by different research groups looking at many different types of lignocellulosic feedstocks (biomass types). Each pre-treatment type normally functions via a slightly different chemical mechanism and targets different components within the biomass. Therefore it was essential to evaluate a wide range of potential reagents and protocols in order to find out which pre-treatment system worked best for BSG. This is especially true as the pre-treatment step has been concluded to be the most energy intensive step of the whole biofuel production process (Yang & Wyman, 2007). As a consequence an inefficient or non-optimised pre-treatment system would be hugely costly (both financially and energetically) if attempting to operate on an industrial scale. For this

reason a large proportion of the research presented in this thesis has investigated the pre-treatment step for BSG (based bioethanol production) in detail. A number of specific pre-treatment systems were investigated which included dilute acid and alkali catalysed hydrothermal techniques (Chapter 4), alkaline peroxide and caustic based techniques (Chapter 5) and microwave based autohydrolytical techniques (Chapter 6).

In addition, the enzymatic saccharification step was investigated in order to attempt to maximise the achieved glucose yields from the digestion of pre-treated BSG (Chapter 7). A large number of different factors were investigated. Ultimately, a novel combined saccharification protocol was developed that incorporated many of these optimisations in an attempt to further maximise achieved glucose yields and produce highly fermentable feedstocks from the BSG.

As an alternative option to the conventional chemical engineering based approach to biofuel production from lignocellulosic biomass, the use of consolidated bioprocessing (CBP) was investigated using a range of different fungal species in various consortia (Chapter 8). This involved using a filamentous fungus effectively as a saccharification enzyme factory and a yeast species as a fermentative ethanol producer utilising any sugars liberated. This evaluated ethanol production rates (using just BSG and water alone) without the need for any energy intensive thermo-chemical pre-treatment step and without the need for expensive exogenous cellulolytic enzyme preparations.

Finally, biorefining approaches were investigated in order to attempt to expand the potential high value product streams that could be extracted from BSG as opposed to purely targeting the carbohydrate component for biofuel production. In particular, attempts were made to successfully fractionate the considerable protein component in BSG. This could generate an additional high value product that could be used as part of an animal feed or ‘recycled’ back

into the brewing or bioethanol process as a nitrogenous adjunct for high gravity brewing (Chapter 9). In addition to simply fractionating the protein component, attempts were made to evaluate whether direct cellulolytic enzymatic saccharification could be conducted afterwards without any additional pre-treatment.

10.2 Preliminary investigations

10.2.1 Compositional analysis

The predominant components where shown to be cellulose, hemicellulose, protein and lignin (ca. 22%, 15%, 27% and 9% w/w respectively for lauter tun derived BSG) which was in line with other published literature (Aliyu & Bala., 2011; Forssell et al., 2008; Mussatto et al., 2006; White et al., 2008). Overall this confirmed a significant quantity of potentially high value components were still present in BSG. This justified the subsequent research and development of strategies that attempted to either fractionate or utilise many of these components and provide potentially higher value uses for BSG.

Mash filter derived BSG (which also incorporated a wheat adjunct) contained ca. 40% less cellulose than lauter tun derived BSG. This would suggest that lauter tun based brewing with an all malt grist would be better as a lignocellulosic biofuel substrate, due to the higher cellulose (and therefore higher potential glucose) concentration. In addition, this also showed the requirement for specific compositional analysis of BSG from each different brewery or even each different brew stream within the same brewery. This would be in order to accurately track any compositional changes that occur in relation to material processing of the biomass (such as attempted generation of higher value products such as fuel ethanol).

Sieving was also used to determine the size distribution of the fragments of dried BSG. This found the largest single size band of fragments were ca. 425 μm in size, accounting for ca.

66% (w/w) of the total mass of fragments present. This sieve analysis also confirmed there was a considerable range of sizes of fragments overall (from <0.17 μm to >2 mm in size).

10.2.2 Direct attempts at enzymatic saccharification without any prior thermo-chemical pre-treatment

Attempts to liberate a significant quantity of fermentable sugars directly from BSG (without any pre-treatment step) using both Novozymes Ondea® Pro and Novozymes Cellic® CTec2 were conducted. Ondea® Pro was shown to be highly ineffective with negligible sugars liberated and negligible mass loss achieved (<1% w/w). This was considered to be due to the majority of enzymes within this preparation being designed for use on barley and not BSG. It was suggested that the majority of the components that the Ondea® Pro was designed to solubilise (liberate) had already been extracted through the mashing step of the brewing process. In contrast the use of Cellic® CTec2 (although in very high doses; up to 160 FPU/g biomass was tested) had some limited success in liberating fermentable sugars. However, the % theoretical glucose yields were very low (<35% theoretical glucose yields at 160 FPU/g biomass enzyme dosing) which would not be commercially viable given the high cost of the enzyme preparation. In conclusion, this unfortunately but not surprisingly confirmed the need for some form of thermo-chemical pre-treatment step for BSG prior to any attempts at enzymatic saccharification in order to achieve suitably high glucose yields in any feedstocks produced.

10.2.3 Ultrastructural analysis

The use of scanning electron microscopy (SEM) for ultrastructural imaging purposes provided some insight into the heterogeneous mixture of different material fragments that BSG was composed of. Ultrastructural imaging conducted on fragments of BSG that remained after direct attempts at enzymatic saccharification using cellulolytic enzymes (Novozymes Cellic® CTec2) was able to detect morphological changes that appeared as an

overall ‘smoothing’ effect of the surface topography of the endosperm fragments (in response to the enzymes action). However the overall size of the fragments was not significantly reduced by the action of the enzyme mixture. This suggested that only limited physical changes were occurring through the enzyme activity. It further confirmed the need for some form of thermo-chemical pre-treatment prior to any attempts at enzymatic saccharification in order to achieve suitably high sugar yields in any feedstocks produced from BSG.

10.2.4 Protein fractionation attempts using of dry sieving

This approach aimed to replicate the results described by Schwencke (2006) who used vibrational wet sieving technology, but in this case through a less technically demanding, and simplified dry sieving protocol. Some degree of success was seen with this dry sieving protocol with an enrichment of the protein content (ca. 15% - 20% w/w more protein) in the smaller sized fragments (less than 212 μm in size). A corresponding enrichment of the structural, complex carbohydrate content (cellulose and hemicellulose polysaccharides) was also seen in the larger sized fragments, which was as expected.

10.3 Thermo-chemical pre-treatment conclusions

Our objectives in developing a pre-treatment were to minimise chemical usage, minimise energy input and also maximise the solids loading used (in order to minimise water usage). Also, the operation at high solids loadings was recognised as a pre-requisite for achieving suitably high biomass processing rates that would boost the efficiency of any large scale operation.

10.3.1 Dilute acid and alkali hydrothermal pre-treatments

A range of different dilute acid and alkali reagents were evaluated using a 121°C reaction temperature. The best performing candidates were shown to be 1% HCl from the acid reagents and 3% NaOH from the caustic reagents. Glucose concentrations achieved after

subsequent enzymatic saccharification (using an excess of Celluclast® cellulase enzyme) were ca. 86 % theoretical for the 1% HCl 121°C pre-treatment and ca. 55 % theoretical for the 3% NaOH 50°C pre-treatment. Whilst these high sugar yields were achieved using an excess of cellulase enzyme (which would not be commercially viable on a large industrial scale) it did however conclude the effectiveness of the pre-treatment using both reagents at this reaction temperature (121°C). This specific temperature was used primarily as it allowed the use of the laboratory autoclave which enabled relatively large volumes of biomass (BSG) to be pre-treated easily but also as it was considered to be a realistically achievable reaction temperature within a brewery (if on-site processing options were sought) as pressurised steam at a similar temperature is already available within most large breweries. These two best performing candidates (1% HCl and 3% NaOH at 121°C) both produced fermentable feedstocks that achieved acceptable attenuation rates and rapid fermentation rates. Although numerous reagents were shown to be effective in terms of high sugar liberation the 1% HCl reagent was considered superior as it also liberated a significant quantity of sugars (especially C5 pentose sugars) into the liquid fraction (the pre-treatment hydrolysate) that was produced directly through the pre-treatment step. In contrast the 3% NaOH reagent did not liberate any significant quantity of low molecular weight sugars that could be detected. In addition, the 3% NaOH reagent generated a product (after pre-treatment) that had a problematically high viscosity (ca. 13 Pas) that was ca. 10-fold higher compared to the product attained using the 1% HCl reagent (which had a viscosity of ca. 1.2 Pas). This would present technical problems for handling this product stream on an industrial scale and would require the use of very high energy, high torque motors on any pumps used to move the product stream around a processing plant (or within a brewery if processed on-site). From the remainder of the reagents screened 1% phosphoric acid was shown to also be somewhat effective as a pre-treatment reagent when also used at 121°C. Glucose yields of ca. 50% theoretical yield were

obtained after enzymatic saccharification using an excess of Celluclast® cellulase. Phosphoric acid was of particular interest as it was both present in some breweries in relatively large quantities (as ortho-phosphoric acid used in brewery CIP) which could enable on-site processing of BSG but also as it could offer a food grade pre-treatment option for the processing of BSG.

Further optimisation of this phosphoric acid catalysed hydrothermal pre-treatment system was discussed later in Chapter 6 (sections 6.2.4 and 6.3.5) as it involved the use of higher reaction temperatures that were beyond those achievable by the laboratory autoclave used here. These higher required reaction temperatures involved using a laboratory microwave reactor. Glucose yields of greater than 90% theoretical were attainable when reaction temperatures of either 160°C (with 3% phosphoric acid) or 190°C (with 5% phosphoric acid). Whilst these yields were impressive the protocol would be less favourable than using the 1% HCl reagent at 121°C (which was shown to achieve ca. 86% theoretical glucose yield) as both the reagent concentration and energy requirements would be considerably higher.

10.3.2 Alkaline reagent based pre-treatments

Alkaline peroxide (5% H_2O_2 at pH 11.5 at 100°C) was shown to be effective at enhancing the subsequent enzymatic saccharification sugar yields. Glucose yields of >85% theoretical were obtained. However, no correlation was seen with the increased enzymatic saccharification yields and any detectable drop in lignin concentration present in the insoluble residues that remained after pre-treatment. Also the protocol did not work as effectively at lower temperatures (20°C) which confirmed the requirement for moderately high temperatures and therefore a higher thermal energy input. In addition, another negative factor for alkaline peroxide pre-treatment was that as solids' loading was increased beyond 25% (w/v) the efficacy of the pre-treatment decreased significantly. This would limit the overall process efficiency if operating on a large commercial scale. The alkaline peroxide reagent itself also

presents health and safety issues associated with its use and handling on a large scale due to its reactivity and stability.

Caustic pre-treatment (5% NaOH at 50°C) was also shown to be effective at enhancing the subsequent enzymatic saccharification sugar yields. Glucose yields of >90% theoretical were obtained. However unlike with alkaline peroxide pre-treatment, with the caustic pre-treatment a correlation was seen between increased enzymatic saccharification and reduced lignin concentration present in the remaining insoluble residues. In addition the caustic protocol was shown to maintain its effectiveness as a pre-treatment reagent even at very high solids loadings (>45% w/v) achieving ca. 70% theoretical glucose yields.

Both of these alkaline pre-treatment systems produced feedstocks with suitably high glucose concentrations (when combined with an effective enzymatic saccharification step) which then allowed for trial fermentations to be conducted. Whilst specific optimisation of the subsequent enzymatic saccharification and the fermentation steps was not conducted it did provide proof of principle that alkaline pre-treatments were a genuine viable option for any large scale processing of BSG. However, these trial fermentations demonstrated problematically long fermentations with long initial lag phases especially when compared to the feedstocks produced using the 121°C hydrothermal pre-treatments (such as using the 1% HCl or 3% NaOH reagents). Further investigations looking at nutrient supplementation of a feedstock produced using 5% NaOH pre-treatment (at 50°C) revealed the addition of peptone and zinc (as $ZnSO_4$) were the best individual additives tested (at 10 g/L and 1.5 mM concentrations respectively). The use of both these supplements together significantly improved the fermentability of the 5% NaOH feedstock through a ca. 50% reduction in the time to attenuation.

Overall the caustic based system (NaOH) was the preferred option of the two alkaline pre-treatment protocols developed, as it liberated higher sugar concentrations whilst operating at a lower temperature and was capable of functioning at very high solids loadings. In addition it was considered that the discharge temperature of spent grains (BSG) from a brewery lauter tun would be close to the caustic pre-treatment temperature of 50°C. This would allow for rapid and effective processing of the biomass on site (within a brewery) as large quantities of caustic are already present within all breweries (for CIP use) and also minimal additional thermal energy input would be required as the temperature change (ΔT) would be minimal.

10.3.3 Autohydrolytical and dilute acid hydrothermal microwave based pre-treatments

Preliminary screening concluded that reaction temperatures of ca. 200°C were required for the successful enhancement of enzymatic saccharification of BSG when using autohydrolytical pre-treatment. When using an excess of Celluclast® cellulase (ca. 50 FPU/g biomass) and at very low solids loading (0.5% w/v) for the subsequent saccharification step after pre-treatment, high glucose concentrations were attained (>300 mg glucose/g biomass). Further optimisation of the autohydrolytical pre-treatment system also confirmed its ability to operate effectively at high solids loadings (up to 35% w/v) whilst still enhancing enzymatic saccharification (>90% theoretical glucose yields). When combined with a more commercially viable enzymatic protocol using a low dose of Novozymes Cellic® CTec2 (designed to more closely reflect a large scale, commercial enzymatic saccharification step) a fermentable feedstock was produced that contained suitably high glucose concentrations for some degree of ethanol production (ca. 80% theoretical ethanol yield with good attenuation and minimal initial lag phase by the yeast). Whilst specific optimisation of the subsequent saccharification and the fermentation steps was not conducted it did provide proof of principle that autohydrolytical pre-treatment was a genuine viable option for any large scale

processing of BSG. Although the procedure used dielectric heating via a microwave reactor the process could be considered in essence 'high temperature steam pressure cooking' and given that breweries have an abundant supply of relatively high pressure steam it would be suggested that with minimal modifications to the existing brewhouse engineering a heated pressurised reactor system could easily be constructed that would be highly effective for autohydrolytical pre-treatment of BSG on-site within the brewery. The ability of the microwave reactor to achieve higher reaction temperatures than the limited maximum temperature of ca. 121°C of the laboratory autoclave (previously used for investigation of dilute acid catalysed hydrothermal pre-treatments) then warranted further evaluation of the catalytic properties of acid reagents at higher temperatures similar to those used for the autohydrolytical pre-treatment. The objective was clearly to establish if in the presence of a 1% HCl acid catalyst, how much lower the reaction temperature could be dropped from the autohydrolytical optima (200°C) yet still provide a high degree of enhancement of enzymatic saccharification. As with most other pre-treatment investigations conducted, attempts were also made to determine the maximum solids loading the protocol could effectively function at in order to evaluate its viability for a large scale process option. Experiments found that a 140°C-160°C lower reaction temperature range could now be used with the 1% HCl acid catalyst and still liberate ca. 80% theoretical yield when operating at high solids (30% w/v). This denoted a 40°C temperature reduction from the autohydrolytical optima which (when operating on a large scale) could represent a huge energy and therefore cost saving.

10.3.4 Pre-treatment energy calculations

An approximate calculation of the total energy required to conduct autohydrolytical pre-treatment is given in Table 10.1 A. This calculation was based upon the assumption that the process would be conducted on-site within a brewery. It also assumed that brewery hot liquor (at 80°C) would be used as the water source. However, should this not be the case the energy

requirements would obviously be higher if the water temperature needed to be raised from ambient temperature to the target reaction temperature. Therefore the approximate energy requirements calculated here are for illustrative purposes only to show the variation between different pre-treatments. The energy required to conduct autohydrolytical pre-treatment at 200°C (and at 25% w/v solids loading) was estimated to be ca. 56.2 MJ/hL. A similar calculation for a dilute acid catalysed system at 160°C is given in Table 10.1 B. In this case the energy was estimated to be 41.4 MJ/hL which represented a considerable reduction in total energy (a reduction of ca. 15 MJ/hL or almost 600 MJ per metric tonne BSG processed) when the reaction temperature was dropped by 60°C (Table 10.1 C). As suspected (due to the high specific heat capacity of water relative to that of BSG) the bulk of the energy requirement for achieving the target reaction temperatures is used for heating the water component. This highlights the importance of operation at high solids loading during the pre-treatment step as the lower the quantity of water present the lower the thermal energy requirement overall. Table 10.2 illustrates the estimated variation in thermal energy requirements for autohydrolytical pre-treatment at 200°C as solids' loading is varied. For each metric tonne (1000 kg) BSG pre-treated, operation at 10% (w/v) solids loading would require ca. 5600 MJ energy. If the solids loading was increased to 20% w/v the energy requirement would then drop by 50% to only ca. 2800 MJ (for the same quantity of BSG processed).This not only represents a huge potential energy saving but also greatly reduces the water usage therefore improving the life cycle assessment (LCA) favourability. This is likely to apply to any pre-treatment systems that use elevated reaction temperatures and not just autohydrolytical systems alone. In addition, the investigation of the specific dielectric properties of BSG could also allow for the specific tuning of microwave based heating systems to allow for an even more efficient heat transfer. The phosphoric acid catalysed hydrothermal pre-treatment system (as discussed in section 4.3.2) was also further developed

using the microwave reactor as it could achieve higher reaction temperatures. This offered a potentially food grade pre-treatment system that could achieve >90% theoretical glucose yields if sufficient reaction temperatures and acid concentrations were used. Optimal reaction conditions were found to be 160°C and 3% H_3PO_4. However, problematically high sugar degradation product concentrations were generated with the most effective pre-treatment reaction permutations (furfural and HMF concentrations of ca. 20 g/L and 5 g/L respectively). Dealing with the problematic concentrations of 5-HMF and furfural would require some form of detoxification or separation, such as vacuum evaporation or filtration through activated carbon media (Mussatto & Roberto, 2004) in order to avoid any deleterious inhibitory effects in any downstream processes such as any fermentation conducted. Whilst this developed protocol did potentially provide a food grade pre-treatment option it would likely be more costly on a large scale than other pre-treatment options that achieved similar % theoretical glucose yields such as 1% HCl 121°C hydrothermal pre-treatment due to the higher pre-treatment reagent quantities and higher reaction temperatures required.

10.3.5 Overall pre-treatment conclusions

From all of the pre-treatment systems evaluated the 1% HCl 121°C and 5% NaOH 50°C protocols were considered to be the top choices (Table 10.3). This was in terms of being highly effective yet commercially realistic pre-treatments that could possibly be conducted on-site within a brewery. The relatively low chemical reagent usage and realistically achievable reaction temperatures (within a brewery etc.) justify this. However the 1% HCl 121°C pre-treatment system is still favoured overall due to its lower overall chemical usage (both lower initial pre-treatment reagent usage and also lower neutralisation reagent after pre-treatment) and also the higher liberated sugar concentration in the feedstock ultimately produced after enzymatic saccharification (especially when also considering the separate C5 pentose sugar rich hydrolysate generated directly from the pre-treatment step).

Table 10.1 Approximate calculation of the total energy requirement for attaining the required hydrothermal pre-treatment reaction temperatures at A) 200°C and also at B) 160°C with a dilute acid catalyst. Calculation based upon 1000 hL volume at 25% w/v solids loading and an initial water temperature of 80°C (SABMiller research brewery hot liquor temperature). All calculations based upon a simplified version of the model described in Mafe et al (2015) with similar assumptions and omissions such as reaction energy ignored as likely exothermic process and maintenance energy also ignored as likely very low. For further simplification calculations based on the assumptions of 5% evaporation and sensible heating phase from brewery hot water temperature to target reaction temperature. Due to high system pressures (ca. 14.6 bar for 200°C and ca. 5.4 bar 160°C) these target reaction temperatures were used as the water boiling temperatures for simplicity. For 160°C system acid heating energy ignored as also likely to be very small quantity. In addition no compensations are made for heat loss as optimised vessel design and use of heat recovery systems (which are commonly used within a brewery already) could minimise this. Specific heat capacity (c) for BSG taken from Tang et al (2004).

A) 1000 hL autohydrolytical pre-treatment at 200°C and 25% w/v solids loading:

Sensible heating calculation:

Brewery hot liquor Temp:	80	°C
Target Temp.:	200	°C
ΔT:	120	°C
Water density at 80°C:	0.9718	g/cm^3
	25000	kg BSG
	97180	kg liquid
Specific heat capacity (c_p) (approx.)		
BSG	1.24	KJ/kg
Liquid (water)	4.1	KJ/kg
Sensible heating:	47843560	KJ
($Q = m \times c_p \times \Delta T$)	47844	MJ

Latent heating calculation:

Density of water at 200°C:	0.864	g/cm3
Pressure at 200°C:	14.6	bar
5% of 100,000 L:	5000	L
mass of water:	4320	kg
H_{fg}	1941	kJ/kg
Latent heat of vaporization:	8385120	KJ
($L_v = m \times H_{fg}$)	8385	MJ
TOTAL:	56229	MJ (per 1000 hL)
	56.23	MJ/hL

B) 1000 hL dilute acid (3% H_3PO_4) hydrothermal pre-treatment at 160°C and 25% w/v solids loading:

Sensible heating calculation:

Brewery hot liquor Temp:	80	°C
Target Temp.:	160	°C
ΔT:	80	°C
Water density at 80°C:	0.9718	g/cm^3
	25000	kg BSG
	97180	kg liquid
Specific heat capacity (c_p) (approx.)		
BSG	1.24	KJ/kg
Liquid (water)	4.1	KJ/kg
Sensible heating:	31906040	KJ
($Q = m \times c_p \times \Delta T$)	31906	MJ

Latent heating calculation:

Density of water at 160°C:	0.907	g/cm3
Pressure at 160°C:	5.2	bar
5% of 100,000 L:	5000	L
mass of water:	4535	kg
H_{fg}	2080	kJ/kg
Latent heat of vaporization:	9432800	KJ
($L_v = m \times H_{fg}$)	9433	MJ

TOTAL:	41339	MJ (per 1000 hL)
	41.34	MJ/hL

C) Pre-treatment at 25% w/v solids loading:	MJ/hL	MJ/1000 kg BSG
160°C dilute acid	41.3	1652
200°C autohydrolytical	56.2	2248
Energy saving of acid catalyst:	**14.9**	MJ/hL
	596	MJ/1000 kg BSG

Table 10.2 Variation of the thermal energy input required for 200°C autohydrolytical pre-treatment of BSG over a range of solids loadings.

Autohydrolytical pre-treatment at 200°C						
Solids loading (w/v)	10%	15%	20%	25%	30%	35%
Energy usage (MJ) per 1000 kg BSG	5621	3748	2811	2249	1874	1607

In addition the viscosity of the final product from 1% HCl 121°C pre-treatment was much more manageable than that produced when using 5% NaOH 50°C pre-treatment. Finally the fermentation rate of the feedstock produced using the 1% HCl 121°C pre-treatment system was much faster than that of the feedstock produced when using 5% NaOH 50°C pre-treatment.

Table 10.3 Overview of the best performing pre-treatment systems developed.

	% theoretical glucose yields[1]	Thermal energy input	
		(MJ/hL)[2]	(MJ/1000 kg BSG)[3]
1% HCl 121°C hydrothermal	86 %	26.7	1070
5% NaOH 50°C	92%	N/A	N/A
Autohydrolytical 200°C	90%	56.2	2248
3% Phosphoric 160°C hydrothermal	90%	41.3	1652
5% Alkaline peroxide 100°C	87%	18.8	753
3% NaOH 121°C hydrothermal:	70%	26.7	1070

[1]*from the insoluble residue remaining after pre-treatment. Enzymatic saccharification using 50 FPU/g biomass Celluclast® at 0.5% (w/v) solids loading.*

[2]*Approximate energy value based upon an initial brewery hot liquor temperature of 80°C and pre-treatment at 25% (w/v) solids loading. No calculation adjustments are made for heating efficiency and any heating losses or reagent heating.*

[3]At 25% w/v solids loading pre-treatment operation

All steam table values obtained from Engineering Toolbox: http://www.engineeringtoolbox.com/water-thermal-properties-d_162.htmL

10.4 Enzymatic saccharification conclusions

Preliminary enzymatic saccharification work using pre-treated BSG (conducted for the screening of the efficacy of various pre-treatment reagents; Chapters 4 - 6) was conducted at very low solids loadings (0.5% w/v; very dilute reaction systems etc.) and using excessive enzyme doses (ca. 50 FPU/g biomass) purely to evaluate pre-treatment efficacy. As a result high % theoretical glucose yields could be achieved (>80% theoretical glucose yields for all optimised pre-treatment systems). However, these saccharification reaction conditions (i.e. the very large enzyme doses used and very low solids loading used) would not be economically viable on a commercial scale. Enzyme doses would need to be minimised and the operational solids loading pushed as high as possible in order to:

- Minimise water usage
- Maximise the quantity of biomass processed per unit time
- Produce feedstocks with sufficiently high glucose concentrations

The latter was especially important as whilst dilute enzymatic saccharification reactions attained high % theoretical glucose yields, the total glucose yields (g/L glucose concentrations) were low. As such feedstocks produced this way would not have sufficient glucose concentrations to justify any large commercial scale fermentations or subsequent distillations as it would not be economically viable. Therefore, a range of experiments was designed to look at various reaction parameters in an attempt to maximise achieved sugar yields.

High torque mixing when compared to more ‘passive’ mixing systems such as shaking incubation was shown to provide a small enhancement of glucose liberation (from ca. 39% theoretical glucose to ca. 44% when operating at high solids loading; 15% w/v). This was further enhanced by adding auxiliary aeration to the mixing system (that resulted in an

increase to ca. 45% theoretical glucose yield) which may have supported the redox dependant gH61 CAZy enzymes that were present in Novozymes Cellic® CTec2 which assist in the deconstruction of cellulose when combined with other enzymes.

Shorter reaction times (ca. 24 h) were found to be optimal in terms of maximal glucose liberation and time efficiency when compared to longer saccharification times of ca. 72 h as the use of the additional 48 h only liberated an additional 18% more glucose overall. Also when operating at very high solids loadings (ca. 25% w/v) it was found that low enzyme doses (ca. 10 FPU/g biomass) were optimal in terms of cost efficiency as a x16 dose increase only increased % theoretical glucose yields by 8%. From the large range of ancillary (supplementary) enzymes tested to augment Novozymes Cellic® CTec2 the best candidates were identified as ferulic acid esterases, additional xylanases (Cellic® HTec) and acetyl xylan esterases as they all boosted % theoretical glucose yields to 52% - 58% (from the original ca. 45% yield). Batch-fed protocols in which the biomass was split into two separate aliquots with the second added after 24 h saccharification (reaction time) were also shown to liberate an additional quantity of glucose (ca. 12% more) when conducted at greater than 20% w/v solids loadings. From all of the experimental work conducted, a combined enzymatic saccharification protocol was developed that attempted to further maximise the achieved glucose yields and incorporated aerated high-torque mixing, batch-fed biomass addition and the 3 best performing supplementary enzymes (ferulic acid esterase, acetyl xylan esterase and xylanases from Novozymes Cellic® HTec) alongside the Novozymes Cellic® CTec2. This was evaluated at various high solids loadings (5% w/v to 25% w/v) and compared against an aerated high-torque mixing, non-batch-fed saccharification system using only the benchmark Novozymes Cellic® CTec2. Whilst an increase in glucose yields was achieved (ca. 19% more glucose) with the combined saccharification system (when operating at 15% w/v solids loading) the improvements were not sufficiently high to justify their

inclusion in any large scale commercial process, due to the prohibitive costs of the supplementary enzymes and added complexity of processing. Overall, these experiments confirmed the complexity of enzymatic based saccharification of pre-treated biomass such as BSG, as even the combined saccharification system could still only achieve less than 43% theoretical glucose yields when operating at high solids loading (>15% w/v). In contrast, using the exact same pre-treatment system (1% HCl at 121°C) for BSG but conducting the enzymatic saccharification at much lower solids loading (0.5% w/v) and using an enzyme dose of ca. 40 FPU/g biomass (as discussed in section 4.3.2) liberated ca. 86% theoretical glucose. This illustrated the importance of optimising of the enzymatic saccharification step. It also confirmed that future work was still needed to produce feedstocks containing high g/L glucose concentrations for economically viable fermentations (and distillation) yet with suitable efficiency; whilst also maintaining a high % theoretical glucose yields.

10.5 Consolidated bioprocessing (CBP) conclusions

There is a high cost in terms of energy usage, chemical reagent usage and exogenous enzyme usage that is associated with the conventional procedure for production of liquid biofuels from lignocellulosic biomass. As such other options will always be sought that offer improved LCA (through lower energy and lower chemical inputs) or superior 'green credentials'. CBP appeared to offer the perfect purely biological solution if sufficient ethanol yields could be produced and with a suitable volumetric productivity (within a rapid enough time period). With 3 permutations of primary fungal species and secondary yeast species tested, the saké fermentation combination of *A.oryzae* and *S.cerevisiae* NCYC479 was by far the most promising in terms of its production of ethanol from just BSG and water. Ethanol yields of ca. 37 g/L were produced within 10 days incubation. This gave a volumetric productivity of 3.5 g ethanol/L/day (Table 10.4). In addition temperature control was shown to be optional which rendered the process energetically favourable at ambient temperature in

many places around the globe. Cellulolytic enzyme supplementation of the saké CBP system was conducted (using Novozymes Cellic® CTec2) to determine if enzyme secretion by the primary fungal species was rate limiting in terms of saccharification (and therefore rate limiting in terms of ethanol production). However no additional ethanol was produced with the enzyme supplementation which suggested fungal enzyme secretion was not rate limiting. In additional experiments, the original *S.cerevisiae* NCYC479 yeast strain was substituted for the *S.cerevisiae* NCYC2592 strain (the high ethanol tolerance phenotype distillers strain) to investigate if ethanol yields could be increased further. However, significantly lower final ethanol yields were actually produced with this alternate yeast strain (only ca. 20 g/L ethanol concentrations after 10 days incubation; ca. 45% less than the NCYC479 variant). Whilst a range of investigations were conducted, no explanation could be found for the apparent superiority of the original NCYC479 saké yeast strain in terms of the high ethanol yields attained from the CBP trials.

qRT-PCR was conducted using a representative selection of CAZy (carbohydrate degrading) genes from the *A.oryzae*. From the order of the up-regulation of the target genes, hemicellulose was identified as the initial macromolecule targeted then followed by cellulose. Due to the lower recalcitrance of hemicellulose this was expected to be the initial target as it could be depolymerised more energetically easily than crystalline cellulose. These findings were in line with those of Delmas et al (2011) that proposed the related fungal species *A.niger* also targeted hemicellulose first when cultured on wheat straw, an alternative lignocellulosic biomass type.

Overall the saké fermentation system was by far the best performing permutation of the CBP systems tested.

Table 10.4 Maximal ethanol yields (g/L) and volumetric productivity (g/L/day) achieved from all CBP and SSF variants tested.

CBP/SSF system:	Mean maximal ethanol yield: (g/L)	Volumetric productivity[1] (g/L/day)
A.niger + S.cerevisiae NCYC2592	0.7	0.1
S.cerevisiae NCYC2592 + Novozymes Cellic®CTec2	3.1	1
S.cerevisiae NCYC479 + Novozymes Cellic®CTec2	5.6	2
A.niger + S.cerevisiae NCYC479	6.7	2.3
H.insolens + S.cerevisiae NCYC2592	8.7	0.9
A.oryzae + S.cerevisiae NCYC479 (BSG direct from lauter tun)	9.8	1.0
A.oryzae + S.cerevisiae NCYC479 (1% HCl 121°C pre-treated BSG BSG)	12.2	1.2
A.oryzae + Kluyveromyces Spp. NCYC548	14.8	1.5
A.oryzae + Kluyveromyces Spp. NCYC546 + Novozymes Cellic®CTec2	16.5	1.7
A.oryzae + Kluyveromyces Spp. NCYC179	18.2	1.8
A.oryzae + Kluyveromyces Spp. NCYC179 + Novozymes Cellic®CTec2	18.3	1.8
H.insolens + S.cerevisiae NCYC479	18.5	1.9
A.oryzae + S.cerevisiae NCYC2592	20.3	2.0
A.oryzae + Kluyveromyces Spp. NCYC1426	20.8	2.1
A.oryzae + Kluyveromyces Spp. NCYC1426 + Novozymes Cellic®CTec2	22.6	2.3
A.oryzae + S.cerevisiae NCYC479 + Novozymes Cellic®CTec2 (30°C)	24.6	2.5
A.oryzae + S.cerevisiae NCYC479 + Kluyveromyces Spp. NCYC1426 + Novozymes Cellic®CTec2	25.0	2.5
A.oryzae + S.cerevisiae NCYC479 + Kluyveromyces Spp. NCYC179 + Novozymes Cellic®CTec2	26.1	2.6
A.oryzae + S.cerevisiae NCYC479 + Kluyveromyces Spp. NCYC546 + Novozymes Cellic®CTec2	26.2	2.6
A.oryzae + H.insolens + S.cerevisiae NCYC479 (30°C)	30.0	3.0
A.oryzae + S.cerevisiae NCYC479 + Novozymes Cellic®CTec2 (15°C)	32.6	3.3
A.oryzae + H.insolens + S.cerevisiae NCYC479 (15°C)	33.0	3.3
A.oryzae + S.cerevisiae NCYC479	36.8	3.7

[1]*Volumetric productivity calculations based on day 10 mean ethanol yields for all CBP systems tested with the exception of A.niger + S.cerevisiae NCYC479 and the SSF systems (S.cerevisiae + Novozymes Cellic®CTec2) which both used day 3 yields as these were more applicable. All BSG was dried and ground unless otherwise stated.*

10.6 Protein fractionation: conclusions

The relatively high protein content of BSG (ca. 25% w/w) led us to investigate fractionation of this potentially valuable component, as it could possibly be used as a food additive or a nitrogenous adjunct for high gravity brewing. With the primary objective of this research project being biofuel production from the structural carbohydrate components (cellulose and hemicellulose) the formulation of a protein fractionation protocol which could be incorporated into this biofuel production (successfully joined together without any deleterious effects on the efficacy of either processes etc.) was our ultimate aim.

Initial work evaluated the use of enzymes to hydrolyse protein from the substrates using both the brewing industry specific Alphalase NP (Danisco) and Pronase (a complex protease mixture of bacterial origin from Sigma-Aldrich). Preliminary evaluation of these enzymes involved measuring total protein and free amino nitrogen (FAN) concentrations that were liberated into the liquid fraction that was generated after incubation. This work showed that the initial BSG was the optimal substrate to treat with these enzymes as a higher degree of protein hydrolysis was achieved. It was suggested that the severity of the thermo-chemical pre-treatments conducted would have destroyed or denatured a significant proportion of the protein present in BSG. As such, fractionation of the protein before this stage was considered optimal. However, only relatively low total protein fractionation levels were achieved (ca. 130 μg/mL protein) even when using a synergistic combination of both enzyme preparations. Even optimisation of the best performing candidate (Pronase) using very high enzyme doses (10 g/L) still did not achieve a significant degree of protein solubilisation (a maximum of 800 μg/mL protein). Given the poor performance and also the high cost of the enzyme preparations ($120/g) a commercially viable protein fractionation protocol could not be developed using these enzymes.

In light of the poor performance of all the enzymatic protein fractionation strategies, alternative chemical based strategies were then considered. Using the protocol outlined in Celus et al (2007) as a starting point, attempts were made to replicate the procedure using a dilute caustic reagent (0.3 M NaOH with a reaction temperature of 60°C). However, poor protein fractionation yields were achieved (only ca. 4% mass loss of the total protein concentration present in the BSG). Further optimisation work was then conducted, evaluating a range of stronger caustic concentrations (0.3M - 0.9M NaOH) and higher reaction temperatures (60°C – 100°C). This work concluded that 0.6M NaOH at 80°C was optimal, achieving >65% total protein extraction efficiency. Overall this provided a much more effective (and also much lower cost) protein fractionation system compared to the enzymatic strategies. Unfortunately due to practical time constraints of the project it was not possible to successfully link this protein fractionation step with an optimised biofuel production system. The previous optimised pre-treatment and enzymatic saccharification steps (Chapters 4-7) would have unlikely still been optimal and as such it would have required a new set of specific optimisation experiments for both the subsequent pre-treatment and enzymatic saccharification steps. However, this could be conducted as future work if it was deemed necessary.

10.7 Concluding remarks: Best overall processing options

As the primary objective of this research project was the production of bioethanol from BSG the final conclusions drawn from all investigations undertaken are based around the achievement of maximal ethanol yields per unit of mass of starting BSG. The chosen units used were kg pure ethanol achieved per metric tonne of BSG (1000 kg). Both conventional advanced generation biofuel production systems (using the separate pre-treatment, enzymatic saccharification and then fermentation steps etc.) and the best performing consolidated bioprocessing (CBP) system were compared.

Volumetric productivity was also considered as an important factor (kg ethanol produced per hour) as an excessively slow process would unlikely be economically viable on a large commercial scale even if the final achieved ethanol yields were high. In addition % theoretical ethanol yields were calculated for each of the best process options. This approximate calculation was based around BSG having a starting cellulose concentration of ca. 20% (w/w) and under the assumption that any ethanol produced was purely from fermentation of glucose of cellulosic origin (in order to simplify the calculations). Another factor considered was the energy input required to reach the required reaction temperatures (for any thermo-chemical pre-treatments and also the enzymatic saccharification steps) as one processing option may have appeared to be optimal in terms of ethanol output but may have had a prohibitively high energy cost due to a high reaction temperature required for the pre-treatment step, for instance. Other factors considered were; the water usage requirements, as a process that consumed an excessive quantity of water would be unlikely to represent a green, sustainable processing option and also the quantities of pre-treatment and neutralisation reagents and exogenous saccharification enzymes required were considered when evaluating all process options. If absolute minimal chemical and energy inputs were the primary driving factors in choosing a bioethanol production system from those evaluated here then the CBP system (*A.oryzae* and *S.cerevisiae* NCYC479) would appear optimal with a 94 kg pure ethanol yield per metric tonne BSG (Table 10.5). This equated to ca. 79% theoretical ethanol yield. Although the biological complexity of the fungal consortia used (the numerous hydrolytic enzymes secreted from the *A.oryzae* etc.) would very likely mean that at least a small proportion of the ethanol produced by this system was not of cellulosic origin but rather another carbon source. Other possibilities could have been the use of gluconeogenesis pathways (from substrates such as protein) which could have very likely artificially inflated the quoted theoretical yield from this system. However, the absolute ethanol yields were

never the less impressive when considering no energy or chemically intensive pre-treatment or expensive exogenous enzymes were used whatsoever and the system could function at ambient temperature. In addition this CBP system only required 36 hL of water (per metric tonne BSG) which was only ca. 50% of the water requirement needed for many of the best conventional advanced generation systems developed here. However the downfall of the CBP system may be the low volumetric ethanol productivity (0.5 kg ethanol/h) when compared to the conventional systems (>1 kg ethanol/h). This low volumetric productivity of the CBP system relates to its requirement for ca. 10 days (ca. 240 h) to achieve maximal ethanol yields. This would likely be prohibitively slow when compared to other processing options. In addition, it would require a huge footprint in order to operate at scale due to the significant numbers of tanks or silos required to process the large quantities of BSG that are generated by many large international breweries (if this were to be considered as an option for processing on-site within a brewery). In addition on-site processing using this CBP system could pose a risk of *A.oryzae* contamination within a brewery which would be highly detrimental and risk raw materials (malt and hops etc.) being lost or batches of beer spoilt.

Life cycle assessment (LCA) would need to be conducted to determine if the energy and chemical savings (low operational cost and technical simplicity) could compensate enough for the low volumetric ethanol productivity. The highly complex nature of LCA was considered to be outside the remit of this research project but could be conducted as future work. In addition it must be noted the CBP system was not fully optimised and further work could be conducted in order to attempt to increase maximal ethanol yields or increase the volumetric productivity.

Table 10.5 Overall conclusions and process yields of the best performing process options for bioethanol production from BSG

	Pure ethanol yield (kg/metric tonne BSG)	Ethanol yield[1] (%) (% theoretical)	Volumetric productivity[2] (kg ethanol/h)	Water usage[3] (hL/metric tonne BSG)	Energy usage[4] (pre-treatment; MJ/hL)
Conventional advanced generation biofuel production					
1% HCl 121°C hydrothermal	79.4	77.8	1.9	40/59	233
5% NaOH 50°C	49.7	48.7	0.50	59	N/A
200°C autohydrolytical	51.5	50.5	1.1	43/59	248
Consolidated bioprocessing					
A.oryzae + *S.cerevisiae* NCYC479	94	79	0.5	36	N/A

All calculations based around dry weight of BSG.
[1]Calculation based around assumption from cellulose component only (at 20% w/w in starting BSG)
[2]based around CBP yields on day 10 and final ethanol yields achieved after *S.cerevisiae* fermentations of feedstocks produced using the three pre-treatments.
[3]Based off dry BSG as optimised pre-treatments were developed using dried BSG.
[4]Pre-treatment energy usage per hL slurry (at 25% w/v solids loading). Estimation of only the energy required to achieve the target temperature.
All pre-treatments conducted at 25% w/v solids loading.

When moving on to evaluate the conventional biofuel production systems (using the separate pre-treatment, enzymatic saccharification and then fermentation steps etc.) it immediately becomes apparent that the volumetric productivity is no longer an issue as most systems developed could achieve greater than 1 kg ethanol per hour. The exception here was the caustic based system which was only capable of producing ca. 0.49 kg ethanol per hour. This represented at least a 50% reduction in volumetric productivity when compared to the acid hydrothermal (1% HCl at 121°C) and autohydrolytical systems (200°C). Whilst most of the % theoretical ethanol yields were lower than for the CBP system (ca. 51 – 78% theoretical ethanol yield in the case of the conventional systems) it was likely that these calculations were more accurate (therefore more representative) in that the vast majority of ethanol was from cellulosic origin. In addition, all conventional systems produced reasonable ethanol yields of >49 kg pure ethanol/metric tonne BSG (dry weight). However, the key factor here is that all conventional systems required chemically or energetically intensive pre-treatment steps and expensive enzyme use (for the saccharification step) which combine to significantly increase the operational costs (OPEX). The estimated energy requirement for both the 1% HCl 121°C hydrothermal and 200°C autohydrolytical based systems was considerable at ca. >40 MJ/hL of pre-treatment slurry (calculated based upon operation at 25% w/v solids loading) or >1000 MJ/metric tonne BSG. This would represent a considerable financial cost and given that the energy density of fuel ethanol (E100) is 26.4 MJ/kg, the production of only 49 kg pure ethanol would only derive ca. 1294 MJ in total, which would put the process into a net energy loss overall if using the autohydrolytical pre-treatment system (only ca. 58% of the energy input required for the pre-treatment step alone). In contrast the 5% NaOH 50°C system was considered to have a negligible energy requirement as the discharge temperature of the spent grains (from the lauter tun within the brewery etc.) was considered to be suitable for immediate caustic based pre-treatment at ca. 50°C. This would represent a considerable

financial saving in terms of energy input. In addition, the low reaction temperature of this system (50°C) would negate the requirement for specialised pressurised reactors (such as those needed to achieve and safely maintain 200°C reaction temperatures) which would reduce the capital expenditure (CAPEX) required. However, the problematically high viscosity of the product generated after caustic pre-treatment needs to be considered, as this would bring with it additional technical difficulties and financial implications associated with processing (high energy requirements for pumping a highly viscous gel-like slurry etc.). In addition the caustic based system also required the largest volume of neutralisation reagent as the product was ca. pH 12 after pre-treatment. This needed adjusting to ca. pH 5 for the subsequent enzymatic saccharification step (Table 10.6) which represents both an additional cost and an additional problem due to the large volume of neutralisation metal salts forms (with potentially inhibitory activity). In contrast the 200°C autohydrolytical system required no neutralisation reagent whatsoever, thus reducing costs. The 5% NaOH 50°C system also involved a longer pre-treatment reaction (>12 h) which added to the overall process time (when compared to the 200°C autohydrolytical and 1% HCl 121°C hydrothermal systems which took less than 1 hour). This, when combined with the problematically slow fermentation of the feedstock (produced after the enzymatic saccharification step) gave the 5% NaOH 50°C caustic based system a problematically long cycle time of ca. 100 h even when the optimal nutritional supplements (zinc and peptone; which would also represent an additional cost) were added to improve the fermentation rate.

Table 10.6 Reagent usage (pre-treatment and neutralisation) for the best performing process options for bioethanol production from BSG.

	Reagent usage[1] (Pre-treatment; L/hL)	Reagent usage[2] (Neutralisation; L/hL)
Conventional advanced generation biofuel production		
1% HCl 121°C hydrothermal	2.7 (37% HCl)	0.5
5% NaOH 50°C	12.5 (40% NaOH)	1
200°C autohydrolytical	N/A	N/A
Consolidated bioprocessing		
A.oryzae + *S.cerevisiae* NCYC479	N/A	N/A

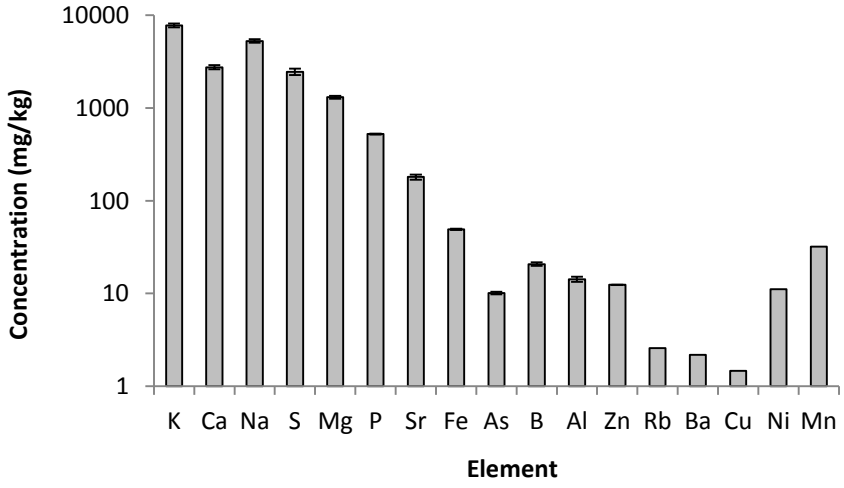
[1]Pre-treatment reagent usage per hL slurry (at 25% w/v solids loading).

[2]Neutralisation to pH 5.0 (Cellic® CTec2 enzyme optima) via 40% NaOH or glacial acetic acid (>99%).

In contrast the process cycle times for the 1% HCl 121°C and the 200°C autohydrolytical systems were only ca. 40 h. This significantly reduced the relative volumetric productivity of the 5% NaOH 50°C caustic system when compared to these systems. Overall, choosing a single processing system over all others is extremely difficult. Many factors would need to be considered such as cost and availability of chemical reagents, the availability to generate the required reaction temperatures for certain pre-treatments (and CAPEX and OPEX; requirement of specialised pressurised reactors etc.) and the desired site of processing for the BSG. From evaluation of all of the developed protocols the proposed best solution is considered to be the 1% HCl 121°C conventional bioethanol system. This was due to many factors including the low overall reagent usage (both pre-treatment and neutralisation reagent requirements: Table 10.6), realistically achievable reaction temperature and short pre-treatment time. In addition the ease of processing post pre-treatment (the relatively low viscosity) was another factor and ultimately as it yielded the equally highest quantity of ethanol (ca. 78 kg ethanol/tonne BSG) but with the most efficient volumetric productivity of almost 2 kg/h. Whilst these calculated ethanol yields accounted for use of the glucose that was present in the hydrolysate generated directly from the pre-treatment step using 1% HCl at

121°C, there would also be a significant quantity of C5 pentose sugars that could be used as a fermentative (or growth) substrate should a suitable organism be found. This would further boost the LCA favourability of the 1% HCl 121°C system in comparison to the other developed protocols. In addition ICP-MS based elemental analysis of the final remaining insoluble waste residue after using the 1% HCl 121°C system (the residue remaining after the subsequent enzymatic saccharification step etc.) concluded that the mineral content was extremely high. Concentrations of K, Ca, Na, S, Mg and P were all present at concentrations between ca. 500 – 8000 mg/kg (Figure 10.2). This might suggest a use for this final waste product as a fertiliser, due to this high mineral content and this strengthens the case for use of the 1% HCl 121°C system overall. As such the 1% HCl (at 121°C) pre-treatment system for bioethanol production from BSG was considered the best overall processing option.

Figure 10.1 Multi element analysis of the final insoluble waste residue (after bioethanol production using the 1% HCl 121°C pre-treatment system) by Inductively Coupled Plasma Mass Spectrometry (ICP-MS).



Multi-element analysis of the final insoluble waste residue that remained after bioethanol production from BSG using the 1% HCl at 121°C pre-treatment system (and subsequent enzymatic saccharification using Novozymes Cellic® CTec2). Analysis conducted using ICPMS (Thermo-Fisher iCAP-Q) with a 'Flatopole collision cell' (typically charged with helium gas) upstream of the analytical quadrupole to reduce polyatomic interferences. Data are the mean ± SD of two replicate experiments and only major elements found to be present are included..

11 Future work

11.1 Pre-treatment based studies

11.1.1 Detailed investigation of the morphological and ultrastructural changes that occur in response to the different pre-treatment strategies

In an attempt to fundamentally understand the changes that occur in terms of the reduction in recalcitrance that is seen in the BSG biomass when different pre-treatments are conducted, detailed structural analysis through some form of imaging or microscopy (possibly with additional staining techniques used to highlight any changes) could prove very useful. Whilst basic compositional analysis of the starting BSG and then the new composite materials produced after the different pre-treatments was conducted and this could sometimes predict the degree of enhancement of the subsequent enzymatic saccharification step, this was not always the case. A more detailed understanding of the changes that occur in response to each pre-treatment (and specifically how each different pre-treatment protocol reduces overall recalcitrance) may assist in the formulation of new, novel pre-treatment strategies (that were specifically tailored and optimised for BSG) or further optimisation of existing pre-treatment strategies through this additional knowledge.

11.1.2 Optimisation of pre-treatment protocols for BSG that is derived from grist that contains cereal adjuncts in addition to the malted barley

The modern brewing process used for many of the light beers and lagers that are produced on a global scale (which would therefore account for a substantial proportion of the global BSG production) involves a significant amount of use of cereal adjuncts in the grist charge such as maize, rice or wheat in addition to the malted barley. In addition, modern brewing methods have also been developed that use unmalted barley with commercial enzyme preparations used instead for modification of the grain. Overall these strategies offer a lower cost

fermentable sugar source than brewing with 100% malted barley and can confer substantial cost savings when brewing on such a large scale. As such the BSG that is the co-product from these brewing streams will be of different composition in terms of the concentration of the various polymers (such as lignin, hemicellulose, cellulose and protein) when compared to BSG originating from 100% malt based brewing. With cellulose being found in both the primary and secondary cell walls of the BSG biomass and as the relative proportions of the primary and secondary cell walls will be different for the BSG that originated from a different grist mixture it would be expected that the recalcitrance would therefore be somewhat different. Whilst many thermo-chemical pre-treatments have been shown to have a similar degree of efficacy when tested on a range of different lignocellulosic biomass types (such as wheat straw, miscanthus and corn stover etc.) there may be subtle differences for each different biomass types. Therefore, the grist differences (when using adjuncts) may have some degree of effect on the efficacy of various pre-treatments which may require further optimisation of the process to maximise the sugar liberation and attempt to minimise energy and water usage and keep process costs down where possible.

11.1.3 Determination of the specific dielectric properties of BSG to optimise microwave based pre-treatment strategies.

If the specific dielectric properties of BSG where known it may then allow for the modification and therefore further optimisation of a microwave based autohydrolytical or microwave based dilute acid catalysed hydrothermal pre-treatment step (such as those described in Chapter 6). This optimisation of the heating process through the specific tuning of a microwave based heating reactor could potentially reduce the energy required to attain the required temperatures for the successful pre-treatment of BSG. Even a small reduction in energy usage could represent a significant cost reduction if operating on a very large scale.

11.1.4 Ionic liquid based pre-treatment

The relative newest pre-treatment reagent class to be used as a deconstructive catalyst for lignocellulosic biomass is the use of ionic liquids. This class of non-volatile compounds is purported to be capable of dissolving cellulose using very mild reaction conditions (Gräsvik et al., 2014). The effectiveness of ionic liquids lies with the amphiphilic nature of cellulose. Ionic liquids are claimed to be capable of breaking both the hydrophilic and hydrophobic linkages present in crystalline cellulose due to their liquid salt nature. At the time of writing little if any published scientific literature exists regarding the use of ionic liquids as pre-treatment reagents specifically for BSG. However there is research using ionic liquids with other lignocellulosic biomass types. This would possibly justify their evaluation on a laboratory small scale to establish their effectiveness with BSG.

However there are considerable issues with the use of ionic liquids due to their relative high cost compared to other pre-treatment reagents such as acids and alkalis. This renders some form of reagent recycling absolutely necessary for the cost effective usage of ionic liquids on a large scale. This obviously adds an additional technical hurdle which must be overcome and would require further experimentation.

11.1.5 Steam explosion based pre-treatment

The prevalence of steam explosion pre-treatment systems for many pilot plant lignocellulosic bioethanol facilities suggests it to be a highly effective protocol for achieving suitable deconstruction for enhancement of the subsequent enzymatic saccharification. Therefore, comprehensive optimisation trials using BSG may yield interesting results which may confirm its suitability as a large scale pre-treatment option.

11.1.6 Carbonic acid based hydrothermal pre-treatment systems

As even weak acids were shown to still provide some catalytic action when combined with hydrothermal temperatures as a pre-treatment strategy (Chapter 4) an interesting idea would be the evaluation of carbonic acid as a potential weak acid based pre-treatment reagent. An optimisation study looking at the carbonic acid concentrations and corresponding hydrothermal temperatures that would be required to successfully deconstruct the lignocellulosic matrix in BSG could yield interesting results. With the CO_2 recovery systems that are already present in many large scale breweries, the on-site production of carbonic acid from this captured CO_2 should not be difficult in the presence of a suitable catalyst or the enzyme carbonic anhydrase (Lindskog, 1997). This could provide a constant low cost supply of pre-treatment reagent for processing BSG on-site.

11.2 Enzymatic saccharification studies

11.2.1 Comprehensive optimisation of large scale variants of the combined protocol for enzymatic saccharification

Chapter 7 described attempts at maximising the sugar yields from enzymatic saccharification reactions that digested the insoluble residues that remained after the pre-treatment of BSG. Whilst glucose yields were somewhat improved from using an amalgamation of different optimisations combined together (into one single protocol) there is still room for further improvement as % theoretical yields of only ca. 43% where achieved at best.

As described numerous operating parameters where individually optimised and then combined to formulate a basic consolidated enzymatic saccharification process. However, when combined (into the single protocol) each parameter may not have then still been optimal. Therefore comprehensive optimisation of each parameter when used in synergy may then suggest a sweet spot that could boost achieved glucose yields.

In addition to further optimisation of the reaction parameters alternative commercial cellulolytic enzyme preparations (other than Novozymes Cellic® CTec2) could be tested. An alternative enzyme preparation may perform better when used specifically on pre-treated BSG due to differences in the specific cocktail of enzymes or the specific activity of a particular cellulolytic enzyme within it.

11.2.2 Investigation of the fundamental issues affecting the limited final glucose yields when operating at high solids loadings

A deeper more comprehensive investigation of the rate limiting factors that appear to 'cap' the glucose yields when functioning at high solids loadings could then allow for either novel bioreactor designs or process options that may assist in the production of high glucose concentration feedstocks. Whilst Chapter 7 attempted to functionally investigate some of these issues through variation of a selection of parameters it did not address the fundamental issues that currently restrict cellulolytic enzymes from effectively functioning at high solids loadings. Investigations at the molecular level may be required in order to decipher specifically what was impeding the cellulolytic enzymes from functioning as well as they do at low solids loading. Alternatively the use of ultrasonic technology to enhance the saccharification yields could be evaluated in an attempt to increase sugar yields.

11.3 BSG biorefining studies

11.3.1 Development of brewery based hardware required for on-site processing of BSG for biorefining purposes

Within this thesis the research has described a number of potential processing options for BSG that could offer a higher value product (s) than the current predominant uses (cattle feed and directly burnt for energy). However, due to the rapid microbial spoilage of BSG (in the absence of some form of energy intensive drying step) should any such processing be

conducted then it would ideally need to occur either very close to the brewery in geographical terms, or ideally within the brewery itself. This would negate the substantial transport costs for very large quantities of BSG or negate the substantial footprint space needed to store large quantities of BSG as it could be processed immediately upon its egress from either the lauter tun or the mash filter. Depending on the exact processing options that were chosen (the chosen pre-treatment and enzymatic saccharification protocols and whether or not an initial protein fractionation step was conducted) the development of small scale or pilot scale reaction vessels or bioreactors could prove very useful for identifying any bottlenecks in the process (or identifying any excessively high capital costs for the equipment that would be required) but also for truly evaluating the feasibility of on-site processing within a brewery. Should a cost effective and technically feasible process be practically developed it could allow almost complete recycling of BSG within a brewery. Combined heat and power systems or steam generation systems could be powered (or partly powered) from bioethanol derived from BSG. Delivery trucks could also be powered using this produced bioethanol. Fractionation of the protein content prior to bioethanol production could then also be utilised within the brewery as a nitrogenous supplement for high gravity brewing which would often involve a large proportion of cereal adjuncts where the quantity of free amino nitrogen (FAN) can be problematically low which can lead to fermentation issues if not resolved. The final remaining insoluble residue could then either be burnt directly or put into a plasma gasification or pyrolysis unit for the recovery of a final small amount of energy. The development of a small pilot scale complete system within a brewery could then allow for detailed energy balance calculations, life cycle assessment (LCA) and economic modelling to be conducted which could then identify the feasibility of the large scale roll out of this technology. Even if currently not deemed economically viable or not currently sustainable (with the current economic climate) this data could prove invaluable for the future as the

global fuel crisis worsens and a paradigm shift in economics and priorities may change the outlook sufficiently to then justify the protocols usage.

11.3.2 Investigation of other alternative value added product streams from BSG

Whilst this research project predominantly concentrated on the production of bioethanol as the target carbon end point there are other potentially high value end products or platform chemicals that could be chosen instead. Whilst the same base feedstock would be used a different microbial species may be employed other than the use of a yeast strain that specifically specialised in high ethanol production (as was predominantly utilised within this research project). The production of a pharmaceutical intermediate platform chemical or bioplastic monomeric compound (such as lactic acid, which could be polymerised to form polylactic acid, a commercially useable plastic) could be targeted. These products would then likely have a higher value than that of fuel ethanol (bioethanol) which could then improve the LCA favourability of the thermo-chemical pre-treatment and enzyme saccharification protocols advocated here and possibly render it's wide scale roll out more economically viable. Alternatively food grade compounds could be sought from biorefining BSG for incorporation into and use as human or animal food products that may have potential high value.

Also further development (facilitated through GM approaches) of microbial species that can more efficiently utilise other non-glucose compounds (e.g. C5 pentose sugars) and produce an alternative, non-ethanol carbon end product would be highly advantageous.

11.3.3 The final insoluble waste residue after all biorefining steps

Ultimately after any and all processing or fractionation steps have been conducted on the BSG there is still some remaining biomass left over as a ‘final’ insoluble waste residue. The

actual amount of material (biomass) that remains after bioethanol production (after the pre-treatment and enzymatic saccharification steps) is surprisingly high.

For the 1% HCl (at 121°C) and 5% NaOH (at 50°C) pre-treatment based bioethanol production systems 27% and 45% (respectively) of the starting biomass remained (after both the pre-treatment and enzymatic saccharification steps). This represents a huge quantity of material when thinking about these processes operating on a large industrial scale. Therefore, investigation of novel uses for these residues would be essential in order to improve LCA favourability and add further value. Various energy technology options could be trialled to attempt to recover a small quantity of energy from this final waste residue. Pyrolysis, plasma gasification or even possibly anaerobic digestion of the residue could be used. In the specific case of when using the 1% HCl (at 121°C) pre-treatment system the highly insoluble final remaining waste residue had an enriched mineral ash content (due to the fractionation of so many other components). Therefore, should any of these energy techniques prove ineffective or inefficient at energy recovery then incorporation into or use as a form of crop fertiliser could be investigated due to the high inorganic mineral (ash) content.

11.3.4 Complete optimisation of the biorefining process (linkage of the protein fractionation and biofuel production systems)

Section 9.3.2.2 described a developed process for the somewhat successful fractionation of the protein in BSG. Whilst as expected this caustic based system did provide some small degree of a 'pre-treatment' effect (as subsequent glucose yields after enzymatic saccharification were higher than yields achieved directly from unmodified BSG) it was not considered sufficient that a biofuel production process (the direct linkage of an enzymatic saccharification step to produce a suitable feedstock) could be directly conducted immediately afterwards. Therefore, the successful linkage of the initial protein fractionation

step and subsequent biofuel production would still require some form of a pre-treatment step (after the protein fractionation). The initial protein fractionation step produced a new composite material (that differed to the starting BSG due to the reduced protein content and any other chemical modifications as a result of the action of the caustic reagent) with a lower recalcitrance. As a result this material would have behaved differently when thermo-chemical techniques (such as subsequent pre-treatments) were applied to it. Therefore, a milder pre-treatment may have been possible which could have both improved LCA favourability (improved the green credentials of the process) and also possibly enhanced the subsequent enzymatic saccharification yields to a greater extent through a lower degree of formation of degradation products (that excessively severe pre-treatments could generate etc.). However, practical time constraints of the research project unfortunately did not allow for the investigation of 'linked' pre-treatments (post protein fractionation step etc.).

11.4 Fermentation studies

11.4.1 Development of novel yeast strains that can effectively produce high ethanol concentrations from pentose (C5) sugars

The significant concentration of hemicellulose (and therefore arabinoxylan) that is present in BSG represents a large pool of pentose (C5) sugars that can be quite effectively fractionated into the liquid phase (hydrolysate) that is produced during the use of either autohydrolytical or dilute acid catalysed hydrothermal pre-treatments. This hydrolysate does also contain a small concentration of glucose and other hexose (C6) monosaccharides that most fermentative species can use to produce ethanol. However, the bulk of the sugars present are pentose based and as such are not readily utilised by most of the efficient ethanol producing microbes such as *S.cerevisiae*. The discovery or the development of a novel yeast strain which had the capacity to both utilise these C5 sugars and also produce a liquid biofuel such

as ethanol as the main carbon end point (in a significant quantity) would be highly prized. In the absence of such a yeast strain (or bacterium) the pentose sugars are largely unused and wasted. Some research groups have produced *S.cerevisiae* strains that are capable of utilising pentose sugars for ethanol production (Bellissimi et al., 2009; van Maris et al., 2006; Van Maris et al., 2007). However, within the scope of the research presented here in this thesis only a small proportion of time was directed at attempting to find a yeast strain that could produce ethanol from pentose sugars. Whilst various *Kluyveromyces* strains were shown to uptake pentose sugars none actually produced any significant concentration of ethanol. The use of various techniques including accelerated evolution or genetic modification could enable a yeast strain to utilise pentose sugars and produce ethanol. Alternatively the use of a microbe that utilised the C5 sugars present but produced a non-ethanol end product (as discussed in section 11.3.2) such as a platform chemical could be another possibility.

11.4.2 Development of inhibitor resistant yeast strains that can effectively utilise pre-treatment generated hydrolysates as feedstocks

Along the same lines as discussed in section 11.4.1, the development of a yeast strain that could tolerate high concentrations of known inhibitors (from lignin and/or sugar degradation products etc.) would prove very useful. It may allow the utilisation of the sugars present in either inhibitor laden pre-treatment hydrolysates or it may allow for a reduction in the degree of residue washing (washing of the pre-treated biomass) that was required after the pre-treatment step. The latter would obviously reduce the water usage considerably and therefore improve the process efficiency. Although within this research project it has been shown that pitching yeast at a suitably high concentration can overcome moderately significant inhibitor concentrations when attempting to ferment pre-treatment hydrolysates (section 4.3.8). However, this would have cost implications associated with the use of so much yeast per individual fermentation. Therefore, increased inhibitor tolerance may allow the use of lower

pitching rates to be used and may allow the re-use of yeast through serial re-pitching as the health and vitality of the cultures would likely be adequate. Again accelerated evolution technology that constantly exposed the yeast to increasing concentrations of various key inhibitors or genetic modification routes could possibly be used to achieve this.

11.4.3 Comprehensive investigation of the cause of the fermentation issues associated with using the NaOH pre-treatment derived feedstock

The optimised caustic alkaline pre-treatment protocol (5% NaOH at 50°C) developed and described in Chapter 5 (section 5.3.3) was shown to be highly effective at enhancing the subsequent enzymatic saccharification yields from BSG. However, whilst the feedstock ultimately produced using this system contained a sufficiently high glucose concentration there were issues regarding the fermentability. In particular the attenuation time was problematically long when compared to a feedstock of similar glucose concentration but produced using a hydrothermal pre-treatment system (as discussed in section 4.3.5).

A small quantity of work was conducted using potential supplements to augment any possible nutritional deficiencies that may have been present in the NaOH derived feedstock. Peptone and various metal salts were shown to make a significant improvement in reducing the problematic fermentation length before attenuation was achieved when using this feedstock. However, should this pre-treatment protocol be considered for scaling up to pilot plant (or larger) scale then further research could be conducted to specifically identify the cause of the slow fermentation progression. This may allow a more targeted approach as to exactly which supplements may be necessary and at what specific concentration to achieve the required enhancement effect. Then only the absolute minimum supplementation could be used in order to reduce the operational process costs or alternatively modification of the upstream process could be conducted in an attempt to minimise the nutrient deficiency (or minimise the

generation of the inhibitory compound that the supplements may have assisted in detoxifying) if the fundamental knowledge of the exact issue was known.

11.5 Consolidated bioprocessing (CBP) studies

11.5.1 Comprehensive optimisation of the saké based CBP system for ethanol production from BSG

The saké based CBP system was shown to significantly outperform most other fungal based CBP system permutations tested (Chapter 8). However, no real optimisation work was conducted to attempt to maximise the ethanol concentrations produced. Various parameters including the specific yeast pitching rate, quantity of koji mould added, solids loading used (quantity of BSG relative to water used), nutrient or micronutrient supplementation and comprehensive temperature optimisation (including variation of the temperature throughout the process) could be investigated. Even in the absence of an improvement in the final ethanol yields there may be some modification that could speed up the process so that maximal ethanol concentrations were attained sooner (<10 days) as this was considered one of the main drawbacks of the process if contemplating it's usage as a viable large scale option.

In addition to the parameter based optimisation that could be conducted (for the saké based CBP system) genetic manipulation based approaches could be conducted in order to attempt to maximise the ethanol yields produced. The inactivation of the metabolic pathways that allow the *A.oryzae* to utilise glucose (or other hexose based sugars) could then free more of the available sugars for the *S.cerevisiae* to ferment to ethanol. This could in effect force the *A.oryzae* to switch to entirely C5 pentose sugar metabolism utilising all the carbon sources that *S.cerevisiae* could not. Alternatively it may further encourage the *A.oryzae* to utilise other non-sugar carbon sources (such as proteins or lipids) through gluconeogenesis.

Investigation of the ethanol tolerance of *A.oryzae* could prove interesting and highlight a potential issue if it was determined to be lower than the ethanol concentrations reached during the course of using this CBP system. The saké fermentation system, when producing the saké beverage, is capable of attaining high ethanol concentrations (ca. 20% ABV) that exceed those achieved with the CBP system. However, this is the presence of an abundance of freely available starch from the rice substrate. As such the metabolic 'health' of the *A.oryzae* would likely be much better in the presence of a surplus of glucose. The starvation response (when used for the BSG based CBP system) may have reduced the ability of the fungus to tolerate ethanol. This may have reduced its capacity as a cellulolytic 'enzyme factory'. As with other strategies discussed for the development of novel or adapted yeast strains, work could be conducted that aimed to improve (increase) the ethanol tolerance of *A.oryzae* which may allow it to keep functioning as an enzyme factory or possibly continued hyphae penetration into the lignocellulosic matrix which may further reduce recalcitrance and possibly push the maximal ethanol yields achieved further. Alternatively, the genetic manipulation of the *A.oryzae* in order to encourage the overexpression of saccharification enzymes may prove useful. Attempts could be made to minimise the amount of new biomass produced by the *A.oryzae* and shift the carbon flux further towards enzyme production for enhanced saccharification.

11.6 Overall process efficiency studies

11.6.1 Life cycle assessment (LCA) of both the best performing CBP based ethanol and second generation ethanol production systems presented here

In addition to conducting life cycle assessment (LCA) on a brewery based pilot plant scale conventional bioethanol system (utilising a separate pre-treatment, enzymatic saccharification

and then fermentation step) it would be very interesting to also conduct LCA on the saké based CBP system as a direct comparison. Whilst the saké based CBP system was much slower in terms of ethanol volumetric productivity it was significantly more energy efficient through no thermo-chemical pre-treatment or expensive exogenous enzyme use. LCA may confirm that a CBP based bioethanol approach could be the way forward for the future if further optimisation could speed up the process (increase the ethanol volumetric productivity) somewhat.

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Appendix 1: Publications during research

- Wilkinson, S., Smart, K., Cook, D. 2014a. A comparison of dilute acid and alkali catalysed hydrothermal pre-treatments for bioethanol production from Brewers Spent Grains. J. Am. Soc. Brew. Chem., 72(2), 143-153.
- Wilkinson, S., Smart, K., Cook, D. 2014b. Optimisation of alkaline reagent based chemical pre-treatment of Brewers Spent Grains for bioethanol production. Ind. Crops Prod., 62, 219-227.
- Wilkinson, S., Smart, K., Cook, D. 2015. Optimising the (microwave based) hydrothermal pre-treatment of BSG for bioethanol production. Journal of Fuels. (in publication).

Appendix 2: Run tables and data for designed experiments and 3D response surface models (RSM)

D-optimal experimental designs were created using DesignExpert (Stat-Ease, USA) using a collection of reaction combinations (such as reaction temperatures, reaction times, solids loadings and reagent concentrations etc.) from which the D-optimal algorithm chose the treatment combinations to include in the design. This reduced the total number of experiments needed to be conducted from that of a full factorial experimental design yet still provide a statistically valid data set. All subsequent run tables and data presented in this appendix refer to the figure of the same number within the main body of the thesis.

Table 1(for Figure 4.2 and 4.3): Experimental design used to evaluate the impacts of variation of % solids loading in both the pre-treatment and enzyme hydrolysis stages of the saccharification of BSG, according to a D-optimal design space. Hydrothermal pre-treatment utilized 1% HCl (121°C 30 min) and the pre-treatment solid residue was digested with Celluclast® cellulase from *Trichoderma reesei*.

Run	**Factor 1** Pre-treatment step A: Solids loading (% w/v)	**Factor 2** Saccharification step B: Solids loading (% w/v)	**Response 1** Pre-treatment hydrolysate Arabinose (g/L)	**Response 2** Pre-treatment hydrolysate Galactose (g/L)	**Response 3** Pre-treatment hydrolysate Glucose (g/L)	**Response 4** Pre-treatment hydrolysate Xylose (g/L)	**Response 5** Saccharification Sugar yield Glucose (g/L)	**Response 6** Saccharification Sugar yield Xylose (mg sugar/g biomass)	**Response 7** Saccharification Sugar yield Arabinose (mg sugar/g biomass)
1	37.5	22.5	6.342	0.98	11.44	12.83	31.2	38.11	14.28
2	50	30	7.299	1.38	11.38	13.99	24.8	37.65	12.18
3	50	15	7.232	1.36	11.38	13.58	42.1	48.77	11.98
4	25	15	4.767	0.7	9.97	10	41.1	39.99	13.89
5	50	15	7.17	1.38	11.57	13.79	40.9	47.89	11.65
6	37.5	22.5	5.96	0.88	10.8	11.79	31.6	39.73	14.21
7	25	15	4.98	0.73	10.02	10.34	40.4	40.83	13.64
8	37.5	22.5	6.35	1.27	10.91	12.85	30.6	39.06	13.21
9	50	22.5	7.2	1.34	11.25	13.59	31.1	39.95	13.47
10	25	30	5.04	0.73	9.97	11.01	25.9	33.15	24.19
11	25	22.5	5.36	0.78	10.12	10.83	31.2	34.4	18.04
12	37.5	30	6.21	0.96	11.46	12.54	25.7	35.4	17.49
13	37.5	15	6.43	1.3	11.36	13	42.7	49.96	10.88
14	50	30	7.26	1.36	11.42	13.78	24.1	35.09	12.14
15	32	19	5.63	0.85	10.65	11.49	36.2	40.96	13.09
16	44	19	6.37	0.94	11.013	12.66	35.9	45.48	13.79
17	32	26	5.64	0.86	10.68	11.69	29.2	37.55	18.38
18	44	26	6.58	1.27	10.78	12.95	27.7	38.07	13.23
19	25	30	4.94	0.73	10.02	10.65	24.8	30.56	21.87

Table 2 (for Figure 4.4): Experimental design used to evaluate the impacts of variation of solids loading (% w/v) and NaOH concentration on the pre-treatment of BSG, according to a D-optimal design space. NaOH pre-treatment conducted at 121°C for 30 min and remaining solid residue digested with Celluclast® cellulase from *Trichoderma reesei*.

Run	**Factor 1** Pre-treatment A: NaOH Concentration (w/v)	**Factor 2** Pre-treatment B: Solids loading (% w/v)	**Response 1** Saccharification yield Glucose (mg sugar/g biomass)	**Response 2** Saccharification yield Xylose (mg sugar/g biomass)	**Response 3** Saccharification yield Arabinose (mg sugar/g biomass)	**Response 4** Remaining residue Hemicellulose (% w/w)
	%	%	121	49	3.5	18
1	1	15	134	56	4.6	18
2	1	20	101	36	5	16
3	1	20	108	37	5.9	17
4	1	25	81	29	4.6	15
5	1	25	102	29	4.6	19
6	3	15	357	143	4.8	17
7	3	20	330	149	5	18
8	3	20	310	138	5.8	17
9	3	20	329	147	5.3	16
10	3	25	277	119	6	17
11	5	15	413	123	4.9	11
12	5	15	373	104	4.7	13
13	5	20	341	131	5.4	13
14	5	20	336	110	5.3	13
15	5	25	286	117	6.5	16

Table 3 (for Figure 6.3): Experimental design used for preliminary screening of the simultaneous impacts of reaction temperature (110°C - 200°C) and reaction time (1 – 10 min) during microwave based autohydrolytical pre-treatment of BSG according to a D-optimal design space. Autohydrolytical pre-treatment utilized a Monowave 300 microwave synthesis reactor with G30 vessels (Anton Parr). 2.5% (w/v) solids loading. Each of the 20 experimental runs in the design was conducted twice for additional replication to allow for calculation of the standard deviation values displayed for the 2D chart presented in Figure 6.3. The response factor data tabulated here is the mean of the two replicates.

Run	Factor 1 A:Temp. (°C)	Factor 2 B:Time (min)	Response 1 Saccharification yield arabinose (mg/g biomass)	Response 2 Saccharification yield galactose (mg/g biomass)	Response 3 Saccharification yield glucose (mg/g biomass)	Response 4 Saccharification yield xylose (mg/g biomass)
1	110	1	4.5	0	24.5	24.4
2	110	4	4.8	0	24.4	24.3
3	110	7.75	4.5	0	24.3	21.7
4	110	10	4.7	0	26.9	24.3
5	133	3.25	4.5	0	24.6	24.4
6	133	7.75	6.3	0	28.5	27.6
7	155	1	5.9	0	27.8	27.1
8	155	5	10.5	0.2	39.0	34.2
9	155	10	8.2	0	42.8	30.5
10	178	3.25	6.0	0	41.3	24.1
11	178	7.75	5.1	0	61.5	26.9
12	200	1	3.8	0.1	99.8	21.5
13	200	5.5	3.5	0.1	235.2	20.6
14	200	10	2.2	0.4	304.1	13.3
15	200	15	2.0	0.2	242.2	11.6

Table 4 (for Figures 6.4 and 6.5): Experimental design used to simultaneously evaluate the impacts of variation of the % solids loading (10% - 35% w/v) and reaction time (5 min – 15 min) during microwave based autohydrolytical pre-treatment of BSG at 200°C according to a D-optimal design space. Autohydrolytical pre-treatment utilized a Monowave 300 microwave synthesis reactor with G30 vessels (Anton Parr).

Run	Factor 1	Factor 2	Response 1	Response 2	Response 3	Response 4	Response 5	Response 6	Response 7	Response 8	Response 9	Response 10
	A:Solids loading	B:Time	Pre-treatment hydrolysate	Pre-treatment hydrolysate	Saccharification yield	Saccharification yield	Saccharification yield	Saccharification yield	Inhibitors	Inhibitors	Remaining residue	Remaining residue
			Glucose	Xylose	Glucose	Glucose	Xylose	Xylose	5-HMF	Furfural	Cellulose	Hemicellulose
	% (w/v)	(min)	(g/L)	(g/L)	(g/L)	(% theoretical)	(g/L)	(% theoretical)	(g/L)	(g/L)	(% w/w)	(% w/w)
1	35	5	4.3	3.87	0.95	69.4	0.057	44.7	1.7	3.8	23.2	11
2	10	10	2.2	1.74	1.08	71.0	0.115	18.8	0.8	1.1	26.7	4
3	22.5	10	4.2	3.02	1.16	89.1	0.069	62.4	2.2	2.9	24.7	6
4	22.5	5	3.6	2.66	0.76	53.5	0.054	14.1	0.9	1.4	24.6	8
5	35	15	2.2	0.14	1.14	95.0	0.029	97.9	3.7	5.1	16.5	9
6	10	5	1.7	0.70	0.73	51.3	0.109	21.7	0.3	0.4	27.8	5
7	10	15	2.4	1.62	1.24	87.9	0.125	42.2	1.2	1.7	24.0	3
8	35	15	2.2	0.16	1.13	94.5	0.022	80.8	3.5	5.3	16.8	8
9	35	5	4.5	3.89	0.77	56.3	0.048	38.0	1.6	2.3	22.9	12
10	22.5	10	4.2	3.17	1.08	82.8	0.057	51.4	2.6	2.6	24.1	6
11	22.5	15	2.9	0.31	1.23	83.1	0.074	55.2	3.2	4.9	27.2	7
12	22.5	10	4.5	3.36	0.87	67.0	0.044	39.9	2.2	4.5	20.9	6
13	35	10	4.1	1.86	1.04	95.5	0.035	93.6	2.9	5.6	20.2	10
14	10	5	1.6	0.72	0.75	52.7	0.110	21.8	0.4	0.4	25.3	5
15	16	7.5	3.0	2.35	0.81	49.9	0.069	49.1	1.1	1.9	29.1	4
16	29	7.5	4.4	3.88	0.92	84.8	0.034	68.8	2.1	4.4	18.4	9
17	16	12.5	3.1	2.28	1.68	87.8	0.156	79.1	1.6	2.6	31.1	4
18	29	12.5	3.5	0.55	1.23	98.9	0.035	83.5	3.1	6	22.6	8
19	10	15	2.3	1.55	0.94	66.7	0.105	35.3	1.1	1.8	27.0	3

Table 5 (for Figure 6.9): Experimental design used for preliminary screening of the simultaneous impacts of reaction temperature (120°C - 200°C) and phosphoric acid concentration (1% – 5% H_3PO_4) during microwave based hydrothermal pre-treatment of BSG according to a D-optimal design space. Phosphoric acid catalysed hydrothermal pre-treatment utilized a Monowave 300 microwave synthesis reactor with G30 vessels (Anton Parr). All reactions conducted at 25% w/v solids loading.

Run	Factor 1 A: Temp (°C)	Factor 2 B: Acid concentration (H_3PO_4: % v/v)	Response 1 Glucose yield (% theoretical)
1	120	2	14
2	120	2	16
3	120	2	15
4	120	2	19
5	120	3	20
6	120	3	17
7	120	3	17
8	120	3	17
9	120	4	25
10	120	4	24
11	120	4	19
12	120	4	23
13	120	5	28
14	120	5	30
15	120	5	37
16	120	5	32
17	140	1	21
18	140	1	17
19	140	1	22
20	140	1	19
21	140	2	33
22	140	2	29
23	140	2	28
24	140	2	34
25	140	3	39
26	140	3	38
27	140	3	35
28	140	3	36
29	140	4	32
30	140	4	45
31	140	4	42
32	140	4	35
33	140	5	66
34	140	5	66
35	140	5	86

36	140	5	89
37	160	1	56
38	160	1	48
39	160	1	54
40	160	1	51
41	160	2	56
42	160	2	67
43	160	2	61
44	160	2	72
45	160	3	89
46	160	3	78
47	160	3	76
48	160	3	95
49	160	4	76
50	160	4	88
51	160	4	94
52	160	4	91
53	180	1	65
54	180	1	70
55	180	1	51
56	180	1	48
57	180	2	67
58	180	2	71
59	180	2	70
60	180	2	70
61	180	3	76
62	180	3	66
63	180	3	85
64	180	3	67
65	180	4	81
66	180	4	83
67	180	4	52
68	180	4	61
69	200	1	75
70	200	1	76

Table 6 (for Figure7.7): Experimental reaction conditions for evaluation of various solids loadings (5% - 25% w/v) and various enzyme doses (10 – 160 FPU/g biomass; Novozymes Cellic® CTec2) during enzymatic saccharification of pre-treated BSG. Pre-treatment: 1% HCl hydrothermal protocol; 121°C 30 min at 25% w/v solids loading. Modelled according to a D-optimal design space.

	Factor 1:	**Factor 2:**	**Response 1**	**Response 2**
			Saccharification yield	Saccharification yield
Run	A: Enzyme dose:	B: Solids loading:	Glucose	Glucose
	FPU/g biomass	(% w/v)	(g/L)	(% theoretical)
1	40	5	15	42
2	160	5	19	53
3	10	5	11	30
4	160	25	61	34
5	10	25	46	26
6	40	15	37	34
7	10	15	33	30
8	40	25	56	31
9	40	15	45	41
10	40	15	37	34
11	160	5	19	52
12	10	5	11	31
13	160	15	40	36
14	160	25	63	35
15	80	10	33	46
16	80	10	33	45
17	20	10	15	21
18	20	10	24	33
19	80	20	56	39
20	80	20	53	36
21	20	20	59	41
22	20	20	50	34
23	160	15	42	39
24	40	25	63	35
25	10	15	36	33
26	10	25	50	28
27	80	15	44	40
28	20	15	34	31
29	80	15	41	38
30	20	15	34	31

Table 7 (for Figure 7.8): Experimental reaction conditions for evaluation of various solids loadings (5% - 25% w/v) and various hydrolysis times (10-72 h) during enzymatic saccharification of pre-treated BSG. Pre-treatment: 1% HCl hydrothermal protocol; 121°C 30 min at 25% w/v solids loading. Enzymatic saccharification: Novozymes Cellic® CTec2 (20 FPU/g biomass) modelled according to a D-optimal design space.

	Factor 1	**Factor 2**	**Response 1**	**Response 2**
			Saccharification yield	Saccharification yield
Run	A: Solids loading (% w/v)	B: Hydrolysis time (h)	Glucose (g/L)	Glucose (% theoretical)
1	25	10	36	20
2	5	72	15	42
3	25	72	56	31
4	25	48	51	28
5	5	72	14	40
6	20	48	54	37
7	15	48	37	34
8	15	48	39	36
9	5	48	18	49
10	20	72	51	35
11	15	10	22	20
12	15	24	26	24
13	25	10	39	22
14	10	24	30	41
15	25	72	57	32
16	5	10	11	32
17	5	24	13	35
18	10	24	36	50
19	25	72	57	32
20	5	10	12	34
21	10	48	32	45
22	20	24	54	38
23	15	72	40	37
24	15	24	28	26
25	5	72	14	40
26	25	10	37	20
27	10	72	33	46
28	10	10	27	38
29	20	24	41	29
30	25	24	42	23

Table 8 (for Figure 9.1): Experimental design used to investigate the simultaneous impacts of Pronase protease enzyme dose, operational solids loading and reaction time on protein fraction from BSG, according to a D-optimal design space. Pronase digestion conducted at 37°C and at pH7 .5. Protein concentration in the generated hydrolysate quantified via the Bradford reagent method.

Run	Factor 1 A: Enzyme dose (g/L)	Factor 2 B: Reaction time (h)	Factor 3 C: Solids loading (% w/v)	Response 1 Protein (μg/mL)
1	5	4	5	140
2	0.63	64	5	276
3	5	34	10	314
4	5	34	5	251
5	10	32	10	302
6	0.63	4	5	95
7	0.63	32	25	264
8	10	4	25	197
9	5	17	25	199
10	0.63	4	25	202
11	0.63	64	15	304
12	5	32	25	275
13	5	64	15	500
14	10	64	15	602
15	5	32	15	330
16	10	32	10	259
17	10	30	25	188
18	10	32	15	322
19	0.63	4	15	103
20	0.63	32	5	216
21	10	64	5	545
22	5	34	15	350
23	10	4	5	147
24	0.63	4	15	100
25	5	34	10	325
26	10	64	5	515
27	10	32	5	251
28	0.63	64	5	280
29	0.63	32	15	237
30	10	4	15	149
31	5	4	15	166
32	10	32	25	389
33	0.63	64	25	323
34	0.63	4	25	145
35	2.5	4	5	126
36	2.5	4	5	112

37	2.5	16	10	332
38	2.5	16	10	364
39	2.5	32	10	364
40	2.5	32	10	399
41	2.5	32	15	406
42	2.5	32	15	426
43	2.5	64	25	550
44	2.5	64	25	550
45	5	64	5	435
46	5	64	25	663
47	10	64	25	788
48	10	64	25	786