

Viability of Autoclaved Municipal Solid Waste as a source for Biofuels and other products

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Abstract

Energy is an important requirement for population growth, technological progress and urbanisation. Worldwide energy demand has been projected to increase by 5-fold by 2100. Fulfilment of these energy requirements cannot be solely from fossil fuels, such as oil, coal and natural gas, on account of their adverse environmental impacts and concomitant depletion of natural resources. As a result multiple approaches for generating alternative energy are being explored globally. In this study, processed municipal solid waste (MSW) fibre was assessed as a substrate for the production of various bio-energy related products. The MSW was characterised and the results showed that there was, as expected, a wide compositional variation between samples.

There was a significant amount of lignocellulosic material found in some samples and the potential to exploit this to generate fermentable sugars was explored. Direct enzyme hydrolysis using 30 FPU (Filter Paper Units)/g Cellic® CTec2 gave a 30 % release of available glucose. Pre-treatments- using hot water, dilute acid and dilute alkali are all applied to enhance sugar release but were all found to be ineffective.

The possibility of using MSW as a substrate for cellulase enzyme production via solid state fermentation (SSF) by *Trichoderma reesei (T. reesei)* and *Aspergillus niger (A. niger)* was explored. Both fungi grew well on the substrate and following optimization a cellulase activity of 26.10±3.09 FPU/g could be produced using *T. reesei* at 30 °C with a moisture content of 60 % with inoculation of 0.5 million spores/g and incubation for 168 hr. Addition of extra nitrogen and/or carbon did not improve cellulase accumulation. Acid or alkali pre-treatment of MSW led to reduced cellulase production. Crude enzymes produced from MSW by *T. reesei* were evaluated for their ability to release glucose from MSW. A cellulose hydrolysis yield of cellulase was 24.7 % achieved, which was close to that

obtained using a commercial enzyme. Results demonstrated that MSW could be used as an inexpensive lignocellulosic material for the production of cellulase enzymes.

High concentrations of toxic heavy metals were found in all MSW samples tested and this precludes the use of this material as a soil enhancer. Thus studies were undertaken to explore the potential for bioleaching as a means to generate compost that meets environmental standards for safe use. *A. niger* and *Saccharomyces cerevisiae* NCYC2592 were used and the impact of growth medium and pH tested. Both organisms were effective at solubilising the heavy metals and this may be related to their ability to synthesise organic acids into the fermentation medium that act as chelators.

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List of Abbreviations

ABE	Acetone Butanol Ethanol
AD	Anaerobic Digestion
ADF	Adenosine Di phosphate
AFEX	Ammonia Fiber Explosion
AM	Animal Manure
AMF	Sub Merged Fermentation
APS	Anaerobic-Phased Solids
ATP	Adenosine tri phosphate
BSA	bovine serum albumin
BTA	Biotechnische Abfallverwertung
C/N ratio	Carbon/ Nitrogen ratio
CBH	Cellobiohydrolase
CBM	Cellulose Binding Modules
CCME	Canadian Council of Ministers of the Environment
COD	Chemical oxygen demand
CRI	Crystallinity Index
CSTR	Continuous Stirred Tank Reactors
DEPH	Di (2-ethylhexyl) phthalate
DGE	Diesel Gallon Equivalent
DMSO	Dimethyl Sulfoxide
DNS	Dinitrosalicyclic
DOC	Dissolved Organic Carbon
DOW	Domestic Organic Waste
DP	Degree of Polymerization
DTPA	Diethylene Triamine Penta Acetate
DW	Distilled Water
E85	Ethanol 85
EC	Electrical conductivity
EDTA	Ethylene Diamine Tetra Acetate
EEC	Estimated Environmental Concentration
EG	Endo-B-1-4-Glucanase
EH	Enzyme Hydrolysis
EPA	Environmental Protection Agency
EU	European
FFA	Free Fatty Acid
FOG	Fat, Oil and Grease
FPA	Filter paper assay
FPU	Filter Paper Units
FT	Fischore-Tropsch
GGE	Gasoline gallon equivalent
GH	glycosyl hydrolases
GHG	Green House Gases
HDPE	High Density PolyEthylene
HMF	hydroxymethylfurfural
HPAEC	High Performance Anion Exchange Chromatography
HPH	High Pressure Homogeniser
HPLC	High Performance Liquid Chromatography
HRT	Hydrolytic Retention Time
HW	Household Waste
IBE	Isopropanol, Butanol, Ethanol

ICP-MS	Inductively Coupled Plasma Mass Spectrometer
ICS	Ion chromatographic
IU	International Unit
JGI	Joint Genome Institute
Ktoe/y	Kilotonne/year
LAS	Linear alkyl benzene sulphonates
LCM	Lignocellulosic Material
LHW	Liquid Hot Water
LPG	Liquefied Petroleum Gas
MBT	Mechanical Biological Treatment
MC	Moisture content
MeO	Metal Oxide
MHT	Mechanical heat treatment
MOPS	3-(N-Morpholino)propane sulfonic acid, 4-Morpholinepropanesulfonic acid
MPT	Mechanical pre-treatment
MSW	Municipal Solid Waste
MSWm	Municipal Solid Waste management
NP	Nonylphenol
NPE	Nonylphe-nolethoxylates with 1-2 etoxy
NPK	Nitrogen, Phosphorus, Potassium
NTA	Nitrilotriacetic Acid
ODW	Oven Dry Weight
OFMSW	Organic Fraction Municipal Solid Waste
OLR	Organic Loading Rate
PAH	Polycyclic Aromatic Hydrocarbons
PCA	Principle Component Analysis
PCB	Polychlorinated Biphenyls
PEG	PolyEthylene Glycol
PET	PolyEthylene Terephthalate
pH	Potential Hydrogen
pHc	pH during fermentation
pHm	initial pH medium
PTE	Potentially Toxic elements
PVC	Polyvinyl chloride
RDF	Refuses Deprival Fuel
RDR	Rotary Drum Reactor
RPKM	Reads Per Kilo base of transcript per Million mapped reads
S/L ratio	Solid/ Liquid Ratio
SBG	Spent Brewing Grains
SSF	Solid State Fermentation
SWM	Solid Waste Management
TIE	Toxicity Identification Evaluation
TOC	Total Organic Compounds
TS	Total solid
UASB	Up flow Anaerobic Sludge Blanket
UCSC	University of California Santa Cruz
VOC	Volatile Organic Compound
VS	Volatile Solid
WHO	World Health Organization

Chapter 1 Introduction

1.1 Environmental impact of Municipal Solid Waste (MSW)

The earth's climate has changed and the temperature has risen since the beginning of the twentieth century due to increases in atmospheric concentrations of greenhouse gases. Climate change is a long term concern. Bio energy is considered to be one solution for solving environmental issues such as water, soil and air pollution, as well as decreasing reliance on petroleum.

There are many resources available for producing bio energy, such as agricultural crops and their waste, animal waste, food processing and MSW. These are considered as potential renewable and sustainable energy sources. Generally, MSWs are considered to be one of the most sustainable resources around the world (Murtala *et al.,* 2012). Additionally, MSW presents an environmental problem in relation to its disposal. Furthermore, MSW treatments play a critical role in getting bio-energy with a high quality of gases; biofuels and fertilisers. For that, nowadays, there are many available technologies applying for bio-energy production, such as Anaerobic Digestion (AD), incineration, Refuses Deprival Fuel (RDF) and fermentation for biofuel productions (Potts and Duncan, 2009).

MSW is a complex refuse whose composition is heterogeneous in nature, within MSW some of the components are stable, while others degrade as a result of biological and chemical processes causing potential pollution problems (Alhassan, 2012), also waste generation is becoming increasingly significantly as shown in Figure 1.1 which leads to increasing pollution problems. For this reason, Solid Wastes Management (SWM) is an important consideration in order to decrease the effect of pollution.

In most countries solid waste landfill is the most common means of disposal. Landfill sites are unsightly, unsanitary, and generally smelly, and attract animals and insects (Kumar and Alappat, 2003). To overcome the problem of MSW,

incineration is wildly applied; incineration produces a safe substrate called ash which can be used for further applications (Dhadse *et al*., 2008; Lam *et al*. 2010)

Unmanaged MSWs have various impacts on the environment and on human health. For instance, water may become polluted by leachate. If the leachate enters surface and ground water before sufficient dilution, it may lead to serious pollution which affects animal and human life (Mage *et al.,* 1996), such as cholera and dengue fever via rodents and vector insects at landfill sites, or through air and water (Kreith and Tchobanoglous, 1994). Also using water polluted by MSW for bathing, food irrigation and drinking water can also expose individuals to disease causing organisms and other contaminants (Alam and Ahmade, 2013).

Regarding atmospheric pollution, MSW can emit Green House Gases (GHG) during composting of solid waste in landfill (Kreith and Tchobanoglous, 1994); managing this could decrease the GHG emission as shown in a study carried out in Europe (EU) where by time total annual net of CO_2 was decreasing (see Figure 1.2). In addition, the World Health Organization (WHO) estimates that about a quarter of the diseases facing mankind today occur due to prolonged exposure to environmental pollution (Kimani, 2007).

Generally, the impacts of bio-energy include a negative impact on living organisms by the increase in erosion, depletion of soil nutrients and soil quality. These problems are related to the cultivation of annual crops; a further impact is in the use of pesticides and fertilisers. These agriculture inputs can affect people's health by leaching residues into ground water (Herzog *et al*., 2001).

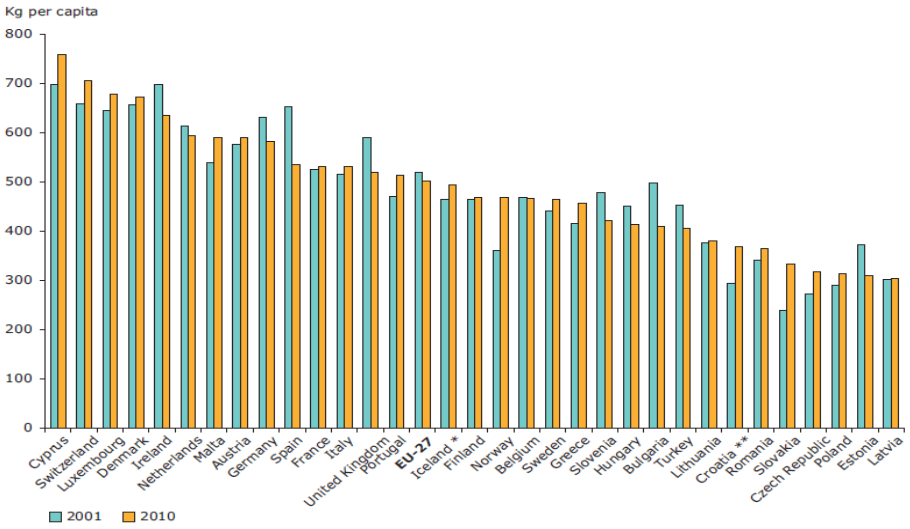


Figure 1.1 Municipal wastes generated per capita, 2001 and 2010 (Eurostat, 2012a; ETC/SCP, 2013a)

Note: (*) 2008 data used for 2010. (**) 2004 data used for 2001. According to Eurostat the comparability of the data over time is high. However, some breaks in the time series are documented, which can influence the comparability between countries and within a country. Generally, the quality of the data has improved during the period 2001–2010.

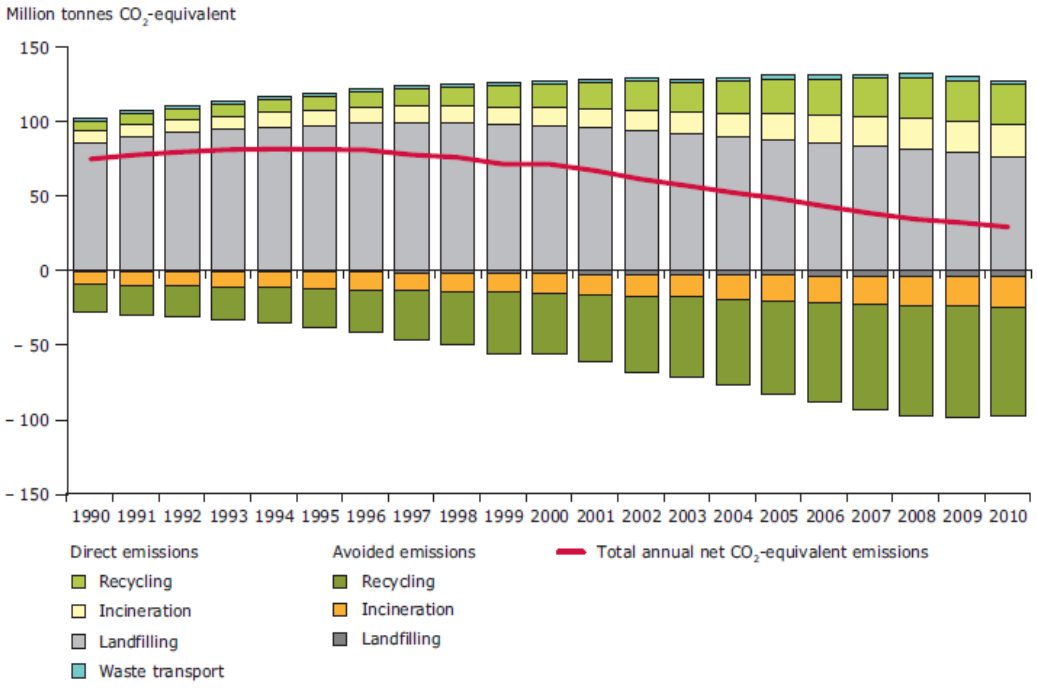


Figure 1.2 GHG emissions from municipal waste management in the EU, Switzerland and Norway (ETC/SCP., 2011; ETC/SCP., 2012)

Note: Excluding Cyprus due to lack of data. GHG emissions before 1995 are calculated based on back casted waste data.

The concentration of CO_2 in the atmosphere responsible for global worming had risen to 391 ppm by the end of 2001; an increase of about 6 % compared to records for 2000. Comparing GHG production, in particular CO_2, with the world total population, the 2010 world energy statistics (Petroleum, 2010) show that 44 % of total CO_2 emission comes from 17 % of the world total population (developed nations) while the remaining 83 % of the world population (developing and least developed) contribute half of the total emissions (Khatib, 2011) (see Figure 1.3).

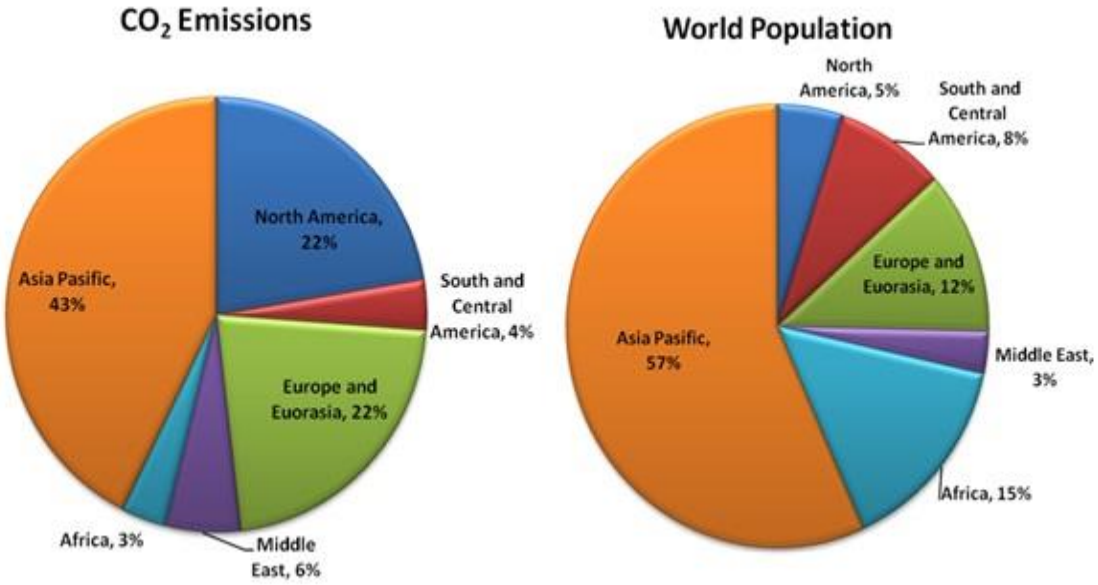


Figure 1.3 Comparison of World Population and CO_2 emission (BP, 2010)

1.2 Current problems with MSW

Municipal Solid Waste Management (MSWM) issue; MSW is primarily waste produced by households, which is usually managed by local authorities. Many points have to be taken into consideration about Solid Waste Management's (SWMs), including the amount of waste generated and the types of materials that make up this waste; whether hazardous or non-hazardous. Furthermore, the rapid changes in the composition of waste and its properties could cause some management issues. To overcome these issues an insight into the impact of

waste generation, collection, transportation and disposal methods is needed to find a suitable methods to reduce and eliminate environmental and human health impacts, to support economic development and, finally, attempt to improve quality of life by using this waste for bio-energy production (Technologies, 2000).

In addition, MSWM is one of the key topics in environmental protection. In England, around 23.7 million tons of household waste was generated in 2009/10, which is equivalent to 1036 kg per household (DEFRA report, 2010; Municipal waste management statistics for England 2009/10) (DEFRA, 2010). The current technologies for MSW treatment are incineration, landfill, composting, biofuel and AD. Recently, anaerobic conversion of MSW into biogas and biofuel has attracted growing interest as a promising method to reduce environmental impact and to generate renewable fuel at the same time (Li *et al*., 2012).

Cost of converting MSW to biofuel; nowadays biofuel production is applied in most EU countries. Figure 1.4 compares the price of biofuel with diesel. Some MSW contain a high lignocellulosic content 51.2 % (Barlaz *et al*., 1989) 63.4 % (Bookter and Ham, 1982) and are emerging as an attractive alternative to other biomass feed stocks, such as energy crops and agricultural wastes, because of lower costs and higher potential for fossil fuel displacement as a result of reducing GHG emissions (Tomas-Pejo *et al*., 2008). MSW can be utilized for AD but there may be more technically and commercially advantageous approaches. One of the alternative approaches is to hydrolyse the lignocellulosic component in the MSW into simple sugars and then ferment these sugars into ethanol. It has been estimated that around 152 L of ethanol could be generated from a tonne of processed MSW (Li *et al*., 2012). However, as with other lignocellulosic biomass there are technical problems to overcome not least the inherent recalcitrance of the material to degradation. Pre-treatments can make cellulose more accessible for microbial and enzymatic attacks (Harmsen *et al*., 2010). Biological, acid or

alkaline pre-treatments are usually required; the last two are corrosive and not eco-friendly if they are used at high concentrations. Furthermore, biological processes have some drawbacks such as the process takes several days and the cost of the enzymes required. Despite these problems the biological conversion of cellulosic biomass to fuels and chemicals through enzymatic hydrolysis, following hydrothermal pre-treatment, offers an environmentally friendly option and potential for high yields, higher selectivity, lower energy costs and milder operating conditions than chemical processes. The effective saccharification of lignocellulosic biomass requires a complex mixture of enzymes, and fungi are a good source for most of these (Binod *et al*., 2011). Industrially the filamentous fungus *T. reesei* (*teleomorph Hypocrea jecorina*) has been used for the production of cellulolytic enzymes cocktails (Singhania *et al*., 2010). "Cellulase activity" results from a combination of enzymes that catalyse the reaction of water with cellulose to release shorter chains and ultimately soluble glucose. The mechanism of hydrolysis of cellulose is still not fully understood (Himmel *et al*., 2010). Enzymatic hydrolysis is known to be influenced by both the structural features of cellulose and the mode of action of the enzymes which in turn is influenced by factors such as enzyme source, thermal inactivation, activity balance for synergism, specific activity, nonspecific binding and enzyme compatibility. Other, challenges for the effective hydrolysis of substrate to fermentable sugars. Include the production of reactive cellulosic fibre available for enzymatic attack, avoiding both the destruction of hemicelluloses and cellulose, and the formation of possible inhibitors for hydrolytic enzymes and fermenting microorganisms, minimizing the energy demand, reducing the cost of size reduction of the feedstock, reducing the cost of material for construction of pre-treatment reactors, producing less residues, consumption of little or no chemical (Taherzadeh and Karimi, 2008).

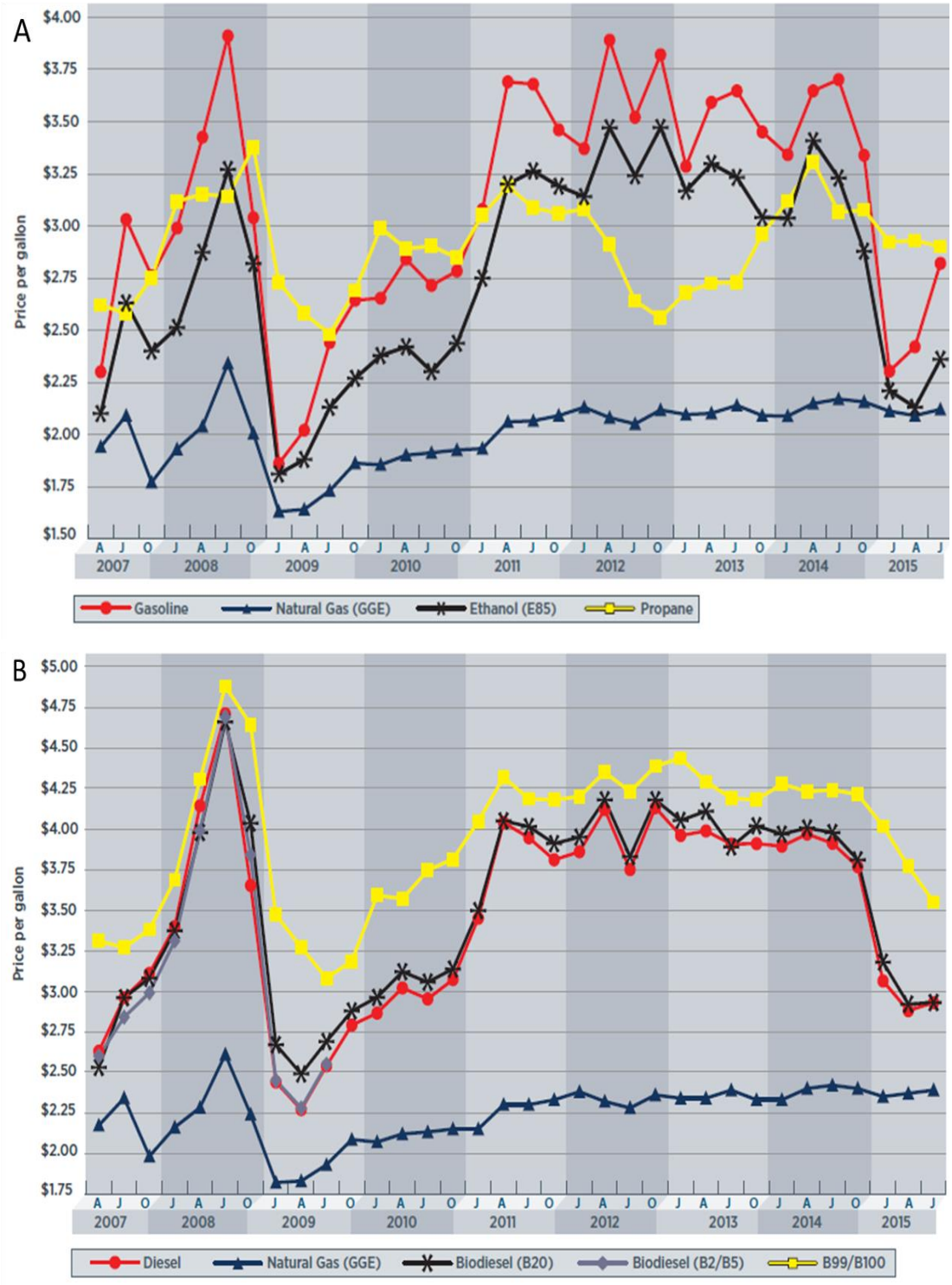


Figure 1.4 Cost of for alternative biofuels which began in September 2005 to July 2015 (natural gas, propane, ethanol (E85), and biodiesel) relative to gasoline and diesel. Natural gas in [GGE (Gasoline gallon equivalent)], propane, and ethanol (E85) have been compared against gasoline prices, while natural gas in [DGE (Diesel Gallon Equivalent)] and biodiesel have been compared against diesel prices (A) Alternative fuel price versus gasoline (B) Alternative fuel price versus diesel (Energy, 2015)

Heavy metals; Toxic metals can have an effect on the environment in different ways: (1) they can remain in the environment for long periods; (2) low toxicity compounds contain heavy metals that can be transformed from low toxic species into more toxic forms such as mercury (3) they can effect human health by the bioaccumulation and bio-augmentation of heavy metals into the food chain; (4) metals can be transformed and changed in valence and species, but cannot be degraded by any methods including bio-treatment; (5) the toxicity of heavy metals occurs at low concentration of about 1.0–10 mg/L. Some strong toxic metal ions, such as Hg and Cd, are very toxic at concentrations of 0.001–0.1 mg/L (Alkorta *et al*., 2004; Dold, 2008)

MSW derived compost and/or fertiliser plays an important role in replenishing levels of organic material in the soil, providing nutrients such as nitrogen, phosphorus, and potassium. These act as essential micronutrients for plants, help to improve soil structure and water holding capacity and have beneficial effects on microbial biomass and activity (Barry *et al*., 2004; Bell *et al*., 2004). Composting is one of several methods for treating bio solids to create a marketable end product that is easy to handle, store, and use. The end product is usually a Class A, humus-like material without detectable levels of pathogens that can be applied as a soil conditioner and fertiliser to gardens, food and feed crops, and rangelands (TRENCH, 2002; ÖZSOY, 2006; Lu *et al*., 2012).

Wastes can contain heavy metals at high concentrations, for this reason the waste has to pass through a special treatment to reduce the amount of heavy metals (Solisio *et al*., 2002). Removing Heavy metals from aqueous solution can be carried out using many methods such as chemical precipitation, ion exchange, electrochemical treatment membrane technologies, adsorption on activated carbon etc. However, chemical and electrochemical method are inefficient, as they produce large amounts of sludge which is hard to treat, while the rest are

very expensive which make them inapplicable for large scale use (Veglio and Beolchini, 1997; Solisio and Lodi, 2002).

An alternative method is microbial leaching (Hong *et al*., 2000). Bio-hydrometallurgical (bioremediation) is considered as a green technology, with low cost, low energy requirement and that can effectively sequester dissolved metal ions out of dilute complex solutions quickly and with high efficiency (Wu and Ting, 2006).

Heavy metal removal by bio-sorption has been extensively investigated (Volesky 1990). Some reviews have been published focusing on different aspects of heavy metal bio-sorption (Kapoor and Viraraghavan, 1995; Volesky and Holan, 1995; Davis *et al*., 2003; Gavrilescu, 2004). From these reviews, it can be seen that the research on bio-sorption has focused on the following three major fields. First, the bio-sorbents (Kratochvil and Volesky, 1998); secondly, the mechanism of bio-sorption; and thirdly, up-scaling the bio-sorption process. The bio-sorption process is basically at the stage of laboratory-scale studies and presents great difficulties in attempting to apply bio-sorption process into practice (Tsezos, 2001).

1.3 Objectives of this project

The objectives of this project are to investigate potential approaches that could improve the use of MSW for various products:

The first aim was to investigate MSW composition and variability, especially in terms of lignocellulosic and heavy metal content (chapter 3).

The second aim of this project was to explore the potential for using MSW as a source of fermentable sugars. The efficacy of enzyme hydrolysis on "as received" MSW was determined and the effect of a range of pre-treatments to optimise the hydrolysis step explored (chapter 4).

The third aim was to develop solid state fermentation methods using MSW as the substrate for the production of cellulolytic enzymes (chapter 5).

The forth aim was to explore the use of fungi and yeast to decrease the amount of heavy metals in the MSW to be able to generate a safe compost or fertiliser (chapter 6).

1.4 Thesis outline

The thesis is structured and presented as the following way:

Chapter 1 Introduction: this chapter provides an overview of the project explains the effects of waste on the environment and current issues facing companies to manage the waste safely and suggests some ways to solve them.

Chapter 2 Literature review: a review of bioethanol, biochemical, cellulase enzyme and fertiliser production is described.

Chapter 3 Compositional analysis of MSW: reports the physical and chemical properties for three substrates and compares their properties.

Chapter 4 Optimizing conditions for sugar release from MSW: will explore various acid and alkaline pre-treatments followed by enzymatic hydrolysis and their effect on sugar release.

Chapter 5 Optimizing "cellulase" production from MSW following a Solid State Fermentation (SSF) by *Trichoderma reesei* and *Aspergillus niger.*

Chapter 6 Bioleaching of MSW by Fungi and Yeasts: in this chapter, heavy metal leaching from MSW has been optimized for fertiliser production.

Chapter 7 Conclusion and future work: in this chapter, I will summarise all the findings of this study and suggest some potential future work that can be further explore.

Chapter 2 Literature Review

2.1 Municipal solid waste

Waste has become defined as "any substance or object in the categories which the holder discards or intends or requires discarding" (Murtala *et al*., 2012). Solid waste has become defined as including "all domestic refuse and non-hazardous wastes such as commercial and institutional wastes, street sweepings and construction solid waste may be categorised according to its origin (e.g. domestic, industrial), content (e.g. glass, paper) and hazardous potential (e.g. toxic, flammable, infectious)" (Murtala *et al*., 2012). Waste sources includes domestic and/or residential, municipal, commercial, institutional, garbage (a term that is used for animal and vegetable waste from various activities), rubbish (a general term which is applied to solid waste from households, businesses and institutions), ash, and bulky wastes (any household waste that cannot be stored in regular storage containers) (Murugesan, 2008).

2.1.1 Characteristics and composition of MSW

The rate of waste generated is an index of socio-economic development and economic prosperity of the region. Extending urbanized areas and increasing industrialisation has led to a greater use of resources and production of increasing amounts of waste. Waste composition and quantity depends on economic activity, income and resource consumption (DBSSR, 2012). Figure (2.1) represents the difference in waste generation as a % according to a country income.

Determination of the chemical properties helps in the selection of the most suitable method for utilization of ash or any other substrate (Dhadse *et al*., 2008; Lam *et al*., 2010).

MSW collected from households contains various percentages of waste including glass, textiles, metals, food scraps, yard wastes, wood and plastics. The waste may contain RDF too (RDF is when raw MSW is transformed into RDF that can be

used to generate electricity) as shown in Figure 2. The main purpose of the RDF method is to produce an improved solid fuel or pellets from MSW (Sharholy *et al*., 2008). While, Figure 2.3 shows a compositional study commissioned by Defra in England (DEFRA, 2010).

Solid waste is generally divided into three broad categories: **Putrescible waste,** this decomposes rapidly, secretes a bad odour and is visually unpleasant. This kind of waste is mainly composed of waste from food preparation and consumption; **Fermentable waste**, this decomposes rapidly and is the result of substances, such as crop and market debris; finally, **Non-fermentable waste**; this resists decomposition and breaks down very slowly (Kreith and Tchobanoglous, 1994).

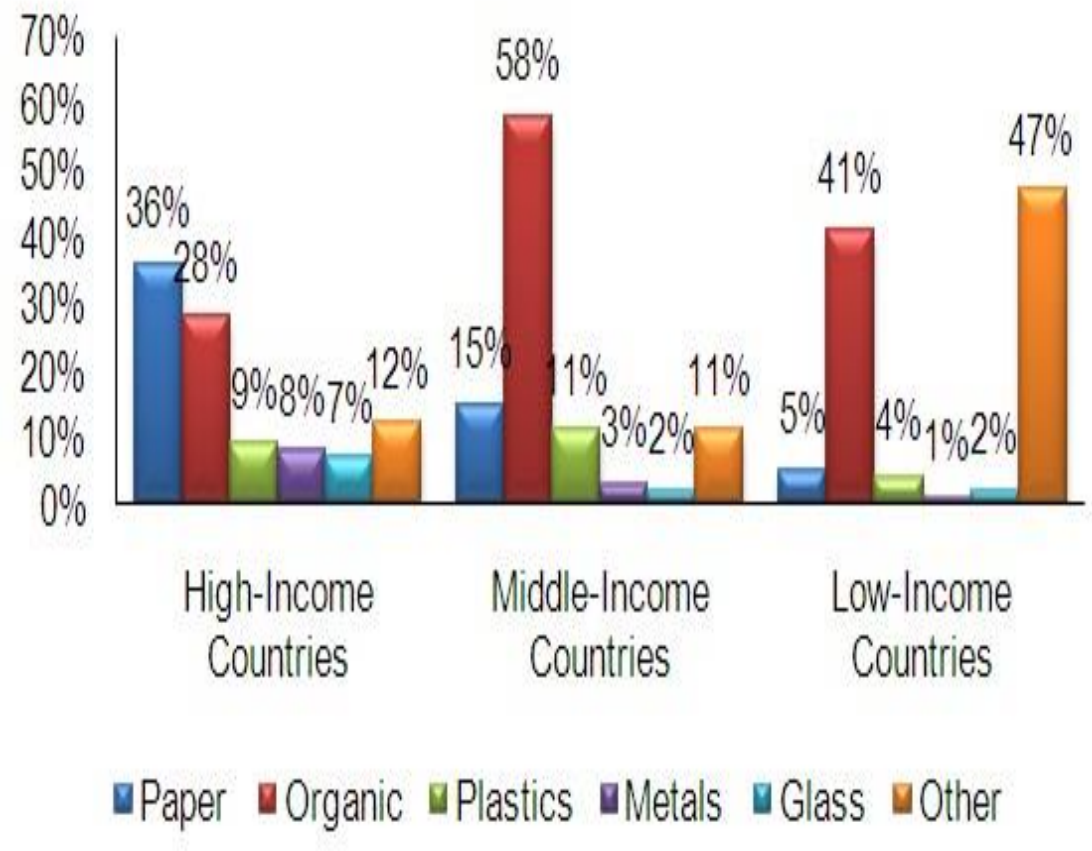


Figure 2.1 Comparison of MSW streams among high income countries (developed and some developing), medium income countries (most developing) and low incomes countries (some developing and least developed countries) (UNDESA; 2010; Chandak, 2010)

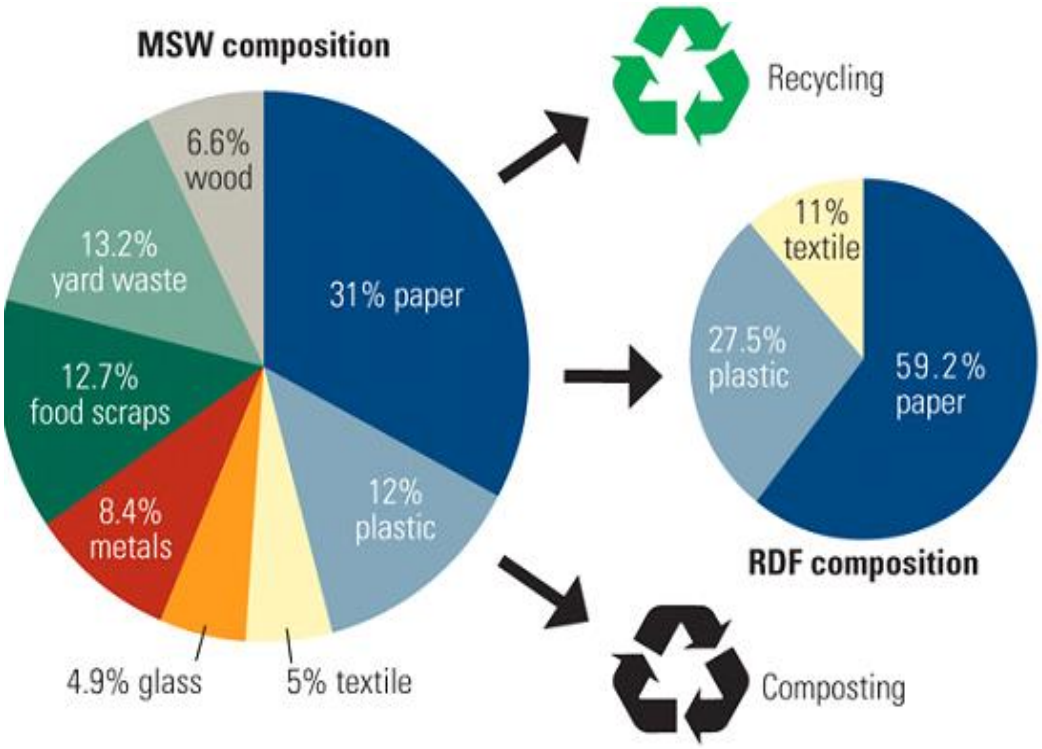


Figure 2.2 MSW Composition and Types the diagram shows the typical composition of MSW (Energy Matter, 2012)

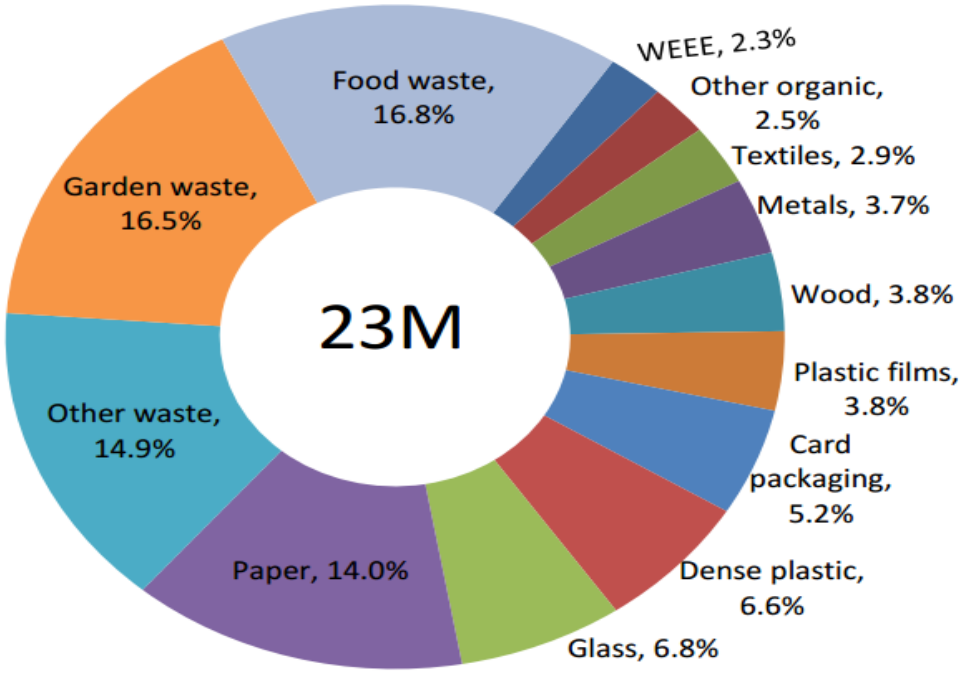


Figure 2.3 Composition of local authority collected waste, England 2010/11 (DEFRA, 2010)

In general about 50 – 60 % of MSW consists of organic matter (Gellens *et al*., 1995; Veeken and Hamelers, 1999), and the Organic Fraction MSW (OFMSW) contains typically 40-50 % cellulose, 12 % hemicellulose, and 10-15 % lignin by weight (Wang *et al*., 1994). The properties of the MSW substrate can be divided into chemical and physical characteristics (He *et al*., 2004).

Physical properties:

Particle size, generally lignocellulosic materials consist of two different types of surface areas- internal and external - with the external area being related to the size and shape of the particles (Fan *et al*., 1980). A smaller particle size provides a greater surface area for enzymatic attack (Palmowski and Miller, 2000). Hence, a reduced particle size could increase the hydrolysis rate and shorten digestion times (Juanga, 2005). Thus smaller particles have been shown to provide better digestion for biogas production (Zhang and Zhang, 1999), while some other studies have shown that there is no significant effect in reduction of biomass particle size in terms of enzyme hydrolysis (Negro *et al*., 2003). Other studies have suggested that grinding MSW from 2.2 to 1.1 mm did not affect mesophilic digestion (Ghosh *et al*., 2000). However, to get smaller particles energy consumption would be required (Bergman *et al*., 2005).

Moisture content is a physical parameter that is used to determine the liquid content in a substrate. Moisture content in a solid biomass effects the specific surface area and crystallinity of natural cellulose, by increasing wet content the specific surface area also increases (Fan *et al*., 1980).

Chemical properties: MSW consists of lignocellulosic substances plus some bulk particles (e.g. glass , plastic, and wood), cellulose is a structural component of the plant cell wall, and makes up between 35 to 50 % cellulose of the plant cell wall (Takara and Khanal, 2012). The structure of cellulose is a linear polysaccharide polymer of glucose molecules linked together via β-1, 4 glucosidic bonds. This type of structure allows the polymer to exist in long chains. Furthermore, the degree of polymerisation of cellulose, which refers to the

number of glucose units making up one polymer molecule, can range from 800-10,000 units (Harmsen *et al*., 2010). Cellulose is present in two forms: unorganized amorphous and organized crystalline, in the later several cellulose polymers are linked together in parallel by hydrogen bonds and coalesce to form cellulose micro fibrils (Chandra *et al*., 2012; Martin-ryals, 2012).

Hemicellulose is a structural component of plant cell walls making up 20 to 35 % of the structure (Takara and Khanal, 2012), hemicellulose binds to cellulose micro fibrils via hydrogen bonds. Hemicellulose is present in amorphous polysaccharide forms (various 5- and 6- carbon sugars, including arabino-xylans, gluco-mannans, galactans, and others). The most common polysaccharide family present in hemicellulose is xylan (composed primarily of the 5-carbon sugar xylose and some 6-carbon sugars, such as glucose). Typically, the number of sugar units in hemicellulose, does not exceed 200 units, and is much shorter than cellulose (Harmsen *et al*., 2010; Martin-ryals, 2012).

Lignin is the third major structural component of plant cell walls and can represent 10 to 20 % of plant cell wall content (Takara and Khanal, 2012). Lignin is present as a complex, three-dimensional amorphous polymer which is composed of various phenolic monomer units (Harmsen *et al*., 2010; Chandra *et al*., 2012). Lignin essentially acts as the glue within the lignocellulosic matrix, binding with cellulose and hemicellulose via a variety of chemical bonds to provide structural support as well as a protective barrier making the plant resistant to chemical and biological degradation (Martin-Ryals, 2012). As a result, removal of lignin via pre-treatment prior to hydrolysis is necessary in order for cellulose and hemicellulose to be accessible for microorganisms and enzymes (Juanga, 2005; Martin-ryals, 2012).

Lipids are characterised either as fats, oils or greases, which are mainly derived from food wastes (Li *et al*., 2002). Lipids are also a substance for anaerobic digestion (AD) because of the methane production yield from lipids is higher than from other forms of organic material. However, excess lipid can be found in some

samples containing organic waste, and an excess of lipid can lead to a low hydrolysis rate and inhibit methanogenic bacteria, due to a synergetic effect on degradation components (Sialve *et al*., 2009). This is because lipids adsorb onto solid surfaces, which may reduce the accessibility of enzymes and as a result delay the hydrolysis process (Hartmann and Ahring, 2006; Neves *et al*., 2008).

Nitrogen is an important constituent of protoplasm, proteins and amino acids; therefore, organisms cannot grow nor multiply in the absence of nitrogen (Kreith and Tchobanoglous, 1994; Diaz *et al*., 1996). Total nitrogen includes all forms of nitrogen, organic nitrogen, ammonium nitrogen (NH_4-N) and nitrate nitrogen (NO_3-N). The measure of total nitrogen content includes both organic and inorganic forms of nitrogen in a sample (Shyamala and Belagali, 2012). Typically, the total amount of nitrogen in MSW compost (fertiliser) varies from 1.2 to 1.8 % (Shyamala and Belagali, 2012). Nitrogen levels above these values can produce a digestate which is unsuitable for agricultural purposes (Zaher *et al*., 2007). Starch which is contained in solid waste has been shown to be easier to process for carbohydrate and gas production (Kapdan and Kargi, 2006). Starch in MSW is readily biodegradable but requires multiple enzymes for complete hydrolysis. There are three main types of enzymes that can act synergistically: alpha-amylases, beta-amylases and gluco-amylases (Palmisano and Barlaz, 1996) .

MSW can contain inhibitory substances such as heavy metals. Identifying toxicity in MSW, especially in the leachate, is difficult due to the restricted number of chemicals detected during routine analysis; however, the US Environmental Protection Agency (EPA) has developed a Toxicity Identification Evaluation (TIE), which is useful for the detection and identification of toxic agents. The TIE method has been divided into three distinct phases: toxicant characterization (Phase I) (Isidori *et al*., 2003), toxicant identification (Phase II) and toxicant confirmation (Phase III) (Burgess *et al*., 2000).

According to (Chen *et al*., 2008) there are several reasons for the wide variations in inhibition/toxicity levels observed for most substances. They seem to be

significantly influenced by various microbiological mechanisms, such as acclimation, synergism and antagonism. Acclimation is the ability of microorganisms to reorganize their metabolic resources to overcome the metabolic blocks that are produced by the inhibitory or toxic substances when concentrations of these substances are slowly increased within the environment. Synergism is an increase in a toxic effect of one substance by the presence of another substance. Antagonism is defined as a reduction of the effect of one substance by the presence of another substance. Several strategies to minimize the effect of inhibitory substances have been reported. These include the removal of potentially inhibitory/toxic substances from the substrate, dilution of the substrate which reduces the concentration of inhibitory substances, addition of chemicals to precipitate or solubilise inhibitory substances, employing pH control to change the chemical form of inhibitory substances, and addition of a material that is antagonistic to inhibitory substances, in order to counteract the inhibitory effect (Bashir and Matin, 2004; Angelidaki *et al*., 2006).

Chemical analysis of MSW has revealed that the major elements are Silicon (Si), Aluminium (Al^{3+}), Iron (Fe^{2+}), Magnesium (Mg^{2+}), Calcium (Ca^{2+}), Potassium (K^{+}), Sodium (Na^{2+}) and Chlorine (Cl^{-}). In ash, the common oxides are SiO_2, Al_2O_3, Fe_2O_3, Na_2O, K_2O. In fly ash derived from MSW, CaO is the most abundant compound, while in bottom ash SiO_2 is the most abundant compound (Lam *et al*., 2010).

Heavy metals, as defined by the EU Directive 67/548/EEC (Estimated Environmental Concentration), are Arsenic (As^{3-}), Cadmium (Cd^{2+}), Chrome (Cr^{6+}), Copper (Cu^{2+}), Lead (Pb^{2+}), Mercury (Hg), Nickel (Ni^{2+}), Selenium (Se^{2-}), Thallium (Ti^{3+}), Tin (Sn^{+4}) and their compounds (oxides, chlorides, etc), and Manganese (Mn^{2+}) and Zinc (Zn^{2+}) are often included. Heavy metals can be found in all MSW fractions at various concentrations (Verma, 2002). The toxic effects of heavy metals have been attributed to their ability to inactivate a wide range of enzyme functions and structures by binding to sulphydryl groups (amino,

carboxylate, imidazole and hydroxyl radicals of enzymes and other proteins) (Chen *et al*., 2008; Lo *et al*., 2010). Metals at the highest concentration in MSW ash are, Cu^{2+}, Hg, Ni^{2+}, Cd^{2+}, Zn^{2+} and Pb^{2+}, with Zn^{2+} and Pb^{2+} present at the highest concentrations (fly ash and bottom ash). Generally, the heavy metals content of fly ash is higher than in bottom ash due to the vaporization of metals during combustion and the process of metal adsorption on the surface of fly ash particles (Lam *et al*., 2010).

Heavy metals are also present in fertilisers produced from MSW using various processes, for example the heavy metals in a digestate. The heavy metals in digestate come mainly from natural and anthropogenic sources. The particular heavy metals in digestate are Cd^{2+}, Cr^{6+}, Pb^{2+}, Cu^{2+}, Ni^{2+}, and Zn^{2+}. Generally, when digestate (fertiliser) is not managed appropriately, the dissolved heavy metals maybe transported into agricultural fields and, as a result, will accumulate in plant roots, then are present in the soil, which can then reach ground water after leaching with rain water (Al Seadi and Lukehurst, 2012).

In addition to inorganic contamination (heavy metals) there can be some organic contaminants in a compost derived from MSW such as PAH (Polycyclic Aromatic Hydrocarbons); DEPH (Di (2-ethylhexyl) phthalate); LAS (Linear Alkyl benzene Sulphonates) and NP and NPE (Nonylphenol and Nonylphenol- nolethoxylates with 1-2 etoxy groups) (Al Seadi and Lukehurst, 2012).

There are some physical impurities that make some substrates unsuitable as a fertiliser. Those impurities consist of non-digestible, digestible, and low digestible materials depending on particle size. This kind of impurity seems to appear in all kinds of biomass feedstock, but most commonly in household waste, food waste, garden waste, straw, solid manure and other solid types of waste. The most common physical particles include: plastic and rubber, glass, metal, stones, sand, larger particle size digestible materials (roots, wood, bark etc.), and other physical contaminants. The presence of these kinds of impurity in feedstock can cause maculation, impediment and heavy wear of plant components. They can

also disturb operational stability and cause economic losses. Moreover, the presence of impurities in digestate can give rise to aesthetic damage and environmental pollution (from PVC (Polyvinyl chloride) and other plastics, glass and metal and so on). There are some methods that can be applied to remove physical impurities to ensure good quality fertilisers. These include source sorting or on site separation, via mechanical, magnetic or other segregation technologies (Al Seadi and Lukehurst, 2012).

2.1.2 Municipal Solid Waste Management (MSWM)

MSWM is a very important topic in environmental protection. In England, around 23.7 million tons of Household waste (HW) was generated in 2009/10, corresponding to just over a ton of HW per household (DEFRA, 2010). While, in the USA, it was estimated that around 752 to 1200 kg per year per capita MSW was generated (Rapport *et al*., 2012).

MSWM is a term usually applied to a heterogeneous collection of waste produced in urban areas whose nature varies from region to region. Generally, this term refer to various activities, such as collection, transfer, treatment, recycling, resource recovery and disposal of solid waste in urban areas (Kreith and Tchobanoglous, 1994).

The aims and principals of MSW management are to conserve the health of the urban population; in particular, that of low-income groups who suffer the most from inferior waste management, enhancing the environment, supporting urban economic development and finally, to generate employment and income in the same sector. The guiding values of sustainable waste management are: to minimize waste generation, maximise waste recycling and reuse, and ensure the safe and environmentally sound disposal of waste (Schübeler *et al*., 1996).

To convert the waste to energy an environmental hierarchy for managing MSW is required as shown in Figure 2.4. The highest value proposition occurs at the top

of the inverse pyramid with the value decreasing towards the bottom of the pyramid.

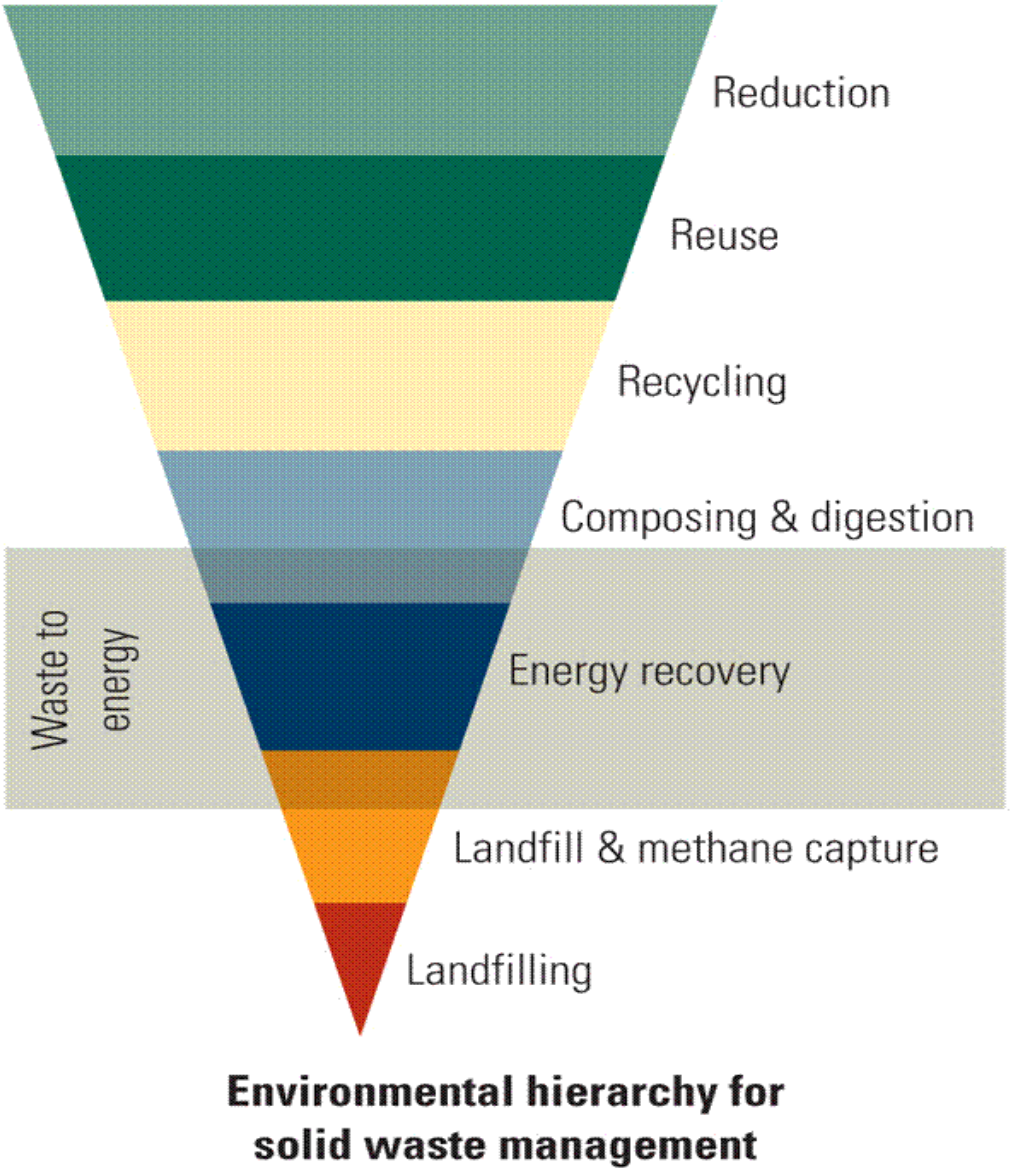


Figure 2.4 Solid waste management hierarchies with environmental efficacy declining from top to bottom (Rosinski, 2012)

The EPA encourages practices that reduce the amount of waste that is required to be disposed of, such as waste prevention, recycling, and composting, MSWM stages include the following aspects:

I. Storage at source

The most critical component in the whole process of MSWM is the segregation and storage of solid waste at its source. This approach has led to the success of MSWM (Sharholy *et al*., 2008).

II. Collection

Generally, waste is collected by local authorities or by special collection systems for recycling (Ludwig *et al*., 2012). There are some problems associated with the collection of solid waste in some developed countries, which include a large number of open communal storage sites, poor methods for collection, and adverse climate conditions, especially high temperature and humidity. The most immediate needs for collection include more efficient vehicle design, and the enclosure of waste at all stage of collection. The frequency of collection is typically governed by a number of factors, such as waste characteristics, climate type, degree of involvement by householders and, finally, the costs of collection (Ludwig *et al*., 2012).

III. Reduction, reuse and recycling the waste

There are many methods used for reduction and sorting such as household sorting methods, this method could be applied by separating or sorting MSW at various collection points; for example, a number of containers on outdoor grounds in selected housing areas. These containers come in various sizes and colours in order to facilitate identification of which container is allocated to which specific waste material. Most collection spots contain between three and six

containers separating glass, packaging, plastic bottles, cardboard and paper (Crichton *et al*., 2003).

In addition, waste typically requires some preparation before treatment, bulky material can be removed by hand and the waste is mechanical separated by splitting open refuse bags, in order to shred and homogenise the waste into smaller particle sizes which can then undergo further separation processes. There are many processes for shredding waste into homogenised particle sizes, such as the use of heat treatment vessels. Waste is heated for 45 min at 120-170 ^{o}C, which is the temperature required to destroy bacteria and spores. As a consequence of the autoclaving process, separation will occur; separation can occur under wet or dry conditions. Wet separation can be conducted in a pulp or wet sieving, which provides both separation and particle size reduction in a single unit. For dry separation, rotating drum pulverizing screens are widely used, with the addition of some water or recycled liquor to moisten and weaken the paper components. Shredding or pulverization is a necessary step to reduce coarser organic matter down to a more homogeneous size. The size reduction process can be done by using screw-cutting, milling, drumming, and pulping or shredding machines too (Bioenergy, 2001).

Waste recycling and waste minimization refers to the removal of some items from the waste stream which can be used as raw materials for the manufacture of new products (Ludwig *et al*., 2012). Recycling has been increasing in most European countries. Figure 2.5 shows how the recycling rate has increased from 2001 to 2010 in the 32 EU countries and Figure 2.6 shows the recycling rates in EU countries in 2008/09.



Figure 2.5 Municipal waste recycling rates as a percentage the total recycling includes material recycling as well as composting and digestion of bio-waste in 32 European countries, 2001 and 2010 (Eurostat, 2012b; ETC/SCP, 2013a; ETC/SCP, 2013b; ETC/SCP, 2013c).

Note: The further from the centre in the radar chart, the better the waste management. According to Eurostat the comparability of the data over time is high. However, some breaks in the time series are documented, which can influence the comparability between countries and within a country.

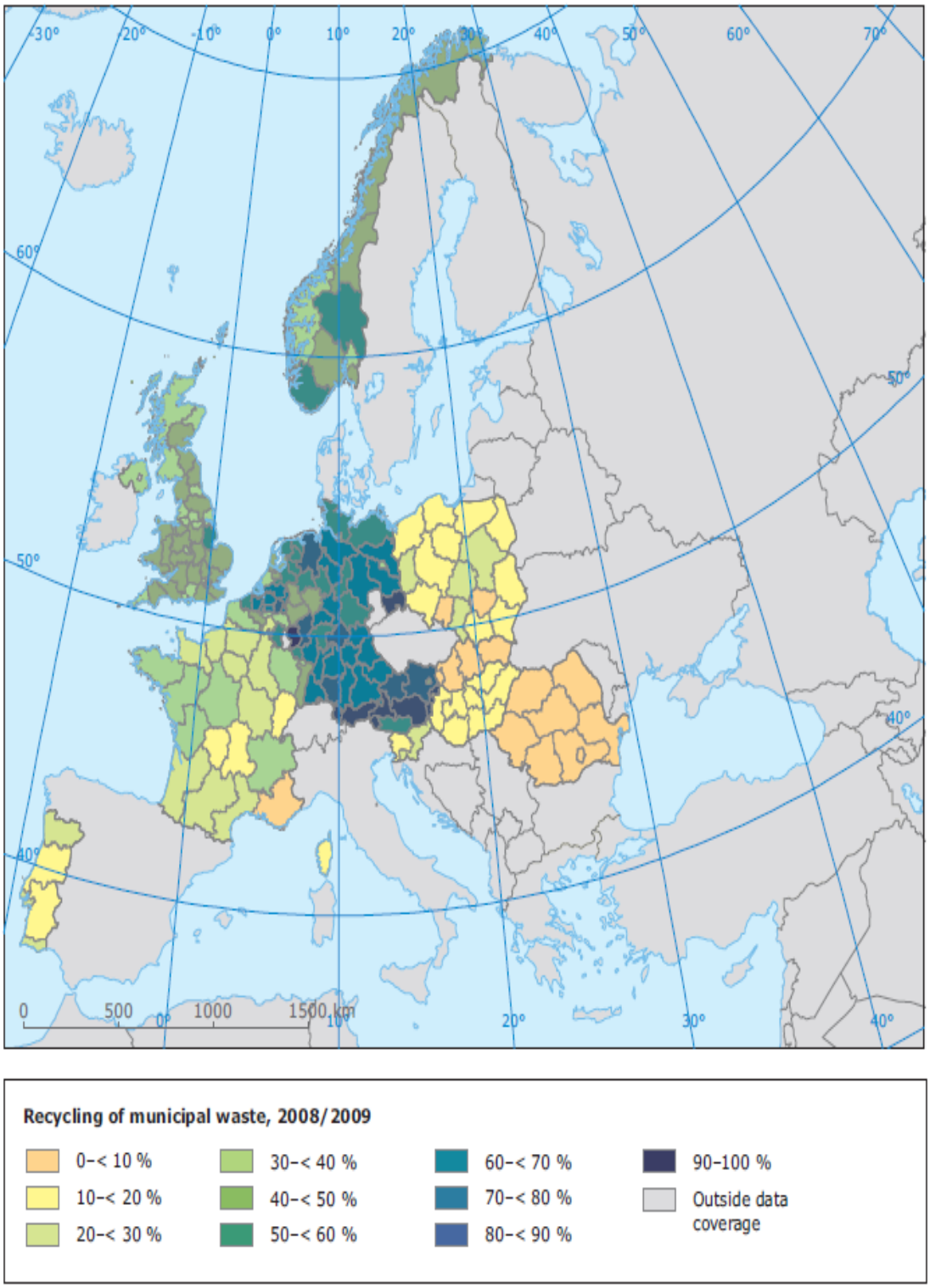


Figure 2.6 Regional recycling rates calculated as a percentage of municipal solid waste generated includes material recycling and composting/digestion, 2008/2009 (Eurostat, 2012b)

Note: 2008 data was used for Belgium, Germany, France, Hungary, Romania and Slovenia. 2009 data was used for the other countries.

IV. Composting

Composting is considered to be the oldest form of recycling, able to transform no-longer-needed organic items into a valuable commodity. The most familiar composting process is the back yard compost pile. There are more than 200 MSW composting facilities operating in Europe. MSW composting has some technical advantages. For example, it can be used with a wide range of household waste and is much faster than other methods due to the use of mechanical processes which include shredding the waste and monitoring it. The composting process can keep up to 60 % of all solid waste from going into landfill and the, compost itself is beneficial to plants and crops helping to retain moisture, allowing good drainage, providing nutrients (which are better able to cling to composted soil particles), and resulting in healthier plant growth. Composting can be conducted through a process known as biodegradation or decomposition, assisted by mechanized control of temperature, oxygen content, and moisture. These are monitored closely to maximise the rate of biodegradation. Furthermore, the composting process is caused by anaerobic bacteria which can break down the organic materials present in MSW (Ludwig *et al*., 2012).

V. MSW treatment and energy recovery

Treatment of solid waste is related to waste volume, toxicity and stability of the waste to be dumped or disposed of in landfills. Treatment is mainly applied in order to recover useful substances. The method of treatment is selected based on the composition, quantity and waste material form. There are different solid waste treatment disposal methods, which are discussed as follows:

Open burning, is a process used to burn unwanted material, without passing through a chimney or stack. This method reduces the volume of waste, extends the lifetime of a dumpsite and is cheap. However, open burning has some

disadvantages, such as emitting pollutants to the atmosphere including dioxin, particulate matter, polycyclic aromatic compounds, volatile organic compounds, carbon monoxide, hexachlorobensene and ash. All of these have the ability to cause serious risks to human and animal health by releasing acidic gases, such as the halo-hydrides of nitrogen and carbon (Ludwig *et al*., 2012).

MSW components are suitable for open burning. However, there are some factors that should be taken into consideration, such as the conditions on the day (winds which will disperse the emissions); manageable volume of waste, geographic conditions and ground conditions. There are some materials that should be removed before open burning in order to reduce harmful emissions, such as waste from building demolition, plastics, asbestos, and non-woody waste. Non-segregated open burning is not an acceptable option for the management of MSW. Open burning subcategories include open burning of residential municipal (household) solid waste, land clearing waste, and yard waste (Steffen *et al*., 1998).

Incineration, is a common technique for treating waste, as it can reduce waste mass by 70 % and volume by up to 90 %, as well as providing recoverable energy to generate electricity (Lam *et al*., 2010). This process requires highly skilled personnel and careful maintenance. However, this method can be effective when other methods are not available. The main advantage of this method is to reduce waste volume for landfill sites. Moreover, the incinerator can be built close to the centre of waste generation, and hence can reduce transportation costs. The ash produced from this process can provide a low cost aggregate, and can help to decrease the greenhouse effect, as well as reducing the original volume of combustibles by 80 to 95 %. Incineration has some disadvantages, including high operating costs, the need for skilled staff, and the need for disposal of the remains in controlled and well-operated landfill sites, in order to prevent ground and surface water pollution (Rand *et al*., 2000). Generally, MSW incineration produces two main types of ash, bottom ash and fly ash (Lam *et al*., 2010). The

MSW incineration process has three main parts: incineration, energy recovery and air pollution control (Lee *et al*., 2007).

For incineration MSW is fed into the furnace continuously at a temperature of at least 850 ^{o}C and with a residence time of more than two seconds, aeration should be sufficient to ensure complete combustion of the waste and to prevent the formation of dioxins and carbon monoxide. To enhance combustion the three T's guideline requires: high temperature which increases burnout, increased turbulence which exposes more waste surface and increases burnout, and a longer residence time (Lam *et al*., 2010) as shown in Figure 2.7.

For energy recovery, the generated heat from the process is used to produce steam; then the steam drives the turbine to generate electricity. Air pollution is a major problem for incineration. Nowadays an advanced controlled system designed to decrease air pollution such as dry/wet scrubber (spray fine atomized slurry or lime powder into the hot exhaust gas to neutralise the gas) are used. Also incorporated into the process is the use of an activated carbon column (to absorb the heavy metals and organic pollutants such as Polychlorinated Biphenyls (PCB) and volatile organic compounds (VOC) in the exhaust gas (Chang and Ku, 1998) and non-catalytic reduction systems (used to remove nitrogen oxides by adding urea or ammonia for reaction to nitrogen, carbon dioxide and water). The bag filter system acts to filter and remove the fine particulates and dust particles (Tchobanoglous *et al*., 1993).

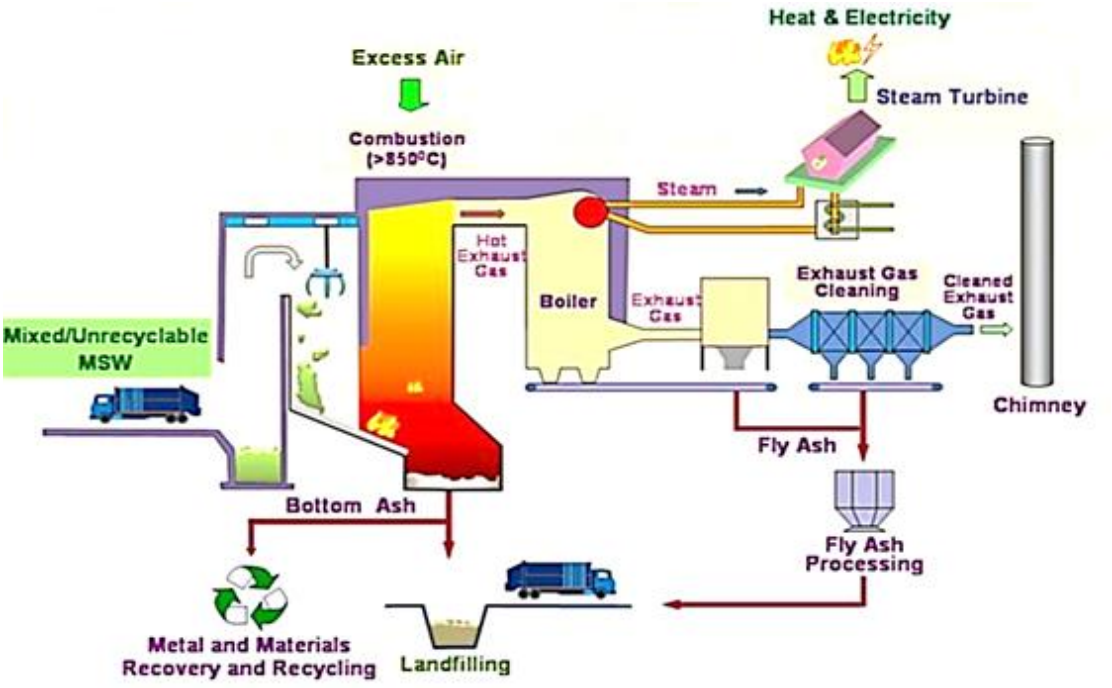


Figure 2.7 Schematic Diagram of the MSW incineration process published by Environmental Protection department, the government of the Hong Kong (Herraiz, 2009)

VI. Landfill

Disposal of MSW, through landfill sites or open dumping, is considered as the last resort for disposal of waste. The method for disposal depends on several factors, including the availability of facilities, the volume of the waste material, hydro geological characteristics, costs and national policies. There are some common disposal points, such as land disposal, uncontrolled tipping and controlled tipping sanitary landfill, disposal to water bodies, fresh water and lakes, coastal zone, marine areas (Ludwig *et al*., 2012). Sanitary landfill and dump sites are both important for reducing and eliminating health risks to the public, as well as risks to environmental quality. Most of these problems can be prevented by using soil to cover the dumping site. Moreover, this land feature will then act as a natural buffer between the landfill and the environment. For example, to prevent the risk of water contamination, adding clay can help due to the fact that it is fairly impermeable, because clay soil particles bind tightly to other particles. In

addition, the bottom and sides of landfills can be lined with a layer of clay or plastic to keep in the liquid waste, known as leachate, in order to prevent it from escaping into the surrounding soil. The leachate can be collected and pumped to the surface for treatment. Another method of protecting landfill sites from rain and wind exposure is to divide the landfill site into a series of individual cells. When the landfill cells have reached a specific capacity, they can be capped with an impermeable seal composed of clay and soil. Bioreactor landfill sites are considered to be a new technology, which is used to enhance the microbiology process for accelerating waste decomposition. This process includes adding re-circulated landfill leachate. Furthermore, there are many advantages that come from enhancing the microbial processes, like rapidly reducing the volume of waste, maximising the production and capture of methane, and reducing costs associated with leachate management (Ludwig *et al*., 2012). In addition, increase in recycling of municipal waste has led to a reduction in the percentage of municipal waste landfilled across Europe (See Figure 2.8).



Figure 2.8 Municipal waste landfilling rates calculated as a percentage of municipal waste landfilled per municipal waste generated in 32 European countries, 2001 and 2010 (Eurostat, 2012a; ETC/SCP, 2013a; ETC/SCP, 2013b; ETC/SCP, 2013c)

Note: The quality of the data has improved during the period 2001–2010.

2.2 Current and future applications for MSW

Nowadays industrial chemical synthesis needs to consider impacts that include energy use, economy and effects on the environment. So any commercial products must be produced with minimum energy requirement. One way to satisfy the above constraints is by using biochemical processes. These processes can be environmentally friendly, cost effective and carried out at ambient

conditions (Burgess *et al*., 2000). Examples are methane and biofuel production (Li *et al*., 2006) and bio-compost production from MSW (Hassen *et al*., 2001).

2.2.1 Methane gas production by anaerobic digestion

AD is a process that has attracted increasing attention in both developing and developed countries as a promising approach for the conversion of organic waste into biogas. AD is a biological treatment method that relies on microbial activity to digest the waste (Potts and Martin, 2009). It was originally used mainly to manage the accumulation of organic wastes and/or for organic fertiliser production, but the emphasis is now shifting to renewable energy generation. These facilities generally treat organic materials abundant in their geographical locations. Consequently, waste from farm animals is the predominant feedstock for AD in China, India and North America, wastewater in North America, while MSW and industrial food processing wastes are utilised in Europe, (Arsova, 2010; Levis *et al*., 2010) reported the presence of almost 120 plants in Europe between 1998 and 2008, with a total operational capacity of around 4.6 million tonnes per annum with the highest production in Germany Figure 2.9. The EU was the highest biogas producer in the world in 2012 and is predicted to maintain this position until 2022 as shown in Figure 2.10.

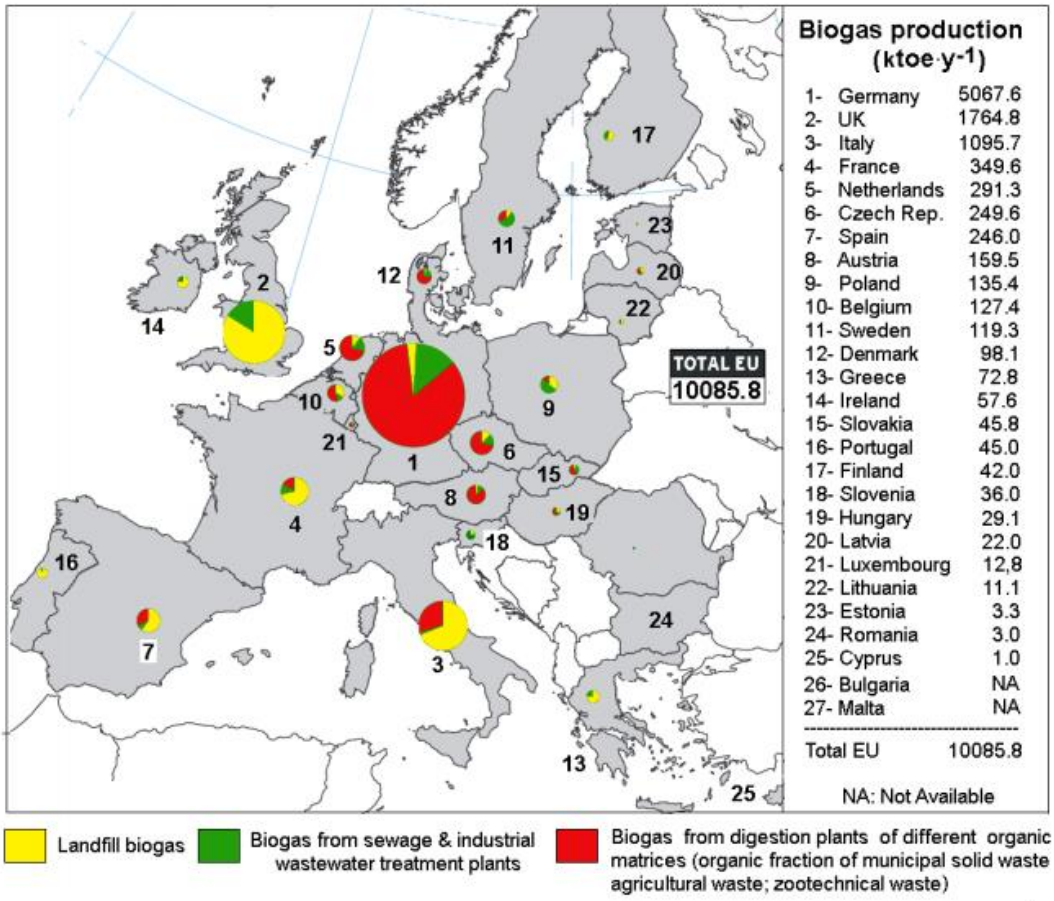


Figure 2.9 Primary energy production of biogas in the European Union in 2011 (ktoe /y) (Eurobserver, 2012)

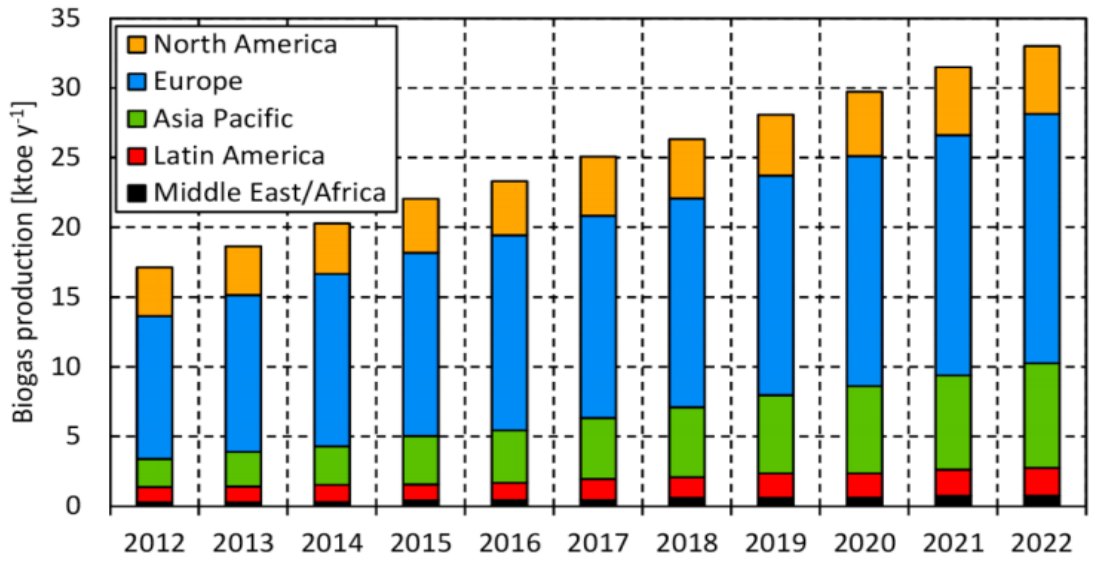


Figure 2.10 Biogas production at 2012 and predicted trend to 2022 in different areas of the world (Reserch, 2012)

In Europe, thermophilic digestion of MSW accounts for around 30-40 % of market share. The Hydraulic Retention Time (HRT) in thermophilic digestion is around 7-21 days (Rapport *et al*., 2012; Walker *et al*., 2012), which is shorter than that for mesophilic digestion 15-25 days (Rapport *et al*., 2012). Biogas productivity is higher in thermophilic digestion, ranging from 1.1-8.1 m^3/m^3d than in mesophilic digestion systems which range from 0.8-2.8 $m^3/m^3.d$ (Nasir *et al*., 2012; Rapport *et al*., 2012). The methane production yield in thermophilic digestion varies from 0.10 m^3/kg volatile solid (VS)(Güelfo *et al*., 2011) to 0.43 m^3/kg VS (Angelidaki *et al*., 2006). Those are similar to that reported in mesophilic digestion of MSW 0.11-0.3 m^3/kg VS (Nasir *et al.,* 2012). Although psychrophilic AD has been investigated for municipal wastewater treatment it has been rarely used for AD of MSW due to the low methane production yield and low organic removal rate (Mrowiec and Suschka, 2010).

Generally, an AD with over 20 % initial total solid (TS) loading is considered as a dry digestion. The initial moisture content in MSW varies from 0.9 to 80.6 % (Adhikari, 2006), depending on the source of the MSW and pre-treatment method. As most MSW have moisture contents of less than 80 %, process water is generally required to make up the designated solid loading rate. Dry digestion is usually operated in a plug flow reactor, which could have a solid loading rate up to 40 % (Vandevivere *et al*., 2003).

Regarding the impact of solid loading rate on methane gas production yield, Igoni (2006) developed a mathematical model and simulated methane production with solid load rates from 4 to 10 %. Within this range, the amount of biogas produced increased as a power function of the percentage of solid loading. Fernández *et al*., (2008), investigated the influence of substrate concentration on mesophilic AD of the OFMSW at a high solid loading range. The experiment was conducted in a batch reactor at 35 °C, during a period of 85–95 days at solid concentrations of 20 or 30 % TS. Their findings indicated that the reactor with 20 % TS achieved a higher yield of 0.11 m^3 CH_4/kg. VS removal compared to 0.07 m^3CH_4/kg for the

30 % TS reactor. In addition, the 20 % TS digestion provided the highest Dissolved Organic Carbon (DOC) removal of 80.69 %, compared to the 30 % TS digestion which had a DOC removal of 69.05 %.

Zhu *et al*. (2010) investigated the AD of MSW in a two-stage digestion system at an OLR of 9.2 gVS/L/d, and obtained around 3.5 L/L.d biogas with CH_4 yield of 0.19 L/gVS. Ramesh *et al*. (2009) carried out a two-stage AD of OFMSW using an acidogenic fermenter and an Up flow Anaerobic Sludge Blanket (UASB) reactor at 30 °C with short HRT of 6-24 hr. The volatile acids generated were then transferred to the UASB reactor for biogas generation. The maximum biogas production was 82.29 L/L.d with maximum Chemical Oxygen Demand (COD) removal efficiency of 86 %.

Also, Rao and Singh (2004) generated biogas in the range of 62–72 % with the overall average methane content of the biogas, applied food wastes emanating from fruit and vegetable markets, households, and hotels using batch digestion reactor, the experiment was carried out for 100 days at 26 ^{o}C degree.

In 2009, Trzcinski and Stuckey (2009) used continuous two-stage anaerobic membrane process for biogas (methane) production at mesophilic condition from OFMSW: Wastes from Kitchens (41 %); Gardens (11 %); and Paper Waste 48 %, the production yield was 69 %. Furthermore, Trzcinski *et al*. (2010) developed a three-stage system, including a hydrolytic reactor, an anaerobic membrane bioreactor for the treatment of leachate, and an aerobic membrane bioreactor for the treatment of permeate. Up to 75 % VS removal was achieved at an OLR of 4 gVS/L/day. The methane production yield was 208 mL CH_4 STP/g VS.

Typically, anaerobic reactors of solid waste can be categorized into several categories: mainly dependant on the moisture content, feeding mode and temperature, also depends on reactor shapes (vertical, horizontal rectangular or cylindrical) and mixing methods (gas injection or mechanical stirrers) (See Table 2.1).

The characteristics of some of the principal technologies used around the world for biogas production are shown in Table 2.2. These include the Kompogas (one stage dry system), Valorga, RosRoca, BTA (Biotechnische Abfallverwertung GmbH) (one stage wet system), Dranco, Cites and Linde (two stage dry system) and Schwarting_Uhde (two stage wet system) each technology gave various biogas production (Nayono 2010).

Table 2.1 Types of AD Reactors for biogas production (Griffin, 2012)

Reactors types	Advantage	Disadvantage	Number of reactors
Single stage system	-More predominant AD applied in full scale for OFMSW	Energy production is lower	The digestion process occur in one reactor vessel
	-Suffer less frequent technical failures	The waste can cause clogging in pipes in single-stage,	
	-Smaller investment costs and all of the bacteria existed in the same volume and the environmental conditions are kept at equilibrium	wet digesters	
Two or multiple Stage system (Sequential system)	Two stages are different in the viewpoint of nutrient level, growth capacity and activities in various environment background.	More complex and expensive	This system are filled once with fresh substrate or digestate sample, with or without stirring method,
Batch Process and continuous process.	-Simple -Low tech -Robust Reliable process due to niches and use of several reactors.	Large tank volume required due to needs longer RT the low organic loading rate and the formation of a scum layer which used only 1/3 of the tank volume	Divided into single stage, sequential stage and hybride batch –UASB.

Table 2.2 Operating conditions, biogas production rate and total capacity of the most commonly used AD processes (Arsova, 2010)

AD technology	Retention time days	Biogas production Nm^3/Mg of feed	Total annual biogas production Nm^3
Kompogas (Continuous single step)	15-20	100	53,350,000
Valorga (Horizontal Plug flow)	18-25	80-160	263,640,000
BTA (Single stage)	12 to 17	85-95	27,045,000
Deanco (single stage)	20	100-200	94,050,000

Biogas production can be carried out using sole or co-digestion with other substrates, (Co–digestion or Co-Fermentation) this process includes mixing and treating different waste materials together. The successful key factor for co-digestion is balancing macro and micro nutrients (Nayono, 2010). Co-digestion has the following advantages: it improves biogas yields due to positive synergisms established in the digestion medium, it supports the process by adding missing nutrients, it helps to establish the required moisture content and, finally, it makes handling mixed waste easier (Khalid *et al*., 2011); for example MSW has high total solids content and is rich in various carbon sources, which makes it a potentially ideal substrate to be co-digested with nitrogen-rich but low total solids content waste streams. The co-digestion process is generally considered to improve nutrient balance, improve process stability, dilute potentially toxic compounds, allow for a higher Organic Loading Rate (OLR) and thus overall and leads to increased methane production (Sosnowski *et al*., 2003). The most common types of Co-substrate may help to improve OFMSW characteristics are: **Sewage sludge**, which contains micro and macro nutrients which leads to a better Carbon/Nitrogen Ratios (C/N) and facilitates the adjustment of moisture content (Hartmann *et al*., 2003); **Animal Manures (AM),** which have many beneficial points, making them suitable for AD, such as abundance and high buffering capacity, mainly due to the levels of ammonia.

Furthermore, AM has a low TS content, which can be used to adjust the moisture of OFMSW. Animal manure also contains a wide variety of nutrients, which are necessary for optimal bacterial growth (Nayono, 2010); and **Organic industrial wastes**: That can have a stimulatory effect on synthesis of methane (Ağdağ and Sponza, 2007).

One of the earliest trials on co-digesting OFMSW with sewage sludge was carried out by Cecchi and co-workers in 1988 (Cecchi *et al*., 1988). Following Cecchi's study, several researchers repeated this experiment with their local MSW and sewage sludge (Sosnowski *et al*., 2008; Dereli *et al*., 2010). Furthermore, another investigation carried out using co-digestion of MSW with Fat, Oil and Grease (FOG) wastes from a sewage treatment plant (Martín-González *et al*., 2010). Compared with the sole digestion of MSW, the co-digestion of MSW and FOG enhanced methane yield from 0.36 to 0.49 LgVS. In addition to sewage sludge, MSW has been widely co-digested with wastewater (Elango and Pattnaik 2007, Shanmugam and Horan, 2009), fruit and vegetable waste (Gómez *et al*., 2006), farm slurry (Callaghan *et al*., 1999) and animal manure. Some key findings on AD of MSW for biogas production have been summarised in Table 2.3.

Table 2.3 Applying MSW for biogas production using AD processes (Gunaseelan 1997)

Feed	Fermenter	Temperature °C	HRT (days) (days)	OLR Kg/VSm/d	CH_4 yield m^3/kg vs	References
MS-OF MSW	Laboratory plant	50-55	16-21	10	0.286	
30-35 % TS	Dranco process			12.1	0.282	(Baere *et al*., 1986)
				13.2	0.283	
MS-OF MSW	Pilot plant					
81 % TS	SEBAC process	55	42	3.2	0.19	(O'Keefe *et al*., 1993)
			21	6.4	0.16	
MS-or MSW fresh	Pilot plant	55.8	5.7	17.8	0.192	
16-22 % TS	semi- dry fermentation	55.5	7.4	12.9	0.215	(Mata-Alvarez *et al*., 1993)
		56.2	11.7	9.7	0.179	
MS-or MSW pre-composed	Pilot plant	51.5	6.1	19.9	0.131	
16-22 % TS	semi- dry fermentation	54.6	7.8	13.5	0.159	
		54.8	11.7	6.9	0.254	
MS-OF MSW 17 % TS	CSTR pilot pant semi dry fermentation	55	8.5	13.4	0.188	(Cecchi *et al*., 1993)
MS-OF MSW Algae 9.1 % TS	CSTR pilot pant semi dry fermentation	55	7.5	13.4	0.212	
MS-OF MSW	CSTR	35	16.2	4.1	0.145	
Pre-composed			32	3.4	0.162	(Mata-Alvarez *et al*., 1990)
			15.6	6.8	0.114	
			21.3	4.8	0.14	

2.2.2 Running an anaerobic digestion process

The process is divided into four stages, in each stage there are different microorganisms that can decompose OFMSW and produce various products (Arif, 2010), as shown in Figure (2.11).

A. Hydrolysis

This is considered an important step in the anaerobic biodegradation process, especially for green waste, which is usually composed of cellulosic and lignocelluloses materials (Wang *et al*., 2009). Methane yield directly depends on the efficiency of the hydrolysis reaction. The rate of hydrolysis is affected by a number of factors, for example pH, temperature, concentration of hydrolytic bacteria and physiochemical properties of the organic substrates (Neves *et al*., 2006). Hydrolytic bacteria are heterogeneous, obligate and facultative anaerobic bacteria strains (Rilling, 2005). These bacteria are able to achieve the depolymerisation of organic polymers (Arsova, 2010).

In this step, the main components in MSW such as cellulose, hemicelluloses and lignin start to decompose. These are biodegradable and make up more than 90 % of the biochemical methane potential of the MSW, while phenolic groups in lignin are inhibitory to the enzymatic effects. **Cellulose**, the celluloytic enzyme system is composed of endoglocanases, exoglucanases and glucosidases. The main anaerobic bacteria for degrading cellulose include *Bacterioides succinogenes, Clostridium lochhadii, Clostridium cellobioporus, Clostridium thermocellum, Clostridium stercorarium, Ruminococcus flavefaciens, Ruminococcus albus, Butyrivibrio fibrosolvens,* and *Micromonospora bispora* (Palmisano and Barlaz 1996). **Hemicelluloses**, this has a simpler structure and is more readily degraded than cellulose. Despite that, it still requires a complex enzyme system for depolymarisation due to the various monomers that comprise it. The predominant bacteria found to degrade the hemicelluloses in rumen are *Bacterioides ruminicola. B. fibrisolvens, Ruminococcus flavefaciens,* and *R. albus.*

Pectin is considered to be another important cell wall polymer, so several *Clostridium* species have been identified as pectinolytic as well as rumen bacteria such as *Bacteroidesrumenicola,* and *Streptococcus bovis* (Palmisano and Barlaz, 1996). **Lignin**; this is a highly branched aromatic polymer whose depolymerisation leads to monomers that can be used by the anaerobic bacteria in several anaerobic energy-yielding processes. These include oxygenic photosynthesis, denitrification, sulphate reduction, fermentation and methanogenesis. However, it is still doubtful whether lignin can be depolymerised to its monomers under AD conditions (Arsova, 2010). **Proteins and lipids**; these both serve as a source of energy in MSW substrates. Protein is the main source for carbon, nitrogen and energy for bacterial growth, and is hydrolysed by protolytic enzymes within specialized groups of anaerobic bacteria such as *Clostridium perfringens, C. bifermentans, C. histolyticum,* and *C. sporogenes*. These are responsible for protein degradation in digesters (Palmisano and Barlaz, 1996). In addition to these organisms, numerous other species of anaerobic bacteria, such as *Bacterioides, Butyrivibrio, Fusobacterium, Slelnomonas Peptococcus, Campylobacter* and *Streptococcus,* are capable of depolymerisations of the proteins to amino acids and, moreover, to acetic, propionic and butyric acid (Gill *et al*., 2014). Meanwhile, **lipids** are catalysed by esterase enzymes, leading to saturated and unsaturated long chain fatty acids and glycerol, which are more easily metabolized by bacteria. Anaerobic microorganisms are capable of decomposing lipids usually found in MSW. These include *Anaerovibrio lipotyca* and *Syntrophomonas wolfei* (Palmisano and Barlaz, 1996). Also, various species of *Clostridium* and *Micrococcus* are able to degrade lipids. **Starch;** some of the microbes found in anaerobic digesters which are able to degrade starch are *Streptococcus bovis, Bacteriodes amylophilus, Succinomonas amylolytica, B. ruminocola*, and a number of *Lactobacillus* species (Palmisano and Barlaz, 1996).

B. Fermentation (Acidogenesis)

In the second stage, hydrolysed compounds are fermented into volatile fatty acids (acetic, propionic, butyric, valeric acids), neutral compounds (ethanol, methanol), and ammonia. The microorganisms responsible for biological conversion of carbohydrates into CO_2 and H_2 are obligate anaerobic bacteria and facultative bacteria. The specific concentration of products formed in this stage differ according types of bacteria, and culture conditions (temperature and pH) (Arsova, 2010).

C. Acetogenesis or obligatory hydrogen producing Acetogens (OHPAs) S-oxidation.

In this stage, the products of hydrolysis are processed and simple molecules (propionate, butyrate, ethanol), formed from acidogenesis are further digested to hydrogen, carbon dioxide, and acetate. For example, hydrogen plays a specific role in AD reactions, by inhibiting the oxidation process, especially when hydrogen partial pressure is low enough to ensure thermodynamic feasibility and conversion of all acids. For this reason, they live in close symbiosis with methanogenic and sulfidogenic bacteria, which use hydrogen as an energy source (Sahm, 1981), As a result, the concentration of hydrogen is an indicator of the health of the digester (Mata-Alvarez, 2003).

D. Methanogenesis

Is the last stage of AD, which includes the production of methane from the raw material, formed in the previous stage. Methane is produced from a number of simple substances, including acetic acid, methanol or carbon dioxide and hydrogen. The most important source for methane production (75 %) is acetate (Evans, 2001).

The microorganisms responsible for methanogenesis can be divided into two groups. Firstly, the acetate consuming bacteria, which utilize acetic acid known as

acetoclasic methanogenesis. Secondly, hydrogenotrophic bacteria which utilise hydrogen and carbon dioxide (Rilling, 2005).

Methane producing bacteria can decompose compounds that have a low molecular weight by utilizing hydrogen, carbon dioxide and acetic acid to form methane and carbon dioxide (Rilling, 2005). The most common methanogensis genera in the biogas reactors are *Methanobacterium*, *Methanothermobacter* (formerly *Methanobacterium*), *Methanobrevibacter*, *Methanosarcina*, and *Methanosaeta* (formerly *Methanotrix*) (Arsova, 2010).

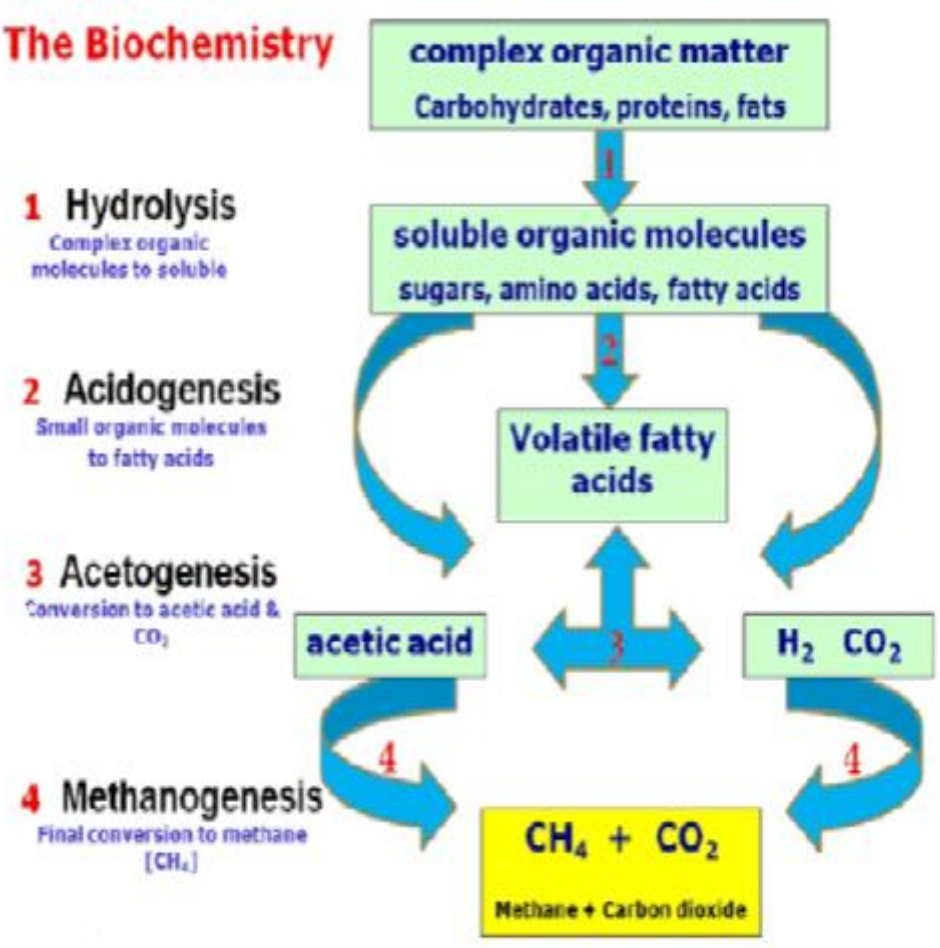


Figure 2.11 Schematic representation of the main conversion processes in anaerobic digestion (Amaya *et al*., 2013)

The main product from this process is biogas, which is including methane (CH_4) 55-60 %, carbon dioxide (CO_2) 35-40 %, water (H_2O) 2-7 %, hydrogen sulphide (H_2S) 20- 20,000 ppm (2 %), ammonia (NH_3) 0-0.05 %, nitrogen (N_2) 0-2 %, oxygen (O_2) 0-2 %, and hydrogen (H_2) 0-1 %. However, the composition of biogas depends upon the feed material (Vij, 2011). The biogas requires further treatment to get an appropriate gas ratio. This is especially true of the ratio of hydrogen to carbon monoxide (Boerrigter and Rauch, 2006). Some examples of purification include sulphates in solid waste, which help to produce H_2S in the biogas. The systems being used to remove H_2S from biogas are based on chemical, bio-chemical or physical processes. Furthermore, there are many other impurities which could affect gas quality, depending on the type and composition of the feedstock and the type of gasification process and gas purification process. Gas post-treatments are divided into removing particles, organic impurities (i.e., tar and hydrocarbons), and inorganic impurities (metal halides, and nitrogen and sulphur-containing compounds) (Boerrigter *et al*., 2004). There are some handling methods for following impurities.

The other major product is the residual digestate. The digestate usually requires sanitising before it can be applied as a fertiliser or soil amendment. However, the quality of digestate depends upon the quality of the feedstock, the method of digestion the extent of post-treatment, and the energy consumption in fertiliser manufacturing (Monnet, 2003). The main cause of problems in the digestate is the presence of inorganic contaminants (heavy metals), organic contaminants and lack of nutrients like nitrogen, phosphorus and potassium (NPK). In general, the digestate contains fibre and liquor that has to be separated (Chaudhary, 2008). There are some popular methods for treatments:

Wet process: The product can be spread directly onto farmland or indirectly by separating the product into solid and liquid fractions. The solid fraction can take about two to four weeks to mature into dry and fully stabilized compost. Either the liquid fractions may be recycled for the dilution of fresh waste and

implemented directly to farm Land as a liquid fertiliser, or else sent to a waste water treatment plant (Chaudhary, 2008).

Dry process: The digestate material is usually dewatered and matured to compost (the composting of digestate is done to get a high quality product as a result of the breakdown of organic components, fixing mineral nitrogen into the humus, and reducing nitrogen loss). The liquid part which is produced from AD process is recycled in order to moisten and inoculate the raw incoming MSW. The most common methods that can be used for the dry process are screw press, wire presses, centrifugation, decanter, and cyclone. However, the fibre that is produced is bulky and contains low levels of plant nutrients, so it can be used as soil conditioner. Nutrients in the liquor digestate consist of a large proportion of nutrients through conventional irrigation methods (Chaudhary, 2008).

2.3 Second generation biofuels

2.3.1 Bio-energy and Biomass

Bio-energy is energy derived from biomass. It is an attractive, controllable and storable form of renewable energy (Bridgwater, 2003). Bio-energy can be generated by biological, chemical or physical processes. Energy derived from sunlight is stored as chemical bonds between carbon, hydrogen and oxygen atoms. These bonds can be broken down by digestion, combustion or decomposition to release the stored energy (McKendry, 2002).

The difference between bio-energy and biomass is that "biomass is considered a source of organic matter for the production of bio-energy, while bio-energy is a renewable energy produced from organic matter through the conversion of complex carbohydrate" (Morton and Brown, 2010). Depletion of low extraction cost fossil fuel reserves, and climate change have converged to create a requirement to develop more sustainable energy systems based on renewable lignocellulosic biomass (Agbor *et al*., 2011).

There are many resources for the production of bio energy such as forestry crops and residues (Klass, 1998). Agricultural crops and resources are another source of bio-energy production because they do not reduce available farmland and are readily available. In addition, corn and wheat residues left in the field could potentially be collected and combusted to produce energy (McKendry, 2002). Sewage sludge is an organic material produced during activated sludge waste water treatment. Sewage sludge is also considered to be a reusable biomass as it contains organic material and micro-organisms (Rulkens, 2007). Industrial residues such as those produced by the petroleum industry, can be used to successfully produce bio-diesel from waste oils, bio-diesel can be used as a biofuel for vehicles (Ramadhas *et al*., 2004). MSW is an excellent source for production of bio-energy. Food and paper are the major biomass components present in MSW. A number of biological or thermal processes are used in order to recover and produce energy, for example the 'average gas recovery rates ranges from 120 to 150 m^3/ton of dry MSW equivalent to a heat value of 2500 m^3/ton" by AD process (Rosillo-Calle, 2012).

2.3.2 Types of biofuel productions from various substrates

The various forms of bio-fuel are described below:

I. Liquid bio-fuels

Ethanol C_2H_5OH (ethyl alcohol) can be made through fermentation of sugars, in particular glucose. Ethanol is using for accelerating the energy efficiency of vehicle engines, and is less flammable and less effect than petrol on the environment, avoiding problems from oil spills, because ethanol contains oxygen. In addition, it has low carbon emissions.

Bio diesels (methyl esters), is produced with almost no sulphur and, as a result, can reduce the level of sulphur emissions to the atmosphere. To get bio-diesel fuels, the reaction requires a catalyst; usually a strong base such as sodium or potassium hydroxide. This reaction produces new chemical compounds

called methyl esters. The benefits of using biodiesel are that it produces less global warming than fossil fuels. It does so by lowering carbon monoxide emissions, unburned hydrocarbons, particulate matter, and air toxins (Van, 2005).

Methanol (CH_3OH) called methyl alcohol or wood alcohol; this is considered to be the simplest alcohol fuel. It is a light, volatile, flammable liquid; biodegradable and less toxic than other biofuels. It can be used as a fuel, blended with gasoline in internal combustion engines and for fuel cell vehicles. Anaerobic metabolism can naturally produce methanol. Furthermore, modern methanol production can be very efficient through the use of catalysts capable of working under low pressure. Nowadays, the global methanol production capacity is about 40 M ton/year, while the actual production or demand is about 32 M tonne (Hamelinck and Faaij, 2002).

II. Biogas

Biogas is comprised mainly of methane and carbon dioxide which are produced by AD. Biogas with high levels of methane can be used for heating or electricity generation, and the gas that is produced from conversion of animal waste to methane can bring significant environmental and health benefits (Pettus, 2009).

Electricity produced from biomass may be regarded as more environmentally acceptable due to carbon dioxide being sequestered when the biomass crop grows (Carpentieri *et al*., 2005). Electricity production from biomass can be produced from various technologies *such* as: direct combustion, co-firing, gasification, pyrolysis, and AD. These technologies are dependent on the source (plant or animal) and the physical state (solid, liquid, gas) of the biomass (Bauen *et al*., 2004). Bioelectricity production provides a number of advantages; it can use available biomass which can be stored so the electricity can be generated on demand. However, drawbacks include the fact that biomass has lower energy densities than fossils fuels, and has higher transportation costs (Paolo, 2004;

Monforti-Ferrario *et al*., 2010). Hydrogen can be produced using a variety of sustainable processes, like the biological or chemical reforming of bio methane. The potential biological process for direct hydrogen production include bio photolysis of water by algae, dark and photo-terminative and two stage dark/ photo stage in AD (Williams, 2007). Biomass gas is produced by heating biomass in low oxygen, high temperature environments in order to break it down and to release a flammable energy which is rich in synthesis gases or syngas (Cherubini, 2010).

III. Solid bio fuels

Can be divided into woody biomass, herbaceous biomass, fruit biomass and a mixture or blend of these three (Alakangas, 2010). The solid bio fuels typically derive from wood, sawdust, grass cuttings, charcoal, agricultural waste, non-food energy crops, and dried manure. Solid biofuel energy is mainly used for electricity production and heating. Biomass can be burnt directly in a stove or furnace to supply heat or steam, provided the raw biomass is in a suitable form. However, it can also be shaped into pellets and burned in a pellet stove when solid biomass is in a less convenient form, such as sawdust (Fitzmorris *et al*., 2009).

2.3.3 Pre-treatment of the substrate for biofuel production

The best and most effective pre-treatments are those that require no reduction in particle size, preserve the pentose (hemicellulose) fraction, avoid formation of possible inhibitors of hydrolytic enzymes and fermenting microorganisms, minimise energy use, have low running costs (operating costs, capital costs, and biomass costs), low catalyst cost, consume of little or no chemicals and which use cheap chemicals (Palmqvist and Hahn-Hägerdal, 2000; Taherzadeh and Karimi, 2008). Pre-treating lignocellulosic material aims to produce a material that is

more reactive than the original material and can produce soluble fermentable sugars (Hendriks and Zeeman, 2009).

Several methods have been introduced for pre-treatment of lignocellulosic material prior to enzymatic hydrolysis or digestion. These methods are classified into: physical pre-treatment, physico-chemical pre-treatments, chemical pre-treatments, and biological pre-treatments (Fan *et al*. 1982; Wyman, 1996).

2.3.3.1 Physical pre-treatments

Physical pre-treatment includes size reduction such as chipping, shredding, grinding, and milling. These methods have been used to enhance the digestibility of lignocellulosic biomass (Palmowski and Miller, 2000). Harvesting and preconditioning reduces lignocellulosic biomass from logs to coarse sizes of about 10–50 mm, chipping reduces the biomass size to 10–30 mm the process reduces heat and mass transfer limitations. Grinding and milling can further reduce the particle size to 0.2–2 mm and these processes can start to impact cellulose crystallinity. However, it has been shown that further reduction of biomass particle size below 40 mesh (0.4 mm) has little effect on the rates and yields of biomass hydrolysis (Chang *et al*., 1997). Reduction of particle size hasn't been studied widely in terms of hydrolysis (Nguyen, 2000), but some studies have shown that milling increases biogas, bioethanol and bio-hydrogen yields. Using milling will increase the cost of production, however, milling is still seen as economically feasible (Hendriks and Zeeman, 2009).

I. Thermal pre-treatments

Are applied in order to solubilise the hemicellulose thereby improving rates of hydrolysis of lignocellulose material (Harmsen *et al*., 2010). There are various thermal pre-treatment methods such as use of steam (~ 240 °C and high pressure for a few minutes) (Hendriks and Zeeman, 2009), steam explosion (a rapid release in pressure which causes a disruption in the structure of the

material) (Hendriks and Zeeman, 2009), liquid hot water treatment (where water is maintained as a liquid at high temperatures (160 to 230 °C) and under high pressures (>5 MPa) (Weil *et al*., 1998; Carvalheiro *et al*., 2008; da Costa *et al*., 2009).

As the temperature rises above 150–180 ºC, hemicellulose and lignin become solubilised (Garrote *et al*., 1999). There are two main components of hemicelluloses (xylan and gluco-mannans), the xylans are the least thermally stable when compared with gluco-mannans. If temperatures during pre-treatment exceed 180 ºC, an exothermal reaction (probably solubilisation) of the hemicellulose begins (Beall and Eickner, 1970), the thermal reactivity mainly depends on the composition of the lignocellulosic biomass and has an influence on the temperature at which this exothermal reaction begins (Hon and Shiraishi, 2000).

Generally, during thermal processing, the hemicellulose portion of the plant cell wall becomes hydrolysed and forms acids which catalyse the further hydrolysis of the hemicellulose (Gregg and Saddler, 1996). Furthermore, thermal processes can cause an increase in the Crystallinity Index (CrI) of cellulose, though no increase was observed when the CrI was already high (Weimer *et al*., 1995).

The thermal pre-treatments also produce phenolic compounds as a result of the solubilisation of lignin, these phenolic compounds have been shown to be toxic or inhibitory to the growth of bacterial, yeast and methanogens/archae (Gossett *et al*., 1982). Furthermore, if these phenolic compounds are not removed quickly they have been shown to re-condense as a precipitate onto the biomass (Liu and Wyman, 2003). Use of severe pre-treatment conditions enhances the condensation and precipitation of solubilised lignin compounds (Negro *et al*., 2003).

Steam pre-treatment is characterised by the use of a large vessel, steam at temperatures up to 240 ºC and high pressure, moisture content of the biomass

during pre-treatment with steam is an important factor, for example the higher the moisture content, the longer the optimum time required for steam pre-treatment (Brownell and Saddler, 1987). Steam pre-treatment has been shown to solubilise a fraction of the hemicelluloses, this process is referred to as 'auto-cleave'. A common term used in steam pre-treatments is the so called 'severity factor' (log R0), which is a measure for the severity of the pre-treatment (Overend *et al*., 1987; Hendriks and Zeeman, 2009).

Steam-explosion is the most commonly used pre-treatment method, the process includes injecting high pressure saturated steam into a reactor, as a result leading to the temperature rising to 160-260 ºC. Pressure is suddenly reduced and the biomass undergoes an explosive decompression which lead to the destruction of the fibre structure decreasing crystallinity of the cellulose and increasing substrate surface area (Zhu *et al*., 2005).

Liquid Hot Water (LHW) biomass pre-treatment is a hydrothermal process which does not require addition of chemicals, the process uses water under high pressure which has been shown to penetrate into the biomass, hydrate cellulose, and remove hemicellulose and part of the lignin, this makes the process cheap and more applicable industrially (Mosier *et al*., 2005). Use of hot water also reduces the requirement for reducing the size of the lignocellulosic prior to pre-treatment and produces lower amounts of neutralization residues. In this process, hemicellulosic carbohydrates are dissolved as liquid-soluble oligosaccharides and can be separated from insoluble cellulosic and lignin fractions (Taherzadeh and Karimi, 2008). This process increases enzyme accessibility by increasing surface area of the cellulose (Zeng *et al*., 2007). Pre-treatments with steam and LHW are both hydrothermal pre-treatments characterised by higher pentose recovery and lower formation of inhibitory components (Dien *et al*., 2006).

II. Mechanical pre-treatment (MPT)

MPT is a process which includes waste sorting and homogenisation, and is followed by biological treatment (Visvanathan *et al*., 2005). The method depends on stressing the substrate cell wall without addition of any chemical substances (Hartmann *et al*., 2000; Li, 2012). The MPT can break down the crystalline structure of cellulose thus increasing the reactant surface area following fine milling (nano-milling) (Zhu *et al*., 2005). Other pre-treatments using mechanical treatment are: High Pressure Homogenisation (HPH), stirred ball mills, and the jetting and colliding method (Engelhart *et al*., 2000; Toreci *et al*., 2010).

Autoclaving is a heat treatment. The autoclave is an instrument using high temperatures, and pressure. This process has been applied previously to sterilise hospital wastes and some animal wastes (Stringfellow *et al*., 2011). The process as applied on MSW is a relatively recent innovation and the commercial process is shown in Figure 2.12.

The main functions of autoclaving unsorted MSW include destroying bacteria, reducing the size of waste by 60 %, reducing moisture content, removing recyclable materials from the waste stream and, finally, increasing the quality of recyclable metals (by stripping away label glue from food cans and heat shrink packaging). However, heat can have an adverse effect on some recyclable plastics, such as polyethylene terephthalate (PET) and high density polyethylene (HDPE) (Barton, 1983; Glenn, 1991).

Autoclaving is conducted within a large, enclosed vessel. The process consists of collecting waste from resources, followed by injecting unsorted MSW automatically into the autoclave (Papageorgiou *et al*., 2009). To run any autoclaving method, some points need to be taken into consideration such as pressure (6.2 bar is maintained for between thirty and sixty minutes). Aeration can be supplied via a blower directed from the bottom through the material, and gasses are collected at the top in order to analyse them. Steam is injected at pressure and the temperature increased to 130 ^{o}C. This temperature is

considered to be the optimal temperature for pre-treatment of total solids when compared to 160 ^{o}C and 200 ^{o}C (Stentiford, 2010).

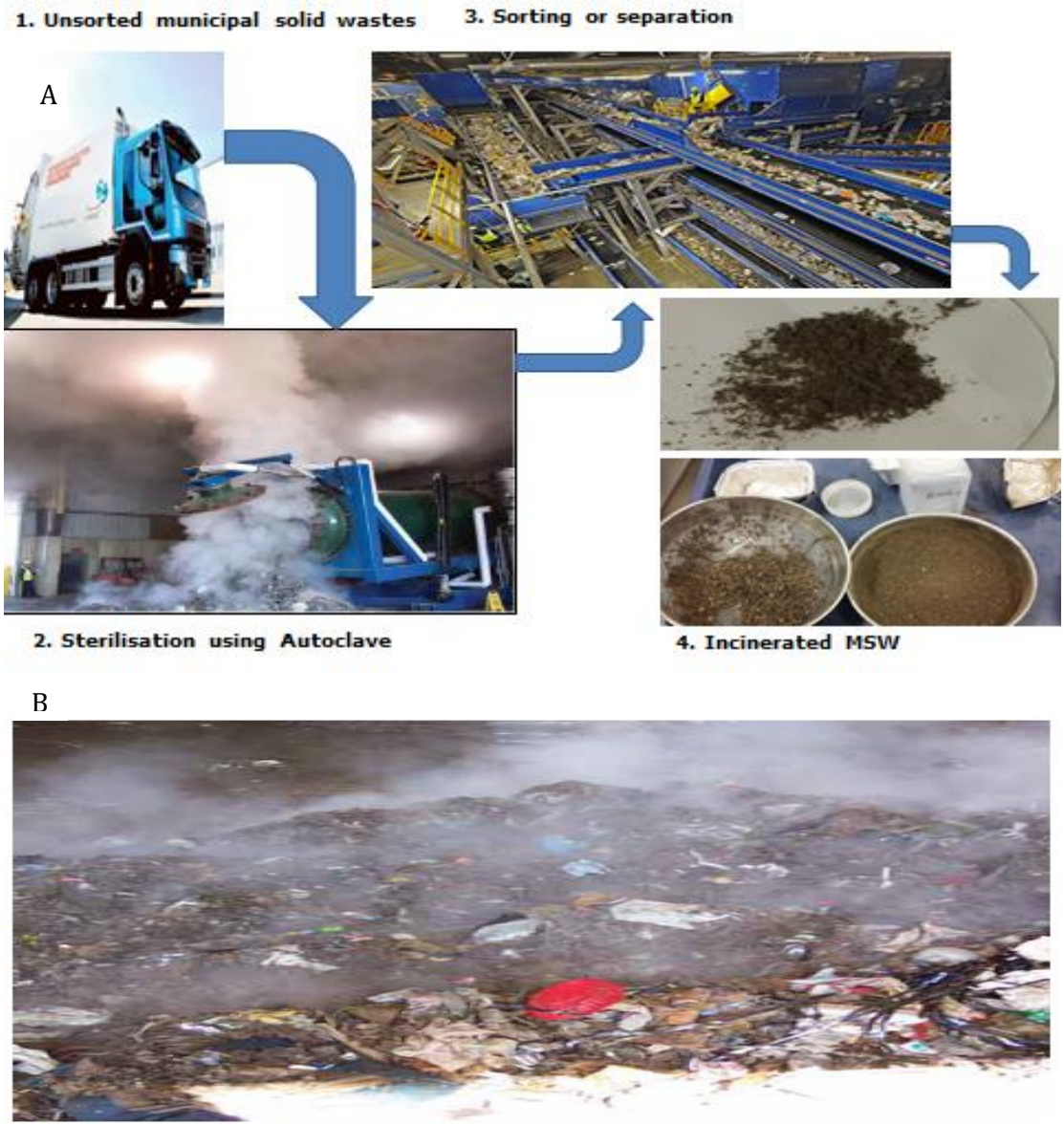


Figure 2.12 Process of Autoclaving MSW using autoclave in a current study (A) the autoclaving process (B) autoclaving process output

2.3.3.2 Chemical pre-treatments

There are many chemical substances which can be used for the pre-treatment of biomass such as oxidizing agents, alkali, acids and salt (Mtui, 2009).

2.3.3.2.1 Acid hydrolysis or pre-treatment

Acid hydrolysis is one of the most common methods used for the pre-treatment of lignocellulosic biomass to attain higher sugar yields (ye Lee *et al*., 2013). The main goal of this method is to solubilise the hemicellulosic fraction of the biomass, which increases the accessibility of the enzymes to the cellulosic fraction (Alvira *et al*., 2010).

Either dilute or strong acid can be used in this type of pre-treatment to hydrolyse hemicellulose and solubilise or precipitate lignin (Hendriks and Zeeman, 2009). With regards to suitable acid concentration for releasing sugar from the substrate some studies have revealed that 0.5 % H_2SO_4 is an optimal acid concentration for treatment of waste from vegetables and rice straw (Karimi *et al*., 2006). While, higher acid concentrations up to 2.5 M is capable of separating lignin and other organic components (Miller and Hester, 2007; Rahman *et al*., 2007).

Weak acid hydrolysis (dilute acid treatment)

The most efficient pre-treatment for lignocellulosic substrates with low lignin content is dilute acid which offers a good recovery in terms of sugar. The aim of this process is to remove hemicelluloses thus increasing porosity and as a result improving enzymatic digestibility (Chen *et al*., 2007). Some disadvantages are the further degradation of hemicelluloses sugars which can be corrosive and degraded further to furfural and hydroxymethyl furfural (HMF), these compounds are strong inhibitors of microbial fermentations (Harmsen *et al*., 2010). Indeed for some years dilute sulphuric acid has been added to biomass to manufacture furfural commercially (Zeitsch. 2000). The production process hydrolyses

hemicelluloses to xylose then condenses the xylose into furfural which is recovered by distillation (Mosier *et al*., 2005).

Various acids can be applied for pre-treating different lignocellulosic substances including dilute sulphuric acid (Gil *et al*., 2010), dilute nitric acid (Salvi *et al*., 2010), dilute hydrochloric acid (Kurakake *et al*., 2005), dilute phosphoric acid (Goshadrou *et al*., 2011), peracetic acid (Zhao *et al*., 2009) and dilute oxalic acid (Scordia *et al*., 2011).

Among these, the most commonly applied is dilute sulphuric acid due to its availability, cost, safety and low environmental concerns (Chandel *et al*., 2011). Pre-treatment with sulphuric acid helps to achieve high yields of xylose from xylan (Da Costa *et al*., 2009). and increases the enzymatic digestibility of cellulose (Tucker *et al*., 2003). However, use of sulphuric acid produces inhibitors such as furfural (Dagnino *et al*., 2013), dilute acid pre-treatments have also been found to be suitable for a wide range of feed stocks including softwood, hardwood, herbaceous crops, agricultural residues, waste paper and MSW (Chung *et al*., 2005).

However, use of dilute acid has some drawbacks such as corrosion, the need for neutralisation before the fermentation process, formation of degradation products and the acids or chemical price should be taken into consideration (Singh *et al*., 2015). Dilute acid pre-treatments can increase the cellulose conversion by enzymes to sugar but doesn't fully remove lignin which precipitates on the cellulose surface and may inhibit the hydrolysis process (Zhang and Lynd, 2004). To decrease the negative impact of the lignin on the Enzyme Hydrolysis (EH) process some studies have added surfactants such as Tween-80, dodecylbenzene sulfonic acid and polyethylene glycol 4000 (PEG, 4000), to acid pre-treated corn Stover biomass at 140–220 ^{o}C. The Presence of these surfactants enhanced lignin removal and improve cellulose digestibility by increasing the hydrophobicity of the biomass (Qing *et al*., 2010).

To further improve digestibility dilute acid pre-treatments can be combined with other pre-treatments such as combining acid and alkaline pre-treatments (strong acid–strong alkali or weak acid–weak alkali), these combined pre-treatments have been shown to remove most of the non-cellulosic materials (Kim *et al*., 2012). Generally there are two types of weak acid hydrolysis:

- High temperature and low-solids loading (T>160 °C, 5-10 % wt substrate concentration).
- Low temperature and high-solids loading (T≤160 °C, 10-40 % substrate concentration) (Harmsen *et al*., 2010).

Strong acid hydrolysis; Concentrated H_2SO_4 and HCl have been used for treating lignocellulosic substrate due to their ability to directly hydrolyse cellulose and thus not require any use of enzymes (Sun and Cheng, 2002). This method has some advantages such as high monomeric sugar yield and mild temperatures are required. However, drawbacks for this process are the corrosive nature of the acid and the need to recycle acid in order to lower costs, toxicity, and the requirement for expensive construction materials (Talebnia *et al*., 2010). Some companies have commercialised the use of strong acids for microbial fermentation purposes (Harmsen *et al*., 2010).

2.3.3.2.2 Alkaline hydrolysis or pre-treatment

For alkaline pre-treatments, the most common chemicals used are calcium and sodium hydroxide for solubilising lignin (Da Costa *et al*., 2009). Sodium hydroxide (NaOH) is mainly used because it is a safer chemical substance and can be recycled (Chang and Holtzapple, 2000). However, it is expensive and needs to be removed because of salt production (González *et al*., 1986). This method has not been applied industrially (Agbor *et al*., 2011). Sodium hydroxide has received the greatest attention due to its outstanding delignification capacity which is essential to achieve high biomass digestibility (McIntosh and Vancov

2011). The main goal of alkaline pre-treatment is to increase the internal surface area of the lignocellulosic material due to swelling induced by the alkali (Agbor *et al*., 2011). This method is more effective with low lignin containing biomass such as agricultural residues, herbaceous crops and hardwoods than on softwood which have a higher lignin content (Zheng *et al*., 2009). Furthermore, due to the low severity of the alkaline pre-treatment little sugar decomposition occurs and hemicellulose is retained in the biomass, the method can remove acetyl and various uronic acids which can lower enzyme accessibility (Chang and Holtzapple, 2000). However, use of strong alkali concentrations leads to dissolution, 'peeling' of end-groups, alkaline hydrolysis and decomposition of dissolved polysaccharides (Fengel and Wegener, 1983). This peeling has advantages but could be at risk of degradation and loss of polysaccharides or carbon in the form of carbon dioxide. To prevent peeling, the temperature is kept low during the extraction process (room temperature or lower) (Hon and Shiraishi, 2000). Research has revealed that applying NaOH at room temperature for 24 hr preserves most of the carbohydrates but caused substantial lignin degradation (Singh *et al*., 2015). Other research (Wang, 2009) applied 121 ºC autoclaving using NaOH as a pre-treatment on biomass and the result showed that this was an impractical temperature, along with the use of pressure of 15 psi, for large scale industrial applications. However, alkaline pre-treatment at room temperature seems to be the best pre-treatment method using caustic materials (Liu *et al*., 2012).

The advantages of alkaline pre-treatment are the use of lower temperatures, pressures and residence times when compared to other pre-treatment technologies (Himmel *et al*., 1994). Alkali pre-treatments also have lower running costs when using chemicals such as sodium hydroxide, ammonia, peroxide and lime (Sendich *et al*., 2008). While, alkaline pre-treatment drawbacks include that this type of pre-treatment has little impact on the solubilisation of cellulose and

hemicelluloses (Gírio *et al*., 2010) and conversion of alkali into irrecoverable salts during the pre-treatment (Hendriks and Zeeman, 2009).

The efficiency of this process for hydrolysing the organic fraction of MSW has been investigated by using 0.5 – 2 M alkali (NaOH, $Ca(OH)_2$, NaOH-urea, Na_2CO_3) at 120-200 °C. Use of alkali at these concentrations substantially facilitated saccharification and improved enzymatic hydrolysis (Agbor *et al*., 2011). Recent studies have shown that a combined acid/alkaline pre-treatment of lignocellulosic wastes was more efficient than acid or alkaline individually (Damisa *et al*., 2008).

Alkaline treatment can also be separated into two types on the basis of the alkali employed. These include: Pre-treatment with calcium, sodium and potassium hydroxide and pre-treatment with ammonia. Ammonia Fiber Explosion (AFEX) treats lignocellulosic biomass (40–50 % moisture content) with pure liquid ammonia at mild temperatures (80–100 °C) and high pressures (40–50 atm) followed by explosive pressure release, pre-treatment helps to disrupt the fibre structure and increases the surface area. The advantage of this process is lower moisture content, lower formation of sugar degradation products and ability for ammonia to reduce lignin inhibitory effect on enzymatic hydrolysis. Disadvantages included costs due to recycling and treatment of chemicals (Brodeur *et al*., 2011).

The mechanism of alkaline hydrolysis depends on solvation and saphonication of intermolecular ester bonds crosslinking xylan hemicelluloses and other components such as lignin (Sun and Cheng, 2002). Solvation and saponification causes the removal of these cross-links and enhances the porosity of the lignocellulosic materials (Laureano-Perez *et al*., 2005). There are a number of important aspects of alkaline pre-treatment which cause low lignin removal and cellulose swelling; first is that the higher the monomeric hemicellulose fraction, the lower the total recovery of the hemicellulose (Laser *et al*., 2002), because the

monomeric forms are easily degradable to other volatile compounds for example furfural, which leads to losses of digestible substrate for the ethanol process (Bobleter, 1994); secondly, alkali extraction can also cause solubilisation, redistribution and condensation of lignin and modifications in the crystalline state of the cellulose; thirdly, alkaline pre-treatment changes the cellulose stricture to a form a denser and thermodynamically more stable form than the native cellulose (Gregg and Saddler, 1996).

2.3.3.3 Biological processing

The main biological method for the generation of fermentable sugars is through enzymatic hydrolysis usually after a hydrothermal or chemical pre-treatment. The enzymes are normally produced by microorganisms (fungi and bacteria) and the products of the hydrolysis are usually reducing sugars such as glucose. Cellulase enzymes are mainly used for the hydrolysis of lignocellulosic substrates (Chandra et al., 2007).

The most common microorganisms able to produce hydrolytic enzymes are bacteria belonging to the *Clostridium, Cellulomonas, Bacillus, Thermomonospora, Ruminococcus, Bacteriodes, Erwinia, Acetovibrio, Microbispora,and Streptomyces* genera (Bisaria and Martin, 1991), and fungi such as *Sclerotium rolfsii, Phanerochaete chrysosporium* and species of *Trichoderma, Aspergillus, Schizophyllum and Penicillium (*Sternberg, 1975; Duff and Murray, 1996). In the fungal kingdom, *Trichoderma* has been most extensively studied for cellulase production (Sternberg, 1975). However, white rot fungi are thought to be the most efficient for lignin hemicellulose degradation in waste material (Pérez *et al*., 2002). White and soft rot fungi attack both cellulose and lignin, the fungi can degrade lignin using enzymes such as peroxidases and laccase, while brown rots mainly degrade cellulose (Kumar *et al*., 2009).

Enzymes which participate in the hydrolysis of cellulose consist of three major groups: (1) endoglucanase (endo-1,4-glucanohydrolase) which have been shown

to degrade low crystalline structures within the cellulose fibre, creating free chain ends; (2) exoglucanase or cellobio hydrolase (1,4-glucan cellobiohydrolase) which remove cellobiose unit from the free chain ends and (3) glucosidases which hydrolyse cellobiose to produce glucose (Sun and Cheng, 2002). While, for hydrolysing hemicelluloses, a number of enzymes such as glucuronidase, acetyl esterase, feruloyl esterase, xylanase, xylosidase, galacto mannanase and gluco mannanase are used (Aranda *et al*., 2004; Georgieva *et al*., 2008).

The rate and degree of the EH is influenced by: mass transfer resistance, including surface film resistance around cellulose, bulk phase resistance and the resistance through the capillary pores of the cellulose particles (Yeh *et al*., 2010). Two major factors contribute to lower hydrolysis rates during EH. Firstly cellulose may be transformed into a less digestible form for enzyme during hydrolysis (Lee and Fan, 1983). Secondly, the soluble products, including glucose and celluobiose, may have a profound inhibiting effect on the action of cellulosic enzymes (Lee and Fan, 1983; Wen *et al*., 2004) (See Figure 2.13).

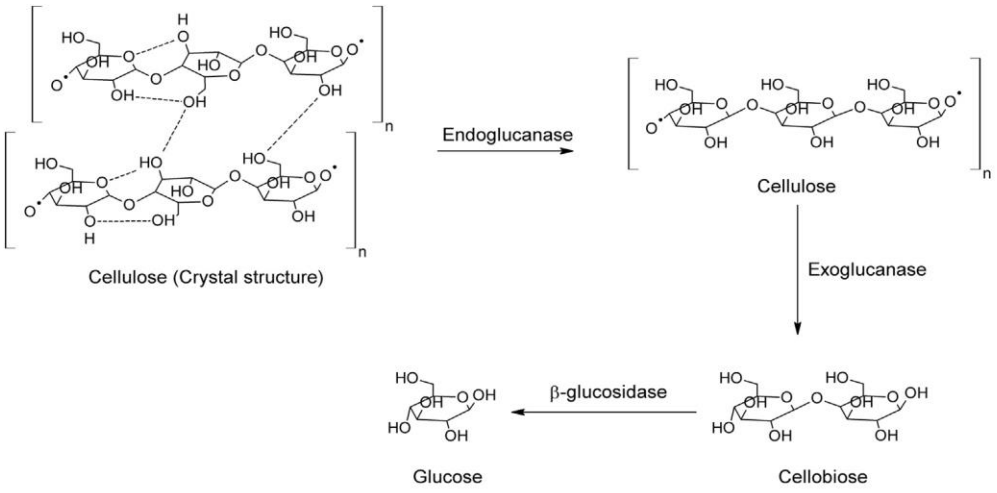


Figure 2.13 Mechanism of cellulose hydrolysis by cellulase (Yoon *et al*., 2014)

The high cost of commercial "cellulose" cocktails is one of the largest obstacles to the economic bio-refinering of biomass which requires large amounts of enzyme (Zhu *et al*., 2009). There have been attempts to improve cellulase activity such as direct evaluation and rational design for each cellulase and the reconstitution of designer cellulosome or cellulase mixtures (cocktails) which are directly active on the substrate (Zhang and Zhang, 2013). These improvements include basic studies on fungal physiology and chemistry, cellulase gene regulation and expression, recombinant enzymes, protein engineering of cellulase and development of cellulase enzyme cocktails (Mathew *et al*., 2008). The genome of *T. reesei* has revealed that this fungus contains fewer cellulases and hemicellulases than any other sequenced fungi despite being the best known commercial producer of cellulases (Martinez *et al*., 2008). Thus other fungal species may harbour more effective enzymes.

Factors affecting the enzymatic hydrolysis of cellulose including substrate condition (pre-treated and unpre-treated), environmental conditions (pH and temperature), and cellulase activity (Sun and Cheng, 2002).

- **Substrate concentration** is one the main factors that affects the initial rate of enzymatic hydrolysis and yield. Increasing substrate concentration has resulted in an increase in yield and hydrolysis reaction rates (Cheung and Anderson, 1997). However, high substrate concentrations can cause substrate inhibition depending on the ratio of total substrate to total enzyme (Huang and Penner, 1991). A 5 % w/v substrate concentration achieved the highest rates of hydrolysis, further increase in the substrate concentration decelerated the rate of hydrolysis due to stirring difficulties and reduction in the aqueous mobile phase (Lee and Fan, 1982; Szczodrak, 1988). The study showed for MSW that the hydrolysis increased from 27 % at 10 g/L to 53 % at 50 g/L with a sugar yield of 385 mg sugar/g fibre (Xu *et al*., 2007).

- The **structural features** of the substrate including cellulose crystallinity, degree of cellulose polymerization, surface area, and content of lignin can all affect the susceptibility of cellulosic substrates to cellulases (Sun and Cheng, 2002). Pre-treatment of the substrate also influences cellulase enzyme activity (Figure 2.14). The substrate particle size has a significant effect on EH yields, the highest hydrolysis efficiency were observed for the particle size range of 150–300 mm. Hydrolysis efficiency increased from 25 to 37 % by increasing particle size. This was probably due to the grinding process which may change the surface chemistry or morphology of the fibres making them less accessible to the enzyme. Larger particle sizes above 300 mm results in a reduction in sugar yield, this effect can be explained by the longer diffusion of enzyme into the fibre particles suggesting that extensive milling is not needed for the conversion of the organic MSW concentrate (Li *et al*., 2012)**.**

- **Environmental conditions;** increasing the temperature increases the rate of initial hydrolysis, the maximum hydrolysis rate was observed at 50 ^{o}C (Kaar and Holtzapple, 2000). This result could be attributed to the thermal inactivation of endoglucanase I and cellobiohydrolase I (Jiménez *et al*., 1995). Studies investigating the effect of reaction time on the sugar yield during EH indicated that by increasing reaction time sugar yield also increased. However, after 12 hr the hydrolysis rate levelled off indicating that some inhibition may occur after that time (Vlasenko *et al*., 1997). The presence of reducing sugars as well as percentage hydrolysis rates decreased with prolonged time after optimum. This effect may be due to the inhibition of the enzyme action by the accumulated hydrolysis products (Li *et al*., 2012).

- **Cellulase enzyme**, increasing enzyme dosage increases hydrolysis yields and rates; however, increasing enzyme dosage would increase the cost of the process. Cellulose enzyme dosage varied from 7 to 33 FPU/g substrate, depending on the type and concentration of substrates (Sun and Cheng, 2002). Surfactants can be applied to decrease the irreversible adsorption of cellulase on cellulose but may also be partially responsible for deactivation (Converse *et al*., 1988). Surfactants are also used to block lignin binding to the cellulose and thus enhance enzymatic saccharification of cellulose (Tu *et al*., 2009). There are many surfactants that could be applied such as non-ionic Tween 20, 80 (Wu and Ju, 1998), polyoxyethylene glycol (Park *et al*., 1992), Tween 81, Emulgen 147, amphoteric Anhitole 20BS, cationic Q-86W (Ooshima *et al*., 1986), sophorolipid, rhamnolipid, and bacitracin these surfactants are suitable for enhancing cellulose hydrolysis (Helle *et al*., 1993; Sun and Cheng, 2002). In addition, the factors affecting activity of Cellulases include enzyme source and the concentration of enzyme. An effective concentration of

enzyme for cellulose hydrolysis has been determined to be 10 to 60 FPU/gm of dry cellulose or glucan- glucanase- β- D-glucosidase ratio of 1-75-2 IU (International Unit) (Kim and Lee, 2005).

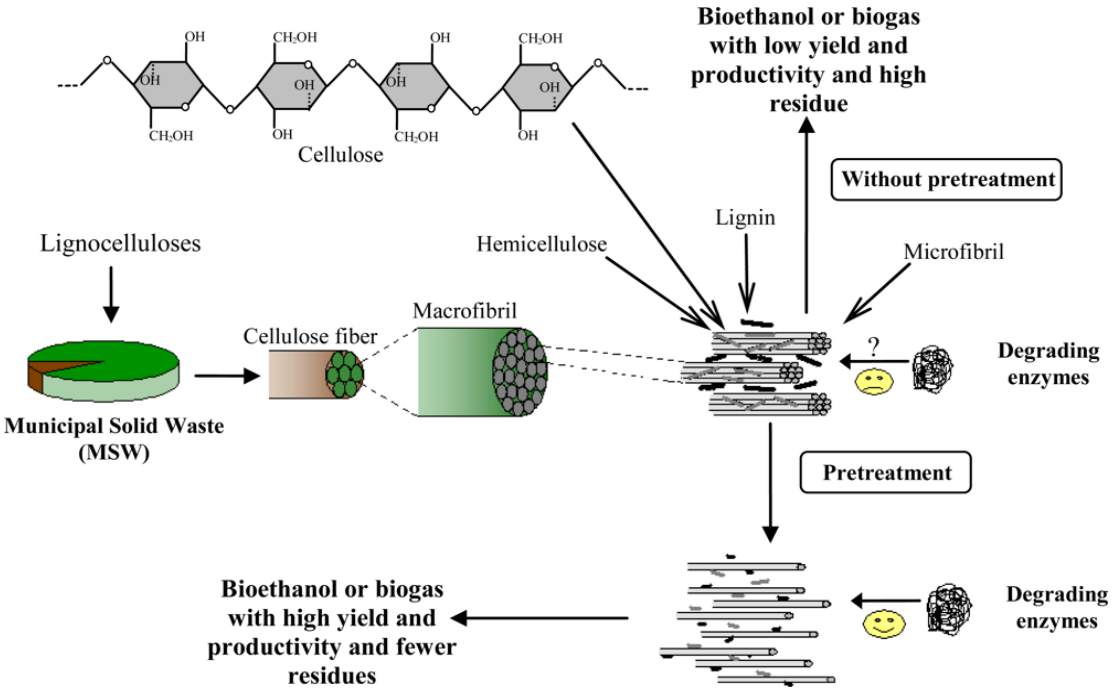


Figure 2.14 Effect of pre-treatment of MSW on accessibility of degrading enzymes (Taherzadeh and Karimi, 2008)

2.3.4 Biofuel and biochemical production from municipal solid wastes

Fuel production from lignocellulosic biomass, has been well researched, however, MSW has not been given as much attention for energy production when compared with other feed stocks (Bellon-Maurel *et al*., 2003). Fuel production from MSW is a promising strategy for energy needs and effective management of MSW. European legislation aims to minimise landfill use in EU countries, and the amount of biodegradable MSW must be reduced by 65 % by 2020 (UNION, 2009). Studies have shown that up to 82.9 billion litres of waste paper-derived cellulosic ethanol could be produced worldwide, replacing 5.36 % of gasoline consumption. The energy independence and security act mandated an increase of 36 billion gallons per year of renewable fuels to be blended into transportation fuels by 2022 (Li *et al*., 2007).

The use of MSW as a biofuel feedstock is dependent on a number of factors technical ones include regional reliability, characteristics of wastes, and compatibility with and efficiency of conversion technologies. The economic once are cost to collect, transport, and waste conversion. Environmental performance including air and water emissions, greenhouse gases, and finally waste generation can affect both costs and public acceptance (Bellon-Maurel *et al*., 2003).

Furthermore, the EPA has defined renewable fuel standards stating that advanced bio-fuels must be derived from feed stock's that meet the definition of renewable biomass. Therefore, MSW must be separated from plastic, rubber, metals and glass (Tengerdy and Szakács, 1998; Bellon-Maurel *et al*., 2003).

There are many types of biofuel such as ethanol, methanol, bio-diesels which could be used as transportation fuels (Li *et al*., 2007).

2.3.4.1 Ethanol production

Production of ethanol or ethyl alcohol has existed since the beginning of recorded history (Otulugbu, 2012). However, since the early 1980's the cost of ethanol production from lignocellulosic biomass was the main concern which was about US $ 0.95 /litre (US $ 3.60 /gallon) (Wright, 1988). However, research has improved ethanol production yield through improved cellulase production, utilisation of a SSF rather than a SMF process, and advances in microorganisms to convert the xylose fraction, as a consequence much better yield and rates have been achieved (Reserves, 1993; Fu and Mielenz *et al*., 2011)

The ethanol production process includes using lignocellulosic substrate such as wheat and corn (Jones *et al*., 1994). However, corn is no longer used for ethanol production because of the wide planting for ethanol production which competes for use of arable land and thus threatens national food securities (Giampietro *et al*., 1997). Lignocellulosic substrates can be used as alternative to corn options

which are low in cost and have a high polysaccharide content (Fujii *et al*., 2009). Nowadays food wastes can be utilised as substrates for ethanol production, research carried out showed that using sweet potato produced 80.23 % ethanol yield (Lee *et al*., 2012).

A few studies has been applied for bioethanol production from MSW, Li and Antizar (2006) showed that using pre-treatment with dilute sulphuric acid followed by steam explosion did increase the rate at which the maximum yield of glucose was formed. However, this pre-treatment did not give higher yields for newspaper wastes.

Another study used selected biodegradable municipal solid waste fractions were subjected to fifteen different pre-hydrolysis treatments to obtain the highest glucose yield for bio-ethanol production. Glucose yields were compared using a factorial experimental design. The highest glucose yield (72.80 %) was obtained with a pre-hydrolysis treatment consisting of H_2SO_4 at 1 % concentration, followed by steam treatment at 121 °C, and enzymatic hydrolysis at 60 FPU/g substrate (Li, 2006).

A study by Yan *et al*. (2012) using enzymatically hydrolysed food wastes showed that batch and fed batch hydrolysates, which contained reducing sugar concentrations of 131.41 and 194.43 g/L respectively, produced 62.93 and 90.72 g/L, ethanol following fermentation using *S. cerevisiae* H058, for 48 hr.

There are three major stages involved in the conversion of lignocellulose to ethanol - pre-treatment, enzymatic hydrolysis and fermentation. This is followed by distillation to extract pure ethanol. The steps, or technologies, required for ethanol production are shown in Figure (2.15).

(1) Pre-treatment

Pre-treatment using physical, physicochemical, chemical and biological methods as mentioned in section 2.7.3, is an important step to make cellulose more accessible in the hydrolysis step. However, this is a costly step, accounting for approximately 33 % of the total cost (Tomas-Pejo *et al*., 2008).

(2) Enzymatic hydrolysis

EH is the process used to convert polysaccharides into simple sugars, which can be fermented by bacteria or yeast (Tong *et al*., 2012).

In the second stage, the conversion of cellulose and hemicellulose can be expressed by the reaction of glucan (for hexoses) and xylan (for pentose) with water:

$(C_6H_{10}O_5)n + nH_2O \rightarrow n\ C_6H_{12}O_6$ .....(1)

$(C_5H_8O_4)\ n + nH_2O \rightarrow n\ C_5H_{10}O_5$ .....(2)

The maximum theoretical yield of hexoses and pentoses is 1.136 kg and 1.111 kg per kg of glucan and xylan, respectively (Kang *et al*., 2014).

(3) Fermentation

The fermentation reaction by yeast and bacteria of simple sugars produces bio-fuels such as ethanol or butanol, CO_2 is also produced during the fermentation process. The simplified reaction equation is: (Kang *et al*., 2014)

The conversion reaction for hexoses (C6) and pentose (C5) is as follows:

$C_6H_{12}O_6 \rightarrow 2\ C_2H_5OH + 2CO_2$..... (3)

$3C_6H_{10}O_5 \rightarrow 5\ C_2H_5OH + 5CO_2$..... (4)

The theoretical maximum yield of ethanol from hexoses and pentose is 0.511 kg ethanol and 0.489 kg CO_2 per kg sugar, respectively (Kang *et al*., 2014).

Generally yeast such as *S. cerevisiae* are used for ethanol production, however, this yeast cannot metabolise xylose efficiently (Kim *et al*., 2012; Sakamoto *et al*., 2012). However, many bacteria and yeast are able to ferment xylose and other pentose sugars either naturally or following genetic manipulation (Mousdale 2008; Deswal *et al*., 2014).

Yeasts when utilised for ethanol production are required to be capable of fermenting all of the sugars present with high ethanol yields. Wild-type *S. cerevisiae* strains are unable to ferment pentose sugars; its capability for xylose utilization has been improved by intensive recent research. During the last fifteen years, research has been focused on finding xylose fermenting microorganisms and understanding the principles behind the utilisation of xylose (Kang *et al*., 2014). *S. cerevisiae* has desirable characteristics such as efficient anaerobic sugar metabolism, toleration of inhibitory industrial substrates better than other microorganisms and ferments hexoses abundantly present in lignocellulosic hydrolysates, such as glucose, mannose and galactose with high yield and productivity (Sun and Cheng, 2002).

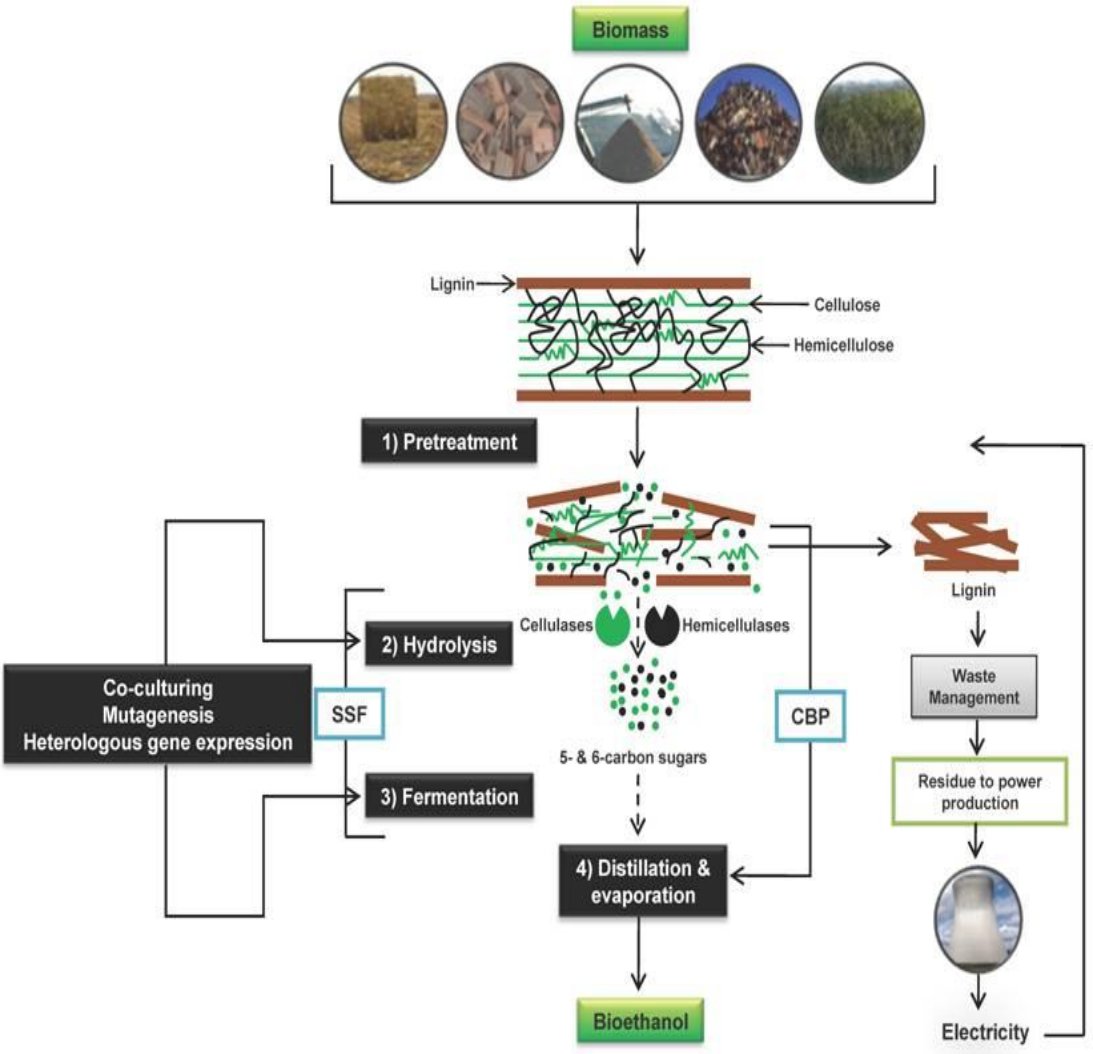


Figure 2.15 Schematic for the conversion of lignocellulosic biomass to ethanol (Dashtban *et al*., 2009)

2.3.4.2 Butanol production

1-Butanol (butyl alcohol or n-butanol) is a four carbon straight chained alcohol with a molecular formula of C_4H_9OH (MW 74.12) and boiling point of 118 ºC (Green, 2011). Butanol is a good source of biofuel (Dürre, 2007), because it has low vapour pressure, can be blended with either gasoline or diesel at any fraction (Raganati *et al*., 2012). Butanol has some advantages over ethanol as a fuel substitute, it has an energy content that is similar to gasoline, lower vapour pressure compared to ethanol and is safer during transport and when used in car engines (Wen *et al*. 2014; Salehi and Taherzadeh, 2015). Butanol can also be used as an important chemical precursor for paints, polymers, plastics, solvents,

plasticizers, butylamines, amino resins, butyl acetates production, etc. (Speight and Speight, 2002;Green, 2011). Therefore, bio-butanol has the potential to substitute for both ethanol and bio-diesel in the biofuel market and is estimated to be worth $247 billion by 2020 (Green, 2011).

For butanol chemical production of Oxo synthesis, Reppe synthesis, and crotonaldehyde hydrogenation are the three most important processes, most of these process rely on petroleum, however, butanol can be produced from biomass (Lee *et al*., 2008). Butanol is currently manufactured from petroleum feedstock (Oxo process). While, Bio-butanol is produced via the Acetone Butanol Ethanol (ABE) fermentation process using renewable resources (biomass) and *Clostridium acetobutylicum* or *Clostridium beijerinckii* in anaerobic conditions) (Raganati *et al*., 2012). Conventional butanol fermentation is carried out by microorganisms in a two-stage batch process: an initial acidogenic stage followed by a solventogenic stage. This fermentation process is known as ABE production (Yen *et al*., 2011).

The microorganisms used for butanol, acetone, ethanol are production usually formed by *Clostridia* bacteria, these bacteria can degrade a number of toxic chemicals by producing chiral products which are difficult to make by chemical synthesis (Francis *et al*., 1994; Spain, 2013).

Currently butanol is produced from the fermentation of corn, cassava or molasses as substrate (Ezeji *et al*., 2013; Wen *et al*., 2014). Different types of biomass such as wheat straw (Qureshi *et al*., 2007; Nanda *et al*., 2014), rice straw (Gottumukkala *et al*., 2014), barley straw (Qureshi *et al*., 2010), corn stover (Qureshi *et al*., 2010), corn cob and fibres (Guo et al., 2013), palm kernel cake (Shukor *et al*., 2014), cassava starch (Li *et al*., 2014), pinewood and timothy grass (Nanda *et al*., 2014), switch grass (Gao *et al*., 2014), have been used as substrates for ABE fermentation by numerous *Clostridium* strains (Salehi and Taherzadeh, 2015). MSW has also been applied to reduce the cost for the biofuel market and has been shown to be more sustainable offering a lower carbon

footprint and reduced GHG emissions (Green, 2011). Some studies have applied domestic organic waste (DOW) as a substrate for butanol production, using steam explosion and enzymatic hydrolysis for the washed and dried DOW produce using *Clostridium acetobutylicum* DSM 1731 produced 1.5 and *C. beijerinckii* B-592 0.9 g/L ABE and *Clostridium* LMD 84.48 1.9 g/L Isopropanol, Butanol, Ethanol (IBE) (Claassen *et al*., 2000).

ABE fermentation is also used with domestic organic waste and *C. acetobutylocum* in a batch fermentation (Claassen *et al*., 2000). Utilization of such waste materials improves the economy of butanol production (Liu *et al*., 2013).

2.3.4.3 Organic acid production from municipal solid waste

Organic acids are other promising bio-energy products that could be produced using renewable carbon sources and microorganisms; these are not currently produced as large-scale processes. However, citric, lactic and succinic acid are three products at different stages of industrial development (Hatti-Kaul *et al*., 2007).

These acids are produced naturally by microorganisms, or are at least natural intermediates in major metabolic pathways. These acids are important for example succinic, fumaric and malic acid could replace the petroleum-derived commodity chemical maleic anhydride (Holladay *et al*., 2007) (Figures 2.16 and 2.17).

To produce organic acids, various cheap substrates has been selected such as red lentil flour in India (Altaf *et al*., 2007), kitchen waste in Japan (Ohkouchi and Inoue, 2007), barley hydrolysates in the EU (Venus, 2006) and oat (Koutinas *et al*., 2007) or liquefied corn starch from cassava bagasse (John *et al*., 2007).

- **Citric acid**

Citric acid is widely use in the food and pharmaceutical industries. 70 % of the food industry is dependent on citric acid followed by about 12 % for the pharmaceutical industry and 18 % for other applications (Shah *et al*., 1993). Citric acid can be obtained by chemical synthesis by the filamentous fungus *A. niger*. In addition to fungi, yeast have been applied and developed as a microbial cell factory for citric acid (Papanikolaou *et al*., 2006). *Yarrowia lipolytica* is also used for the production of citric acid from carbon sources, such as glucose and sucrose (Förster *et al*., 2007).

Various agro-industrial residues such as apple pomace, coffee husk, wheat straw, pineapple waste, mixed fruit, maosmi waste, cassava bagasse, banana, sugar beet cosset and kiwi fruit peel have been investigated for their potential to be used as substrates (Kumar *et al*., 2003).

Nowadays production of citric acid is approximately 1.6 million tons. There are many parameters that help to get highly efficient biotechnological production of citric acid such as "high substrate concentration, low and finite content of nitrogen and certain trace metals, thorough maintenance of high dissolved oxygen, and low pH". Currently the production of citric acid is approximately 1.6 million tons (t) (Berovic and Legisa, 2007).

- **Lactic acid**

Lactic acid has been widely applied in the food, pharmaceutical, leather and textile industries and as a chemical feedstock. Currently, lactic acid is used as starting material to produce biodegradable polymers used in medical, industrial and consumer products (Gross and Kalra, 2002; Couto and Sanromán, 2006; Lahtinen *et al*., 2011). The acid is produced by *Rhizopous oryzae* using SSF with sugarcane bagasse (Soccol *et al*., 1994) or by *Lactobacillus paracasei* in solid-state conditions using sweet sorghum (Naveena *et al*., 2005).

- **Succinic acid**

Nowadays, the succinic acid market is small at around 16,000 tons per year; this acid could replace petroleum derived maleic anhydride, which has a market volume of 213,000 tons/year. Deriving succinic acid from petroleum causes environmental pollution (Song and Lee, 2006). Microorganisms like *Escherichia coli* and filamentous fungi, including *Penicillium simplicissimum,* have been shown to accumulate succinic acid naturally (Gallmetzer *et al*., 2002).

- **Gluconic acid**

Gluconic acid is used widely in the food, pharmaceutical, cement, textile and chemical industries and is in high demand at 50,000–60,000 tons/annum. Gluconic acid is an oxidative product of the glucose industries (Roukas, 2000; Ramachandran *et al*., 2006). Solid state fermentation (SSF) and Sub merged Fermentation (SMF) have been used to produce gluconic acid using *A. niger* (Singh *et al*., 2003). Various substrates have been used for gluconic acid production such as sugarcane molasses which have a high economic benefits in-terms of cost, by using SMF, many studies have applied SSF for gluconic production to reduce the cost (Sharma *et al*., 2008).

- **Oxalic acid**

Oxalic acid and their salts can be used as a bleaching agent, in detergent formulation and as a metal polisher because of its capacity for reducing iron and other metals compounds (Burgstaller and Schinner, 1993). Oxalic acid is also used as a mordant in dyeing processes. There are two ways to produce oxalic acid by either chemical or biotechnological processes, a chemical method uses formic acid salts (heating sodium formate and treating the resulting oxides with sulphuric acid) or by carbohydrate oxidation by nitric acid (Cameselle *et al*., 1998). while the biotechnological methods include using microorganisms such as *Cyanobacteriae* (Heyer and Krumbein, 1991), brown rot fungi (Espejo and Agosin, 1991) and other fungi, such as *Penicillium* (Cunningham and Kuiack, 1992), that

secrete oxalic acid at low concentrations. *A. niger* produces not only oxalic but also citric and gluconic acids according to the operating conditions (Magnuson and Lasure, 2004). Oxalic acid production efficiency depends on factors such as C- and N-source and the initial medium pH as well as the culture/broth pH during fermentation (Strasser *et al*., 1994; Mandal and Banerjee, 2006).

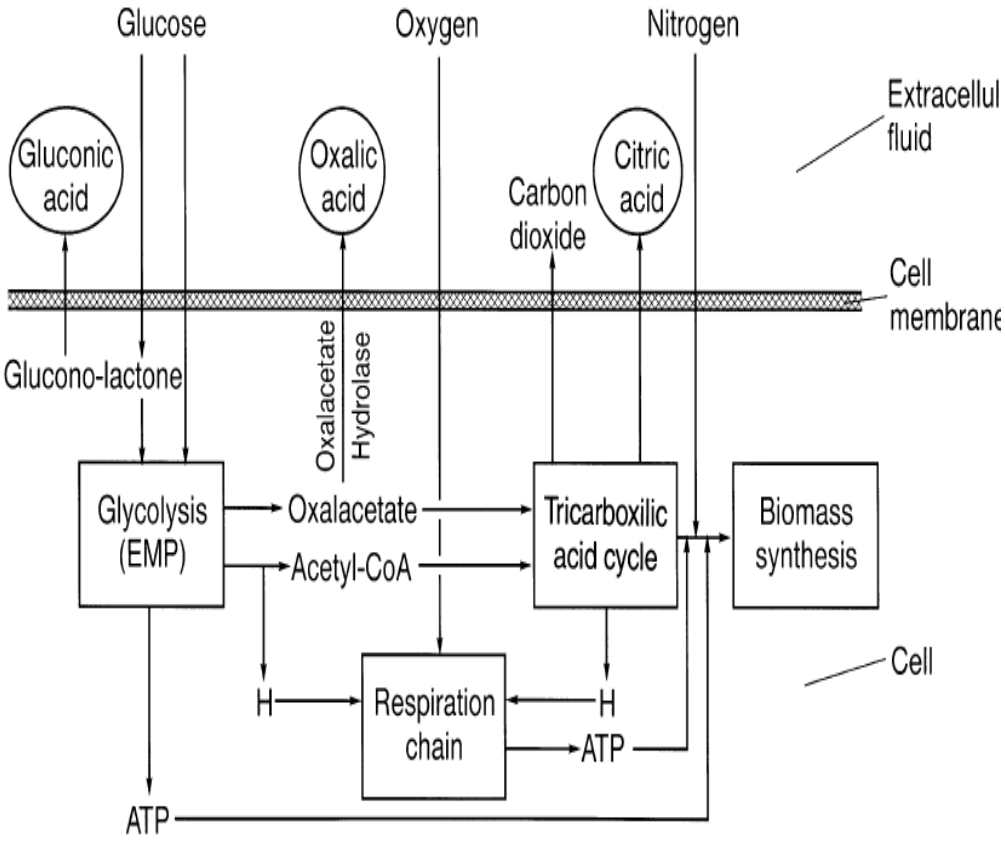


Figure 2.16 Metabolic pathway for citric, gluconic and oxalic acid synthesis in *A. niger* (Cameselle *et al*., 1998)

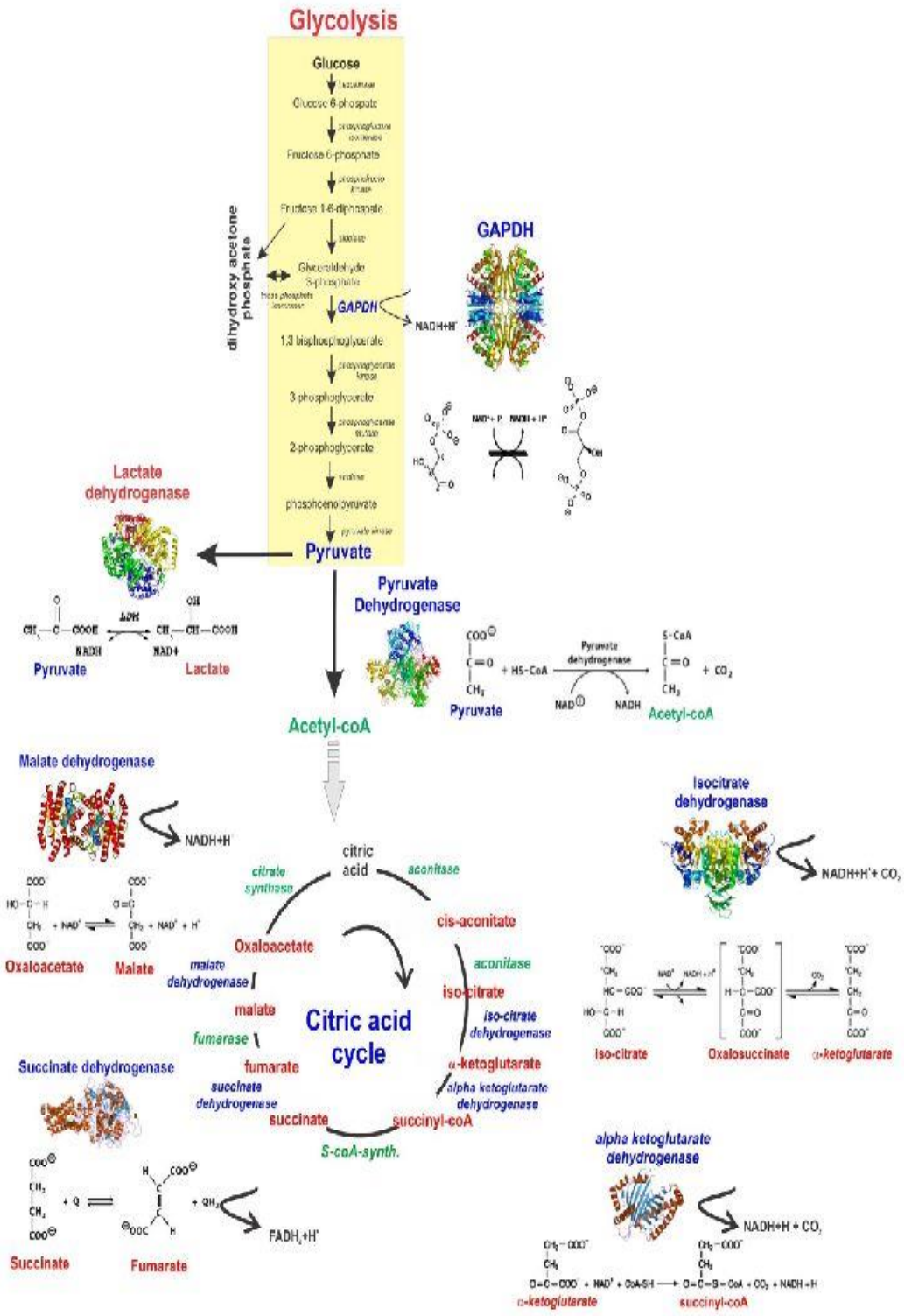


Figure 2.17 Enzymatic pathways of glycolysis and Kreb's cycle (Naik *et al*., 2012)

Note: the DHOs involved in the different metabolic pathways (glycolysis and citric acid cycles)

2.4 Enzyme Production

2.4.1 Use of microorganisms for enzyme production from biomass and MSW

Solid state fermentation (SSF) is currently used for the production of various commercial enzymes (Bellon-Maurel *et al.,* 2003). These include those enzymes involved in the degradation of biomass. The substrates used in SSF can be classified into two categories: inert materials, which only act as an attachment for the microorganism and non-inert materials, which not only function as an attachment but also supply nutrients to the microorganism (Pandey *et al*., 1999). Several parameters need to be taken into consideration to insure a successful SSF process these include environmental parameters (temperature, pH, water content and activity) and the carbon source (biomass, substrate concentration, CO_2) (Burgess *et al.,* 2000).

pH plays an important role for cellulase production and the impact of initial pH of the culture medium has been extensively investigated. Research has revealed that the maximum cellulase activity from corn stover was obtained at pH 2 (Romero *et al*., 1999; Jecu, 2000; Gao *et al*., 2008), at optimal temperature the optimal pH is 3-6 using fungi (Kheng and Omar, 2005). For SSF, it is difficult to monitor the pH and is normally not controlled during the SSF process and can only be adjusted at the beginning of the process (Yoon *et al.,* 2014). It has been reported that in the first 4 days of SSF the initial pH drops and then increases after 8 days using rice straw (Khan *et al.,* 2007). The initial decrease in pH is due to the formation of organic acids and consumption of ammonium salt in the fermentation media (Ingold, 2012).

Temperature, has an obvious effect on germination of spores (Kheng and Omar, 2005). The optimal temperature for *A. niger* growth is room temperature because this is similar to the natural habitat of the fungi, which is classified as a mesophilic microorganisms (Maheshwari *et al*., 2000).

Moisture is a crucial factor which affects metabolite production in SSF, because when the moisture level is low the solubility of nutrients decreases (Archana and Satyanarayana, 1997; Kumar *et al*., 2011). However, high levels of water in the SSF media means that substrate particles will be surrounded by a thick layer of water, these particles stick together and limiting the diffusion of air between the particles and the immediate surroundings (Gervais and Molin, 2003). The presence of water helps to swell the substrate and facilitates absorption of the nutrients from the substrates for growth and metabolic activities (Ibrahim, 2006). The nature of the substrate, porosity, specific surface area the requirements of microorganisms and the type of end product determines the optimal moisture conditions (Kalogeris *et al.,* 2003).

Inoculation size, for SSF inoculums preparation include spore suspension, mycelia disc, and mycelium suspension and pre-inoculated substrate (Yoon *et al*., 2014). Initially spores attach to the outer surface of the substrate particles and grow slowly, multiplying and penetrating into the substrate (Ramachandran *et al.,* 2004). Optimal spore suspension concentration used in most research is approximately 10^6 spore/ mL (Khan *et al*., 2007; Huang *et al*., 2010).

Incubation period has a significant role but maybe affected by several factors, such as the presence of different ratios of amorphous to crystalline cellulose (Ögel *et al*., 2001). The first signs of fungal growth were reported on day two of SSF for cellulase enzyme production and after 7 to 11 days the fungus completely colonised the substrate depending on the type of substrate used (Kumaran *et al*.,

1997; Velazquez-Cedeno *et al*., 2002). During the colonisation phase of fungal growth, extracellulase enzyme was produced to degrade the lignocellulosic substrate into small particles, which helped fungal growth as a nutrient source (Montoya *et al*., 2012).

Supplements - to increase cellulase activities some type of supplements such as carbon and nitrogen sources can be added to the substrate (Gomes *et al*., 2000). Generally cellulose in a lignocellulosic substrate acts as the essential carbon source, also fungal and cellulase production can be stimulated by nitrogen source, peptone can be used as a nitrogen source and is able to increase enzyme production, it's essential to have the proper combination of nitrogen source, lignocellulosic substrate and fungal strain for maximizing cellulase production (Kachlishvili *et al*., 2006). Phosphorus, trace elements and other minerals can also be supplemented into the SSF media and play important roles, phosphorus helps the formation of phospholipid bilayers in the fungal cell membrane (Kumar *et al*., 2008). Addition of a surfactant such as Tween 80 and triton X-100 to the fermentation medium can help to improve the permeability of fungal cell membrane thus allowing the secretion of cellulase in a more rapid manner (Reese and Maguire, 1969; Ahamed and Vermette, 2008). Some trace elements such as Zn^{2+}, Ni^{2+}, Mn^{2+} and Co^{2+} which serve as cofactors, may enhance cellulase enzyme production (Shi *et al*., 2009). The presence of heavy metals could also interfere with energy supplying system for cellulase production for example cellulase of *P. chrysosporium* in liquid medium was inhibited in the presence of 50–150 ppm Cd^{2+}, Cu^{2+}, Pb^{2+}, Mn^{2+}, Ni^{2+}, and Co^{2+}. At 150–300 ppm Mn^{2+} or 300 ppm Cd^{2+} or Co^{2+}, no cellulase activity was detected (Falih, 1998; Baldrian, 2003).

Particle size, the surface area plays an important role for microbial attachment, mass transfer of various nutrients and substrates and subsequent growth and product formation (Prakasham *et al*., 2006).

Type of lignocellulosic substrate, selecting a substrate that is able to support fungal growth, stimulates cellulase production and contains sufficient nutrients is particularly important. Selecting a substrate that enables the anchorage of fungi during fungal growth is also an important criterion before applying it for SSF (Hong *et al*., 2011). In addition, there are many other approaches being taken to enhance cellulase production, such as genetic modification (mutagenesis, heterologous expression) of fungal strains and co-culture of different fungal strains (Yoon *et al*., 2014).

2.4.2 Application of SSF using various biomass

SSF has been employed for the production of antibiotics, surfactants, biocides and enzymes (Pandey *et al*., 1999), these products can be produced using bacteria, yeast and fungi. These microorganisms are capable of growth on solid substrates, among these microorganisms filamentous fungi have been commonly employed due to their physiological capabilities and hyphal mode of growth under conditions of low moisture (Prabhakar *et al*., 2005). Potential applications of SSF included:

I. Production of commercial products.

Industrial residues can be converted into valuable products, for example coffee (pulps and husks), soybeans, cassava husk and bagasse, sugarcane bagasse, sugar beet pulp, fruit wastes, palm tree wastes bio-converted into single cell protein, organic acids like citric and lactic acids, amino acids, pigments, antibiotics, mushrooms, bio-pesticides, gibberellic acid, flavour and aroma compounds (Soccol and Vandenberghe, 2003; Prabhakar *et al*., 2005).

II. Environmental control

The SSF process helps in the biodegradation of hazardous compounds, use of SSF has shown promise for the biological detoxification of industrial wastes and insecticides and for pest control in crops (Pandey *et al*., 1999).

III. Food industry products

SSF has been used in the production of food additives or flavouring compounds (Berger, 1995), these compounds are produced via chemical synthesis or by extraction from natural materials (Janssens *et al*., 1992). Several microorganisms have the ability to produce aroma compounds from agro-industrial wastes (Bramorski *et al*., 1998). Some aroma compounds such as monoterpene alcohols and isoamyl acetate have been produced by *Kluyveromyces marxianus* from Cassava bagasse (Medeiros *et al*., 2001).

IV. Enzyme production

Several enzymes can be produce by SSF using lignocellulosic wastes (Dos Santos *et al*., 2004), recent studies confirmed that SSF is the best system for producing enzymes and better than SMF with regards to yields obtained (Lynd *et al*., 2002; Mathew *et al*., 2008). Generally, the most common industrial enzymes produced using SSF are proteases, cellulase, ligninases, xylanases, pectinases, amylases, glucoamylases; also production of inulinases, phytases, tannases, phenolic acid esterases, microbial rennets, aryl-alcohol oxidases, oligosaccharide oxidases, tannin acyl hydrolase, a -L-arabinofuranosidase (Pandey *et al*., 1999).

The most common enzyme produced is cellulase enzyme **"**Cellulases are a complex enzyme system, comprising endo-1,4-b -D-glucanase (EC-3.2.1.4), exo-1,4-b-glucanase (exocellobiohydrolase, EC-3.2.1.91) and b-D-glucosidase (b-D-glucoside glucanhydrolase, EC-3.2.1.21)"(Pandey *et al*., 1999). Cellulases are one of the largest groups in the structural classification of glycosyl hydrolyses, this classification is based on variability of catalytic domains and does not consider variability in cellulose binding domains (Lynd *et al*., 2002; Mathew *et al*., 2008).

Cellulase is recorded as the third largest industrially produced enzyme, and is applied widely in cotton processing; paper recycling, juice extraction, detergents and as an animal feed additives. Cellulase is also commercially produced for saccharification of biomass (Mathew *et al*., 2008; Singhania *et al*., 2010).

The enzyme is produced by various microorganisms (fungi and bacteria),including *Aspergillus fungi* (Liu *et al*., 2011). Furthermore, the most investigated and genetically improved for fungi enzyme production are the *Trichoderma spp.* (Pajan *et al*., 1997). Generally, *Aspergillus* and *Trichoderma spp*. are well known efficient producers of cellulases (Larroche *et al*., 1988) for example *T. reesei* produces 2 Cellobiohydrolase (CBH), 8(endo-B-1-4-glucanase (EG) and 7 B-glucosidase (Aro *et al*., 2005). Commercially most enzymes are produced from these two strains of soft rot fungi, but *T. reesei* is not capable of producing substantial amounts of B-glucosidase, whilst *A. niger* produces a cellulose system lacking endo and exoglucanase (Ahamed and Vermette, 2008).

In addition, the most important cellulolytic microbes which are able to produce cellulase obtain their energy primarily from carbohydrates and are unable to use lipids or proteins (Lynd *et al*., 2002). Fungi possess the ability to secret large amounts of extracellular protein; such strains are most suited for production of high levels of extracellular cellulase. The most commonly studied cellulolytic organisms is *T. reesei* (Sukumaran *et al*., 2005).

SSF is gaining interest as a cost effective technology for cellulase enzyme production and bioconversion of lignocellulosic biomass using cellulolytic microorganisms. Comparing liquid culture with solid state culture for cellulase enzyme production, SSF has been shown to closely resemble the natural habitat of filamentous fungi, also enzyme titre produced from SSF is superior compared to the titre produced via SMF (Hölker *et al*., 2004; Najafpour, 2015).

Advantages of SSF over SMF: SSF is a process which requires smaller amounts of water and therefore the cost of the process can be greatly reduced (Pandey *et al*., 2000); SSF has been shown to produce higher concentrations of enzymes; higher

fermentation productivity and has a lower demand on the sterility of the equipment (Raghavarao *et al*., 2003; Hölker *et al*., 2004). Finally, the crude enzyme extracted from SSF can be applied directly to hydrolyse the lignocellulosic substrate (Yoon *et al*., 2014). Studies have reported a higher yield of cellulase from *T. reesei* using SSF compared with SMF processes (Chahal, 1985). Furthermore, some studies have proposed that SSF application could be a better technology for commercial production of cellulase with low cost, and by using naturally available cellulose sources (Pandey *et al*., 1999).

2.4.3 Mechanisms of SSF for cellulase enzyme production

There has been extensive research on cellulase enzyme production (Wilson, 2009). For example the rate-limiting step for crystalline cellulose degradation has yet to be determined. It is not clear which segment of the cellulose fibrils cellulase binds to. A further unknown is how cellulosomes are able to efficiently catalyse the hydrolysis of cellulose and how free cellulose binding modules stimulate cellulase hydrolysis (Moser *et al*., 2008). Finally, how mixtures of cellulases hydrolyse both crystalline and amorphous regions in bacterial cellulose, while most individual enzymes only seem to degrade amorphous regions (Chen *et al*., 2007).

Besides wheat straw, other cheap materials, such as banana peel, rice straw, corn cob residue, rice husk, wheat straw, banana fruit stalk, and coconut coir pith are all being used for cellulase production (Couto and Sanromán, 2005; Klei *et al*., 2012), (Table 2.4).

Table 2.4 Applying various Substrates and Microorganisms for cellulase enzyme production

Substrate	Microorganism	Yield	Reference
Rice barn and corn straw	*Trichoderma reesei*	Cellulase18.5 IU/mL	(Rajesh *et al*., 2012)
Egg shell waste	*Neurosporacrassa*	Cellulase 2.30 U/mL	(Verma *et al*., 2012)
Water hyacinth	*Trichoderma reesei SEMCC-3.217*	Cellulase 13.4 FPIU/g dry solid	(Zhao *et al*., 2013)
Xylose industry	*Trichoderma reesei ZU-02.*	Cellulase (158 IFPU/g)	(Xia and Cen, 1999)
Vinegar industry	*Trichoderma koningii AS3.4262*	Cellulase 6.90 IU/g of substrate dry matter (SDM)	(Liu and Yang, 2007)
Wheat bran	*Trichoderma reesei*	Cellulase 2.63 U/ mL	(Maury *et al*., 2012)
Sugar cane bagasse	*Trichoderma reesei NEEL 11460*	Cellulase 154.58 U/gds	(Singhania *et al*., 2006)
Sweet potato	*Bacillus sp*	Amylase and cellulase 28 U/mL	(Zhang *et al*., 2004)
Oil palm in the form of empty fruit bunches	*Trichoderma harzianum*	Cellulase 8.2 U/gm	(Alam *et al*., 2009)
	T2008		
Saw dust and bagass	*Aspergillus niger*	Cellulase Sawdust gave the best result with an enzyme activity value of 0.0846 IU/mL while bagasse gave 0.0682 IU/mL	(Guruchandran and Sasikumarand, 2010)
Rice bran	*Trichoderma reeseii QM9414 and T. reesei MCG77*	Cellulases 1.1635 U/g	(Latifian *et al*., 2007)
Rice straw	*Acremoniumcellulolyticus*	Cellulases and hemicellulases DBMc, 10.8 FPU/mL and WDMc, 10.4 FPU/mL	(Hideno *et al*., 2011)
Cocoa (Theobroma cacao) meal	*Aspergellus niger*	Cellulase 14.18 U/mL and xylanase11.86 U/mL	(Dos Santos *et al*., 2013)
Banana waste	*Bacillus subtilis (CBTK 106),*	CellulaeThe optimal ®lter paper activity (FP Ase) of 2.8 IUgdsÿ1, CMCase activity of 9.6 IUgdsÿ1 and cellobiase activity of 4.5 IUgdsÿ	(Krishna, 1999)

2.5 Composting and fertiliser

2.5.1 Compost and fertiliser production from municipal solid waste

Waste contain various levels of metals, some of them are discharged directly or indirectly in to the environment, which can cause serious environmental pollution, and threaten life (Bishop, 2000; Wang, 2002). These metals are classified into the following three categories: toxic metals (such as Hg, Cr^{6+}, Pb^{2+}, Zn^{2+}, Cu^{2+}, Ni^{2+}, Cd^{2+}, As^{3-}, Co^{2+},Sn, etc.), precious metals (such as Pd^{2+}, Pt, Ag^{+}, Au, Ru etc.) and radionuclides (such as U, Th, Ra, Am, etc.) (Bishop, 2000).

In recent years, leaching which is used to remove metals from aqueous solution has been carried out using methods such as chemical precipitation, ion exchange, electrochemical treatment membrane technologies, adsorption on activated carbon etc (Fu and Wang, 2011).

The major advantages of bio-sorption over other methods include low cost, high efficiency, minimisation of chemical and biological sludge, no additional nutrient requirement, regeneration of bio-sorbent, and possibility of metal recovery (Kratochvil and Volesky, 1998). The bioleaching process has many advantages: economically, the process is cheap and simple to operate, has lower energy requirement; environmentally, the process is environmentally friendly because there are no by-products e.g. gaseous pollutants are produced in bio-hydrometallurgy (Brombacher *et al*., 1997). In addition, bioleaching has potential for the environmental clean-up of mining sites, treatment of mineral industrial waste products, detoxification of sewage sludge and for the remediation of soils and sediments contaminated with heavy metals (Bosecker, 2001; AUNG, 2005).

Microbial bioleaching is based on the natural ability of microorganisms to transform solid compounds to a soluble and extractable form. This may involve enzymatic oxidation or reduction of the solid compound, or an attack on the solid compound by metabolic products (Ehrlich, 1997).

Bioleaching has been defined as the interaction between metals and microorganisms, which leads to solubilisation of metals in a solid form.

Additionally, the term "bio-oxidation" is also used (Brandl *et al*. 2001). In addition, leaching can be direct (i.e., physical contact between microorganisms and solid material) or indirect (e.g., bacterial oxidation of Fe^{2+} to Fe^{3+} which catalyses metal solubilisation as an electron carrier) (Tributsch, 2001).

Various microorganisms can be applied for bioleaching processes such as autotrophic bacteria, heterotrophic bacteria, and fungi. Fungi belonging to the *Aspergillus* and *Penicillium* genus have been the most extensively studied (AUNG, 2005), marine algae (eg *Sargassum natans)*, yeast (eg. *S. cerevisiae*) have also been studied. Fungi are able to solubilise metal compounds by excreting acid, mainly in the form of organic acids, using heterotrophic fungi results in a faster leaching process and with a shorter lag phase; for example by using MSW fly ash as the substrate *Aspergillus thiooxidans* required 1-3 months, while *A. niger* only requires 2-3 weeks to complete the leaching process (Krebs *et al*., 1997). Addition of organic acids helps to increase the solubility of metal ions at non-acidic pH values by chelating, in addition, complexation between metal ions and organic acid anions may reduce their toxicity (AUNG, 2005).

Fungi can withstand a much wider pH range, typically from 2 to 7, media composition and leaching environment (Burgstaller and Schinner, 1993; AUNG, 2005). The organic acids are produced by fungi in complexes with metal ions and enhance metal solubilisation, these complexes help to reduce the toxicity of heavy metal ions to the fungi, and thus the fungi are fairly resistant to heavy metals (Burgstaller and Schinner, 1993). Microorganisms are able to mobilize these metals by various reactions (Brandl, 2001).

Currently, *S. cerevisiae* is also used for heavy metal bioremediation, yeast bioleaching strains are affected by many factors such as pH, redox potential, presence of anions and soluble organic compounds (Machado *et al*., 2010). *S. cerevisiae* has advantages for bioremediation as the yeast is a mediocre bio-sorbent, easily cultivated at large scale, has a high yield of biomass, can be easily manipulated genetically and the complete genomic sequence is available (Wang

and Chen, 2006). In addition, research has shown that *S. cerevisiae* has the ability to remove toxic metals by accumulating metals in an external layer of the cell wall (Soares and Soares, 2012).

Due to ash being rich with nitrogen, phosphorous and potassium which are the main nutrients for plant growth, these MSW leachates could be used as a replacement for commercial fertilisers. In addition, application of lime or alkali substance can reduce soil acidity. However, the heavy metals present in MSW ash are toxic for plants and animals, removing metals makes it more applicable in the agriculture field (Giordano *et al*., 1983). One study has shown that MSW fly ash, bottom ash and combined ash can influence plant growth in a positive manner. Growth of alfalfa and Swiss chard in ash-amended soils was similar to that in soils amended with phosphorous and potassium fertiliser, indicating that MSW ash can supply essential nutrients for plant growth (Rosen *et al*., 1994; Lam *et al*., 2010).

2.5.2 Mechanisms of Bioleaching

In the environment, heavy metals are present at low concentrations in the soil; however, these metals can be toxic at higher level such as zinc and copper, while others like aluminium and lead are only known for their toxicity. Soil acidity helps to dissolve metal containing minerals and increases uptake by plants, which causes metal toxicity as the plasma membrane of root cells is often damaged by exposure to toxic metals, resulting in leakage of cellular solutes. However, there are some plants called edaphic ecotypes which can tolerate the presence of heavy metals in the soil (Hodson and Bryant, 2012).

Nowadays, there are many causes increasing the concentration of heavy metals in the environment (Farrell and Jones, 2009). The composting process has been defined as the biological decomposition of organic matter by adverse population of microbes under controlled aerobic condition to form stable humus –like end products (Swan *et al*., 2002).

Fungi can tolerate metals using two mechanisms: firstly, extracellular (chelation and cell-wall binding) sequestration, this step avoids metal entry into the cell (Hall, 2002). The second mechanism is the intracellular physical sequestration of metal either by binding to proteins or other ligands preventing damage to cellular targets. In this mechanism metal transport proteins may be involved in metal tolerance, either by extruding toxic metal ions from the cytosol out of the cell or by allowing metal sequestration into the vascular compartment (Khan and Maqsood, 2007; Maghsood *et al*., 2009), (Figure 2.18).

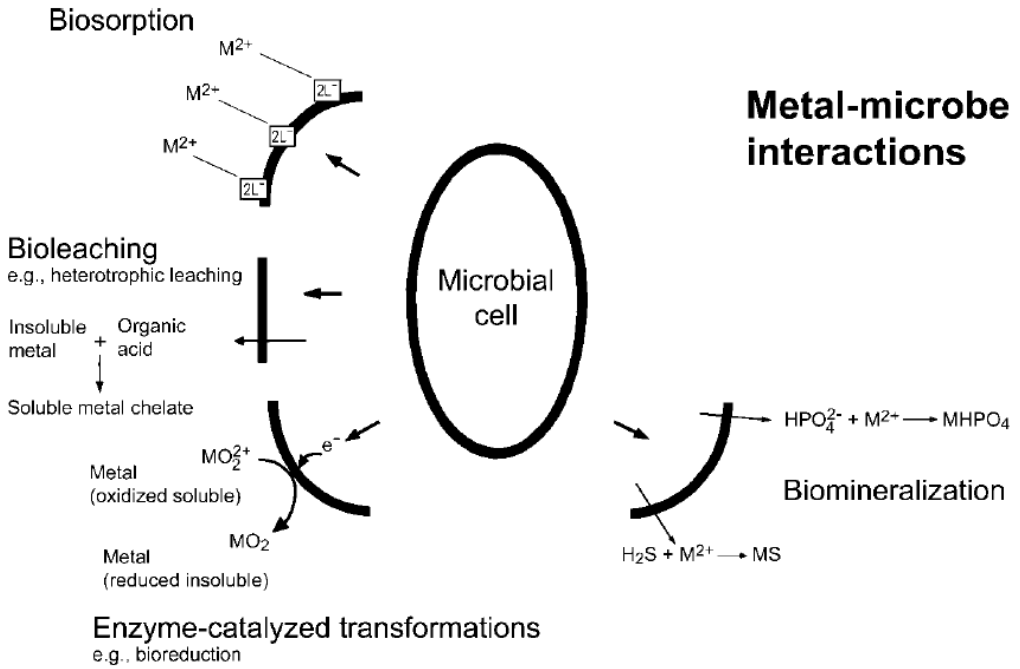


Figure 2.18 Mechanism of metal-microbe interactions that can be harnessed for bioremediation application (Lloyd, 2002)

The ability of microorganisms (bacteria and fungi) to mobilize and leach metals from solid materials is based mainly on three principles (Brandl, 2001):

I. Redoxolysis (oxidation and reduction reactions)

Divided into direct and indirect mechanisms: **direct mechanism**: metals are solubilised through enzymatic reaction, through physical contact between the leaching materials and microorganisms. Leaching a metal from a solid structure may occur through oxidation or reduction reactions. This involves the transfer of

electrons either from the solid structure to an electron acceptor like O_2 called oxidation or the injection of electrons into the solid structure from an electron donor like H_2 termed reduction (Ehrlich, 1997).

Direct bacterial leaching can be described according to the following reaction:

$$MeS + 2\,O_2 \xrightarrow{\text{bacteria}} MeSO_4$$

In the above, MeS is the metal sulphide (Bosecker, 1997), direct leaching benefits the autotrophs, because they conserve energy during the process (Ehrlich, 1992).

Indirect redox mechanism causes oxidation of ions originating from the microbial oxidation of ferrous iron (Fe^{2+}) compounds, which helps to dissolve metals from the solid chemically. Ferric iron is an oxidising agent (Brandl, 2001). Redoxolysis of fungal bioleaching is a reduction of ferric iron and manganese, mediated by oxalic acid in an acidic environment (Burgstaller and Schinner, 1993). Indirect leaching of metal sulphides can be described by the following reactions (Ehrlich, 1997):

$$2\,Fe^{2+} + 2\,H^{+} + \tfrac{1}{2}\,O_2 \xrightarrow{\text{bacteria}} 2\,Fe^{3+} + H_2O$$

$$2\,Fe^{3+} + MeS \xrightarrow{\text{abiological}} Me^{2+} + S + 2\,Fe^{2+}$$

$$S + H_2O + 3/2\,O_2 \xrightarrow{\text{bacteria}} H_2SO_4$$

II. Acidolysis (the formation of organic or inorganic acids)

In acidolysis, organic acids are formed by bacterial metabolism resulting in organic acidolysis, complex and chelate formation (e.g. production of citric acid or gluconic acid by *A. niger* or P. *simplicissimum*, and sulphuric acid by *A. ferrooxidans* and *A. thiooxidans*) (Scragg, 1999). The acidolysis mechanism is solubilization of heavy metals by bio-produced acids this step plays the most important role in bioleaching process (Bosshard *et al*., 1996).

Mineral solubilisation occurs simultaneously in the presence of ligands under acidic conditions. A kinetic model of the coordination chemistry of mineral solubilisation has been developed which explains the dissolution of oxides by protonation of the mineral surface and the surface concentration of suitable complex forming such as oxalate, malonate, citrate and succinate (Scragg, 1999). The protons and the oxygen combine with water and the metal is therefore detached from the surface (Burgstaller and Schinner, 1993).

$$MeO + 2\,H^{+} \longrightarrow Me^{2+} + H_2O$$

In the above, MeO is the metal oxide.

Protons are obtained from the acids produced, and the maximum amount available determines the amount of metal oxides solubilized. This process is usually fast and it is the most important mechanism for fungal bioleaching. In the above, MeO is the metal oxide (Burgstaller *et al*., 1992).

III. Complexolysis (the excretion of complexing agents)

The third mechanism including extraction of metals by complexing agents, to form soluble metal complexes, organic acids can leach metals through complexation. Complexolysis is a slower mechanism when compared with acidolysis, metal dissolution depends on the complexing capacity of molecules and bonds in the solid particles, so if the bond between metal ions and ligands are stronger than the lattice bonds between metal ions with solid particles, the metal will be successfully leached out from the solid particles (Ehrlich, 1992). Additionally, the complexation of heavy metals can reduce metal toxicity to the fungi when high concentrations of metals are present (AUNG, 2005).

IV. Bioaccumulation

Bioaccumulation is another important mechanism or process in fungal bioleaching. in this process the mycelium functions as a "sink" for the metal ions

and causes continuous solubilisation of the metals by the accumulation of metal ions from the leaching solution through active metabolic reactions and passive adsorption, this continuous solubilisation upsets the equilibrium between the solid and dissolved metal. In addition, the fungal cell wall contains many different functional groups (e.g. hydroxyl, amine, carboxyl, phosphate and sulphydryl groups), which are able to bind metal ions to a greater or lesser extent (Kapoor and Viraraghavan, 1995).

2.5.3 Factors Influencing Bioleaching

The effectiveness of leaching depends largely on the efficiency of the microorganisms, chemical and mineralogical composition of the material to be leached and leaching conditions. The maximum metal extraction can be achieved only when optimum conditions are employed (Bosecker, 1987).

Nutrient Culture Media - Effective microbial growth, biosynthesis of new cells and metabolism requires nutrients in order obtain to get maximal growth some selective nutrients help in the production of the necessary metabolites for bioleaching. The presence of ammonium, phosphate and magnesium salts have been shown to increase growth rates; inorganic iron and sulphur compounds are required for chemo-litho-autotrophs (Bosecker, 1987). For leaching metals some nutrients help to increase the production of organic acids and scavenge metals (Banfield *et al*., 1999). Research has revealed that potassium deficiency increased oxalic acid production significantly by tree seedlings colonised by the fungus *Paxillus involutus*, while Mg^{2+} deficiency increased oxalate production in both mycorrhizal and non-mycorrhizal tree seedlings in the same experiment (van Schöll *et al*., 2008). Furthermore, carbon source plays an important role in the determination of quality and quantity of organic acid production (Couto and Sanromán, 2006).

Microbial type: There's a diverse range of mechanisms that microbes can adopt in bioleaching processes because of difference in their metabolic activities. These differences can be intra- or inter-species, depending on other factors such as exposure to high levels of heavy metals. For example, *Aspergillus* and *Penicillium* have mutants that can withstand heavy metals and a genetic adaptation (mutation) (Couto and Sanromán, 2006). Aerobic and anaerobic microorganisms are both involved in many biohydrometallurgical processes; these microorganisms require adequate oxygen or CO_2 to get their optimum growth and activity. Aeration, shaking or stirring are some of the common methods employed in the laboratory to supply oxygen or CO_2 to the microbes because of insufficient oxygen or CO_2 cause slow microbial growth as a result there is a decrease in the metals leaching rate (AUNG, 2005).

pH and temperature: Optimal microorganism growth is pH dependent along with solubilisation of metals. Its known that low pH is the most favourable condition for metal solubilisation (Karaivko *et al*., 1977; Jain and Sharma, 2004). Temperature plays a role in a bioleaching process, an optimal temperature should be maintained according to the optimal microbial growth condition. Mesophilic microbes grow at temperatures of 28-35 °C, while thermopiles grow at temperatures above 50 °C (Bosecker, 1987; AUNG, 2005).

Metal Resistance of Microorganisms: Leaching metals from the substrate is accompanied by an increase in metal concentration in the leachate. Generally heavy metals exhibit toxic effects due to four factors: (1) the blocking of functional groups of biologically important molecules, (ii) the displacement and /or substitution of essential metal ions from biomolecules and functional cellular units, (iii) the induction of conformational changes of polymers, and (iv) the influence on membrane integrity and transport processes (Gadd and White, 1993). In addition, highly toxic metals in a substrate could inhibit microbial growth, and thus decreases the bioleaching rate and efficiency (Kida *et al*., 1996). A high content of carbonate in the solid residue, increases the pH of the

leaching solution and influences microbial growth on substrates such as fly ash (Bosecker, 1997).

Particle Size, decreasing particle size increases the surface area, which leads to an increase in the contact area between the leaching agents and the solid particles; as a result there is an increase in the leaching yield. Research has revealed that the highest solubilisation rate occurs with a particle size of a few tens of microns (AUNG, 2005). Also, solid liquid ratio is another factor. Increasing the solid mass causes an increase in the amount of a toxic metals in the leaching environment thus, an optimum pulp density must be determined for the bioleaching process (Bosecker, 1987; AUNG, 2005)

Bioleaching Period, bioleaching requires a longer period to leach metals when compared with chemical leaching i.e., *Thiobacilli* is a slow growing bacterium that may require a few weeks to complete the bioleaching process. Fungi generally show a shorter lag phase and hence may bioleach at a faster rate (Bosshard *et al*., 1996).

Physicochemical factors, other physical factors include shaking, and aeration. Most of these remaining factors are interconnected (Couto and Sanromán, 2006).

2.5.4 Fertiliser or compost production from MSW

MSW is composed largely of kitchen and yard waste; these wastes have been composted by many municipalities (Otten, 2001). The composting process converts organic waste material into a low cost product, that is suitable for agriculture (Wolkowski, 2003). For compost production, many factors have to be taken into the consideration such as economic and environment, municipal landfill capacity; costs associated with land filling and transportation of materials; adoption of legislation to protect the environment; decreasing the use of commercial fertilisers; increasing the capacity for household waste recycling and improved quality of compost products (Hansen *et al*., 2006).

The main advantages of compost production are: reduction in volume of the wastes, kill pathogens, prevents germination of weeds in agricultural fields, and destroys malodorous compounds (Jakobsen, 1995). There is rising interest in organic composter production from MSW for agricultural use due to positive effects on biological, physical, and chemical soil properties (Iglesias-Jiménez and Alvarez, 1993; Hargreaves *et al*., 2008).

1) **Physical soil properties,** the primary benefits of MSW compost is that it has a high content of organic matter and low bulk density (Soumare *et al*., 2003). MSW compost contains a humic acid to fulvic acid ratio of 3.55 (He *et al*., 1995). MSW compost has some other advantages such as increasing soil organic matter; increasing soil C/N ratio; (Walter *et al*., 2006); the compost has a higher water holding capacity than the soil; it improves soil structure (Soumare *et al*., 2003); and increases aggregate stability (Annabi *et al*., 2007).

2) **Biological soil properties** soil quality is determined by soil microbiological properties (Crecchio *et al*., 2001). Addition of MSW compost to the soil increases N, C and S immediately and for up to one month, while for P, biomass requires five months (Perucci, 1990). Other advantages of adding compost are an increase in soil microbial biomass and soil respiration (an index of general metabolic activity of soil microorganisms) (Bhattacharyya *et al*., 2003). Another measure of soil microbial health is the activity of soil enzymes involved in the transformation of the principal nutrients (Crecchio *et al*., 2004). Research has shown that trace metals have an effect on the biological activity in a soil after being applied with compost derived from MSW, due to the high level of trace metals, this effects depends on the time of application, their concentration, and soil characteristics (Crecchio *et al*., 2004). To study the effects of MSW compost on soil biology should include metal analysis (Crecchio *et al*., 2001).

3) Chemical properties

pH Applying MSW helps to increase soil pH and has been highlighted as a major advantage. This increase in soil pH is due to the mineralization of carbon and the subsequent production of OH ions by ligand exchange as well as the introduction of basic cations, such as K^+, Ca^{2+}, and Mg^{2+} (Mkhabela and Warman, 2005).

Electrical conductivity EC and salt effects, increasing salt content has a negative effect on soil which effects plant growth, the EC of the soil solution relates to the dissolved solutes content and salt content in the soil. Agricultural soils EC levels range from 0 to 4 dS/m, while MSW composts range from 3.69 to 7.49 dS/m (Brady and Weil, 1996). Applying MSW compost at rates ranging from 40 to 120 Mg/ha has led to an increase in the EC content of soil EC (Walter *et al*., 2006).

Nutrients (N,P,K), differences in leaching rate or availability for plants depends on the feedstock and compost maturity (Ring and Warman, 2000). MSW compost contains nitrogen which could become available for the plant this availability of N in MSW compost has been estimated at 10 % in the first year after application with some reports of N release in the second year after application (Zhang *et al*., 2006; Hargreaves *et al*., 2008). Some other study reported N in MSW compost be available 6 months after application (Hadas and Portnoy, 1997). While, for P from MSW compost requires three consecutive years (Bar-Tal *et al*., 2004). Some other studies report 10-50 % P in MSW was available in both first and second year after application (Soumare *et al*., 2003).

Phosphorus and nitrogen content in MSW compost are not regulated by the Canadian Council of Ministers of the Environment (CCME) or the United States EPA (USEPA, 2000; CCME, 2005)

Potassium is another important mineral for plant growth and was found to be increased even when low amounts of MSW derived compost was

applied around 36–48 % of total K in the MSW compost was found to be available to the plant (Soumare *et al*., 2003). The total concentration macronutrient and metals that has been found in MSW composts is shown in Figure 2.19.

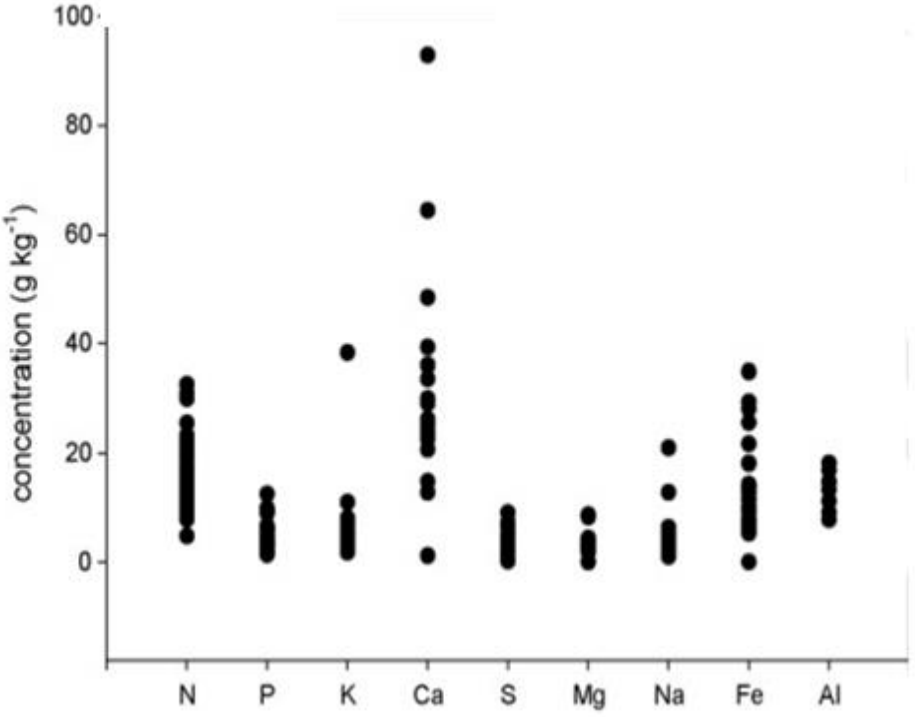


Figure 2.19 Total concentrations of the macronutrients and metals present in MSW compost (Hargreaves *et al*., 2008)

Chapter 3 Characterisation of Municipal Solid Waste (MSW)

3.1 Introduction

In general MSW is made up of solid and semi-solid waste from cities and is likely to be quite variable and may contain both bio-degradable (organic) (OFMSW) and non-biodegradable substances (inorganic). This variation in composition may prove problematic for its use as a bio-energy feed stock although this can be overcome to some extent by the introduction of specifications for the feedstock a process that is routinely used for RDF.

Substrate composition analysis thus plays an important role prior to bio-energy production; the main components of OFMSW are lipids, proteins, and carbohydrates (cellulose and hemicelluloses) and lignin. These substances can all be degraded, except lignin which can cause resistance and be limiting for bio-energy production (Mosier *et al*. 2005; and Fernandes *et al*. 2009). Cellulose and hemicellulose are normally responsible for more than 50 % of the total dry mass in most of the MSW.

The main objective of this study was to investigate the feasibility of using MSW for bio-energy production. The first objective was thus to determine the chemical composition and physical properties of the MSW. Compositional analysis (cellulose, hemicellulose, lignin, starch, lipids, protein and total nitrogen) was carried out to determine the major components present in the MSW. This was accompanied by analysis of trace elements and assessment of physical properties such as particle size and cellulose crystallinity.

Three separate samples of MSW have been analysed to determine the potential heterogeneous nature of this OFMSW substrate.

3.2 Materials and method

3.2.1 Procurement of MSW

Municipal solid waste (MSW) was kindly supplied by Wilson Steam Storage Ltd based in Northern Inland (UK). The MSW which called black bag waste had been subjected to magnetic separation to remove big particles, then autoclaving at 165 ^{o}C for 2 hr, the autoclaving consist of screw big drum contain blades, followed by steam injection this process called physical agitation which breaks up organic materials. The MSW as received was sieved using a 2 mm sieve (powder form), and the fraction that passed through the sieve was collected and dried in an oven at 70 ^{o}C overnight. The first batch was stored dry at room temperature and used for all the experiments in this thesis. The other two batches were used for compositional analysis only.

3.2.2 Sample preparation for analysis

The MSW samples as received were first sieved to remove large fragments of plastic and metal which were discarded, using 2 mm-mesh sieve (Rump and Krist, 1988). Three batches of MSW were processed the first batch was a powder. The second and third batches of MSW were different with a larger average particle size compared to the first substrate. All three samples were oven dried immediately, as described in section 3.2.1, and stored at room temperature.

3.2.3 Physical characteristics

3.2.3.1 Moisture Content (%MC)

The moisture content of the MSW samples was measured by weighing 1 kg of representative MSW onto a pre-weighted aluminium tray, which was, then placed into an oven at 70 ^{o}C for 24 hr and until a constant weight was achieved (Juanga, 2005). Moisture content was then calculated using the following formula:

$$\% \text{ MC} = \frac{1000 - \text{Wo}}{1000} \times 100\ \%$$

Where Wo= Sample weight after drying

MC= Moisture content

3.2.3.2 Determination of cellulose crystallinity in MSW

Cellulose crystallinity of the MSW materials was measured by wide angle X-ray diffraction using a Siemens S5000 system with a vertical theta-theta goniometer, using powder reflection geometry, with Cu^{2+}, Kα incident radiation (1.542 nm), with fixed detector slit. The raw diffraction curve was recorded from 5 to 50° 2θ, at 0.05° resolution, which was then subjected to a linear baseline correction over these limits. Total biomass crystallinity was estimated by an amorphous subtraction procedure, according to equation (1) using an experimental profile of a highly ball milled sample, where the amorphous intensity was adjusted so it just touched the sample profile in the region of 17° 2θ. Estimation of biomass crystallinity was complicated by the presence of non-biomass related mineral diffraction peaks, from inorganic components of the municipal waste which were subtracted manually from the broader biomass profile.

$$\text{Total crystallinity } (\%) = \frac{100 \times (\text{corrected total intensity} - \text{amorphous intensity})}{\text{Corrected total intensity}} \qquad (1)$$

A further procedure was adopted where the main known cellulose diffraction peaks were fitted to the corrected sample profile by modelling as Gaussian shapes. The combined integral intensities of these peaks, from the [110], [0-10], [200] and [102] reflections represented the crystalline cellulose contribution. The modelled biomass crystallinity was then calculated using equation (2).

$$\text{Modelled crystallinity } (\%) = \frac{100 \text{ x integral of fitted cellulose peaks}}{\text{Fitted cellulose integral} + \text{Amorphous integral}} \qquad (2)$$

An estimation of the lateral dimensions of the cellulose fibrils in the cell walls of the biomass component of the MSW was determined using the Scherrer equation (3), where L is the dimension corresponding to the cellulose-I [200] reflection, IB

is the [200] integral breadth, following the curve fitting of this diffraction peak, λ is the X-ray wavelength (Cu Kα= 1.542 nm) and θ is the diffraction angle of the [200] peak in radians.

$$L = \frac{0.9\lambda}{IB\cos(\theta)} \qquad (3)$$

3.2.3.3 pH (potential Hydrogen)

A fresh sample was made up in five times its volume ratio (1:5) of Ro water. The suspension was shaken for 1 hr using a mechanical shaker and the final pH recorded (WI, 2005).

3.2.3.4 EC (Electrical Conductivity)

EC at equilibrium in a water suspension of soil, sludge, or bio waste is expressed as milli Siemens/meter (mS/m) or micro Siemens/meter (µS/cm). The sample was tested by using an EC module (conductivity meter FE30/FG3).

20 g of sample was added to 200 ml distilled water, followed by shaking at 20 ^{o}C, for 30 min. After shaking, the samples were filtered using filter paper (with low ash content and high retentive properties) and the EC recorded as mS/cm (WI, 2005).

3.2.3.5 Alkalinity

Alkalinity measures "the ability of a solution to neutralize acids and can be expressed using several different units" (typically calcium carbonate mg $CaCO_3$/L).

50 mL distilled water was added to 10 g of dried MSW to prepare a suspension (1:5 w/v). The suspension was shaken for 30 min. The sample was titrated potentiometrically to an end-point of pH 4.5 with 0.1 N H_2SO_4 or HCL (Noble et al., 1996). Alkalinity was calculated using the following equation:

$$\text{Alkalinity mg } CaCO_3/L = \frac{A \times N \times 50.000}{\text{mL sample}}$$

A = mL of acid used for titration to pH 4.5.
N = Normality of acid (0.1 N H_2SO_4).

3.2.4 Chemical characteristics

3.2.4.1 Total sugar, cellulose and hemicellulose

Total sugar, cellulose and hemicellulose were measured using the acid hydrolysis method; 30 mg of MSW was placed into a 20 mL Pyrex boiling tube, and 1 mL of 12 M sulphuric acid added. The sample was placed in a water bath at 37 ^{o}C for 1 hr.

After incubation, 11 mL of demineralised water was added and the sample incubated at 98 ^{o}C for 2 hr, allowed to cool and centrifuged 5 min at 4472 g. Finally 1 mL of the diluted sample was transferred to a high performance liquid chromatography (HPLC) vial for sugar analysis.

Monomeric sugars along with suitable sugar standards were analysed by high performance anion exchange chromatography (HPAEC). The HPAEC was used to identify and quantify monomeric sugars (arabinose, galactose, glucose and xylose). A Dionex ICS-300 Ion chromatographic was used for the detection of monosaccharides (arabinose, galactose, glucose and xylose). The samples were prepared 1:10 ratio (sample:RO water), all samples and standards were filtered using 0.2 µm pore size Whatman GD/X syringe filters (GF/C 25 mm filter diameter; Whatman International Ltd., UK).

Reagent-Free™ ion chromatography equipped with the instrument system, electrochemical detection using ED 40 and computer controller. A caroPacTM PA 20 column (3*150 mm) was used and the mobile phase was 10 mM NaOH with a flow rate of 0.5 mL/min. The injection volume was 10 µL and the column temperature was 30 °C. The sample analysis was completed in 14 min.

The chromatogram of standards and retention time of each sugar (Arabinose 6.92, Galactose 7.2, Glucose 8.1, and Xylose 9.2), the concentration of standards were 0.5-20 g/L The peak area of sugar in standard solution was plotted against sugar concentration to draw a standard curve. Sugar concentration of sample was calculated by interpolation on the standard curve. The analysis can give arabinose, galactose, glucose and xylose concentrations and from these results the amount of cellulose and hemicellulose concentration formulas can be determined (Sluiter and Sluiter, 2005; Sluiter *et al*., 2008). The concentrations of glucose and xylose were used to calculate the cellulose and hemicellulose contents, respectively. For cellulose this was after correction for the glucose contribution from the starch. These concentrations of polymeric sugars were calculated from the concentration of the corresponding monomeric sugars, using a hydro correction of 0.88 (132/150) for C-5 sugars (xylose) and a correction of 0.90 (162/180) for C-6 sugar (glucose) (Sluiter *et al*., 2007).

3.2.4.2 Total lignin

Total lignin was determined using the acetyl bromide method. A 100 mg sample of MSW was added to 4 mL of a solution of 25 % acetyl bromide in glacial acetic acid and incubated in a water bath (50 ^{o}C) for 2 hr. After cooling a further 12 mL of glacial acetic acid was added and the sample centrifuged at 1609 g for 5 min, 0.5 mL of supernatant was transferred to a new falcon tube and 2.5 mL of glacial acetic acid, 1.5 mL of 0.3 M NaOH, 0.5 mL (0.5 M) hydroxylamine hydrochloride (Hamcl) solution added, the volume was made up to final volume of 10 mL by the addition of glacial acetic acid. Standard lignin solutions at 0.4, 0.6, 0.8, 1.0 and 1.2 mg/10 mL, were prepared and used as standards following the same procedure as above. Absorbency of the samples was read at 280 nm (Sluiter *et al*., 2008).

3.2.4.3 Starch

A MOPS buffer (3-(N-Morpholino) propanesulfonic acid, 4-Morpholinepropanesulfonic acid) was prepared by dissolving 11.5 g MOPS in 900 mL D.W. and adjusting the pH to 7.0 using 1 M HCL; then adding 0.74 g calcium chloride dehydrate and 0.2 g sodium azide, finally the solution was brought to 1 L with DW.

An acetate buffer was prepared by adding 11.8 mL glacial acetic acid to 900 mL distilled water, adjusting the pH to 4.5 using 1 M sodium hydroxide, then adding 4 g sodium acetate bring the final volume to 1 L.

Determination of starch was carried out using the NERL method (Sluiter and Sluiter, 2005), 100 mg MSW was added to 0.2 mL ethanol and 2 mL dimethyl sulfoxide (DMSO), and placed into a water bath (100 ^{o}C) for 5 min. Following this incubation, 2.9 mL MOPS buffer was added to the sample. Then 0.1 mL thermo stable alpha amylase (150,000 U/g) was added. Samples were incubated at 100 ^{o}C for 6 min, mixing every 2 min. The sample was then transferred to a 50 ^{o}C incubator and 4 mL sodium acetate buffer and 0.1 mL amyloglocosidase, (20 units) added and incubated for 30 min. After incubation samples were centrifuged at 1609 g for 10 min. Samples were prepared for analysis by mixing 1 mL with 9 mL RO water and the glucose content determined by HPLC Dionex ICS-300SP) as mentioned in section 3.2.4.1. Starch content was calculated using the following formulas (Sluiter and Sluiter, 2008):

$$\mathrm{ODW} = \frac{\text{weight (air dry sample)} \times \%\text{Total solids}}{100} \qquad (1)$$

$$\%\ \mathrm{R\ starch} = \frac{\text{Conc.detected by HPLC, mg/mL}}{\text{Known weight of starch before analysis, mg/mL}} \times 100 \qquad (2)$$

$$\%\ \mathrm{Starch} = \left(\mathrm{CHPLC} \times \left(\frac{\text{Vol.solution.ml}}{\mathrm{ODW}}\right)\middle| 1.11 \times \%\ \mathrm{R\ starch}\right) \times 100 \qquad (3)$$

Where:

C_{HPLC} = cConc. of glucose as determined by HPLC, mg/mL.
Vol. Solution = 9.3 mL, total volume of liquid added to solids.
ODW = Oven Dry Weight of the sample, mg

1.11 = Is the glucose to starch oligomer correction factor
% R_{starch} = Is the starch recovery

3.2.4.4 Total Lipid analysis

The lipid content was measured using the Folch method as described by (Cequier-Sánchez *et al*., 2008). A sample of 400 mg MSW was added to 12 mL of dichloromethane/methanol (2:1,v/v) and incubated for 2 hr at room temperature with occasional mixing. Following incubation samples were centrifuged at 178 g for 5 min and the upper organic phase was carefully removed using a glass syringe and transferred into a clean 50 mL glass centrifuge tube, 2.5 mL KCl (0.88 %, w/v) was added and the sample centrifuged at 178 g for 5 min and the lower organic phase carefully transferred into a pre-weighed glass tube. The lower phase was dried under nitrogen gas until all liquid had evaporated. The tube was reweighed to give the total lipid content (Cequier-Sánchez *et al*., 2008).

3.2.4.5 Total Nitrogen/Protein

The analysis was carried out using a thermo scientific flash EA1112 Nitrogen/ protein analyser; (Thermo Scientific Flash EA1112) with L-aspartic acid as the standard using the method as described by (Campbell and Plank, 1992). The procedure started by placing a 50 mg sample into a sealed tin capsule, which was dropped from the auto sampler into the combustion chamber. This was maintained at 900 ºC and packed with an oxidation catalyst. When the sample had been injected into the combustion chamber, a fixed amount of oxygen was added to the helium carrier gas. Under these conditions the highly exothermic oxidation of tin brings the local temperature up to around 1800 ^{o}C, ensuring the complete combustion of the sample. This process is called dynamic flash combustion. The combustion gases are then swept through the reduction reactor which contains reduced Co^{2+} and is maintained at 680 ºC. Here, oxides of

nitrogen are converted to elemental nitrogen and excess oxygen from the combustion process is trapped.

The helium stream then passed through filters of soda lime, magnesium perchlorate, and a molecular sieve to remove CO_2, SO_2, and H_2O. The stream was then passed through a nitrogen separation column at 50 ^oC and into a thermal conductivity detector. Analysis used the Eager 300 software, using L-aspartic acid as standard (Campbell, 1992; Fisher, 2007).

Determination of protein from total nitrogen data using conversion factor (Hames *et al*., 2008), for the current study The calculation factor from nitrogen is 6.25. Nitrogen conversion factors depend on the amino acid composition of the protein. Generally, a specific Jones factor for nitrogen content of the food being analyzed should be used to convert nitrogen to protein when the specific factor is known. When the specific factor is not known, N x the general factor 6.25 should be used (Maclean *et al*., 2003).

3.2.4.6 Trace element analysis

The sample was digested as follows; firstly all the glassware was washed and dried. Concentrated HNO_3 15 mL of was added to 2 g of MSW and a blank (acid without sample), were then both digested on a hot plate, until the volume reached around 5 mL. Samples were filtered using filter paper (No.42) then distilled water added to make a final volume of 100 mL. The sample and blank were analysed using the Inductively Coupled Plasma Mass Spectrometer (ICP-MS), which can be used for one of three (multi-element) analytical applications: Trace-element analysis at the ppb (µg/L) level; detector operating in pulse-counting mode but with a mass-dependent cross calibration factor to convert analogue signals. Major elements (Ca^{2+}, Mg^{2+}, K^+, and Na^+) were analysed at the ppm (mg/L) level; detector operating in analogue mode only, and determination of isotope ratios for individual elements; detector operating in pulse-counting mode only. Starting the instrument by Check air conditioner; change temperature

setting to 18 ^{o}C and put fan on full; Check that cylinders of Collision Cell gas (7 % H_2 in He and H_2 alone) are pressurized; Put 50 mL tube with 5 ppb Tune-A solution in 2 % HNO_3 in Autosampler position 0,1,10; Put bottle of 2 % Aristar nitric acid wash on 'internal standard' line if in use; Set peri-pump speed to 17 rpm for a 2-line feed (T-piece in use) and 30 rpm for a single line feed (e.g. Burgener Miramist) if necessary; Turn the plasma on, open correct operating conditions file (usually the latest standard tune conditions in the list and Leave to warm up for 5-10 min aspirating 2 % nitric acid (Hokura *et al*., 2000).

3.3 Results

3.3.1 Moisture content, pH, EC and alkalinity

The moisture content, pH and alkalinity of three MSW batches were determined (Table 3.1). Results revealed that the first batch was completely dry, on receipt, whilst batches two and three had much higher moisture as received. The pH values of the three samples were identical with all three being just below neutral. EC was more variable and showed that the first batch was higher than the other two batches indicating that the first sample had more ions than the other two batches. For alkalinity determination, the levels of alkalinity of 3 batches samples were 9.82, 8.54 and 7.89 mg $CaCO_3$/L, respectively.

Table 3.1 Physical characteristics three of batches of MSW

Samples	MC (%)	pH	EC (mS/cm)	Alkalinity $CaCO_3$/L
Batch 1	0.87±0.054	6.51±1.8	4.92±0.38	9.82±0.065
Batch 2	42.6±4.5	6.3±0.98	3.5±0.096	8.54±0.058
Batch 3	25.4±3.5	6.4±0.88	2.5±0.066	7.89±0.045

3.3.2 MSW cellulose crystallinity

The X-ray diffractograms for the three MSW samples are shown in Figure 3.1. These revealed distinct differences between the three batches. The peaks for Batch 1 between 20-25 (Angle 2θ) are evidence for the presence of the main native cellulose-I allomorph, and these could be fitted successfully to the equations as described in the methods section. The total organic crystallinity in batch one was calculated to be 17.8 %. This is lower than that typically expected for other lignocellulosic material, such as wheat straw, which usually have crystallinities around 23-25 %. This indicates, as expected, the presence of relatively more non-crystalline material in the MSW. The chemically determined

cellulose content of the first batch of MSW was 27.8 %, from which it is possible to estimate the crystallinity of the cellulose fraction as 64 %. This is a similar value for crystallinities found for native cellulose in woody materials; In addition an estimation of the average fibril lateral dimension could be established from the Scherrer equation, which gave a value of 2.7 nm. This again is typical for fibrils in woody materials, which are relatively thin compared to other sources of cellulose such as cotton or flax. Therefore from these XRD results it is reasonable to assume that the major cellulosic contribution to the MSW in Batch 1 maybe coming from paper card packaging or office waste. The X-ray diffractograms for batches two and three were both very different to that for Batch 1. In both cases the peaks between 20-25 (Angle 2θ) are very much reduced. This indicates an absence of crystalline cellulose and is consistent with the compositional analysis of these two batches which indicated a cellulose content of 11.04 and 10.06 %, respectively.

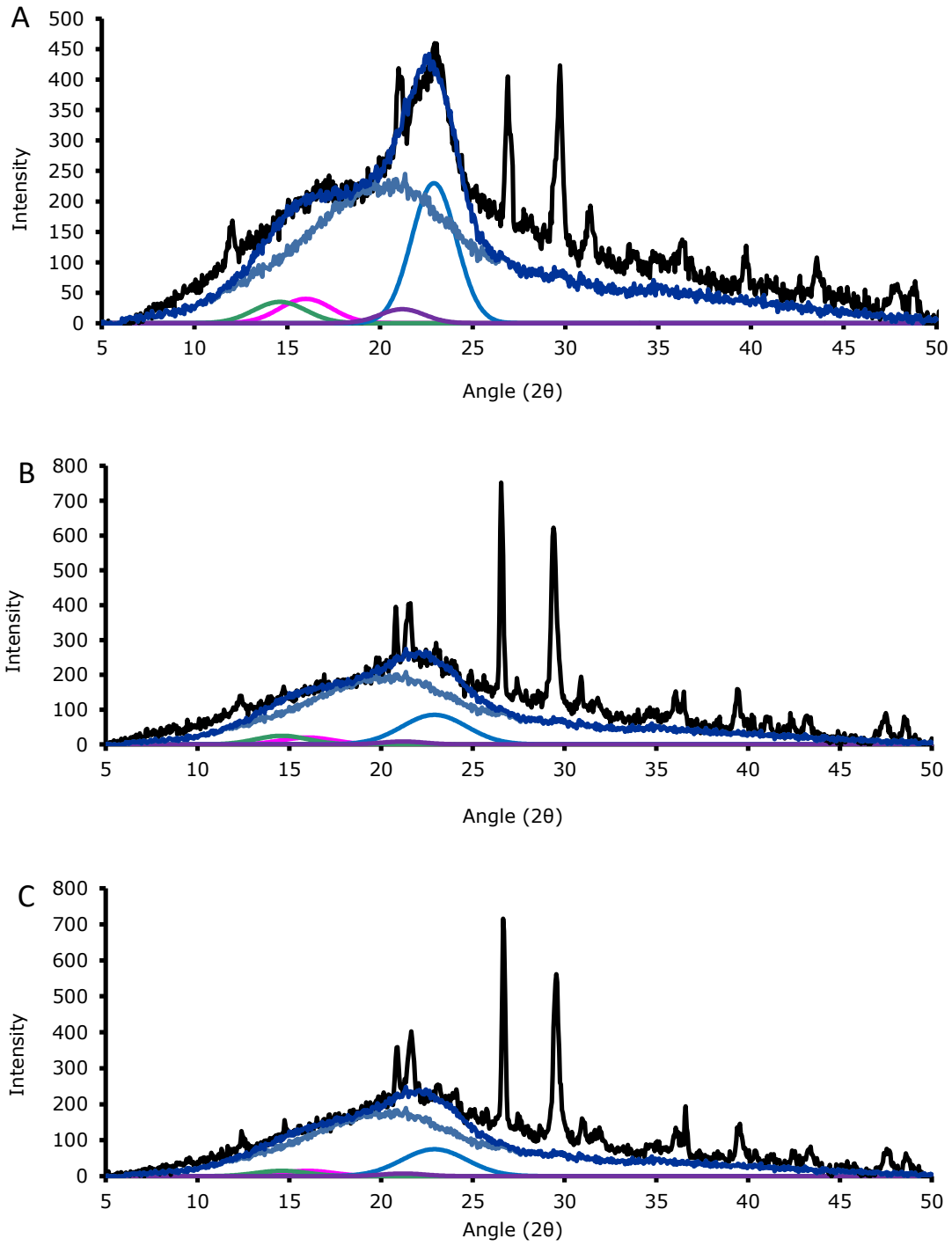


Figure 3.1 X-ray diffraction result of the untreated MSW. The experimental data were then fitted with Gaussian deconvolution interpolation (A) Batch 1 (B) Batch 2 (C) Batch 3

The black line shows the experimental result; the blue line shows the curve fitted data computed where the curve can be treated as a combination of 4 sub peaks shown in green and pink using peak deconvolution method.

3.3.3 Chemical Composition

3.3.3.1 General compositions

The amounts of cellulose, hemicellulose, starch, lipid, protein and total nitrogen in the three MSW samples were determined and are summarised in Table 3.2 Similarly, the sugar monomer compositions are shown in Table 3.3. It is apparent that there were marked differences in composition between Batch 1 and the other two batches. All three batches had similar levels of protein and lipid whilst Batch 1 had approximately twice the level of starch as the other two batches. Lignin in batches two and three was slightly higher than in Batch 1 but the most striking differences were in the levels of cellulose and hemicellulose. The results indicated that only the first batch was enriched with these two polymers. The Batch 1 has about 82 % of the mass accounted for by these components but Batches 2 and 3 were 61 % and 52 %, respectively. Remain part probably small fragments of glass and melted plastic.

Table 3.2 Chemical composition of the three batches of MSW (g/100 g)

Samples	Cellulose	Hemicellulose	Lignin	Starch	Lipid	Total Nitrogen	Protein
Batch 1	27.82±0.1	12.66±0.07	17.78±0.05	6.28±0.02	11.23±0.1	1.88±0.07	11.75±0.2
Batch 2	11±0.04	2.34±0.04	23.72±0.07	3.2±0.02	12±0.05	1.3±0.038	8.06±0.03
Batch3	10±0.17	2.11±0.06	21.57±0.24	3.3±0.06	7.3±0.02	1.2±0.032	7.5±0.05

Table 3.3 Sugar composition of the three batches of MSW (g/100 g)

Samples	Arabinose	Galactose	Glucose	Xylose
Batch1	2.877±0.08	0.68±0.03	30.8±0.05	14.36±0.01
Batch 2	2.07±0.05	0.80±0.02	12.26±0.07	2.66±0.05
Batch 3	2.10±0.054	0.731±0.025	11.11±0.08	2.40±0.012

3.3.3.2 Trace element analysis

Elemental analysis for the three MSW samples was divided into major and minor elements and these are shown in Tables 3.4 and 3.5, respectively: In terms of the major elements (Na^{+}, Mg^{2+}, P^{3-}, S^{2-}, K^{+} and Ca^{2+}) all of these were similar except for S which was much higher in Batch 1 than in the other two batches. A total of 22 minor elements were detected in the MSW. Batch 1 generally had higher levels of these elements and in particular had higher levels of Zn^{2+}, Cu^{2+}, Ni, Co^{2+}, Mn^{2+} and $Al^{+3.}$

Table 3.4 Major element analysis of the three batches of MSW (mg/kg)

Samples	Na^{+}	Mg^{2+}	P^{3-}	S^{2-}	K^{+}	Ca^{2+}
Batch 1	2509.27±145	2722.43±254	1113.66±151	5392.43±299	2190.69±120	25064.74±1550
Batch 2	1846.55±219	2182.37±200	1426.67±223	2037.69±239	1722.23±231	21075.73±2278
Batch3	2177.40±102	2385.02±438	1442.55±48	2177.92±187	1944.97±105	22168.96±1789

Table 3.5 Minor element analysis of the three batches of MSW (mg/kg)

Samples	B^{3}	Ti^{3}	Al^{3+}	V^{2}	Cr^{6+}	Mn^{2+}	Fe^{2+}	Co^{2}	Ni^{2+}	Cu^{2+}	Zn^{2+}
Batch1	41.56±4.8	21.88±1	3120.61±119	23.88±0.9	15.96±0.7	397.73±3	3851.89±200	11.27±0.6	93.47±28	1844.95±116	612.68±43
Batch 2	6.17±0.7	12.17±1	1876.54±335	26.25±3	25.95±2.4	120.40±15	3258.52±548	1.83±0.23	12.69±2	122.08±63	346.84±99
Batch 3	6.96±0.3	12.50±1	1954.41±133	26.71±1	26.88±2.3	137.24±5	2817.18±274	1.83±0.08	12.37±1	70.37±5	388.93±117

Samples	As^{3-}	Se^{3-}	Rb^{3-}	Sr^{2-}	Mo^{3+}	Ag^{+}	Cd^{2+}	Cs^{+}	Ba^{2+}	Pb^{+}	U^{4+}
Batch 1	2.98±0.4	0.13±0.02	2.32±0.14	63.41±7	3.39±0.08	3.15±0.38	33.92±2	0.15±0.012	291.22±24	161.19±9	0.23±0.018
Batch2	1.57±0.1	0.15±.028	1.74±0.25	44.71±6	1.15±0.14	0.48±0.06	1.35±0.08	0.12±0.02	62.93±12	123.66±25	0.16±0.025
Batch3	1.59±0.2	0.14±0.021	1.92±0.14	45.92±3	1.22±0.17	0.56±0.05	2.64±0.6	0.12±0.007	61.97±5	123.37±13	0.15±0.011

3.4 Discussion

The physical characteristics of the MSW samples used in this study are summarised in Table 3.6 and compared to values in the literature.

The moisture content of our samples was very variable. The first batch being very low compared with the other two batches. This may be due to the fact that the samples were pre-treated using an autoclave and dried before despatch. The last two batches had been stored for a period and thus may have either not been dried or stored properly. This could have resulted in a change in composition. Batch 1 was thus used for further experiments and Batches 2 and 3 only for compositional analysis.

The solid content of the first batch was very high compared with other studies (Poggi-Varaldo *et al*., 1997), these other studies used MSW that had been dried by air and sun exposure, and their results may have thus varied from those obtained here as they did not use an autoclave (Wang *et al*., 2001)

pH and alkalinity, pH values were almost the same for all three batches, being just below neutral in each case. Previously reported pH values for MSW vary considerably between 4.59 and 8.6 (Ibrahim *et al*., 2011; Kasthuri *et al*., 2011), thus the values obtained here are within this range. The low pH observed in the MSW sample analysed in this study may be due to the fact that they may contain more organic acid and thus make the substrate acidic as suggested by (Ibrahim *et al*., 2011). Furthermore, the presence of acidic compounds depends on substrate composition such as organic fraction composition and separation technology (Liu *et al*., 2008). Whilst, an alkaline pH above 8 might be due to lower levels of organic acids (Hartmann and Ahring, 2005). Determination of alkalinity revealed that alkalinity of the first batch was 9.82 mg $CaCO_3$/L. Alkalinity value from previous studies ranged from 14 to 5.1 mg $CaCO_3$/L (Fernández *et al*., 2008; Forster-Carneiro *et al*., 2008), respectively. The high

alkalinity value may be attributed to the degradation of protein present in the waste, degradation releases NH_3 which reacts with CO_2 forming ammonium carbonate which is alkaline (Adhikari, 2006). Generally, alkalinity increases or decreases depending on pH and, as a result, depend on the same reasons as for pH and indeed the alkalinity value in the 3 batches were almost the same.

Determination of the EC in the 3 batches of MSW samples revealed a higher value of 4.92 mS/cm in Batch 1 compared with the other two batches. These samples showed a lower EC value when compared to (Kasthuri *et al*., 2011) who determined that EC in a MSW substrate was 10.2 mS/cm. The lower values in this study compared to the literature may be due to the presence of a lower concentration of soluble salt (which inhibit biological activity and may be unsuitable for land application if large quantities of the material are used) (Kasthuri *et al*., 2011).

Table 3.6 Comparison of physical characteristics of municipal solid waste (MSW) in this study with literature values

References	MC (%)*	pH	EC * (mS/cm)	Alkalinity $CaCO_3$/L
Batch 1	0.87	6.51	4.92	9.82
Batch 2	42.6	6.3	3.5	8.54
Batch3	25.4	6.4	2.5	7.89
(Kasthuri et al., 2011)	*	8.6	10.4	*
(Fernández *et al*., 2008)	57.17	6.51	*	14
(Poggi-Varaldo *et al*., 1997)	*	5.83	*	*
(Ibrahim *et al*., 2011)	74.68	4.59	*	*
(Rao and Singh, 2004)	85	*	*	*
(Adhikari, 2006)	80.6	*	*	*
(Forster-Carneiro *et al*., 2008)	*	7.3	*	5.1
(Abu-Qudais and Abu-Qdais, 2000)	63.3	*	*	*
(Durlak *et al*., 1997)	25.2	*	*	*
(Wang *et al*., 2001)	0.55	*	*	*
(Saikia *et al*., 2007)	*	10.5	*	*

MC= Moisture Content.

EC= Electrical Conductivity.

The chemical composition of the MSW samples used in this study are summarised in Table 3.7 and compared to values reported in the literature.

Cellulose: This study revealed that the cellulose in the first batch MSW was 27.82 g/100 g, while in Batch 2 and 3 were 11.0 and 10.0 g/100 g, respectively.

In a current research X-ray experiment applied to describe the crystallinity of the substrate in a first batch showed crystallinity substances comparing with other two batches, present crystallinity in the substrate has a branched structure and random amorphous with little resistance to hydrolysis (Ademark *et al*., 1998), the effects of crystallinity in a MSW biomass describe in a detail at chapter 2.1.1 and 2.3.3.1. Comparing the cellulose value of first batch in the present study with other studies, it can be seen that they have almost the same values (Jones *et al*., 1983; Barlaz *et al*., 1997; Ivanova *et al*., 2007). One study reported MSW as containing 33 % cellulose and it has been suggested that this is mainly because the samples contained a high proportion of newspaper waste (Poggi-Varaldo *et al*., 1997). Some other literature data has shown cellulose content of MSW at around 40-50 % on a dry weight base (Wang *et al*., 1994), while (Barlaz *et al*., 1989) recorded 51.2 g/100 g cellulose The cellulose content of MSW depends on the amount of paper, wood and plant products which are considered as the dominant biodegradable polymers in municipal waste. The low cellulose content found in Batches 2 and 3 in this study may have been due to a low levels of cellulosic substance in the MSW substrate values as low as 15.50 g/100 g have been reported (Rao and Singh, 2004). Alternatively, this may have been due to the fact that the MSW may not have been stored adequately and the cellulose components degraded by microbial action prior to receipt.

Hemicellulose in MSW typically makes up 12 % of the total dry weight (Wang *et al*., 1994). In the current study, the hemicelluloses content for Batches 1, 2 and 3 were 15.45, 2.3 and 2.1 g/100 g, respectively. The last two batches were very low compared to the first batch.

While, comparing the level of hemicellulose of the first batch with previous studies, the highest reported value was 11.9 g/100 g which was recorded by (Jones *et al*., 1983), while the lowest value was recorded by (Ham *et al*., 1993) which was 5.14 g/100 g, these variations and low hemicellulose content may have been caused the same factors that lead to low cellulose content due to the same reason as described for cellulose.

The lignin contents were 17.78, 23.72 and 21.57 g/100 g, respectively in the three batches, for the first batch, the amount of lignin falls within the reported range for MSW of 10-15 % lignin on a dry weight bases (Wang *et al*., 1994). This data revealed that the lignin content in the first sample was within the expected range. Whilst 10-15 % is the most common range for lignin in MSW other research indicates the highest recorded lignin content was 25.1 g/100 g (Price *et al*., 2003), while the lowest value was recorded by (Jones *et al*., 1983) at 7.2 g/100 g. Thus the values for batches 2 and 3 fall within this range but are still higher than normally expected. This again may have been influenced by the storage conditions. If cellulose and hemicellulose contents of batches 2 and 3 have been reduced during storage this would result in the lignin (which is more resistant to microbial degradation) have a higher final percentage composition.

In this study, the amounts of starch for the three batches were 6.283, 3.2 and 3.3 g/100 g, respectively. The amount of starch in MSW ranged from 10 to 19 % according to (Davidsson *et al*., 2007). This previous study used 17 types of domestically-sourced sorted OFMSW (only food residue and some other parts), each waste type was identified by its origin and by pre-sorting, collection and pre-treatment methods. This range is higher than my data probably due to the fact that the MSW used in this study was unsorted and contained plastic and glass particles so as a result contained lower amounts of the organic fraction. Determination of **lipids** revealed that the samples of MSW had lipid contents of between 7 and 12 g/100 g. The lipid content in the first batch was almost the same as that reported by (Ponsá *et al*., 2011) which was 11.52 g/100 g, and (Rao and Singh, 2004) determined the amount of lipid in MSW substrate at 8.5 g/100 g value. Generally, waste that contain food contain higher amount of lipids (Gallert and Winter, 2005; Hartmann and Ahring, 2006). Thus the lipid content in all three batches was consistent with reported values.

Determination of total **nitrogen and protein** revealed that the amount of total nitrogen in first batch sample was 1.88 g/100 g and last two batches were almost close from the first batch value 1.3, and 1.2 g/100 g, respectively.

These values compared with other values reported in a previous studies showed that the current data within the range with previous studies (Rao and Singh, 2004; Cuetos *et al*., 2008; Ponsá *et al*., 2011)

Nitrogen may be found in the form of ammonia. So, ammonia is one of the total nitrogen forms which range in MSW from 50 to 200 mg/L and can stimulate the growth of microbes, whereas, if its concentration rises above 1500 mg/L, it can inhibit the AD process (Juanga, 2005).

A significant proportion of the nitrogen present in biomass is found in form of protein. So biomass waste contains proteins as a source of nitrogen and sulphur (Yokoyama and Matsumura, 2008). Proteins are bio-macromolecules that consist of amino acids (molecules that contain both amine and carboxylic acid functions) joined by peptide bonds (amide bond) and have a variety of biological roles, such as structure, storage of nutrients, etc (Becidan, 2007).

Regarding MSW, the protein content of Batch 1 was 11.75 g/100 g. Comparing the present data with Batches 2 and 3 were 8.06 and 7.5 g/100 g, respectively. Also comparing with previously published data, it was found that study data was higher to other researchers' data (Rao and Singh, 2004; Cuetos *et al*., 2008; Ponsá *et al*., 2011). The total organic content in a present study is almost 80 % by totalling up the amount of organic are generally associated with measured protein content and, consequently, higher available nitrogen content (Khalid *et al*., 2011) (See Table 3.7).

Table 3.7 Comparison of chemical characteristics of solid waste (MSW) in this study with literature values. All values are in g/100 g (w/w %)

References	Cellulose	Hemicellulose	Lignin	Starch	Lipid	Protein	Total nitrogen
Batch 1	27.8	15.45	17.8	6.283	11.23	11.75	1.88
Batch 2	11.0	2.3	23.7	3.2	12	8.06	1.3
Batch 3	10.0	2.1	21.6	3.3	7.3	7.5	1.2
(Ham *et al*., 1993)	17.28	5.14	12.67	*	*	*	*
(Barlaz *et al*., 1997)	28.8	9	23.1	*	*	*	*
(Barlaz, 2006)	42.4	6.6	10.9	*	*	*	*
(Jones *et al*., 1983)	25.6	11.9	7.2	*	*	*	*
(Bookter and Ham, 1982)	63.4	9	15.7	*	*	*	*
(Barlaz *et al*., 1989)	51.2	8.7	15.2	*	*	*	*
(Price *et al*., 2003)	43.9	5.8	25.1	*	*	*	*
(Ivanova *et al*. 2007)	24.8	6.7	*	*	*	*	*
(Ponsá *et al*., 2011)	*	*	*	*	11.52	4.37	1.83
(Rao and Singh, 2004)	15.5	9.5	*	*	8.5	6.87	1.1
(Eleazer *et al*., 1997)	28.8	9	*	*	*	*	*
(Cuetos *et al*., 2008)	*	*	*	*	*	8.3	1.33
(Poggi-Varaldo *et al*., 1997)	33	*	*	*	*	*	*
(Komilis and Ham, 2003)	47.79	6.89	*	*	4.79	*	*

Heavy metal analysis , The main sources of heavy metals in MSW are batteries (Ni, Zn^{2+}, and Cd^{2+}); and reasons for their retention are poor availability of recycling facilities for hazardous wastes and poor public attitudes to waste management (Richard and Woodbury, 1992; Slack *et al*., 2007). In addition other materials such as paints, electronics, ceramics, plastics and inks/ dyes can all contribute to the heavy metal burden of MSW (Richard and Woodbury, 1992; Déportes *et al*., 1995). Generally, paper fractions contain the highest concentration of these metals (Hasselriis and Licata, 1996). However, information about quantity and sources of hazardous metals in MSW are lacking (Slack *et al*., 2004; Farrell and Jones, 2009). Heavy metals can be classified into three classes based on their biological function and effects: (1) the essential metals (Na^{+}, K^{+}, Mg^{2+}, Ca^{2+}, V^{2+}, Mn^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+} ,Mo^{3+} and W); (2) toxic metals (Ag^{+}, Cd^{2+}, Sn, Au, Hg, Ti^{3+}, Pb^{2+}, Al^{3+} and metalloids, Ge, As^{3-}, Sb, and Se^{2-}) and (3) nonessential , nontoxic metals (Rb, Cs, Sr and T) (Roane and Pepper, 2000).

Heavy metals can also be classified according to their volatility, when MSW leaves the furnace and passes through the heat recovery boiler, volatile metals such as lead and cadmium can condense and attach to the substrate, the highly volatile metals are mercury and arsenic and could be present in the fumes (Hasselriis, 1994). Therefore, the temperature, residence time and present of reactive compounds can have significant effects on heavy metal condensation and release from and to MSW (Abanades *et al*., 2002).

In the present study major and minor elements were measured for all three batches. Levels in general in the first batch were higher than the last two batches. For the first batch of the major elements (Na^{+}, Mg^{2+}, P^{3-}, S^{2-}, K^{+}, and Ca^{2+}) the highest concentration found was for Ca^{2+} (25064.75 mg/kg). The presence of calcium was probably due to the formation of metal oxides from the lime spray treatment during the incineration process. Sulphur was recorded as the second highest major element (5392.43 mg/kg) possibly due to S being a constituent of volatile elements which are formed due to the high temperature and efficient burnout during the incineration process (Eighmy *et al*., 1995).

For instance, comparing the Ca^{2+} content in a first batch with the other studies, showed that it was higher than some previously published values (Bosshard *et al*., 1996; Park and Heo, 2002; Yang *et al*., 2009), but lower than other values (Eighmy *et al*., 1995; Xu and Ting, 2004; Wu and Ting, 2006; Rodella *et al*., 2014). While, for K content the composition was higher than other two batches, and lower than some of the data published previously (Eighmy *et al*., 1995; Xu and Ting, 2004; Wu and Ting, 2006; Rodella *et al*., 2014).

Other major elements were present at less than (>10,000 mg/kg) including K^{+}, Mg^{2+}, Na^{2+}, S^{2-} and P^{3-}. It was noted that most of these major constituents are volatile elements which are also formed due to the high temperatures and efficient burnout during the incineration process (Eighmy *et al*., 1995).

The levels of the major components found in this present study were higher when compared with some other studies as shown in Table 3.8. This is probably due to a high concentration of salty food waste and plastic material in the MSW used in this study, the major source of chloride in the MSW is NaCl and KCl the current results agree with (Park and Heo, 2002). In addition, incinerated MSW contains soluble salts and mineralogical phases such as anhydrite ($CaSO_4$), Quartiz (SiO_2), gehlenite ($Ca_2Al\ (AlSi)O_7$), hematite (Fw_2O_3) and portlandite ($Ca\ (OH)_2$) (Quina *et al*., 2011).

The highest minor elements in the three batches and most other research were Fe^{2+}, Al, Cu^{2+}, Zn^{2+}, and Mn^{2+}, for the first batch the value were (3851, 3120, 1844.9, 612.68 and 397.73 mg/kg respectively). While, for other minor elements showed various values when compared with previous studies. The levels of these minor elements found in this study have been compared with previous reports as shown in Table 3.9.

Fe^{2+} level was the highest among other minor metals in the current study which is agree with Verma (2002), reported that Fe^{2+} represented more than 50 % of the total metal products found in the MSW samples that used. The second highest element in the current study was Al^{3+}, which is another important metal located in can, foils, etc. Some types of plastic and glass packaging contain high levels of heavy metals and contribute heavily to their total output (Verma, 2002; Dien *et al*., 2006).

Comparing this study with other studies, concentrations of Fe^{2+} and Al^{3+} were lower than recorded by (Rodella *et al*., 2014). Also Al^{3+} was lower than (Wu and Ting, 2006), the levels for the rest of the metals were similar to those previously reported compared with other studies is shown in Table 3.9

When looking at the presence of minor elements in the sample of MSW analysed here contained more lithophilic elements such as Ca^{2+}, and Al^{3+} and contained less volatile elements such as K^{+}, Na^{+}, Cd^{3+}, Zn^{2+} and Pb^{2+} than some other studies. This relative enrichment is probably due to the high combustion temperature and efficient burnout at the MSW incineration facility (Li *et al*., 2004).

Table 3.8 Major elements composition of Municipal solid wastes (mg/kg)

References	Na^{+}	Mg^{2+}	P^{3-}	S^{-2}	K^{+}	Ca^{+2}
Batch 1	2509.3	2722.4	1113.7	5392.4	2190.7	25064.7
Batch 2	1846.6	2182.4	1426.7	2037.7	1722.2	21075.7
Batch 3	2177.4	2385.0	1442.6	2177.9	1945.0	22169.0
(Rodella *et al*., 2014)	*	*	3500.0	6200.0	16700.0	205000.0
(Yang *et al*., 2009)	*	*	*	*	*	114.3
(Wu and Ting, 2006)	*	10820.0	*	*	23020.0	258000.0
(Xu and Ting, 2004)	*	9110.0	*	*	11500.0	404000.0
(Park and Heo, 2002)	23.1	2.0	1.7	5.7	13.6	9.8
(Bosshard *et al*., 1996)	1.1	*	*	*	*	13.2
(Eighmy *et al*., 1995)	84000.0	1100.0	*	*	109000.0	46300.0

Table 3.9 Minor elements in municipal solid waste (mg/kg)

References	B^{3+}	Tl^{3+}	Al^{3+}	V^{2+}	Cr^{6+}	Mn^{2+}	Fe^{2+}	Co^{2+}	Ni^{2+}	Cu^{2+}	Zn^{2+}	As^{3-}	Se^{3-}	Rb^{3-}	Sr^{2-}	Mo^{3+}	Ag^{+}	Cd^{2}	Cs^{+}	Ba^{2+}	Pb^{+}	U^{4+}
Batch 1	41.6	21.9	3120.6	23.9	16.0	397.7	3851.9	11.3	93.5	1845.0	612.7	3.0	0.1	2.3	63.4	3.4	3.2	33.9	0.2	291.2	161.2	0.237
Batch 2	6.2	12.2	1876.5	26.3	26.0	120.4	3258.5	1.8	12.7	122.1	346.8	1.6	0.2	1.7	44.7	1.2	0.5	1.4	0.1	62.9	123.7	0.2
Batch 3	7.0	12.5	1954.4	26.7	26.9	137.2	2817.2	1.8	12.4	70.4	388.9	1.6	0.1	1.9	45.9	1.2	0.6	2.6	0.1	62.0	123.4	0.2
(Rodella *et al.*, 2014)	*	9300	24300	40	300	470	12300	*	140	650	5900	*	*	30	370	*	*	720	*	*	900	*
(Yang *et al.*, 2009)	*	*	35.67	*	0.18	0.74	22.94	*	*	0.55	5.25	*	*	*	*	*	*	0.062	*	*	2.15	*
(Wu and Ting , 2006)	*	*	19210	*	72	309	5240	14	22	570	6288	*	*	*	151	*	*	95	*	539	2000	*
(Wang *et al.*, 2006)	*	*	*	*	318.4	*	*	36.94	185.7	976.7	6090	82.43	*	*	*	*	11.75	72.02	*	*	4770	*
(Xu and Ting, 2004)	*	*	1860	*		71	2200	*	*	326	7890	*	*	*	185	*	*		*	*	2070	*
(Li *et al.*, 2003)	*	*	*	*	366.2	*	0.435	*	74.85	1286	5622	130.7	*	*	*	*	*	289.7	*	*	4451	*
(Li *et al.*, 2003)	*	*	*	*	1657	*	0.123	*	554.9	2641	7118	295.1	*	*	*	*	*	1596	*	*	6793	*
(Park and Heo, 2002)	*	0.8	2.6	*	332	680	*	*	*	1653	3	*	*	*	*	*	*	629	*	*	758	*
(Wang *et al.*, 2001)	*	*	*	*	326	2035	*	*	*	1048	6240	*	*	*	*	*	*	47.5	*	*	1393	*
(Pascual *et al.*, 1999)	*	*	*	*	365		*	*	*	237	650	*	*	*	*	*	*	2	*	*	235	*
(Glasse,r 1999)	*	*	*	*	0.07	0.05	*	*	*	0.06	1.7	*	*	*	*	*	*	0.03	*	*	0.8	*
(Krebs *et al.*, 1997)	*	1.1	*	*	0.06	*	*	*	0.014	0.09	2.7	*	*	*	*	*	*	0.04	*	*	0.8	*
(Hasselriis and Licata, 1996)	*	*	*	*	93.5	*	*	*				*	*	*	*	*	*	13.5	*	*	163.4	
(Bosshard *et al.*, 1996)	*	*	7	*	0.07	0.077	2.8	*	0.014	0.11	3.1	*	*	*	*	*	*	0.049	*	*	0.89	3.05
(Eighmy *et al.*, 1995)	*	6100	20800	*	494	448	1600	13.3	69.8	2220	*	960	17.4	206	*	47.1	*	1990	*	2400	27000	*

Chapter 4 Optimising conditions for sugar release from MSW

4.1 Introduction

MSW contains cellulose and hemicellulose which are tightly bonded to lignin via hydrogen or covalent bonding (Yang *et al*., 2011). To access the sugars pre-treatments are required which convert cellulose and hemicellulose into available monomeric sugars (Hamelinck *et al*., 2005).

A biological process can be defined as making use of enzymes as bio-catalysts for the breakdown of cellulose into monomeric sugars, use of enzymes speeds up the liberation of monomeric sugars but has drawbacks in terms of time and cost (Scheper *et al*., 2008). Despite this the enzymatic hydrolysis of cellulosic biomass to sugars which can be further converted into fuel or chemicals offers an environmentally friendly option with potential for higher yields, selectivity, lower energy costs and milder operating conditions than employing chemical processes (Lee *et al*., 2014). The most common enzyme used for enzyme saccharification is cellulases, which are produced by fungi including the filamentous fungus *T. reesei* (*teleomorph Hypocrea jecorina*). This enzyme has the capacity to hydrolyse cellulose into glucose (Singhania *et al*., 2010; Binod *et al*., 2011). Furthermore, cellulase which is produced by *T. reesei* is a cocktail of enzymes which catalyses the reaction of water and cellulose with the end-product being glucose. Due to the complex nature of cellulose, enzymatic hydrolysis is influenced by both the structural features of cellulose and related factors such as enzyme source, thermal inactivation, and activity balance for synergism, specific activity, nonspecific binding, and enzyme processibility /compatibility. However, the mechanism of hydrolysis of cellulose is not fully understood due to the fact that lignocellulosic biomass is a multi-scale, complex and highly heterogeneous material (Himmel *et al*., 2010; Binod *et al*., 2011; Meng and Ragauskas, 2014).

Enzyme hydrolysis is a promising method for the conversion of lignocellulosic cellulose into glucose, however, there are many factors which can affect the hydrolytic efficiency of the cellulosic substrate such as crystallinity of cellulose, the lignin content, the presence of heavy metals, degree of polymerization (DP), moisture content, and particle size (Alvira *et al*., 2010). Furthermore, microbial contamination of the enzyme can affect the efficiency of the hydrolysis process (Gregg and Saddler, 1996; Chang *et al*., 2009). Research has focused on decreasing the adverse effects of these factors on the process, for example cellulose crystallinity, cellulose contains regions of crystallinity where parallel cellulose chains are tightly held together via hydrogen bonds, these crystalline sections have been shown to make cellulose less accessible to the hydrolytic enzymes; this observation could explain why substrates with a high degree of crystallinity are more resistant to enzymatic degradation when compared to more amorphous substrates (Fan *et al*., 1980; Viikari *et al*., 2012).

Presence of lignin in the substrate could have a negative effect on hydrolysis (Li *et al*., 2014). Addition of surfactants has been reported to enhance cellulose hydrolysis (Eriksson *et al*., 2002; Zhou *et al*., 2015) Adding surfactants has some advantages such as preventing enzyme denaturation during agitation; and can change the nature of the substrate (e.g. by removing inhibitory lignin) (Rosen and Kunjappu, 2012). Non-ionic surfactants such as the fatty acid esters of sorbitanpolyethoxylates (Tween80, Tween20) and PEG have been shown to enhance hydrolysis (Eriksson *et al*., 2002), addition of surfactants such as non-catalytic bovine serum albumin (BSA) have been observed to have similar effects on enzyme hydrolysis (Binod *et al*., 2011).

The main approach for liberating fermentable sugars from lignocellulosic materials (LCM) is to employ hydrolytic enzymes following a pre-treatment. Use of chemicals such as strong H_2SO_4, at high temperatures, can liberate fermentable sugars directly (Taherzadeh and Karimi, 2007), whilst weaker acid or alkali treatments can make the substrate more accessible to enzymes. However,

such processes can generate potentially inhibitory compounds such as weak acids, furan and phenolics (Sun and Cheng, 2002; Mosier *et al*., 2005; Greetham *et al*., 2014).

In general, use of any pre-treatment leads to changes to the biomass in terms of a decrease of the cellulose crystallinity, polymerisation, partial or complete hydrolysis of hemicellulose; a result of these changes being that enzymes can access and hydrolyse cellulose more rapidly (Sun and Cheng, 2002). Use of acid concentrations (sulphuric acid 1-10 %) and a temperature range of 100-150 ^{o}C can break down the cellulose and hemicellulose fraction of the plant cell wall into monomeric sugar molecules (Wingren *et al*., 2003; Mosier *et al*., 2005). While, Use of (> 3 %) sulphuric acid as a pre-treatment is characterised by a lower recovery of monomeric fermentable sugars (about 50 %) because sugars released from the cellulosic polymer during the process are further converted into less desirable compounds; less severe pre-treatment conditions are required to release pentose sugars from hemicellulose (Wyman *et al*., 2005). Use of (> 4 %) sulphuric acid can liberate over 90 % of monomeric sugars from hemicellulose and cellulose polymers. However, pre-treatment with strong acids can be corrosive, toxic, and hazardous (Badger, 2002).

During initial decomposition of hemicellulose in an acid pre-treatment, monosaccharides are converted into less desirable compounds including furfural which is a product of dehydration of pentose sugars or hydroxymethylfurfural (HMF) a product of the dehydration of hexoses and acetic acid. Hydrolysis of acetyl groups linked to sugar monomers inhibits fermentation, leading to reduced ethanol yields (Romero *et al*., 2007).

Use of alkaline compounds such as NaOH (Silverstein *et al*., 2007), CaOH (Kaar and Holtzapple, 2000; Chang *et al*., 2001), KOH (Chang and Holtzapple, 2000), or aqueous ammonia (Kim *et al*., 2003) has been shown to enhance the release of fermentable monomeric sugars during subsequent enzyme hydrolysis. Pre-treatment of biomass using an alkaline has advantages in that it utilises lower

temperatures and pressures when compared with other pre-treatment strategies (Chang *et al*., 2009).

Alkali pre-treatment removes lignin and part of the hemicellulose, thus increasing the accessibility of enzymes to cellulose. The pre-treatment has also been found to cause swelling of the microfibril leading to an increase in internal surface area, decrease in polymerisation, crystallinity, and a breaking of the ester linkages which joins hemicellulose (Gupta, 2008). However, all of the cellulose and most of the hemicellulose is left as an insoluble polymeric form, therefore alkali pre-treatment works most efficiently with lignocellulosic material with high lignin content (Hamelinck *et al*., 2005).

The main objective of the present work was to optimize the efficient conversion of available lignocellulosic material present in MSW into fermentable sugars, such a step is key for efficient production of biofuel(s). Experiments will look into addition of antibiotics, optimal enzyme activity, adding surfactants and optimal loading rates. MSW was subjected to sulphuric acid and alkaline pre-treatments, followed by enzyme hydrolysis to determine release of fermentable sugars. Levels of inhibitory compounds were also assessed and the impact of the resultant hydrolysates on the metabolic activity of yeast was determined using a phenotypic microarray assay.

4.2 Materials and Methods

4.2.1 Yeast strain and growth conditions

Saccharomyces cerevisiae NCYC2592 was used in this study. This strain was maintained on agar containing 10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose, and 20 g/L agar (YPD agar) and grown on 10 g/L yeast extract, 20 g/L peptone, and 20 g/L glucose (YPD) in an orbital shaker (1800 rpm) at 30 ºC under aerobic conditions.

4.2.2 Cellulase Assay

The filter paper cellulase activity protocol (Filter paper assay FPU) was used to assess enzyme activity. Enzyme solution (0.5 mL) was mixed with 0.5 mL of 50 mM citrate buffer (pH 4.8). Strips (1 x 6 cm, approximately 50 mg) of Whatman No. 1 paper (cellulose substrate) were placed in the tubes, and incubated at 50 ^{o}C for 1 hr. After incubation, 3 mL of Dinitrosalicyclic (DNS) reagent prepared according to (Adney and Baker, 1996), was added to each tube and the mixture boiled for 5 min and cooled on ice (5 min). Samples were diluted (0.2 mL of sample was mixed with 2.5 mL of distilled water), the released reducing sugar (as glucose) was measured by using DNS method (Miller, 1959), and absorption was read at 540 nm using a Jenway 7315 spectrophotometer. The filter paper activity (U/ml) was calculated using the following equation.

$$\text{Filter paper activity}, \left(\frac{\text{U}}{\text{ml}}\right) = \frac{0.37}{\text{Concentration of enzyme that release 2.0 mg glucose}} \qquad (1)$$

Then it was then converted to Unit (U) per gram of dry weight MSW using the following equation.

$$\text{Filter paper activity}, \left(\frac{\text{U}}{\text{g}}\right) = \frac{\text{Filter paper activity}, \left(\frac{\text{U}}{\text{ml}}\right) * \text{Total volume of the fungal extract}, (\text{ml})}{\text{Dry weight of the substrate used in SSF}, (\text{g})} \qquad (2)$$

4.2.3 Enzyme hydrolysis method

The digestibility of MSW, either as obtained or following pre-treatment, was assessed using the NREL (Ghose, 1987; Adney and Baker, 1996). 0.5 g MSW was mixed with 100 mL sodium citrate buffer (50 mM, pH 4.8). Cellic® CTec2 (Novazyme) cellulose cocktail was added at a concentration of 15, 30 or 60 FPU/g MSW. The samples were incubated for 72 hr at 50 ^{o}C on a rotary shaker at 150 rpm. 0.5 ml of samples was taken at time-points from 0 to 72 hr. To determine sugar content, filtered through 0.45 µM Whatman filter paper and the amount of glucose released determined using (Dionex ICS-300SP) as described in section 3.2.4.1. by Davis (1998); and Cataldi *et al*. (2000).

Also the effect of bacteriosataic agents has been studied such as Antibiotic (AB) (tetracycline) and sodium azide (SA) (both supplied by Sigma Aldrich) and surfactants such as Bovine Serum Albumin (BSA), Triton X-114 (1,1,3,3-Tetramethylbutyl) phenyl-polyethylene glycol, Polyethylene glycol tert-octylphenyl ether) Triton X-100 (4-(1,1,3,3-Tetramethylbutyl) phenyl-polyethyleneglycol, t Octylphenoxypolyethoxy ethanol, Polyethylene glycol), Tween 20 (poly(oxyethylene) 20 sorbitan-monolaurate Polyethylene glycol sorbitan monolaurate, Polyoxyethylenesorbitan monolaurate)and Tween 80 (poly(oxyethylene) 20- sorbitan-monooleate POE (20) sorbitan monooleate), Polyethylene glycol sorbitan monooleate, Polyoxyethylenesorbitan monooleate, Polysorbate 80 (Merck). Enzyme yield was calculated as:

$$Glucose\ (\frac{g}{ml}) = \frac{Amount\ of\ dry\ substrate \times amount\ of\ glucose\ in\ \ MSW}{100}$$

$$\text{Saccharification yield } (\%) = \left(\frac{\text{Glucose in solid residue } (\frac{\text{g}}{\text{ml}})}{\text{Glucose } (\frac{\text{g}}{\text{ml}})} \right) \times 100$$

4.2.4 Pre-treatment of MSW

4.2.4.1 Acid treatment using an autoclave

For pre-treatments with sulphuric acid concentrations (0-4 %) 2 g samples of MSW were added to 100 mL of acid and autoclaved for 15 or 30 min at 121 ^{o}C Samples were then centrifuged at 4472 g for 10 min.

Post treatment samples were centrifuged at 4472 g for 10 min to separate the solid and liquid fractions; the sugar (glucose) concentrations in the liquid fraction were determined as described in section 3.2.4.1. The solid fraction was dried at 25 ^{o}C room temperature, weight loss and sugar content were determined following addition of 1 mL of 12 M sulphuric acid (after washing the pre-treated samples using distilled water) and incubation at 37 ^{o}C for 1 hr, then 100 ^{o}C for 2 hr (Sluiter *et al*., 2008; Sluiter *et al*., 2011). The sugars composition was determined using (Dionex ICS-300SP) as described in section 3.2.4.1. All experiments were conducted in duplicate and compared with controls.

The liquid fraction following low sulphuric acid pre-treatment were presence of glucose as described above, inhibitory compounds were determined too. Liquid hydrolysates were taken forward for assessment using phenotypic microarrays (Biolog, CA, US). As described below.

For inhibitors determination, hydrolysate was centrifuged at 4472 g for 10 min. The supernatant was filtered through a 0.2 µm syringe filter (Whatman, UK) and put into a glass vial (Chromacol, UK). Concentrations of inhibitory compounds generated by the various pre-treatment methodologies were determined by HPLC. Hydrolysate samples were analysed using a Waters 2695 separation module with UV detection at 270 nm detector. A C18 techsphere column 259 X 4.6 mm id analysis column operating at atmospheric temperature and 4000 psi pressure with elution 1 % acetic acid as a mobile phase and helium de gas for

eluent was used. Standards- Stock inhibitory solutions prepared from acetic, formic, levulinic, vanilic acid, furfural, and hydroxymethylfurfural (HMF) (Sigma-Aldrich) were also used to calibrate the HPLC and store at -20 °C until required.

Also Liquid hydrolysates were taken forward for assessment **using phenotypic microarrays** (Biolog, CA, US). Assessment of yeast metabolic activity, yeast growth, preparation and assays under control conditions (6 % glucose, 0.67 % YNB) for phenotypic microarray analysis were according to (Greetham *et al*., 2014). Hydrolysates from enzyme hydrolysis were examined by adding hydrolysate to PM plates pH was established at pH 4.8-5.

The yeast were maintained on YPD slants (2 % (W/V) glucose, 2 % (W/V) Peptone, 1 % (W/V) yeast extract, and 2 % (W/V) agar at 4 ^{o}C, then a small quantity was transferred from each tube culture to broth medium (2 % (W/V) glucose, 2 % (W/V) Peptone, 1 % (W/V) yeast extract). The hydrolysate from previous experiment used to examine the resistance of yeasts and pH of the hydrolysates.

PM plates were prepared using 140 µL of 0.67 % (w/v) yeast nitrogen base (YNB) supplemented with 450 µL 6 % (w/v) glucose, and 25 µl of dye D (Biolog, Hayward, CA, USA) mixed them with 900 µL of enzyme hydrolysate, plus control by adding all the previous growth media except hydrolysate which replaced by RO sterile water.

The second part is strain preparation for inoculation into the PM assay plates as follows. Incubated cells in broth medium at 25 ^{o}C were inoculated into sterile water in 20 x 100 mm test tubes and adjusted to a transmittance of 62 % (~5x106 cells/mL) using a Biolog turbid meter (Biolog), to prepare cell suspension.

Then the culture 3.5 mL were mixed with 17.5 mL IFY buffer ™ (Biolog), to mix it with previous medium. Next 90 µl of the above mix was inoculated into each well in a Biolog 96-well plate, under aerobic condition. The prepared plates were then placed in the OmniLog reader and incubated for 96 hr at 30 ^{o}C.

Hydrolysates from pre-treatment of MSW were analysed for their impact on yeast performance using a phenotypic microarray assay (Biolog, CA, US). Prior to the phenotypic microarray hydrolysates were adjusted to pH 5.5 using 3 M NaOH.

The principle of the OmniLog reader is that the PM plates are photographed at 15 min intervals, and the pixel density in each well converted to a signal value reflecting dye conversion. Dye reduction reflects metabolic activity of the cells which has been defined here as the redox signal intensity.

After completion of the run, the signal data was compiled and exported from the Biolog software using Microsoft® Excel. In all cases, a minimum of three replicate PM assay runs were conducted, and the mean signal values are presented.

4.2.4.2 Pre-treatment using a microwave reactor

A microwave was used to establish the pre-treatment conditions for pre-treatments using sulphuric acid (0.1-2 %) plus control (RO water only). A laboratory microwave 300, Anton paar was used to pre-treat the substrate; each vessel was filled with 2 % MSW (0.2 g MSW plus 9 mL of water or sulphuric acid (0- 2 %). Samples were heated to 160 ^{o}C with three ramped temperature times (5 – 20 min). Once final temperature was reached, temperature was held for 1 min. The solid fraction was separated from liquid fraction as described in 4.2.4.1, the optimum selected condition followed by enzyme hydrolysis as described in section 4.2.3.

4.2.4.3 Alkaline pre-treatment

Pre-treatment were carried out using either dilute NaOH (0.1-4 %) and autoclaving for 30 and 15 min at 121 ^{o}C or strong (5 - 50 %); NaOH at 25 ^{o}C for 12 hr. All experiments used 2 % w/v MSW (2 g MSW in 100 mL).

Solid were separated from the liquid as previously described (4.2.4.1). The solid fraction was dried at 25 ^{o}C and weight loss was determined. Sugars present in the solid residue were determined using Dionex ICS-300SP as mentioned previously (3.2.4.1).

4.3 Results

4.3.1 Enzyme hydrolysis of untreated MSW

MSW as received was first assessed for digestibility by hydrolysis using Cellic® CTec2 and the standard NREL assay. The glucose released during enzyme hydrolysis is shown in Figure (4.1A). It was observed that glucose concentration increased in the first 12 hr of the hydrolysis but then dropped significantly thereafter at the end of the hydrolysis (72 hr) no free glucose could be detected in the samples, and it was suspected that the hydrolysate had become microbially contaminated.

To test this hypothesis the hydrolysis was repeated but with the addition of either 30 mg of antibiotic (tetracycline) or a chemical disinfectant (sodium azide). In addition the effect of autoclave sterilisation of the MSW prior to hydrolysis was also tested. The results from these hydrolyses are shown in Figure 4.1B. Under all three conditions glucose accumulated throughout the hydrolysis period and there was no eventual reduction observed.

Glucose yields after 72 hr represented 34.077, 28.42 and 33.57 % of that available in the original MSW in the presence of antibiotic, disinfectant or following autoclaving of the sample, respectively. Autoclave sterilisation of the MSW was used routinely for all the remaining experiments.

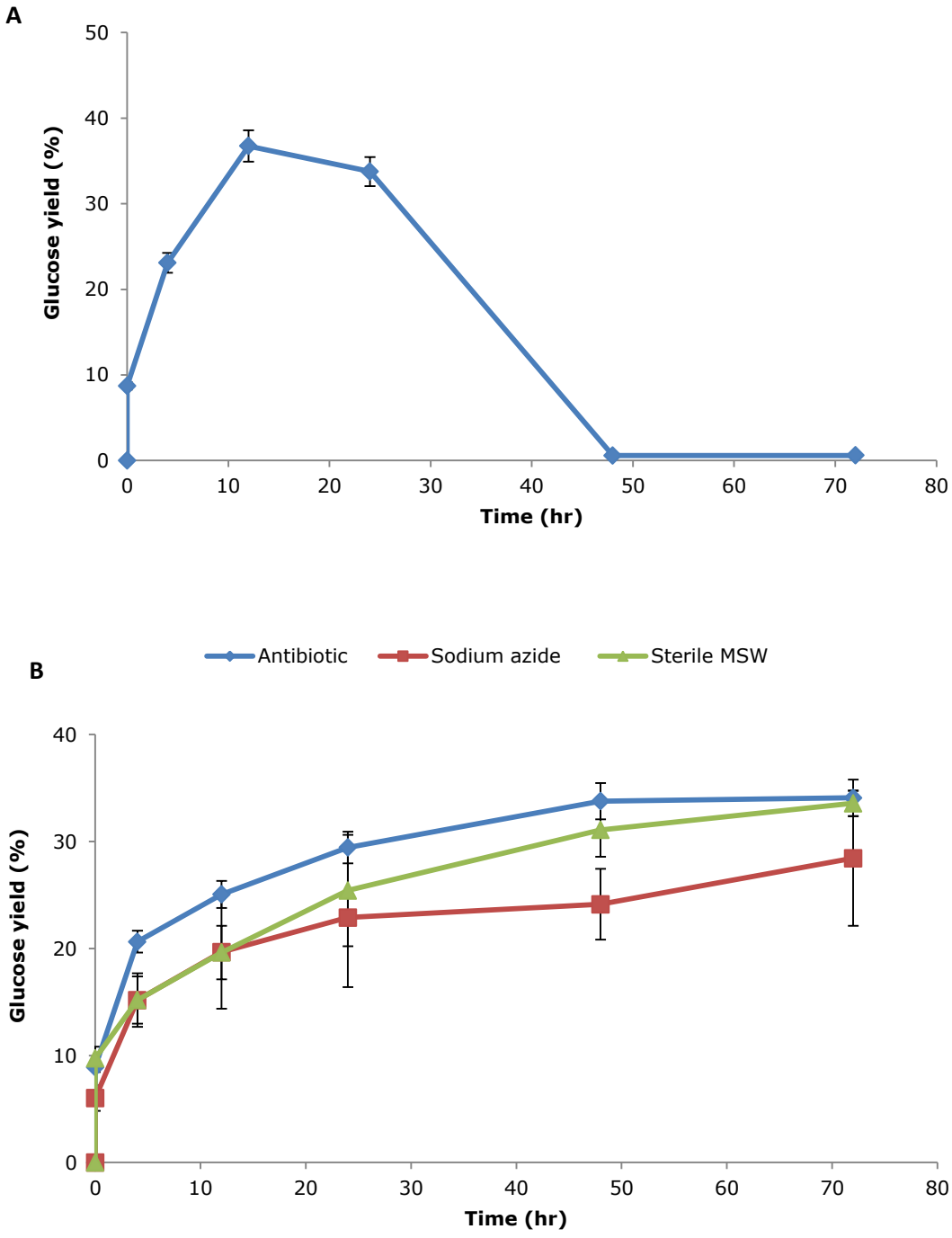


Figure 4.1 Glucose release (%) during enzyme hydrolysis of MSW

MSW was subjected to enzyme hydrolysis either as received (A) or following sterilization by autoclaving or the addition of anti-microbial agents (B). Results are presented as % available glucose released and are the mean ± SD (n=3).

4.3.2 Optimization of enzyme loading rate for hydrolysis with Cellic® CTec2

The effect of enzyme dosage on hydrolysis of MSW was determined by carrying out a standard hydrolysis with 0.5 % W/V MSW and various enzyme loadings (15, 30 and 60 FPU/g MSW). The results are shown in Figure 4.2. The maximum final glucose concentration was achieved with a cellulase loading of 30 FPU/g substrate. Analysis revealed that glucose released using 15 and 30 FPU/g (25.44 and 31.22 %) were significantly different (p=0.0001). Assays using 60 FPU resulted in a slight increase in glucose concentration (0.51 ± 0.09 g/L) (31.26 %), however, the increase was not significantly higher than with the use of 30 FPU/g (p=0.154).

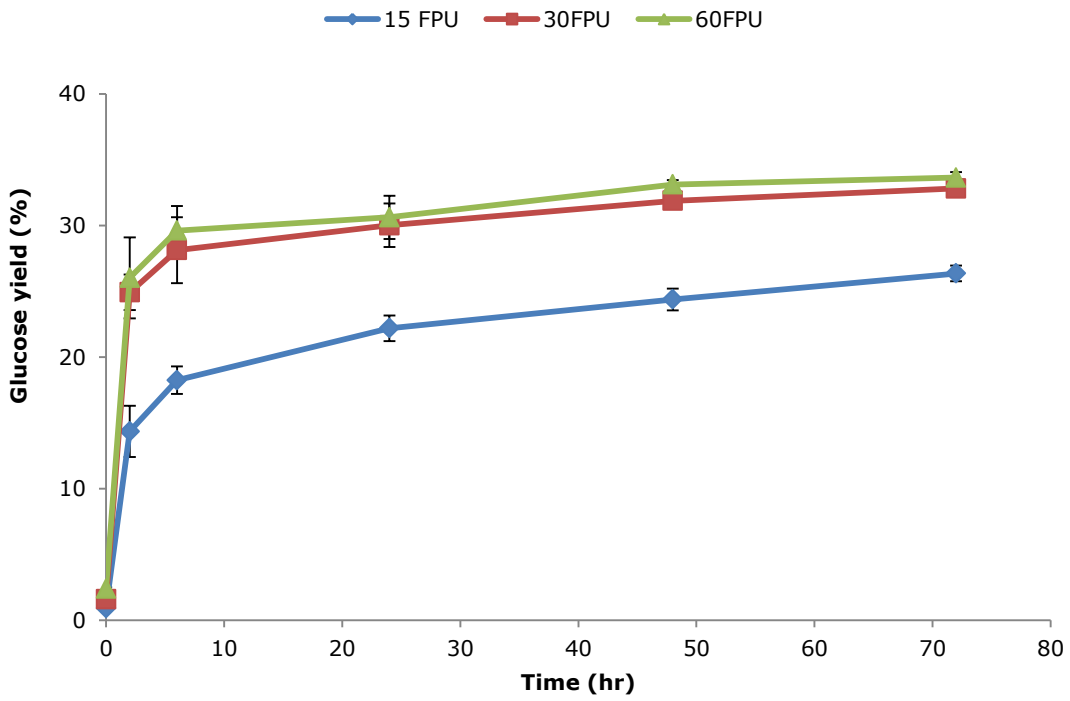


Figure 4.2 The effect of enzyme loading on hydrolysis of MSW

Sterilised MSW, as received, was hydrolysed using various enzyme loading rates and glucose release (as % total available) recorded over 72 hr. Results are the mean ± SD (n=3).

4.3.3 Effect of addition of surfactants on the enzymatic hydrolysis of MSW

Five surfactants, as described in section 4.2.3, were used to investigate their impact on MSW hydrolysis. These experiments were performed with commercial enzyme Cellic® CTec2 at 30 FPU/g MSW (autoclaved). 30 mg of each surfactant was added to the hydrolysis system.

A study by Kurakake *et al*. (1994), showed that application of 3.33 wt % of Tween 20 (the non-ionic surfactant) during pre-treatment of bagasse at 170–190 °C enhanced subsequent enzymatic hydrolysis by 22–27 % compared to those treated with water (control), Generally in a current study the addition of surfactant does not improve efficiency of EH dramatically.

Release of glucose from autoclaved MSW following the addition of surfactants was determined (Figure 4.3) and compared with control (no surfactant added). There was a significant difference in the release of sugars between surfactants ($P=0.0056$). The highest release of sugars was observed using tween 80 and PEG, 47.8 and 48.66 %, respectively. While, a significantly reduced level was recorded using either of the triton surfactants. There was no significant difference between the two tritons themselves. The only really major effect seen was the reduction in rate and final level displayed in the presence of triton X100. The final conclusion is that the addition of surfactants would not really be beneficial commercially.

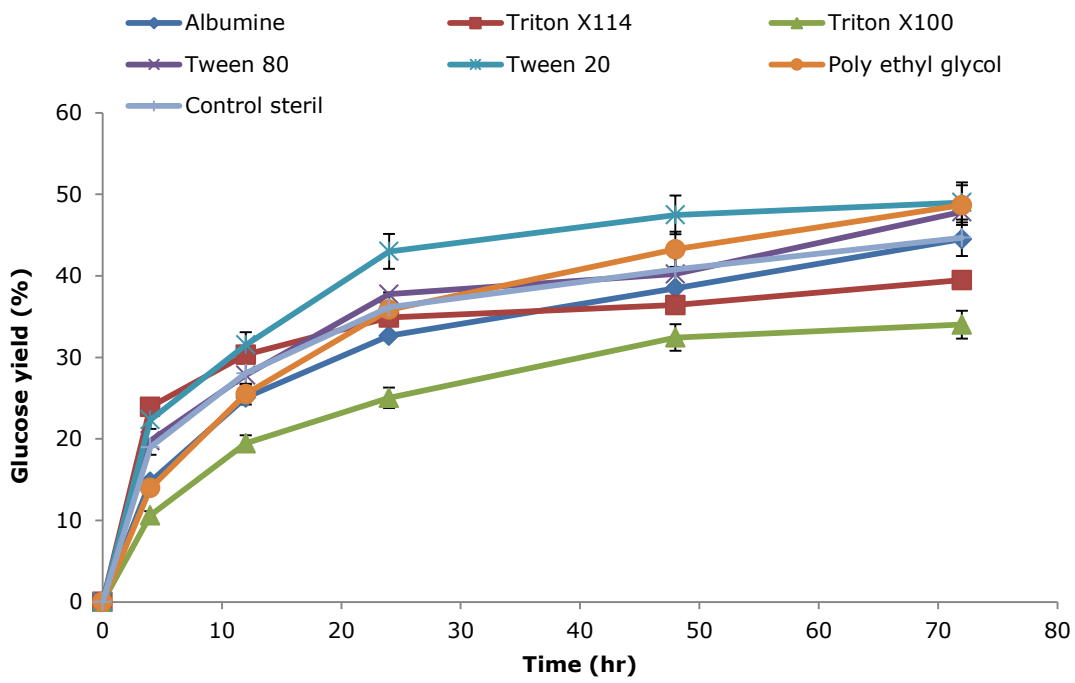


Figure 4.3 Effect of surfactants on hydrolysis of MSW

Sterile MSW as received was hydrolysed in the presence of various surfactants monitored over 72 hr and comparing them with a control (no surfactant). Results are the mean ± SD (n=3).

4.3.4 Effect of pre-treatments of MSW on glucose release

4.3.4.1 Acid pre-treatments

In an attempt to improve sugar production from MSW, the effect of pre-treatments on subsequent EH was investigated. In the first instance the impact of acid catalysed hydrothermal pre-treatments was investigated. Firstly, by examining the effect of autoclaving for 15 or 30 min with different concentrations of sulphuric acid (0 -4 %) and secondly using the microwave reactor to investigate the impact of higher temperatures.

The impact of the pre-treatments themselves on the release of glucose into the acid hydrolysate, and retention within the solid residue was first determined. The results for autoclaving MSW in 0-4 % sulphuric acid are shown in Figures 4.4 A and 4.5 B. Retention of glucose in the solid residue remained at around 85 % with pre-treatments up to 1 % sulphuric acid after which it declined (Figure 4.4

A). Glucose release into the liquid fraction showed a steady increase with acid concentration reaching around 20 % of available glucose following a 3 % sulphuric acid pre-treatment (Figure 4.4 B).

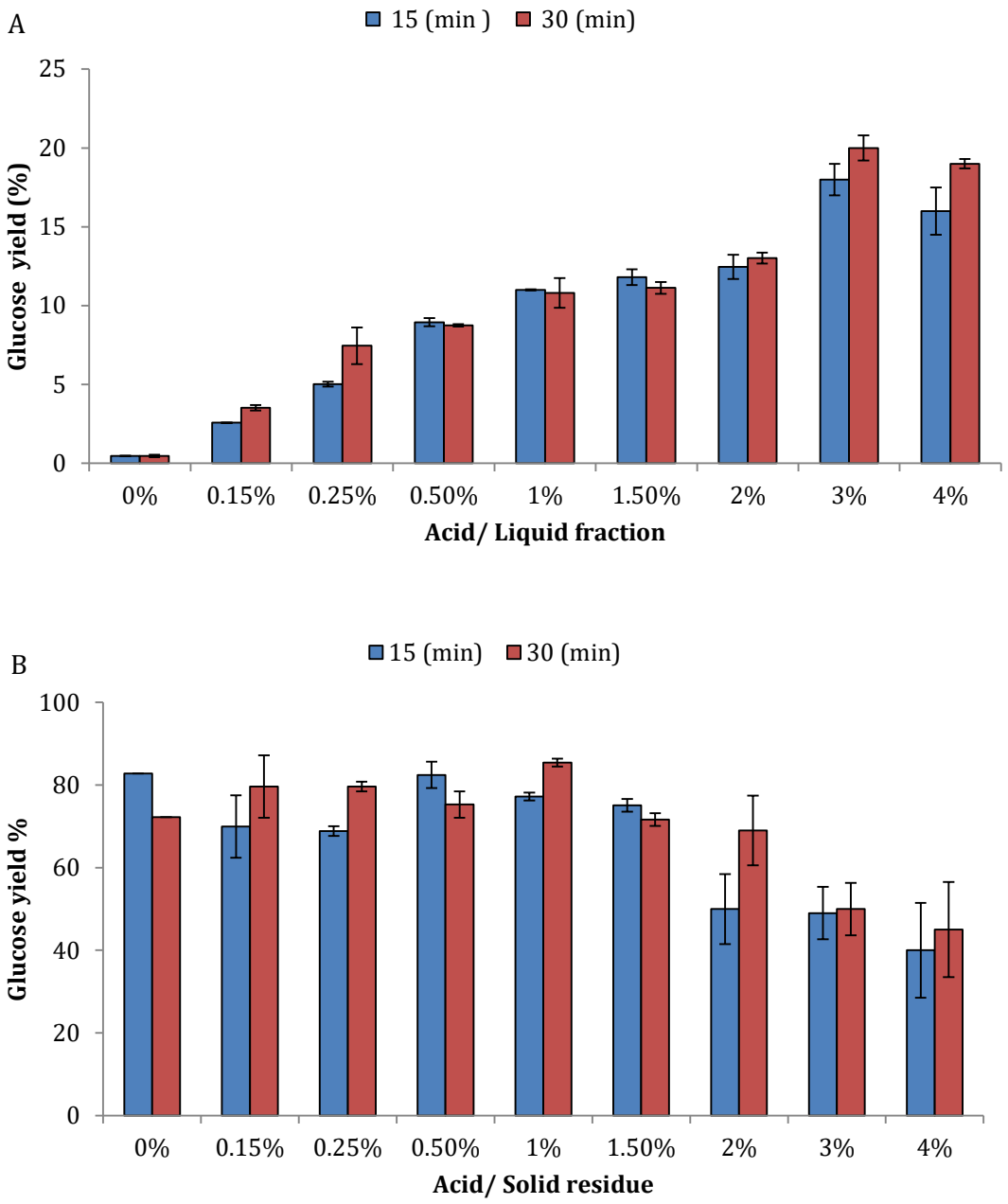


Figure 4.4 Effect of acid pre-treatments on release of glucose yield % from MSW

MSW was pre-treated at (2 % w/v) by autoclaving for 15 or 30 min at various sulphuric acid concentrations (0-4 %). The recovery of glucose the solid (A) and liquid (B) fractions were determined. Results are mean ± SD (n=3).

The microwave reactor was used to assess the impact of higher temperatures for the pre-treatment of MSW. MSW at 2 % (w/v) was treated at 160 ^{O}C using sulphuric acid concentrations from 0-2 %. The retention of glucose in the resultant solid residue and release into the liquor are shown in Figures 4.5 A and B. As seen previously the retention of glucose in the solid residue was around 80-85 % in all cases and release of glucose into the liquor increased with acid concentration. It proved technically impossible to explore temperatures above 160 ^{o}C due to the pyrolysis of the plastic particles in the sample which caused blowouts of the glass tube used for the experiment.

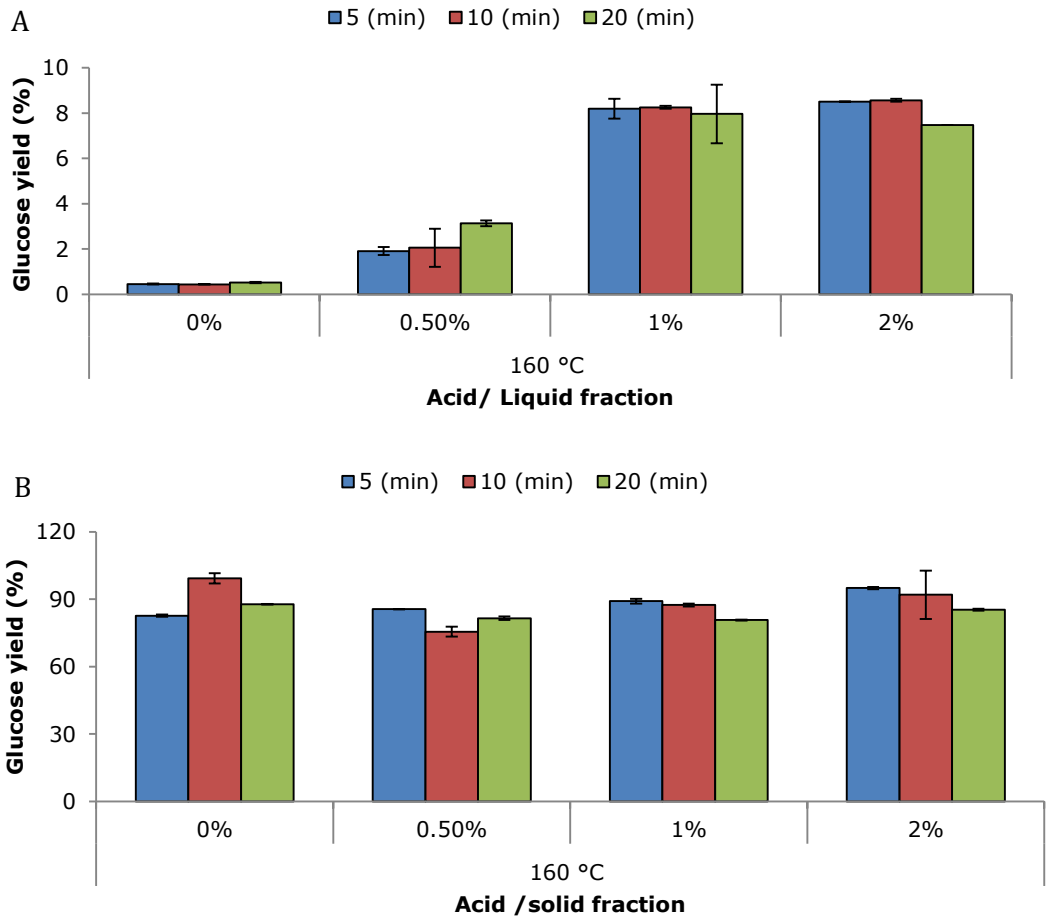


Figure 4.5 Effect of low sulphuric acid pre-treatments on release of glucose from MSW

MSW was pre-treated at (2 % w/v) by microwave at various sulphuric acid concentrations (0-2 %). The recovery of glucose in the solid (A) liquid (B) fractions were determined. Results are mean ± SD (n=3).

In the next step the impact of selected pre-treatments on the subsequent enzyme saccharification was assessed. The treatments chosen were autoclaving for 30 min either with no acid or with 1 % sulphuric acid and with similar conditions at 160 ^{o}C in the monowave. The results are shown in Figure 4.6. Autoclaving or monowave treatment without acid gave the highest glucose release 27.66 and 27.12 % respectively. There was no significant difference (p=0.098) between these values demonstrating that increased temperature does not impact digestibility of the MSW. In both cases addition of acid actually resulted in a reduced release of glucose to 22.68 and 23.39 %, respectively. These differences were statistically significant indicating that the addition of acid has actually impaired the subsequent enzymatic hydrolysis.

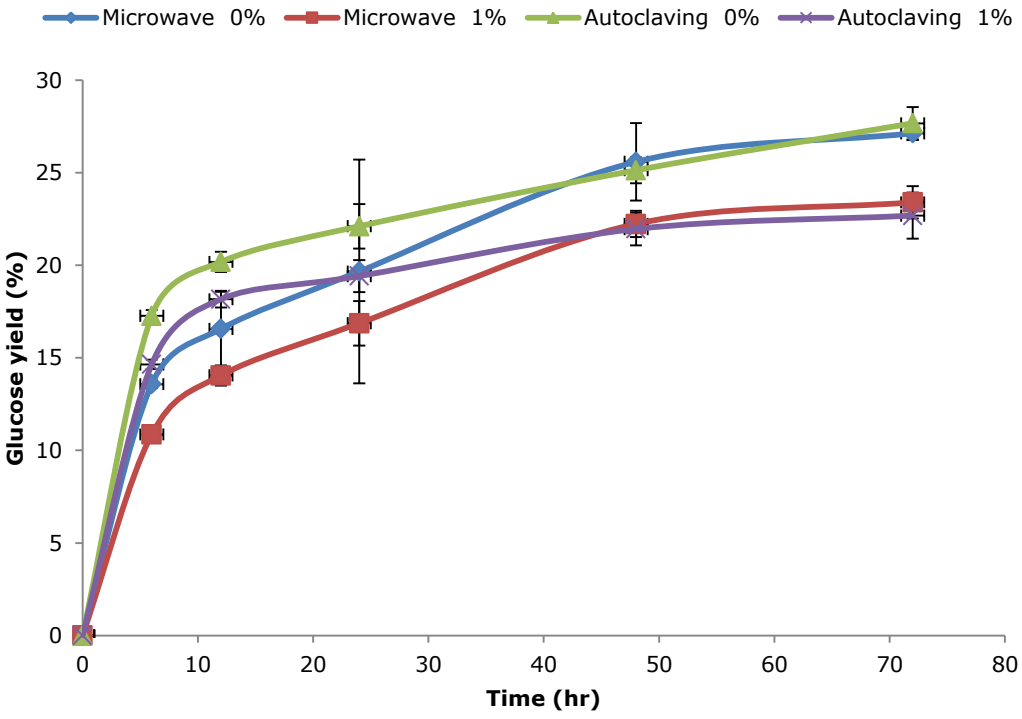


Figure 4.6 Glucose release (%) during enzyme hydrolysis of pre-treated solid residue MSW using low sulfuric acid concentrations

Enzyme hydrolysis using sulphuric acid pre-treated solid residue (0 or 1 %) 30 FPU for pre-treated solid 2 % solid liquid ratio MSW followed by autoclaving (30 min autoclaving) or use of a microwave (10 min at 160 ºC) to release fermentable sugars from MSW and comparing with control).

The impact of acid pre-treatments on the production of inhibitors was assessed. MSW (2 % w/v) was subject to autoclaving in sulphuric acid concentrations from 0.1–4 % and the liquor analysed for furfural and HMF (Figure 4.7). Furfural production was minimal, at below 0.01 mM, until treatment with 3 % sulphuric acid at which point it increased dramatically to 0.05 -0.07 mM at 3 and 4 % sulphuric acid, respectively. In contrast production of HMF increased steadily from 0.05 to 3 % sulphuric acid, reaching a maximum production of 0.0245 ± 0.001 mM. Interestingly treatment with 4 % sulphuric acid appears to result in a reduction in HMF levels.

Inhibitor production following pre-treatment of MSW in the microwave at 160 °C and 2 % sulphuric acid is shown in Figure 4.8. The highest production of HMF was after 10 min and furfural after 20 min the recorded concentrations being 0.0447± 0.089 and 0.089 ± 0.004 mM, respectively.

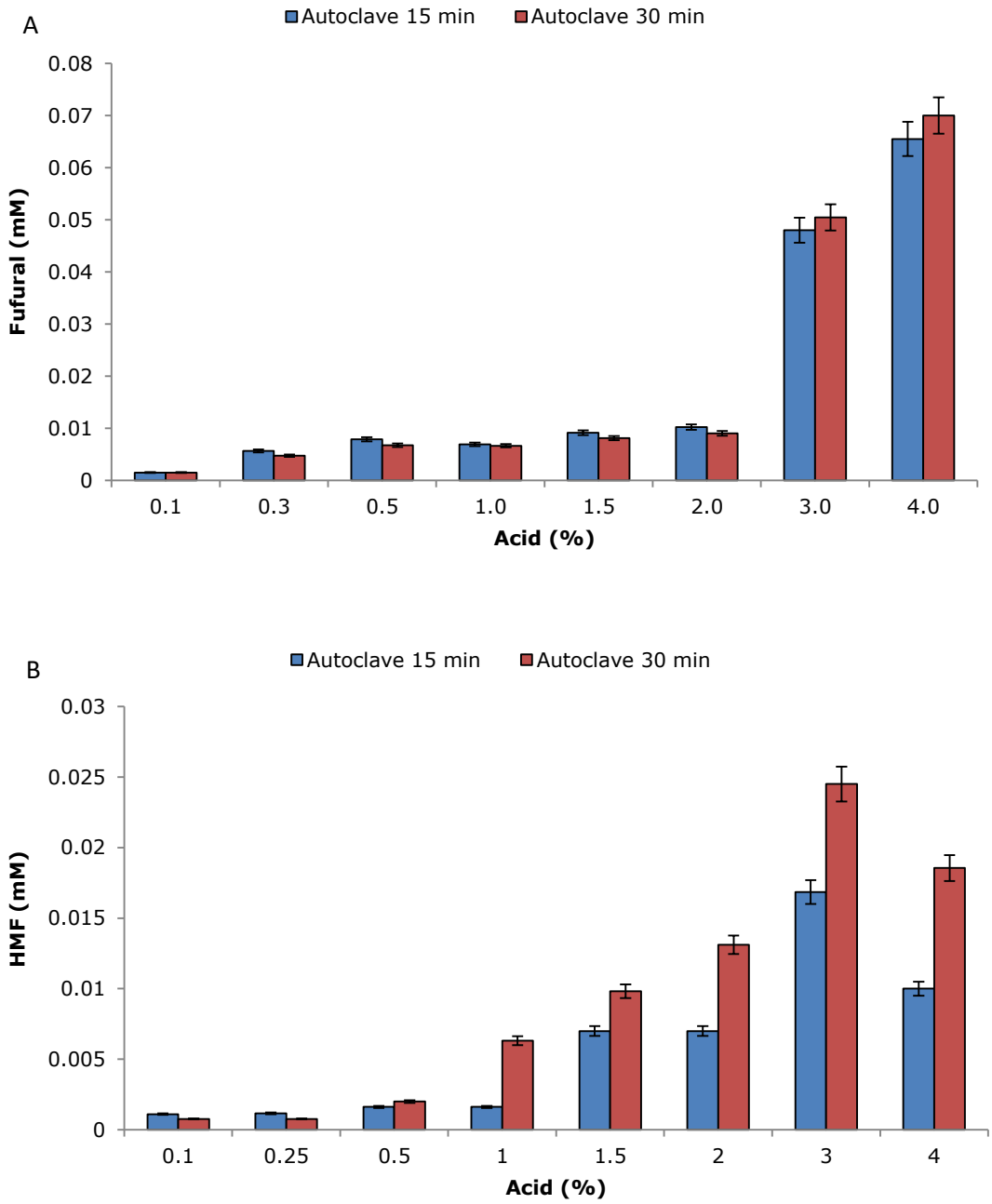


Figure 4.7 Production of inhibitors (Furfural and HMF) following autoclaving pre-treatment

2 % w/v MSW was treated with sulphuric acid (0.1-4 %), at 121 ^{o}C, using two different retention times and concentrations of inhibitors assessed (A) Furfural; (B) HMF Mean ± SD (n=3).

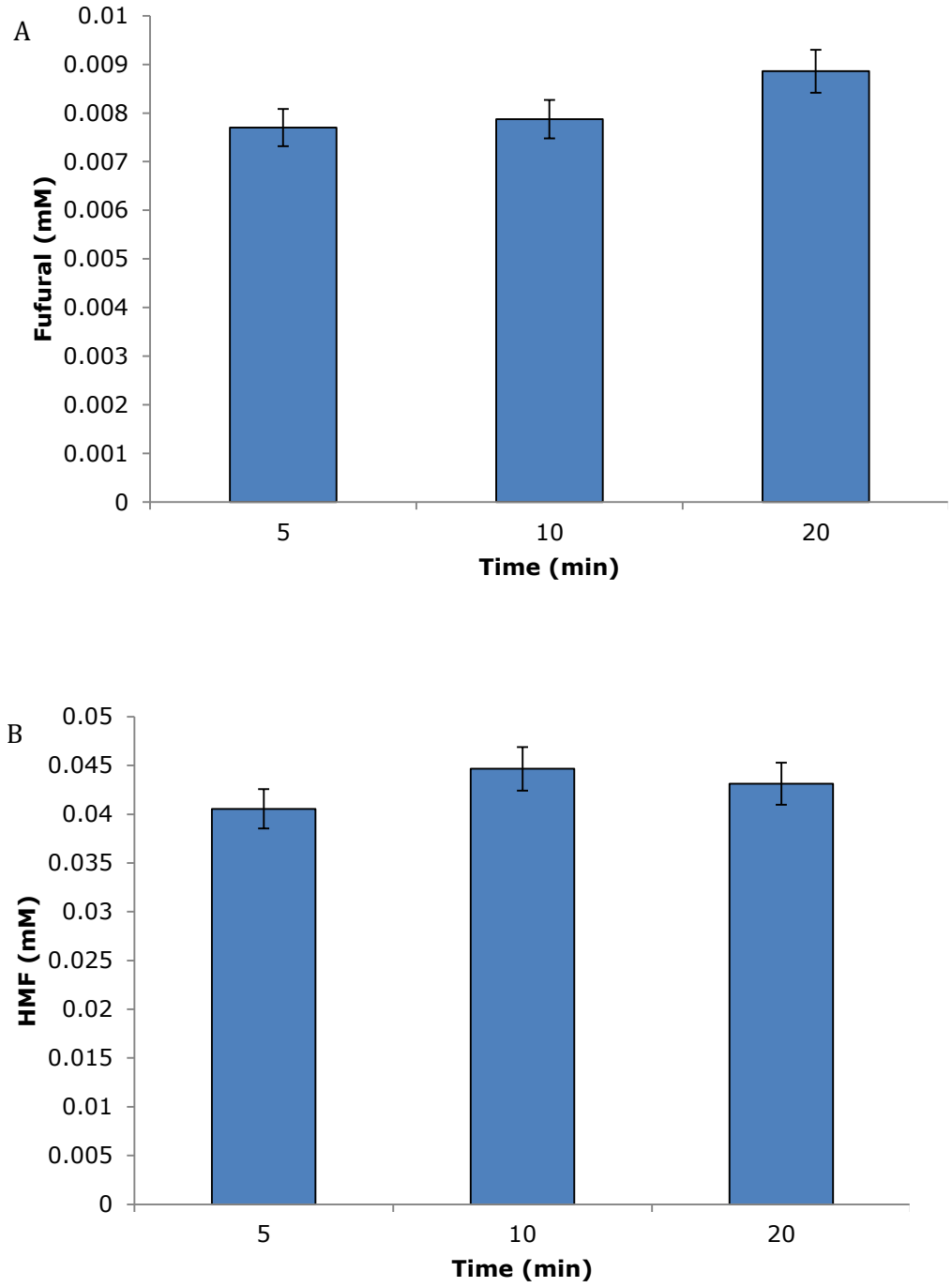


Figure 4.8 Production of inhibitors (Furfural and HMF) following microwave pre-treatment

2 % w/v MSW was treated with sulphuric acid (2 %) sulphuric acid, at 160 °C, using three different retention times and levels of inhibitors assessed (A) Furfural ; (B) HMF Mean ± SD (n=3).

To assess the potential impact of these inhibitor concentrations on yeast performance a phenotypic microarray assay was carried out in the presence of hydrolysates derived from 2 % MSW and pre-treated by autoclaving for 30 min in 0-4 % sulphuric acid. The results are shown in Figure 4.9. Metabolic activity of the yeast was reduced, compared to the control (no hydrolysate) which showed a significant difference between them, in all cases. However, this was minimal and constant until 3 % sulphuric acid at which point activity declined further.

A similar experiment was carried out using the hydrolysate from microwave pre-treated MSW (160 ^{o}C with 0 or 2 % sulphuric acid for 5, 10 and 20 min). The highest metabolic output was observed using a hydrolysate which was derived from a 2 % MSW, 20 min retention time with 2 % sulphuric acid but this still did not shown better performance comparing with the experimental control, there was no significant difference between 2 % at 20 min using acid and 0 % at different retention time, while has a significant difference with 5 and 10 retention time using 2 % sulphuric acid results are shown in Figure 4.10.

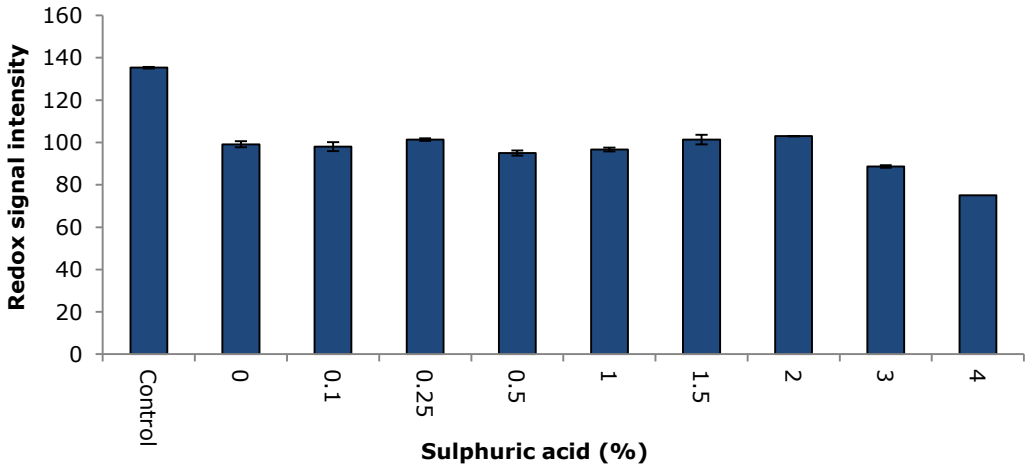


Figure 4.9 Effect of MSW hydrolysates on metabolic output (Redox Signal Intensity) of *S. cerevisiae*

MSW was treated at 121 ^{o}C for 30 min using various acid concentrations (0-4 %) The resultant hydrolysates were then added to growth medium and the effect on yeast metabolic activity assessed. Mean ± SD (n=3).

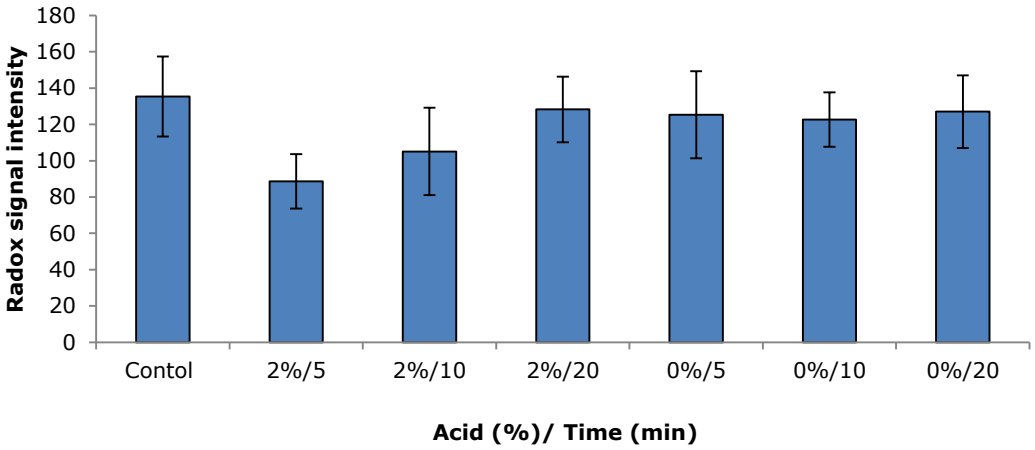


Figure 4.10 Effect of MSW hydrolysates on metabolic output (Redox Signal Intensity) of *S. cerevisiae* using hydrolysate derived from MSW use of a microwave at 160 ºC and various incubation times using 0 and 2 % sulphuric acid. Mean ± SD (n=3)

4.3.4.2 Alkaline pre-treatments

The impact of MSW pre-treatment using alkaline was also investigated. MSW (2 % w/v) was either pre-treated with NaOH (5-50 %) at room temperature for 12 hr or with (0-4 %) NaOH at 121 ^{o}C for 15 or 30 min. In each case the impact on retention of glucose in the solid residue was assessed Figures 4.11 and 4.12, respectively. Treatment with high NaOH at room temperature resulted in a low recovery of glucose in the solid residue. In contrast, the retention was relatively constant at around 70-85 % in all treatments using the autoclave and the lower concentrations of NaOH up to 2 % and then declined slightly at higher concentrations. Greatest glucose retention was obtained using 2 % alkaline and 15 min autoclaving and so this was used for further analysis. As expected only trace levels of glucose were observed in the hydrolysate (liquid fraction).

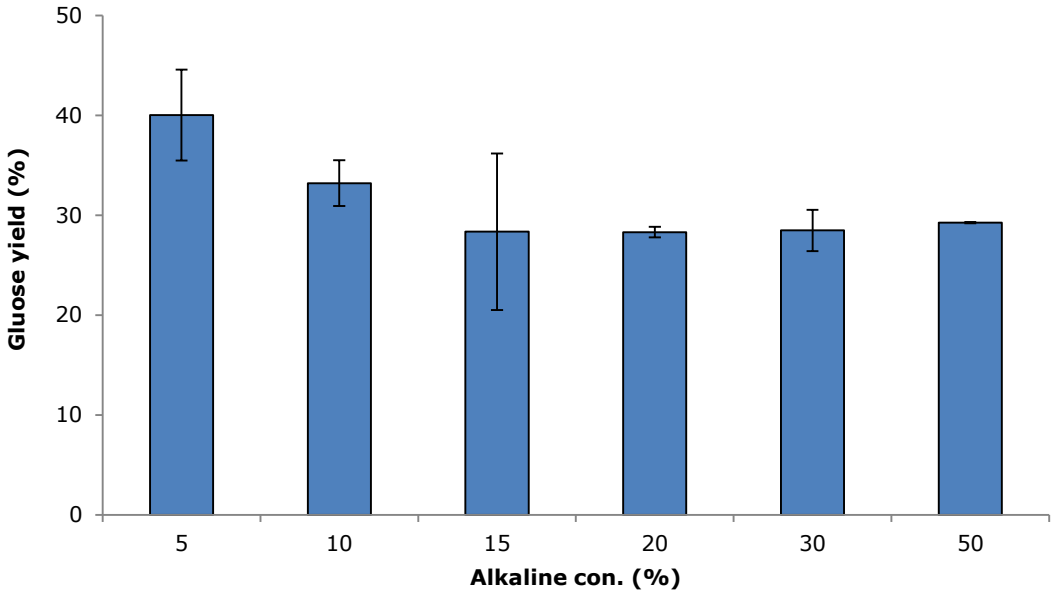


Figure 4.11 Effect of high concentrations of sodium hydroxide at room temperature on glucose retention in the solid residue

MSW (2 % w/v) was incubated with NaOH concentrations (5-50 %) at room temperature for 12 hr. The resultant residue was dried and glucose retention measured. Results are presented as % glucose recovered and are the mean ± SD (n=3).

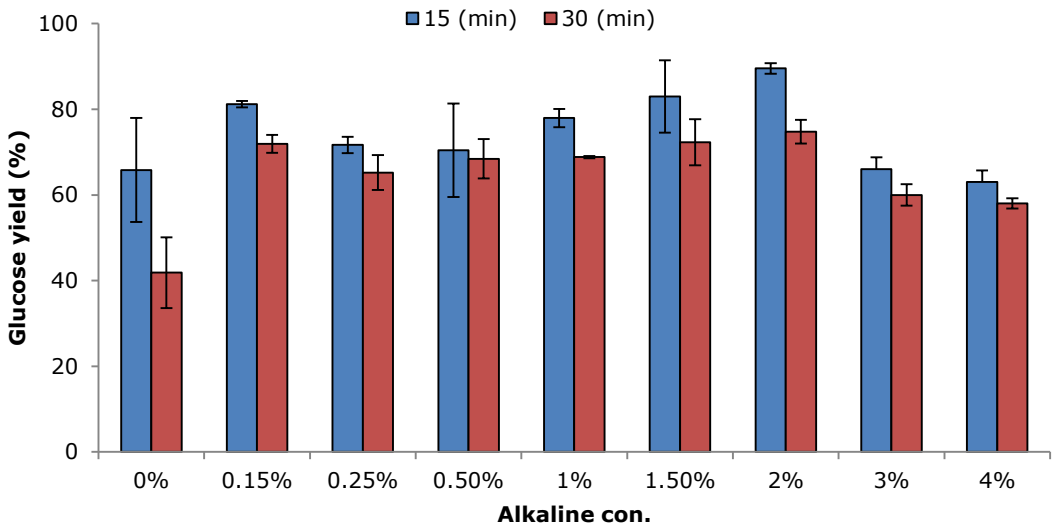


Figure 4.12 Effect of low concentrations of sodium hydroxide at 121 °C on glucose retention in the solid residue

MSW (2 % w/v) was autoclaved with NaOH concentrations (0-4 %) at 121 °C for 15 or 30 min. The resultant residue was dried and glucose retention measured. Results are presented as % glucose recovered and are the mean ± SD (n=3).

The impact of alkaline pre-treatment the subsequent EH of MSW was assessed (Figure 4.13). After 72 hr of EH the alkali pre-treated MSW was characterized by a higher glucose release (38.3 ± 1.38 %) when compared with control but this was not statistically significant (p=0.087). This indicates that use of an alkaline pre-treatment may increase the conversion of MSW into monomeric free sugar when compared with control conditions. However, the resultant yield, at below 40 % is still too low to be considered commercially effective.

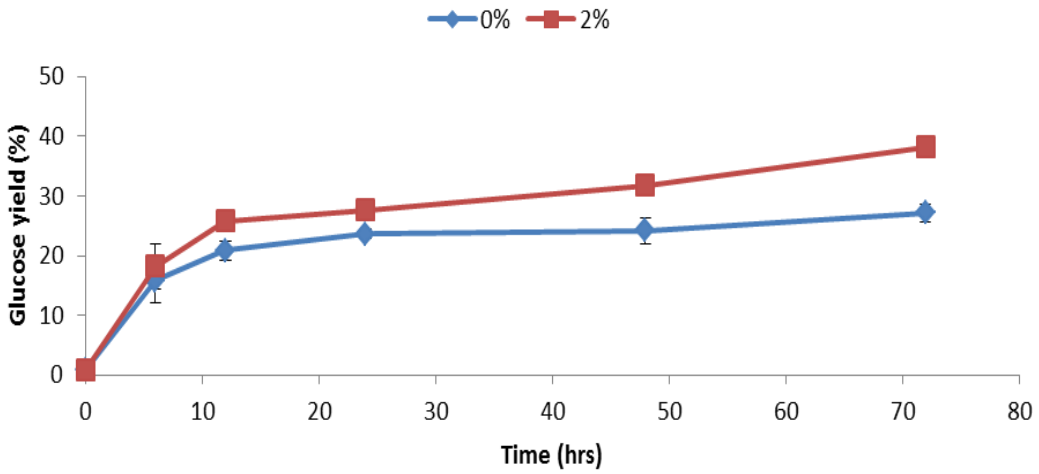


Figure 4.13 Effect of alkaline pre-treatment on glucose release from MSW

2 % w/v MSW was autoclaved at 121 °C in 2 % NaoH for 30 min. The residue was subjected to enzyme saccharification and the amount of glucose (% available) in released into the hydrolysate assessed. Results are the mean ± SD n=3)

4.3.5 Effect of solid to liquid (S/L) loading ratio on enzymatic hydrolysis of MSW

The experiments reported above were all carried out at low substrate loading for the EH and as such generated very low final glucose concentrations of around 0.5 g/L. In order to generate the higher levels required commercially it is common practice to use higher substrate loadings at the hydrolysis stage. Thus the impact of substrate loading ratio on the EH of MSW was investigated, Solid loading rates of 0.5-6 % (w/v) were used in a standard EH experiment with the enzyme

loading held constant at 30 FPU/g MSW. The results are shown in Figure 4.14. A, B, and Figure 4.15.

Loading above 6 % S/L led to the reaction system becoming too viscous for adequate stirring. Statistical analysis revealed a significant difference between S/L loading rates (p=0.0012), with glucose conversion decreasing as S/L ratio's increased from 0.5 to 6 %. Finally by increasing the substrate loading rate glucose concentrations in a hydrolysate did not increase significantly compared with 0.5 % MSW, and were still unsuitable for fermentation, the highest yield measured was 32.81 % using 0.5 % MSW, while the glucose concentrating using 6 % was higher comparing with the others S/L rates was 2.54 g/L.

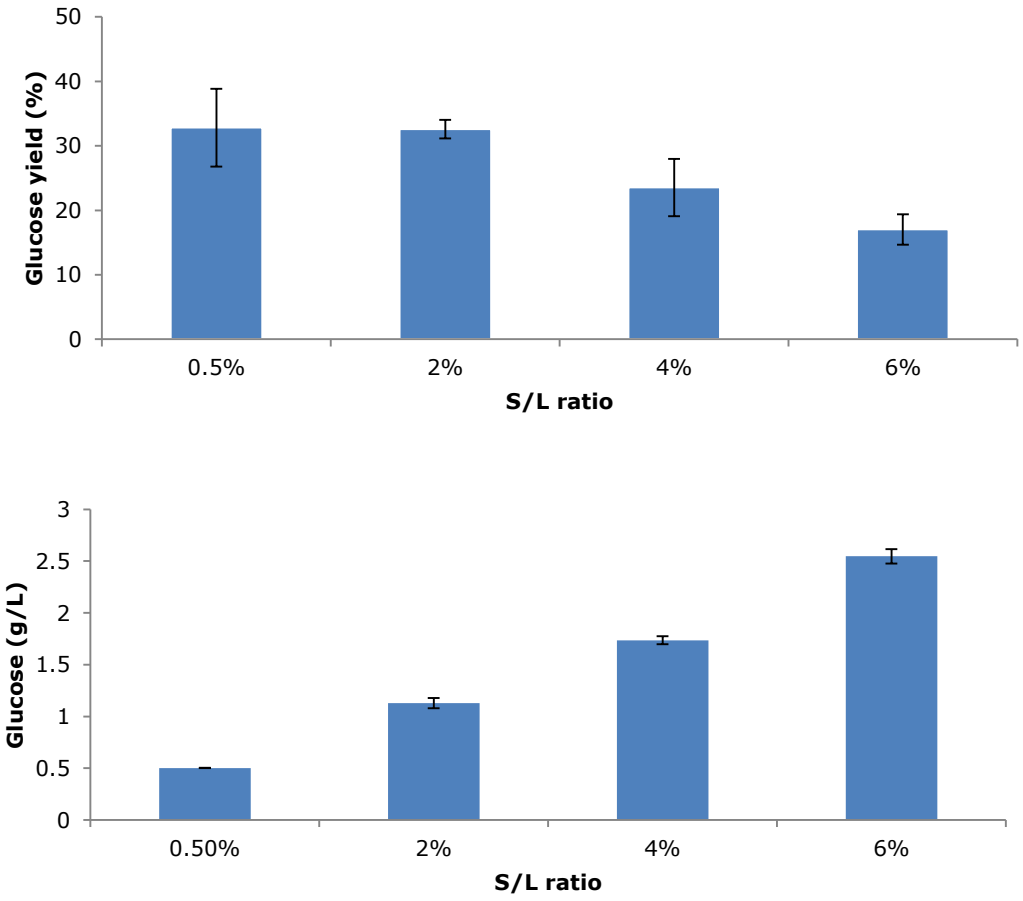


Figure 4.14 Effect of various solid liquid ratio of un-pre-treated MSW on sugar releasing using 30 FPU of cellulase enzyme at 72 hr (A) Amount of glucose yield % (B) amount of glucose by g/L

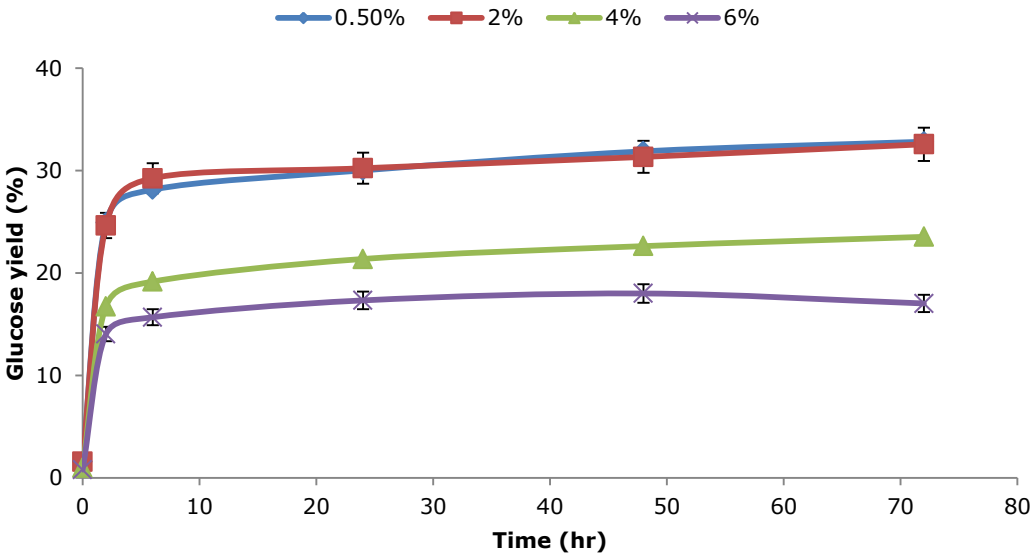


Figure 4.15 Glucose release (%) during enzyme hydrolysis of various S/L ratio of un-pretreated MSW

Enzyme hydrolysis using cellulase enzyme pre-treated various MSW solid ratio (0.5 or 6 %) monitored over 72 hr. Results are the mean± SD (n=3).

4.4 Discussion

Initial studies on the hydrolysis of the MSW as received showed a reduction in glucose yield over time. It was postulated that this was due to microbial contamination of the substrate. This was indeed shown to be the case since addition of either tetracycline or sodium azide, or sterilisation by autoclaving, were all able to prevent this glucose utilisation during the EH phase.

In order for biofuel production to be cost-effective a low operating cost is very important (Modenbach and Nokes, 2012). The cost of enzymes represents a significant proportion of these operating costs. A quantitative economic analysis of some pre-treatment methods was given by (Eggeman and Elander, 2005). The main factors that affect EH of lignocellulosic materials are substrate concentration and quality, pre-treatment method, cellulose activity, and hydrolysis conditions such as pH and mixing (Taherzadeh and Karimi, 2007; Kristensen *et al*., 2009).

Increasing the cellulase dosage rates from 15 to 30 FPU/g increased glucose release from MSW. Results from this study thus suggest that an enzyme loading rate of 30 FPU/g MSW may be optimal. Cellulase loading rate is generally between 5 to 35 FPU/g for a range of substrates and anything higher than this range would significantly increase the cost (Taherzadeh and Karimi, 2007).

In this present study applying doses above 30 FPU/g appeared to correlate with a reduction in release of glucose which agrees with a previous observation where high enzyme loadings were found to be impractical for example, sugar yield decreased from 70 to 68 % (40 to 20 FPU/g substrate) using mixed hardwood chips from a mill as substrate (Xue *et al*., 2012). In addition, Higher enzyme loading has been correlated with overcrowding on the cellulose chains thus reducing rates at which cellulose is hydrolysed (Olsen *et al*., 2011).

The surfactants used included amphiphilic compounds that contain a hydrophilic head and a hydrophobic C tail. They are capable of self-assembling into micelles and adsorbing onto surfaces depending on the surfactant structure and the

polarity of the surface (Helle *et al*., 1993). The main advantage of addition of a surfactant is to cause a surface structural modification or disruption of the lignocellulose that increases accessibility of the enzyme(s) to cellulose, or their addition may affect enzyme-substrate interaction by preventing non-productive adsorption of enzymes they may also act as enzyme stabilizers. Furthermore, they adsorb at the air-liquid interface and thus prevent enzyme denaturation during agitation in the hydrolysis mixture (Kim *et al*., 1982; Eriksson *et al*., 2002).

During EH, cellulases tend to irreversibly bind to lignin through hydrophobic interactions causing a loss in enzyme activity with the amount and composition of lignin affecting the formation of soluble sugars during EH (Binod *et al*., 2011). To decrease the effects of the presence of lignin adding surfactant can show a positive effect on EH by significantly increasing hydrolysis efficiency, allowing for either a faster hydrolysis rate or lower enzyme dosage. The addition of surfactants may also facilitate efficient recycling of cellulases after saccharification, a process step that ideally needs to be considered to reduce the cost of lignocellulosic ethanol production (Binod *et al*., 2011).

In the current study, a number of surfactant were examined for their ability to improve EH including non-ionic surfactants such as fatty acid esters of sorbitanpolyethoxylates (tween 80, tween 20) and PEG in the current study tween 20 and PGE gave almost the same yields but tween 20 showed better performance during the EH process; addition of these surfactants have been shown previously to enhance EH (Binod *et al*., 2011). Addition of non-catalytic proteins in the current experiment such as BSA gave almost the same yield as the control. BSA has been shown to adsorb to surfaces, reducing unspecific binding by "filling up" adsorption sites on lignin surfaces (Pan *et al*., 2005; Börjesson *et al*., 2007; Binod *et al*., 2011).

Using detergents such as Triton X100 and X114 resulted in a reduced efficiency. This result contradicts that of other studies which showed that adding surfactant

such as triton X100 and protein-based biopolymers improved the EH yield in a substrate containing lignin (Eriksson *et al*., 2002; Yang and Wyman, 2006; Eckard *et al*., 2013). While, other study showed that when enzymatic hydrolysis of mixtures of cellulose and lignin was conducted in the presence of Triton X-100 at 50 ^{o}C cellulose was observed to settle in a water bath; however, lignin and Triton X-100 remained suspended in solution, and as a result did not improve the EH process (Börjesson *et al*., 2007). The results from adding surfactant in the present study showed that this would not be commercially viable.

Pre-treatment using hot water, acid and alkaline, the MSW as received (if sterilised) had 30-40 % saccharification efficiency. This is relatively high compared to completely untreated LCM which would normally have only around 10 % of its glucose released without any pre-treatment. However, the MSW could be considered to have already been subjected to a pre-treatment through steam autoclaving. The steam treatment may have made a fraction of the MSW accessible but there was obviously a significant proportion that remained recalcitrant. The impact of further pre-treatments on MSW digestibility was thus investigated to find out whether treatments had any major effects on the subsequent glucose release during enzyme hydrolysis.

Pre-treatment is considering as a necessary step to alter some lignocellulosic characteristics to increase glucan and xylan accessibility to enzyme attack (Öhgren *et al*., 2007). This type of pre-treatment has a great effect on EH (Kumar *et al*., 2009). Furthermore, the selection of a specific pre-treatment for a particular raw material to increase biomass digestibility depends on several factors such as the combination of composition of the substrate, type of pre-treatment, dosage and efficiency of the enzyme used for the hydrolysis although the individual impacts of these factors on the EH are still unclear (Alvira *et al*., 2010). The pre-treated biomass can be filtered to obtain two fractions: one solid

cellulose-enriched fraction and a liquid fraction rich in hemicellulose derived sugars (Mosier *et al*., 2005).

Use of water at high temperature (121-180 °C) also called liquid hot water, is a pre-treatment of LCM often termed autohydrolysis (the reaction is "automatically" initiated by the hydro ions [H^3O^+] generated from the dissociation of water molecules), the major advantages of this pre-treatment are cost, less equipment corrosion, a reduction in xylose degradation and as a cause a reduction in the presence of undesirable by products including inhibitory compounds in the hydrolysates (Huang *et al*., 2008).

The results from the current study showed a high retention of glucose (and hence cellulose) in the solid fraction following hot water treatment. This is in agreement with Mok and Antal (1993) which, showed that by using liquid hot water around 4–22 % of the cellulose, 35–60 % of the lignin and 90 % the hemicellulose being removed in the process for up to 15 min at temperatures of 200–230 ^oC due to higher water input, and subsequently concentration of degradation products is reduced (Alvira *et al*., 2010). Also, pre-treatment of herbaceous feed stocks, such as corn stover by liquid hot water has been shown to remove up to 80 % of the hemicellulose and to enhance subsequent enzymatic digestibility (Mosier *et al*., 2005a) of sugarcane bagasse (Laser *et al*., 2002) and wheat straw (Pérez *et al*., 2008).

In this study a hot water pre-treatment resulted in a reduction in glucose yield in the subsequent enzyme saccharification this is contradictory to most previous studies on LCM, probably because of the steam autoclaving making a fraction of the cellulose irreversibly recalcitrant for some reason. Also, hot water under pressure as a pre-treatment has been shown to penetrate the cellular structure of biomass, hydrate cellulose, and remove hemicellulose, all these physical alterations should improve the enzymatic digestibility of cellulose (Hsu *et al*., 1980).

Acid pre-treatment, the main function of an acid pre-treatment of lignocellulosic material is to hydrolyse hemicellulose polymers into sugar monomers, these monomers are then recovered in the liquid fraction after filtration. Remaining in the solid fraction is cellulose and lignin which require further pre-treatment such as EH (Taherzadeh and Karimi, 2008).

Acid cone and temperature had an effect on increase sugar release, in the present study comparing the microwave with autoclave methods the latter showed significant improvement in being able to retain glucose in the solid residue. The maximum sugar retention obtained was using 5 min, 2 % MSW, 160 ^{o}C, 2 % acids as a pre-treatment, compared with autoclaving at 121 ^{o}C and using various acid concentrations. This might be due to the fact that sugar production and decomposition reaction rates increase dramatically with rising temperature and acid, also it takes less time to get maximum yield (Dien *et al*., 2006; Yang *et al*., 2009). In addition, use of a microwave at 160 ^{o}C as a pre-treatment was better than higher temperatures (< 180 ^{o}C) in terms of releasing fermentable sugars from MSW. However, very high temperatures lead to rapid degradation of sugar and a profound influence on inhibitor reaction kinetics. So as the temperature increases the rate of inhibitor production increases rapidly, in addition the energy requirements for the process are also increased (Lenihan *et al*., 2010).

Following the dilute acid pre-treatment EH efficiency was slightly improved but was still below the control so the optimum was with 0 % acid Treatment at 1 % concentration gave the highest yield among the acid treatments used in this study and this would agree with previous studies which revealed that optimum yield followed a pre-treatment with 1 % sulphuric acid. One study showed that using relatively low sulphuric acid concentrations (0.5 - 1.5 %) as a pre-treatment of lignocellulosic material was an economically and environmentally feasible approach for the liberation of fermentable sugars (Wingren *et al*., 2003; Binod *et al*., 2011). In addition this low efficiency may be because after pre-

treatment MSW substrate was made up of very fine particles and repeating washing process may remove some of the sugars. This observation agrees with previously published work which highlighted that use of a dilute acid pre-treatment can have a negative impact on pre-treated biomass namely the production of inhibitory products and reduced glucose yields (Selig *et al*., 2007). Whilst, some other studies have revealed that treatment of lignocellulosic materials with diluted acids can improve EH of the solid residue (Lee *et al*., 1999; Ramos, 2003).

Alkaline pre-treatment removes lignin and part of the hemicellulose, thus increasing the accessibility of enzymes to cellulose because alkaline pre-treatment has also been found to cause swelling, leading to an increase in internal surface area. Furthermore decrease polymerisation, crystallinity, breakage of the ester linkages that join xylan and hemicelluloses can all influence hydrolysis rates (Fan *et al*., 1987; Fox *et al*., 1989). However, all of the cellulose and most of hemicellulose is left in an insoluble polymeric form. Commonly, alkali pre-treatment works more efficiently with a biomass with a high lignin content (Hamelinck *et al*., 2005; Gupta, 2008).

In the present study pre-treatment with dilute alkaline was characterized by a higher glucose yield (using 2 % MSW) and 2 % NaOH comparing with other alkaline concentrations, assessment of solid fractions revealed that percentage weight loss was low. The current study agreed with some other studies which revealed that the low severity of the alkaline pre-treatment resulted in low sugar decomposition and that hemicellulose was retained in the biomass, the method can remove acetyl and various uronic acid substitutions in the hemicellulose which can lower enzyme accessibility (Chang and Holtzapple, 2000; Zhu *et al*., 2005). Furthermore, results here correlate with published observations reporting that use of an alkali pre-treatment led to an increase in production of sugars (Tutt *et al*., 2012). In addition the reason for low efficiency may be due to the degree of washing of the samples, as washing has been shown to reduce glucose

concentrations (Weimer *et al*., 1986; Chou, 1987) or the particulate size where small particulate size has been shown to enhance xylan or glucan conversion rates (Elshafei *et al*., 1991; Chundawat *et al*., 2007)

In contrast to the current study, pre-treatment with dilute NaOH has been reported to enhance production of sugars from LCMs (Azizul *et al*., 2012). Zhang, Shahbazi *et al*. (2010), recommended using low concentrations of NaOH (1 to 2 %) since the chemical is expensive, thus pre-treatment with NaOH increased cellulose digestibility from 14 to 55 %, this pre-treatment also decreased the lignin content from 25 to 20 %.

In this study, increasing alkaline concentration above 4 % at room temperature led to a reduction of glucose recovery in the solid residue fraction. This could be due to the biochemical nature of the samples, alkali pre-treatment has been shown to work more efficiently with LCM's which have a high lignin content, which makes it an unsuitable pre-treatment for MSW (Hamelinck *et al*., 2005; and Mosier *et al*., 2005; Gupta, 2008). The poor performance of alkaline pre-treatments has been reported previously leading to a reduction in liberation of sugars (Tutt *et al*., 2012). Also, Studies have revealed that by increasing the concentration of alkaline (6-20 % NaOH) there is an increase in biomass delignification which results in cellulose dissolution and reduced lignin removal (Silverstein *et al*., 2007; Mirahmadi *et al*., 2010).

The experiment showed that alkaline pre-treatment of MSW in combination with thermal pre-treatment caused considerable release of sugars when compared with other alkali pre-treatments at room temperature. The higher sugar yield in solid residue correlated with the severity of applied temperature to increase sugar or glucose yield %.

Alkaline pre-treatment can play a significant role in exposing the cellulose to EH (Fan *et al*., 1980). In a current study enzyme hydrolysis applied for the best selected alkaline concentration of pretreated MSW (2 % alkaline plus control), so the alkaline pretreated substrate applied for EH, pre-treatment with dilute NaOH

has been reported to enhance production of sugars from LCMs (Taherzadeh and Karimi, 2008; Azizul *et al*., 2012), which agrees with the present study. This is due to the pre-treatment causing swelling of substrate particles, increase internal surface area, and porosity, as a result makes EH more efficient as a resulting increase in sugar yield (Agbor *et al*., 2011).

Determination of inhibitors, during pre-treatment, many compounds are formed by degradation such as acetic acid, formic acid, and levulinic acid. When pre-treatment conditions are stringent furfural and 5-HMF are produced from pentose and hexose sugars, respectively and lignin degradation produces vanillin, syringaldehyde, and 4-hydroxybenzaldehyde (Binod *et al*., 2011), Acetic acid is ubiquitous in hydrolysates where hemicellulose and components of the plant cell wall have acetyl groups which can undergo hydrolysis (Cantarella *et al*., 2004; Taherzadeh and Karimi, 2008; Zhang *et al*., 2013). Formic acid may be formed as a by-product of sugar and lignin breakdown, whereas levulinic acid is generated from the degradation of HMF (Klinke *et al*., 2003; Cantarella *et al*., 2004; Taherzadeh and Karimi, 2008). Generally, concentrations of inhibitory compounds such as HMF, furfural, vanillin and formic acid are around 5 mM, while for some others -levulinic, coumaric acid and acetic acid- levels of around 25-50 mM have been reported and these have been shown to exhibit a significant inhibitory effect on the fermentation process (Martinez *et al*., 2000; Palmqvist and Hahn-Hägerdal, 2000; Klinke *et al*., 2004) These levels agree with those found in the present study. Presence of inhibitory compounds in a hydrolysate may lead to a reduction in growth of microorganism affecting the rate of sugar uptake while simultaneously reducing production of desired end-products (Chandel *et al*., 2011).

In the present study only HMF and furfural were detected in the hydrolysates following sulphuric acid pre-treatment at 121 °C. The use of sulphuric acid and relatively low temperature (121 °C) has been shown to avoid production of inhibitors (Greetham *et al*., 2014). Furthermore use of relatively high

temperature with a microwave was characterized by presence of inhibitors in the hydrolysates higher levels were found in 160 ^{o}C treated hydrolysate compared with other pre-treatments conducted at 121 ^{o}C. The relationship between increasing temperature of the pre-treatment and increased production of inhibitory compounds has been noted previously (Lenihan *et al*., 2010; Chandel *et al*., 2011; Greetham *et al*., 2014). Furthermore, the concentration of sulphuric acid used as the pre-treatment had less of an effect on the production of inhibitors this observation agreed with previously published data (Lenihan *et al*., 2010).

Solid loading rate: in this study solid loading rate was examined for its effect on EH and it was found that an S/L ratio of 6 % w/v substantially increased final glucose concentration but not the glucose yield %. Use of solid to liquid ratios above 6 % can cause problems in mixing and mass transfer (Sun and Cheng, 2002). Furthermore, at high solids loading a major challenge is lack of liquid in the reactor, as water is essential for effective hydrolysis (Hodge *et al*., 2009; Kristensen *et al*., 2009).

Studies have shown that conversion of cellulose decreases as S/L ratios increased, however, these results differed from the data observed in this study (Cara *et al*., 2007; Jørgensen *et al*., 2007; Kristensen *et al*., 2009; and (Ingram *et al*., 2011). Generally, sugar concentrations correlated with biomass composition density, indicating that the total sugar content in the biomass is not the primary contributing factor for sugar availability in untreated biomass (Serapiglia *et al*., 2013).

The final conclusion appears to be that none of the pre-treatments resulted in a major increase in saccharification efficiency. The reason for this is unclear. It is possible that during pre-treatment of the substrate with acid, alkaline and water / steam lignin can be release into the solution, which then reacts to form compounds with limited solubility. These compounds precipitate onto the surface

of the substrate (Liu and Wyman, 2003; Yang and Wyman, 2004). The lignin deposition can then effect irreversibly on enzyme adsorption (Donohoe *et al*., 2008). Among the different methods, alkali was the most effective and may represent the most promising technology for industrial application.

Chapter 5 Optimising cellulase production from municipal solid wastes (MSW) using solid state fermentation (SSF) by *Trichoderma reesei* and *Aspergillus niger*

5.1 Introduction

The reliable supply of cheap cellulase is crucial to reduce the cost of hydrolysing lignocellulosic raw materials. Enzyme production through SSF can have many advantages over SMF, such as higher yield and higher product stability (Hölker and Lenz, 2005; Singhania *et al*., 2009). SSF has the advantage of bringing the cultivated microorganism into closer vicinity to the substrate and achieving the highest substrate concentration for fermentation and it reproduces the natural microbiological processes like composting and ensiling. The disadvantages of SSF are that it has a longer lag time, and it has different optimal conditions for spore germination and vegetative growth and larger inoculation size compared to SMF (Gowthaman *et al*., 2001). SMF is technically easier to perform as compared to SSF (Hölker and Lenz, 2005), while SMF can be considered as a violation to their natural habitat, especially of fungi (Singhania *et al*., 2009). SSF was chosen as the method of investigation in this study.

The factors associated with SSF have been described in detail in the literature review (Bellon-Maurel *et al*., 2003; Dos Santos *et al*., 2004). Cellulase enzymes are produced by various bacteria such as *Bacillus subtilus, Bacillus circulans* (Ray *et al*., 2007) *Bacillus sphaericus-JS1* (Singh *et al*., 2004), *Cellulomonas flavigena* (Rajoka, 2004) and fungi like *Aspergillus sp.* (CORAL *et al*., 2002). *T. reesei* has a long history of safe use in industrial scale enzyme production, is considered non-pathogenic, and it has been shown not to produce fungal toxins or antibiotics under conditions used for enzyme production (Rajesh *et al*., 2012). Research has revealed that *T. reesei* can produce cellulase when grown on bagasse (Aiello *et*

al., 1996), egg shell waste (Verma *et al*., 2012), water hyacinth (Zhao *et al*., 2013), lignocellulosic waste from the xylose industry (Xia and Cen, 1999), waste from the vinegar industry (Liu and Yang, 2007), wheat bran (Maurya *et al*., 2012), sugar cane bagasse (Singhania *et al*., 2006), domestic waste water sludge (Alam *et al*., 2008), sweet potato (Zhang *et al*., 2004), oil palm lignocellulosic biomass in the form of empty fruit bunches (Alam *et al*., 2009), Soyhull (Krishna, 1999), saw dust (Guruchandran and Sasikumarand, 2010), rice bran (Latifian *et al*., 2007), rice straw (Hideno *et al*., 2011) potato peel (Dos Santos *et al*., 2013), and banana waste (Krishna, 1999).

There has been some research using MSW for cellulase enzyme production (Stutzenberger, 1971; Gautam *et al*., 2011), but this was not using MSW as prepared in the current study. No research has been carried out using autoclaved MSW as a substrate for cellulase enzyme production.

MSW can contain a high lignocellulose content; which indicates that it could be used for cellulase production. In this chapter, we report an example of using actual industrial MSW for cellulase production and the use the cellulase cocktail for the EH of MSW. *Aspergillus niger* and *T. reesei* were selected due to their track records for cellulase production. The final aim was RNAseq of *Trichoderma* grown on MSW to determine global enzyme expression over different time points (3, 5 and 7 days) and to investigate the biochemical response to the substrate.

5.2 Materials and Methods

5.2.1 Fungal strains and growth conditions

The microorganisms used in this study were the fungi *T. reesei* and *A. niger* N402 which were kindly donated by Professor David Archer (University of Nottingham, UK). Procedures for the storage and cultivating of *A. niger* have been described by (Ries *et al*., 2013). *A. niger* (spore suspension) was maintained on PDA (Potato Dextro Agar), potato starch (4 g), dextrose (20 g) and agar (15 g) slant, while propagation of *T. reesei* used a mixture of PDA and 4 % MSW. Spores were harvested from slants incubated at 28 °C for 7 days by adding 5 mL/ slant tube reverse osmosis (RO) water. The spore suspension was counted and diluted to a final concentration of 10^6 spore/1 mL. The spore count was performed on a microscope (100X , Optica microscope).

Mineral solutions, two stock solutions of trace metals were prepared; Mineral solution 1 contained (g): 0.5 KH_2PO_4; 0.5 K_2HPO_4; 1.0 $(NH_4)_2SO_4$; 0.2 $MgSO_4$; in 1000 mL distilled water. Mineral solution 2 contained (g): 26 KCl; 26 $MgSO_4$;76 KH_2PO_4 in 1000 mL of distilled water.

5.2.2 Solid state fermentation (SSF) process

5 g (dry weight) of MSW was placed into a 250 mL Duran bottle. Sufficient distilled water was then added to the MSW to give a final moisture content of 60, 70 or 80 %. The Duran bottles were autoclaved at 121 ^oC for 15 min. After sterilisation the substrate was cooled down to room temperature. Spore suspensions of *T. reesei* or *A. niger* (0.5, 1 or 2 x10^6 spores/g of dry MSW) were then added. The mash was mixed using a sterilised spatula and approximately 2.0 g of inoculated MSW mash was distributed to Petri dishes. The Petri dishes were then incubated at 25, 28 or 30 ^oC using a static incubator for up to 168 hr.

5.2.3 Crude enzyme extraction

Fermented solid samples from Petri dishes containing 2.5 g of original dry substrate were mixed with 20 mL of 0.05 M sodium citrate buffer (pH 4.8). The mixture was transferred to a blender and blended for 10 s. The suspension was poured into a beaker, stirred on a magnetic stirrer (300 rpm) for 30 min at 4 ^{o}C, and then transferred into 50 mL conical tube for centrifugation (4472 g) for 10 min. Supernatants containing crude enzymes were retained after centrifugation and enzyme activity determined were explained in detail later section 5.2.4.

All the SSF experiments were carried out in triplicate. Microsoft Excel was used to calculate data means and standard deviations. The significance of the different experimental group was determined using Student's t-test at the significance level of p=0.05.

5.2.4 Measurement of cellulase activity

The filter paper cellulase activity protocol (Adney and Baker, 1996) was used to assess enzyme activity during the purification process. Enzyme containing samples (0.5 mL) were mixed with 0.5 mL of 50 mM citrate buffer (pH 4.8). Strips (1 x 6 cm, approximately 50 mg) of Whatman No. 1 paper (cellulose substrate) were placed in the tubes, and the tubes were incubated at 50 ^{o}C for 1 hr. After that, 3 mL of DNS reagent (prepared according to (Adney and Baker 1996), was added to each tube and the mixtures were boiled for 5 min, cooled on ice (5 min), samples were diluted (0.2 mL of sample mixed with 2.5 mL of distilled water) and absorption was read at 540 nm.

A tube without enzyme an addition of 0.5 mL of citrate buffer was added instead) and another tube with enzyme sample but without substrate (no Whatman paper added) were used as controls. Absorption of control was deduced from the absorption of samples and each sample run in duplicate. Data means and standard deviation were calculated using Microsoft Excel. Glucose standards were

prepared using a glucose stock solution (10 mg/mL), serially diluted and were incubated with DNS as described above.

The FPU in the enzyme solution was converted to Unit per gram of MSW using the equation mentioned previously (refer to section 4.2.2)

5.2.5 Enzymatic hydrolysis of MSW

Fresh fungal extract obtained from SSF of MSW using *T. reesei* at 30 ^{o}C, 60 %, 0.5 x10^{6} spore/g dry MSW, harvested after 120 hr of fermentation, was used to hydrolyse non-treated MSW. 8.3 mL fungal extract was mixed with 91.7 mL citric acid buffer solution (50 mM, pH 4.8) to achieve an enzyme loading rate of 30 FPU/g dry MSW. A commercial cellulase Novozyme Cellic® CTec2 (Novozyme, Nottingham, UK) was used as a control. The control enzyme solution was prepared by diluting 25 µl of Novozyme Cellic® CTec2 in 100 mL citrate buffer (50 mM, pH 4.8). The enzyme loading ratio was again 30 FPU/g dry MSW.

The hydrolysis was started by adding 0.5 g (dry weight) MSW into the fresh fungal extract or control enzyme solutions, and then the samples were shaken at 150rpm in a shaking incubator at 50 $^{\circ}$C for 72 hr. Samples were taken at 0, 2, 4, 12, 24, 48 and 72 hr. After centrifuged at (16,249 g) for 10 min, the supernatants were analysed for glucose concentration using Dionex ICS-300. As described in section 4.2.4.1

For the time-resolved hydrolysis experiments, glucose concentration curves were fitted to a single exponential growth function, described by equation (1), where Cf is the final concentration at the assymtote of the function, Ct is the concentration at time t, and k is a rate constant.

$$C_t = C_f(1\text{-}\exp[-kt]) \quad \text{.....(1)}$$

Monosaccharides (arabinose, galactose, glucose and xylose) were determined using a Dionex ICS 300. As described in section 4.2.4.1.

5.2.6 Acid and alkali pre-treatments

MSW (2 g) was added to 100 mL of either 1 % H_2SO_4 or 2 % NaOH and autoclaved at 121 ^oC for 30 min or 15 min, respectively. After autoclaving, samples were neutralized to pH 7.0 using either 1 M NaOH or 1 M H_2SO_4 as appropriate. The samples were then centrifuged at 4472 g for 10 min. The solid fraction (pellet) was rinsed three times by adding distilled water to the pellet and re-centrifuge. The solid fraction was then dried overnight at room temperature.

5.2.7 Transcriptome analysis of *T. reesei* during SSF on MSW

T. reesei, was cultured on MSW incubated at 30 ^oC under optimal conditions and fungal samples collected at three time points 96,120 and 168 hr and frozen until required. The mycelia were frozen using liquid N_2 and ground using a pestle and mortar. The ground mycelium was then gradually added to 1 mL of Trizol (invitrogen) in a 2 mL tube to give a final volume of around 1.5 mL. The samples were, left at room temperature for 10 min. Then 200 µl of chloroform was added to each sample, which were then vortexed and left for 2-3 min. Samples were then vortex again, then centrifuged at 37788.4 g for 10 min. 750 µl of the upper aqueous phase was removed and placed into a 1.5 mL tube. 750 µl of isopropanol was added and samples left for 20 min on ice to precipitate the RNA, Samples were centrifuged at 37788.4 g for 10 min this resulted in a gel like pellet being formed on the side and bottom of the tube. The supernatant was removed and pellet washed with 700 µl of 70 % ethanol, centrifuged at 37788.4 g for 10 min. The ethanol was removed and the pellet left to dry in a laminar airflow cabinet for 10-15 min. The pellet was resuspend in 100 µl of DEPC (Diethylpyrocarbonate) treated water. RNA clean up kit (Qiagen) was used to remove DNA following the manufacturers "on column DNase digest" protocol.

RNA samples were sent for quality control and RNAseq to the service centre. The University of Nottingham. Annotation of the reads and the initial global analyses was carried out by Dr Martyn Blythe. Three biological replicates were prepared for

each time point. Unfortunately, one of the three replicates from day 5 failed the internal RNAseq quality control and as such was not used in the subsequent analysis.

A Filtering Pipeline was used to filter reads with low sequencing score and reads aligned to adaptor sequences. Initially raw reads were trimmed for adapter sequences using Sythe. This was followed by quality trimming using Sickle. Reads that passed the filter were then aligned to the reference and reads were removed which mapped to the rRNA and tRNA genes (as annotated in the genome reference). Then reads that passed the rRNA and tRNA filter were mapped onto the reference genome in the context of known gene exon coordinates by tophat mapping tool. The reference genome used was *T. reesei Version2 as* provided by the Joint Genome Institute (JGI) through their WEB site. It was found however, that the reference genome had poor annotation.

Read counts for each gene were calculated using 'htseq-count'. This program determines the number of uniquely aligned reads per gene. MAPQ30 (Unique) was then used to correctly mapped reads and to generate counts per gene. Gene expression was expressed as Reads Per Kilo base of transcript per Million mapped reads (RPKM) values. The RPKM is simply a normalized read count (stranded/sense reads) for a given gene as defined by the University of California Santa Cruz (UCSC) ref Gene database. The read count of the exon-space of a gene is normalized against the total number of mapped reads (Uniquely and correctly mapped reads with rRNA excluded) in that particular alignment file, and against the total length of the gene's exon-space.

5.3 Results

5.3.1 Optimization of moisture content and temperature

The combined impact of moisture content and temperature on cellulase production by both *T. reesei* and *A. niger* was investigated. A series of SSF were set up with moisture contents of 60, 70 or 80 %, and fermentation temperatures of 25, 28 or 30 ^{o}C. These were inoculated with either *T. reesei* or *A. niger* spores at a concentration of 1x10^{6} spores/g and left to ferment for 120 hr (5 days). Cellulase was extracted and assayed at the end of the fermentation and the results are shown in Figure 5.1. It can be seen that *T. reesei* produced significantly higher amounts of cellulase than *A. niger* under all conditions. There was a statistically significant effect of moisture content (p=0.003) with 60 % giving the highest enzyme activity, at all three temperatures, for both *T. reesei* and *A. niger*. There was also a significant effect of temperature (p=0.0092) on cellulase enzyme production. In this study, the best temperature for *T. reesei* was 30 °C, whilst 25 °C resulted in maximum production of cellulase by *A. niger.*

The highest cellulase activity obtained was 18.98±0.65 FPU/g, and this was at 60 % moisture content, 30 ^{o}C, and using *T. reesei*. As *T. reesei* performed better in SSF of MSW, it was selected for further optimization experiments.

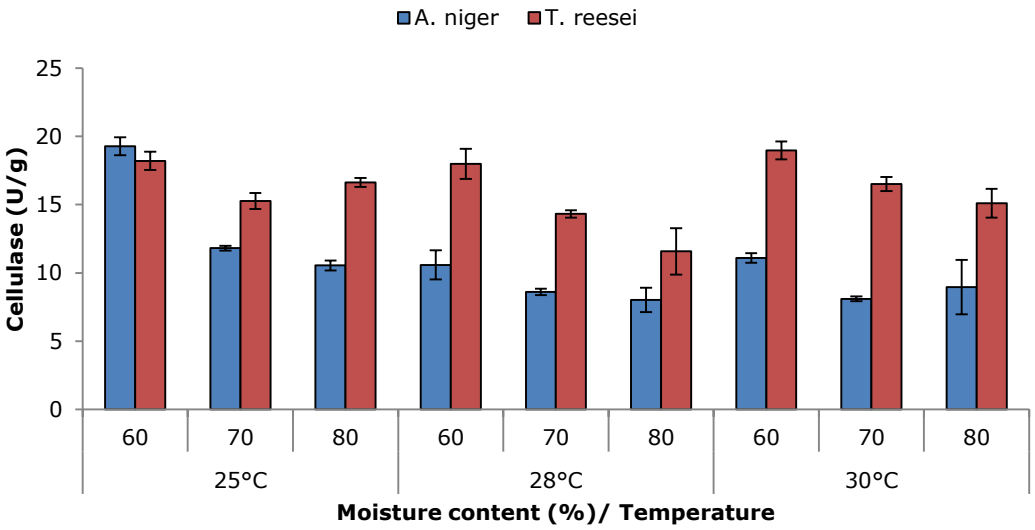


Figure 5.1 Impact of moisture and temperature on cellulase production

Solid state fermentations of MSW were set up at 60, 70 or 80 % moisture content. These were inoculated with either *T. reesei* or *A. niger* at a spore density of 10^6**.** Plates were then incubated at 25, 28 or 30 ^{o}C for 120 hr and cellulase production determined. Results presented are the mean ± SD (n=3).

5.3.2 Optimisation of inoculation size (*Trichoderma,* 30 ^{o}C, 60 %, 120 hr)

Using the moisture and temperature condition determined above the effect of inoculation size on cellulase production by *T. reesei* was examined using spore suspension inoculations of $0.5x10^6$, $1x10^6$ and $2x10^6$ spores/g dry weights MSW. The results are shown in Figure 5.2. The maximum cellulase activity (19.13±1.5 FPU/g) was obtained in fermentations using $0.5x10^6$ spores/g. increasing the inoculation size from $0.5x10^6$ to $1x10^6$ and $2x10^6$ spores/g, resulted in a significant decrease in recovered cellulase activities to 17.17±2.91 and 15.03 ±2.81 FPU/g, respectively.

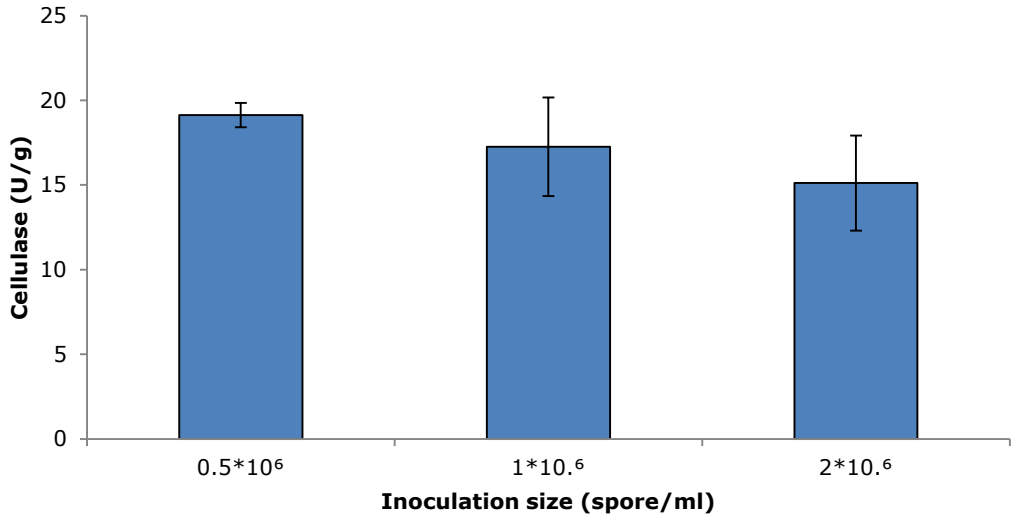


Figure 5.2 Impact of inoculation dose on cellulase production

SSF of MSW using *T. reesei* at three different inoculation doses was carried out using 60 % moisture at 30 °C. Crude enzyme was extracted after 120 hr and analysed for cellulase activity. Results presented are the mean ± SD (n=3).

5.3.3 Effect of additions on cellulase production

Using the optimal conditions determined above the effect of adding additional mineral supplements, additional carbon and nitrogen sources or the inclusion of a mineral clay to absorb potentially toxic elements were examined. The addition of mineral solution 1 did not increase cellulase production, whilst addition of mineral solution 2 resulted in a statistically significant 50 % reduction in enzyme activity ($p = 0.0044$). The results for the addition of the other supplements are shown in Figure 5.3.

In the case of the other additions, additional carbon (cellulose powder), nitrogen (peptone) or the addition of clay did not result in any significant changes in cellulase production as compared with the control. These results might imply that the MSW has sufficient intrinsic nutrients and that additional supplementation is not required. The only significant differences observed in Figure 5.3 were between cellulase production in the presence of 0.2 % clay and with some of the fermentations with the addition of carbon and/or nitrogen. The addition of clay

may result in the removal of inhibitory heavy metals and thus encourage fungal growth, there was a slight increase in cellulase production following the addition of 0.2 % clay but this was not significant. Similarly the addition of alternate carbon or nitrogen sources may act to deflect production of cellulase by the fungus. There was a general decrease in cellulase production observed with the addition of these nutrients but again this was not significant when compared to the control.

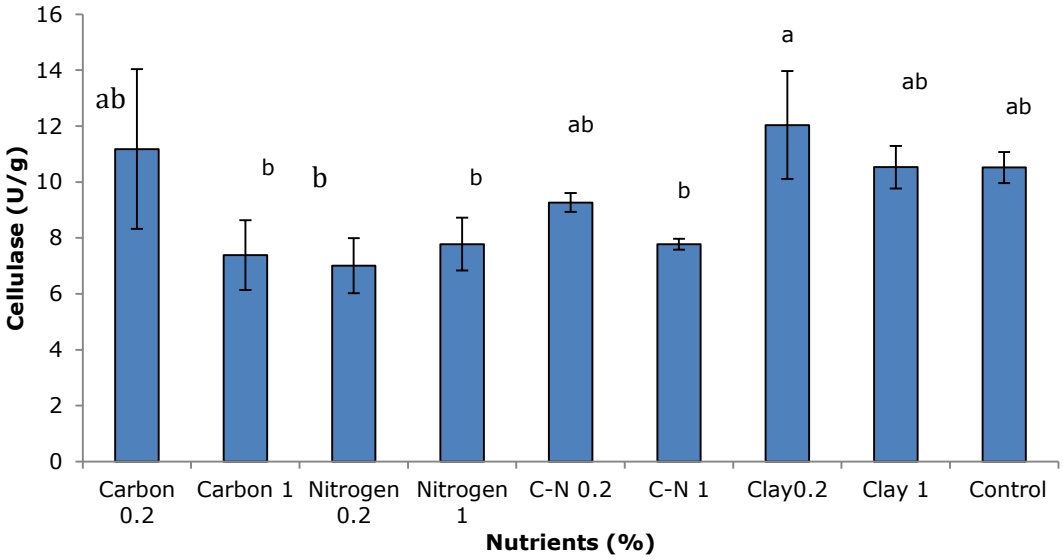


Figure 5.3 Impact of additional carbon, nitrogen or clay on cellulase production

SSF of MSW using *T. reesei,* at an inoculation dose of 0.5 *10^6 Spore/ml*,* was carried out using 60 % moisture at 30 ^{o}C. The effect of adding additional carbon (cellulose powder) or nitrogen (peptone) nutrient sources, either individually or in combination was examined. The addition of clay at either 0.2 or 1 % (w/w) was also tested. All fermentations were compared to a control. Crude enzyme was extracted after 120 hr and analysed for cellulose activity. Results presented are the mean + SD (n=3). Bars with different letters indicate significant difference (p=0.05).

5.3.4 Effect of pre-treating MSW on cellulase production

The impact of pre-treatments, designed to increase the accessibility of the cellulose, on cellulase production was examined. The MSW was subjected to either an acid or alkali pre-treatment prior to SSF. The results showed that in both cases pre-treatment actually resulted in reduced cellulase production compared to non-treated MSW. Pre-treatment with acid resulted in a significantly

lower cellulase production of 47.8 % of that obtained in the control fermentations, whilst that with alkali was only slightly higher at 65.2 % of the control.

5.3.5 Cellulase production under optimal conditions

The optimization of conditions for cellulase production described above utilized a fixed SSF incubation of 120 hr. The effect of extending this culture time from 5 to 7 days was thus examined. This extended fermentation increased cellulase production from 18.53±0.19 to 26.10±3.09 FPU/g. longer fermentation times were not explored as they were felt to be commercially insignificant. The length of incubation period is a prime concern for the development of a commercial cellulase production process and 7 days may not be viable.

5.3.6 Hydrolysis of MSW using crude fungal extract

The crude fungal extract obtained from an optimum fermentation was collected. This was then used for the EH of a fresh MSW sample with a cellulase enzyme loading rate of 30 FPU/g dry weight. A commercial cellulase cocktail, Cellic® CTec2 (Novazyme), was used as a control at the same enzyme loading rate. Glucose release with time for the hydrolysis is shown in Figure 5.4. The glucose concentration in the hydrolysis using Cellic® CTec2 reached 90 % of its final concentration with 24 hr. This represents a 32.8 % yield of available glucose in the MSW. In the hydrolysis using the fungal extract from the SSF, the initial glucose release rate was slower compared to that with Cellic® CTec2 and the final yield was around 24.7 %. Although lower than the commercial enzyme, the hydrolysis achievable with the cellulase extract from *T. reesei* grown on MSW was nonetheless significant.

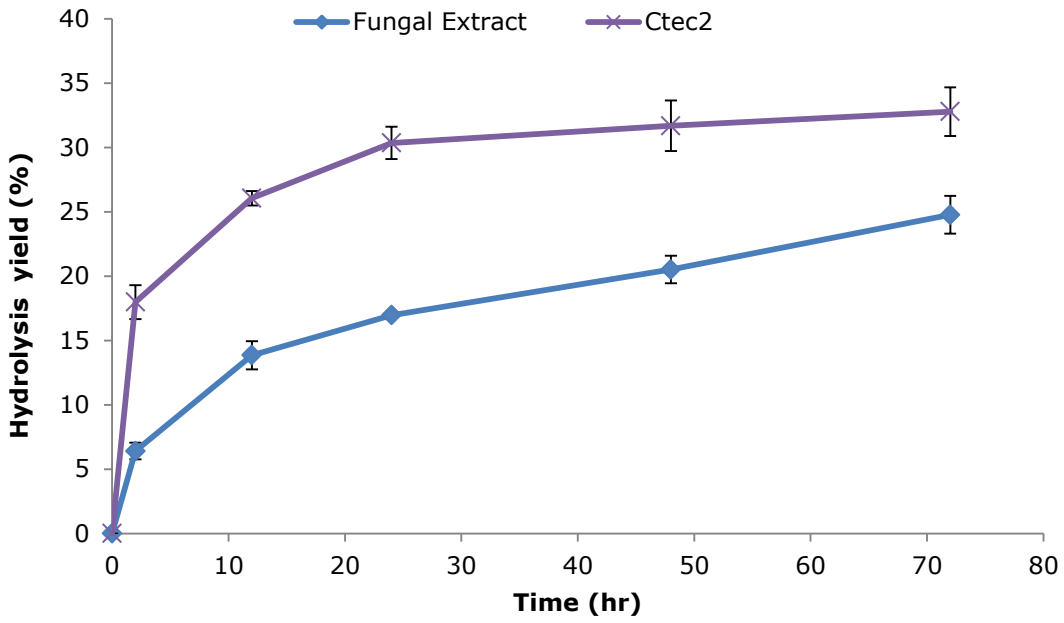


Figure 5.4 Glucose release during the hydrolysis of MSW using either commercial or MSW derived cellulase cocktails

Untreated MSW was incubated with either a commercial enzyme (Cellic® CTec2) or the crude enzyme as obtained from SSF of MSW (MSW). In each case at an enzyme loading rate of 30 FPU/g dry weight. Results presented are the mean ± SD (n=3).

5.3.7 Comparison of hydrolysis kinetics for commercial and MSW derived cellulase enzymes

A further study to compare the efficiencies of the commercial cellulase enzyme cocktail (Cellic® CTec2) with a crude enzyme preparation extracted from MSW was carried out over 50 hr of incubation and at two enzyme loadings of 15 and 30 FPU/g.

The glucose release in each case is shown in Figure 5.5. In this instance reducing the enzyme loading resulted in a reduced yield when the commercial enzyme was used but not with the fungal derived crude extract. This might imply that cellulase activity is not rate limiting in this case. Constant rate curves were fitted to the experimental data as described by equation (1) in section 5.2.5, from which rate constants and asymmtote (Ci) for sugar yields were determined. These are summarized in Table (5.1) and predicted curves compared to actual in (Figure 5.5). These values confirm the conclusions drawn above.

Table 5.1 Kinetic parameters for enzyme hydrolysis of MSW material

Enzyme	Ci : 15 FPU	Ci : 30 FPU	k : 15 FPU	k : 30 FPU
product	**(%)**	**(%)**	**(hr^{-1})**	**(hr^{-1})**
Cellic® CTec2	21.5	30.5	0.4	0.31
MSW	19	19	0.4	1

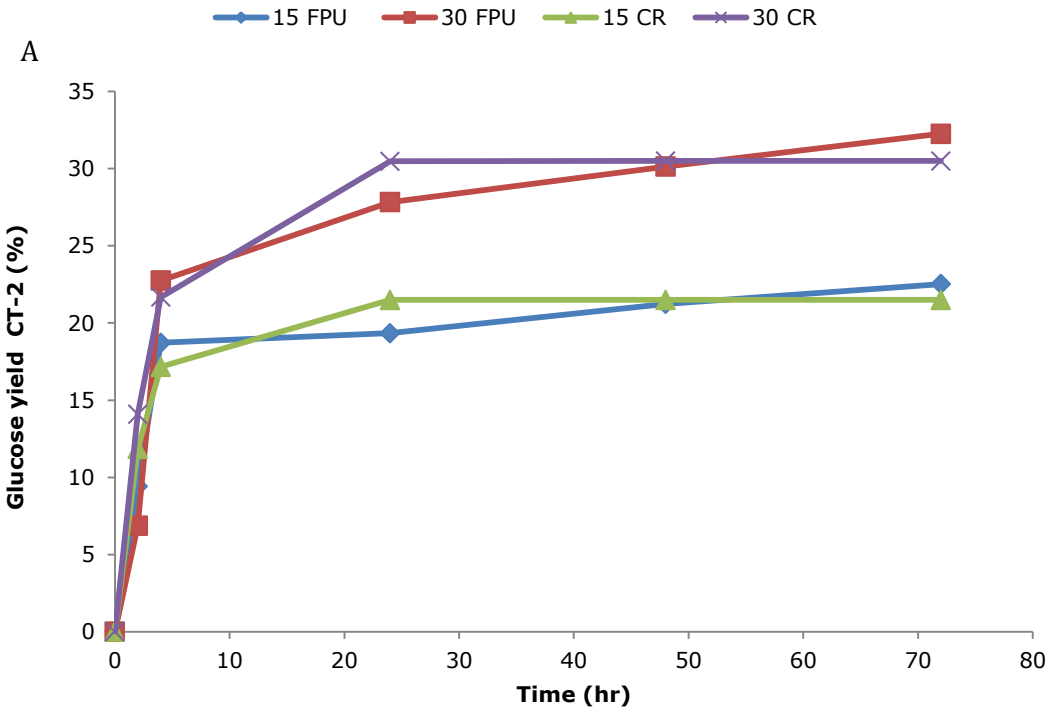


Figure 5.5 Glucose yield (%) with the rate constant of each enzymes using 15 and 30 FPU of crude and Cellic® CTec2 enzymes for hydrolysing autoclaved MSW (A) Cellic® CTec2 enzyme for hydrolysing MSW (B) crude enzyme extracted from MSW for hydrolysing MSW. Mean ± SD (n=3). FPU= Filter Paper Assay; CR = constant rate

5.3.8 Transcriptome analysis

Using *T. reesei*, SSF was carried out under optimal conditions and fungal mycelium harvested at 3, 5 and 7 days. RNA was extracted from the mycelium and sent to the service centre at The University of Nottingham for RNAseq analysis. Initial filtering and mapping of the data was carried out by the service centre. Unfortunately one of the three replicates for day 5 failed the quality control and as such has been removed from this analysis. Figure 5.6 shows the results of the trimming and mapping exercises as carried out by the bioinformatics service. S1-3 represent the triplicate samples from day 3, S4 and 6 the samples from day 5 and S7-9 the triplicate samples from day 7. S5 is missing as this failed the quality control. S9 data was not provided by the bioinformatics centre Raw counts ranged from 40 to 68 million and in all cases the filtering and mapping to genes has reduced the number. However, there were still 20 to 38 million reads that could be mapped to genes and a total of 9,143 individual genes were finally identified and quantified.

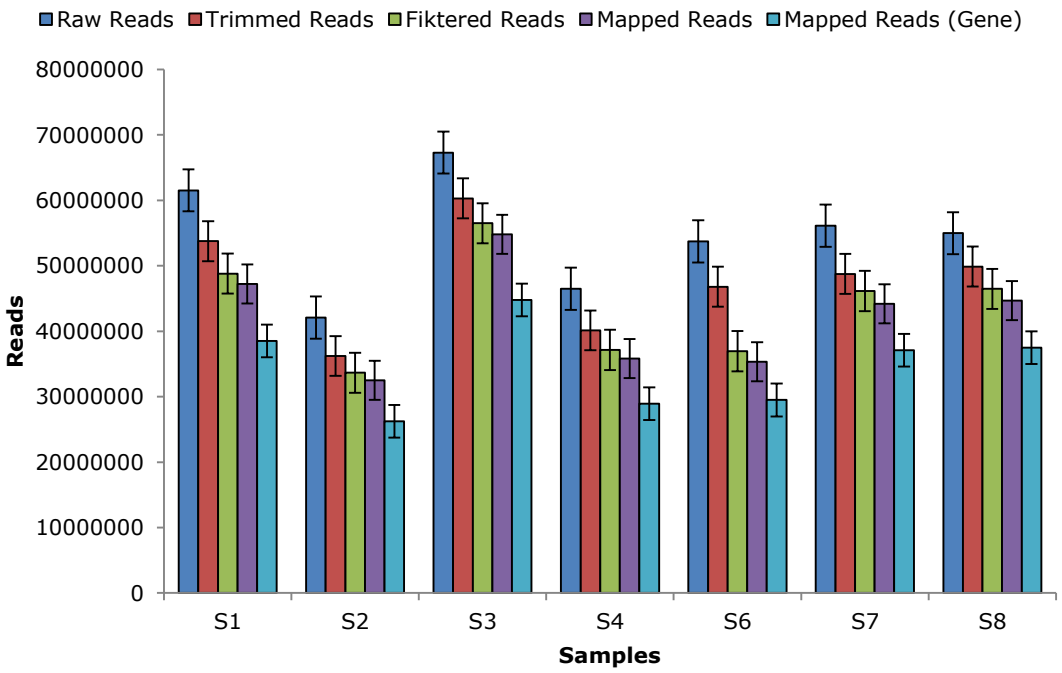


Figure 5.6 Filtering and mapping data for individual samples. Quality control and initial filtering and mapping were carried out by the service center as described in the method

PCA analysis of the mapped genes from the individual samples showed three clear clusters of samples 1,2 and 3; 4 and 6 and 7,8 and 9. Indicating that there was good replication between the biological replicates and that overall gene expression on the three sampling days was significantly different (data not shown).

This RNAseq data was made available at the end of the PhD programme and as such detailed analysis of the data has not been possible for this thesis.

The RPKM values for all 8 samples was transferred to an excel spread sheet that has been generated by Dr Paul Daly (The University of Nottingham) for further analysis. A copy of this spread sheet with the expression levels of all 9,143 genes is included on the CD attached to this thesis.

The spread sheet was designed to specifically analyse those carbohydrate active enzymes that are listed on the CaZy website. Thus the preliminary analysis of gene expression on the three days of sampling was restricted to this class of enzymes and in particular to the glycosyl hydrolases (GH). The analysis was carried out using the nomenclature of Hakkinen *et al.* (2012). Of the 9,143 individual genes 228 of these mapped onto carbohydrate active enzymes. The number of genes that returned a RPKM of above 0 was 221, 219 and 198 for days 3, 5 and 7, respectively. Of the 228 identified genes 200 were identified as GH. The number of GH genes returning a RPKM value of greater than 0 was 195, 192 and 175 for days 3, 5 and 7, respectively. It is not possible to analyse all of these genes, however a large number were found to be expressed at very low levels with RPKM values below 10. Also for this thesis we are really only interested in those genes that are expressed very highly since it is assumed that the high level of RNA will correlate with a high protein expression. It is noted that this need not be the case. In that case an arbitrary threshold for the RPKM of 100 was selected and only those genes with a value in excess of this chosen for analysis. It was

found that 28, 38 and 10 of the GH genes fell into this category for day 3, 5 and 7, respectively. The highly expressed GH genes fell into 25 classes according to the CAZy database. These and the number of genes highly expressed in each class at each of the three time points is shown in Table 5.2. Along with their annotated enzyme activities from the CAZy data base

Table 5.2 Highly expressed GH genes during *T. reesei* fermentation on MSW (RPKM>100)

CAZy group and suggested enzyme activity	Number of genes expressed on day 3	Number of genes expressed on day 5	Number of genes expressed on day 7
GH1 (β-glucosidase)	1	1	0
GH2 (β-mannosidase)	1	1	0
GH3 (β-glucosidase)	0	2	0
GH5 (β-1-3-glucosidase)	1	3	0
GH6 (Cellobiohydrolase)	0	1	0
GH7 (Endo-β-1,4 glucanase)	0	1	0
GH11 (β- 1,4 xylanase)	0	1	0
GH12 (β-1,4 glucanase)	0	1	0
GH13 (amylase)	0	1	0
GH15 (Glucoamylase)	0	1	0
GH16 (glucanosyl transferase)	6	3	1
GH17 (β- 1,3 glucosidase)	3	3	2
GH18 (Chitinase)	1	2	1
GH25 (N.O diacylmuramidase)	1	1	0
GH31 (α- glucosidase)	1	1	0
GH37 (α-trehalase)	0	1	0
GH47 (α-1,2 mannosidase)	1	1	0
GH61 (Cu dependent polysaccharide monooxygenase)	3	3	0
GH71 (α- 1.3 glucanase)	1	1	0
GH72 (β-1,3 glucosyl transferase)	3	3	3
GH74 (Xyloglucanase)	0	1	0
GH76 (α-1,6 mannase)	4	5	2
GH92 (α-1.2 mannase)	1	0	0
GH104 (unassigned)	0	0	1

Expression pattern for selected CAZy genes

The second analysis was to look at the expression pattern of those CAZy genes thought to be most significant. These were those involved in starch metabolism (GH13-amylase and GH15 glucoamylase), hemicellulose metabolism (GH11-xylanase and GH 74 xyloglucanase) and cellulose metabolism (GH6 cellobiohydrolase and GH7 endo glucanase). The data base was searched for all genes in these 6 categories and the expression levels of these at the three sampling points (See Figure 5.7).

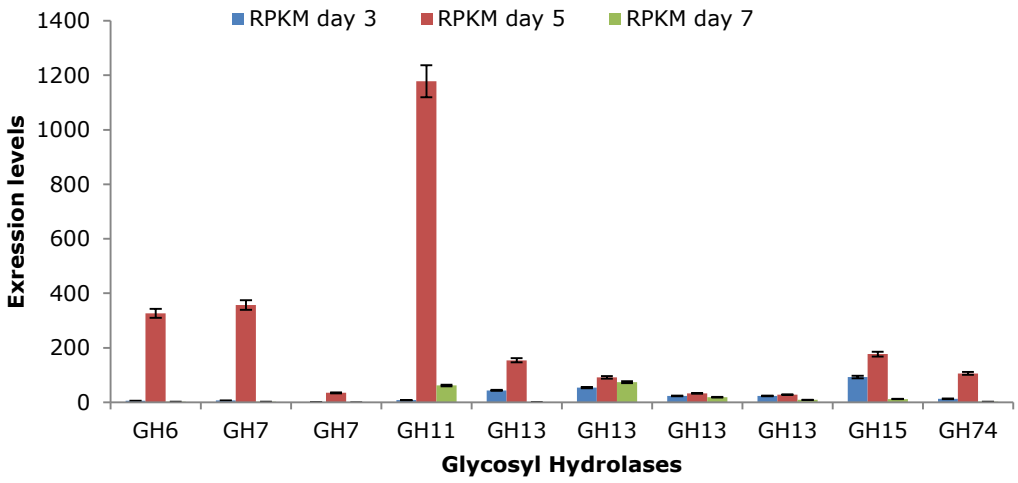


Figure 5.7 Expression levels for selected GH genes in *T.reesei* during SSF on MSW

In the case of amylase (GH13) the data base returned four independent genes. These along with the other gene involved in starch metabolism (GH15) showed co-ordinated expression. In all cases there was relatively high expression at day 3 and this remained relatively constant throughout the sampling period. The two genes involved in hemicellulose expression (GH11 and 74) also showed co-ordinated expression being low but detectable on day 3 and peaking on day 5. For the genes involved in cellulose metabolism the data base returned two independent genes for endo glucanase (GH7) and the expression of both these was co-ordinated with the other gene (GH6). In this case expression was extremely low on day 3 and peaked on day 5. The overall pattern is consistent

with the hypothesis that the fungus is utilising starch, as the most easily degradable substrate, in the early stages of the fermentation but attempts to exploit the hemicelluloses and then cellulose later on. Expression levels at day 7 were all relatively low suggesting that the fungal population is under severe stress at this stage and maybe dying off.

General highly expressed genes

The top 20 most highly expressed genes were identified for each sampling point. These are listed in Table 5.3. This list is in ascending order of gene ID number, not in order of expression, to allow easier comparisons between the three time points.

Table 5.3 Gene ID numbers for the 20 most highly expressed genes at each time point. Green highlights are those identified at all three times. Red those common to days 3 and 5; yellow highlights those common between days 5 and 7

Day 3	Day 5	Day 7
3007	44700	44700
53947	45971	45971
56118	49366	49366
64667	53947	61078
65718	61078	65718
68107	65718	66092
68909	68107	66276
70840	70840	70840
72137	72137	72137
73516	73516	73516
74060	81136	81136
81136	82374	103498
82510	106516	106516
105533	106591	106591
111890	109296	109296
119989	111362	111362
121605	121163	121163
123029	121439	121439
123650	121653	121653
124210	123650	12350

Six genes are represented across all three time points. 2 are common between days 3 and 5 and 11 between days 5 and 7. This supports the global analysis of changes in gene expression where there was a larger variation between days 3 and 5 than between days 5 and 7 and again may reflect the acclimatisation into a steady state.

The JGI WEB site was used to obtain putative enzyme identifications for these genes.

Of the 31 genes identified in the search above 18 returned hypothetical proteins of unknown function. The putative identification of the remaining 13 (See Table 5.4).

Table 5.4 Putative functions for the gene products

Gene ID	Putative function
49366	Protein turnover
56118	Acetyl C0 binding protein
68107	Ribosomal protein
68909	Ribosomal protein
73516	Glucose repressible gene protein-related protein
81136	Membrane bound protein of unknown function
82510	Histone H4
106516	Glucose/ribitol dehydrogenase
111890	Phosphate transporter
119989	Hydrophobin 2
121605	Actin regulatory protein
123029	Cu^{2+}/ Zn^{2+} superoxide dismutase
124210	Histone H3

As might be expected many of the genes encode for house-keeping genes e.g. ribosomal proteins and histones.

The database can also be searched for expression of specific target enzymes with potential commercial value. Of interest would be lipases and proteases. The *T. reesei* database on the JGI WEB site lists 136 gene IDs as having potential lipase activity and 268 with potential protease activity. A manual search for all of these would not be feasible but future work could explore this further. However, a manual search for the lipase genes has identified at least five that are expressed (Table 5.5) and one of these has an expression level above 100.

Table 5.5 Expression of selected putative lipase genes in *T .reesei* during SSF fermentation on MSW

Gene ID	EC number	RPKM Day 3	RPKM Day 5	RPKM Day 7
32364	3.1.-.-	11.77651	17.56082	0.417827
66324	3.1.1.23	131.8513	56.24179	22.14281
75989	3.1.-.-	10.71609	13.56596	3.480861
119742	3.1.1.3	36.70678	35.01845	20.04622
56427		10.79305	12.6074	1.494388

5.4 Discussion

SSF processes have been shown to be particularly suitable for the production of enzymes by fungi (Pandey *et al*., 1999), and the use of an abundantly available substrate such as MSW would reducing the overall cost of cellulase production.

Fungi that have been reported to produce cellulases include *Trichoderma*, and *Aspergillus spp* (Sternberg, 1975; Fang *et al*., 1987). *Trichoderma* has been most extensively studied for cellulase production and has been shown to be able to hydrolysis various substrates (Sternberg, 1975), In this study conditions for the production of cellulase from MSW have been determined. The novelty of the present study is that use of SSF with steam autoclaved MSW for the production of cellulase enzyme hasn't been reported previously. Furthermore, cellulase can be produced without adding supplements or pre-treatment with acid or alkali chemicals.

Moisture content; is generally considered to be a crucial factor that affects oxygen transfer and nutrient accessibility in SSF. High moisture encourages fungal growth, nutrient transportation and enzyme activities, but limits oxygen transfer and facilitates contamination (Mekala *et al*., 2008; Mrudula and Murugammal, 2011) A wide range of moisture content from 50-89.5 % have been used in various studies for cellulase production using *A. niger* (Pensupa *et al*., 2013).

In the current study 60 % moisture was the minimal moisture content that could be tested due to the water absorbing properties of the substrate, and this proved to be the most effective. In addition, to the preference of the microorganism, optimum moisture content may also be influenced by properties of the substrate such as porosity and particle size (Mekala *et al*., 2008; Maurya *et al*., 2012)

The present study showed that for both *Aspergillus* and *Trichoderma* 60 % moisture produced the most cellulase. Similar effects of moisture have been reported in the past. High moisture ratios of 1:2 (w/v) encourages fungal growth, nutrient transportation and enzyme activities, but limits oxygen transfer and facilitates contamination (Mekala *et al*., 2008; Mrudula and Murugammal, 2011). In the current study SSF using *T. reesei,* when the moisture content was increased to 80 % from 60 %, this resulted in lower cellulose production. A study found that increasing moisture in rice bran resulted in lower cellulase production because the increase in moisture level was believed to reduce the porosity of the rice bran, thus limiting oxygen transfer (Maurya *et al*., 2012). Another study showed the maximum yield of the enzyme, 2.29 U/mL, was obtained at 70 % moisture level. Use of relatively small volumes of water in SSF substantially reduces effluent volume and consequent contamination (Genckal and Tari, 2006).

Temperature is another important factor that affects fungal growth and enzyme production. The combined impact of temperature and moisture content has also been reported in similarly studies using spent brewers grains (SBG), rice bran and soybean hulls supplemented with wheat bran (Francis *et al*., 2003; Latifian *et al*., 2007; Brijwani *et al*., 2010).

In this study, the best temperature for *T. reesei* was 30 °C, while 25 °C was best for *A. niger*. SSF of *T. reesei* and *A. niger* is normally operated in the temperature range from 25 to 30 °C (Singhania *et al*., 2006) and is often dictated by the growth kinetics of the microorganism employed rather than enzyme produced (Lonsane *et al*., 1985). An optimum temperature of 30 ^{o}C was reported for cellulase production by *A. niger* YL128 (Ja'afaru and Fagade, 2010). Studies have shown that 30 °C is the optimal growth conditions for *T. reesei* and *A. niger* with higher temperatures altering cell wall composition and stimulating protein catabolism causing cellular death (Ikram-ul-Haq and Khan, 2006; Bansal *et al*.,

2012) However, some other studies have shown that temperature has little or no impact on cellulase production (Jecu, 2000).

During the optimisation of moisture and temperature it was observed that *T. reesei* produced more cellulase when compared with *A. niger* (Rajesh *et al*., 2012) reported optimum cellulase production at 28 ^{o}C utilising substrates such as rice bran and corn straw and *T. reesei*. Other reports have shown that temperatures in the range of 25-30 ^{o}C were optimal for enzyme production. (Singhania *et al*., 2006; Gautam *et al*., 2011). Some studies have reported maximum cellulase production at 30 ^{o}C (Mrudula and Murugammal, 2011). However, optimal temperature depends on the microorganism (Murai *et al*., 1998; Lu *et al*., 2003).

Inoculation size can also impact SSF performance. The decreased cellulase productivity as the inoculation size increased observed in this study could be explained by the high concentration of biomass depleting the nutrient pool and the available oxygen at the early stages of growth, which then affected cellulase formation (Bansal *et al*., 2012). Alternatively, this might be due to the creation of anaerobic conditions or a nutritional imbalance as a result of increased growth of the microorganisms (Kashyap *et al*., 2002; Kumar *et al*., 2008).

In contrast several other studies have shown that a lowering in cellulase production was correlated with lower inoculant. In these cases this may have been due to fewer conidial cells being present in the fermentation. However, the decreased in cellulase production even after inoculating the medium with higher spore concentrations might be due to the creation of anaerobic conditions or a nutritional imbalance as a result of growth of microorganisms (Haq *et al*., 1993; Iqbal *et al*., 2010).

Following optimisation of cellulase production for moisture etc in the current study it was observed that addition of mineral salt solutions, peptone or cellulose powder actually resulted in lower cellulase production when compared with controls. However, addition of minerals has been successfully used for enzyme

production from various fruit industry residues using different fungal strains (Pandey *et al*., 1999).

Pre-treatment or modification of substrate is a common practice to increase the accessibility of the substrate to microorganisms (Krishna, 2005; Koppram *et al*., 2014). In this work pre-treatment of MSW, with either acid or alkali resulted in reduced cellulase production in SSF. A similar negative impact of pre-treatment on cellulase production has been reported by several other researchers (Aiello *et al*., 1996; Brijwani and Vadlani, 2011; DoMyoung *et al*., 2014), other study showed an adverse effect on enzyme production in orange peel in an SSF process (Kannakar and Ray, 2010). In this study, results showed that more cellulase was obtained from untreated substrate than pre-treated substrates, suggesting that some unknown compounds involved in the induction of cellulase expression may be removed during pre-treatment and therefore inhibit cellulase induction (Kim *et al*., 2014). The optimal cellulase production in untreated MSW was high probably due to the presence of adequate nutrients, higher porosity and efficient aeration (Babu and Satyanarayana, 1995; Baysal *et al*., 2003)

In addition, The decrease of cellulase activity could be attributed to (1) structure changes to the lignocellulosic raw materials caused by the pre-treatment (Aiello *et al*., 1996; Kannakar and Ray, 2010); (2) the generation of inhibitory compounds formed during the pre-treatment (Aiello *et al*., 1996); (3) the removal of certain nutrients, such as nitrogen by the pre-treatment (Brijwani and Vadlani, 2011; DoMyoung *et al*., 2014) and (4) the release of toxic heavy metals that were originally in the non-soluble form in the MSW.

Furthermore, some other studies have reported low cellulase yields following alkaline pre-treatment with changes in lignin, hemicelluloses and modification of cellulose structure countering the positive effects of alkali pre-treatment, low cellulase production has also been shown after acid pre-treatment (Brijwani and Vadlani, 2011; Kim *et al*., 2014).

In contrast a previous study at Nottingham found that acid modification of wheat straw significantly enhanced cellulase production in SFF by *A. niger* (Pensupa *et al*., 2013), Pre-treatment changes the chemical and physical structure of biomass, and may improve fungal access during the SSF process (Sun and Cheng 2002; Dwivedi *et al*., 2009)

Pre-treatment of cellulosic biomass has been observed to enhance cellulase production the reason being most likely the removal of lignin from the lignocellulosic substrates (Milala *et al*., 2009; Youssef and Berekaa, 2009).

Time of incubation is a prime commercial concern for the production of cellulase enzymes. Generally, the optimum incubation time depends on the type of medium, growth rate of microorganisms on the particular carbon source and enzyme production pattern (Kuhad and Singh, 1993). Extending culture time from 5 days to 7 days increased cellulase yield in SSF using MSW. However, increasing incubation time is not always commercially viable and would be costly. Various studies have shown that maximum cellulase production from *T. reesei* could be achieved within 72-96 hr, the optimal was at 72 hr using four different agro industrial residues- cassava bagasse, sugar can bagasse, wheat barn and rice straw (Singhania *et al*., 2006), also the maximum cellulase produced using apple pomace (2.3 FPU/g) was at 120 hr (Sun *et al*., 2010). The elongated cellulase production period maybe due to the characteristics of this non homogenous nature of the MSW substrate, fungal cells may thus require longer time to both grow and express cellulase enzymes.

In this study, higher enzyme production was observed after 168 hr for *T. reesei* when compared with 120 hr, increased cellulase production over longer incubation periods maybe due to the composition of the MSW substrate which contained various carbon sources and therefore required longer exposure for enzyme production compared with a with homogenous substrate. Results here disagree with some studies which have recorded a maximum enzyme production after 96 hr, with enzyme productivity declining gradually afterwards probably due

to the release of proteases and a drop in pH of the medium (Bansal *et al*., 2012). Studies using *T. reesei* have shown that yields of cellulase remained constant over 72-96 hr, with maximum production observed after 96 hr (Singhania *et al*., 2006). Also, by using apple pomace as a substrate and *T. reesei* showed an increase in enzyme production over an incubation period of 0-120 hr (Sun *et al*., 2010). With regard to cost of cellulase production, short incubation periods for enzyme production offer the potential for inexpensive production of enzyme (Muniswaran and Charyulu, 1994; Silva *et al*., 1995). Generally, there is a direct relationship between the production of enzyme and metabolic activity within the incubation period; *A. niger* and *T. reesei* are active cellulolytic species within a 4 to 6 day incubation period, which is commercially suitable (Kang *et al*., 2004; Gautam *et al*., 2011). After this incubation period production declines possibly due to the depletion of macro and micronutrients in the fermentation medium resulting in the inactivation of the secretary machinery of the enzymes (Nochur *et al*., 1993). Also, maximum cellulase yield has been observed after five days of incubation by (Kang *et al*., 2004; Grover *et al*., 2013).

Transcriptome analysis

Preliminary analysis of the RNAseq data would suggest that there is a much greater degree of variation between days 3 and 5 of SSF as compared to between days 5 and 7. This may reflect the *Trichoderma* becoming "acclimatised" to the complex MSW substrate. It would be anticipated that over the first few days of culture the fungus would be exploiting a range of potential substrates including the starch, protein and possibly the hemicelluloses since these are more readily available than the cellulose. After five days these nutrients may have become exhausted and the fungus has established the biochemical machinery to exist on the more abundant cellulose and hemicellulose fractions.

More detailed analysis identified the expression of genes for a large number of carbohydrate active enzymes. Expression of amylase (GH13) and glucoamylase (GH15) genes appears to be an early event and levels are consistent across the SSF. This might be as expected as starch was identified as a component of the MSW and is the most readily digestible substrate. Expression of cellulytic enzymes- GH6. GH7 and possible GH12 seemed to peak at day 5. This might be expected as cellulose is going to be a substrate that is used towards the end of the fermentation. Those genes associated with hemicellulose digestion (GH11 and 74) were also highly expressed at day 5 again indicating that hemicellulose may be acting as a secondary substrate. GH61 has been implicated in cell wall breakdown and is thought to code for lytic polysaccharide monoxygenase enzymes (LPMO)(Levasseur *et al*., 2013). Several genes (GH2; 47; 76 and 92) were identified that are involved with metabolism of mannans, especially, on day 3 and 5. This suggests a source of mannose in the MSW but this was not identified in the compositional analysis is chapter 3. The chitinase (GH18) and N.O diacylmuramidase (GH25) enzymes may be involved in turnover of fungal cell walls. Of the other glucanosyl transferases GH16 and 72 were also highly represented although how this may be related to the MSW composition is unclear. More detailed analysis of the RNAseq data was not possible as this was provided after the PhD programme had been completed. However, examples of how this may be exploited are to identify other potentially important enzymes such as lipases and proteases that could be generated using the MSW substrate.

Chapter 6 Bioleaching Municipal Solid Wastes by Filamentous Fungi and Yeasts

6.1 Introduction

To avoid disposal of MSW through landfill it can be used as a compost or fertiliser in order to improve the physical, chemical and biological properties of impoverished soils (Baldantoni *et al*., 2010). Compost containing municipal bio solids, and other organic wastes has been shown to help improve the physical and chemical properties of soil (Barzegar *et al*., 2002; Mkhabela and Warman, 2005; Šimon *et al*., 2013); and applying compost derived from MSW has been correlated with a positive effect on soil quality. Composting is one of several methods for treating bio solids to create a marketable end product that is easy to handle, store and use (Elherradi *et al*., 2005; Silva *et al*., 2007).

Compost production can be carried out under either aerobic or anaerobic conditions, and recently an anaerobic pre-treatment of MSW followed by an aerobic curing step has been utilized, (Vogt *et al*., 2002; Lopes *et al*., 2004). Compost returns large quantities of organic matter and nutrients such as phosphorus, potassium, nitrogen, and organic carbon back into the soil, improving soil texture, and elevating the soil's cation exchange capacity (an indication of the soil's ability to hold nutrients). Furthermore, compost can also be used in various land applications such as wetland and mined land restoration (Peot, 1998); however use of sewage sludge, mineral fertiliser and, MSW compost are major sources of metal input in agriculture (Smith, 2009; Lakhdar *et al*., 2010).

Soil contamination by the presence of heavy metals is thus an issue which can affect plant viability (Phil-Eze, 2010), and this could arise from the use of MSW as the main component of a compost (Muir and Howard, 2006; Weber *et al*., 2007; Carbonell *et al*., 2009). Presence of contaminants like Cd^{2+}, Ni^{2+}, Le+, Zn^{2+} and

Co^{2+} can alter soil chemistry and have an impact on the viability of organisms and plants which are dependent on the soil (Shayler *et al*., 2009).

There are many methods for the removal of metals from MSW or soil such as hydrothermal (Guerrero *et al*., 2000; Zhang and Itoh, 2006), subcritical water treatment (Zhang and Itoh, 2006), chemical process using inorganic mineral acids such as sulphuric acid, hydrochloric acid, and nitric acid (Nagib and Inoue, 2000), or chelating reagents like nitrilotriacetic acid (NTA) (Guerrero *et al*., 2000; Hong *et al*., 2000), ethylene diamine tetra acetate (EDTA), and Diethylene Triamine Penta acetate (DTPA) (Hong *et al*., 2000), ammonium or sodium hydroxides (Wang *et al*., 2006) and fungal bioleaching (Bosshard *et al*., 1996). However, they all have disadvantage such as high cost, and related toxicity of potential bio-products (Wu and Ting, 2006).

Heavy metal removal by bio-sorption (microbial leaching) has been extensively investigated (Mudhoo *et al*., 2012). Biohydrometallurgical (bioremediation) is considered a green technology, with low cost, low energy requirement and the process can sequester dissolved metal ions out of dilute complex solutions quickly and with high efficiency (Wu and Ting, 2006). There are three principal groups of microorganisms which have been used for bioleaching these are autotrophic bacteria (e.g. *Thiobacillispp*.), heterotrophic bacteria (e.g. *Pseudomonas spp., Bacillus spp*.) and heterotrophic fungi (e.g. *Aspergillus spp., Penicillium spp*.). The most important commercial yeast is *S. cerevisiae* (Da Silva *et al*., 2005) which is currently used for many industrial applications (e.g. bioethanol) and as a bio-absorbent for the removal of heavy metals. However, other yeast such as *Candida* spp or *Pichia* spp (*P. guilliermondii*), have been recognised as potentially efficient bio-sorbants (Wang and Chen, 2009). Yeasts have some advantages over filamentous fungi, and have been shown to have unique characteristics when compared with other microorganisms in regards to removal of heavy metals from a substrate (Wang and Chen, 2006). Currently, very little is known about the bio-sorption of heavy metals from autoclaved MSW using either filamentous fungi or

yeast. Advantages of using *S. cerevisiae* as a bio-sorbent in metal, bio-sorption are that the cells are easy to cultivate and can be obtained from various food and beverage industries, yield of biomass is high, they are considered a safe organism, also bio-sorbent made from *S. cerevisiae* has been readily accepted by the public when applied and *S. cerevisiae*, is an ideal model organism to identify the mechanism of bio-sorption for metal ion removal. Compared to bacterial and fungal leaching has the following advantages: (i) ability to grow at higher pH (higher than 5.5), thus making yeast a more suitable microbe for the bioleaching of solid wastes with an alkaline pH; (ii) generally faster leaching process rates characterised by a shorter lag phase; and (iii) ability of excreted metabolites (e.g. organic acids) to form complexes with metal ion, thus reducing its toxicity to the biomass (Burgstaller and Schinner, 1993; Castro *et al*., 2000). However, the operating cost is relatively high for fungal leaching (by hetrotrophs) when compared with bacterial leaching (by autotrophs) due to a requirement for an organic carbon source for growth and organic acid excretion (Wu and Ting, 2006). Acidolysis is the principal mechanism in bioleaching when using fungi, with organic acids such as citric, oxalic and gluconic acids produced during bioleaching (Bosshard *et al*., 1996; Lopes *et al*., 2004). The most important species of fungi are *A. niger* and *Penicillium simplicissimum* for their ability to produce organic acids (Ren *et al*., 2009). Currently research on bio-sorption is focused on the following topics; (i) bio-sorbents; and (ii) mechanism of bio-sorption (Kratochvil and Volesky, 1998).

This study focused on the capacity for filamentous fungi and yeast to act as bio-sorbents for autoclaved MSW, correlating removal of heavy metals from MSW with design parameters such as pH, various media (artificial, clay and hydrolysates derived from an acid pre-treatment of Sorghum medium) and bio-sorption. The levels of heavy metals in the solid and liquid phase were determined after bioleaching and weight loss after bioleaching was also determined. Microbial bioleaching was also compared with the use of chemicals

for bioleaching including various weak and strong acids. The resultant metal levels in the MSW biomass were compared with standards set for safe limits in compost in the UK.

6.2 Materials and Methods

6.2.1 Microorganisms

Aspergillus niger N402 and *T. reesei* was kindly donated by Professor David Archer (University of Nottingham, UK). *A. terreus* was obtained from the DSMZ culture collection (Germany) and was cultured according to the procedure in (Bosshard, Bachofen *et al*., 1996).

Yeast strains -*Saccharomyces cerevisiae* (NCYC2592) *Kluyveromyces marxianus, Schefforsomyces stipitis, Candida succiphilia* (NCYC1403)*, Pichia gulliermondii* (NCYC 443)*,* and *Candida shehatae* (NCYC2389) were obtained from the National Collection of Yeast Cultures (www.ncyc.co.uk). Culturing conditions were described in a section 4.2.1.

All flasks were incubated at 30 °C with mixing at 120 rpm (Wu and Ting, 2006). Six-days-old conidia were harvested from potato dextrose agar surface and number of spores counted using a haemocytometer and standardized to 1×10^7 spores/mL of spore suspension. One mL of spore suspension was added to 100 mL medium, of 10 % MSW for fungi bioleaching and 2 % MSW for yeast experiment.

6.2.2 Artificial medium used to establish the tolerance to metals in yeast

Stock solutions for each metal individually ranging between 0.1-4 mM, for Mn(II) ($MnSO_4$), Fe(II) ($FeSO_4$), Pb(II) ($Pb(NO_3)_2$), Zn ($ZnSO_4$), Al $Al_2(SO_4)_3$, and Cu^{2+} (II) , $CuSO_4$ were prepared by dissolving in RO water. Chemicals were provided at analytical grade by Sigma-Aldrich. A working metal solution of 0.1-3 mM concentration was prepared from a 4 mM stock solution by serial dilution for each metal individually. The pH was maintained at 5.5 by adding 3 M NaOH. The solution used for phenotypic microarray assays was as described in section 4.2.4.1).

6.2.3 Media used for bioleaching

6.2.3.1 Artificial medium

Fungal artificial medium: The constituent of this artificial medium were based on the study by Wu and Ting (2006). Medium was prepared containng g/L at two different pH (5.5 and 2.5): glucose (60), $NaNO_3$ (1.5), KH_2PO_4 (0.5), $MgSO_4 \cdot 7H_2O$ (0.025), KCl (0.025), yeast extract (1.6), autoclaved at 121 ^oC for 15 min, the medium was used immediatly.

Yeast Artificial medium – the yeast medium included two artificial medium the first medium called **Medium1** contained (g/L): 120 glucose, 10 yeast extract, 20 peptone.

The second medium called **Medium** 2 was based on the study by Damodaran, Suresh *et al*. (2011) the medium contained (g/L) prepared at two different pH (5.5 and 4): 20 glucose, KH_2PO_4, 2.72; K_2HPO_4, 5.22; $(NH_4)_2SO_4$, 2.0; $MgSO_4$ $7H_2O$, 0.5; $FeSO_4 \cdot 7H_2O$, 0.0022; $ZnSO_4 \cdot 7H_2O$, 0.004, $MnSO_4$ $4H_2O$, 0.004; $CuSO_4 \cdot 5H_2O$, 0.004.

6.2.3.2 Other Media

A 100 % pure clay called Terracotta Clay supplied by http://www.piscesart.co.uk/shops, the clay sample was dried in an oven at 70 ^oC. 10 g of clay was mixed with 100 mL DW and the pH adjusted according to the experimental requirment, after the pH was adjusted the solution was sterilised by autoclaving (121 ^oC and for 15 min), the clay was mixed well before transfering into the flasks.

Sorghum bran medium: sorghum was added to 1 % H_2SO_4 to give a 20 % w/v solid loading and autoclaved at 121 ^oC for 30 min. The medium was adjusted to the required pH before being sterilised by vacuum filter sterilisation (0.45 μM), the resultant sample contained 56 g/L glucose, pH for all medium was adjusted to 5.5 and 2.5 for fungi and 5.5 and 4 for yeast.

6.2.4 Chemical leaching

Chemical leaching of the heavy metals was carried out using organic acids (oxalic and gluconic) and inorganic acids (H_2SO_4) at concentrations of 0.5 M. Experiments were performed under the same conditions as for bioleaching i.e., 30 ^oC and 120 rpm and 10 % w/v MSW. The organic and non-organic acids were selected according to the literature (Wu and Ting, 2006). Samples were collected after 7 and 15 days (Yang *et al*., 2008) and separated into the solid and liquid fractions.

After 15 days, samples were centrifuged at 4472 g, for 20 min, the liquid fraction was decanted and retained, whilst the solid fraction was washed three times using RO water and dried at 70 ^oC, weight loss (%) in the solid fraction was determined and presence of heavy metals in both fractions determined.

The samples were filtered through 0.45 nm member filters before the following analysis (1) organic acids (only fungi) using ICP-MS, (2) heavy metals (28 heavy metals) were selected in this study Boron (B^{3+}) , Sodium (Na^{2+}), Magnesium (Mg^{2+}), phosphorus (P^{2+}), Sulphure (S^{2-}), Potassium (K^{+}), Calcium (Ca^{2+}), Thalium (Ti^{3+}), Aluminum (Al^{3+}), Vanadmium (V^{2+}), Chromium (Cr^{6+}), Manganese (Mn^{2+}) , Iron (Fe^{2+}), Cobalt (Co^{2+}), Nickel (Ni^{2+}), Copper (Cu^{2+}), Zinc (Zn^{2+}), Arsenic (As^{3-}), Selenium (Se^{2-}), Rubidium (Rb^{+}), Strontium (Sr^{2+}),Molybdium (Mo^{3+}), Silver (Ag^{+}), Cadmium (Cd^{2+}), Cesium (Cs^{+}), Barium (Ba^{2+}), Lead (Pb^{2+}), and Uranium (U^{4+}), finally the yield was determined in the solid and liquid fractions (Refer to section 3.2.4.6).

The metal extraction efficiency was calculated as the % recovery in the MSW:

1) Starting value was calculated mg/kg
2) Treatment –solid residue for analysis mg/kg but the recovery of solid residue is Net 100 %
3)

(A) Calculate Solid residue retention (%): MSW 1 kg of MSW has X amount of Fe^{2+} for example; Treat 10 g of MSW has :

$$\text{Formula (A)mg} = \frac{\text{Fe}\left(\frac{\text{mg}}{\text{kg}}\right) * 10}{1000}$$

Recover Yg of solid residue (dry weight) which has Z mg/kg of Fe, so total in residue is :

$$\text{Formula (B)mg} = \frac{\text{Z} * \text{Y}}{1000}$$

Retained in residue is:

$$\text{Formula (C) \%} = \frac{\text{B}}{\text{A}} * 100$$

(B) 10 g MSW so A is same

100 ml collected and then this T mg/L

So total in 100 mL is:

$$\text{Formula (D)mg} = \frac{\text{T} * 100}{1000}$$

$$100\ \%\ \text{Solubilities} = \frac{D}{A} * 100\ \%$$

(C) Mass balance is

$$\text{Mass balance (100 \%)} = \frac{\text{B}}{\text{A}} * 100\% + \frac{\text{D}}{\text{A}} * 100\ \%$$

$$Or\ \text{B} + \text{D} = \text{A}$$

6.2.5 Bioleaching process

For the first experiment using fungi and yeast bioleaching was carried out using 250 mL Erlenmeyer flasks with 10 % w/v autoclaved MSW in 100 mL of medium.. Two bioleaching processes were performed: (i) One-step process: the MSW was incubated with the organism for 15 days; (ii) Two-step process: this was as decribed by Wu and Ting (2006), the organism was pre-cultured for 7 days then the MSW was added and the experiment continued for another 7 days (total experiment time was 15 days) .

All process carried out with the control MSW with DW only (No Microorganisms), Also a test applied MSW with microrganisms (No medium) to see if any medium is required or whether MSW alone can support fungal growth.

All culture media were autoclaved at 121 °C for 20 min prior to inoculation and all experiments were carried out in triplicate. Samples (1 mL) was taken daily using disposable sterilised pipettes and, volume was made up by sterile deionised water if necessary to keep the moisture level stable in the medium. Then after 15 days of experiment the solid and liquid fraction separated as described in section 6.2.4.

6.2.6 Analytical methods

Heavy metals – the dried solid fraction was digested using concentrated nitric acid the liquid fraction taken forward to measure heavy metals released. Heavy metals analysis was as described in section 3.2.4.6.

Organic acids (analysed as citrate, oxalate, gluconate) were determined by HPLC using a Jasco AS-2055 Intelligent auto sampler (Jasco, Tokyo, Japan) and a Jasco PU-1580 Intelligent pump (Jasco) and RI detectors, using Aminex HPX-87H column with a cation H guard column (Biorad), mobile phase of 0.0085 M H_2SO_4, and flow rate of 0.4 mL/min at ambient temperature.

Data analysis PCA (Principal component analysis) and design expert were used for the statistical analysis of the data. The amount of heavy metals using various conditions such as organisms, pH and media were analysed by the PCA. While,

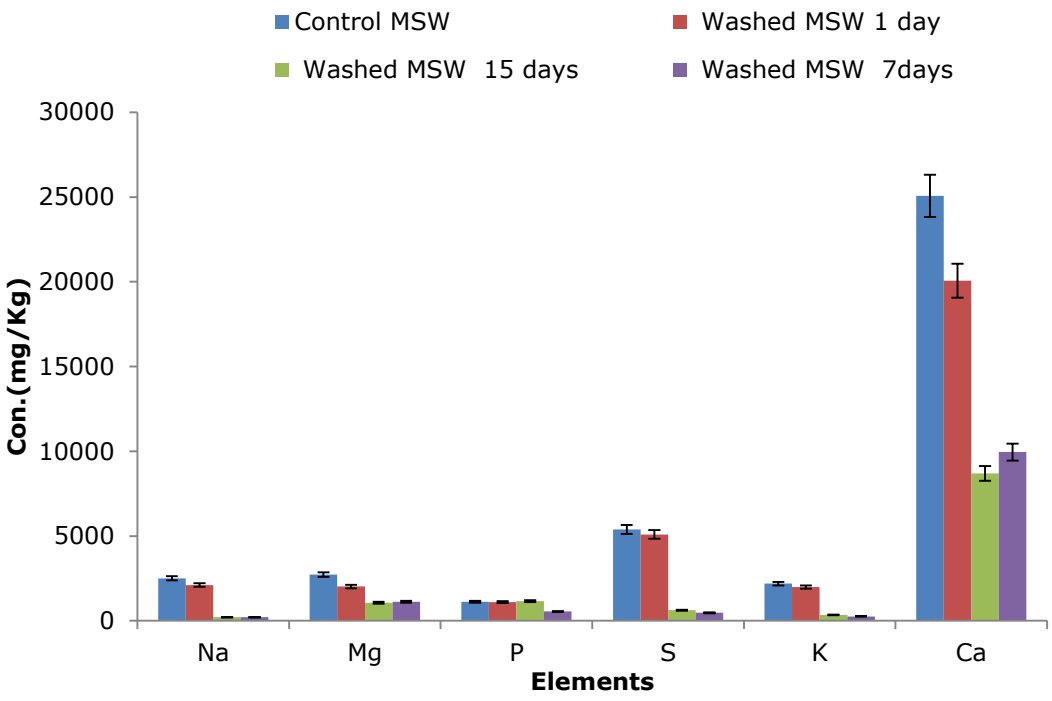
the analysis of variance (ANOVA) was used to summarise the calculation for both fungi and yeast using the design expert program supplied by University of Nottingham.

6.3 Results

6.3.1 Heavy metal content of MSW

The elemental composition of MSW was determined previously and is shown in Tables 3.4 and 3.5.

The first experiment carried out looked at the efficiency of simply washing the MSW with RO water, shaking and incubation for 1, 7 and 15 days, the results shown that washing decreased the metal concentrations in the solid residue especially comparing control (non-washed) with MSW washed for 15 days as shown in Figure (6.1). However, levels of some heavy meals, in particular Al, Fe, Cu^{2+} and Zn^{2+} were still very high even after 15 days. The washing has also reduced beneficial elements such as S^{2-} and Cu^{2+}. Thus further experiments were carried out in an attempt to improve the leaching of these metals.



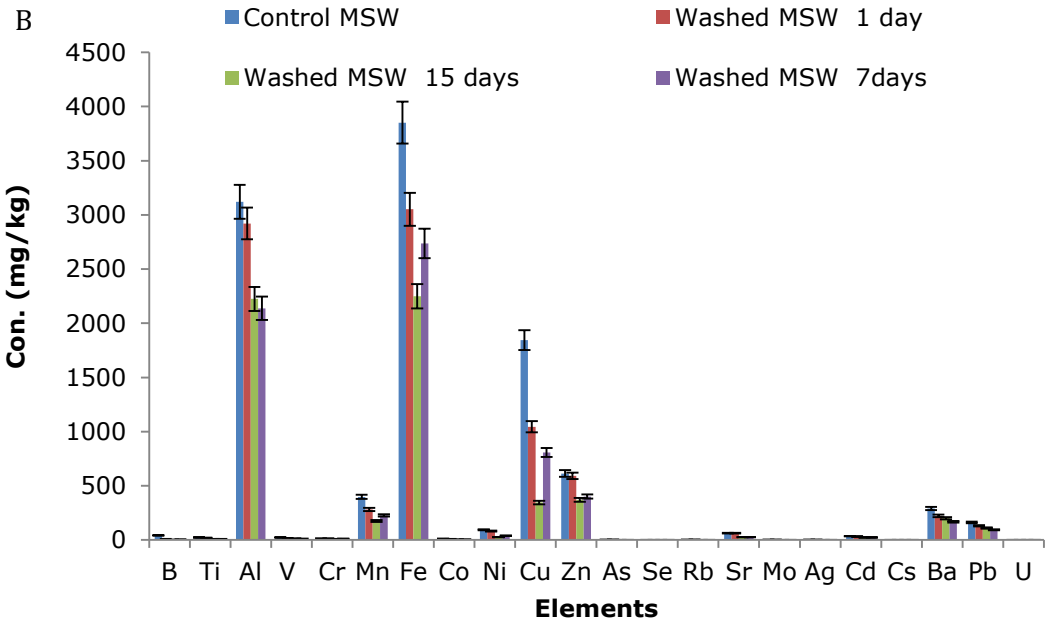


Figure 6.1 Effect of washing with RO water on the concentration of elements in MSW. MSW was incubated with water for 1 and 7 and 15 days, the solid residue was collected, dried and elemental composition determined. This was compared to the control (unwashed MSW) (A) Major elements; (B) Minor elements

6.3.2 Leaching metals from MSW using various media and conditions

6.3.2.1 Chemical leaching of MSW using organic and non-organic acids

To compare the effect of chemical leaching of MSW, a study was carried out using sulphuric, oxalic and gluconic acids to leach the elements including heavy metals from MSW. The MSW was incubated with the organic acid for 7 or 15 days and the elemental composition of the dried residue determined. This was compared to control incubation in water. The data has been divided into two parts 7 days (Figure 6.2 A) and 15 days (Figure 6.2 B). For ease of comparison the amount of each element remaining in the solid residue has been expressed as % of that in the original unwashed MSW. Overall for 7 and 15 the experiment H_2SO_4 appears to have given the best performance (lowest retained yield % in a solid residue). A statistical analysis of these data was carried out for the top 10 highest elements on both days 7 and 15 require focusing on them to decrease their concentration in a solid residue (Table 6.1). The results revealed that gluconic acid showed highest % yield when compared with the other organic acids in terms of leaching elements from the solid fraction of MSW at 15 days. However, they also suggest that using any of these acids did not show a big difference in terms of bioleaching compared with the control (using only RO water with MSW). There was no significant difference between 7 and 15 day values for all metals. Whilst there was a significant different between acids except for leaching of Se^{2-} and Cs^{+}. Some metals are still being retained at high levels, further experiment were thus carried out to improve leaching from the solid fraction.

It was noticed from the above data that the different acids tended to show a differential pattern in terms of which elements were leached. Thus a PCA analysis was carried out to further investigate this observation. PCA assays looking at the leached yield (%) into the liquid fraction revealed that there was a correlation between the use of gluconic acid (7 and 15 days) and leaching of Ca^{2+}, Ba^{2+}, Mo^{3+}, Se^{2-}, Ag^{+} and Na^{+} metals. While, use of sulphuric acid correlated with the leaching of elements such as Pb^{2+}, Ti^{3+}, Zn^{2+}, Mn^{2+}, Cd^{2+}, Ni^{2+}, Mg^{2+}, Co^{2+}, Al^{3+}, V^{2+}, Cr^{6+}, U^{4+}, Cu^{2+}, and S^{2-} from the MSW. Assays revealed that the highest leachate yield (best condition) into the liquid fraction was obtained using Oxalic acid and H_2SO_4 (7 and 15 days) for elements such as P^{3-}, B^{3+}, Rb^{+}, Cs^{+}, $K^{+,}$ Cr^{6+}, V^{2+}, Mg^{2+}, Cu^{2+}, Co^{2+} and Fe^{2+} and results showed that addition of oxalic acid released more elements from MSW into the liquid fraction compared with the other acids (Figure 6.3).

PCA was also used to look at retention (%) of elements including heavy metals in the solid fraction of MSW following addition of organic or inorganic acid (Figure 6.4). This revealed that the lowest metal yield % (high performance) was obtained with sulphuric acid (7 and 15 days), this data correlated with only leaching of Mo^{3+} and S^{2-}. However, use of oxalic acid (7 days) or RO water (7 and 15 days) correlated with leaching of Ca^{2+}, Ti^{3+}, Pb^{2+}, Sr^{2+}, Ni^{2+}, Cd^{2+}, Ba^{2+}, Zn^{2+}, U^{4+}, Co^{2+}, Mn^{2+} , and Ag^{+} from the solid fraction. An assessment of leaching revealed that the highest leaching defined as % yield followed use of Ro water (15 and 7 days), Gluconic acid (7 and 15 days) and correlated with leaching of Na^{+}, As^{-3}, Rb^{+}, Cr^{6+}, K^{+}, Cu^{2+}, Se^{2-}, B^{2+}, Cs^{+}, Mg^{2+}, Al^{3+}, V^{2+}, Fe^{2+}, P^{3-} and Rb^{+} .

Mass balances calculation;. The liquid phase was collected and the amount of metals leached (mg/kg) was determined. The raw data is presented as an appendix on the enclosed CD. This amount of metal was added to that determined in the residual solid (mg/kg). This was then compared to the initial amount of metal in the MSW to give a mass balance. The original data for both fungi and yeasts mass balances are attached as an appendix (Data present on a compact disc). This mass balance was not always 100 %. There was a big various in terms of both part yield %.

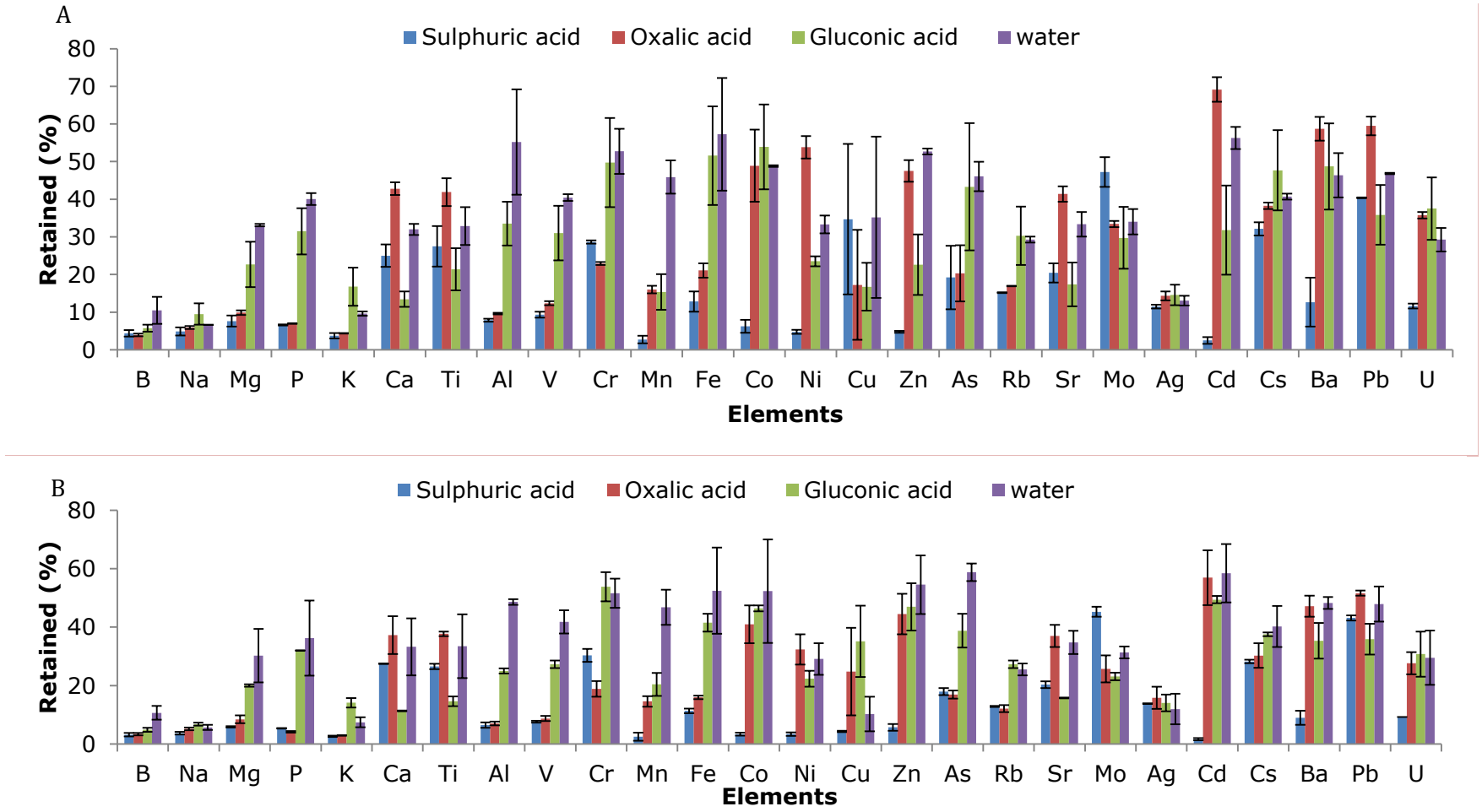


Figure 6.2 Effect of incubating MSW in either water or acids on elemental composition of MSW.

MSW was incubated with water or acids for (A) 7 days or (B) 15 days. The solid residue was dried and elemental composition determined. Results are expressed as % remaining compared to original MSW.

Table 6.1 ANOVA table for the effect of three organic and inorganic chemicals on amount retained in the solid fraction at two time points

	Treatments mean solid yield value %									
	7 days				15 days				P= value	
Metals	**Sulphuric acid**	**Oxalic acid**	**Gluconic acid**	**Water wash**	**Sulphuric acid**	**Oxalic acid**	**Gluconic acid**	**Water wash**	**A factor (Acids)**	**B factor (time)**
Se^{2-}	146.1	148.7	160.1	134.5	120	106.4	120.6	103.1	*	0.0002
Pb^{2+}	40.4	59.5	35.9	46.8	43.1	51.6	35.9	47.9	0.015	*
Cd^{2+}	2.5	69.1	31.8	56.2	1.6	56.9	49.4	58.4	0.001	*
Cr^{6+}	28.7	22.9	49.8	52.7	30.3	18.8	53.9	51.6	0.005	*
Ba^{2+}	12.7	58.7	48.7	46.3	9	47.1	35.3	48.3	0.001	*
Co^{2+}	6.3	48.9	53.9	48.8	3.3	41	46.4	52.3	0.002	*
Cs^{+}	32.1	38.3	47.7	40.6	28.2	30.2	37.6	40.2	*	*
Zn^{2+}	4.8	47.5	22.6	52.7	5.7	44.5	46.9	54.5	0.001	*
S^{2-}	114.3	7.4	9	7.05	113.7	6.1	6.6	6.6	0.001	*
Mo^{3+}	47.2	33.4	29.7	34.03	45.2	25.7	23.1	31.3	0.006	*
Average	43.5	53.5	48.9	51.9	40	42.8	45.6	49.4		

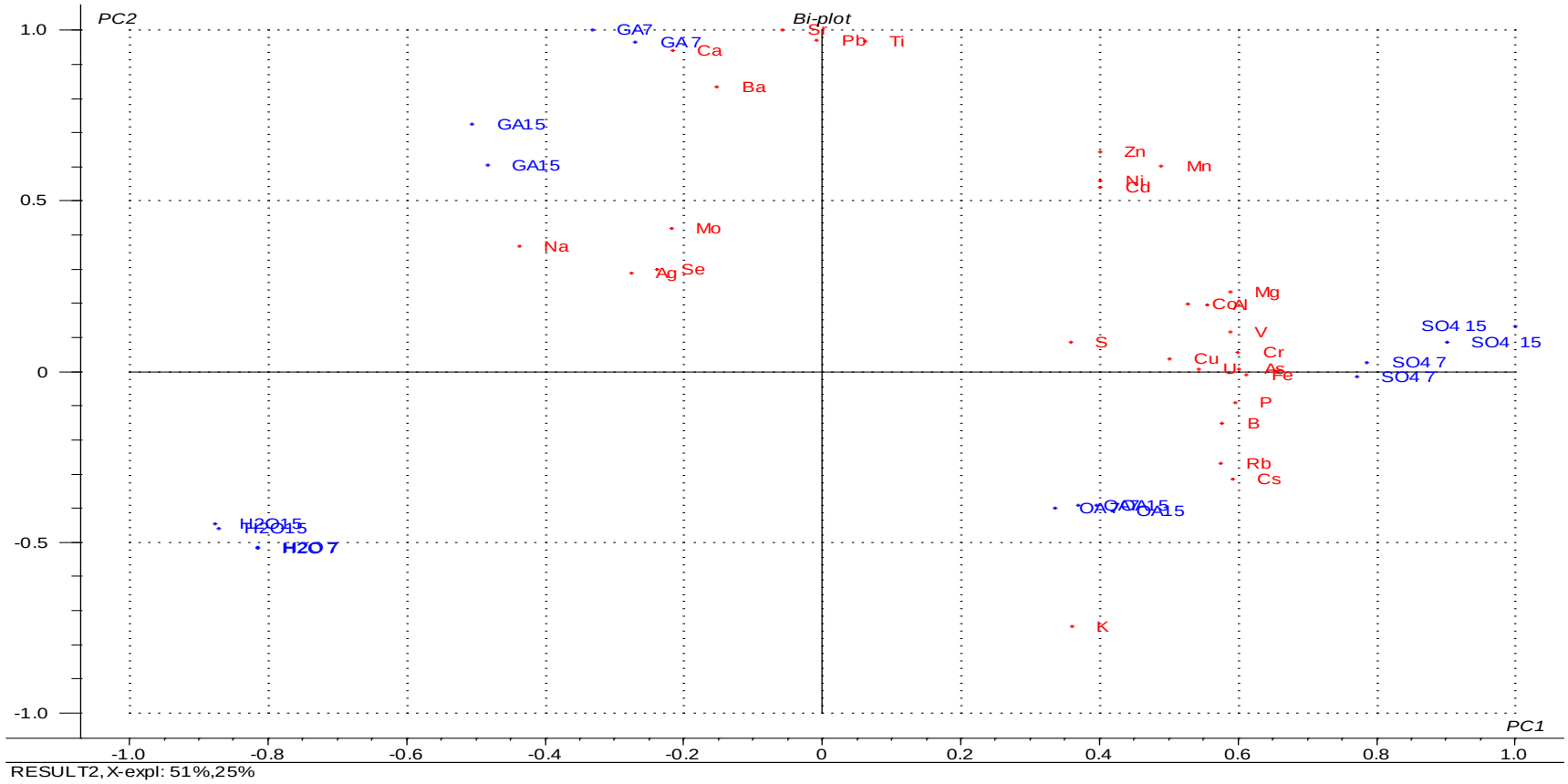


Figure 6.3 PCA analyses of the amount leached elements into the liquid part following chemical leaching using organic and inorganic acids for 15 days

(SO_4 7=H_2SO_4 7 days leaching; SO_4 15=H_2SO_4 15 days leaching; OA 7 =Oxalic acid 7 days; OA 15 =Oxalic acid 15 days; GA 7= Gluconic acid 7 days; GA 15= Gluconic acid 15 days; H_2O 7= sterilised Ro water 7 days; H_2O 15= sterilised RO water 15 days).

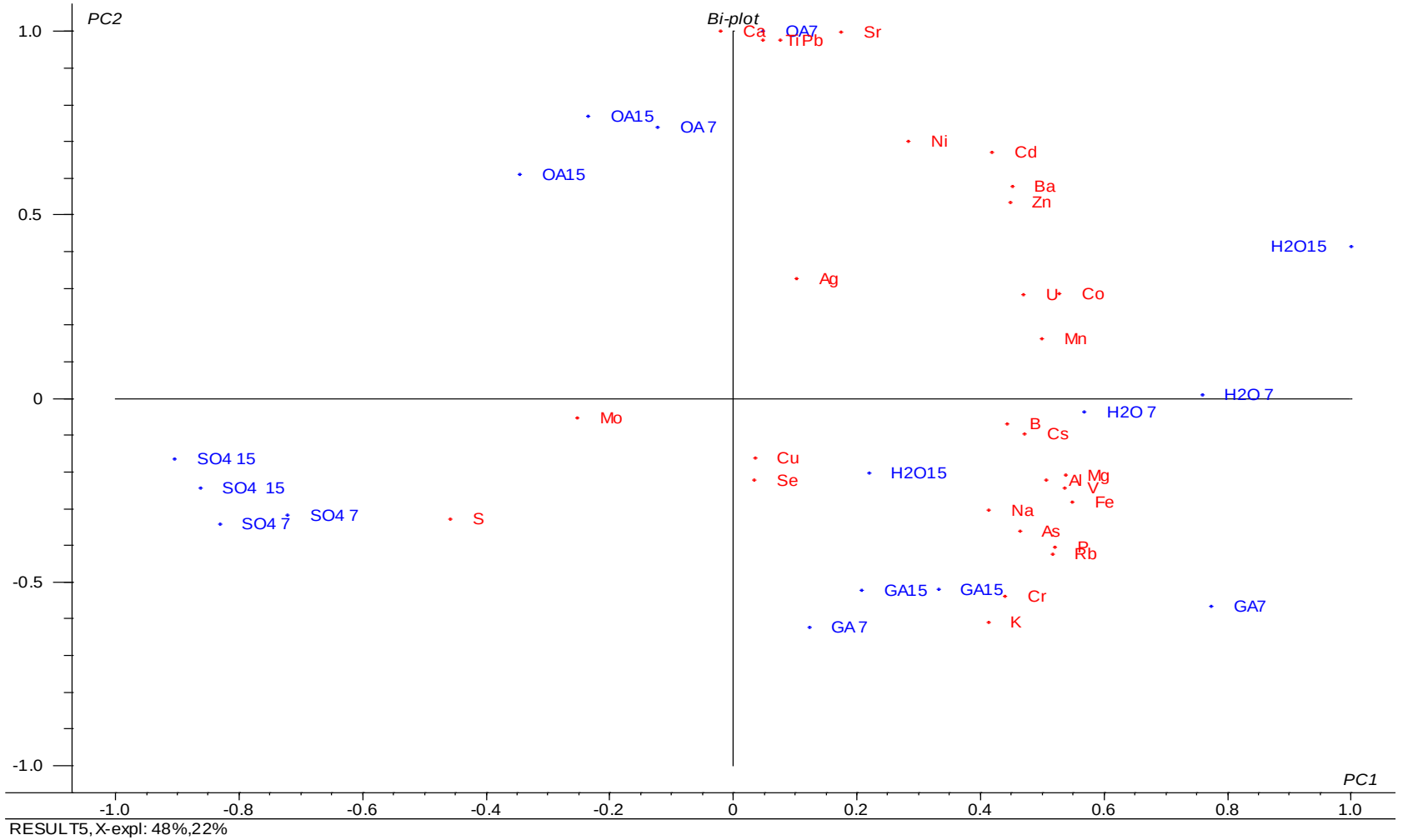


Figure 6.4 PCA analyses for the amount of retained elements in the solid fraction following chemical leaching using organic and inorganic acids at 15 days experiments time duration

6.3.2.2 Comparison of fungal strains for the bioleaching of metals from MSW using one and two-way methods

The next set of experiments explored the application of bioleaching for the improvement of the elemental composition of MSW. For this experiment three strains of fungi (*A. niger*, *T. reesei* and *A. terrus*) were used. Spores of fungi were inoculated into medium whose pH had been adjusted to pH 5.5. Bioleaching was assessed using either a one step method (mixing MSW with the medium and strain at the same time) or a two step method (mixing medium with strain for 7 days then transfering to the autoclaved MSW). Following 15 days incubation the liquid and solid phases were collected and elemental analysis carried out on each. The raw data sets are again provided as an appendix on the enclosed CD.

Controls were prepared as mentioned in section 6.2.5, also a test mixing MSW with microorganism in the absent of nutrients or medium, the aim being to see if these organisms can grow without extra glucose in the medium. Results are again presemnted as % of original amount retained in the solod residue.

Comparison of the retained (%) of metals within the MSW solid residue revealed that the one-way method was the best and that *A. niger* was the most efficient microorganism in terms of leaching ability (Figure 6.5).

Data from the bioleaching experiments for the ten metals with the highest retained yield % using three fungal strains was analysed using Design Expert and ANOVA to calculate the relationship between factors (fungi) for each metal (Se was excluded due to instrumentation error). Generally, the order of bioleaching was P^{3-}<Ba^{2+}< Rb^{+}<Cd^{2+}< Fe^{2+}< Cs^{+}<Al^{3+}< Co^{2+} <As^{3}, however, in general the retained %) was still not very low comparing with the RO control, further optimisation was required (Table 6.2). For the remaining sections a similar analytical approach was taken.

To assist with the analysis the results were again compared using PCA. Analysis of the metals present in the liquid fraction revealed that assays using only RO water shows there was no clear clustering of the metals, which might indicate differential leaching efficiencies. Addition of *T. reesei* and *A. terreus* (using both methods) correlated with increased leaching of Cs^{2+} , B^{2+}, S^{2-}, Na^{+}, K^{+}, Rb^{+}, Ca^{2+}, Mg^{2+}, Co^{2+}, Mn^{2+}, Sr^{2+}, As^{3-}, and Ti^{3+} from MSW; while, addition of *A. niger* (one way method only) was highly correlated with leaching of Se^{2-}, Zn^{2+}, P^{3-}, Ni^{2+}, Cr^{6+}, B^{3+}, Cd^{2+}, Pb^{2+}, Mo^{3+}, U^{4+}, and Ba^{2+} when compared with the other experiments. Analysis revealed that in terms of the liquid fraction the highest overall level of metal leaching from MSW was following incubation with *A. niger* using the one way method (Figure 6.6).

A similar analysis was carried out on the retained % in the solid fraction. PCA analysis of this data (Figure 6.7) revealed that addition of *A. niger* and *T. reesei* (using both one and two way methods) showed improvement in a retained yield % of Se and Cu and that addition of *A. terreus* correlated with the lowest yield % for retained metals. However, addition of *A. terreus* (using one and two way methods) correlated with improved in a retained yield % of Na^{+}, K^{+}, P^{3-}, S^{2-}, As^{3-}, B^{3+}, Ag^{+}, Rb^{+}, Mg^{2+}, Pb^{2+}, and Fe^{2+} elements from MSW.

Comparing retained % yield of metals from the solid phase under control conditions with those using the three fungi revealed that the three fungi were more successful at removing U^{4+}, Cs^{+}, Ba^{2+}, Cd^{2+}, V^{2+}, Ti^{3+}, Cr^{6+}, Co^{2+}, Zn^{2+}, Ca^{2+}, Mn^{2+}, Al^{3+}, and Ni^{2+} from MSW.

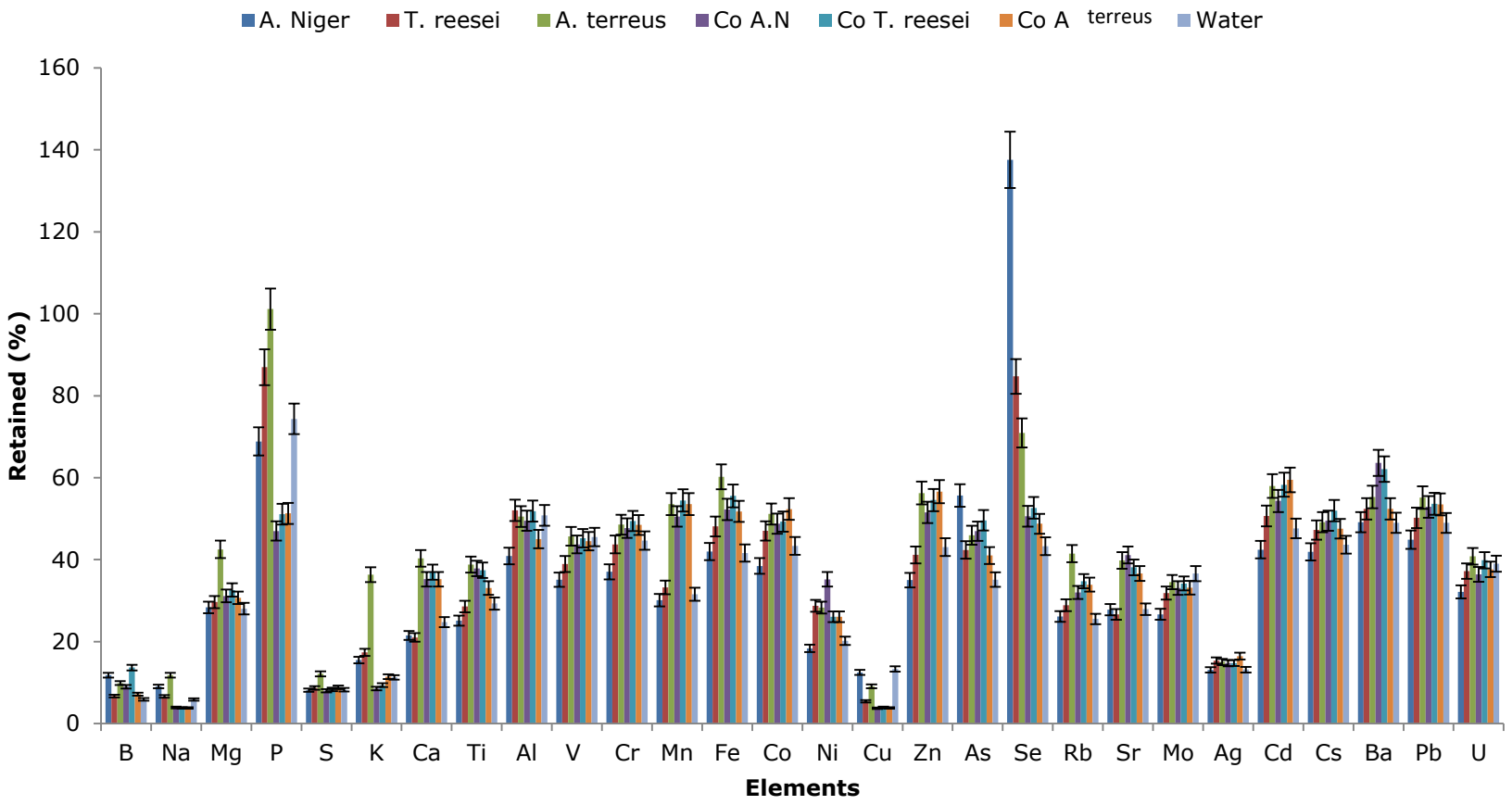


Figure 6.5 Effect of bioleaching on MSW elemental composition using three fungal strains

Retained yield (%) of elements in a solid residue of MSW using one-way method and three fungal strains in a present of artificial medium at pH 5.5 at 15 days' time point

Co = control (no nutrient addition to the medium, only MSW and microorganisms in a RO water).

Table 6.2 ANOVA table represents the amount of retained elements in the solid fraction of MSW using one-way method and three fungal strains in a present of artificial medium at pH 5.5 for the 15 days duration

	Treatments mean value for solid part yield %				
Metals	***A. terreus***	***T. reesei***	***A. niger***	**Washed by water**	**P= value A factor (Fungi)**
Se^{2-}	70.9	84.7	137.6	43.29	0.001
P^{3-}	101.1	87	68.8	74.33	0.026
Ba^{2+}	55.3	52.4	49.2	49	*
Rb^{+}	41.5	28.9	26.1	25.5	0.0001
Cd^{2+}	58	50.6	42.4	47.64	0.0042
Fe^{2+}	60.2	48.1	42	41.6	0.02
Cs^{+}	49.2	47.2	41.9	43.6	*
Al^{3+}	50.5	52	40.9	29.29	*
Co^{2+}	51.1	47	38.5	43.34	0.041
As^{3-}	46	42.4	55.7	35.13	*
Average	58.3	54.04	54.3	43.2	

*= no significant difference

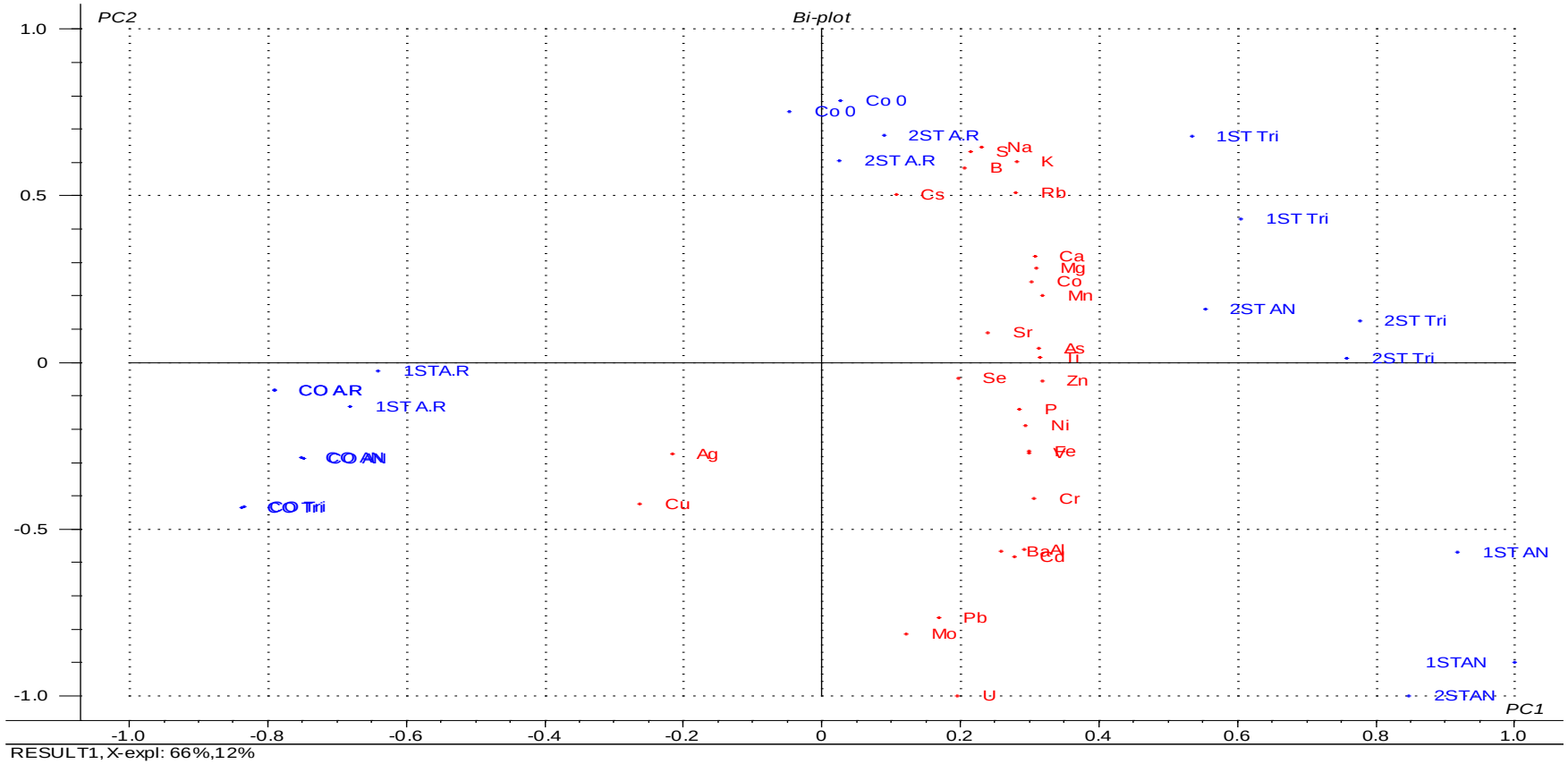


Figure 6.6 Amount of leached elements into the liquid of MSW using PCA under different conditions, one way and two method and pH 5.5 for 15 days duration, with three different organisms *A. niger, A. terreus* and *T. reesei* add in to the artificial medium

(1STAN= one way method *A. niger*; 2 STAN= two way method *A. niger*, 1 STAT= one way method *A terreus*; 2 STAT= two way method *A. terreus* ; 1 STTri= one way method *T. reesei* ; 2 STTri= two way method *T. reesei*; CO AN= control *A. niger* no medium ; COAT= control *A. terreus* no medium; COTri= control *T. reesei* no medium; CO 0 = no medium and no inoculation).

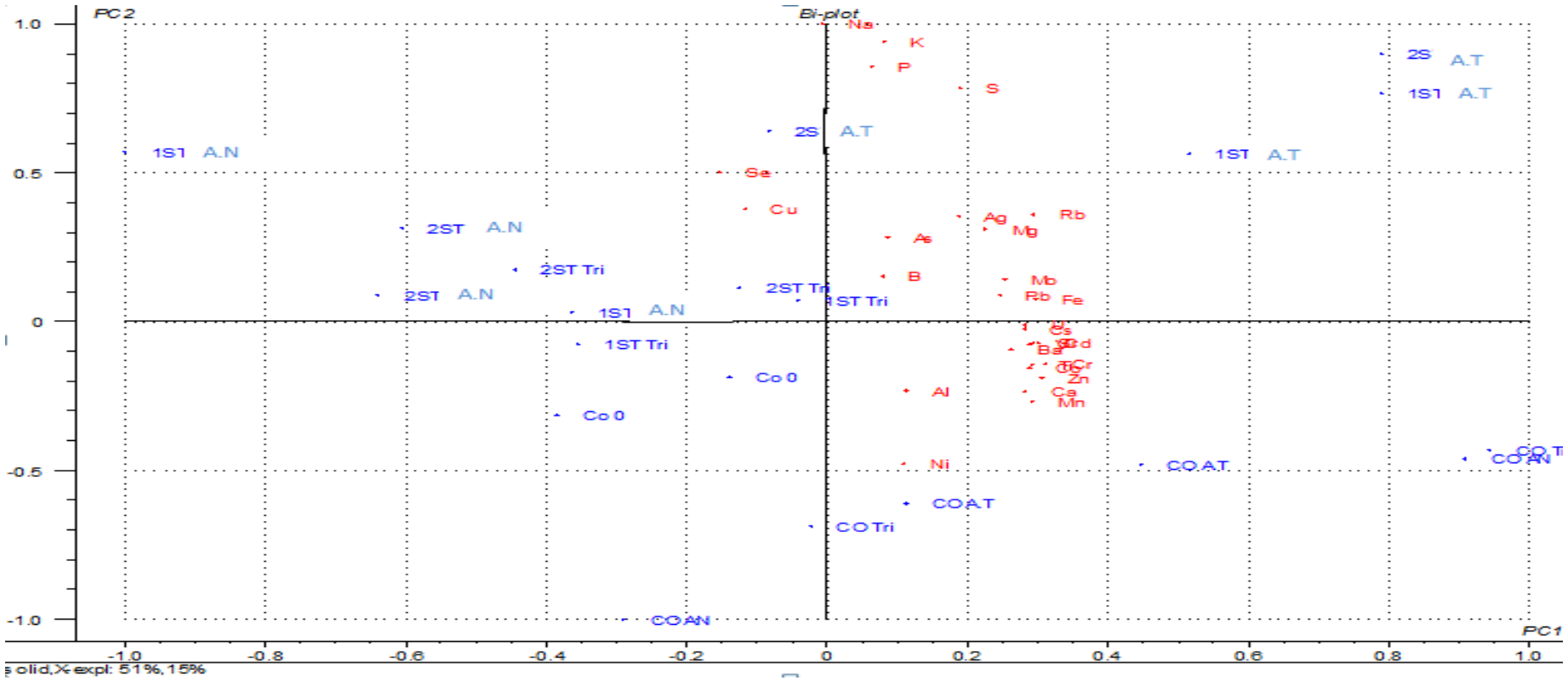


Figure 6.7 Amount of elements retained in the solid residue of MSW using PCA under different conditions, one way and two method and pH 5.5 for 15 days, with three different organisms *A. niger, A. terreus and T. reesei* add in to the artificial medium

6.3.2.3 Effect of pH and media compositions on bioleaching elements from MSW

From the previous experiment *A. niger* and the one way method was selected as the best condition. Three media as mentione previously in sections 6.2.3.1 and 6.2.3.2 were adjusted to either pH 5.5 or 2.5 and inoculated with *A. niger*. 1 mL of samples were taken after 4, 6, 8, 10, 12, and 15 days to determine the levles of elements in the liquid and solid phases as described above. Controls containing no fungal inoculant were also included and samples taken out at the same time point as for inoculated conditions and compared. The raw dara is again presented as an appendix on the enclosed CD

In the first set of experiment pH was fixed at 5.5 and the impact of the three different media- artificial, clay and sorghum media was assessed. Incubations were carried out as previously described and the liquid and solid phases collected for elemental analysis (data presented on CD)

The results for retained yield % in the solid phase are shown in Figure 6.8. These show that the artificial and clay medium resulted in the lowest retention (best performance) compared to the other media for all 28 metals analysed.

The ANOVA comparison for the top 10 highest elements required to work on them to get lowest retention yield is shown in Table 6.3. Results revealed that Se had the highest yield in terms of bioleaching; the clay medium showed better performance in terms of lowest retention yield (best performance) but still not better than control (washed by water). These elements had a significant difference in terms of using various media ($Se^{2-,}$ P^{3-},Cs^{+} ,Cd^{2+} ,Zn^{2+} ,Co^{2+} , and Pb^{2+}).

Again the data was analysed by PCA. The results were compiled to give a measure of leaching efficiency in the liquid and retained yield % for the solid for these are shown as Appendix A.1 and 2. The results for the liquid part showed that artificial medium, when using *A. niger*, one way method, and pH 5.5 was the best condition to remove metals from MSW (highest retention yield %). While for the solid par clay medium showed lowest yield % (best performance) as shown in Appendix A. 2. Bioleaching of metals at starting pH's of pH 5.5 did not improve retained yield (%) it was still higher comparing with washed water sample, so the impact of lowering the pH to 2.5 was assessed.

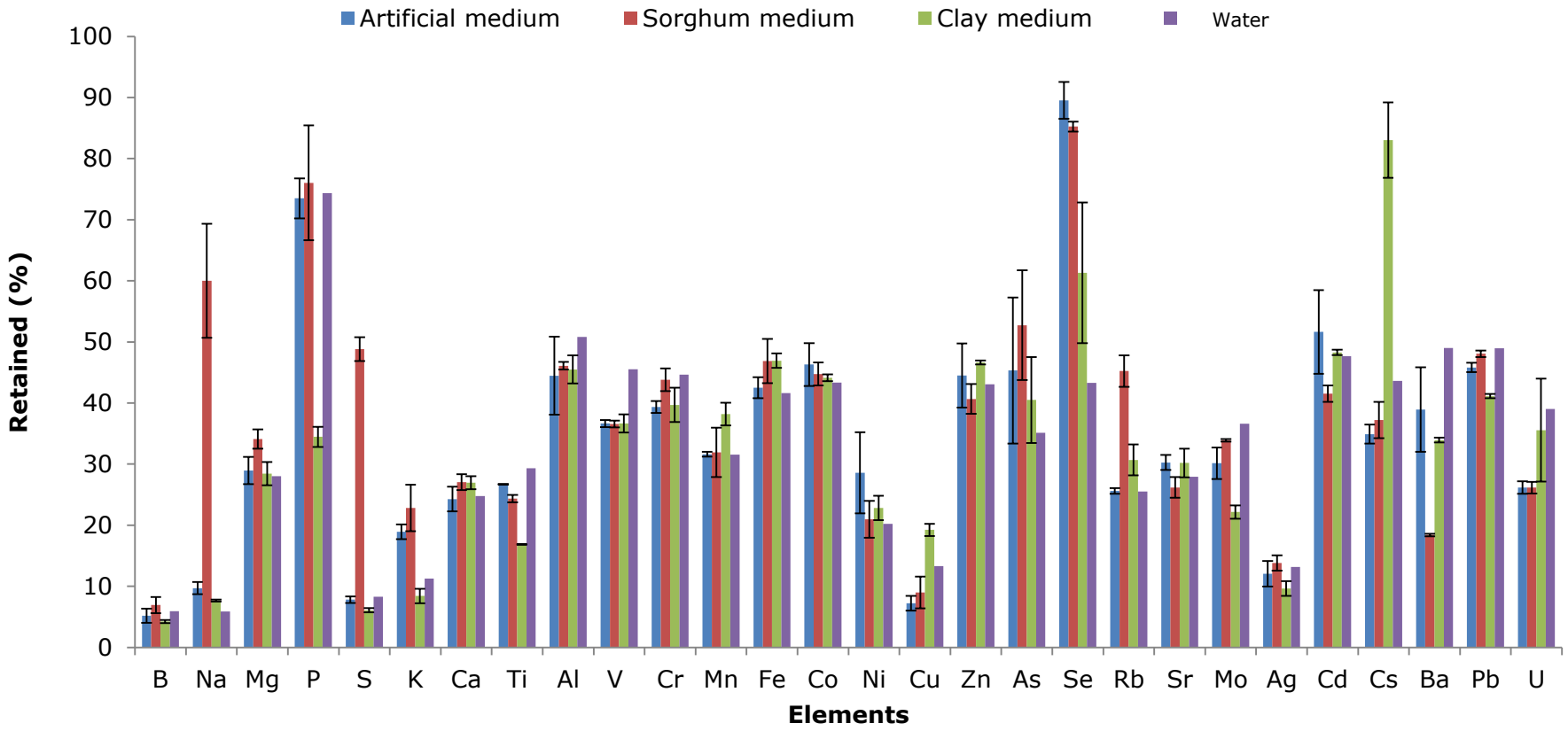


Figure 6.8 Amount of retained elements in the solid residue was assessed at pH 5.5 and the impact of three different media- artificial, clay and sorghum media using *A. niger*

Table 6.3 P-value of analysis of variance (ANOVA) table of the amount of retained elements in the solid fraction in a MSW using three media and *A. niger* at starting pH 5.5

	Treatments mean value solid yield %				
Metals	**Artificial Med.**	**Sorghum Med.**	**Clay Med.**	**Washed by water**	**P= value A factor (media)**
Se^{2-}	89.5	85.2	61.3	43.2	0.0076
P^{3-}	73.5	76	34.5	74.3	0.0001
Cs^{+}	34.9	37.2	83	43.6	0.0001
Cd^{2+}	51.6	41.5	48.3	47.6	0.0092
Zn^{2+}	44.5	40.7	46.7	43	0.004
Al^{3+}	44.5	46.1	45.5	50.8	*
Fe^{2+}	42.5	46.9	46.9	41.6	*
Co^{2+}	46.3	44.8	44.1	43.3	0.001
Pb^{2+}	45.8	48.1	41.1	48.9	0.0003
As^{3-}	45.3	52.7	40.5	35.1	*
Average	51.9	51.9	49.2	47.1	

*= not significant

A similar approach to that above was carried out using the same three media but at pH 2.5. Generally, more acidic conditions helped to dissolve elements easier than at pH 5.5.

Using bar charts, the elements retained yield (%) in the solid fraction for assays only containing inoculated medium was determined and results revealed that sorghum medium has the lowest elements retained when compared with the other media (Figure 6.9).

The analysis followed by drawing ANOVA table for highest ten elements retained in the solid residue require to decrease them using three different media revealed that there was significant different between media for the P^{3-}, Cd^{2+}, Cr^{6+} and As^{3-}, elements. In addition, some metals like Cs^{+} recorded non-correct data is probably instrument error. The results showed that sorghum medium showing better performance comparing with the other media (Table 6.4).

PCA analysis of, heavy metal leaching from liquid fractions at pH 2.5 revealed that assays which included artificial medium (inoculation) correlate with highest leaching yield % for all metals. As shown in Appendix A.3. Analysis revealed that assays containing *A. niger* using an artificial medium had the highest leaching of elements from MSW such as Cd^{2+}, Ni^{2+}, U^{4+}, As^{3-}, Se, Cr^{6+}, Ti^{3+}, V^{2+}, Sr^{2+}, Ca^{2+}, Pb^{2+}, Ag^{+}, Cs^{+}, and Cu^{2+}. Results revealed that use of artificial medium had the highest correlation for release of elements from the solid into the liquid fraction when compared with other media. However, addition of other media such as sorghum regardless of the addition of fungi showed a strong correlation with leaching elements including heavy metals; correlation was particularly evident in assays which had a fungal inoculant when compared with non-inoculated assays. The leached elements were Na^{+}, S^{2-}, P^{3-}, Rb^{+}, B^{3+}, K^{+}, Mg^{2+}, Fe^{2+}, Mn^{2+}, Zn^{2+}, and Mo^{3+} elements respectively, as shown on CD drive

Bioleaching of heavy metals from the solid fraction of MSW using various media at pH 2.5 analysing the solid fraction of MSW following bioleaching with a starting pH of 2.5 it was revealed that use of sorghum as a medium (inoculate and non-inoculant) correlated with lowest retained (best performance) of Na^{+} and S^{2-} (defined as % yield), however, addition of artificial medium (inoculated and non-inoculated) correlated with retained yield % of P^{3-}, K^{+}, B^{3+}, Cu^{2+}, Ti^{3+}, Ag^{+}, Mo^{3+}, Se^{2-}, Pb^{2+}, Co^{2+}, Ba^{2+}, Cd^{2+}, Ni^{2+}, V^{2+}, Fe^{2+}, and Cr^{6+}. Addition of clay as a medium correlated with the highest retained % from MSW such as Zn^{2+}, As^{3-}, Mg^{2+}, Rb^{+}, Al^{3+}, U^{4+}, Sr^{2+}, Mn^{2+}, Cs^{+} and Ca^{2+} (Appendix A.4).

From the result revealed that using sorghum medium with *A. niger*, pH 2.5, with inoculated or non-inoculated recorded highest elements leached (% yield) when compared with use of other media and control.

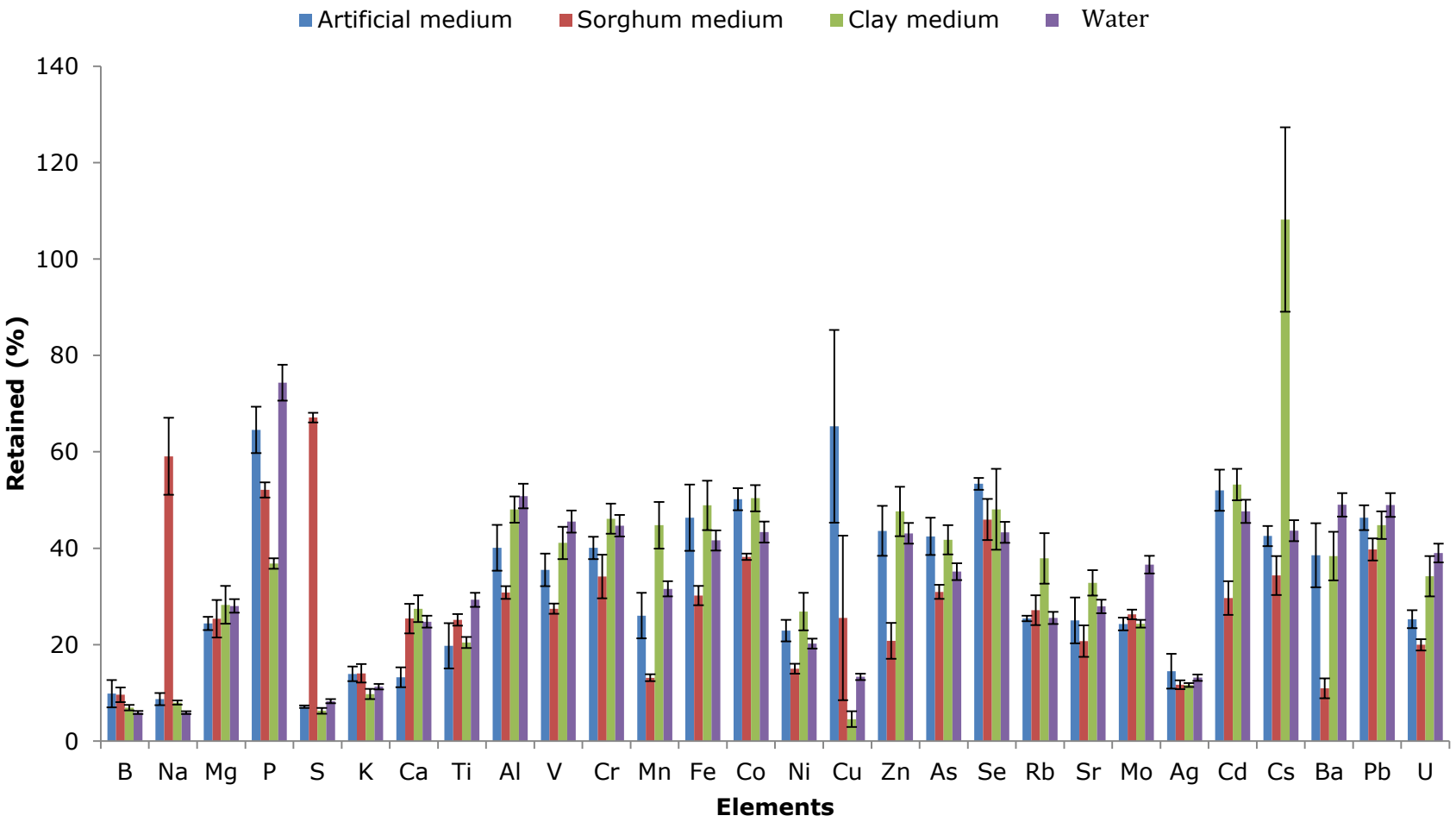


Figure 6.9 Amount of retained elements in the solid fraction of MSW using *A. niger* at pH 2.5 and three media (Artificial, Sorghum and Clay media) compared with (washed MSW)

Table 6.4 ANOVA table for the amount of retained elements in the solid fraction of MSW using three media and *A. niger* at starting pH 2.5

	Treatments mean value %				
Metals	**Artificial Med.**	**Sorghum Med.**	**Clay Med.**	**Washed by water**	**P= value A factor (media)**
Cs^{+}	42.5	34.4	108.2	43.6	0
P^{3-}	64.6	52.1	36.8	74.33	0.001
Se^{2-}	53.4	45.9	48.1	43.9	*
Co^{2+}	50.2	38.2	50.4	43.3	0
Pb^{2+}	46.3	39.7	44.8	48.9	*
Cd^{2+}	52	29.6	53.2	47.6	0.007
Fe^{2+}	46.3	30.2	48.9	41.6	0
Cr^{6+}	40.1	34.1	46.1	44.6	0.005
Al^{3+}	40.1	30.8	48	50.8	0
As^{3-}	42.5	30.9	41.8	35.1	0.01
Average	47.8	36.6	52.6	47.3	

In order to compare bioleaching at two different pH's (2.5 and 5.5) the average value of total elements retained (% yield) from the solid fraction was compared, in general bioleaching was better at pH 2.5 when compared with bioleaching at pH 5.5. However, aside from this generalisation, there were exceptions such as use of sorghum as a medium which performed better at pH 2.5 when compared with pH 5.5 (lowest retention yield in a solid fraction). Generally, sorghum medium at pH 2.5 showed better performance when compared with pH 5.5 the best retained yield was 28.9 % using sorghum medium at pH 2.5 comparing with washed water retained yield % was 33.28 % (Figure 6.10).

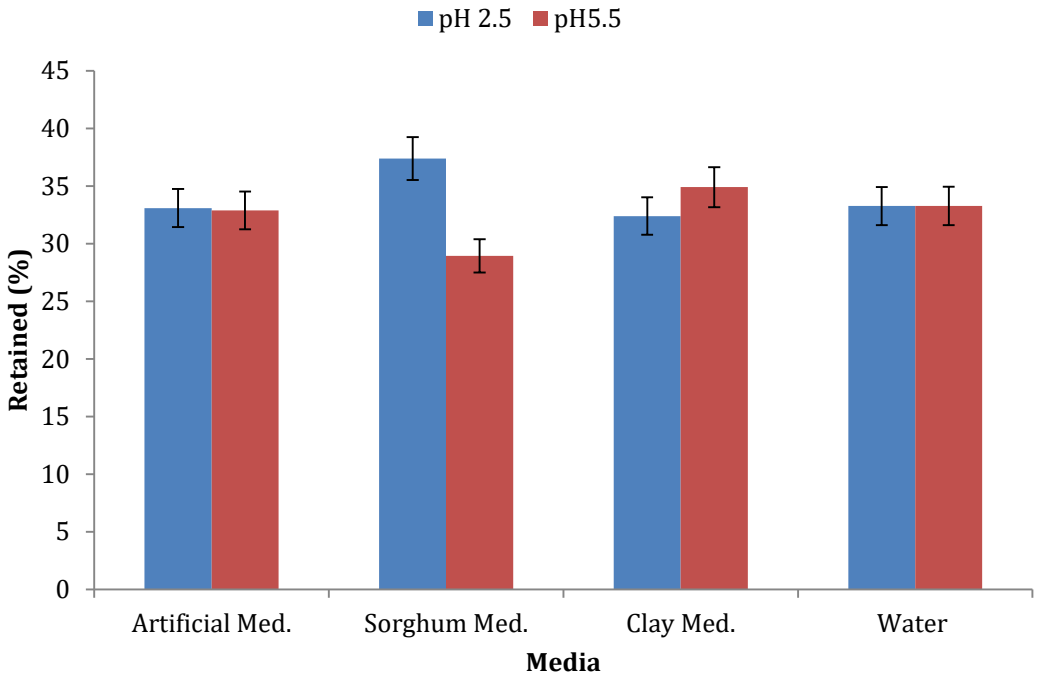


Figure 6.10 Comparing amount of retained yield % in the solid fraction of total elements in MSW with a starting pH of either 2.5 or 5.5 and three media using *A. nger* fungi

6.3.3 Organic acid production as a by-product using fungi and different media

Fungi often produce organic acids such as gluconic, oxalic or citric acids as part of the bioleaching mechanism. These acids could be used for chemical leaching of MSW, or indeed represent by products of the process. Use of these acids could decrease the cost of chemical leaching and at the same time could add value to the liquid fraction. Thus in addition to bioleaching the production of organic acids was also assessed in the experiments.

A bioleaching experiment was carried out using a starting pH of 5.5, with three media- artificial, clay and sorghum an using addition of *A. niger*. The production of three organic acids –gluconic, oxalic and citric was then determined over 15 days. The results are shown in figure 6.11. *A. niger* was shown to produce all three organic acids and experiments here revealed that gluconic acid was detected at the highest concentrations when compared with oxalic and citric acid. Results revealed that addition of either sorghum or artificial medium resulted in an increased organic acid production. Gluconic acid production was initially highest on artificial medium but decreased after the first few days. Production on sorghum medium was also high initially and again declined with time. There was little production observed with the clay medium. However, citric acid production was initially low in all media tested but increased transiently in the sorghum medium after about 10 days. However, this may have been due to instrument error. Oxalic acid production was only observed in the sorghum medium and again this was transient. Acid production in all three cases was very low.

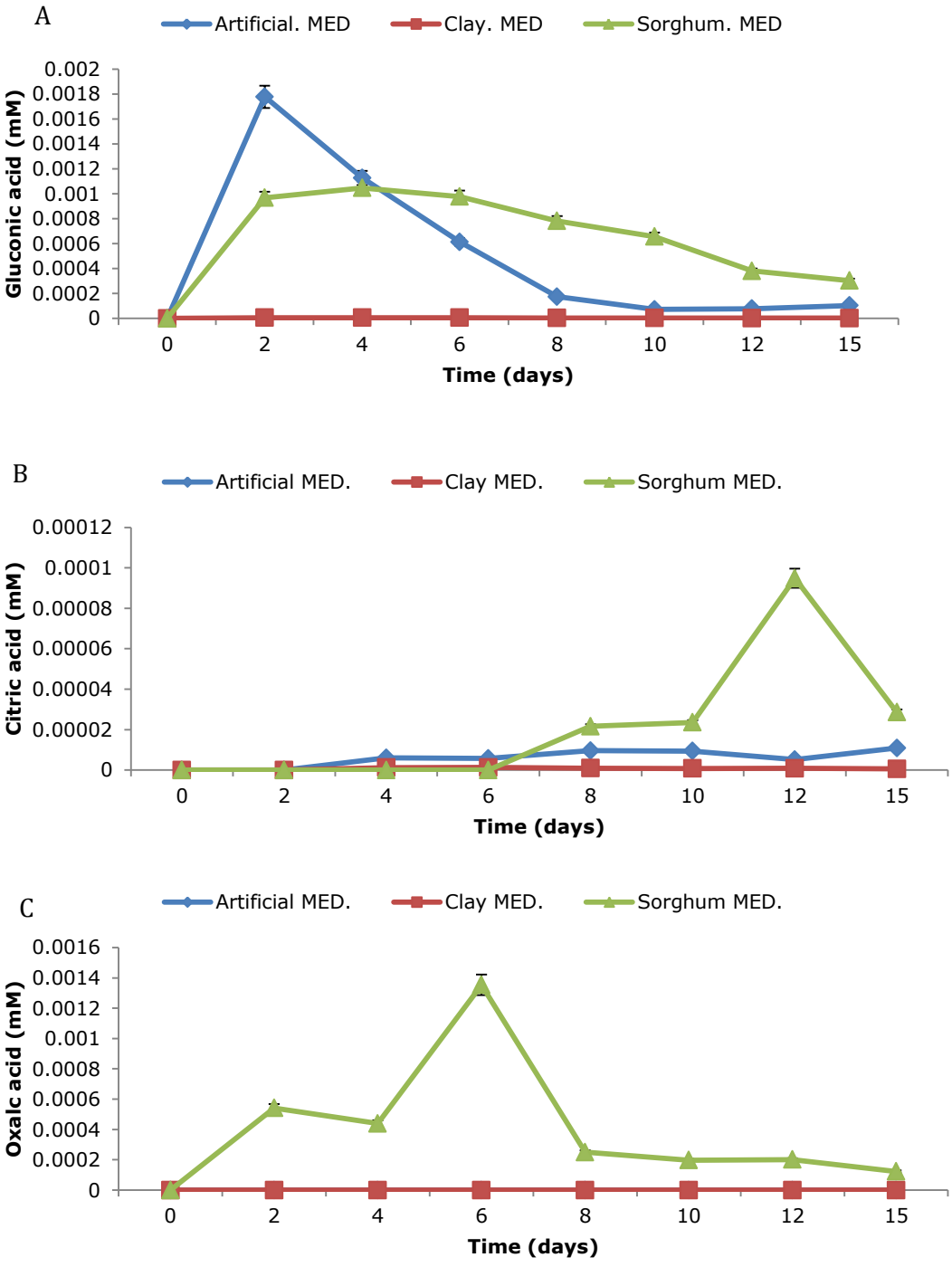


Figure 6.11 organic acid productions during bioleaching of MSW

MSW was incubated at a starting pH of 5.5, using three media inoculated with *A. niger* to determine the production of three organic acids (A) Gluconic acid (B) Citric acid and (C) Oxalic acid, Data represented as a triplicate values (Med = Medium)

The experiment was repeated using a starting pH of 2.5 (Figure 6.12). Assays with a starting pH of 2.5 required the addition of 25 mL sterile RO water at day 9 to keep the moisture levels stable in the flasks, Gluconic acid production profiles were similar to those seen at pH 5.5 but the absolute concentrations obtained were much higher. Production of the other two acids was again very low and sorghum medium showed the highest levels for both acids.

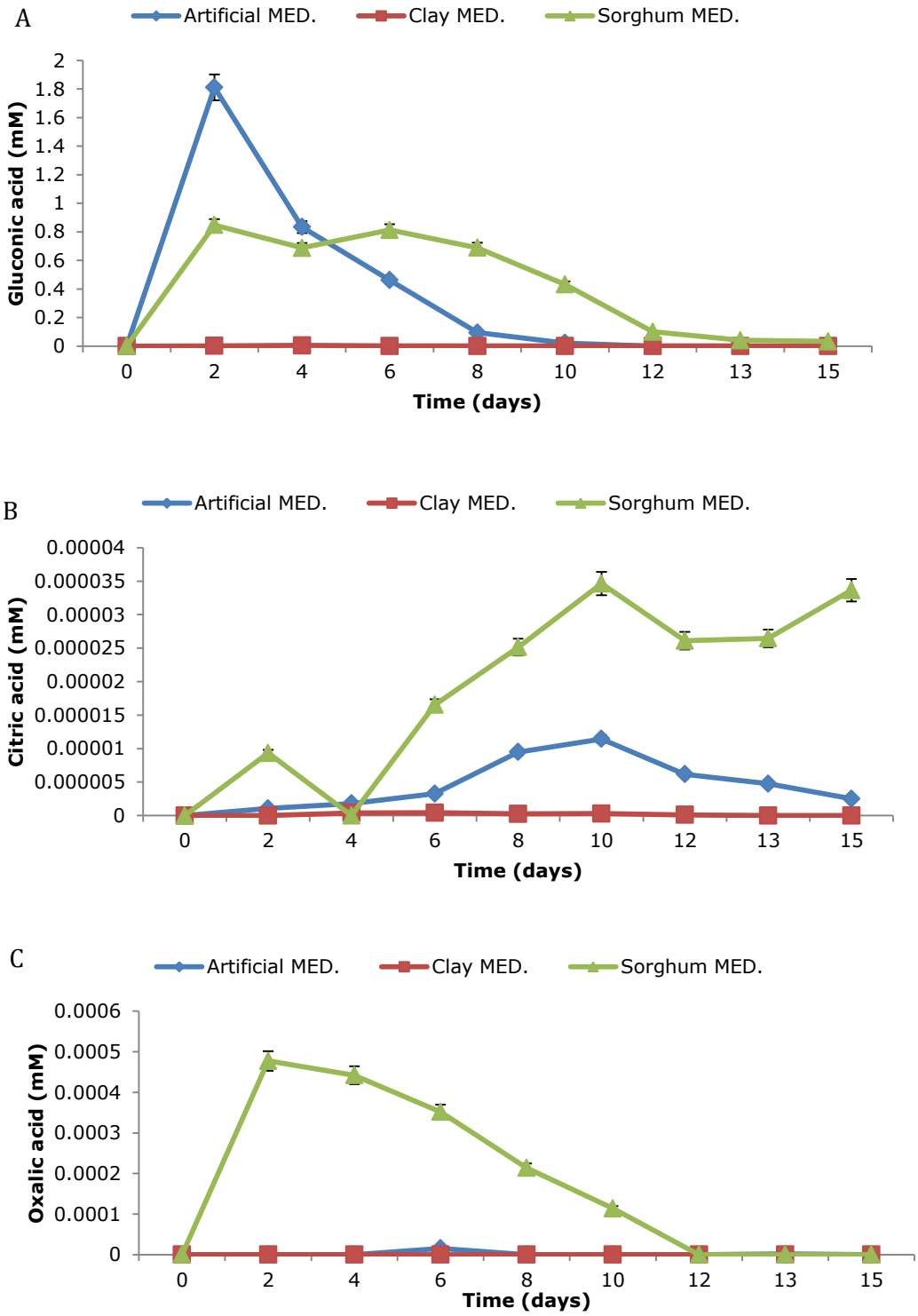


Figure 6.12 Organic acid production at pH 2.5 and three media over time., using three media inoculated (*A. niger*) to determine three organic acids (A) Gluconic acid (B) Citric acid and (C) Oxalic acid. Data represented as a triplicate values

6.3.4 Effect of using yeast for bioleaching process

The bioleaching above utilised filamentous fungi but was not particularly effective. Further experiments were thus carried out to explore whether the use of yeast would be more effective.

6.3.4.1 Effects of heavy metals on yeast metabolism

Yeast can be used as agents for bioleaching of heavy metals from MSW; however, tolerance to heavy metals in yeast is not yet fully understood. The effect of heavy metals on yeast metabolism was thus determined using a phenotypic microarray assay. The results are shown in appendix A 5. It was determined that *S. cerevisiae* NCYC2592 displayed tolerance to Mn^{2+}, Zn^{2+} or Al^{3+} up to 4 mM. However the presence of other metals such as Pb^{2+}, Fe^{2+} and Cu^{2+} at 4 mM reduced metabolic output when compared with control conditions. The presence of Cu^{2+} at 0.1-0.5 mM actually improved metabolic output when compared with control conditions. These results suggest that the yeast strains used in this study could tolerate the presence of Mn^{2+}, Zn^{2+} and Al^{3+} at concentrations relevant to those found in the MSW.

6.3.4.2 Bioleaching with *S. cerevisiae* NCYC2592 using either a one or two way method.

Yeast were incubated with MSW as described in the methods using the two media and the the two bioleaching methods and the impact on bioleaching monitored as for the filamentous fungi. The constitution of basal medium was based on the study by (Norris and Kelly, 1977; Blackwell and Tobin, 1999; Damodaran *et al*., 2011).

Comparing Retained yield (%) it was shown that P using a one way method had the highest yield when compared with other elements in all conditions, the results shown from total metals average that lowest retained yield % was by using medium one and two way method. The results are shown in Figure 6.13.

The highest 28 elements retained from MSW were taken forward for ANOVA testing and results revealed that there was no significant difference between methods and media in terms of retained yield of elements (Table 6.5).

PCA analysis for the solid fraction revealed that use of a two-way method, medium 1 and 2 correlated with retaining of Na elements also showed the lowest retained yield % (best performance). Also revealed that using a one-way method using medium 1 and 2 correlated with retention of Cu^{2+}, Ca^{2+}, Ag^{+}, Ti^{3+}, Ba^{2+}, Pb^{2+}, Sr^{2+}, U^{4+}, Cs^{+}, As^{3-}, Ni^{2+}, and Mn^{2+}. In conclusion, using a two-way method and medium 1 correlated with the highest (lower performance), Se^{2-}, Zn^{2+}, Cd^{2+}, Cr^{6+}, U^{4+}, Al^{3+}, Fe^{2+}, Mo^{3+}, Co^{2+}, Mg^{2+}, S^{2-}, P^{3-}, K^{+}, Rb^{+}, and B^{3+} retaining from the solid residue (See Figure 6.14).

These results suggest that the two way method and medium 1 was the best combination as long as shown no significant differences between methods and media. Comparing the data with the control yeast with medium 1 and 2 way method showed the lowest retaining yield comparing with water. Further experiments were carried out to increase the retained yield %. For the remaining sections a similar analytical approach was taken in addition of determining the amount of elements in a liquid part too.

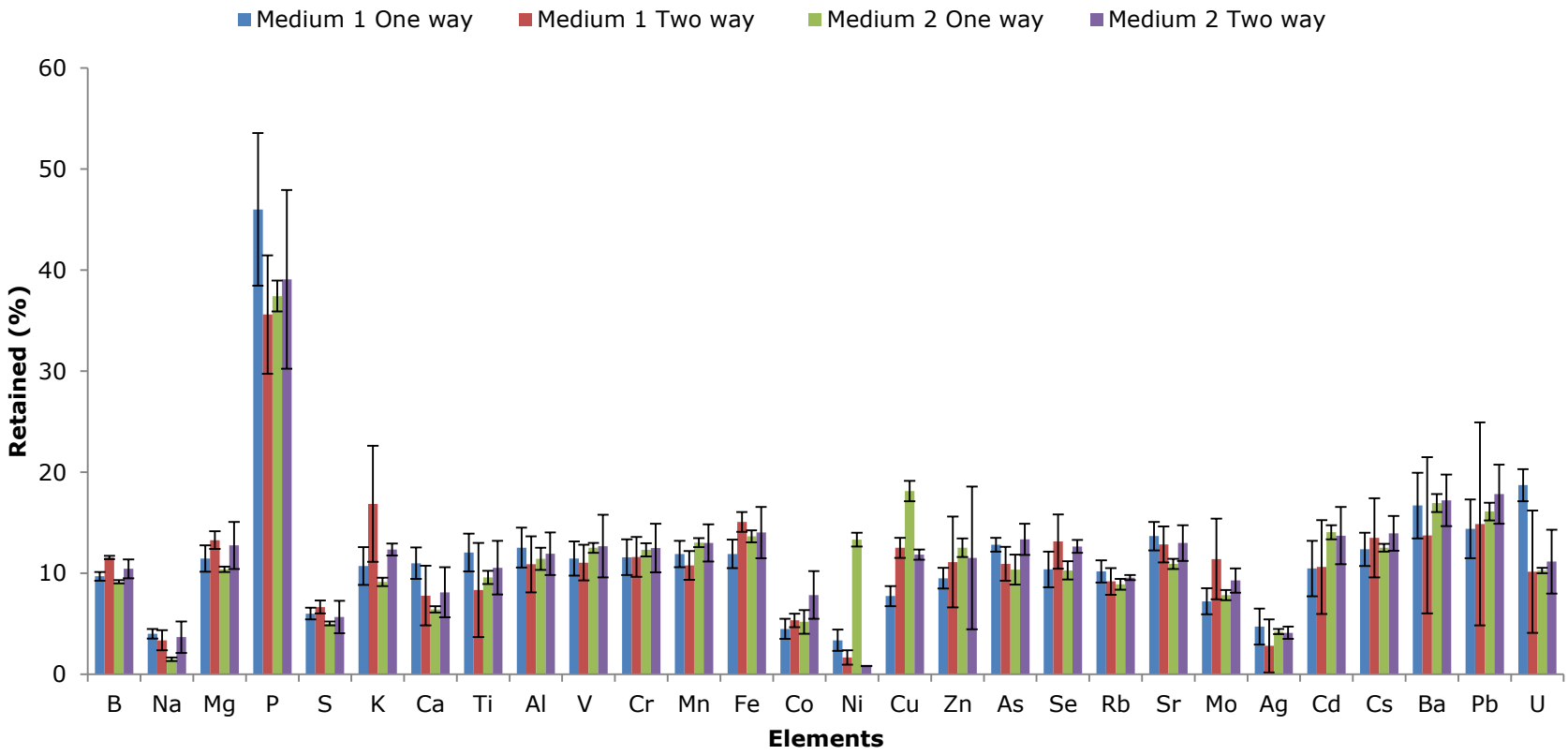


Figure 6.13 Percentage of retained elements in the solid fraction of MSW using two media and two methods (one and two way methods) in the presence of *S. cerevisiae* at pH 5.5 for 15 days duration

Table 6.5 ANOVA table for amount of retained elements in the solid fraction of MSW for evaluate P- value using two media and two different methods at pH 5.5 in a presence of *S. cerevisiae* for 15 days duration

	Treatments mean value of solid yield %						
	Medium 1		Medium 2		Washed by water	P= value	
Metals %	One method	Two method	One method	Two method		A factor (Media)	B factor (method)
P^{3-}	46	35.6	37.4	39.1	74.3	0.053	*
Ba^{2+}	16.7	13.7	16.9	17.2	49.9	*	*
Pb^{2+}	14.4	14.9	16.1	17.8	48.9	*	*
Zn^{2+}	7.7	12.5	18.1	11.8	43	*	*
Co^{2+}	11.1	15.1	13.6	14	43.3	*	*
Cd^{2+}	10.5	15.6	14.1	13.7	47.6	*	*
Al^{3+}	13.7	12.8	10.9	13	50.8	0.047	*
Cs^{+}	12.4	13.5	12.5	13.9	43.6	*	*
Fe^{2+}	11.9	10.7	13	13	41.6	*	*
Cr^{6+}	11.4	11	12.5	12.7	44.6	*	*
Average	15.5	15.5	16.51	16.6	48.7		

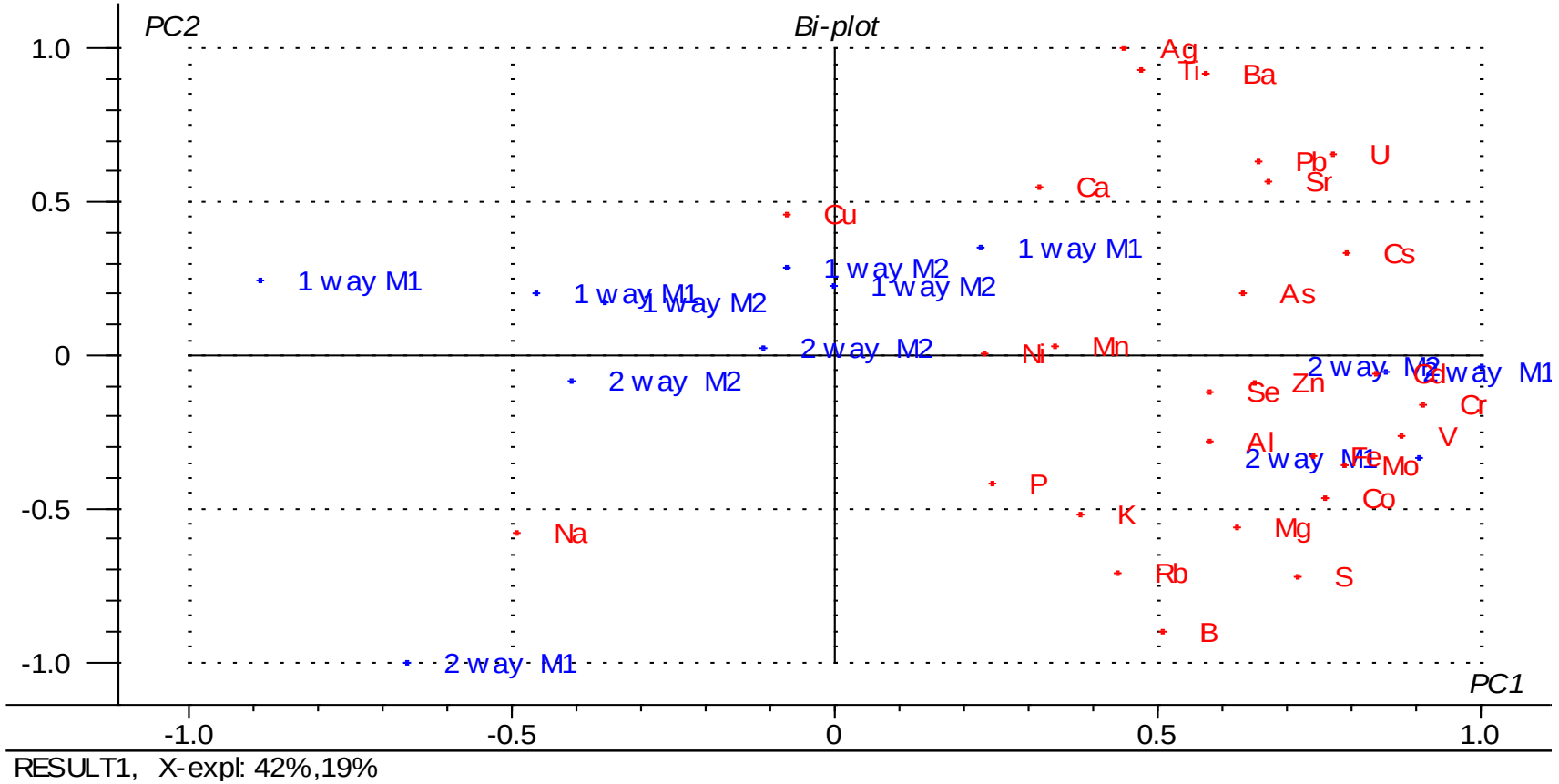


Figure 6.14 PCA analyses for 28 elements for the amount of retained elements in the solid fraction using two media and two different methods at pH 5.5 in the presence of *S. cerevisiae* for 15 days duration

(1wayM1=one way method, and medium one; 2way M1= two way method and medium one; 1 way M2= one way method and medium two; and 2 way M2 = two way method and medium two).

6.3.4.3 Comparing yeast strains for bioleaching of MSW

From the previous experiments the two way method and medium 1 was found to be the best combination. In this section five yeast strains plus *S. cerevisiae* were compared using these growth conditions as mentioned previously in section 6.2.1.

The results revealed that the retained yield % was lowest using *S. stipitis* when compared with other strains. In terms of elements retained from the solid fraction the highest for all strains were P^{3+} and Se^{2-} (Figure 6.15).

The experiment was followed by ANOVA analysis of the top 10 highest elements retained in the MSW residue, the results revealed no significant difference between yeast strains and also showed that *S. stipitis* has lowest retained yield % (best performance), the lowest retained yield showed a significant improvement comparing with the control (Table 6.6).

PCA analysis for the liquid fraction revealed that *C. shehatae* correlated with leaching of only Pb^{2+} and Se^{2-} which related to lowest yield %, while *S. stipitis, C. succiphilia, and K.marxianus* correlated with leaching highest and most elements of when compared with other strains (See Appendix A.6).

Using PCA to analysis the ability of each strain to remove metals from the solid fraction of the MSW (retained yield %) revealed a strong correlation between ability of *S. stipitis* and *S. cerevisiae* correlated with retaining lowest yield % for most of elements (See Appendix A.7). Form the result *S. stipitis* and *S. cerevisiae* applied for further experiment to get the lowest retained yield % in MSW.

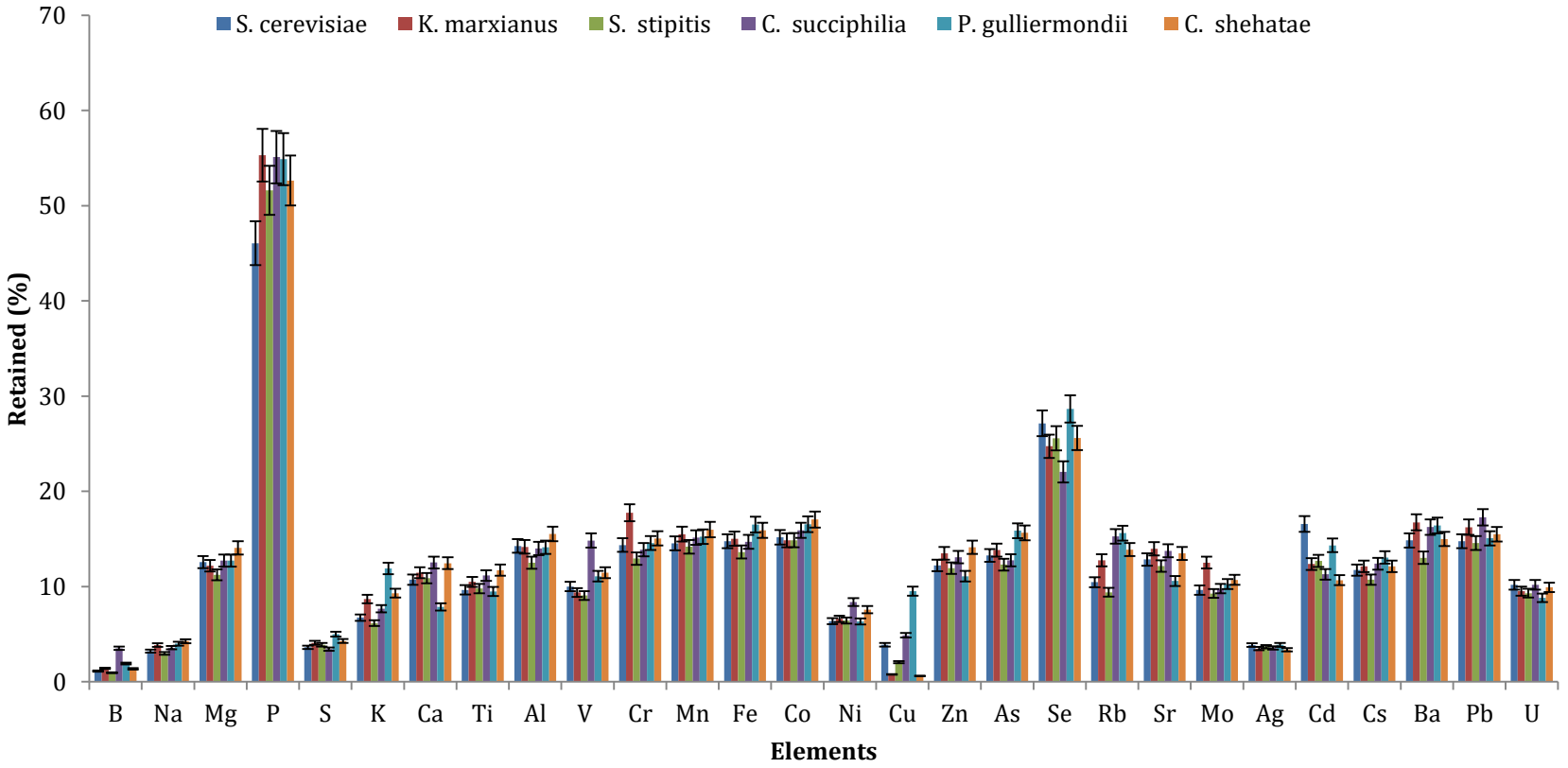


Figure 6.15 The Percentage of retained elements in the solid fraction for 28 elements using various yeast strains, and medium number one with two way method at pH 5.5 for 15 days duration time

Table 6.6 ANOVA table for amount of retained highest 10 elements in the solid fraction using various yeast strains, and medium number one with two way method at pH 5.5 for 15 days duration

	Treatments mean value for solid part yield %							P= value
Metals %	***S. cerevisiae***	***K.marxianus***	***S. stipitis***	***C. succiphilia***	***P.gulliermondii***	***C. shehatae***	***Washed by water***	**Factor A (strains)**
P^{3-}	46.1	55.3	51.6	55.1	54.9	52.6	74.3	*
Se^{2-}	27.1	24.7	25.6	22	28.7	25.6	43.2	*
Co^{2+}	15.2	14.9	14.9	15.9	16.5	17	43.3	*
Pb^{2+}	14.8	16.2	14.6	17.3	15.1	15.5	48.9	*
Ba^{2+}	14.8	16.7	13	16.2	16.4	15	49	*
Mn^{2+}	14.5	15.5	14.2	15.1	15.3	16	31.5	*
Fe^{2+}	14.8	15	13.6	14.7	16.5	15.9	41	*
Cr^{6+}	14.4	17.8	12.9	13.9	14.6	15.1	44.6	*
Al^{3+}	14.3	14.2	12.5	14	14.1	15.5	50.8	*
As^{3-}	13.3	13.8	12.3	12.8	15.9	15.6	35.1	*
Average	18.9	20.4	18.5	19.7	20.8	20.4	46.17	

6.3.4.4 Effect of artificial and non-artificial media and pH

Yeast strains, *S. cerevisiae* NCYC2592 and *S. stipitis* NCYC 1541 were inoculated in to the best selected medium 1 and non-artificial medium prepared at either pH 5.5 or 4 and the bioleaching experiment carried out for 15 days.

Comparing retained yield % in the solid residue it was observed that sorghum medium (*S. cerevisiae)* and artificial medium (*S. stipitis)* showed better performance in terms of removing metals from solid (Figure 6.16).

ANOVA analysis for the highest ten retained elements, determined that use of artificial medium and *S. stipitis* as a strain had the highest ability in terms of removing elements from MSW as shown in ANOVA table (Table 6.7), but still for some elements did not hit the point which require to be applicable to use as a compost, further optimisation carried out using lower pH (PH = 4).

PCA applied to the leachate (liquid fraction) revealed that addition of clay (*S. cerevisiae* and *S. stipitis*) did not correlate with leaching of any elements due to the fact that the elements may be adhering to the surface of the clay, however, addition of sorghum as a medium correlated with improved leaching of Ba^{2+}, Sr^{2+}, Ca, Mg, Mn^{2+}, As, Al, and P, addition of artificial medium correlated with the highest leaching yield % of Fe^{2+}, Se^{2-}, Pb^{2+}, Ni^{2+}, Cr^{6+}, Mo^{3+}, Cd^{2+}, Co^{2+}, Zn^{2+}, V^{2+}, and Rb^{+} (Appendix A.8).

PCA analysis on the solid fraction as retained yield % revealed that sorghum medium using both yeast strains correlated with the lowest retaining yield % of As^{3-}, Co^{2+}, Mn^{2+}, Cu^{2+}, Mo^{3+}, Zn^{2+}, P^{3-}, B^{3+}, S^{2-}, Mg^{2+}, and Na^{+} elements, followed by artificial medium and use of *S. cerevisiae* which correlated with removal of Ba^{2+}, V^{2+}, Al^{3+}, Sr^{2+}, Se^{2-}, Ti^{3+}, Ca^{2+}, Cr^{6+}, Pb^{2+}, Ni^{2+}, and Co^{2+}, the result showed that sorghum medium using two strains has the best performance when compared with other two media (Appendix A.9).

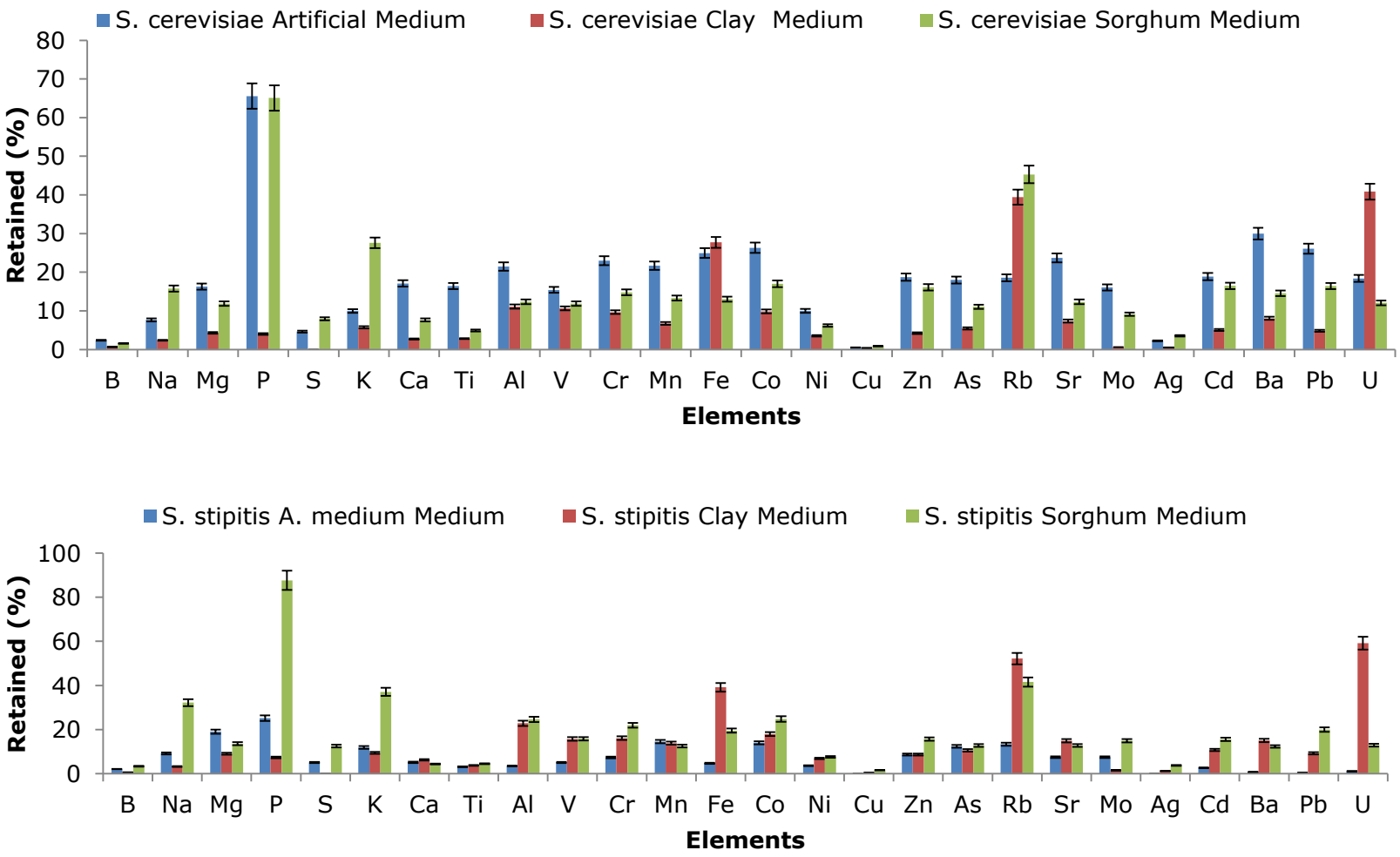


Figure 6.16 The amount of retained 28 elements in the solid fraction in assays with a starting pH of 5.5 following addition of three media with two yeast strains (A) *S. cerevisiae* and (B) *S. stipites* for 15 days

Table 6.7 ANOVA table for top ten highest elements retained in the solid fraction of MSW *using S. cerevisiae and S. stipites* yeast strains, two way method and three media at pH 5.5 for 15 days duration

	Treatments mean value for solid part yield %								
	Artificial Med.		**Clay Med.**		**Sorghum Med**			**P=value**	
Metals %	***S. cerevisiae***	***S. stipitis***	***S. cerevisiae***	***S. stipitis***	***S. cerevisiae***	***S. stipitis***	**Washed by water**	**A factor (media)**	**B factor (strains)**
Se^{2-}	268.4	190.4	184.5	170.9	142	159.1	43.2	0.0004	0.078
Cs^{+}	25	9.7	168	233.4	16.9	21.4	43.6	*	*
P^{3-}	65.6	25.2	4	7.3	65.1	87.7	74.3	0.01	*
Rb^{+}	18.6	13.4	39.4	52.2	45.3	41.5	25.5	*	*
U^{4+}	18.4	1	40.9	59.2	12.1	12.9	39	0.0001	0.0033
Fe^{2+}	25	4.7	27.7	39.2	13	19.5	41.6	0.001	0.0018
Co^{2+}	26.3	14	9.8	17.9	17	24.7	43.3	0.05	0.075
K^{+}	10	11.9	5.8	9.4	27.6	37.1	11.2	0.0001	0.0179
Al^{3+}	21.4	3.4	11.1	22.8	12.3	24.6	50.8	*	*
Cr^{6+}	23	7.3	9.7	16.1	14.8	21.9	44.6	*	0.053
Average	50.16	28.1	50.09	62.8	36.6	45.05	41.7		

Effects of a starting pH 4 on removal of metals from MSW. Bar chart analysis for 28 elements carried out using retained yield % in MSW showed that P^{3-}, Rb^{+} and U^{4+}, where the highest elements, the lowest retaining yield % (best performance) recorded using sorghum medium (*S. stipitis*) as shown in Figure 6.17.

Analysis highlighted the top ten elements remaining in the solid fraction of MSW and these were taken forward for ANOVA analysis, Results revealed that by using sorghum medium (*S. stipites)* at a starting pH of 4 showed lowest retained yield % (best condition) comparing with other conditions. The table showed that there is a significant difference between media. ANOVA analysis revealed that use of sorghum as a medium removed more metals from MSW when compared with use of other media while addition of *S. stipitis* correlated with better retained yield when compared with use of *S. cerevisiae* (Table 6.8).

PCA analysis using liquid fraction (% yield) following addition of three different growth media revealed that the highest leached yield was with the addition of sorghum as a medium; addition of sorghum correlated with leaching of Se^{2-}, Ti^{3+}, Mn^{2+}, As^{3-}, P^{3-}, Ca^{2+}, Na^{+}, Mg^{2+}, P^{3-} , B^{2+} and Sr^{2+} from MSW using either yeast strains, then followed by artificial medium also for either strains, this data correlated with leaching of K^{+}, Rb^{+}, V^{2+}, Mo^{3+}, Co^{2+}, Zn^{2+}, Cr^{6+}, Cd^{2+}, Pb^{2+}, Ni^{2+}, and Al^{3+}, finally clay medium whose addition was characterised by the lowest leaching yield had a strong correlation with removal of U and Ba. Results revealed that only addition of sorghum correlated with highest leaching of elements from MSW and release the elements in to the liquid fraction (Appendix A.10).

PCA analysis of leaching from the solid fraction of MSW revealed that use of clay with *S. cerevisiae* correlated with retained yield % of Cs^{+}, Fe^{2+} and U^{4+} elements; and use of sorghum medium (*S. stipites*) correlated with lowest retaining yield % of Na^{+}, S^{2-}, K^{+}, V^{2+}, Zn^{2+}, Ag^{+}, Mo^{3+}, Co^{2+} , Cr^{6+}, Pb^{2+}, As^{3-} and P^{3-} elements (best performance) (See appendix A.11).

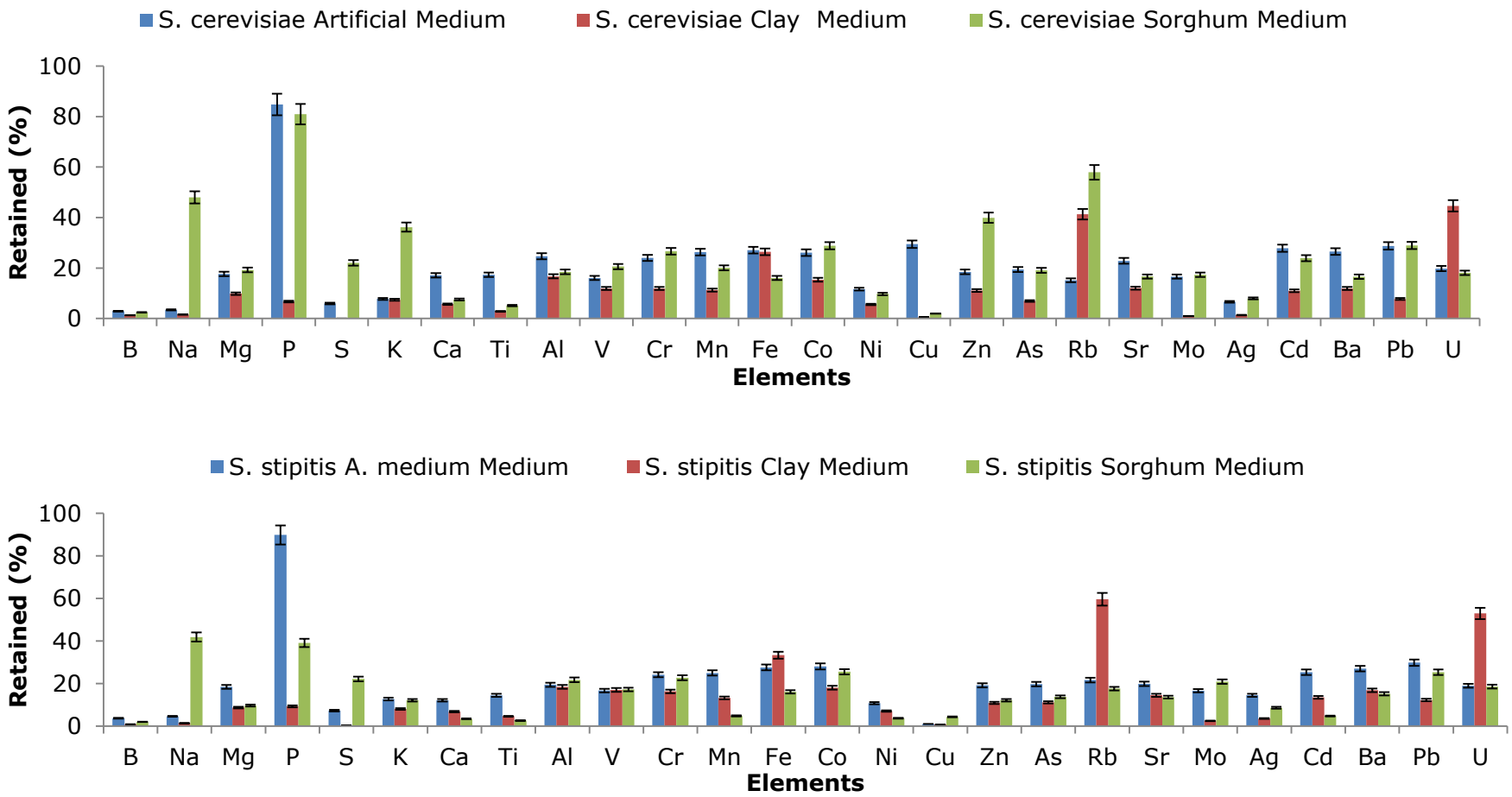


Figure 6.17 The Percentage of retained elements in the solid fraction for 28 elements in a solid residue with a starting pH of 4 and use of three media and addition of two yeast strains (A) *S. cerevisiae* and (B) *S. stipites* the experiment carried out for 15 days

Table 6.8 ANOVA table for top ten highest amount retained elements in the solid fraction of MSW using *S. cerevisiae and S. stipites* yeast strains, two way method and three media at pH 4 for 15 days duration

	Treatments mean value solid yield %								
	Artificial Med.		Clay Med.		Sorghum Med.			P= value	
Metals %	***S. cerevisiae***	***S. stipitis***	***S. cerevisiae***	***S. stipitis***	***S. cerevisiae***	***S. stipitis***	**Washed by water**	**A factor (media)**	**B factor (strains)**
Cs^{+}	21.1	25.1	195.8	327.8	25.5	23.4	43.6	0.038	*
Se^{2-}	139.8	79	71.1	47.8	101.8	50.3	43.2	0.0001	0.0001
P^{3-}	84.8	89.8	6.8	9.2	80.9	39	74.3	0.0001	0.0064
Rb^{+}	15.1	21.6	41.3	59.6	57.9	17.5	25.5	0.0402	*
U^{4+}	19.8	18.9	44.6	52.9	18.1	18.5	39	0.0001	*
Fe^{2+}	27.1	27.5	26.4	33.3	16.1	16.1	41.6	0.0004	*
Co^{2+}	26.1	28	15.3	18	28.9	25.5	43.3	0.0001	*
Pb^{2+}	28.8	29.8	7.7	12.2	29	25.3	48.9	0.0001	*
Cr^{6+}	24	24.1	11.9	16.3	26.7	22.7	44.6	0.0001	*
Al^{3+}	24.7	19.4	16.7	18.4	18.5	21.7	50.8	0.0316	*
Average	41.1	36.3	43.8	59.5	40.3	26	45.48		

Comparing pH 5.5 with pH 4 the results showed that pH 5.5 was better for retaining yield % from the solid fraction of MSW when compared with pH 4. The best retaining yield was 13.8 % using artificial medium pH 5.5 using *S. stipitis* while comparing the lowest retained yield % with the washed MSW, the optimum condition showed a significant improvement comparing with washed MSW sample (the retained yield % using water was 33.25) , as shown in Figure 6.18.

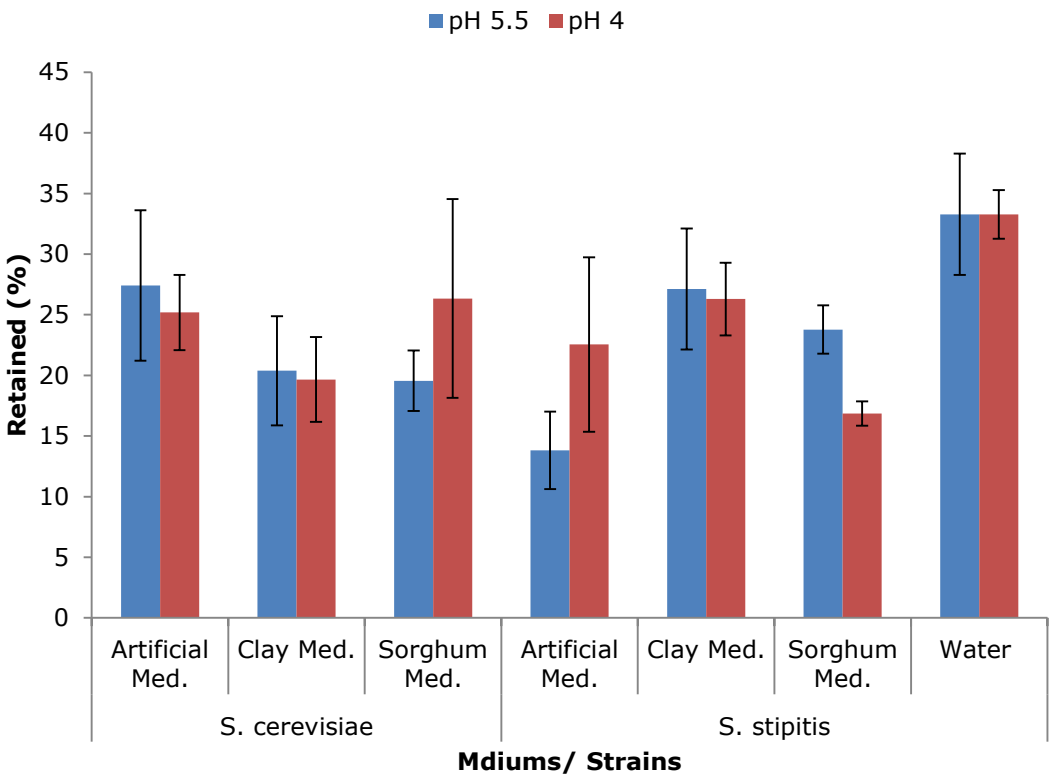


Figure 6.18 Comparison of between amounts retained in the solid fraction by applying three different media at pH 5.5 and 4 using *S. cerevisiae and S. stipitis* for 15 days duration

A.M= Artificial medium

Comparing results of retaining yield % using fungi and yeast. The metal levels achieved under optimum conditions using yeast which almost reached the environmental standards required as shown in Table 6.9, in current study yeast better than fungi in terms of getting lowest retain yield.

6.4 Discussion

MSW could be used for compost production as it contains garden, food and household wastes and has been shown to have a biodegradable proportion varying from 5 to 90 % depending upon the country (Déportes *et al*., 1995). Currently in the UK the biodegradable content is estimated at 68 % but is predicted to fall to 54 % by 2020 due to increased collection of source-segregated waste (SU, 2002).

Compost derived from heterogeneous MSW has a 50-70 % component which is potentially biodegradable (Déportes *et al*., 1995). However, the compost is currently excluded from UK composting standards (BSI, 2005), also there are restrictions in many countries on landfill sites if MSW does not meet quality standards (WRAP, 2002). The reasons behind these restrictions are that even with excellent separation and pre-treatment some contaminants such as glass shards and plastic fragments are not biodegradable. In addition, the presence of other contaminations such as heavy metals (e.g., Cd^{2+}, Cr^{6+}, Cu^{2+},Pb^{2+}, Ni^{2+} and Zn^{2+}) can be a reason for the exclusion especially if applied to arable land for food production (Papadimitriou *et al*., 2008; Farrell and Jones, 2009).

For composting MSW there are three main stages (Stentiford, 1996); the first stage is 'sanitization' which depends on a vigorous aeration regime to accelerate microbial breakdown of substrates (Abu Qdais and Hamoda, 2004), this stage is a necessary stage for most compost standards to ensure destruction of both plant and animal pathogens (Deportes *et al*., 1998). Consequently, a secondary biodegradation phase of 45–55 ^{o}C is used to facilitate rapid substrate degradation (Abu Qdais and Hamoda, 2004). The tertiary phase is similar for both aerated and turned compost, but requires little active management, this stage helps convert potential toxic NH_4 to NO_3; allowing loss of phytotoxic volatile compounds and stabilisation of the microbial community. This tertiary phase also helps to the breakdown and transformation of humic substances and lignin by mesophilic fungi and actinomycetes (Swan *et al*., 2002), furthermore this stage is vital in

determining whether the compost can be applied to arable land for crop/plant production (Ozores-Hampton *et al*., 1999).

The effectiveness of bioleaching is highly dependent on the physical, chemical and biological factors in the system including: carbon and oxygen supply, pH, temperature; pre-culture period and inoculant, microbial resistance, solid residue composition, solid liquid ratio, and bioleaching period (Bosecker, 1997).

In principle, *S. cerevisiae* has higher affinity for uranium, lead and mercury than Co^{2+}, Ni^{2+} or other metal ions. Currently, there is limited information about bioleaching using yeasts. There are many factors which could effects bioleaching capacity; including the states of *S. cerevisiae* (cell age); properties of metal ions; culture conditions such as pH, temperature, contact time, co-ions in solution, initial metal concentration, and micronutrients (Wang and Chen, 2006).

In the current study, assessing the efficiency of chemical leaching it was observed that addition of Gluconic acid gave a higher retained % yield when compared with other acids used in this study, this observation has been reported previously where inorganic acid correlated with increases in the yield (Neale *et al*., 1997). Also, Using commercial organic acid as a leaching agent has been shown to correlate with removal of heavy metals (Yang *et al*., 2008).

6.4.1 Fungal bioleaching

In the present study, the current experiment focused on the biological processes such as using various fungi and optimising conditions to optimise bioleaching defined here as % yield.

The S/L ratio was selected since in the literature it has been shown that optimum S/L ratio for bioleaching of MSW using *A. niger* was 10–20 g/L (MSW mass to culture medium volume) (Bosshard *et al*., 1996; Xu and Ting, 2004; Wu and Ting, 2006; Yang *et al*., 2008). Increasing the solid in the reactor did not

improve leaching presumably due to this fungi's sensitivity to the presence of heavy metals For this reason bioleaching technology has been difficult to apply on an industrial scale (Yang *et al*., 2009) and use of a higher mass content of fly ash leads to lower extracted heavy metals (Yang *et al*., 2008).

Selecting the best fungi for leaching heavy metals using artificial media, mechanisms which lead to microorganisms facilitating mineral biooxidation and bioleaching have been termed contact and non-contact (Rohwerder *et al*., 2003). Adding microbes has been shown to facilitate the process by localising ferric ions in the exo-polymeric material at the mineral surface (Sand *et al*., 2001). Furthermore, fungi have been shown to interact with metals by blocking the functional group of enzymes depending on the type of metal and the organism (Gadd and Griffiths, 1977; Gadd, 1994; Aung and Ting, 2005) leading to an increase in the tolerance of fungi for bioleaching (Yang *et al*., 2009).

In the present study, it was determined that *A. niger* was the best fungi in terms of bioleaching heavy metals, this has been observed previously (Mulligan *et al*., 2004; Aung and Ting, 2005). It has been shown that *A. niger* is more resistant than other fungi for bioleaching (Bosecker, 1997). Organisms under environmental stress have been shown to develop various mechanisms in order to cope with adverse conditions. In fungi severe stresses has been shown to be tolerated by inherent physiological characteristics or by adaptation through temporary alteration in their developmental pattern as demonstrated in detoxification of fly ash MSW using *A. niger* (Bosshard *et al*., 1996; Nazareth and Marbaniang, 2008). Therefore, *A. niger* is a heterotrophic fungus which has become associated with improved tolerance to unfavourable conditions (Brandl *et al*., 2001; Krishna *et al*., 2005; Ren *et al*., 2009).

In the present study, two bioleaching methods were compared (one and two way method), results indicated that element retained yield % following a one-step bioleaching method was better than in two step bioleaching. In assays using 10

g/L MSW concentrations these results agreed with reports published previously (Yang *et al*., 2008). Use of a two-way method was characterised by relatively poor bioleaching and a possible mechanism for this is that calcium oxalate hydrate precipitation in two-step bioleaching was similar to that of one-step bioleaching, however, the on-set of the distortion and swollen structure of the hyphae occurs earlier in a two-step bioleaching when compared with one-step leaching. Despite this, the bioleaching effects appear insignificant because of production of organic acid during a two way method before addition of fly ash and exposure to toxic conditions (Xu *et al*., 2014).

Artificial medium have been applied over many years for the removal of metals from MSW and other contaminated samples. However, the cost of pure glucose or sucrose for the process can raise the cost of compost or fertiliser (Weber *et al*., 2005), for this reason in this study, two non-artificial media were studied; (clay and sorghum). In terms of artificial medium the composition can vary especially for glucose (24-42 g/L) (Atlas, 1995; Krebs *et al*., 1997)

The pH value is also a very important parameter in determining the bioleaching efficiency since the pH changes at different substrate densities (Qu *et al*., 2013).

In the present study, results showed that clay medium inoculated for pH 5.5 correlated with better performance in terms of getting lowest retaining yield % of elements in MSW when compared with other media so the clay showed the better performance , probably because clay's ability to adsorb metals, some researchers such as (Alva *et al*., 2002) have shown that the main attempts for the effective application of red mud are: soil amendment to prevent nutrient loss and reduce heavy metal availability; adsorbents for removal of heavy metal ions and metalloid ions (Cengeloglu *et al*., 2006; Qu *et al*., 2013), in addition clay being mostly composed of clay minerals, stands out because of its high potential to bind heavy metals (Sheoran *et al*., 2009; Fijałkowski *et al*., 2012). Soils having granulometric composition which are characteristics for clay, silt and dust, and those with a high content of organic matter, have a high sorption capacity and a

strong ability to bind metallic elements. Generally, all soils with high sorption capacity for cations, i.e. land containing a large amount of clay minerals, have the ability to accumulate metallic elements (Gworek *et al*., 2000; Kavamura and Esposito, 2010; Fijałkowski *et al*., 2012).

Also the result did not agree with some other researches which showed clay had a negative effects on metals removing from waste, this may be due to minerals present in clay that may migrate with the leaching solution to the bottom of a heap and restrict water flow and access of oxygen (Olson *et al*., 2003); also clay contains metals and has dense particles which have been shown to inhibit fungal growth (Wu and Ting, 2006). Addition of clay also changes the pH of the medium to a more alkaline solution if the medium become alkaline extraction yield has been shown to be reduced (Wu and Ting, 2006).

In assays with a starting pH of 2.5, addition of sorghum medium correlated with lowest retained yield % (best performance) may be because of sorghum hydrolysate is rich in carbon source and its concentration is important for bioleaching as it not only supports microbial growth but also influences the yield of organic acids which are potentially active compounds in bioleaching (Grewal and Kalra, 1995). In the case of *A. niger* for instance, sucrose as a carbon source has been reported to give a higher yield of organic acids than other sugars such as fructose, lactose and galactose (Hossain *et al*., 1984). However, paucity of organic carbon, nitrogen and micronutrients will also limit microbial growth (Hamdy and Williams, 2001; Thiyagarajan *et al*., 2009; Gräfe *et al*., 2011).

In the present study assays with a starting pH of 2.5 were better than 5.5, this effect may be due to the fact that glucose oxidase has an optimum pH at 4.5–6.5 and is inactivated at a pH below 3.0 (Dellweg, 1983). Also, the presence of fly ash increases the pH of the system, and thus activates the enzyme glucose oxidase which converts glucose to hydrogen peroxide, and finally hydrolyses it to gluconic acid (Qu *et al*., 2013).

The study was examined that the bio-produced organic acids was the most important leaching agent in the bioleaching process using fungi (Aung and Ting, 2005). Fungal bioleaching is based on four mechanisms: acidolysis, complexolysis, redoxolysis and bioaccumulation (Schinner and Burgstaller, 1989; Bosshard *et al*., 1996). As the first three processes are mediated through the secondary metabolites produced by the fungus, it is important to determine these metabolites (organic acids) in order to better understand the bioleaching mechanisms (Hong *et al*., 2000). These might also represent valuable compounds if present at high enough levels. Commonly *A. niger* is used for the production of organic acids (citric and gluconic acid) for commercial purpose and production is significantly related to pH and presence of metals in the media (Grewal and Kalra, 1995; Roukas, 2000; Karaffa and Kubicek, 2003). Mohapatra, Pradhan *et al*. (2009), suggested that leaching efficiency depends on the extent of the production of organic metabolites, which decrease the media pH, which then induces the dissolution of metals (Gholami *et al*., 2012). The present study showed that gluconic acid to be the most effective organic acid produced the study agree with (Bosshard *et al*., 1996), Concentration of oxalic acid was quite low in all cases and this has been seen previously (Kubicek *et al*., 1988) and the lowest acid production was citric acid (Xu *et al*., 2014). Assays with *A. niger* at a starting pH of both 5.5 and 2.5 were characterised by production of gluconic acid as the highest by product, then oxalic acid and finally citric acid. At pH 6 *A. niger* causes to accumulate gluconic acid by inhibiting citric acid secretion (Grewal and Kalra, 1995). Studies have shown that using *A. niger* and fly ash produced gluconic acid instead of citric acid due to the inhibition of enzymes in the TCA cycle by manganese. Addition of *A. niger* has been shown to be especially effective for the leaching of lead from fly ash (Bosshard *et al*., 1996). The presence of metal ions, such as Zn^{2+}, Mn^{2+}, Fe^{2+} and Co^{2+} in fly ash has been shown to inhibit the accumulation of citric acid and promote formation of gluconic acid (Burgstaller and Schinner, 1993; Lee and Pandey, 2012), in

particular the presence of manganese is reported to strongly inhibit citric acid production (Grewal and Kalra, 1995). When fly ash was inoculated with spores in an acid medium the alkaline calcium oxide present in the ash was hydrated to form calcium hydroxide which increased the pH, the alkaline medium activates glucose oxidase which converts glucose to gluconolactone which is finally hydrolyzed to gluconic acid (Dellweg, 1983). Furthermore, the preliminary study on the characterisation of MSW in the present study, showed that the pH was 6.8.

Assays revealed that use of a starting pH of 2.5 was characterised by increased production of organic acid when comparing with assays with a starting pH of 5.5, especially in terms of gluconic acid. Organic acid production increasing as pH drops has been observed previously (Bosshard *et al*., 1996; Wu and Ting, 2006). Organic acids are protonated at low pH and hence are membrane permeable, inside the neutral pH cytoplasm, the molecule dissociate, releasing protons and acidifying the cell (Suzuki *et al*., 1999).

There was a reduction in gluconic acid and citric acid levels towards the end of the incubation period. This may be associated with the consumption of the acid in the bioleaching process as well as a drop in the organic acid production, caused by the decrease in the fungus growth rate. It was noted that there was an increase in metal leaching as organic acid production increased (mainly gluconic acid). Therefore, depletion of bioleaching agents could be one reason behind a decrease in the rate of metal extraction as has been suggested previously (Aung and Ting, 2005).

6.4.2 Yeast bioleaching

This study revealed that *S. cerevisiae* displays tolerance to various metals as has been reported previously by (Wang and Chen, 2006) and this has highlighted the potential for the use of *S. cerevisiae* as an agent for removing these metals from MSW.

In this study, concentrations of Cs^{+} and Ca^{2+} in MSW ranged from 0.0032- 517.6 mg/L, respectively. The study also revealed that presence of Cu^{2+}, Fe^{2+} and Pb^{2+} at high concentrations correlated with a reduction in yeast metabolic rates. In addition, an increase in number of metals decreased bioleaching process due to number of metal ions competing for the same binding sites available on the surface of the biomass (Iram *et al*., 2012; Thippeswamy *et al*., 2014).

Both single and two-step processes have been applied for bioleaching using fungi; thus in this study the two methods were also applied for yeast bioleaching. The results revealed that use of a two way method was better than the one way method. There's no published work on the optimisation of bioleaching processes using yeast. One advantage of the two stage method is that the bioprocess and the chemical processes are independent and this makes it possible to optimise each process independently to maximise productivity (Mishra and Rhee, 2010). Also use of a two-step way process permits separate enhancement during each stage, with the biological oxidation stage being fast and effective (Veglio and Beolchini, 1997). In addition, metals from fly ash were bioleached more rapidly using a two-step bioleaching approach compared to a one-step bioleaching approach, resulting in earlier formation of calcium oxalate hydrate, which leads to more rapid decrease in pH occurred in two-step bioleaching since organic acids were already present in the medium prior to the addition of fly ash. However, the addition of fly ash after fungal germination in a two-step bioleaching effectively reduces the toxic effects on the fungal growth, and accelerated the bioleaching process (Wu and Ting, 2006; Xu *et al*., 2014)

With regards to optimising media for bioleaching, use of medium one correlated with better performance when comparing with use of an artificial medium, first medium has been shown to be a natural yeast growth medium (Greetham *et al*., 2014), while in the second medium some minerals have been added which caused an increase in the concentrations of metals or elements and led to less bio-leaching of heavy metals.

Results comparing different yeast strains revealed that *S. stipitis* gave the best performance in terms of leaching metals, this result agrees with published findings, which found that some yeast strains (vacuoled efficient mutants) were better than wild type *S. cerevisiae,* in terms of leaching some metals (Ramsay and Gadd, 1997). Studies have revealed that *Candida* sp. and *K. marxianus* were more efficient than *S. cerevisiae* or *S. pombe* for heavy metal resistance and copper (II) bioaccumulation at higher copper(II) concentrations (Volesky, 1994; Dönmez and Aksu, 1999). While, this study's findings did not correlate with the observation that that *S. cerevisiae* produces a dark melanin pigment and extracellular polymers whose presence as part of a defence mechanism can reduce the toxic effect of heavy metals (Iram *et al*., 2012).

Experiments comparing initial pHs and media for bioleaching of heavy metals from MSW determined that starting the bioleaching (retained yield %) at pH 5.5 was better than at a pH of 4. Bioleaching pH media is consider an important environmental factor which effects yeast performance, site dissociation of the biomass' surface, effects on the solution chemistry of the heavy metals (Esposito *et al*., 2002; Wang, 2002). The improved performance at pH 5.5 could be correlated with higher metal solubility and agreed with a study which revealed that by increasing pH the bio-absorbitive capacity of metals also increased (Volesky, 1990).

In addition, high metal bio-sorption was obtained with an initial metal concentration ranging from 50-100 mg/L at 30 °C temperature and pH 5.0 (Özer and Özer, 2003). Generally, for different bio-sorption systems for metal ions, optimal pH differs depending on the metal e.g. the optimal pH value bio-sorption by *S. cerevisiae* for copper is 5–9, and 4–5 for uranium (Volesky, 1990), and in general the optimum bioleaching pH is above pH 5. However, when pH increases over optimal level, bio-sorption capacity has been shown to decrease which may be due to the fact that the efficiency of the proton at the binding site of yeast is much smaller than that of the metal ion comparing with low pH so the

electrostatic attraction to negatively charged functional groups may be one of the specific bio-sorption mechanisms at higher pH's (Vianna *et al*., 2000; Mapolelo and Torto, 2004). It has been observed that cell wall ligands were closely associated with the hydronium ions [H_3O^+] and restricted the approach of metal cations as a result of the repulsive force at low pH (Özer and Özer, 2003).

While, at pH 5 divalent positive ions could interact with negatively charged groups within the biomass. On the other hand, the outer layer of the cell wall of *S. cerevisiae* consists of coat protein, which can cause a charge through dissociation of ionisable side groups on amino acids (Johncy *et al*., 2010), also inorganic ions such as chloride, may form complexes with some metal ions and thereby affect the sorption process (Price *et al*., 2001). Using higher pH's correlates with higher consumption of hydroxides and precipitation of other metals (Smith *et al*., 2003). Generally speaking, a low pH value is favourable for bio-sorption (Wang and Chen, 2006).

Anions (carbonates, chlorides, fluorides, phosphates and sulphates) in solution can complex metal ions and thus reduce or inhibit their adsorption by *S. cerevisiae* (Hughes and Poole, 1991). Redox potential can also affect the speciation of a given element; for example, chromium exists as Cr(VI) or Cr(III) according to the EH of the solution. Presence in solution of other cations besides the metals of interest can reduce heavy metals accumulation by biomass, by competition for binding sites on yeast cell wall (Ferraz and Teixeira, 1999; Machado *et al*., 2009).

With regards to the selection of the best medium from the current study, it is suggested revealed that presence of artificial medium and *S. stipitis* at pH 5.5 recorded lowest retained yield % because of addition of glucose and other trace elements could enhance cells growth in a system, and facilitate metal bio-sorption (Wang and Chen, 2006). There are some studies that have focused on effects of glucose on bioleaching rates which have revealed that treatment of the yeast cells with glucose increased the amount of Cu^{2+}, Cr^{6+}, Cd^{2+}, Ni^{2+} and Zn^{2+}

removed from electroplating effluent by living cells of *S. cerevisiae* (Stoll and Duncan, 1996).

Direct addition glucose in to the yeast effluent mixed solution had no effects on the amount of bioleaching and this could be a possible reason for the sorghum medium having more effects than the artificial medium in terms of leaching metals from MSW. Furthermore, it has been revealed that addition of cysteine, glucose, ammonium sulphate, phosphate and ammonium chloride in the fermentation media increases uptake of Cr(VI) (Goyal *et al*., 2003). Generally different nutrients clearly led to different functional groups in the corresponding cell surface (Wang and Chen, 2006).

Toxicity characteristic fungal and yeast bioleaching process, are shown in Table 6.9 showed that yeast process almost hit the applicable environmental standards, the table shows MSW before and after the bioleaching process were compared with the identification standard for hazardous wastes identification for extraction procedure toxicity (State Environmental Protection Administration, 1996) and the standard for pollution control on the security landfill site for hazardous wastes (State Environmental Protection Administration, 2001) set by State Environmental Protection Administration, China.

Table 6.9 A comparison of the optimized bioleaching condition results using fungi and yeast against the regulatory level (mg/kg)

	Substrate /standard (mg/kg)		Cd^{2+}	Cu^{2+}	Cr^{6+}	Hg	Ni^{2+}	Pb^{2+}	Zn^{2+}	References
Standards	**UK Compost standards**		1.5	200	100	1	50	200	400	BSI (2005)
	Italian compost standards		3	300		3	100	280	1000	WRAP(2002)
	Green wastes compost		1.4	51	46	0.5	22	87	186	WRAP(2002)
	Bio-waste compost		0.9	96	26	0.6	24	86	289	WRAP(2002)
MSW sample	**MSW**	**Un-pretreated (original)**	33.9	1844.9	16	*	93.4	161.2	612	
	MSW	**water**	22.6	344.5	9.9		26.5	110.6	370.1	
	Sorghum Med. pH 2.5	***A. niger***	10.8	504	5.8	*	15.4	69.1	137.18	
	Artificial Med. 5.5	***S. stipitis***	4.8	2.6	6.4	*	18.7	4.6	290.8	

Chapter 7 Conclusions and Future Work

7.1 Conclusions

From the results obtained the following conclusions for the first batch of MSW can be determined, the physical characteristics are that the MSW was slightly acidic, EC was lower than EC values reported elsewhere, and that the moisture content was very low due to autoclaving the sample, and as a consequence the total amount of solid was quite high. While, for the last two batches the results we really different, due to the preservation and long storing condition leading to samples with inherently higher moisture content and lower sugars indicating contamination by microbes. Chemical characteristics revealed that the amount of cellulose, hemicellulose and lignin were, on average, in line with the normal range of MSW for the first batch of substrate, while for the second and third were quite low. Lipid, protein and total nitrogen values for three batches were almost in the same range found in previous studies. Determination of major elements revealed that the concentrations for the first batch were higher than last two batches. While, for minor elements again the values for the first batch was higher than last two batch, also Fe^{+3} and AL^{+3} elements recorded highest value among other elements. There were high concentrations of heavy metals indicating post-treatment would be required.

Also, in this work, we have investigated the feasibility of using MSW as a feedstock for production of fermentable sugars using an EH method. Firstly, MSW has received optimal 30 FPU/g Cellic® CTec2 showed 30 % release of available glucose. Increasing solid loading to those approaching commercial practice reduced efficiency but final glucose concentration was still too low. Secondly, Explored methods to reduce recalcitrance-surfactants, some enhancement but insufficient to warrant their use commercially. Thirdly, Hydrothermal pre-

treatments- hot water, acid and alkali were generally, not effective and actually decreased yield except for minor improvement with alkali pre-treatment. Fourthly, the reasons for this persistent recalcitrance are unclear. MSW has been subjected to a steam treatment that could be considered as a "pre-treatment" this could account for its higher enzyme saccharificiation efficiency (compared to untreated LCM biomass) but the conditions may have made the residual cellulose more recalcitrant. One possible is the drying method employed since rapid drying at high temperature has been shown to increase cellulose recalcitrance. Finally, improved saccharification may result from modification to the initial steam treatment regime.

The next experiment applied using MSW substrate was cellulase enzyme production, results here show that SSF using MSW as a substrate could represent an economical method for the production of cellulase enzyme with low operational costs as MSW is a cheap and abundant substrate. *T. reesei* recorded the highest production of cellulase enzyme at 30 ^{o}C with a 168 hr incubation period using 60 % moisture content. Crude enzymes derived from this SSF of MSW were able to release sugars from MSW at a rate similar to that of a commercial enzyme preparation.

The final experiment applied using MSW was to examine how growth of microorganisms such as fungi or yeast could correlate with removal of heavy metals from MSW. It was determined that the best fungus for bioleaching of elements was *A. niger*. The *A. niger* using a one-step method and an artificial medium. Artificial medium was chosen as a means of reducing the cost of bioleaching rather than using pure glucose assays using media such as hydrolysates extracted from sorghum, and clay were characterised by good bioleaching and assays to determine optimal pH revealed that pH 5.5 was optimum for clay to remove metals from MSW whereas pH 2.5 was optimum for

addition of sorghum showed a lowest retained yield %. Elements bioleaching (% yield) has been calculated during the process and correlated with production of organic acids as by-products, assays determined that gluconic acid as a highest concentration when comparing with the other organic acids. Use of H_2SO_4 acid as a chemical leaching agent was characterised by better retained yield % than other organic acids.

Bioleaching with yeast has also been studied which showed better performance in compare with fungi, the process was determined that using a two-way methods and *S. stipitis* strain gave the best results then to increase the yield two ranges of pH has been applied pH 5.5 and 4.

Using artificial medium correlated with better performance at pH 5.5, while at pH 4 correlated with sorghum medium which showed lowest retained yield %

Comparison between fungi and yeast bioleaching data revealed that addition of yeast correlated with better retained yield %. Furthermore, using yeast is safer and easy grow and could be separate from MSW by centrifuge.

Generally bioleaching process using either fungi or yeast is better than chemical leaching process in terms of getting lowest retained yield %

7.2 Future work

There was a large variation in the composition of the three batches of MSW. This may have been related to the relative ages of the batches. Batch 1 was received as fresh as possible from the autoclave and was stabilised by drying. The other two had been stored for a considerable time. Analysis of a number of fresh batches (direct from the autoclave) should be carried out to determine the actual extent of variability in practice and explore ways to improve standardisation of the MSW prior to processing. This was not possible in the present study as the production plant was overseas. However, a pilot facility in the UK has recently been established and fresh fibre will now be available.

MSW has received has a low digestibility and this cannot be improved by pre-treatments. This is probably related to the conditions in the autoclaving step (165 ^{o}C). This might be due to plastic in the MSW melting in the autoclave and "coating" the fibres resulting in them being inaccessible to the enzymes. Thus future work might be to look at the effect of changing the conditions in the autoclave. Perhaps reducing the temperature to 120 or 140 ^{o}C or increasing the retention time in the autoclave.

Only very low solid loadings were able to be tested in this study. If a suitable high viscosity enzyme reactor were available then the impact of higher solid loadings say unto about 20 % w/v should be investigated.

Only very limited studies were carried out looking at the ferment ability of the MSW hydrolysate. Once a high loading (more commercial) system was in place the fermentation of the hydrolysate could be examined. In particular to extend the observations on whether there are any inhibitory compounds being produced that may impact the fermentation efficiency. If inhibitors are found to be present then some washing of the MSW may be required at some stage in the process.

The SSF for the production of enzymes could be scaled up. The impact of variation in MSW composition examined. The RNAseq data could be further mined for other commercially useful enzymes and then the actual activities of these monitored.

The use of the MSW hydrolysate, or SSF could be investigated for products other than fuels which may have higher value e.g. succinate.

Finally, the prospect of combining the processes in the thesis in a bio refining process could be explored. Thus MSW could be used to generate enzymes for its own saccharification. The hydrolysate used for fuel or chemical production and the residue for combustion or as compost.

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Appendix

Appendix A

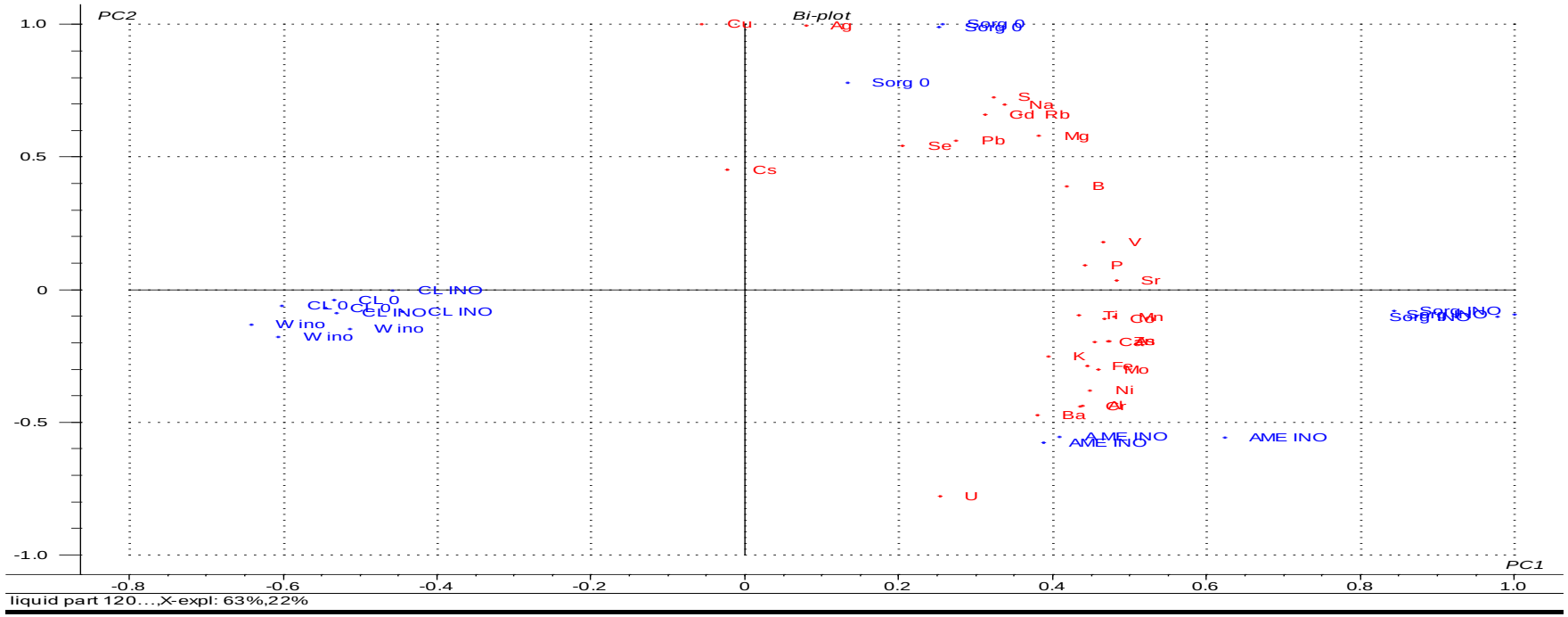


Figure A.1 PCA analysis for the amount of leached elements into the liquid including heavy metals with a starting pH of 5.5 and addition of artificial, clay or sorghum media for 15 days duration time

(AME INO= Artificial medium inoculated; W ino= sterilised water inoculated; Sorg INO= Sorghum medium inoculated; Sorg 0 =Sorghum without inoculation; CL INO= Clay medium inoculated; CL 0 =Clay without inoculation)

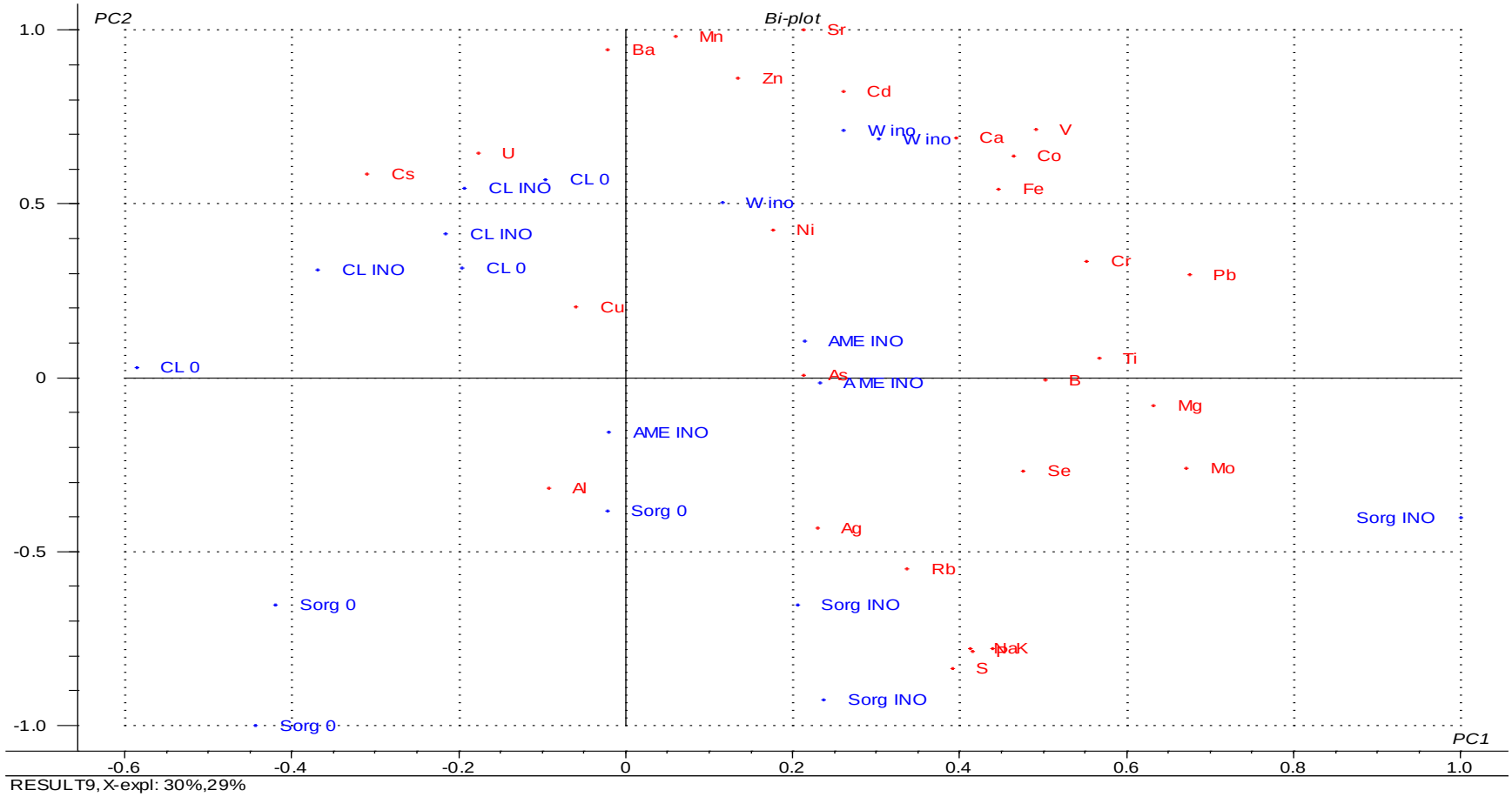


Figure A.2 PCA analyses for the amount of retained elements in the solid fraction of MSW with a starting pH of 5.5 and addition of artificial media, clay or sorghum using *A. niger*

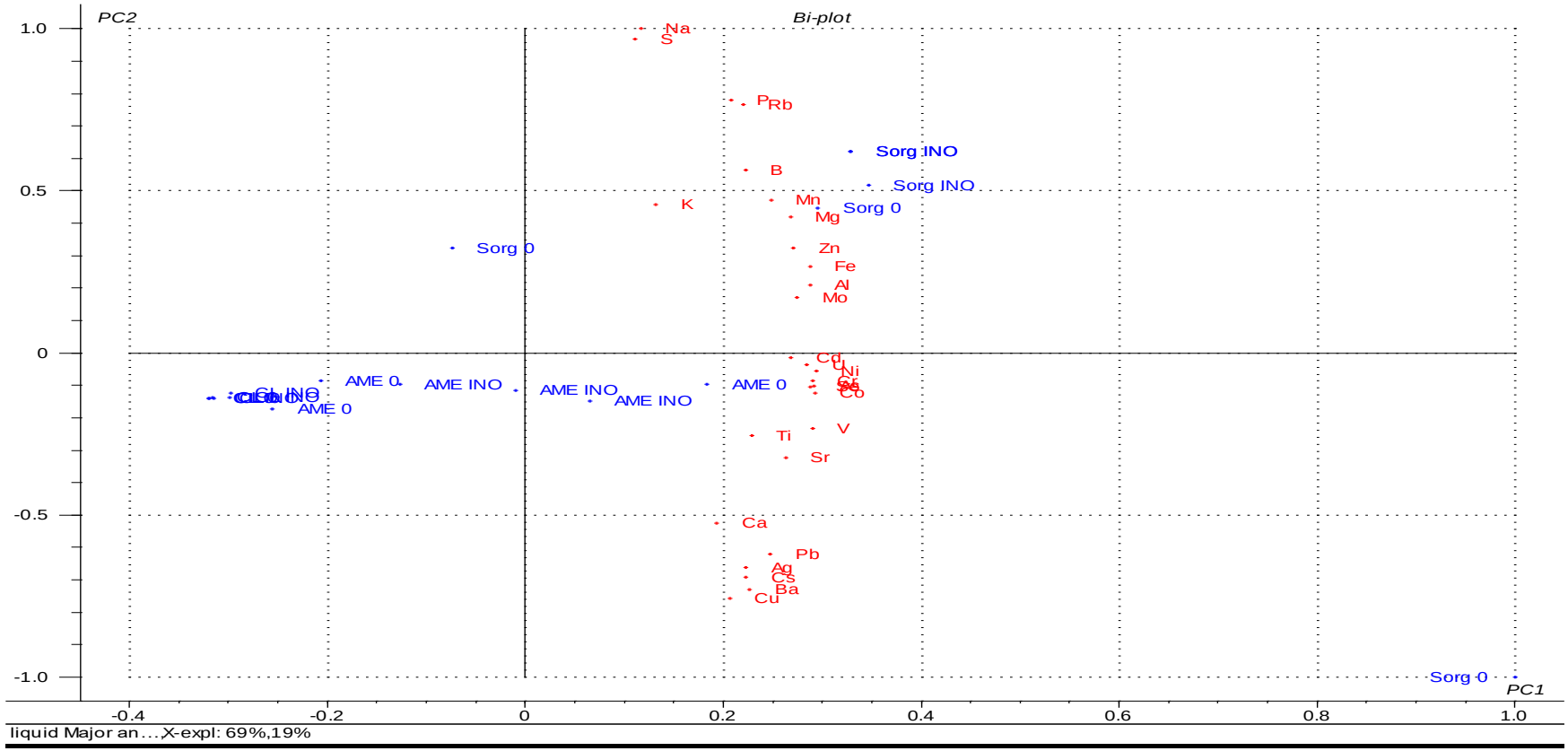


Figure A.3 PCA for the amount of leached elements into the liquid part from MSW with a starting pH of pH 2.5 with addition of artificial, clay and sorghum media, in a presence *A. niger* for 15 days

AME INO= Artificial medium inoculated; AME0= Artificial medium without inoculation; Sorg INO= Sorghum medium inoculated,; Sorg 0 =Sorghum without inoculation; CL INO= Clay medium inoculated; CL 0 =Clay without inoculation .

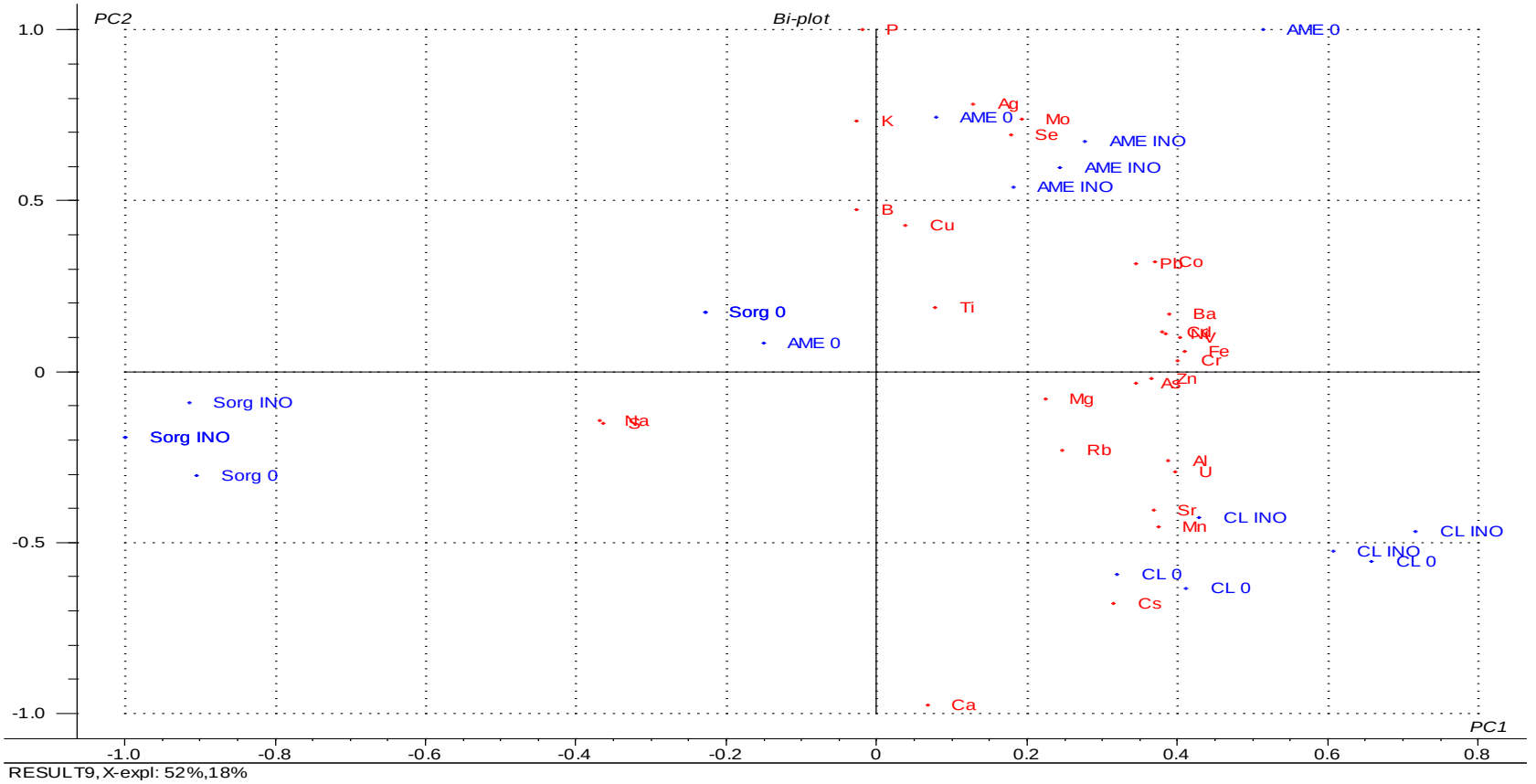
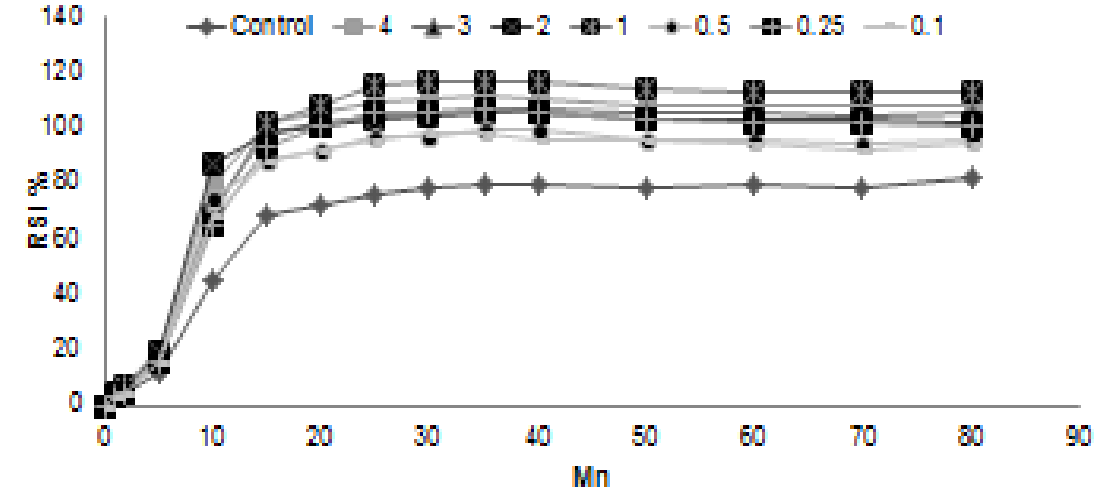
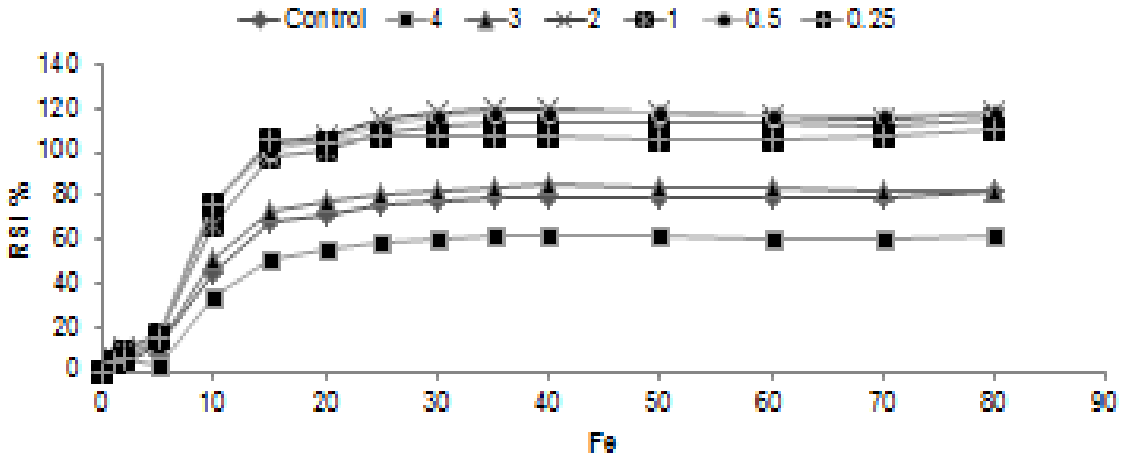
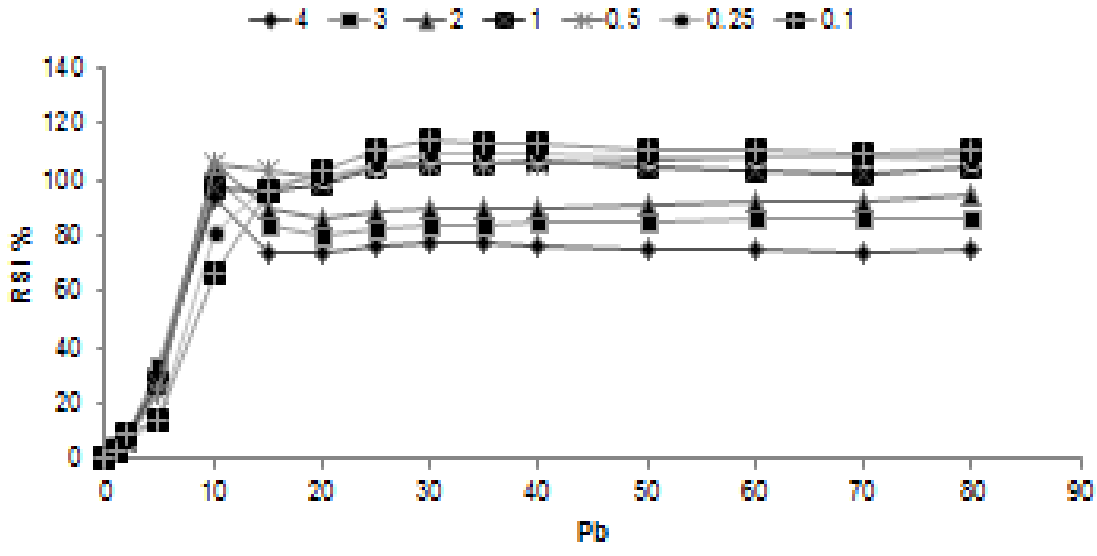


Figure A.4 PCA of the retained elements amount in the solid fraction of MSW with a starting pH of pH 2.5 using artificial, clay and sorghum media in a presence of *A. niger*


Control
4
3
2
1
0.5
0.25
0.1
RSI %
Mn


Control
4
3
2
1
0.5
0.25
RSI %
Fe


4
3
2
1
0.5
0.25
0.1
RSI %
Pb

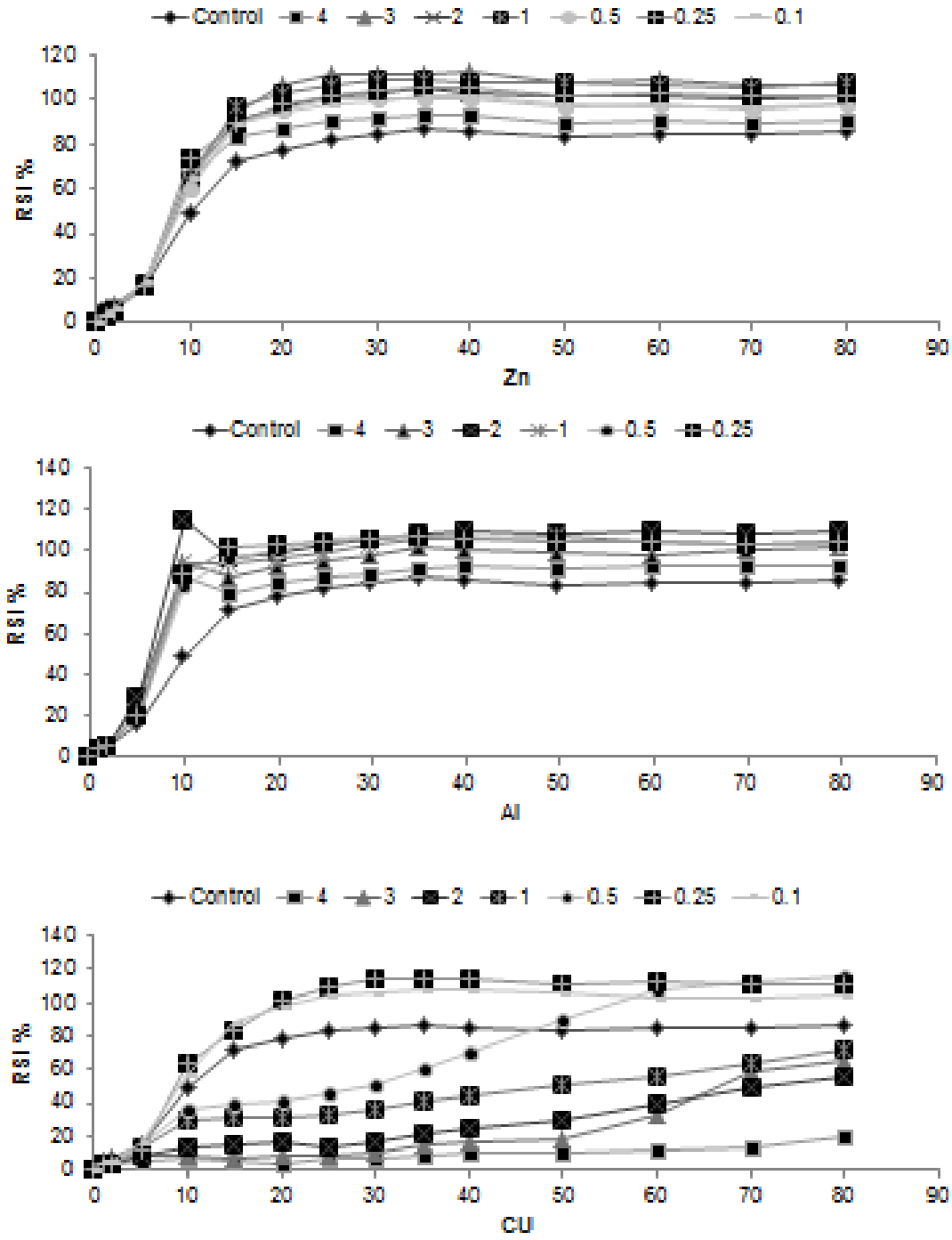


Figure A.5 Effect of some elements stock solutions ranges from 0.1 to 4 mM on S. cerevisiae metabolism performance using microarray (A) Mn^{2+} (B) Zn^{2+} (C) Al^{3+} (D) Pb^{2+} (E) Fe^{2+} (F) Cu^{2+}

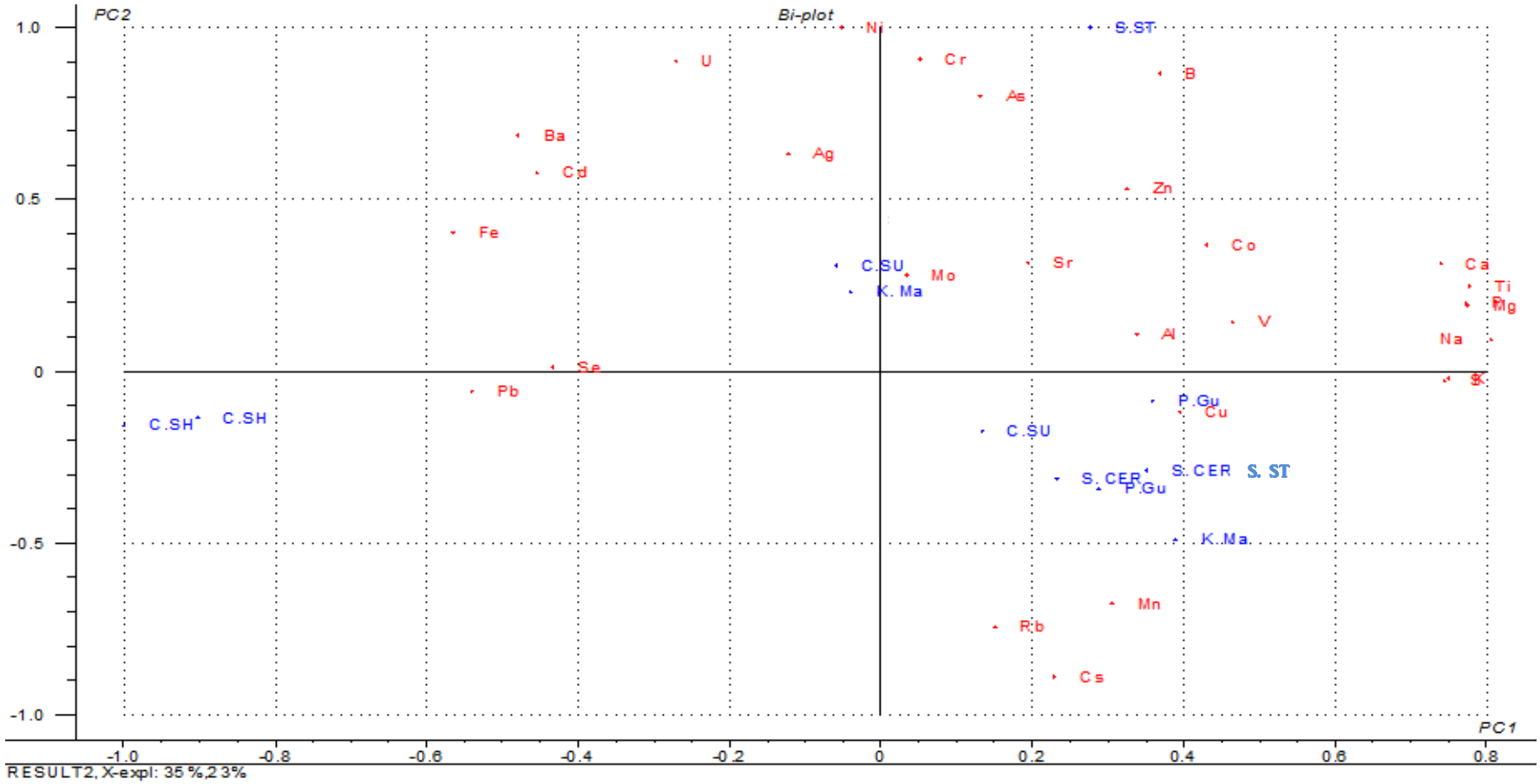


Figure A.6 PCA analysis for the amount of leached elements into the liquid fraction of MSW using various yeast strains at pH 5.5 with medium number one and two way method

(Ref= reference strain *S. cerevisiae* ; K.Mm= *K.marxianus*; S.ST= *S. stipitis*; C.SU= *C. succiphilia*;P.Gu= *P.gulliermondii* ; and C.SH= *C. shehatae*)

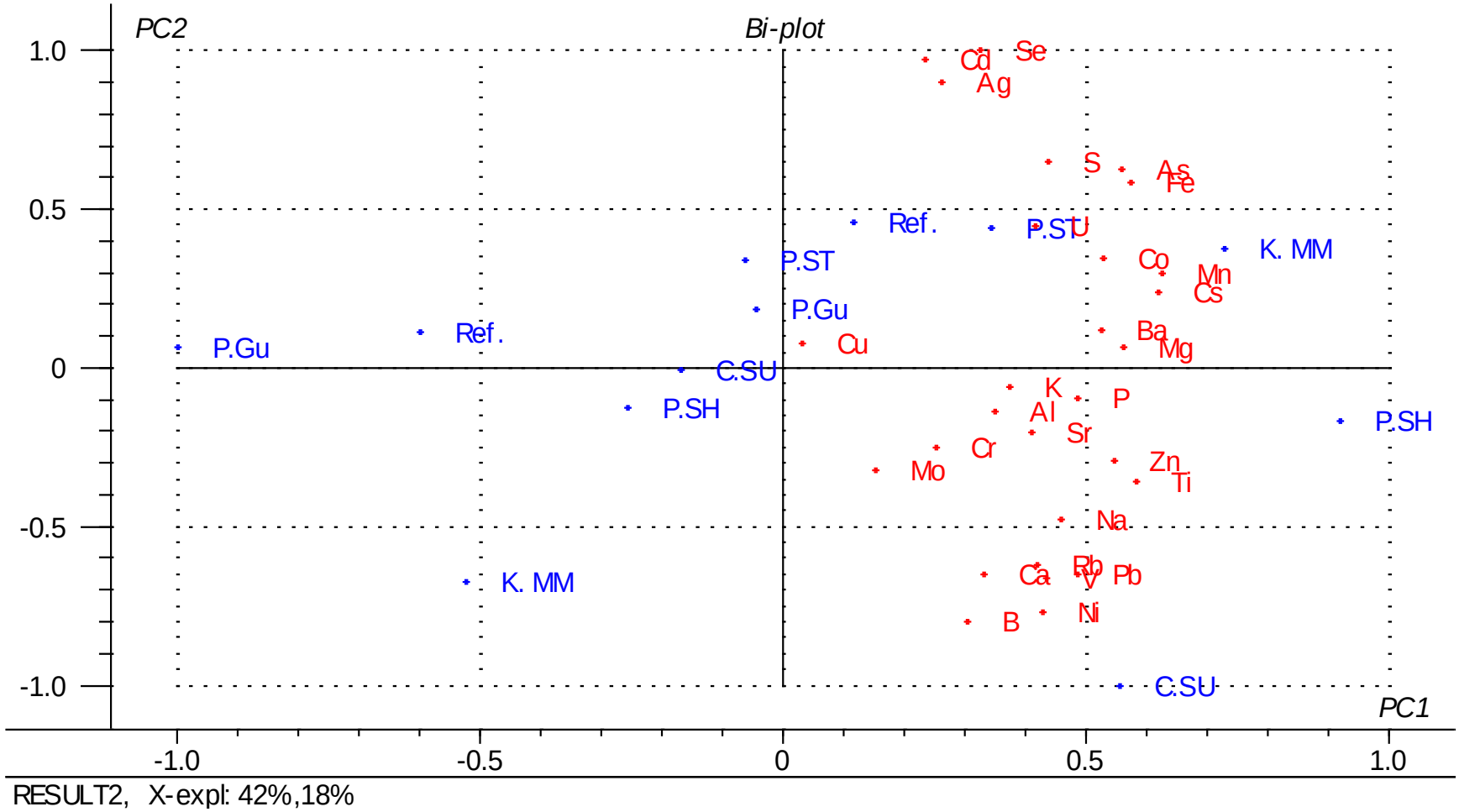


Figure A.7 PCA for the amount retained in the solid fraction of elements in a MSW solid residue using various yeast strains at pH 5.5 with medium number one and two way method

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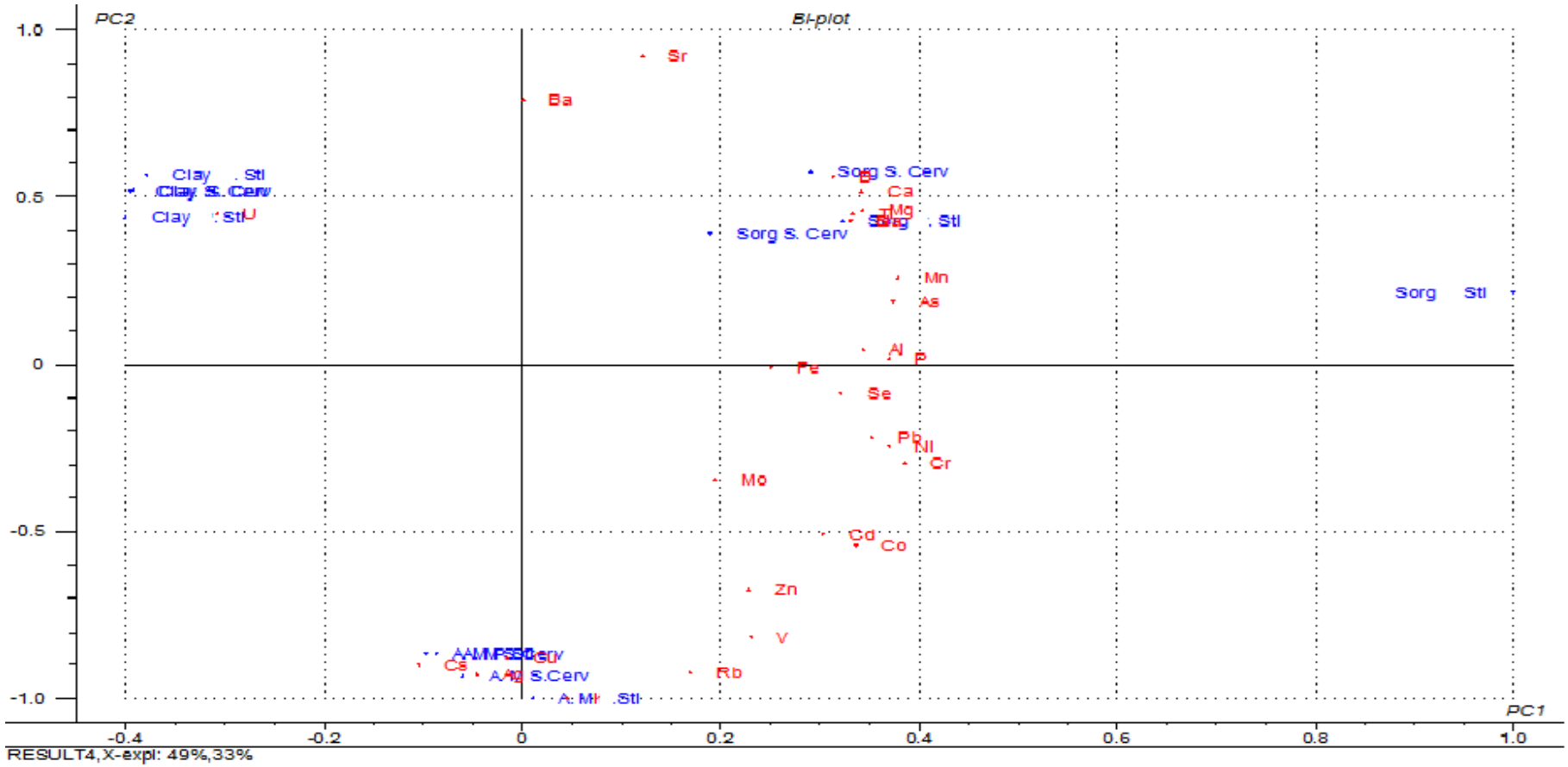


Figure A.8 PCA analysis for the amount of leached elements into the liquid part using three media with a starting pH of 5.5 in a presence of *S. cerevisiae and S. stipitis*

(A. Sti= Artificial medium strain *S. stipitis*; A.M S.cerv = Artificial medium strain *S. cerevisiae*; Sorg sti = Sorghum medium strain *S. stipitis*; Sorg *S. cerv*;= Sorghum *S. cerevisiae*; Clay sti = Clay medium strain *S. stipitis*; and Clay *S. cerv*;= Clay medium *S. cerevisiae*)

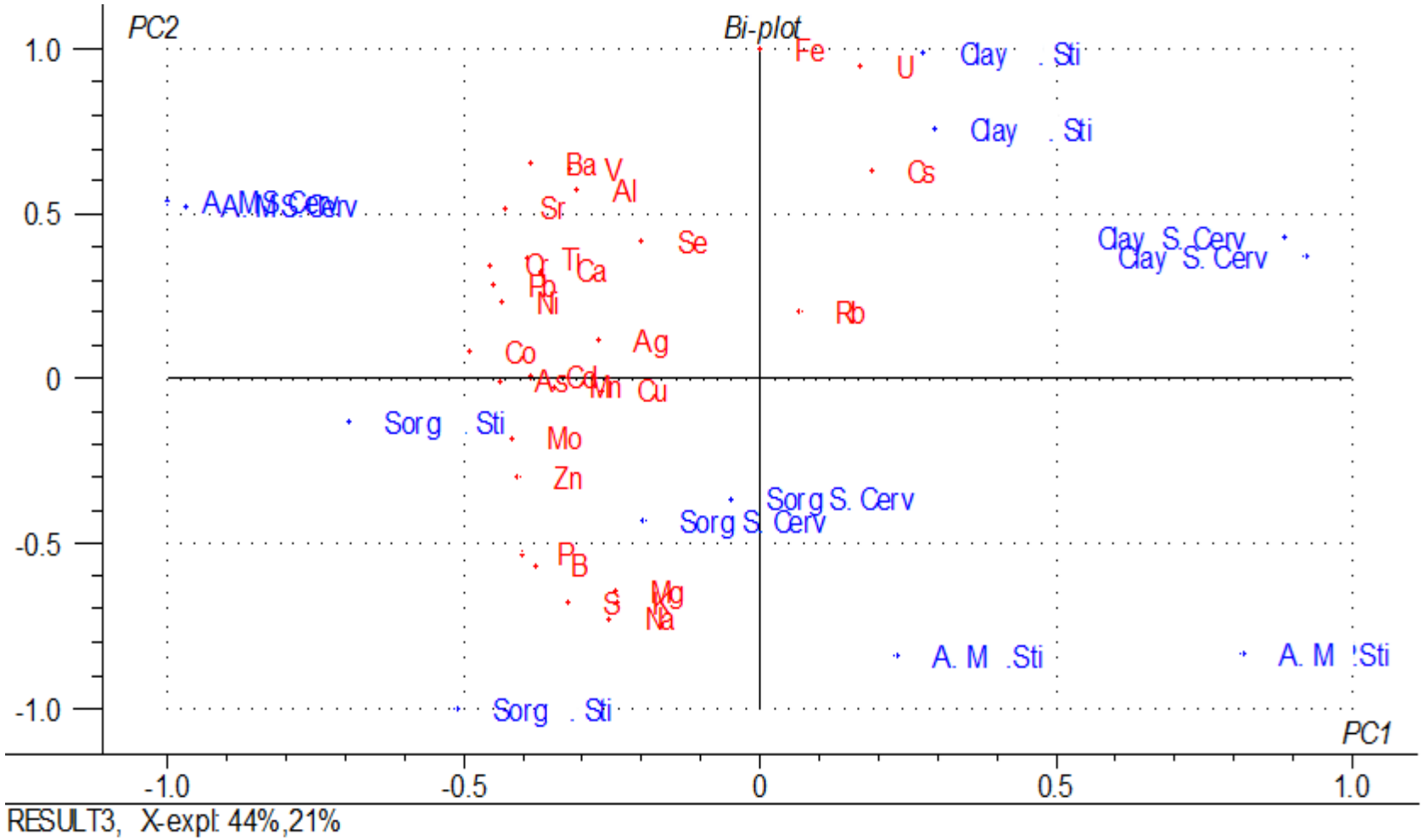


Figure A.9 PCA analysis for the amount of retained elements in the solid fraction using various media with a starting pH of 5.5 using *S. cerevisiae and S. stipitis*

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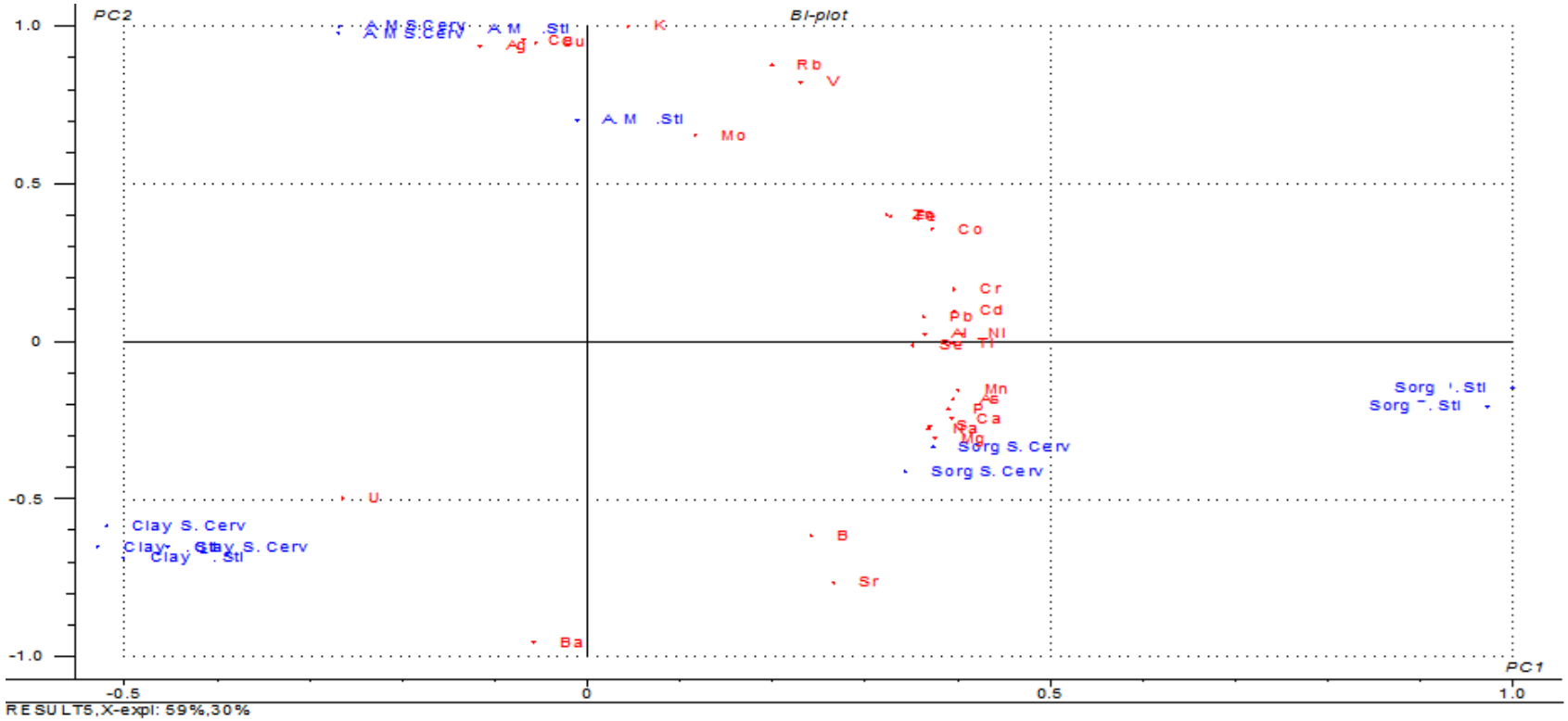


Figure A.10 PCA analysis for the amount of leached elements in a liquid part of MSW using three media at pH 4 in a presence *S. cerevisiae and S. stipitis*

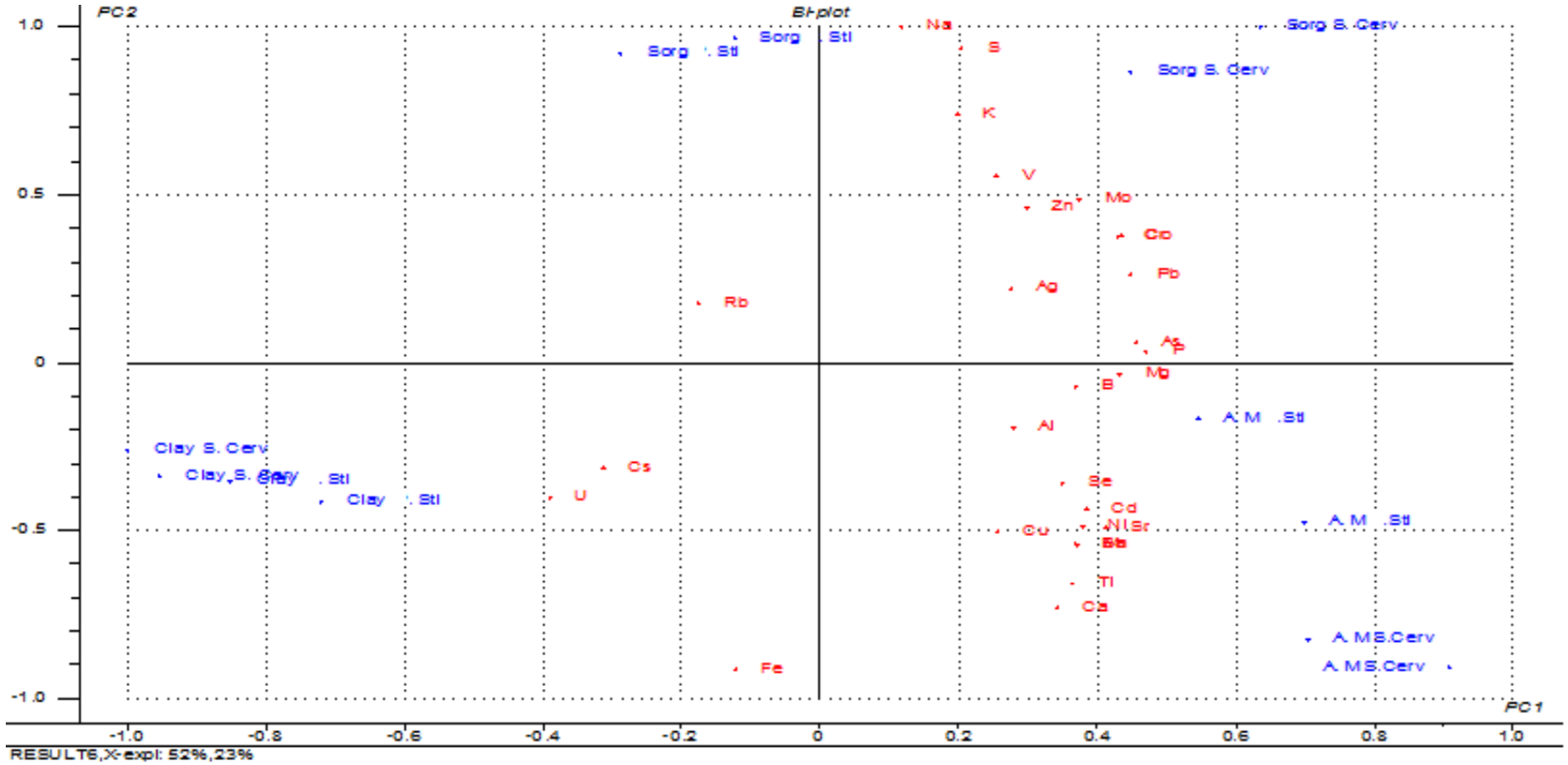


Figure A.11 PCA analysis for the amount of retained elements in the solid fraction of MSW using three media at pH 4 and in a presence of *S. cerevisiae and S. stipitis*