The Generation of Dual Purpose Wheat That Is High Yielding, Adapted To Climate Change And Provides Straw That Is Specifically Adapted For Bio-refining.

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ABSTRACT

The planet we live on is for all intents and purposes a closed environment with finite resources, which over the last few hundred years humans have managed to deplete at an alarming rate. As they take millions of years to form they will eventual run out, and because of this there is a clear need for a sustainable replacement for liquid fossil fuels. Wheat straw is an agricultural waste product that has the potential to produce 70 gallons of ethanol per ton of biomass. The aim of this work was to firstly identify variation in wheat straw tissue and growing conditions with regard to recalcitrance and sugar availability for bio-fuel production; this was carried out using the commercial wheat variety Xi19. Xi19 was fractionated into leaf, peduncle- both upper (stem above 10cm) and lower (stem below 10cm) fractions, and samples of "whole" un-fractionated straw. It was found that the only differences seen with regard to tissue type was between the leaf samples and all other fractions ($p < 0.05$). Xi19, Cordiale and Grafton grown in field conditions under differing plant growth regulator (PGR) and nitrogen applications were then analysed using leaf, stem and "whole" un-fractionated straw and it was shown that neither treatment had any significant impact on recalcitrance and sugar availability ($p > 0.05$).

Wheat is a member of the grass family that has been domesticated over thousands of years. This has caused erosion of the gene pool and, with this in mind, crosses have been created using ancient wild type plants with an aim of increasing genetic diversity. The second aim of this study was to analyse the

first progeny of these potential new wheat lines for traits such as enhanced sugar composition or reduced recalcitrance that improve the straws suitability for bio-refining. Results suggest that the genetic variation within this new population had increased dramatically and showed an impact on morphology, recalcitrance, and sugar availability following enzyme digestion (% available glucose recovered ranging from a low of 15% to a high of 34%). Thus the wild type relatives do appear to be providing traits that would be beneficial for the use of straw in a bio-refining process.

Water consumption for bio-fuel can vary from 2–10 gallons of fresh water for each gallon of bio-fuel produced depending upon the method used. The third aim of this work was to determine if sea water can be substituted for fresh water for each production step. This work showed that acid pre-treatments performed the same in both fresh and sea water, and that the use of sea water for the pre-treatment stage does not appear to effect subsequent enzyme hydrolysis ($p > 0.05$); Enzyme hydrolysis performed in sea water showed a significant reduction to that in fresh water ($p < 0.05$). The effects of salinity on metabolic growth of yeast showed no differences with or without the addition of 6% glucose ($p > 0.05$), and that during fermentation ethanol production was unaffected.

Impact Statement

The earth is a finite closed system that relies on equilibrium to sustain life. This fine balance between nature existed for millions of years before humans arrived, however as the human civilisation has advanced with technology so has the cost to the planet been great. It is now apparent that as a species we have ceased evolving and now change the habitat to fit around us. This has in the main been down to the use of fossil fuels that release large levels of CO_2 that had previously been locked away for millions of years. Since the industrial revolution, CO_2 levels in the atmosphere have increased exponentially, whilst this in itself is bad, the CO_2 levels in the ocean have also increased to the point that acidification is staring to affect some of the corrals, and if this continues the effect on the oceans will be irreversible. To add to this, vast areas of rainforest and jungles have been felled to provide land for agriculture. As the CO_2 levels increase, the earth is becoming warmer and impacting on Polar Regions causing sea levels to rise, which will have an impact on available land that can be used. Whilst this is happening, the human population is also increasing so there is a clear need for a sustainable energy source that does not compete with food and also does not encourage land use changes that could exasperate the problems. The ability to grow a single crop that is drought tolerant and resistant to pests, which can be used as both a food and also converted into liquid bio fuel (possibly with the used of sea water) would be a huge leap forward for humanity.

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TABLE OF CONTENCE

		Page
ABSTRACT		ii
IMPACT STATMENT		iv
ACKNOWLEDGMENTS		v
TABLE OF CONTENCE		vi
LIST OF TABLES		xii
LIST OF FIGURES		xiii
LIST OF ABRIVIATIONS		xviii
LIST OF PUBLICATIONS		xx
CHAPTER		
I	Introduction	1
	1.1: Need for bio-fuels.	1
	1.2: First generation bio-fuels.	3
	1.2.1: Bio-ethanol.	4
	1.2.2: Bio-diesel.	6
	1.3: Problems associated with first generation bio-fuels.	7
	1.3.1: Food competition.	7

1.3.2: Environmental impacts of first generation bio-fuel. 8

1.4: Second generation bio-fuels. 9

1.4.1: Feed stocks available for bio-fuel production. 10

1.4.2: Biomass structure. 11

1.4.2.1: Plant cell wall structure and development. 11

1.4.2.2: Cellulose. 12

1.4.2.3: Hemicellulose. 13

1.4.2.4: Lignin. 14

1.4.2.5: Plant cell growth and structure. 14

1.4.3: Accessing polysaccharides in bio-mass. 17

1.5: Methods for pre-treating biomass. 18

1.5.1: Physical pre-treatments. 19

1.5.2: Pyrolysis**.** 19

1.5.3: Chemical pre-treatments. 19

1.5.3.1: Acid hydrolysis. 20

1.5.3.2: Alkaline hydrolysis. 21

1.5.3.3: Organosolv process. 21

1.5.3.4: Ozonolysis. 22

1.5.4: Physico-chemical pre-treatments. 22

1.5.4.1: Steam explosion (auto hydrolysis). 23

1.5.4.2: Liquid hot water method. 23

1.5.4.3: Ammonia fibre expansion (AFEX). 24

1.5.4.4: Microwave pre-treatment. 24

1.5.5: Biological treatments. 25

1.6: Enzyme hydrolysis. 25

1.6.1: Enzymes required for lignocellulosic break-down. 26

1.6.1.1: Cellulose acting enzymes. 26

1.6.1.2: Hemicellulose acting enzymes. 28

1.7: Yeast and fermentation. 29

1.8: Enzymatic hydrolysis and fermentation. 31

1.9: Wheat as a lignocellulosic feed stock. 33

1.10: Genetic diversity in wheat. 38

1.11: Other Bio fuels. 40

1.12: Water consumption for bio-fuel production. 41

ii Materials and methods. 42

2.1: Wheat samples. 42

2.1.1: Wheat samples for method development. 42

2.1.2: Wheat samples to determine effects of growing condition. 43

2.1.3: Wheat samples to determine genetic influence. 44

2.2: Analysis of sugar composition. 46

2.3: Chemical pre-treatment of straw biomass. 48

2.4: Sea water Pre-treatments. 49

2.5: Digestibility assay. 50

2.6: Sea water enzyme hydrolysis. 51

	2.7: One pot pre-treatment and enzyme digestion.	52
	2.8: Ability of hydrolysate to support metabolic growth.	53
	2.9: Chemical Pre-treatment and digestibility assay (York High throughput (HT)).	54
	2.10: Differential Scanning Calorimetry (DSC).	55
	2.11: Genetic analysis of BC1 samples.	55
	2.12: Statistical analysis.	56
iii	Selection of suitable pre-treatment conditions.	57
	3.1: Introduction.	57
	3.2: Selection of a suitable pre-treatment.	58
	3.3: Analysis of the pre-treated liquor.	64
	3.4: Conclusions.	68
iv	Effect of cultivar and growing conditions on straw properties.	70
	4.1: Introduction.	70
	4.2: Comparison of wheat straw tissue for composition and digestibility.	71
	4.3: Effect of Nitrogen and plant growth regulator (PGR) application on straw digestibility.	76
	4.4: Effect of wheat variety on straw composition and digestibility.	90
	4.5: Conclusions.	94

v	BC1 samples morphology, composition and digestibility.	97
	5.1: Introduction.	97
	5.2: Morphology of the BC1 wheat samples.	98
	5.3: BC1 Composition analysis.	103
	5.4: Chemical pre-treatment and digestibility assay (York High throughput (HT)).	109
	5.5: Genetics analysis.	111
	5.6: Conclusions.	114
vi	Hemicellulose composition of BC1 samples.	116
	6.1: Introduction.	116
	6.2: Analysis of hemicelluloses through xylose extraction and quantification.	116
	6.3: Hemicellulose composition and susceptibility to acid pre-treatment.	117
	6.4: Differential scanning calorimetry (DSC) to assess hemicellulose degradation	125
	6.5: Conclusions.	128
vii	Use of sea water for Bio-fuel production.	132
	7.1: Impact of sea water on pre-treatment and enzyme hydrolysis.	133
	7.2: Impact of hydrolysates on microbial growth.	138

7.3: One pot pre-treatment to fermentation. 141

7.3: Conclusions. 145

viii Discussion. 149

8.1: Future work. 163

References. 166

LITS OF TABLES

Table		Page
Table 1.9.1	Showing the cellulose crystallinity of different fractions of wheat straw	37
Table 2.2	BC1 sample heritage for BC1 samples 1-24.	45
Table 2.3	BC1 sample heritage for BC1 samples 25-48.	46
Table 5.1	Morphological data for BC1 samples	102
Table 6.3.1	Correlations between the monomeric sugars detected in the pre-treated liquor	122
Table 6.4.1	Ranking of the top 10 BC1 samples and the bottom 10 BC1 sample	125
Table 7.1	Composition of Sea water.	132

LIST OF FIGURES

Figure		Page
Figure 1.2.1	US fuel ethanol production	6
Figure 1.4.1	The composition of cell wall polymers	10
Figure 1.4.2	Hemicellulose structure	15
Figure 1.4.3.1	Results from the second CAFI project using corn stover	18
Figure 1.6.1.1	Cellulose structure	27
Figure 1.6.1.2	Arabinoxylan Structure	29
Figure 1.7.2.1	The fermentation pathway	30
Figure 1.8.1	Different processes for bio-ethanol production from biomass	31
Figure 1.9.1	The mature wheat plant	34
Figure 1.9.2	Scanning electron microscope (SEM) images of untreated wheat	35
Figure 1.9.3	SEM of cellulose morphology in wheat straw cell wall epidermal layers	36
Figure 1.9.4	SEM showing the cellulose framework	37
Figure 2.1	Growing position of modern cultivars trialled for PGR and nitrogen applications	43
Figure 2.2	Example of standard curve for calculating sugar quantity	48
Figure3.2.1	Glucose content of the residue from pre-treated straw.	58
Figure 3.2.2	Xylose content of the residue from pre-treated straw	59
Figure 3.2.3	Impact of acid or base treatment on the visual appearance of wheat straw	60
Figure 3.2.4:	Recovery of biomass in the residue remaining following a chemical pre-treatment	61

Figure 3.2.5	% available glucose released from pre-treated wheat straw during digestion	62
Figure 3.2.6	% available xylose released from pre-treated wheat straw during digestion	63
Figure 3.3.1	Arabinose recovered in the pre-treated liquor	64
Figure 3.3.2	Galactose recovered in the pre-treated liquor	65
Figure 3.3.3	Glucose recovered in the pre-treated liquor	66
Figure 3.3.4	Xylose recovered in the pre-treated liquor	67
Figure 4.2.1	Glucose content of wheat straw tissues	72
Figure 4.2.2	Xylose content of wheat straw tissues	73
Figure 4.2.3	Glucose content of the residue from pre-treated wheat straw tissues	74
Figure 4.2.4	Xylose content of the residue from pre-treated wheat straw tissues	75
Figure 4.2.5	Available glucose (%) released from pre-treated wheat straw tissues	76
Figure 4.3.1	Effect of PGR application on glucose content from untreated wheat straw tissues	78
Figure 4.3.2	Effect of PGR application on xylose content from untreated wheat straw tissues	78
Figure 4.3.3	Effect of PGR application on glucose content of the residue from pre-treated wheat straw tissues	79
Figure 4.3.4	Effect of PGR application on xylose content of the residue from pre-treated wheat straw tissues	80
Figure 4.3.5	Available glucose (%) released from pre-treated wheat straw tissues after 72 hours	81
Figure 4.3.6	Effect of nitrogen application on glucose content from untreated wheat straw tissues	82
Figure 4.3.7	Effect of nitrogen application on xylose content from untreated wheat straw tissues	82
Figure 4.3.8	Effect of nitrogen application on glucose content from pre-treated wheat straw tissues	83

Figure 4.3.9	Effect of nitrogen application on xylose content from pre-treated wheat straw tissues	84
Figure 4.3.10	Available glucose (%) released from pre-treated wheat straw tissues after 72 hours	85
Figure 4.3.11	Effect of block on glucose content of the residue from untreated wheat straw tissues	86
Figure 4.3.12	Effect of block on xylose content of the residue from untreated wheat straw tissues	87
Figure 4.3.13	Effect of block on glucose content of the residue from pre-treated wheat straw tissues	88
Figure 4.3.14	Effect of block on xylose content of the residue from pre-treated wheat straw tissues	88
Figure 4.3.15	Available glucose (%) released from pre-treated wheat straw tissues after 72 hours	89
Figure 4.4.1	Glucose content of wheat straw tissues	91
Figure 4.4.2	Xylose content of wheat straw tissues	91
Figure 4.4.3	Glucose content of residue after acid pre-treatment	92
Figure 4.4.4	Xylose content of residue after acid pre-treatment	93
Figure 4.4.5	Glucose (%) released from pre-treated wheat straw	94
Figure 5.2.1	BC1 14 prior to being harvested	99
Figure 5.2.2	BC1 18 prior to being harvested	99
Figure 5.2.3	BC1 40 prior to being harvested	100
Figure 5.2.4	BC1 20 prior to being harvested	100
Figure 5.2.5	BC1 20 prior to being harvested	101
Figure 5.2.6	BC1 38 prior to being harvested	101
Figure 5.2.7	Scatter graph showing the relationship between BC1 plant height and total biomass produced	103
Figure 5.3.1a	Glucose content for stem tissue for BC1 samples	104

Figure 5.3.1b	Xylose content for stem tissue for BC1 samples	104
Figure 5.3.1c	Arabinose content for stem tissue for BC1 samples	105
Figure 5.3.2	Residue remaining (%) following a 1% H_2SO_4 pre-treatment	105
Figure 5.3.3a	Glucose content for pre-treated residue for BC1 samples	106
Figure 5.3.3b	Xylose content for pre-treated residue for BC1 samples	106
Figure 5.3.4a	Available glucose (%) released from pre-treated wheat straw tissues	107
Figure 5.3.4b	Available xylose (%) released from pre-treated wheat straw tissues	108
Figure 5.4.1	Reducing sugars following a high throughput assay of BC1 stem tissue	109
Figure 5.4.2	Scatter graph showing the relationship between glucose recovered and reducing sugars detected	110
Figure 5.5.1	Genetic markers for *Aegilops speltoides* for the 7 chromosomes	112
Figure 5.5.2	Genetic markers for *Triticum urartu* for the 7 chromosomes	113
Figure 6.3.1	Sugar stability during Saemen's hydrolysis	118
Figure 6.3.2	Xylose (%) removed during a standard 1% H_2SO_4 pre-treatment	119
Figure 6.3.3	Arabinose recovered in the pre-treated liquor	120
Figure 6.3.4	Galactose recovered in the pre-treated liquor	121
Figure 6.3.5	Glucose recovered in the pre-treated liquor	121
Figure 6.3.6	Xylose recovered in the pre-treated liquor	122
Figure 6.3.7	Xylose (%) removed during a standard 1% H_2SO_4 pre-treatment	124
Figure 6.4.1	The heat flow and degradation of BC1 samples	126

Figure 6.4.2	HPAEC chromatographs for the liquors from pre-treated BC1 1-6 and BC1-14	127
Figure 6.4.3	HPAEC chromatograph for the liquor for an elite line following	128
Figure 7.1.1	Effect of fresh or sea water on the weight of residue remaining after pre-treatment.	133
Figure 7.1.2	Sugar composition of residue from pre-treatment	134-135
Figure 7.1.3	Sugar recovered in the pre-treated liquor (%)	136
Figure 7.1.4	Effect of fresh and sea water on available glucose (%) released from revelation wheat straw	137
Figure 7.2.1	The ability of hydrolysate to support growth	139
Figure 7.2.2	The ability of hydrolysate to support growth	140
Figure 7.3.1	Sugar composition of the enzyme hydrolysate for revelation wheat straw	142
Figure 7.3.2	Weight loss during fermentation of wheat straw hydrolysate	143
Figure 7.3.3	CO_2 production during fermentation of wheat straw hydrolysate	144
Figure 7.3.4	Ethanol production during fermentation of wheat straw hydrolysate	145

LIST OF ABBREVIATIONS

CO2	Carbon dioxide
Ppm	Parts per million
BC	Before Christ
L	Litre
Min	Minuets
H2SO4	Sulphuric acid
NaOH	Sodium hydroxide
Ca(OH)2	Calcium hydroxide
O3	Ozone
SO2	Sulfur dioxide
Psi	Pounds per square inch
AFEX	Ammonia fiber expansion
CAFI	Consortium for Applied Fundamentals and Innovation
SHF	Separate hydrolysis and fermentation
SSF	Simultaneous saccharification and fermentation
SSCF	Simultaneous saccharification and co-fermentation
C5	5 carbon sugar
C6	6 carbon sugar
H-G-S	p-hydroxyphenyl–guaiacyl–syringyl
SEM	Scanning electron microscope
WAXD	Wide-angle X-ray diffraction
LCs	Landrace cultivars
MWCs	Modern wheat cultivars
CIMMYT	International Maize and Wheat Improvement Centre
BC1	Back cross first generation

BC2	Back cross second generation
BC3	Back cross third generation
HPAEC	High performance anion exchange chromatography
HPLC	High Performance Liquid Chromatography
NHO3	Nitric Acid
KOH	Potassium hydroxide
NREL	National Research Energy Laboratory
mM	milliMolar
FPU	Filter paper units
MFV	Miniature fermentation vestal
RPM	Revolutions per minute
RO	Reverse Osmosis
YNB	Yeast Nitrogen Base
YPD	Yeast Extract Peptone Dextrose Medium
HT	High throughput
DNS	Dinitrosalicylic acid
ANOVA	Analysis of variance
DSC	Differential scanning calorimetry
SNPs	Single nucleotide polymorphisms

LIST OF PUBLICATIONS

PLOSONE|DOI:10.1371/journal.pone.0139129 October1, 2015

Expression of Mitochondrial Cytochrome C Oxidase Chaper one Gene (COX20)Improves Tolerance to Weak Acid and Oxidative Stress during Yeast Fermentation.

VinodKumar, AndrewJ.Hart, Ethiraju R. Keerthiraju, Paul R. Waldron, Gregory A. Tucker, Darren Greetham.

European Biomass Conference and Exhibition (EUBCE) 2016

Improving Wheat Straw Composition and Digestibility by Alien Introgression.

Moirangthem, K., Waldron, P., Tucker, G., King, I., King, J.

Aspects of Applied Biology 131, 2015, Biomass and Energy Crops V.

Dual purpose wheat: for a future that demands more food and energy.

Kamaljit Moirangthem, Paul Waldron, Gregory Tucker, Ian King and Julie King

Biotechnology for Biofuels.

Expression of Aspergillus niger CAZymes is determined by compositional changes in wheat straw generated by hydrothermal or ionic liquid pretreatments.

Paul Daly; Jolanda M van Munster; Martin J Blythe; Roger Ibbett; Matt Kokolski; Sanyasi Gaddipati; Erika Lindquist; Vasanth R Singan; Kerry W Barry; Anna Lipzen; Chew Yee Ngan; Christopher J Petzold; Leanne Jade G Chan; Steven T Pullan; Stephane Delmas; Paul R Waldron; Igor V Grigoriev; Gregory A Tucker; Blake A Simmons; David B Archer.

Chapter 1: Introduction.

1.1: Need for bio-fuels.

With the world population increasing and expected to reach 9.3 billion by 2050 and 10 billion by 2100 (HDR, 2011). This is causing a rapid depletion of fossil fuels, with oil and gas expected to have ran out by 2042 (Shafiee and Topal, 2008). As oil depletes it will become harder and costlier to extract, this will only force up prices of remaining crude oil and other fossil fuels, it is now clear that there is a need to secure both energy and food production.

Additional to this problem there is an increased awareness of the effects that the human population is having on global warming and climate change. The biggest cause of this being the unsustainable burning of fossil fuels, particularly in the industrialised western world (Nema *et al*, 2012), that has resulted in the releasing of tones of previously stored carbon into the atmosphere in the form of the greenhouse gas CO_2. This level has increased steadily from pre-industrial revolution levels of 280ppm, to levels of over 350ppm in 2006 (Agarwal, 2006), rising to a present day level of 402ppm (August 2016) (www.co2.earth, accessed 16/09/16),with estimates that it could rise to between 560ppm and 730ppm by 2100 (Grubb *et al*, 2002).
With many countries around the world committing to reduce CO_2 emissions, the Kyoto Protocol on climate change set the requirement for the reduction of greenhouse gasses for industrialised countries to be 95% of the level in 1990 by the year 2012. This was widely criticised for being weak by scientific

groups and unattainable by economic groups (Grubb *et al*, 2002). Whilst many countries aimed to achieve this there were many that did not, particularly developing countries.

Whilst it has been relatively easy for the clean, renewable, and environmentally friendly production of electricity with wind and solar technologies it has not been so for liquid transportation fuels. With 700 million vehicles on the worlds roads and predicted to increase to 1.3 billion by 2030 (Balat and Balat, 2009) there is a requirement for a sustainable liquid fuel.

Bio-fuels are not a new concept; for thousands of years humans have been fermenting grains and fruits to produce alcohol for therapeutic consumption with sugar cane being converted to ethanol as early as 6000BC (Demirbas, 2009). Ethanol, also known as ethyl alcohol, derived from biomass has been accepted as a suitable liquid fuel for vehicles (particularly if equipped with spark ignition engines) for over a century (Agarwal, 2006; Goldemberg, 2008) and biodiesel was first used over 100 years ago when vegetable oil was used to fuel Rudolf Diesel's compression ignition engine (Ma and Hanna, 1999). Therefore, bio-fuels are a feasible option for liquid transport fuel.

In the USA as early as the 1900s ethanol produced from corn was used as a liquid transport fuel. Henry Ford's model T, a car designed for the masses, had a carburettor adjustment that allowed either petroleum or ethanol to be used as a fuel. Ethanol was used into the 1930s but was abandoned after the Second

World War due to an abundant supply of cheap petroleum fuel (Bothast and Schlicher, 2004).

In 1973 there was an oil crisis that severely interrupted global supplies andthis prompted a resurgence in bio-fuels as a way to replace traditional fossil fuels (Timilsina and Shrestha, 2010). Now, as the demand for crude oil increases and as large scale oil field production is declining annually at a rate of 4-5% (Nigam and Singh, 2010), there has been a renewed interest in bio-fuels.

1.2: First generation bio-fuels.

First generation bio-fuels have rapidly become a reality with large scale cultivation of feed-stocks for liquid bio-fuel production expected to rise due to concerns regarding climate change, greenhouse gas emissions, fuel security, reduction in fossil fuels and an increase in liquid fuel prices (Ruane *et al*, 2010).

The first-generation bio-fuels presently available commercially are typically ethanol produced from sugar crops like sugarcane and sugar beet, from cereal crops like wheat and maize via the fermentation of the sucrose and starch or biodiesel production coming from rapeseed, soybean oil and sunflower oil.

1.2.1: Bio-ethanol.

Since the mid-1970s, and because of the pre mentioned oil crisis, Brazil has had a programme for generating ethanol from sugarcane; this is done by milling the plant and using hot water to obtain as much of the sucrose from the cane as possible (Van den Wall Bake *et al*, 2008). The sucrose is then reduced by hydrolyses in water to α-D-glucopyranose and β-D-fructofuranose, both hexose sugars are then fermented using *Saccharomyces cerevisiae* (baker's yeast) a species of commercial yeast known since the ancient times for converting sugars to alcohol to produce ethanol (Hayes, 2008). This is then distilled to the required concentrations and the water removed to produce "anhydrous ethanol" that can then be blended with petrol to increase the octane value (Ruane *et al*, 2010; Goldemberg, 2008) and to reduce greenhouse emissions.

Around the same time the USA started a similar programme of converting corn into ethanol. Corn contains large amounts of starch; this is a polysaccharide consisting primarily of amylose and amylopectin. Amylose is made of α-(1/4) linked D-glucose units with few or no side chains, compared to amylopectin that has many α-(1/4) glucose side chains attached with α - (1/6) links usually occurring every 24–30 glucose units to the main α-(1/4) polymer (Hu *et al*, 2009; Hayes, 2008). Starch is easily hydrolysed with the use of inexpensive enzymes, mainly alpha-amylase and gluco-amylase (Hayes, 2008).

This is done in one of two ways, dry grinding or wet milling, and whilst the techniques differ greatly they each have their advantages and disadvantages. In dry grinding the whole kernels are ground to a powder that is then mixed with water and the enzyme alpha-amylase for liquefaction, converting the starch into dextrins (short chain glucose polymers) (Gibbons and Hughes, 2009). In contrast in wet milling the kernel is fractionated into its components (starch, corn germ, fibre and protein) the starch is then fermented and the other products converted into other saleable commodities for example corn oil (Hettinga *et al* 2008; Sanchez and Cardona, 2008).

Bio-ethanol production has increased rapidly and since 2003 exponentially. In 1975 there were 551 million L being produced annually, this had increased to 72,072 million L by 2009, jumping 16 million L since 2000. With the vast majority being produced in the USA and Brazil, producing 33,737 million L and 24,261 million L respectively by 2008 see figure 1 (Ruiz *et al*, 2011; Sorda *et al*, 2010).

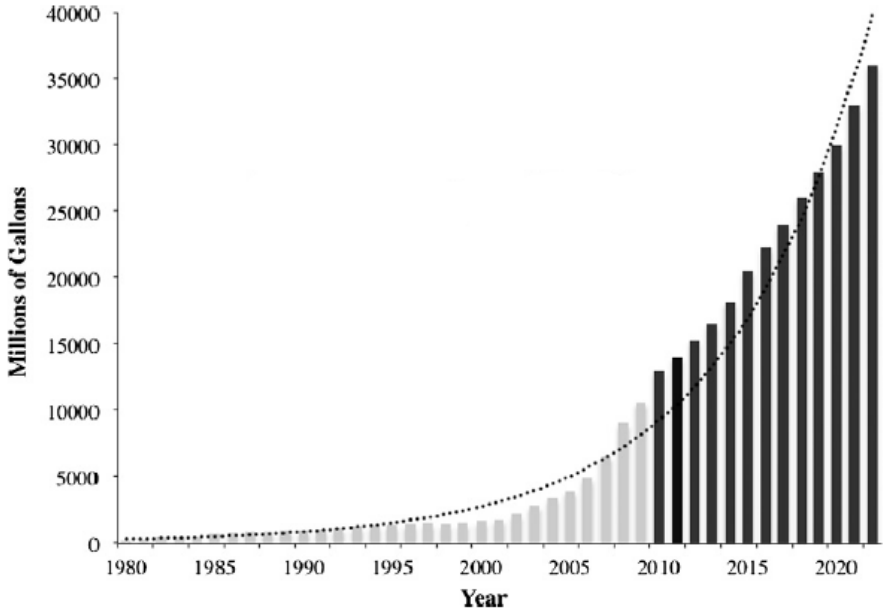


Figure 1.2.1: US fuel ethanol production; Light bars show actual production and dark bars represent estimated production. The points represent the trend model (taken from Ruiz *et al*, 2011).

1.2.2: Bio-diesel.

As previously stated, diesel engines are capable of running on vegetable oils and animal fats but only for a short length of time due to the high viscosity of the oil causing operational problems including the formation of deposits and injector coking (Knothe, 2009).

Biodiesel is made from vegetable or plant oils. Chemically these are triglyceride molecules that consist of three fatty acids groups attached to one glycerol molecule (Demirbas, 2008). These are treated in various ways to reduce the large branched molecules into smaller straight chains that are suitable for modern diesel engines. The most common and cost effective way is transesterification, and in this process the vegetable oil is mixed with an alcohol in the presence of a catalyst, usually an alkali, to produce biodiesel

(with improved viscosity and volatility) and glycerol that are then easily separated (Demirbas, 2010; Pinto *et al*, 2005).

1.3: Problems associated with first generation bio-fuels.

1.3.1: Food competition.

As the majority of all feed-stocks for bio-fuels have the potential to be used for food or animal feed, one of the biggest concerns with first generation bio-fuels is the competition with food and feed and the possible effects it is having on the price of commodities (Ajanovic, 2010). Whilst food production has increased substantially, various global catastrophes (natural and man-made) are at present preventing the global population from attaining their basic nourishment needs. As previously stated, with the estimated increase in human population expected to reach 9.3 billion by 2050, the problem will only get worse unless something is done soon (Escobar *et al*, 2008). In 2006, as a result of the increasing cost of corn (nearly doubling) from $2.25 to $4.00 per bushel resulting in the increased price for food and animal feed, the building of new grain based ethanol refineries was banned in China (Zhang, 2007) at the same time in the USA, 20% of all corn that was grown was converted into bio-ethanol (Sorda *et al*, 2010). In OECD (The Organisation for Economic Co-operation and Development) countries, first generation bio-fuels were used as a way to utilise persistent agricultural surplus and as a way to prop up farmers'

incomes whilst helping to reduce agricultural price fluctuations (Tomei and Helliwell, 2015).

1.3.2: Environmental impacts of first generation bio-fuel.

It has been suggested that the increased use of first generation feed stocks has a negative effect with regards to CO_2 emissions, as the majority of terrestrial carbon is contained in plant biomass or soil (Fargione *et al*, 2008) and this becomes more of a problem when land use change is taken into account. Nema *et al*, (2012) gives the reasons of global land change and deforestation as increased agriculture and urbanisation take effect. Many habitats around the world are being converted from natural to commercial farmland for the production of first generation bio-fuel feedstocks. With the Amazon rainforest being converted for soybean biodiesel, Malaysian and Indonesian peat land tropical rainforest for palm oil biodiesel, and the central grass lands of the USA being converted to corn for bio-ethanol, this is becoming more of a concern (Fargione *et al*, 2008). This has been shown to have both a direct and indirect impact on biodiversity and ecosystem functioning with both loss of habitat caused by land use change and from taking what was once fertile farm land used for food production and changing to energy crops (Hammond and Li, 2015).

1.4: Second generation bio-fuels.

Second generation bio-fuels are considered to be derived from feed stocks that are not in direct or indirect competition with humans for food resulting in little concern that this feed stock will impact on food prices or global food security (Charles *et al*, 2007). The idea of generating bio-fuels, chemicals and bio-products is not new and was first suggested over 70 years ago (Harris and Beglinger, 1946; Zhu and Zhuang, 2012).

Lignocellulosic plant biomass is the most abundant biological resource on the planet and is hugely underutilized (Naik *et al*, 2009; Timilsina, 2010) and is estimated to be in the region of 100×10^9 tonnes per year (Hayes, 2008). Lignocellulosic biomass is made of three parts cellulose, hemicellulose and lignin in the approximate ratio 40-50%, 25-30% and 15-20% respectively (Menon and Rao, 2012; Alonso *et al*, 2010).

The main advantages to utilising lignocellulosic plant biomass are that it is mainly derived from waste that has little to no commercial value. With most potential feed stocks being residual plant matter that results from food production or plants that have been cultivated on marginal land that is not suitable for food production. With this in mind it makes perfect sense to use these waste products to their maximum potential instead of as low grade animal feed or animal bedding as is current practice.

1.4.1: Feed stocks available for bio-fuel production.

There are a vast number of lignocellulosic feed-stocks that are available for conversion into liquid fuels or higher value chemicals. These include woody biomass from forest thinning or fast growing tree species such as willow or poplar grown in short rotation coppicing (SRC), waste agricultural products like sugar cane bagasses or wheat and rice straw or novel crops that can be grown in areas unsuitable for food production like switchgrass, miscanthus and eucalypts (Doherty *et al*, 2010).

Figure 1.4.1 shows four possible feed-stock's for bio-fuels showing their cell wall polymer compositions, this shows that whilst all have comparable lignin, cellulose and hemicelluloses quantities the hemicelluloses fraction differs greatly between feed stocks (Pauly and Keegstra, 2008).

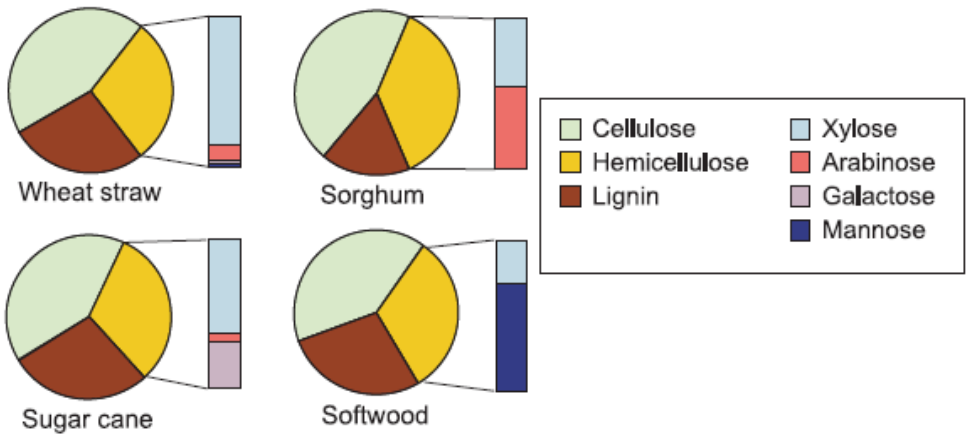


Figure 1.4.1: The composition of cell wall polymers. Cellulose, hemicellulose and lignin are the three main components with the composition of hemicellulose for various plant materials varying. (Adapted from Pauly and Keegstra, 2008)

The food industry has shown large growth in recent years and is now the largest manufacturing sector in the EU.This shows the potential to supply a

continual feed of lignocellulosic waste (Van Dyk *et al*, 2013) that at present is ether sent to land fill as a cheap method of disposal or incinerated at a high-energy cost (Ravindran and Jaiswal, 2015).

1.4.2: Biomass structure.

If a plant material is to be utilised as a feedstock for bio-fuel production it is vital that the plants form and structure is clearly understood. The main thing to note with plants is that each cell has a cell wall that provides structure and strength, without this cell wall plants would resemble slime moulds rather than the variety of plant structures we see (Cosgrove, 2005). The structure of plants is essentially like a building made from osmotically pressurized bricks with each cell pressurized to between 0.1 and 3.0 MPa; this creates rigidity by providing tension within the cell walls (Somerville *et al* 2004). As plants grow they need to reproduce cells, as cell migration is not possible morphogenesis is due to localised cell division and enlargement (Cosgrove, 2005).

1.4.2.1: Plant cell wall structure and development.

Plant cell walls are mainly made up from cellulose, hemicelluloses, pectin and lignin (Pauly and Keegstra, 2008). The quantities and qualities of these have variation between not only different species but also the maturity of the plant

as the composition and architecture are not uniform across the wall as the plant grows (Keegstra, 2010).

1.4.2.2: Cellulose.

Cellulose is the most abundant biological compound on the planet and the main component of plants and is used to maintain their structure (O'Sullivan, 1996). It is made of a linear polymer of glucose units linked via β-(l - 4)-glycosidic bonds. These linear polymers have been observed containing up to 14,000 individual glucose units (Somerville *et al*, 2004); the glucose molecules are arranged in an antiparallel fashion with adjacent chains running in opposite directions forming cellulose molecules that aggregate into long slender microfibrils. These have been estimated to contain between 30 and 36 chains (Somerville *et al* 2004; Wolf-Dieter, 2002) that bind together laterally via hydrogen bonds to form highly orientated crystalline sections. Regions that have a lower level of order and a lower degree of lateral hydrogen bonding are referred to as paracrystalline microfibrils; these amorphous regions are more common where one cellulose chain attaches from one fibril to another (Sun, 2010).

Cellulose is synthesised in rosette-like structures that are embedded in the plasma membrane (Wolf-Dieter, 2002), and as each glucose molecule is added to the chain it is orientated 180^{o} to the previous molecule creating chemically repeating units of β-glucose but structurally repeating β-cellobiose. Whilst

cellulose is a polar molecule with various –OH groups making it theoretically hydrophilic and therefore water soluble, it is not, due to it being capable of forming intra and intermolecular hydrogen bonds, van der Waals forces and hydrophobic interactions giving it a crystal structure that impedes hydrolysis (Alonso *et al*, 2010; Lindman *et al*, 2010) (See image 1.6.1.1).

1.4.2.3: Hemicellulose.

Hemicelluloses are branched amorphous polymers that are made up from several different sugar monomers, D-xylose and L-arabinose that are pentose sugars and D-glucose, D-galactose and D-mannose that are hexose sugars; with D-glucuronic acid, 4-*O*-methyl- D-glucuronic acid and D-galacturonic acid that are sugary acids (Menon and Rao, 2012; Alonso *et al*, 2010). Hemicellulose composition varies dramatically between plant type; with hard woods containing mainly xylans compared to the mostly glucomannan hemicelluloses that are found in softwood (Saha, 2003).

Hemicellulose being non-crystalline, highly branched and containing between 100-200 sugar units (Cheng and Timilsina, 2011) is one of the most complex structures in the cell wall forming hydrogen bonds with the cellulose and covalent bonds (usually α-benzyl ether linkages) with the lignin (Sun, 2008). Hemicellulose and other matrix polysaccharides are produced in the Golgi membranes and it has been estimated that up to 65 different enzymes are required for its synthesis (Keegstra, 2010).

1.4.2.4: Lignin.

Lignin is a complex structure. It is a polyphenolic compound made from p-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol, that gives rise to *p*-hydroxyphenyl, guaiacyl, and syringyl monolignol units when incorporated into the lignin polymers (Weng *et al*, 2008). This creates a hydrophobic, cross-linked aromatic polymer and has a major role in pathogen resistance and water regulation (Pu *et al*, 2013) and helps maintain structure in plant cell walls providing rigidity and strength by cross linking with the cellulose (Menon and Rao, 2012; Neutelings, 2011). Lignin is a significant underlying feature of the biomass of plants that inhibits its digestion. This hydrophobic polymer encases the polysaccharides within the cell wall, providing a strong and durable material resistant to enzymatic attack (Gomez *et al*, 2008). This is because unlike other polymers, like proteins and polysaccharides that have inter unit links that are susceptible to enzyme or chemical hydrolysis, lignin has carbon-carbon bonds and biphenyl ether bonds that are resistant to degradation (Sun, 2008).

1.4.2.5: Plant cell growth and structure.

With higher plants the plant cell wall comprises of three layer types, the primary cell wall, the middle lamella, and the secondary cell wall (Popper, 2008). Primary cell walls are further divided into two types depending on the polysaccharide composition. Type one cell walls are found in the dicots and

non-commelinid monocots where the main hemicellulose is xyloglucan with large amounts of pectic polysaccharides like homogalacturonan, and rhamnogalacturonan I and II (Keegstra, 2010). In type two cell walls, as found in the Poales (true grasses), the main hemicelluloses (image 1.4.2) are arabinoxylan (AX). These are β 1-4 linked D-xylose molecules that have individual α-L – arabinose residues attached with a combination of O-2 and O-3 linkages (Adams *et al*, 2003), and glucuronoarabinoxylans (GAX) these are also β 1-4 linked D-xylose molecules with arabinose and α-(1-2)-glucuronic acid with both arabinosyl and glucuronosyl substitutions on the xylan backbone (Hatfield *et al*, 2016). Type two walls generally contain a higher percentage of cellulose and reduced levels of pectins and proteins (Pauly and Keegstra, 2008).

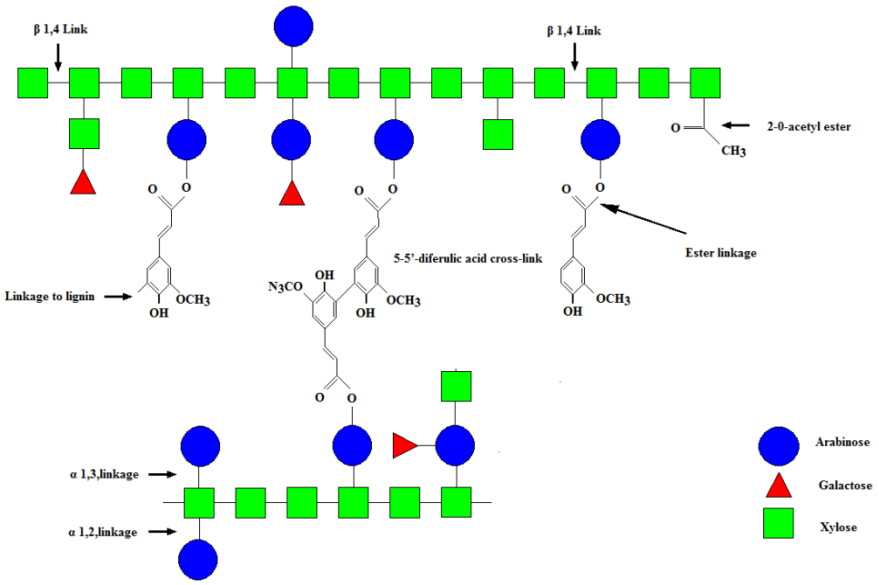


Figure 1.4.2: Hemicellulose structure. Consisting of a β1-4 linked xylose backbone with α-L – arabinose residues, and α-(1-2)-glucuronic acid with both arabinosyl and glucuronosyl substitutions.

The primary cell wall is created whilst the plant cell is elongating and growing and continues to be produced until growth ceases. This is done with the aid of a "scaffold like" structure made from crystalline cellulose and the matrix polysaccharides hemicelluloses that bind to the cellulose to provide strength as well as pectins that form hydrated gels forcing the cellulose microfibrials apart allowing for cell elongation (Cosgrove, 2005). The middle lamella is rich in pectic polysaccharides that hold adjacent cells together whilst allowing for slippage during cell growth and elongation (Cosgrove, 2005). Once growth stops the secondary cell wall is produced which is usually thicker than the primary cell wall and contains significantly more cellulose. Cellulose microfibrils are surrounded by a matrix of hemicelluloses and provide much of the cell wall strength (Marriott *et al*, 2015). The pre-dominant hemicelluloses fraction in the secondary cell wall is glucuronoarabinoxylan(GAX); this accounts for around 50% with minor amounts of xyloglucans and mixed linkage glucans (Scheller & Ulvskov, 2010). As previously stated GAX consist of β 1-4 linked D-xylose molecules with arabinose and α-(1-2)-glucuronic acid with both arabinosyl and glucuronosyl and ferulic acid (FA) residues. These FA residues form a crosslink that binds glucuronoarabinoxylans (GAX) to lignin (Hatfield *et al*, 2016) increasing recalcitrance and impeding enzymatic hydrolysis. The main difference with secondary cell walls is the replacement of water with lignin (Pauly and Keegstra, 2008), that is used to maintain structure and mechanical strength (Cosgrove, 2005) and surrounds specialised cells like vessel elements or fiber cells (Keegstra, 2010). The addition of lignin essentially changes the hydrophilic primary cell wall into a hydrophobic one

and therefore reducing wall porosity thus reducing enzyme degradation (Chesson *et al*, 1997).

1.4.3: Accessing polysaccharides in biomass.

With up to 80% of lignocellulosic biomass being made of polysaccharides the main problem is accessing them sustainably; if the energy yield is equal to or less than the total energy available it can be considered to be unsustainable. Various methods of pre-treatment are available although not all pre-treatments are compatible with all feed-stocks, and whilst pre-treatment is the most energy intensive part of ethanol production, without it there is a significant reduction in the efficiency of ethanol production (Stephen *et al*, 2011). The main purpose of pre-treatment is to open up the cell wall and increase the surface area for enzymatic attack (Ibrahim, 2012). A comparison of some of these methods for the pre-treatment of corn stover has been carried out by the Biomass Refining Consortium for Applied Fundamentals and Innovation (CAFI).The yields of glucose generally ranged between 85 to 95% of theoretical yield, with dilute acid and AFEX pre-treatments the overall monomer sugar yields being only slightly lower than total sugar yield (see figure 1.4.3.1) (Elander *et al*, 2009).

Pretreatment system	Xylose yields			Glucose yields			Total sugar yields		
	Pretreatment	Enzymatic hydrolysis	Combined	Pretreatment	Enzymatic hydrolysis	Combined	Pretreatment	Enzymatic hydrolysis	Combined
Maximum possible	40.4	40.4	40.4	59.6	59.6	59.6	100.0	100.0	100.0
Dilute acid	32.0	1.2	33.2	5.1	49.8	54.9	37.1	51.0	88.1
SO_2 catalyzed steam explosion	15.0	22.1	37.1	2.3	54.3	56.6	17.3	76.4	93.7
Controlled pH	23.4	9.7	33.1	3.3	50.5	53.8	26.7	60.2	86.9
AFEX		32.9	32.9		61.2	61.2		94.1	94.1
ARP	19.0	15.1	34.1	0.8	52.9	53.7	19.8	68.0	87.8
Lime	9.2	19.5	28.7	0.9	57.2	58.1	10.1	76.7	86.8

Figure 1.4.3.1: Results from the second CAFI project using corn stover. Corn stover was used as a feed stock and subjected to a pre-treatment, enzymatic hydrolysis, and combination of both, showing xylose, glucose, and total sugar yields from a variety of pre-treatment methods. (Elander *et al*, 2009)

1.5: Methods for pre-treating biomass.

As stated in 1.4.3, a pre-treatment stage is needed to disrupt the plant material in order to allow enzymes access to the cellulose for efficient conversion to glucose. Pre-treatment methods can be categorised into four types physical, chemical, physicochemical and biological based on their attributes, with some having several variations on the same design (Poornejad *et al*, 2012; Tomas-Pejo *et al* 2008). Each of these methods has both advantages and disadvantages in financial cost, time, the production of inhibitor products, and the need for waste treatment and chemical recovery.

1.5.1: Physical pre-treatments.

Physical pre-treatments generally involve size reduction; this is done through grinding or milling to increase the surface area available, leading to an improvement of enzymatic hydrolysis (Ibrahim, 2012). This method can be energy intensive depending on the desired final particle dimension with the energy input increasing as the particle size decreases (Lynd, 1996) and with moisture content, material properties, and feed rate also increasing the energy requirements (Ibrahim, 2012).

1.5.2: Pyrolysis.

Pyrolysis uses temperatures greater than 300^{o}C, and at this temperature the hemicelluloses and cellulose fractions decompose rapidly. Hemicellulose decompose between around 220 – 315 ^{o}C with cellulose decomposing between around 315 – 400 ^{o}C (Yang *et al*, 2006) producing a gaseous product and residual char. This residual char can then be treated with a mild acid hydrolysis converting the cellulose to reducing sugars (Sun and Cheng, 2001).

1.5.3: Chemical pre-treatments.

Chemical pre-treatments include acids, alkalis and oxidizing agents like peroxide (Ibrahim, 2012). These can be used to disrupt the structure of the

biomass and allow for better enzymatic hydrolysis. The pre-treatment can be carried out at low temperatures with a higher concentration of chemicals or at a higher temperature with less concentrated chemicals; each has their own advantages and disadvantages.

1.5.3.1: Acid hydrolysis.

Acid hydrolysis can be used at either low concentration acids at high temperatures, or with concentrated acids at low temperatures, with the most commonly applied acid being Sulphuric acid (H_2SO_4) although other acids such as hydrochloric and nitric acid have also been used (Chiaramonti *et al*, 2012). Acid pre-treatments work by breaking and reducing the rigidity of the lignocellulosic material (Menon and Rao, 2012) and cleaving the cellulose and hemicellulose polymers into their monomers (Taherzadeh and Karimi, 2007). Hydrolyzing lignocellulosic feedstock with strong acids is highly effective although there are several inherent problems including the need for expensive materials, a potentially hazardous process, corrosion of equipment and the need for acid recovery to reduce costs, although this too is an energy intensive process (Chiaramonti *et al*, 2012; Poornejad *et al*, 2012). Whilst the use of strong acids have been shown to be powerful agents for cellulose hydrolysis (Sun and Cheng, 2001) it has also been shown that weak acids can also be used either at a high temperature with a short reaction time (1-5min) or at a low temperature with a longer reaction time (30-90 min) (Chiaramonti *et al*, 2012).

1.5.3.2: Alkaline hydrolysis.

Alkaline hydrolysis works by breaking the ester bonds that form the crosslink's between lignin, cellulose and hemicellulose with the addition of Sodium hydroxide (NaOH) or Calcium hydroxide ($Ca(OH)_2$) (Chiaramonti *et al*, 2012). Once the ester bond crosslinks have been broken down, the porosity of the lignocellulosic material greatly increases (Sun and Cheng, 2001) allowing for better access to the cellulose. Pre-treatment can be carried out at low temperature but requires a relatively long reaction time and a high base concentration (Chiaramonti *et al*, 2012). However, as with acid hydrolysis there are several problems associated with this method for example the cost of neutralizing the alkalis, their recovery, corrosion problems and difficulty in reusability (Poornejad *et al*, 2012). Whilst there are several problems with alkaline hydrolysis it has been shown to avoid fragmentation of the hemicellulose polymers (Gaspar *et al*, 2007).

1.5.3.3: Organosolv process.

Organosolv process utilises an organic or aqueous organic solvent mixture sometimes with the addition of an inorganic acid catalysts (often Sulphuric acid (H_2SO_4)) (Sun and Cheng, 2001) to decompose the lignin and hemicellulose part of the biomass whilst keeping the cellulose part as a solid (Chiaramonti *et al*, 2012). The most relevant and suitable organic solvents used are methanol, ethanol, acetone, ethylene, glycol and tetrahydrofurfuryl

alcohol, and whilst the use of an inorganic acid catalysts is described when used at high temperature (160-200^{o}C), the use of a catalyst is unnecessary for satisfactory delignification (Chiaramonti *et al*, 2012; Sun and Cheng, 2001). Due to the fact that the solvents inhibit the growth of organisms, that may be required for subsequent enzymatic hydrolysis and fermentation, they must be removed.As a way of reducing costs they can be evaporated, condensed, and recycled (Sun and Cheng, 2001) which can be done via simple distillation (Chiaramonti *et al*, 2012).

1.5.3.4: Ozonolysis.

Ozone (O_3) has been used to degrade hemicellulose and lignin without disrupting the cellulose structure. Ozone pre-treatment is effective at removing the lignin whilst not producing any toxic residues and can be carried out at room temperature and pressure, but due to the high levels of ozone required, is a very expensive process (Sun and Cheng, 2001).

1.5.4: Physico-chemical pre-treatments.

Physico-chemical processes utilise a mixture of a physical treatment with the aid of a chemical catalyst to increase reaction times or reduce the energy required.

1.5.4.1: Steam explosion (auto hydrolysis).

Steam explosion is carried out by applying high pressure steam to the raw material for a length of time in a pressurised vessel and then at a given time rapidly releasing (venting) the high pressure back to atmospheric pressure (Mosier *et al*, 2004). This causes explosive decompression resulting in a breakdown of the lignocellulosic structure, hydrolysis of the hemicellulose and defibration and depolymerisation of the lignin components (Cara *et al*, 2007). Steam explosion can be performed uncatalyzed as described above or with the aid of a catalyst like Sulphur dioxide (SO_2) or Carbon dioxide (CO_2); the addition of a catalyst can improve enzymatic hydrolysis, increase the removal of hemicellulose and limit the production of inhibitory compounds (Sun and Cheng, 2001).

1.5.4.2: Liquid hot water method.

The liquid hot water method consists of immersing the raw material in liquid hot water, typically for 20 minutes at 170-230^{o}C at a pressure of 725 psi (Mosier *et al*, 2004), and works by hydrolyzing the hemicellulose. The hydrothermal pre-treatment method releases a large fraction of hemicellulose sugars in the form of oligomers (Sarkar *et al*, 2011). However, whilst this does not require the need for neutralization, as no chemicals are added, and reduces the need for size reduction as lignocellulose particles break when heated in water (Mosier *et al*, 2004), it does produce small amounts of furfural and

carboxylic acid that are toxic during the fermentation stage by inhibiting microbial growth (Sarkar *et al*, 2011).

1.5.4.3: Ammonia fibre expansion (AFEX).

The process of AFEX is similar to that of steam explosion, as it is an alkaline thermal pre-treatment that exposes the raw material to liquid ammonia at high pressure for a length of time, typically 90^{o}C for 30 min (Sun and Cheng, 2001), and then undergoes explosive decompression. Although this method does not liberate sugars directly it does free the hemicellulose and cellulose polymers for future enzymatic attack (Sarkar *et al*, 2011). Ammonia primarily reacts with the lignin causing a depolymerisation of the lignin and cleaving the lignin-carbohydrate linkages (Mosier *et al*, 2004). Whilst this process does not produce inhibitors that may affect fermentation, a small particle size is needed (Sarkar *et al*, 2011); it is also very expensive with regards to the cost of ammonia and the need for ammonia recovery and is only effective for biomass with a low lignin content (Sun and Cheng, 2001).

1.5.4.4: Microwave pre-treatment.

This method consists of placing the feedstock in a chemical solution and exposing it to microwave radiation for a period of time; this works by increasing the chemical reaction rates due to the high heating efficiency of the

microwaves (Zhu *et al*, 2006). This solubilises the hemicelluloses and lignin fractions allowing for better enzymatic hydrolysis (Kashaninejad and Tabil, 2010).

1.5.5: Biological treatments.

Biological pre-treatments utilise microorganisms such as brown, white and soft rot fungi that have the ability to degrade hemicelluloses and lignin (Sindhu *et al*, 2015). For the effective degradation of lignocellulosic material, a microbial consortium is needed consisting of both fungi and bacteria. This has the advantage of being a cheap method for treatment and with enzymatic hydrolysis has the ability to release near to 100% of the sugars. However, the major drawback is the length of time required (Taherzadeh and Karimi, 2007) and the need for a sterile condition (Sindhu *et al*, 2015).

This problem has been overcome with the development of simultaneous saccharification and fermentation that shall be discussed in a later section.

1.6: Enzyme hydrolysis.

Once the pre-treatment process has been completed and the feedstock washed, neutralised enzymes can be added to release the sugars. Enzymatic hydrolysis requires three steps: 1: the absorption of the enzyme onto the substrate, 2: the

breakdown of substrate via the synergistic actions of endo and exo acting enzymes and 3: the desorption of the enzyme from the substrate (Kuo and Lee, 2008). Whilst this sounds a rather simple process it is far more complex and requires the synergistic action of multiple enzymes to work efficiently.

1.6.1: Enzymes required for lignocelulosic break-down.

For the total breakdown and degradation of all lignicellulosic components, a large variety of enzymes with differing specifities is required (Van Dyk and Pletschke, 2012). The majority of enzymes that have been developed and are currently used for lignocellulosic digestion are derived from bacterial and fungal species (Banerjee *et al*, 2010).

1.6.1.1: Cellulose acting enzymes.

For the degradation of cellulose it is generally accepted that three types of cellulase enzymes are needed, β-glucosidases (EC 3.2.1.21) that acts on the non-reducing and reducing ends of the cellulose fibrils. Endo-1,4-β-glucanases (EC 3.2.1.4) cleave random internal bonds of the cellulose chain creating free ends and reduce the degree of polymerisation. These are then acted upon by exo-1,4-β-glucanases (EC 3.2.1.91 and EC 3.2.1.176) (cellobiohydrolase) (Van Dyk and Pletschke, 2012) that cleave the free ends liberating cellobiose (a disaccharide consisting of two glucose molecules) which are then broken

down into glucose monomers via the action of β- glucosidases (Kumar *et al*, 2008). Lytic polysaccharide monooxygenases (LPMOs) are metalloenzymes that degrade cellulose using a mechanism involving molecular oxygen and an electron donor (Muller *et at*, 2015). These tend to target crystalline cellulose and cleave the β-(1→4) glycosidic bonds; this leaves the C1 or C4 carbon oxidised and allows better access for depolymerising enzymes (Diagram 1.6.1.1).

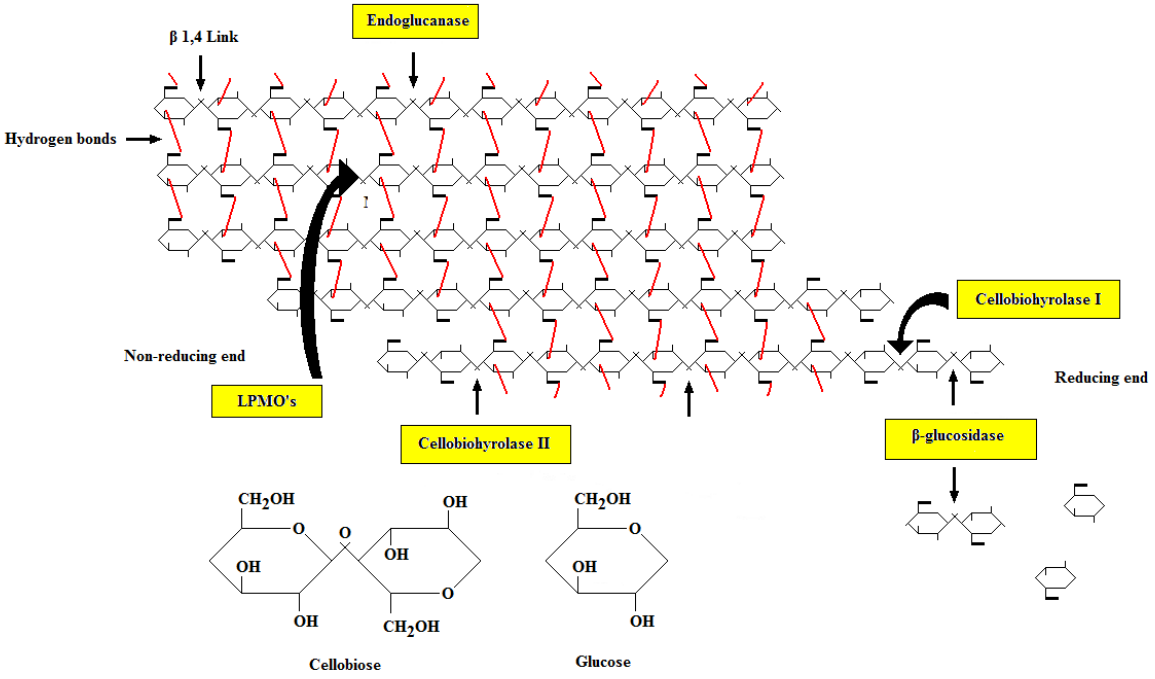


Figure 1.6.1.1: Cellulose structure. Depicting what bonds each enzyme acts upon.

1.6.1.2: Hemicellulose acting enzymes.

The deconstruction of hemicelluloses is far more complex; as stated (section 1.4.2.3) hemicellulose has a highly branched structure made from several different sugar molecules. Due to this varied composition and structure, more enzymes are required for its degradation than are needed for cellulose. Hemicellulases are divided into two types, depolymerising enzymes and enzymes that remove substituent's (Van Dyk and Pletschke, 2012). The degradation of the D-xylose backbone is carried out with the synergistic actions of endo-β -1,4-xylanase and β-xylosidase (Saha, 2003). Endo-β -1,4-xylanase acts on the backbone and cleave it into shorter oligosaccharides that can then be acted upon by the β-xylosidase that then cleave them into xylose monomers. As hemicellulose is a branched structure with large numbers of side chains of arabinose, acetyl, galactose and glucose for example, which may pose steric hindrances to the depolymerising enzymes (Van Dyk and Pletschke, 2012) another set of additional enzymes are required (diagram 0.6.1.2). The hydrolysis of the substituted xylan is carried out by α-L-arabinofuranosidases that cleaves 1-2 linkages or 1-3 linkages; α-glucuronidase, α-galactosidase, acetyl xylanesterase, feruroylesterase, acetyl mannan esterase and ferulic acid esterase that cleave the links between lignin and hemicelluloses (Van Dyk and Pletschke, 2012; Saha, 2003). Agger *et al*(2014) have shown that some LPMO's are also capable of degrading of α- and β-linked polysaccharides.

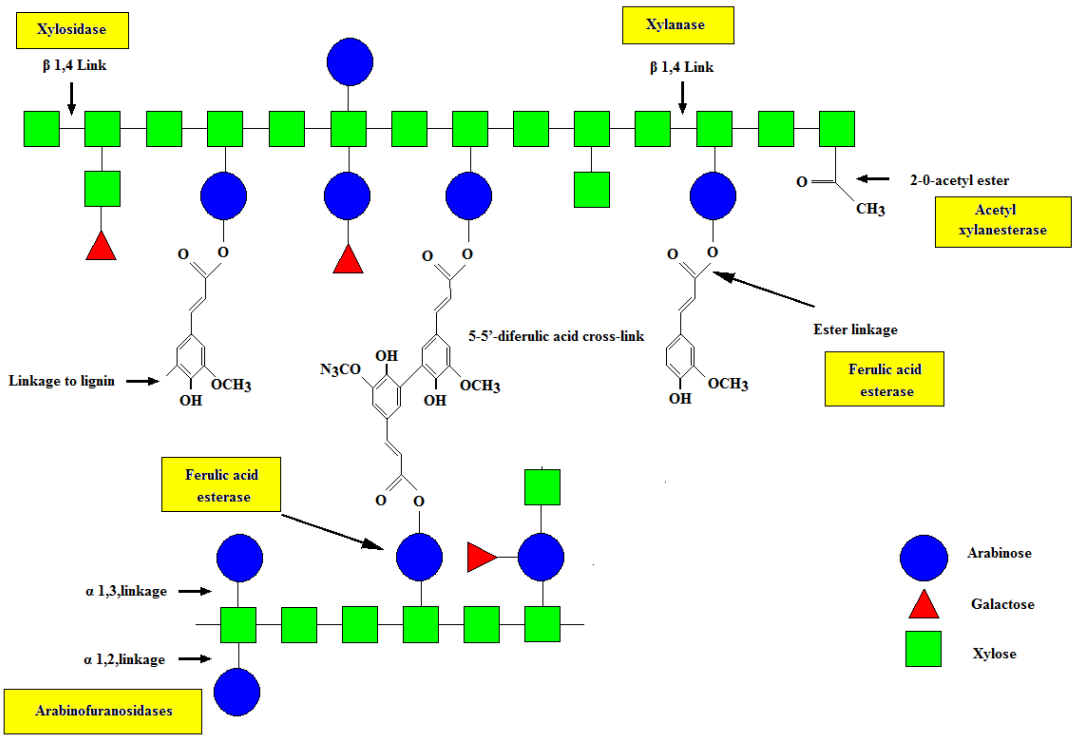


Figure 1.6.1.2: Diagram of arabinoxylan. Structure and the bond's that each enzyme acts upon.

1.7: Yeast and fermentation.

Once the biomass has been broken down into monomeric sugars it can be converted into ethanol, and this can be achieved with the use of yeast via fermentation. Yeasts are eukaryotic ascomycete fungi that originated hundreds of millions of years ago, and are found in all environments on the planet. They usually reproduce asexually in a process known as budding whereby the parent cell devides into two daughter cells by mitosis but have been known to go through sexual hybridization within and between species (Steensels and Verstrepen, 2014). Fermentation is the ability to generate energy without the

use of the electron transport chain, although the term “fermentation” is often used in a broader context. A simplified summarisation of fermentation is

$$C_6H_{12}O_6 \;>>>>\; 2C_2H_5OH + 2CO_2$$

Where one glucose molecule is converted to two ethanol molecules and two carbon dioxide molecules (Sun 2010). Figure 1.7.1 shows the metabolic pathway from glucose to ethanol by *Saccharomyces cerevisiae* (bakers yeast).

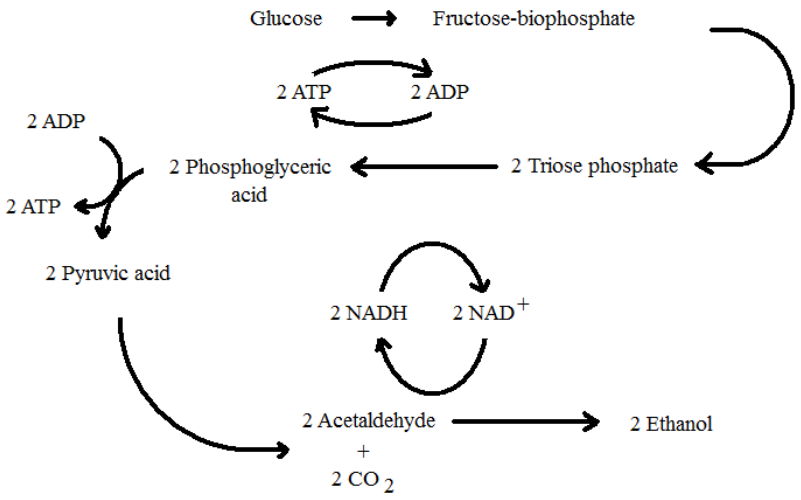


Figure 1.7.2.1. The fermentation pathway. The metabolic pathway from glucose to ethanol by *Saccharomyces cerevisiae.*

Whilst the fermentation step is a necessity for the production of bio-ethanol the traditional method using *Saccharomyces cerevisiae* is not suited due to the fact that current technologies produce a hydrolysate that is of a low glucose concentration with high levels of pentose sugars that are not fermentable at present and high levels of inhibitory products that are generated during the pre-treatment stages (Sun, 2010).

1.8: Enzymatic hydrolysis and fermentation.

The process of enzymatic hydrolysis and fermentation can be carried out in several different ways (See Fig 1.8.1) with each having its own distinct advantages and disadvantages. With separate hydrolysis and fermentation (SHF), the liquid portion from the hydrolysis reactor is first placed into a glucose fermenter where the hexose sugars are converted to ethanol, this is then distilled before the remainder is transferred to a xylose fermenter where the pentose sugars are fermented to ethanol before again being distilled (Balat *et al*, 2007).

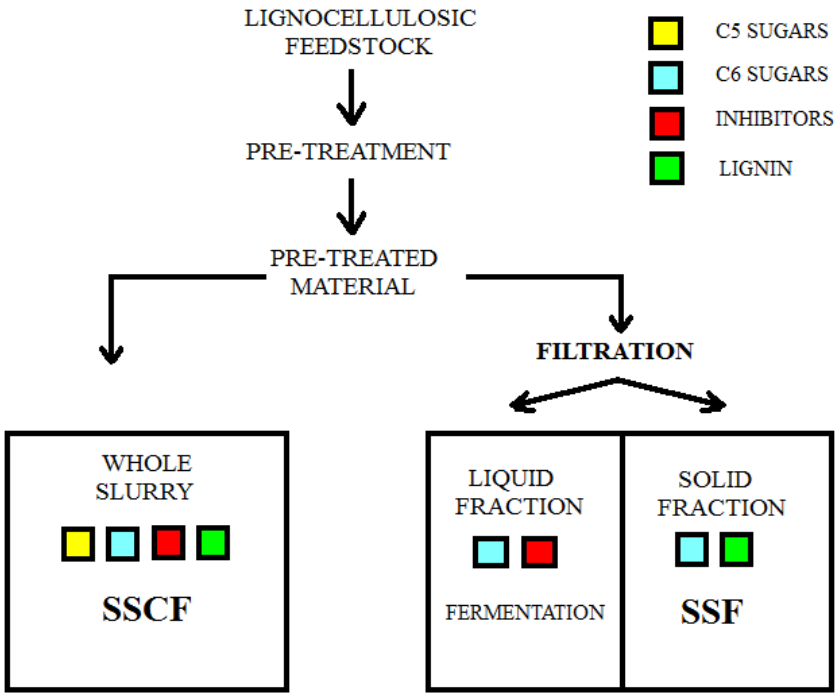


Figure 1.8.1: Different processes for bioethanol production from biomass.

The major advantage to separate hydrolysis and fermentation is that each step can be carried out at the optimum condition (45-50^{o}C for enzymatic hydrolysis

and 30^{o}C for fermentation) (Tengborg *et al*, 2001) with the disadvantage being that cellulolytic enzymes can be subject to end product inhibition, so as glucose and cellobiose accumulate the hydrolysis progressively reduces (Hahn-Hagerdal *et al*, 2006).

Simultaneous saccharification and fermentation (SSF) incorporates both enzymatic hydrolysis and fermentation in the same step, although this reduces the yield compared to SHF. Due to the fact that it is carried out in only one vessel the overall production costs are reduced, thus making it more sustainable and economically viable (Tomas-Pejo *et al* 2008). This method also has the additional advantage that it reduces feedback inhibition as the sugars are converted to ethanol before they build up in significant quantities due to yeast fermentation (Balat *et al*, 2007). The main drawback to this being that as the optimum temperatures are different for each process, a compromise must be made between enzymatic hydrolysis and fermentation (Tomas-Pejo *et al* 2008).

Simultaneous saccharification and co-fermentation (SSCF) can be used if there are both hexose and pentose sugars present, although this is not efficient as most pentose fermenting yeasts such as *Saccharomyces uvarum* are not tolerant to high concentrations of ethanol (Tomas-Pejo *et al* 2008).

Traditionally, *Saccharomyces cerevisiae* (Baker's yeast) and *Zymomonas mobilis* (a natural rod shaped gram-negative bacterium) have been used to convert sugars into ethanol; whilst they can ferment glucose efficiently they are unable to ferment xylose, therefore the fungus *Pachysolen tannophilus* and *Pichia stipitis* must also be used for xylose fermentation (Balat, 2010;

Keshwani and Cheng, 2008). Fermentation can be carried out in either batch form, fed batch or in continuous mode (Palmqvist and Hahn-Hagerdal, 1999). At present the fermentation of C5 and C6 sugars at the same time by the same microorganism is not feasible Although genetically engineered *Saccharomyces cerevisiae* uses the same uptake system for both glucose and xylose this multiple transport system clearly prefers glucose (Carrasco *et al*, 2010)

1.9: Wheat as a lignocellulosic feed stock.

As previously stated, wheat straw biomass can be used for conversion to biofuels and other chemicals. Wheat straw being one of the main cereal crops grown today has a residue/crop ratio of 1.3; in 2008 the annual global crop of wheat was 650 million metric tonnes of grain and would give 850 million metric tonnes of waste straw (Talebnia *et al*, 2009). With a yield of 70 gallons/ton biomass (Zhu and Zhuang, 2012) this could theoretically be used to produce approximately 940 million gallons of bioethanol per year.
With this in mind, wheat straw is an ideal feedstock for bioethanol production without conflicting with food and feed production.

Wheat is a monocotyledon from the order Poales and the family Poaceae (true grasses) and is an annual grass that is now cultivated worldwide. Wheat plants comprise of a single stem with alternating side leaves (figure 1.9.1) (Office of the gene technology regulator, 2008). The stem consists of phytomers

(repeating segments), being made from a node, a hollow internode, and a leaf, with the leaf sheath wrapping around the stem to provide support.

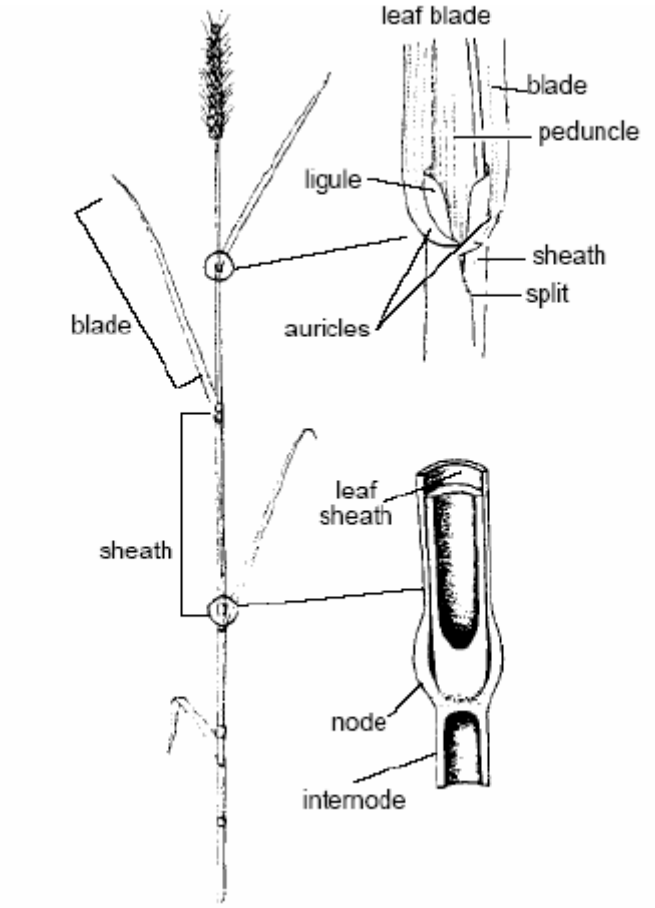


Figure 1.9.1: The mature wheat plant, Wheat plants generally consist of repeating segments of internodes divided by nodes that form the base for the leaf, image shows the stem and leaf structure. (Taken from Office of the gene technology regulator, 2008).

Wheat culms generally consist of five – six internodes with the base internode being very short and each subsequent internode becoming more elongated (Tripathi *et al*, 2003).

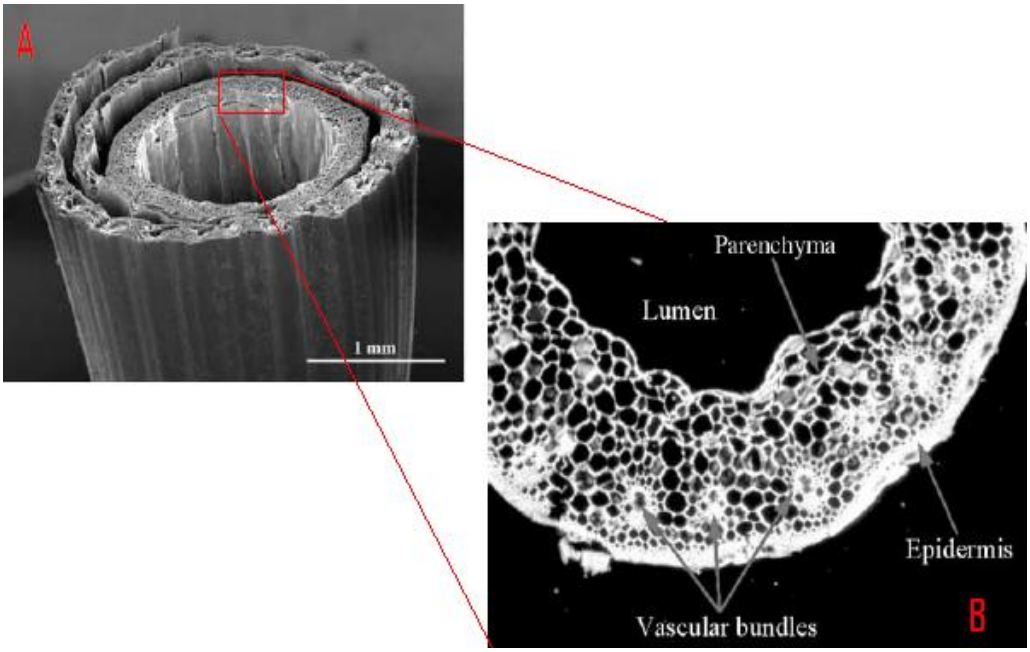


Figure 1.9.2: Scanning electron microscope (SEM) images of untreated wheat. A: showing the stem surrounded by the leaf sheath and B: cross section showing the straw walls various cell types including the epidermis and parenchyma cells, the vascular bundles (phloem and xylem) and the thick wall fibre cells (image A adapted from Kristensen *et al*, 2008; image B adapted from Liu *et al* 2004).

Macroscopically, wheat straw consists of concentric rings surrounding a central lumen (figure 1.9.2a and b). The outer most layer is the epidermis; this has a much denser structure than the parenchyma and contains wax and phytoliths (micro-sized silica particles) (Halvarsson *et al*, 2010). This provides support for the plant and protection from external elements such as wind and rain (Yu *et al*, 2007) by forming a hydrophobic barrier consisting of waxes and cutin that helps reduce desiccation and also provides a defence against pathogenic attacks (Reina-Pinto and Yephremov, 2009). In the wheat straw wall the cellulose is the main component and acts as the framework for the cells and provides tensile strength. Figure 1.9.3 shows the epidermal layers of wheat straw after the lignin and hemicellulose has been removed and shows the two main types of fibers; the first being long and thin and composed of fibrils and the second being twice the width (10 um) and having serrated edges

that connect with other fibers increasing both longitudinal and lateral strength (Liu *et al*, 2004).

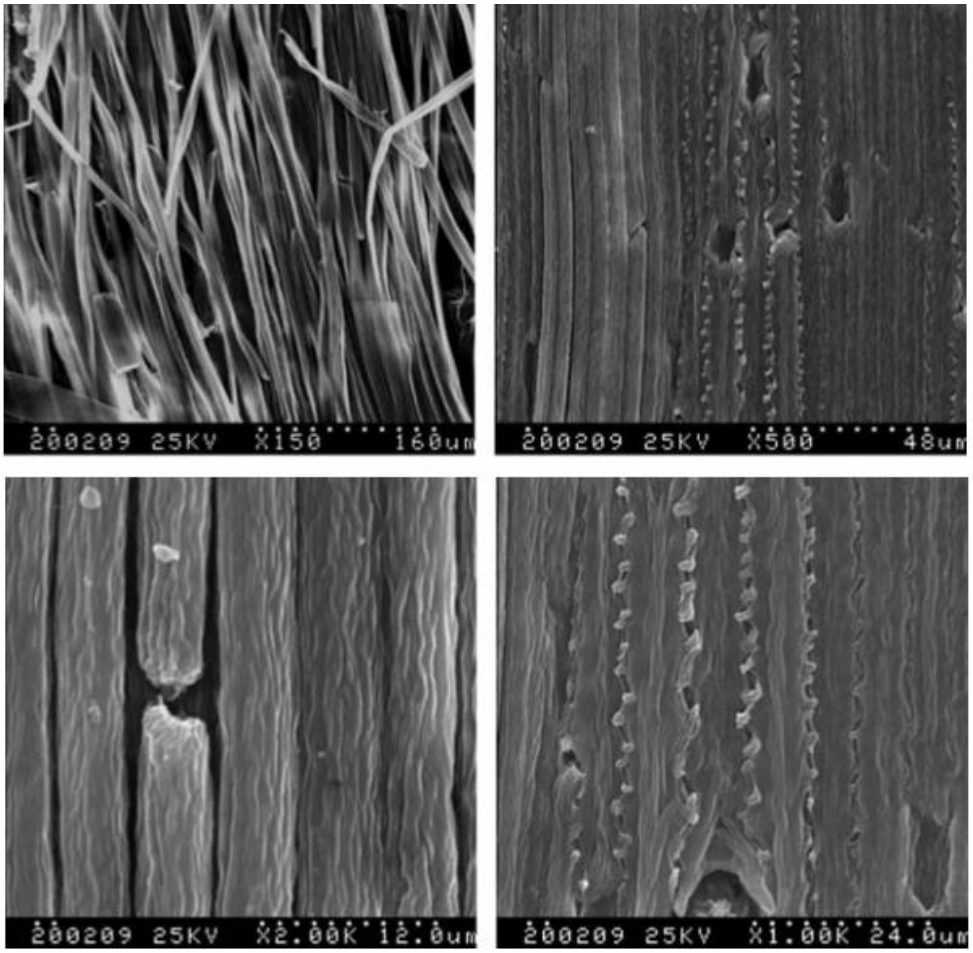


Figure 1.9.3: SEM of cellulose morphology in wheat straw cell wall epidermal layers. The lignin and hemicellulose has been removed Top left showing long thin fibers, top right showing shorter fibers with serrated edges, and below them magnified (adapted from Liu *et al*, 2004)

Beneath the outer epidermal layer is the parenchyma. This is less dense with larger die cell lumen and contains the vascular bundles (Liu *et al* 2004). The frame work for the vascular bundles consists of spiral and annular vessels made of a cellulose framework (figure 1.9.4).

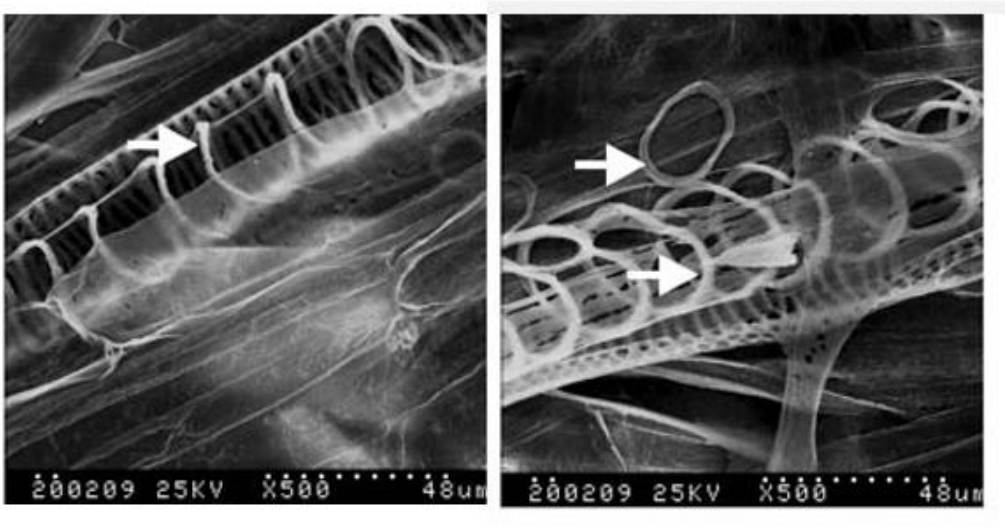


Figure 1.9.4: SEM showing the cellulose framework. The image on the left shows the spiral form vessels and on the right the annular vessels in the vascular bundles (adapted from Yu *et al* 2005).

The structure of cellulose in wheat straw is both crystalline and amorphous,with the different parts of the plant having similar crystallinities (approximately 45%) (table 1.9.1). After hydrolysis for 10 hours at 70^{o}C in a solution of 60% H_2SO_4 (sulphuric acid), hydrolysing the amorphous part, the crystallinity of cellulose increases to 77.2% (Liu *et al* 2004).

Sample	Crystallinity %
Leaf sheath	47.4
Epidermis	44.4
Parenchyma	44.3
Node	43.2
Hydrolysed for 10 h	77.2

Table 1.9.1: Showing the cellulose crystallinity of different fractions of wheat straw. The crystallinity measured using WAXD (Wide-angle X-ray diffraction) and shows that it varies little between fractions but increased dramatically after being hydrolysed (taken from Liu *et al* 2004).

Wheat straw hemicellulose is mainly arabinoxylan and the lignin is made up from all three monolignol units, *p*-hydroxyphenyl–guaiacyl–syringyl (H–G–S) and after thioacidolysis to cleave the ether linkages it shows the proportions of

H-G-S units in lignin to be 5, 49 and 46% respectively (Buranov and Mazza, 2008).

1.10: Genetic diversity in wheat.

As previously stated, lignocellulosic biomass is the most abundant biological resource on the planet and a large proportion of this is made up from agricultural waste. It has been suggested that crop residues, such as wheat straw and corn stover, may be used as a transition between food-based and dedicated energy crops for liquid transport fuels (Heaton *et al*, 2008) and totally renewable and sustainable fuels.

The vast majority of food crops grown in the world today have been domesticated for thousands of years for the production of food only and therefore have been bred to have less biomass and a higher food yield (Gressel, 2007).

Domestication of cereal crops first started 9,600 – 9,000 BC with two wheat species *Triticum monococcum* (diploid einkorn wheat) and *Triticum turgidum* (tetraploid emmer wheat) in an area known as "The Fertile Crescent", and subsequently bred by hundreds of generations of farmers since (Luo *et al*, 2006). This carried on for hundreds of generations with mutations generating new alleles and with recombination creating novel allele combinations; this caused the reduction in genetic variability through random genetic drift, natural selection and the selection by farmers for certain traits eventually resulting in

landrace cultivars (LCs) that are adapted to their local environment (Reif *et al*, 2004).

Landrace cultivars were grown and developed for their ability to provide stable yields in traditional low input farming methods due to each plant within the landrace population having one or more genotypes capable of dealing with various biotic and abiotic stresses (Zeven, 1998). With the advent of modern farming methods and the green revolution, LCs have been replaced by modern wheat cultivars (MWCs) to the point that as of 2005 only 3% of all wheat cultivars were LCs (Reif *et al*, 2004). Over the last 40 – 50 years the International Maize and Wheat Improvement Centre (CIMMYT) has intensively bred spring wheat, and as of 1997 (excluding China) 86% of the spring wheat sown worldwide was CIMMYT or CIMMYT-related germplasm (Reif *et al*, 2004).

Since the 1960s and 1970s, MWCs have increased grain yields substantially. This is due to the introduction of mutant dwarfing alleles that have an abnormal response to gibberellin (a plant growth hormone). Whilst this increases the yield it is at the expense of the straw biomass (Peng *et al*, 1999). It has been suggested that due to the green revolution and subsequent plant breeding, the genetic diversity of wheat has been eroded, creating a narrow genetic base. The vast majority of wheat plants now have the same genetic makeup and this has left them vulnerable to a change in biotic and abiotic stresses and possible mutations in pest populations for example the stem Rust Ug99 (Singh et al, 2011; Moghaddam *et al*, 1997; Rief *et al*, 2004). Evidence is growing to suggest that wheat yields are plateauing and that any increase in yield will require an increase in genetic diversity (Brisson *et al*., 2010; King *et*

al, 2016). Gressel (2007) states that it is naive and ignorant to believe that plants used for bio-fuels that have not been domesticated for that purpose, will grow efficiently and be best suited for their purpose. Neutelings (2011) showed that the manipulation of lignin genes in mutant or transgenic plants gives a high degree of plasticity in the structure (possibly allowing for better saccharification).

1.11: Other Bio fuels.

Third generation bio fuels from algae and microalgae have been suggested as one of the most sustainable options for the production of bio-ethanol and bio-diesel (Maity *et al*, 2014) as not only do they put down large quantities of biomass but also significant quantities of lipids. This is in part due to the fact that they do not have the same levels of lignin and hemicellulose as terrestrial plants as buoyancy in the water provides structure instead (Jones and Mayfield, 2011). Third generation bio-fuels also have the advantage that they do not compete with land space that could be used for food production, thus making them more acceptable to public opinion, (Jambo *et al*, 2015). This could also help with the reduction of water consumption for biofuel production (see section 1.10).

1.12: Water consumption for bio-fuel production.

The water required for second generation bio fuels varies considerably depending upon what feed stock is used. In much of the literature it also takes into account the water needed to grow the feedstock including fertilizing and irrigation, this makes accurate water consumption for the bio-fuel production hard as there will be natural variation depending on where the crop is grown. The fresh water consumption can vary from 2 gallons – 10 gallons of fresh water for every gallon of ethanol produced depending on what method is used (Wu *et al*, 2009). For first generation bio-fuel using corn to ethanol production in the USA, the main water usage is in the growing stages as irrigation and fertiliser production account for around 92.5% with the remainder being used in the conversion process (Lampert et al, 2016). With the use of second generation bio-fuels it should be possible to reduce water consumption as it is derived from waste products that would have been discarded or burned.

Chapter 2: Materials and methods.

2.1: Wheat samples.

All wheat was grown and harvested at the University of Nottingham; it was decided that as many samples would not produce significant biomass to allow for large scale work, commercial varieties would also be used.

2.1.1: Wheat samples for method development.

Orbit wheat variety was grown at the University of Nottingham and harvested during the 2010/2011 growing season, this was the bailed as standard practice and stored inside a dry-store at ambient temperature prior to being knife milled to a 2mm particle size using a Fritch Pulverisette 19 Knife mill, this was then stored in an airtight container until required.

Revelation wheat variety was grown at the University of Nottingham and harvested during the 2013/2014 growing season, this was the bailed as standard practice and stored inside a dry-store at ambient temperature prior to being knife milled to a 2mm particle size using a Fritch Pulverisette 19 Knife mill, this was then stored in an airtight container until required.

2.1.2: Wheat samples to determine effects of growing condition.

The three wheat varieties of Cordiale, Grafton, and Xi19 were all grown outdoors in field 12 at the University of Nottingham and harvested during the 2012/2013 growing season (diagram 2.1). The Xi19 was fractionated into leaf; peduncle and upper (above 10cm) and lower (below 10cm) stem fractions along with whole straw samples prior to knife milling to a particle size of 2mm. Cordiale and Grafton were fractionated into leaf and total stem prior to knife milling. All samples were then stored dry at ambient temperature until required.

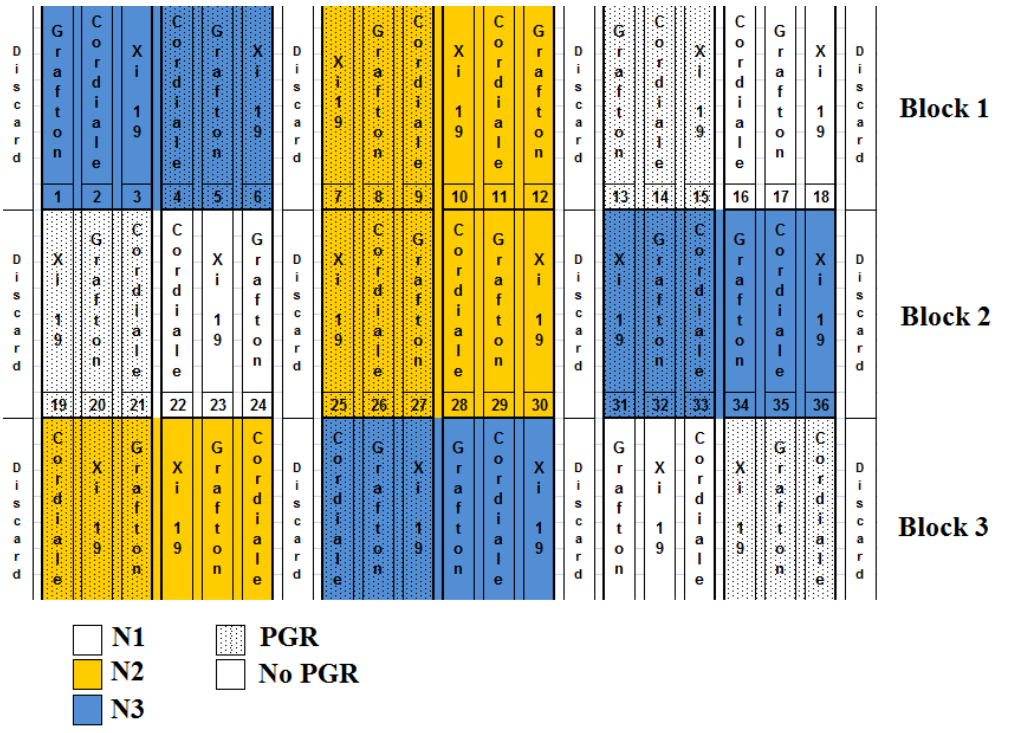


Figure 2.1: Growing position of modern cultivars trialled for PGR and nitrogen applications. Three wheat varieties were grown in field conditions three nitrogen fertilizer regimes nitrogen 1, with no nitrogen; 2 with 50 kg per hectare; and 3 with 100 kg per hectare and either with or without a PGR application

2.1.3: Wheat samples to determine genetic influence.

Back cross (BC1) samples were created by crossing an elite wheat line (Chinese Spring, Paragon, Langdon, Pavon 76, Highbury, Chinese Spring Mutant 84 and Cappelli) with the genome AABBDD that has had the Ph1/Ph1 locus deleted (the Ph1 locus has to be removed before homoeologous recombination between the chromosomes of a wild relative and wheat can occur) with an ancient variety (*Aegilops speltoides*, *Secale iranicum*, *Triticum urartu*, *Thinopyrum elongatum*, *Secale cereal*, *Aegilops comosa*, *Triticum macrochaetum*, *Triticum timopheevii*, *Aegilops triaristata*, *Secale anatolicum*, *Secale montanum* and *Secale vavilovii*) genome RR to create an F1 hybrid (ABD R) and then crossed again with an elite line (AABBDD) with the Ph1/Ph1 deletion (table 2.2 for BC1 – 24 and 2.3 for BC25 - 48), and grown in the glasshouses at University of Nottingham; after the samples had senesced the heads were removed and the seeds collected. Each was then measured for height, weight and leaf:stem ratio. The leaf and stem fractions were then knife milled to a particle size of 2mm. Samples were stored dry at ambient temperature until required.

	BC1 Parents		
BC1 sample	**Female**	**Male**	**Species**
BC1 - 1	CS 94 x Ae speltoides 2140020	Paragon	Aegilops speltoides
BC1 - 2	CS 94 x Ae speltoides 2140018	Paragon	Aegilops speltoides
BC1 - 3	CS mutant 84 x T urartu B14	Paragon	Triticum urartu
BC1 - 4	CS 94 x Ae speltoides 2140008	Paragon	Aegilops speltoides
BC1 - 5	CS mutant 84 x T urartu G65	Paragon	Triticum urartu
BC1 - 6	Paragon mut x S iranicum	Paragon	Secale iranicum
BC1 - 7	CS 94 x Ae speltoides 2140008	Paragon	Aegilops speltoides
BC1 - 8	CS 94	CS mutant 84 x S cereale 390382	Secale cereale
BC1 - 9	CS 94 x Ae speltoides 2140020	Paragon	Aegilops speltoides
BC1 - 10	CS 94 x Ae speltoides 2140008	Paragon	Aegilops speltoides
BC1 - 11	Paragon mut x S iranicum	Paragon	Secale iranicum
BC1 - 12	CS mutant x Ae speltoides 2140066	Paragon	Aegilops speltoides
BC1 - 13	CS 94 x Ae speltoides 2140018	Highbury	Aegilops speltoides
BC1 - 14	Paragon mutant 11	CS mutant P208/511 x Th elongatum 401007	Thinopyrum elongatum
BC1 - 15	Paragon	CS mutant P208/511 x Th elongatum 401007	Thinopyrum elongatum
BC1 - 16	CS 94 x Ae speltoides 2140007	Pavon 76	Aegilops speltoides
BC1 - 17	CS 94 x Ae speltoides 2140018	Paragon	Aegilops speltoides
BC1 - 18	Pavon 76 x Ae speltoides 2140018	Pavon 76	Aegilops speltoides
BC1 - 19	CS mutant x Ae speltoides 2140066	Paragon	Aegilops speltoides
BC1 - 20	Pavon 76 x Ae speltoides 2140018	Pavon 76	Aegilops speltoides
BC1 - 21	Pavon 76 x Ae speltoides 2140018	Pavon 76	Aegilops speltoides
BC1 - 22	CS mutant x Ae speltoides 2140066	Paragon	Aegilops speltoides
BC1 - 23	Paragon	CS mutant P208/511 x Th elongatum 401007	Thinopyrum elongatum
BC1 - 24	CS 94 x Ae speltoides 2140008	Paragon	Aegilops speltoides

Table 2.2. BC1 sample heritage. BC1 samples 1 - 24

	BC1 Parents		
BC1 sample	**Female**	**Male**	**Species**
BC1 - 25	CS 94 x Ae speltoides 2140018	Paragon	Aegilops speltoides
BC1 - 26	CS 94 x Ae speltoides 2140020	Paragon	Aegilops speltoides
BC1 - 27	Paragon mutant 13	Paragon mutant 13 x Th elongatum 401007	Thinopyrum elongatum
BC1 - 28	Paragon	CS mutant P208/511 x Th elongatum 401007	Thinopyrum elongatum
BC1 - 29	CS 94 x Ae speltoides 2140018	Paragon	Secale cereale
BC1 - 30	CS mut 84 x S iranicum P208/11	Pavon 76	Aegilops speltoides
BC1 - 31	CS mutant x Ae speltoides 2140066	Paragon	Secale iranicum
BC1 - 32	CS mutant 84 x T urartu B14	Paragon	Aegilops speltoides
BC1 - 33	CS mutant 84 x T urartu B30	Paragon	Triticum urartu
BC1 - 34	CS mutant 84 x T urartu B30	Paragon	Triticum urartu
BC1 - 35	CS mutant x Ae speltoides 2140066	Paragon	Triticum urartu
BC1 - 36	CS mutant 84 x S cereale 390382	CS 94	Aegilops speltoides
BC1 - 37	CS mutant 84 x T urartu G65	Paragon	Secale cereal
BC1 - 38	CS mutant 84 x S cereale 390382	CS 94	Triticum urartu
BC1 - 39	CS mutant 84 x S cereale 390382	Paragon	Secale cereale
BC1 - 40	CS mutant 84 x T urartu A6	Highbury	Secale cereale
BC1 - 41	CS mutant 84 x S cereale 390382	Paragon	Triticum urartu
BC1 - 42	Paragon	Paragon	Secale cereale
BC1 - 43	Paragon	CS94	Thinopyrum elongatum
BC1 - 44	Paragon	CS94	Thinopyrum elongatum
BC1 - 45	Paragon	CS94	Thinopyrum elongatum
BC1 - 46	Paragon/SELF	CS94	Thinopyrum elongatum
BC1 - 47	Paragon	CS94	Thinopyrum elongatum
BC1 - 48	Paragon	Paragon	Thinopyrum ponticum

Table 2.3. BC1 sample heritage. BC1 samples 25 - 48

2.2: Analysis of sugar composition.

This was carried out using Saemen's hydrolysis (Saeman, 1945) followed by analysis of the monomer sugars by high performance anion exchange chromatography (HPAEC). 30mg of dry biomass was placed into a round-

bottomed Pyrex tube and 1ml of 12M H_2S0_4 was then added, a cap was then screwed on and the sample then placed into a rack and half submerged into a water bath (Grant GD 100) at 37^{o}C for 60 min. The samples were then removed and an additional 11ml of demineralised water was added, the samples were then half submerged in a water bath (Grant GD 100) at 100^{o}C for 120 min, the samples were then removed and placed into a cold-water bath until they became cool enough to handle. 0.1ml of the sample was then placed into a labelled 15ml Falcon tube and 9.9ml of 10mM NaOH was added to make a total of 10ml and the tube capped. Samples were placed into acentrifuge (Thermo Scientific, Megafuge 16 centrifuge) and spun for 5 min at 4472xg. 1-1.5ml of the sample was then taken from the top section of the sample and placed into a numbered HPLC vial for sugar analysis using a Reagent-FreeTM Ion Chromatography equipped with Dionex ICS-3000 system, electrochemical detection using ED 40 and computer controller. A CarboPacTM PA 20 column (3 x 150 mm) was used for the separation with a mobile phase of 10 mM NaOH and a flow rate of 0.5 mL/min. The injection volume was 10 µl and the column temperature was 30^{o}C.

Standard sugar samples consisting of 2g of glucose, 2g of xylose, 2g of arabinose and 2g of galactose dissolved into 1L of demineralised water were diluted to create 1g/L, 0.5g/L and 0.25g/L standards; see figure 2.1 for example.

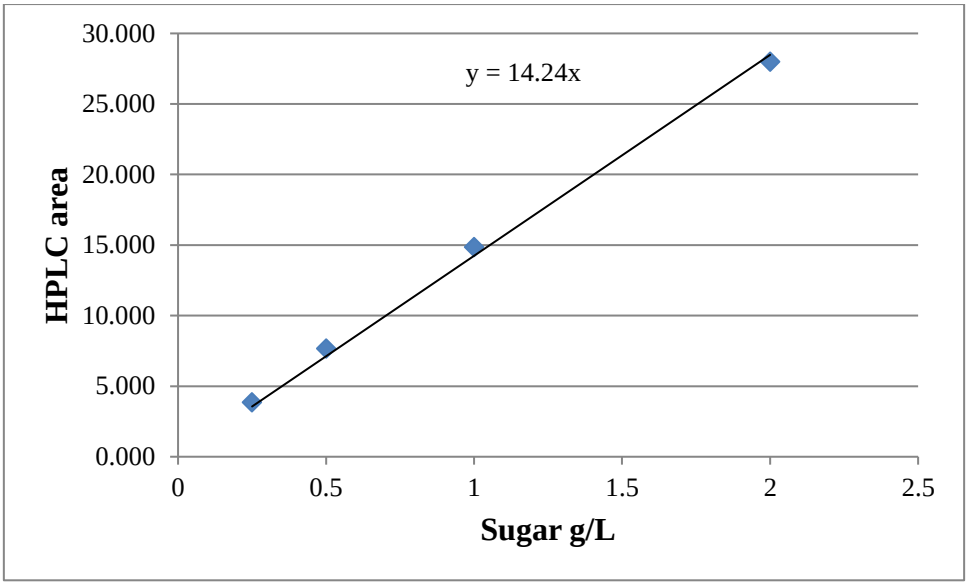


Figure 2.2: Example of standard curve for calculating sugar quantity. Linear graph showing the y value used to convert HPLC area into relevant sugar in g/L (showing glucose), 2 g of glucose was dissolved in 1L of RO water and a serial dilution was made to create 2g/L, 1g/L 0.5g/L and 0.25g/L. They were then diluted1:100 in 10mMNaOH and analysed by HPLC. The peak area was then used to create a standard curve.

2.3: Chemical pre-treatment of straw biomass.

1 g of knife milled wheat tissue was placed into a round bottomed Pyrex tube, 20 ml of acid or base was then added and the top screwed on. Samples were then placed into an autoclave (Priorclave autoclave Tactrol 2) and heated at 121^{o}C for 30 min. The samples were removed when the temperature had cooled to 55^{o}C; samples were then transferred to 50 ml plastic Falcon tubes and centrifuged (Thermo Scientific, Megafuge 16 centrifuge) for 5 min at 4472xg. Liquor was then removed using a 10-ml syringe and stored at -80^{o}C for future analysis.

Samples were then washed by adding 40 ml of de-mineralised water to the solid residue in each tube and vortex mixed (Fisher Scientific TOPMIX FB15024) for 30 s. The samples were then centrifuged again for 5 min at 4472xg and again the liquor removed; this was repeated 4 times. Following the final rinse the solid residue was collected and allowed to air dry for 48 h. Dry samples were then placed into labelled Falcon tubes and frozen until needed. The above method was also carried out using 1% H_2S0_4, 3% H_2S0_4, 5% H_2S0_4, 1% HNO_3, 1% KOH and 1% NaOH to determine the most appropriate pre-treatment.

2.4: Sea water Pre-treatments.

1% H_2SO_4 was made by diluting concentrated H_2SO_4 into sea water that had been collected from Brancaster beach in Norfolk (52°58'29.2"N 0°38'13.9"E) in June 2015 and stored at 4^{o}C until needed; this was used to determine differences between fresh water and sea water for pre-treatments. A standard pre-treatment as described in pre-treatment section 2.3 was carried out substituting de-mineralised for sea water.

2.5: Digestibility assay.

This was carried out using a slight modification to the method described by the National Research Energy Laboratory (NREL) (Selig *et al* 2008). A stock of Cellulase enzyme was prepared by dissolving 200mg of cellulase from *Trichoderma reesei*, (SIGMA ATCC 20921, lot # 31M1283V) in 20ml of 50mM sodium citrate buffer (pH 4.8). This stock was made up as required on the day of use, 200mg of air dried pre-treated biomass was placed into a numbered 50ml Falcon tube and 36ml of 50mM sodium citrate buffer (pH 4.8) was added.

4ml of the stock cellulase enzyme was then added to the numbered 50ml Falcon tube to make a 0.005% wt/wt solid dispersion and a cellulase activity of 40 filter paper units (FPU) per g/ biomass. The samples were then placed horizontally into a shaking incubator (Sarorius Stedim biotech certomat BS1) at 50^{o}C and 150 rpm. After 15 minutes, the tubes were removed and 0.1ml of the sample was transferred into a labelled 15ml Falcon tube and 9.9ml of 10mM NaOH added. This was taken as time zero. Experimental assay samples were then placed back into the incubator whilst the time zero samples were placed into a cold room (4^{o}C) to await further analysis. This sampling was repeated after 4 hours, 24 hours and 72 hours of incubation. All samples were then centrifuged (Thermo Scientific, Megafuge 16 centrifuge) for 5 minutes at 4472xg. 1-1.5ml of the sample was then taken from the top section of the tube and placed into a numbered HPLC vial for sugar analysis using the HPLC (DIONEX ICS 3000) as previously described in section 2.2.

2.6: Sea water enzyme hydrolysis.

This was carried out by adapting the previous method and improving the enzyme used. A stock enzyme was prepared by adding 30ml of Ctec2 (a cocktail of cellulases, ß-glucosidases, and hemicellulase) to 570ml of both sea water and fresh water, 0.1ml of this was transferred into a labelled 15ml Falcon tube and 9.9ml of 10mM NaOH added. This was taken as time zero. 200mg of Revelation straw that had been pre-treated as described in the section 2.1.1, was placed in a 50ml Falcon tube and the stock enzyme added for both sea water and fresh water respectively to give a 10% w/v ratio with an FPU value of 39/g biomass. The samples were then placed into a shaking incubator (Sarorius Stedim biotech certomat BS1) at 50^{o}C and 150 rpm. After 72 hours incubation, the samples were removed and 0.1ml was transferred into a 15ml Falcon tube with9.9ml of 10mM NaOH added. All samples were then centrifuged (Thermo Scientific, Megafuge 16 centrifuge) for 5 min at 4472xg. 1-1.5ml of the sample was then taken from the top section of the tube and placed into a HPLC vial for sugar analysis using aDIONEX ICS 3000 HPLC as previously described in section 2.2.

2.7: One pot pre-treatment and enzyme digestion.

This was carried out using both fresh water and sea water, Revelation straw as bailed was knife milled to a particle size of 0.5mm, 10g of this was placed into a 100ml MFV (miniature fermentation vessel) and 50ml of ether 1% H_2S0_4 made using de-mineralised water or 50ml 1% H_2S0_4 made using sea water was added to give a solid loading of 10%. This was then autoclaved at 121^oC for 15 minutes. Once the samples had cooled 45 ml of 2% NaOH made with either fresh water or sea water added to neutralise before samples were placed into a shaking incubator for 30 min at 50^oC and 150 RPM. The samples were then removed and 5ml of stock CTec 2 (a cocktail of cellulases, ß-glucosidases, and hemicellulase) added to make a 10% solid loading with an FPU value of 392/g biomass and then returned to the incubator. Time zero was taken by adding 50ml of 1% H_2S0_4, 45ml of NaOH and 5ml of stock CTec 2 and vortexing, 0.1 ml of this was transferred into a 15ml Falcon tube and 9.9ml of 10mM NaOH added, due to the viscosity of the sample. After 24 hrs, the sample was analysed by HPLC as previously described in section 2.2. Each sample was then inoculated with yeast (*Saccharomyces cerevisiae* NCYC2592) and weighed to determine time 0, fermentation progression was monitored by weight loss over time to indicate CO_2 evolution as per (Quain *et al*, 1985). Samples were then placed on a stirrer plate at 55RPM and incubated at 35^oC. Samples were weighed to determine weight loss and samples were taken by syringe at selected time points and analysed using a Jasco using a hyplex column (temperature 35^oC with a flow rate of 0.4ml min injection 10 micro) L to measure ethanol production.

2.8: Ability of hydrolysate to support metabolic growth.

The "inhibition capacity" of the pre-treatment hydrolysates was assessed by monitoring their impact on the metabolic activity of yeast using a phenotypic array in the biolog (Greetham, *et al*, 2014). The liquor hydrolysate from the acid pre-treatment, as previously described in section 2.5, was adjusted to pH 4.5 using 1 M NaOH. The metabolic activity of the yeast was determined using a 96 well microtitre plate specifically designed for the Biolog. Each well contained 20.8 µl hydrolysate ,0.2 µl dye and 9 µl glucose from an 80% stock solution (to give a 6% starting glucose concentration) with the control consisting of 18 µl RO water 0.2 µl Dye and 9 µl glucose and 2.8 µl YNB (used as a positive control to show the yeast was active).

Yeast strain (*Saccharomyces cerevisiae* NCYC2592;) was prepared from glycerol stocks stored at -80°C these were streaked onto a YPD (Yeast Extract Peptone Dextrose) plate and incubated at 30°C for 48 hours Colonies were then re-streaked onto a fresh YPD plate and incubated for a further 24 hours.

Yeasts suspensions were prepared by inoculating them from the re-streaked YPD plates into sterile water in 20×100 mm test tubes and adjusted to a density of 5×10^6 cells.ml^{-1} with sterile distilled water using turbidometer. Cell suspensions were prepared by mixing 500 µl of these cells with 2.5 ml of IFY buffer™ (Biolog, USA), to make a total volume of 3ml.

90 µl of this Buffered yeast solution was added to each of the 30 µl hydrolysate or control samples on the 96 well plate to start the assay. Samples

were then placed in an BioLog reader and readings taken every 15 min for 72 hours. The intensity of dye conversion was used to determine metabolic output; all analyses were performed in triplicate.

2.9: Chemical Pre-treatment and digestability assay (York High throughput (HT)).

Untreated BC1 samples were sent to Dr Leo Gomez at York University and a high throughput assay to determine the recalcitrance of straw was carried out as described in Gomez *et al* (2010). The preparation of each sample was done via a robotic arm with the ability to weigh to within 0.1mg. 4 mg of each raw sample was loaded into a 96-well plate and subjected to either a 1% H_2SO_4 at 95^{o}C for 15 minutes or 0.5M NaOH 95^{o}C for 15 minutes. They were neutralized by adding appropriate amount of acid or alkali. CTech 2 (Novozymes) (a cocktail of cellulases, ß-glucosidases, and hemicellulase) was added at a level of 8 filter paper units (FPU) and then incubated at 50^{o}C. The hydrolysis was then analysed for reducing sugar equivalents using dinitrosalicylic acid (DNS) reagent.

2.10: Differential Scanning Calorimetry (DSC).

Untreated straw that had been knife milled to a partial size of 2mm was then passed through a hammer mill to reduce the size to 0.5mm and was mixed with de-mineralised water to yield a 1:1 ratio paste, 15mg was then placed into a gold DSC pan and a lid and cap secured using a torque wrench, the pans were then placed into DSC (Mettler Toledo DSC 823^{e} with a 50801R0 Robot sample placing), and ran from +20^{o}C to 310^{o}C with data collected at 5min intervals.

2.11: Genetic analysis of BC1 samples.

BC1 samples were sent for genetic mapping. Genotyping was performed in collaboration with the University of Bristol. Exon capture of the 10 wild relatives and 4 wheat accessions used in the introgression programme at Nottingham. This data was then used to identify single nucleotide polymorphisms (SNPs) between the wild relatives and the wheat accessions. In total approximately 650,000 SNPs were discovered. Many of these SNPs were then used to produce a 35,000 SNP array (Axiom® Wheat-Relative Genotyping Array) in collaboration with Affymetrix (each of the SNPs selected were polymorphic for at least 6 species). The array was used to genotype BC1, BC2 and BC3 plants produced from several of the wild relative

species. The resulting markers from this analysis were sorted into linkage groups in JoinMap® 4.1 (Van Ooijen, 2011) by the introgression group at the University of Nottingham.

2.12: Statistical analysis.

Statistical analysis was performed using Microsoft Excel 2013, and SPSS for windows. Statistical tests used were ANOVA (Analysis of variance) followed by a Scheffe post hoc test to determine differences between treatments and growing conditions and a Pearson's correlation coefficient test to identify correlations within the BC1 population.

Chapter 3: Selection of suitable pre-treatment conditions.

3.1: Introduction.

As stated in section 1.4.3, a pre-treatment stage is needed to disrupt the plant material in order to allow enzymes access to the cellulose for efficient conversion to glucose. For this, a chemical pre-treatment design was chosen as this will more likely be used in commercial practice in conjunction with a physical treatment. The initial approach was to develop a selection of suitable pre-treatment(s) with which to screen the BC1 wheat populations. This would ideally be a treatment resulting in approximately 20 - 30% of the available glucose being released by digestion from commercial wheat variety. This would allow for the identification of BC1 lines with both improved and impaired digestion. With this in mind, the first aim of this project was to determine the quantity of available sugar in straw biomass; for this, whole orbit as baled was knife milled to a particle size of 2mm as described in section 2.1.1 and the biomass subjected to a total acid hydrolysis and analysed for monomer sugar composition by HPAEC as described in section 2.2.

The results showed glucose to be 0.355 ± 0.05 g per g of biomass and xylose to be 0.23 ± 0.035 g per g biomass.

3.2: Selection of a suitable pre-treatment.

The pre-treatment screen was carried out using whole orbit as baled as a standard wheat sample. The wheat was milled and the biomass subjected to a pre-treatment, as described in methods (section 2.3), except that a range of acids and alkalis were used at varying concentrations (1% H_2S0_4, 3% H_2S0_4, 5% H_2S0_4, 1% HNO_3, 1% KOH and 1% NaOH). The pre-treated biomass was then washed in distilled water and air dried for 72 hrs. It was subjected to a total acid hydrolysis and the resulting monomeric sugars analysed by HPAEC (section 2.2). Results for glucose are shown in figure 3.2.1 and xylose in figure 3.2.2.

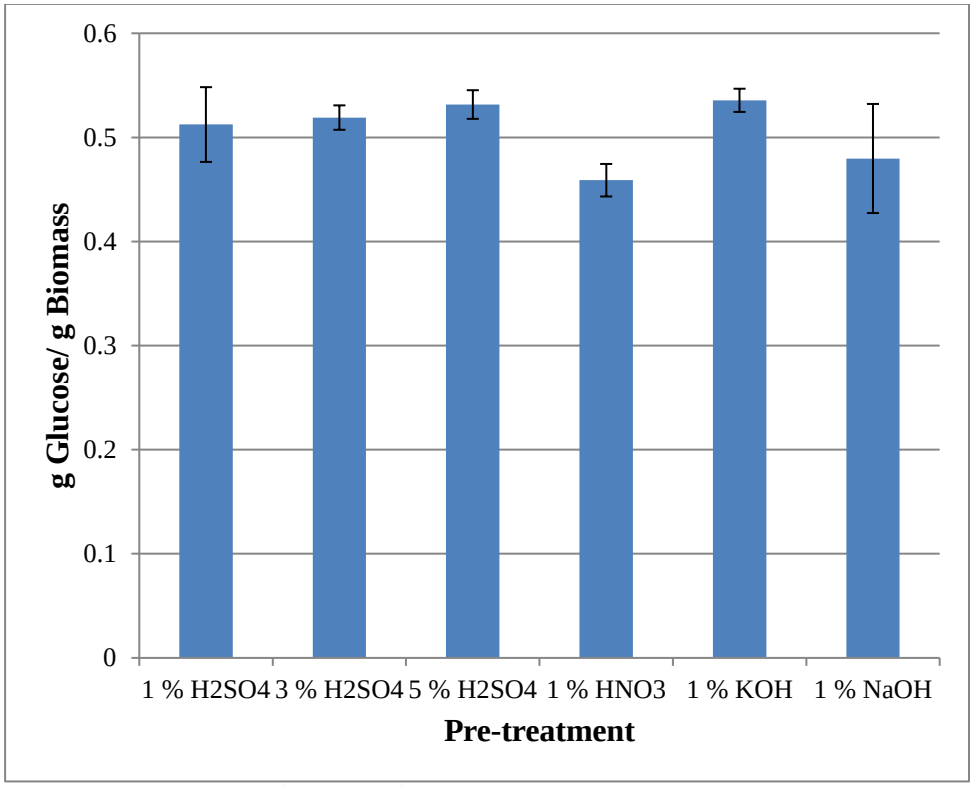


Figure3.2.1: Glucose content of the residue from pre-treated straw. Orbit wheat straw as bailed was milled to a 2mm particle size and subjected to a chemical pre-treatment followed by total acid hydrolysis to determine the quantity of glucose available., Results shown are mean ± SD (n= 4).

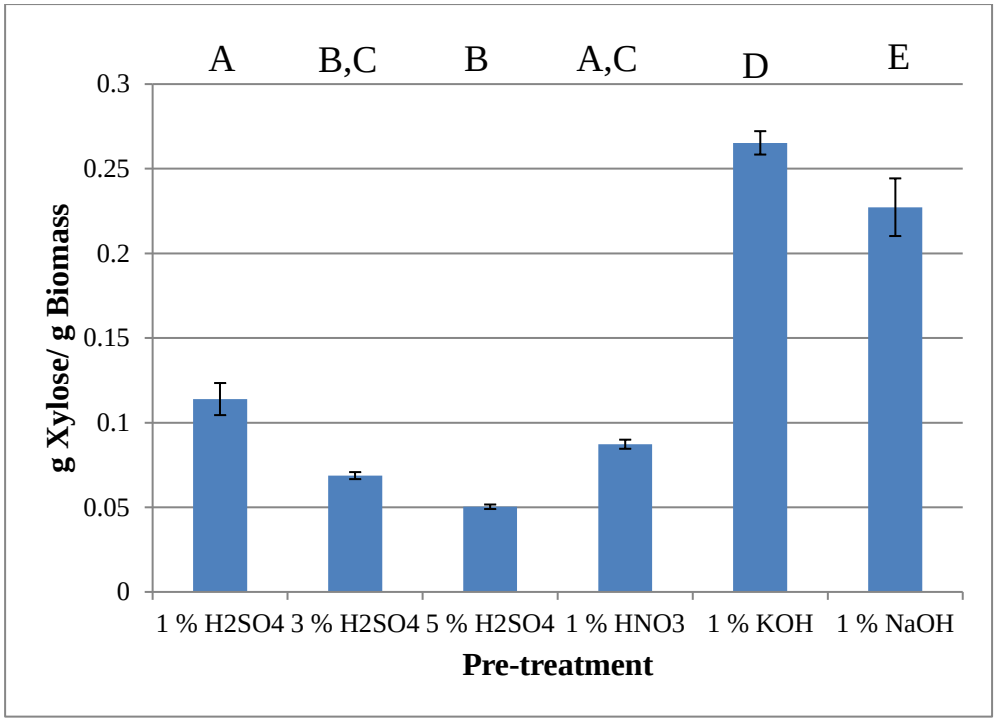


Figure 3.2.2: Xylose content of the residue from pre-treated straw. Orbit wheat straw as baled was milled to a 2mm particle size and subjected to a chemical pre-treatment followed by total acid hydrolysis to determine the quantity of glucose available. Results shown are mean ± SD (n= 4) bars with different letters are significantly different (p<0.05).

For glucose analysis, using ANOVA shows no significant differences between any of the pre-treatments (p>0.5); however, for xylose the analysis using ANOVA showed a significant difference (p< 0.005), post hoc test analysis inferred there was no difference between 1% H_2SO_4 and 1% HNO_3, 3% H_2SO_4 and 5% H_2SO_4, 3% H_2SO_4 and 1% HNO_3. Both 1% KOH and 1% NaOH were significantly different to all other samples. One observation of interest was the vast difference in appearance (colour and texture) between the samples pre-treated with acid compared to samples treated with a base (Figure 3.2.3). This could be due to the lignin fraction being altered with the use of a base in the pre-treatment stage, as stated in section 1.5.3.2. Alkaline treatments are known to disrupt ester bonds that form the cross-links between lignin, cellulose and hemicelluloses.



(A)



(B)



(C)

Figure 3.2.3: Impact of acid or base treatment on the visual appearance of wheat straw. (A) Orbit straw as bailed, knife milled to a 2mm partial size (B) residue following a standard 1 % H_2SO_4 pre-treatment and air dried for 72 hours (C) residue following a standard 1 % NaOH pre-treatment and air dried for 72 hours.

The air-dried samples were weighed after the treatment to determine the % of the original biomass recovered after pre-treatment (figure 3.2.4).

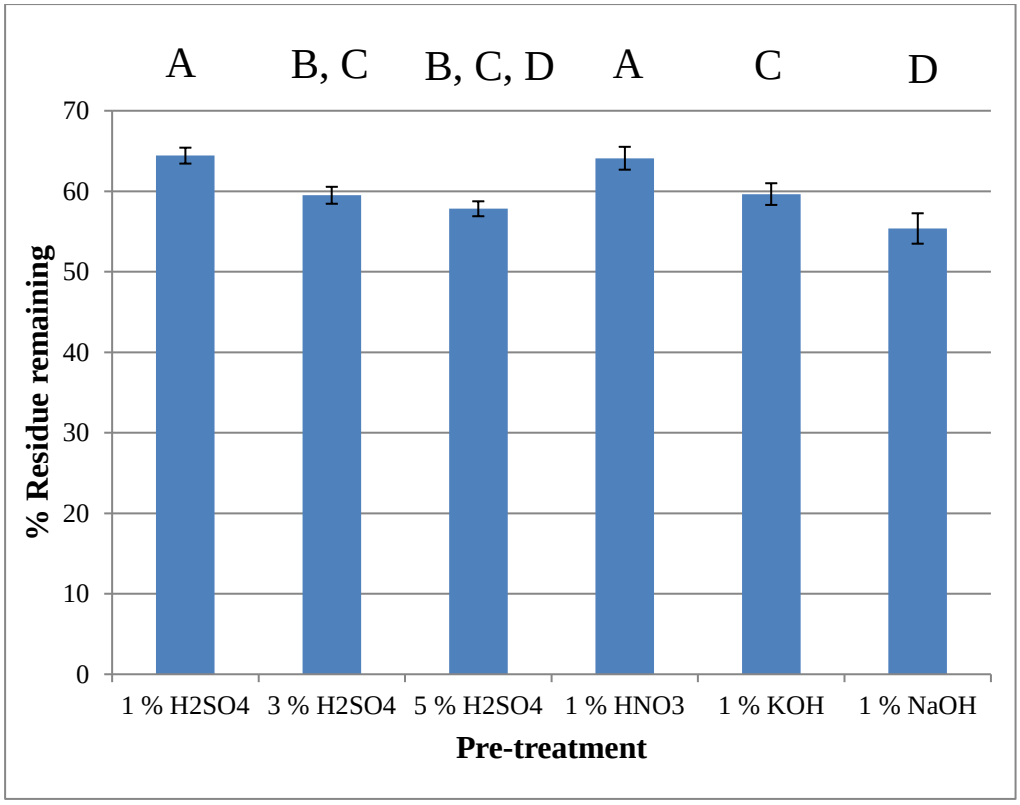


Figure 3.2.4: Recovery of biomass in the residue remaining following a chemical pre-treatment. Orbit wheat straw as bailed was milled to a 2mm particle size and subjected to a chemical pre-treatment and allowed to air dry for 72 hours, samples were then weighed to determine recovery during the pre-treatment. Results shown are mean ± SD (n= 4) bars with different letters are significantly different. (p<0.05)

Analysis of the percentage of remaining residue using ANOVA, showed a significant difference (p<0.05). Post hoc analysis inferred there was no difference between 1% H_2SO_4 and 1% HNO_3, 3% H_2SO_4 and 5% H_2SO_4, 3% H_2SO_4 and 1% KOH, 5% H_2SO_4 and 1% KOH, 5% H_2SO_4 and 1% NaOH. All other samples were significantly different.

The dried residue was then subjected to a standard digestion assay (section 2.5) using cellulase from *Trichoderma reesei* and assessed for glucose (figure 3.2.5) and for xylose (3.2.6) using enzymatic hydrolysis (section 2.5).

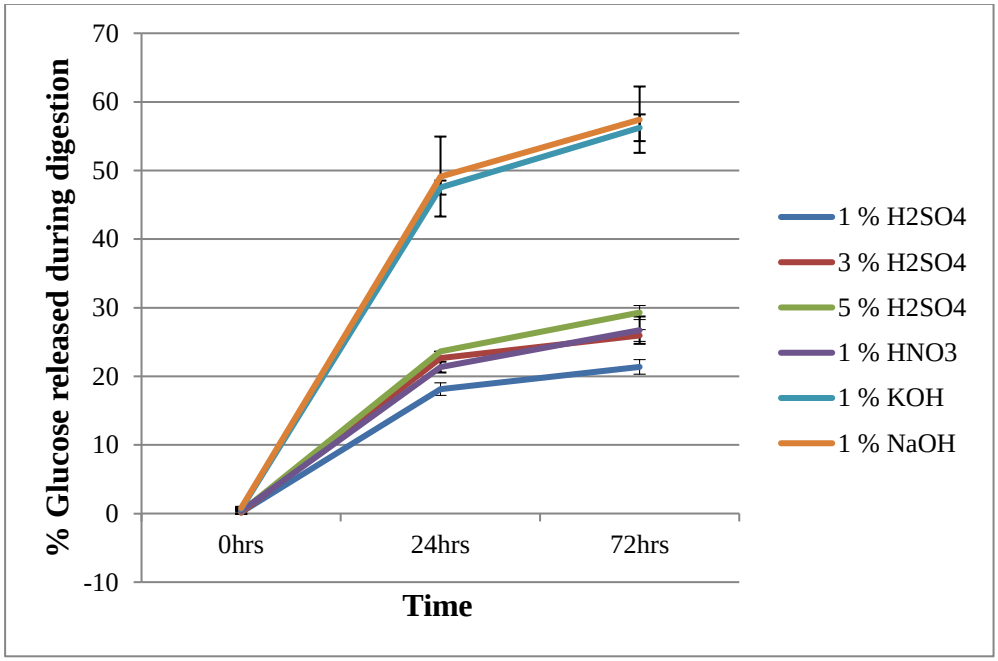


Figure 3.2.5: % available glucose released from pre-treated wheat straw during digestion. Orbit straw as bailed was subjected to various chemical pre-treatments, the solid residue collected and air dried prior to digestion using cellulase from *Trichoderma reesei* at 40 FPU per g/ biomass at 50ºC and sampled over 72 hours. Results shown are mean ± SD (n= 4).

ANOVA for glucose released during digestion shows no significant differences at time 0 hrs (meaning all samples were equal at the start), after 24 hrs a significant difference was seen ($p < 0.05$). Post hoc test infers this is only between the acid pre-treatments and the base pre-treatments. After 72 hrs, the significant difference increased ($p < 0.0005$) with the Post hoc analysis inferring that as well as the acid pre-treatments and the base pre-treatments differing, there was also a significant difference between the 1% H_2SO_4 and the 5% H_2SO_4 treatments.

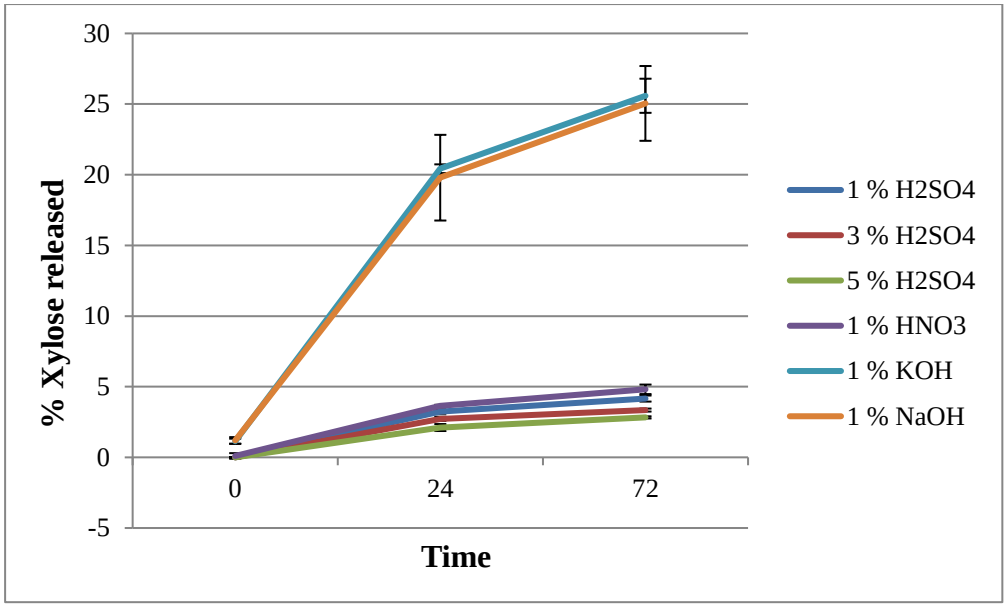


Figure 3.2.6: % available xylose released from pre-treated wheat straw during digestion. Orbit straw as bailed was subjected to various chemical pre-treatments, the solid residue collected and air dried prior to digestion using Cellulase from *Trichoderma reesei* at 40 FPU per g/ biomass at 50ºC and sampled over 72 hours. Results shown are mean ± SD (n= 4).

ANOVA for xylose released during digestion showed a significant difference ($p<0.05$) at all three time points. Post hoc analysis inferred that there was no significant difference between samples treated with acids, and no difference between samples that had been treated with bases but a highly significant difference was seen between acid treated samples and base treated samples.

The high levels of xylose suggest that, as expected, the majority of the hemicellulose fraction was not removed during pre-treatment when a base was used.

3.3: Analysis of the pre-treated liquor.

As seen in figure 3.2.5 and 3.2.6 the levels of glucose and xylose in the pre-treated residue differed greatly between the acid and base treatments. To determine if this was due to sugars being lost during the pre-treatment stage, the liquor from the pre-treatment was analysed for monomeric composition using HPAEC figures 3.3.1 for arabinose, 3.3.2 for galactose, 3.3.3 for glucose and 3.3.4 for xylose.

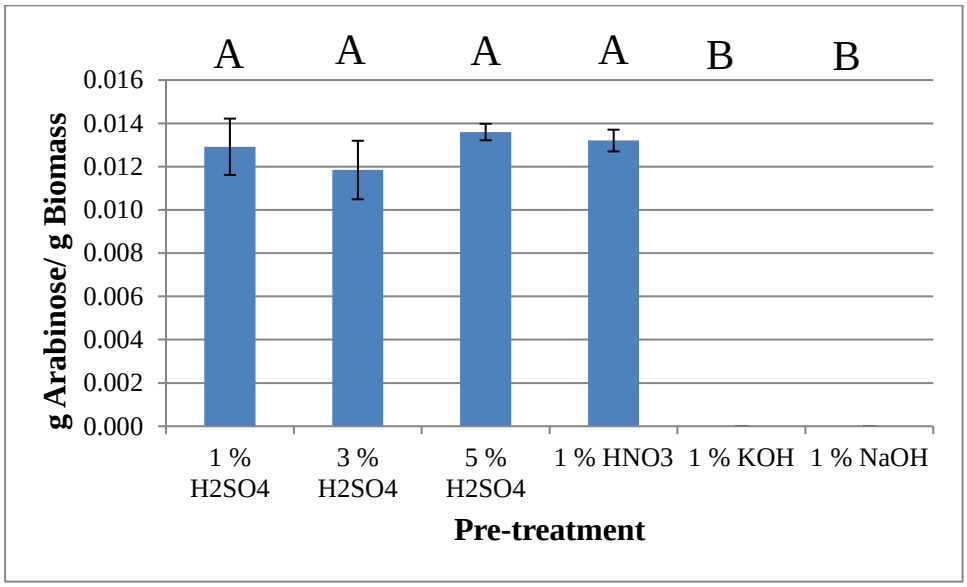


Figure 3.3.1: Arabinose recovered in the pre-treated liquor. Orbit wheat straw as bailed was milled to a 2mm particle size and subjected to a chemical pre-treatment. The liquor fraction was analysed using HPAEC to determine monomeric sugar composition. Results shown are mean ± SD (n= 4) bars with different letters showing significant differences (p<0.05).

Analysis of the data using ANOVA for arabinose in the pre-treated liquor showed a significant difference ($p < 0.005$). Post hoc inferred a difference

between acid treatments and base treatments with all acid pre-treatments being identical and all base treatments being identical.

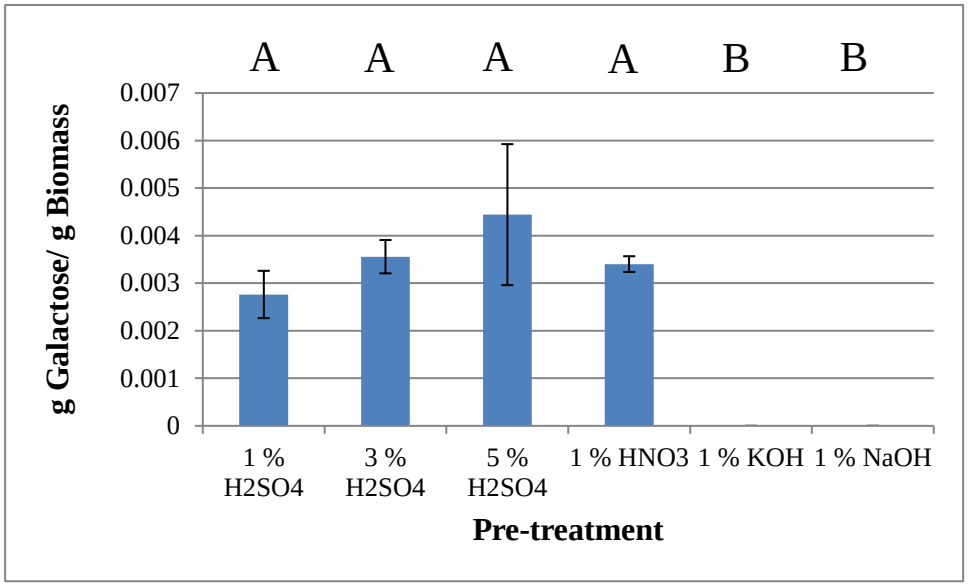


Figure 3.3.2: Galactose recovered in the pre-treated liquor. Orbit wheat straw as bailed was milled to a 2mm particle size and subjected to a chemical pre-treatment the liquor fraction was analysed using HPAEC to determine monomeric sugar composition. Results shown are mean ± SD (n= 4) bars with different letters showing significant differences (p<0.05).

Analysis of the data using ANOVA for galactose yield in the pre-treated liquor showed a significant difference ($p < 0.005$). Post hoc analysis inferred a difference between acid treatments and base treatments with all acid pre-treatments being identical and all base treatments being identical.

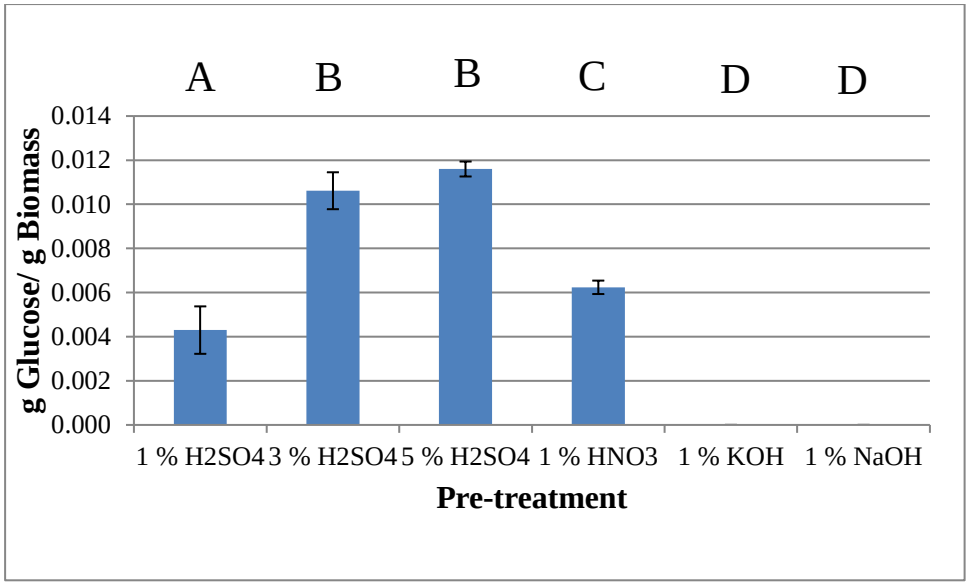


Figure 3.3.3: Glucose recovered in the pre-treated liquor. Orbit wheat straw as bailed was milled to a 2mm particle size and subjected to a chemical pre-treatment the liquor fraction was analysed using HPAEC to determine monomeric sugar composition. Results shown are mean ± SD (n= 4) bars with different letters showing significant differences (p<0.05).

Analysis of data using ANOVA of glucose yield in the pre-treated liquor showed a significant difference ($p < 0.005$). Post hoc analysis inferred a difference between acid treatments and base treatments, the 1 % H_2SO_4 and the 1 % HNO3, the 3 % H_2SO_4 and 5 % H_2SO_4 pre-treatments being identical and all base treatments being identical. As stated in section 1.4.2.2, cellulose has highly orientated crystalline structure but also amorphous regions; the additional glucose seen with the stronger H_2SO_4 pre-treatments could be due to the break down some of the amorphous cellulose.

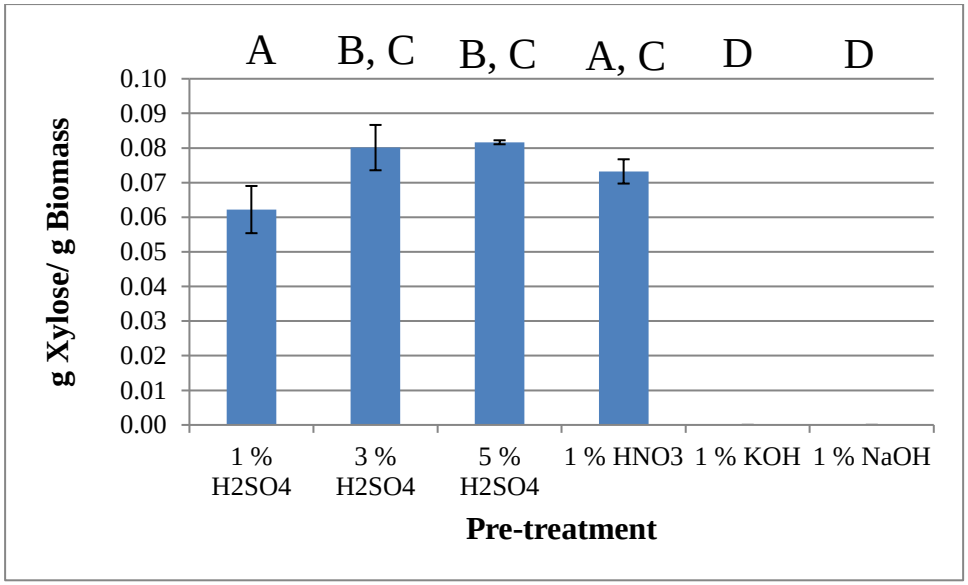


Figure 3.3.4: Xylose recovered in the pre-treated liquor. Orbit wheat straw as bailed was milled to a 2mm particle size and subjected to a chemical pre-treatment the liquor fraction was analysed using HPAEC to determine monomeric sugar composition. Results shown are mean ± SD (n= 4) bars with different letters showing significant differences (p<0.05).

Analysis of data using ANOVA of xylose yield in the pre-treated liquor shows a significant difference ($p < 0.005$). Post hoc analysis inferred a difference between acid treatments and base treatments, with 1 % H_2SO_4 being significantly different to both 3 % H_2SO_4 and 5 % H_2SO_4 as well as the base treatments but identical to the 1 % HNO3 treatment. The 3 % H_2SO_4 5 % H_2SO_4 and 1 % HNO3 treatments were identical as were the 1 % KOH and the 1 % NaOH.

3.4: Conclusions.

It can be seen that the alkaline treatments were significantly better with regards to increasing cell wall digestibility when compared to acids for the yield of glucose and xylose. This could be due to the fact that acid pre-treatments have been shown to break down the hemicellulose fraction whilst leaving both the cellulose and lignin intact (Talebnail *et al*, 2009), in comparison to alkali pre-treatments, where the major reactions have been shown to be much more effective at de-lignification (Kim *et al*, 2015). However, the aim of this work was to develop a treatment that would liberate 20 – 30% of the available glucose and all the acid based treatments were shown to be suitable. In commercial practice the use of minimal acid would be preferred due to financial costs, the need for chemical recovery or neutralisation and corrosion to equipment a 1% H_2SO_4 would seem a good choice for the pre-treatment of wheat straw.

Whilst it was shown that acid treatments and base treatments were significantly different, it should be noted that no monomeric sugars were detected in the pre-treated liquor of the latter. This could be due to them still being in the dried residue (suggested by the amount of xylose in the enzymatic hydrolysis). Alternatively, as the HPAEC is using a CarboPacTM PA 20 column, it was only capable of detecting monomers. As base pre-treatment is known to result in the solubilisation of oligomers, it renders monomeric sugars undetectable.

These results suggest that it may be possible to determine hemicelluloses composition by analysing the liquor from acid based pre-treatments. By using a 1 % H_2SO_4 pre-treatment it may be possible to determine the hemicelluloses composition whilst only removing 0.5% of the glucose content thus still allowing for 20 % to be recovered during the enzymatic hydrolysis. Drawing from this observation, all further work shall be carried out using 1% H_2SO_4.

Chapter 4: Effect of cultivar and growing conditions on straw properties.

4.1: Introduction.

As shown in section 1.9, wheat has a repeating structure (nodes and internodes) that develop at differing stages of plant growth. As it has been shown the composition and architecture of the cellulose, hemicelluloses and lignin are not uniform across the cell wall as the plant grows (Keegstra, 2010). Before analysing the BC1 population, which exhibited very diverse morphology, it was important to ascertain the impact different tissues may have on sugar composition and digestibility. This initial study was carried out on wheat samples cultivated as part of another study being performed at the University of Nottingham. Examining the impact of plant growth regulators (PGRs) and nitrogen input on wheat growth in field conditions during the 2011 season. Three wheat varieties were being tested (Xi19; Grafton and Cordiale) and these were grown in 3 separate replicate blocks with each block being divided into 2. Each plot in the block, being 24 m in length and 1.6 m in width. Seeds were sown at a density of 250 seeds per m^2. The plots were subjected to three nitrogen fertilizer regimes: without nitrogen; with 50 kg per hectare; and with 100 kg per hectare, all either with or without a PGR application (diagram 2.1). After harvest all the plants in each plot were fractionated, as appropriate, and combined to form one sample.

4.2: Comparison of wheat straw tissue for composition and digestibility.

The study to ascertain the impact different tissues may have on sugar composition and digestibility was carried out using one of the wheat cultivars - Xi19. For this analysis, the results from all the nitrogen and PGR applications were pooled n=18. Xi19 is a group 1 wheat that is used mainly for bread production, the export market for food production and as animal feed. It is a high yielding modern elite variety with good rust resistance (wheat stem rust is a fungal disease that can lead to up to 20% yield loss in grain). The straw is quite long and weak however, so requires robust PGR (Plant Growth Regulator) application programmes. It can be sown between the end of September and March.

Xi19 wheat straw was harvested and fractionated into leaf; peduncle; upper (stem above 10cm); lower (stem below 10cm) fractions and samples of "whole" un-fractionated straw prior to knife milling to a 2mm particle size. The fractions were then subjected to Saeman hydrolysis to determine sugar composition as per section 2.2. The results for glucose are shown in figure 4.2.1 and for xylose in figure 4.2.2.

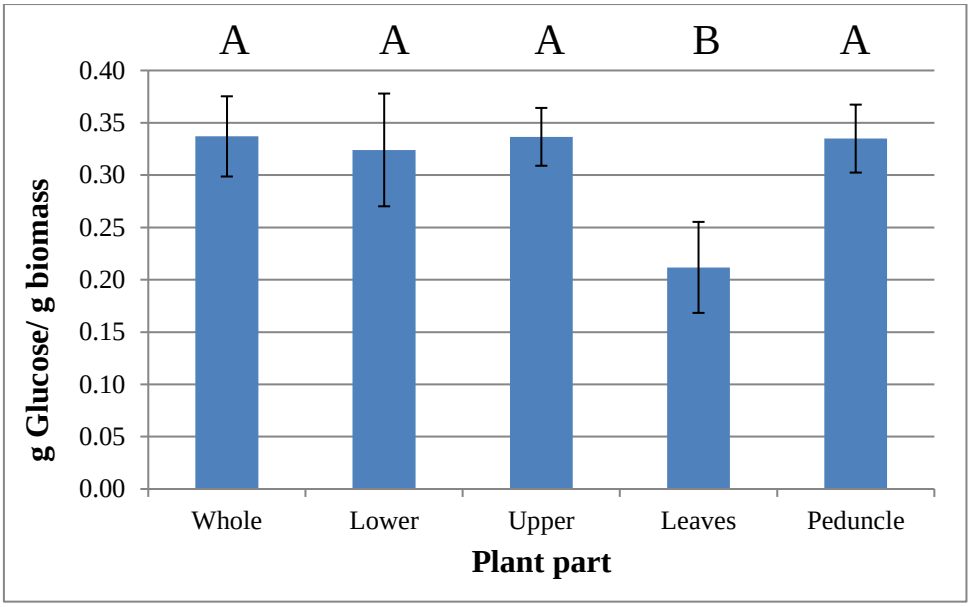


Figure 4.2.1: Glucose content of wheat straw tissues. Wheat Xi19 was harvested and fractionated into leaf, peduncle, upper (stem above 10cm), and lower (stem below 10cm) fractions. Samples of "whole" un-fractionated and individual fractions were then milled to 2mm particle size and sugar composition determined. Results shown are mean ± SD (n= 18), bars with different letters showing significant difference ($p<0.05$)

Analysis of the data using ANOVA for glucose content in raw straw showed a significant difference ($p = 0.0009$) between the different plant fractions. Post hoc analysis inferred that the only significant difference was seen between the leaves and all other fractions with the peduncle; upper (stem above 10cm); lower (stem below 10cm) fractions and samples of "whole" un-fractionated straw being identical to each other.

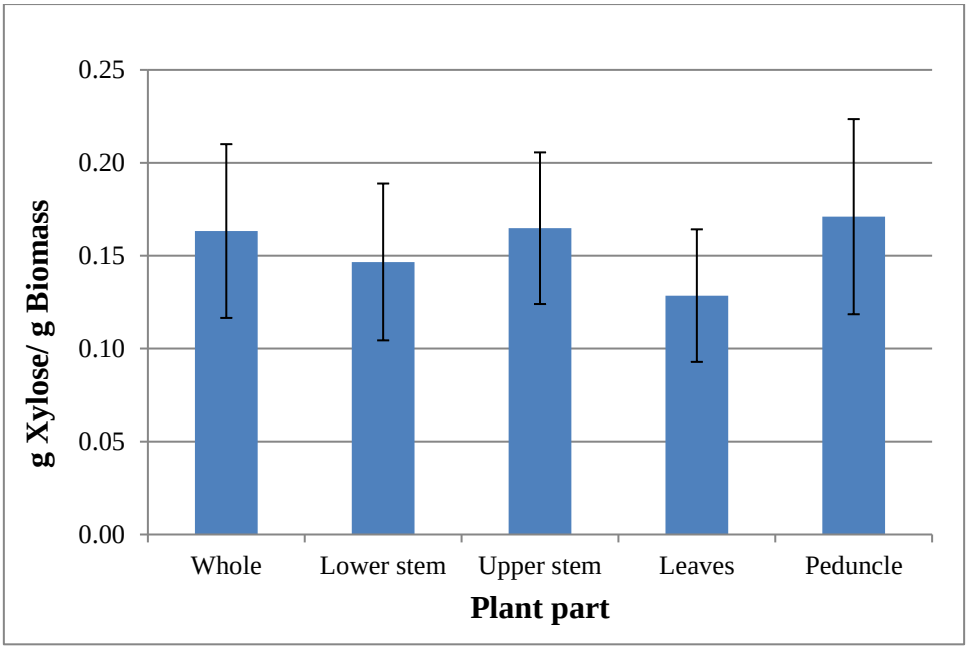


Figure 4.2.2: Xylose content of wheat straw tissues. Wheat Xi19 was harvested and fractionated into leaf, peduncle, upper (stem above 10cm), and lower (stem below 10cm) fractions. Samples of "whole" un-fractionated and individual fractions were then milled to 2mm particle size and sugar composition determined. Results shown are mean ± SD (n= 18).

Analysis of the data using ANOVA of the xylose content in raw straw showed no significant differences between any of the fractions (p>0.05).

The raw straw fractions were then subjected to a standard 1% H_2SO_4 pre-treatment as described in section 2.3; the resulting residue was then subjected to Saemen's hydrolysis as per section 2.2. The results for glucose and xylose are shown in figures 4.2.3 and 4.2.4, respectively.

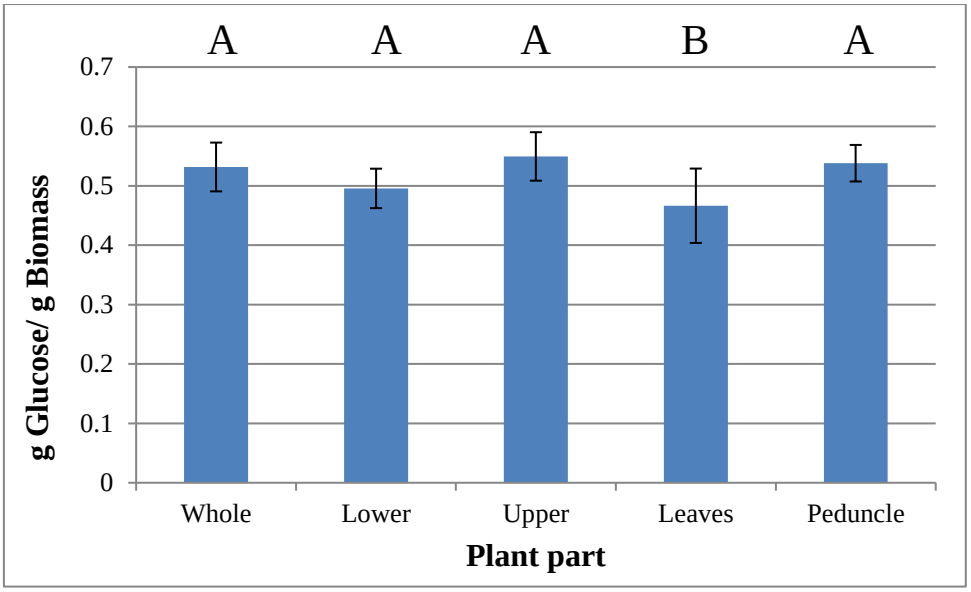


Figure 4.2.3: Glucose content of the residue from pre-treated wheat straw tissues. Wheat was harvested and fractionated into leaf, peduncle, upper (stem above 10cm), and lower (stem below 10cm) fractions. Samples of "whole" un-fractionated and individual fractions were then milled to 2mm particle size, subjected to an acid pre-treatment, the solid residue was collected and sugar composition determined. Results shown are mean ± SD (n= 18), bars with different letters show significant difference (p<0.05)

Analysis of the data using ANOVA of glucose content of the residue showed a significant difference (p <0.001) between the different plant fractions. Post hoc test analysis inferred that the only significant difference was seen between the leaves and the peduncle; upper (stem above 10cm) and samples of "whole" un-fractionated straw. All others being identical with each other.

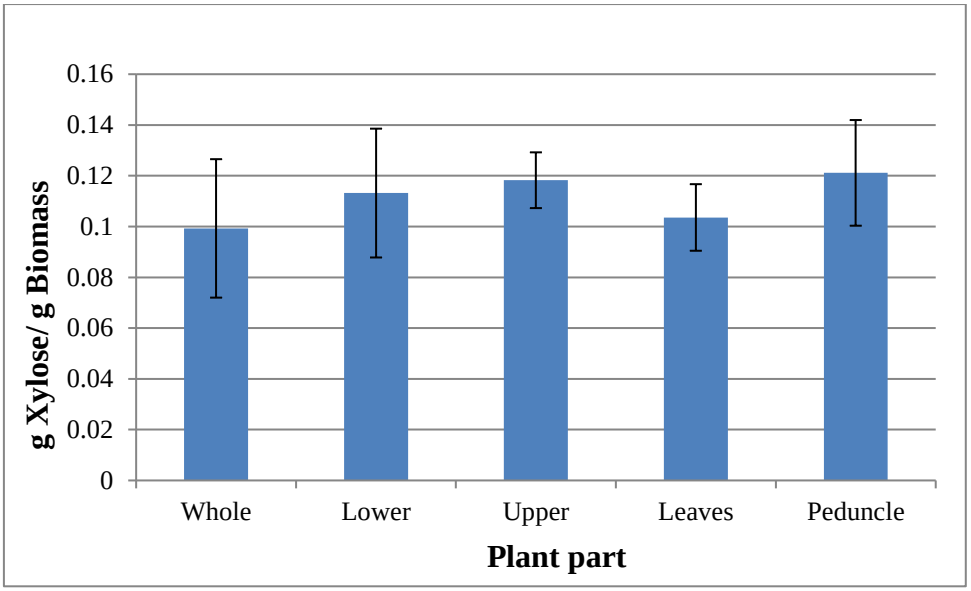


Figure 4.2.4: Xylose content of the residue from pre-treated wheat straw tissues. Wheat was harvested and fractionated into leaf, peduncle, upper (stem above 10cm), and lower (stem below 10cm) fractions. Samples of "whole" un-fractionated and individual fractions were then milled to 2mm particle size, subjected to acid pre-treatment, the solid residue collected and sugar composition determined. Results shown are mean ± SD (n= 18).

Analysis of data using ANOVA for xylose content of the residue showed no significant difference between any of the fractions ($p>0.05$).

The residue from the acid pre-treatment for each tissue type was subjected to the standard digestion assay using cellulase from *Trichoderma reesei* supplied by Sigma as described in section 2.5 and the results are shown in figure 4.2.5.

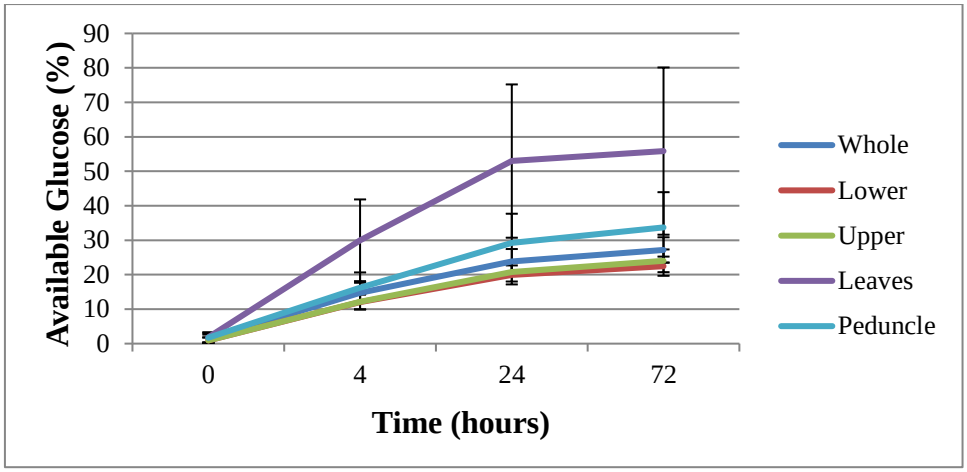


Figure 4.2.5: Available glucose (%) released from pre-treated wheat straw tissues. Wheat was harvested and fractionated into leaf, peduncle, upper (stem above 10cm), and lower (stem below 10cm) fractions. Samples of "whole" un-fractionated and individual fractions were then milled to 2mm particle size, subjected to an acid pre-treatment, the solid residue was collected and digested using cellulase from *Trichoderma reesei* supplied by Sigma at 40 FPU per g/ biomass at 50ºC and sampled over 72 hours. Results shown are mean ± SD (n= 18).

The analysis of data using ANOVA of the digestion assay showed that at time point 0 there was no difference (showing that all samples had the same sugar content at the start) but that this becomes highly significant after 4 hours ($p < 0.001$). Post hoc test analysis inferred the only significant difference was seen between the leaf fraction and all other fractions,

4.3: Effect of Nitrogen, plant growth regulator (PGR) application and growing position on straw digestibility.

It was noted that the standard deviation for the digestibility in section 4.2 was very large. Xi19 was grown in field conditions in three separate blocks each

having differing regimes of nitrogen; without nitrogen; with 50 kg nitrogen per hectare; and with 100 kg nitrogen per hectare, all either with or without a PGR (Plant Growth Regulators) application (diagram 4.1). Since the analysis in section 4.2 was carried out by blocking these treatments together, it would also be interesting to determine if the addition of nitrogen, PGR or external environmental factors had any influence on digestibility and thus contribute to the large standards errors observed. The composition and digestibility data from the Xi19 cultivar above was reanalysed but in this instance leaf, peduncle, upper (stem above 10cm), lower (stem below 10cm) fractions and "whole" un-fractionated samples were analysed together depending on whether they had received PGR applications or not (n=9), followed by the nitrogen regime they had received (n=3) and then finally by the block they were grown in (n=18).

The impact of PGR application on the glucose and xylose content of the untreated straw tissues are shown in figures 4.3.1 and 4.3.2, respectively.

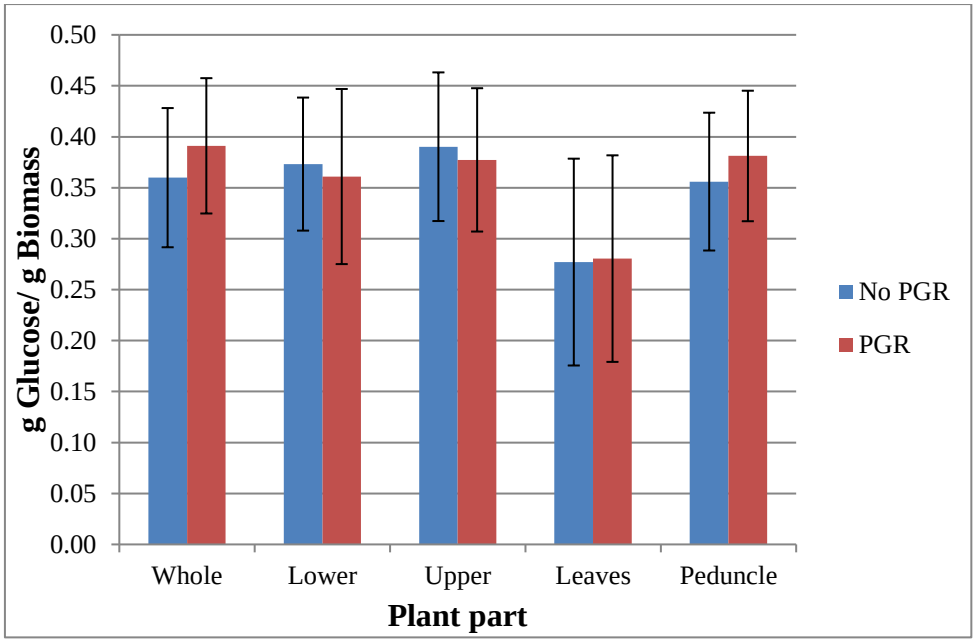


Figure 4.3.1: Effect of PGR application on glucose content from untreated wheat straw tissues. Wheat was harvested and fractionated into leaf, peduncle, upper (stem above 10cm), and lower (stem below 10cm) fractions. Samples of "whole" un-fractionated and individual fractions were then milled to 2mm particle size, subjected to a Saemen's hydrolysis and sugar composition determined. Results shown are mean ± SD (n= 9).

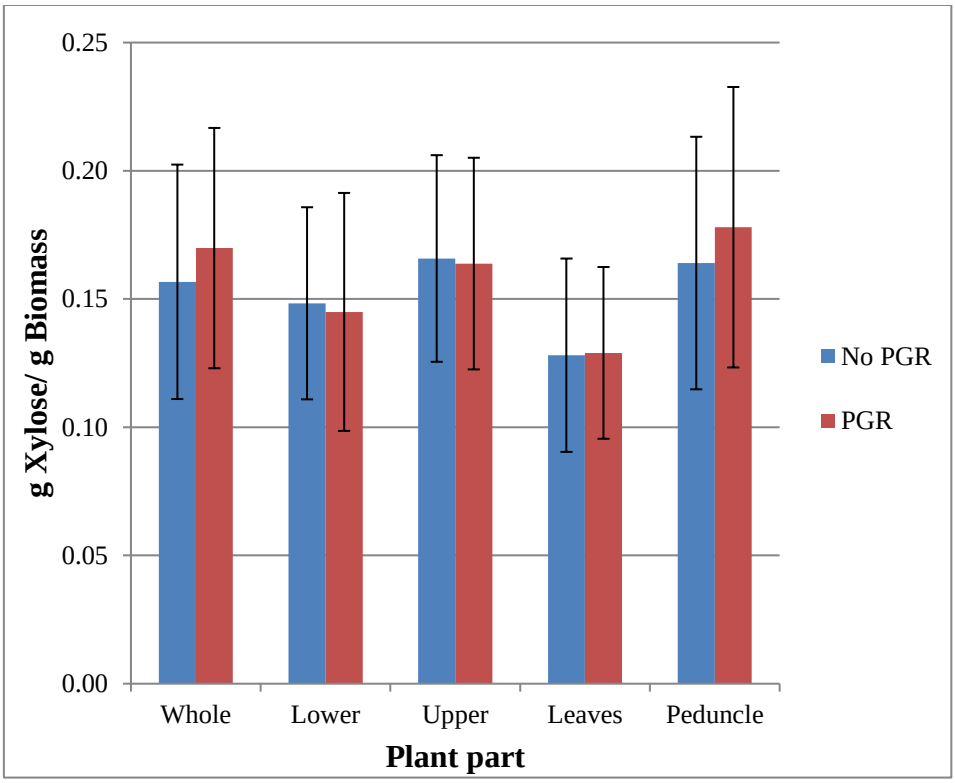


Figure 4.3.2: Effect of PGR application on xylose content from untreated wheat straw tissues. Wheat was harvested and fractionated into leaf, peduncle, upper (stem above 10cm), and lower (stem below 10cm) fractions. Samples of "whole" un-fractionated and individual fractions were then milled to 2mm particle size, subjected to a Saemen's hydrolysis and sugar composition determined. Results shown are mean ± SD (n= 9).

Analysis of the data using ANOVA of both glucose and xylose content of the untreated samples showed no significant difference (p>0.05) between the controls and samples that had PGR applications.

The impact of PGR application on glucose and xylose content of the pre-treated straw tissues are shown in figures 4.3.3 and 4.3.4, respectively.

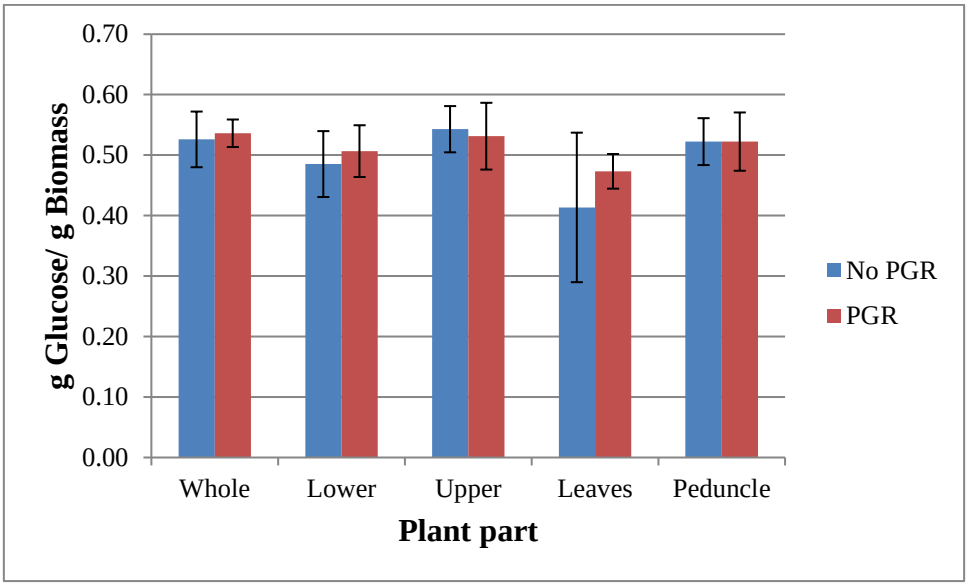


Figure 4.3.3: Effect of PGR application on glucose content of the residue from pre-treated wheat straw tissues. Wheat was harvested and fractionated into leaf, peduncle, upper (stem above 10cm), and lower (stem below 10cm) fractions. Samples of "whole" un-fractionated and individual fractions were then milled to 2mm particle size, subjected to a Saemen's hydrolysis and sugar composition determined. Results shown are mean ± SD (n= 9).

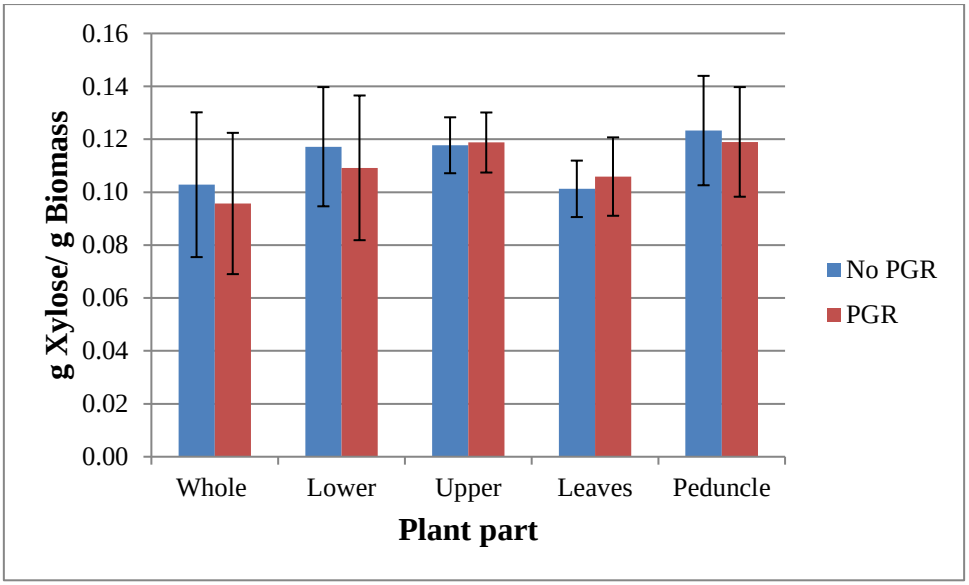


Figure 4.3.4: Effect of PGR application on xylose content of the residue from pre-treated wheat straw tissues. Wheat was harvested and fractionated into leaf, peduncle, upper (stem above 10cm), and lower (stem below 10cm) fractions. Samples of "whole" un-fractionated and individual fractions were then milled to 2mm particle size, subjected to a Saemen's hydrolysis and sugar composition determined. Results shown are mean ± SD (n= 9).

Analysis of the data using ANOVA of both glucose and xylose content of the pre-treated residues showed no significant difference (p>0.05) between the controls and samples that had PGR applications.

The impact of a PGR application on the available glucose after a 72 hour enzyme hydrolysis can be seen in figure 4.3.5.

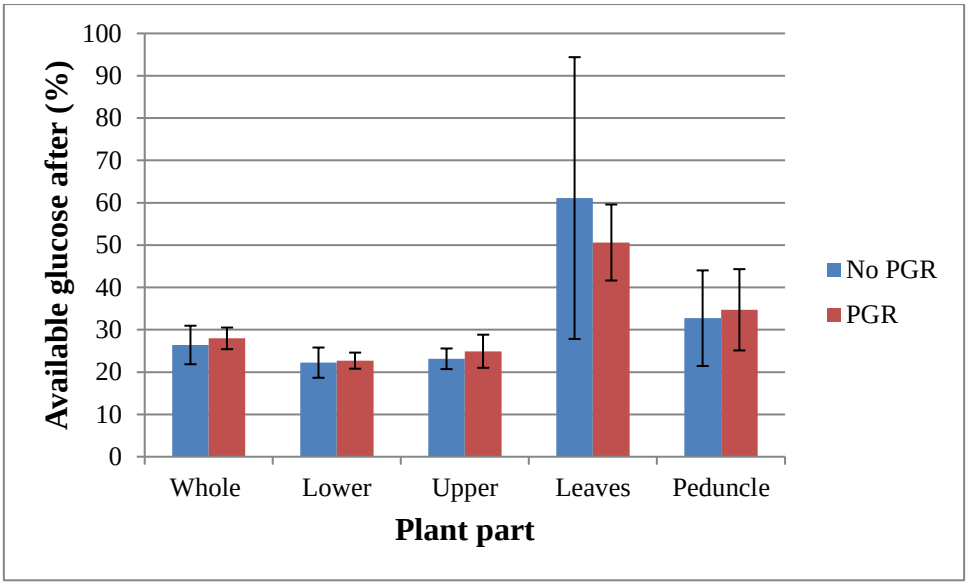


Figure 4.3.5: Available glucose (%) released from pre-treated wheat straw tissues after 72 hours. Wheat was harvested and fractionated into leaf, peduncle, upper (stem above 10cm), and lower (stem below 10cm) fractions. Samples of "whole" un-fractionated and individual fractions were then milled to 2mm particle size, subjected to an acid pre-treatment, the solid residue was collected and digested using cellulase from *Trichoderma reesei* supplied by Sigma at 40 FPU per g/ biomass at 50ºC and sampled over 72 hours. Results shown are mean ± SD (n= 9).

Analysis of the data using ANOVA of available glucose of the treated samples following a 72 hour enzyme hydrolysis showed no significant difference (p>0.05) between the controls and samples that had PGR applications.

The impact of the three nitrogen regimes on glucose and xylose concentrations in the un-treated straw tissues are shown in figures 4.3.6 and 4.3.7, respectively.

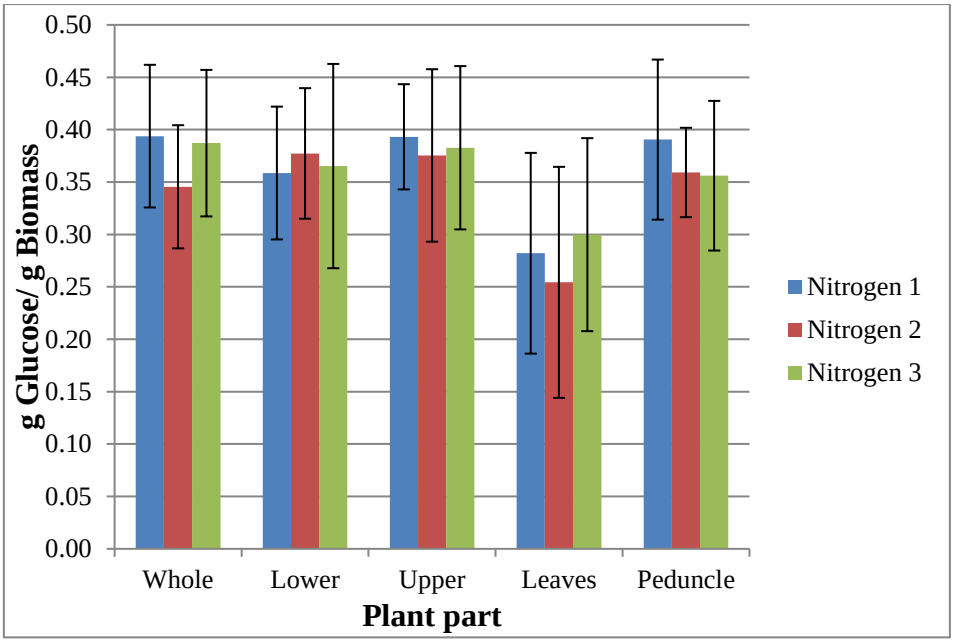


Figure 4.3.6: Effect of nitrogen application on glucose content from untreated wheat straw tissues. Wheat was grown in field conditions with three separate nitrogen regimes; no nitrogen; 50 kg per hectare; and 100 kg per hectare. This was harvested and fractionated into leaf, peduncle, upper (stem above 10cm), and lower (stem below 10cm) fractions. Samples of "whole" un-fractionated and individual fractions were then milled to 2mm particle size, subjected to collected Saemen's hydrolysis and sugar composition determined. Results shown are mean ± SD (n= 3).

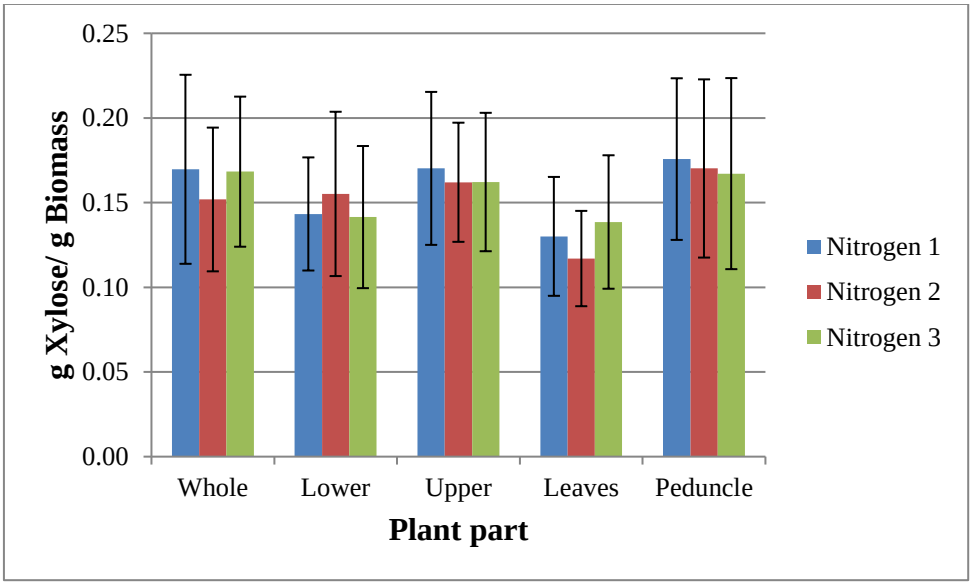


Figure 4.3.7: Effect of nitrogen application on xylose content from untreated wheat straw tissues. Wheat was grown in field conditions with three separate nitrogen regimes; no nitrogen; 50 kg per hectare; and 100 kg per hectare. This was harvested and fractionated into leaf, peduncle, upper (stem above 10cm), and lower (stem below 10cm) fractions. Samples of "whole" un-fractionated and individual fractions were then milled to 2mm particle size, subjected to a Saemen's hydrolysis and sugar composition determined. Results shown are mean ± SD (n= 3).

Analysis of the data using ANOVA of available glucose and xylose content of the untreated samples showed no significant difference (p>0.05) between the three Nitrogen applications.

The impact of the three nitrogen regimes on glucose and xylose concentrations in the pre-treated straw tissue are shown in figures 4.3.8 and 4.3.9, respectively.

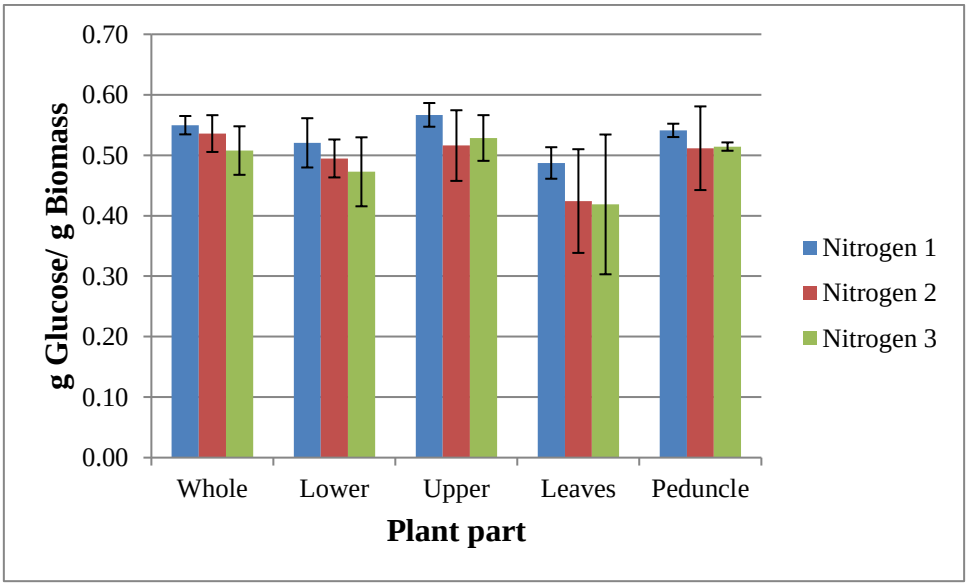


Figure 4.3.8: Effect of nitrogen application on glucose content from pre-treated wheat straw tissues. Wheat was grown in field conditions with three separate nitrogen regimes; no nitrogen; 50 kg per hectare; and 100 kg per hectare. This was harvested and fractionated into leaf, peduncle, upper (stem above 10cm), and lower (stem below 10cm) fractions. Samples of "whole" un-fractionated and individual fractions were then milled to 2mm particle size, subjected to a Saemen's hydrolysis and sugar composition determined. Results shown are mean ± SD (n= 3).

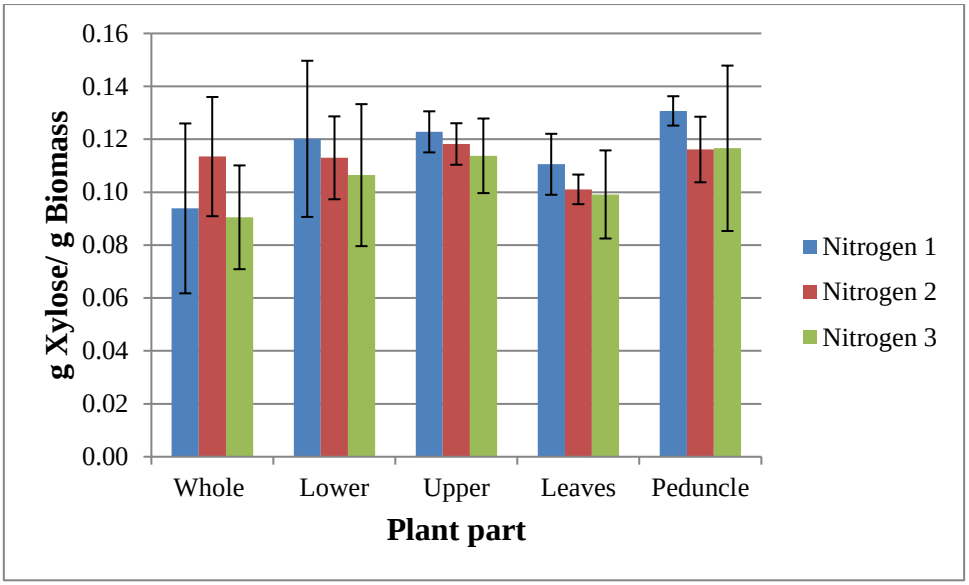


Figure 4.3.9: Effect of nitrogen application on xylose content from pre-treated wheat straw tissues. Wheat was grown in field conditions with three separate nitrogen regimes; no nitrogen; 50 kg per hectare; and 100 kg per hectare. This was harvested and fractionated into leaf, peduncle, upper (stem above 10cm), and lower (stem below 10cm) fractions. Samples of "whole" un-fractionated and individual fractions were then milled to 2mm particle size, subjected to a Saemen's hydrolysis and sugar composition determined. Results shown are mean ± SD (n= 3).

Analysis of the data using ANOVA shows a significant difference between the three nitrogen regimes for glucose ($p<0.05$). Post Hoc analysis inferred the only difference is seen between nitrogen regime 1 and nitrogen regime 3. With ANOVA showing no differences for xylose content ($p>0.05$).

The effects of nitrogen on available glucose after a 72-hour enzyme hydrolysis can be seen in figure 4.3.10.

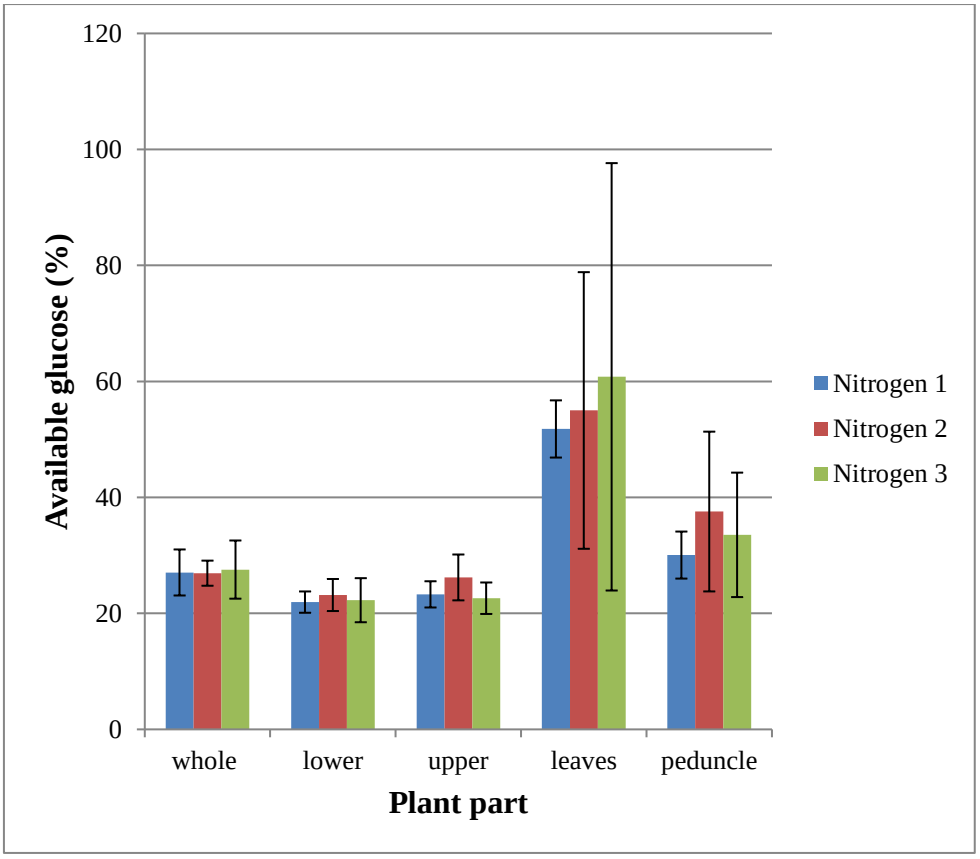


Figure 4.3.10: Available glucose (%) released from pre-treated wheat straw tissues after 72 hours. Wheat was harvested and fractionated into leaf, peduncle, upper (stem above 10cm), and lower (stem below 10cm) fractions. Samples of "whole" un-fractionated and individual fractions were then milled to 2mm particle size, subjected to an acid pre-treatment, the solid residue was collected and digested using Cellulase from *Trichoderma reesei* supplied by Sigma at 40 FPU per g/ biomass at 50ºC and sampled over 72 hours. Results shown are mean ± SD (n= 3).

Analysis of the data using ANOVA for available glucose released after 72 hours showed no significant difference ($p>0.05$) between the three nitrogen regimes.

It can be seen that neither PGR application nor nitrogen regime has any impact on the available sugar content in the pre-treated residues from each of the wheat straw fractions. It can also be seen that the application of PGR and the differing nitrogen regimes have little effect on the glucose released after enzyme hydrolysis for 72 hours, but that the error ranges are still large.

This could be due to the external growing conditions or field position (diagram 2.1). To determine if the growing position had any impact on the composition, the data was analysed based on growing plot. The impact of the growing conditions for glucose and xylose are shown in figure 4.3.11 and figure 4.3.12 respectively.

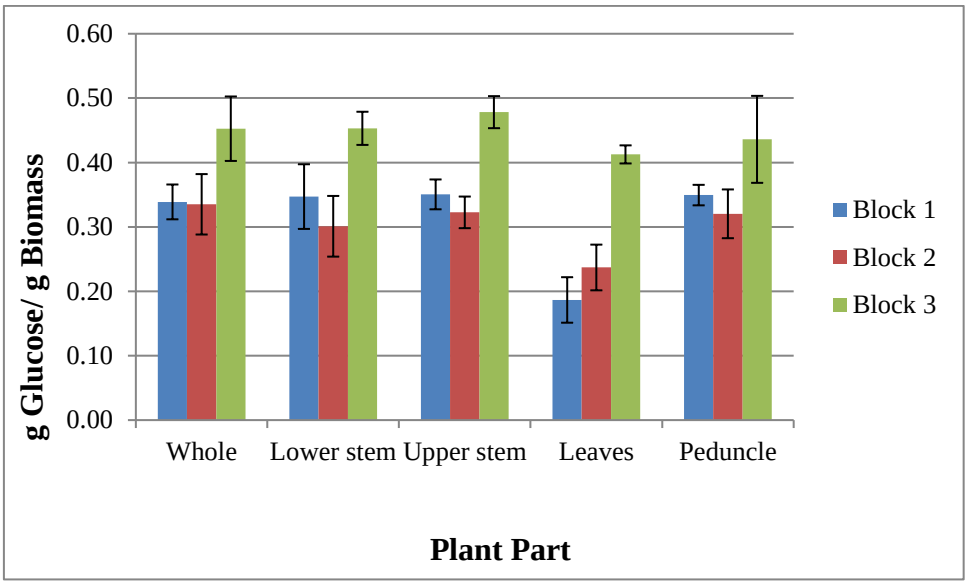


Figure 4.3.11: Effect of block on glucose content of the residue from untreated wheat straw tissues. Wheat was harvested and fractionated into leaf, peduncle, upper (stem above 10cm), and lower (stem below 10cm) fractions. Samples of "whole" un-fractionated and individual fractions were then milled to 2mm particle size, subjected to a Saemen's hydrolysis and sugar composition determined. Results shown are mean ± SD (n= 6).

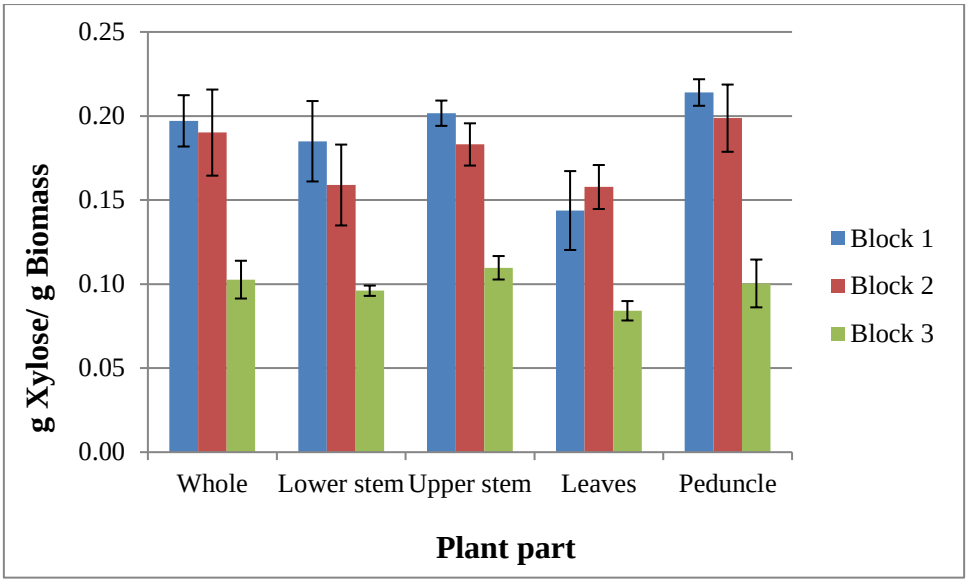


Figure 4.3.12: Effect of block on xylose content of the residue from untreated wheat straw tissues. Wheat was harvested and fractionated into leaf, peduncle, upper (stem above 10cm), and lower (stem below 10cm) fractions. Samples of "whole" un-fractionated and individual fractions were then milled to 2mm particle size, subjected to a Saemen's hydrolysis and sugar composition determined. Results shown are mean ± SD (n= 6).

Analysis of the data using ANOVA of glucose and xylose content in untreated wheat straw showed that there was a significant difference ($p < 0.01$). Post hoc test analysis inferred no significant difference between the first two blocks ($p>0.05$) but the third block was significantly different for both glucose and xylose content ($p<0.05$).

The impact of the three growing conditions on glucose and xylose concentrations in the pre-treated straw tissue are shown in figures 4.3.13 and 4.3.14, respectively.

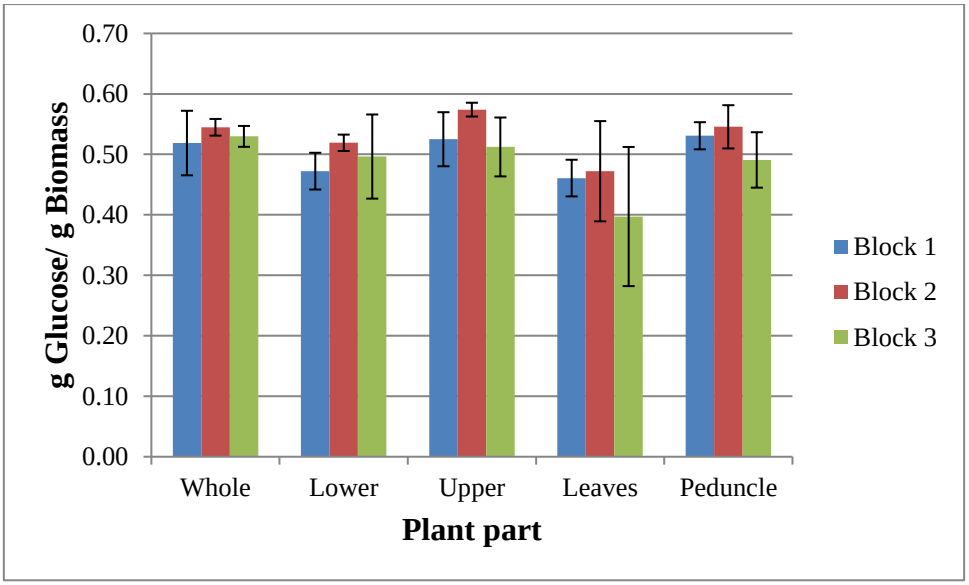


Figure 4.3.13: Effect of block on glucose content of the residue from pre-treated wheat straw tissues. Wheat was harvested and fractionated into leaf, peduncle, upper (stem above 10cm), and lower (stem below 10cm) fractions. Samples of "whole" un-fractionated and individual fractions were then milled to 2mm particle size, subjected to a Saemen's hydrolysis and sugar composition determined. Results shown are mean ± SD (n= 6).

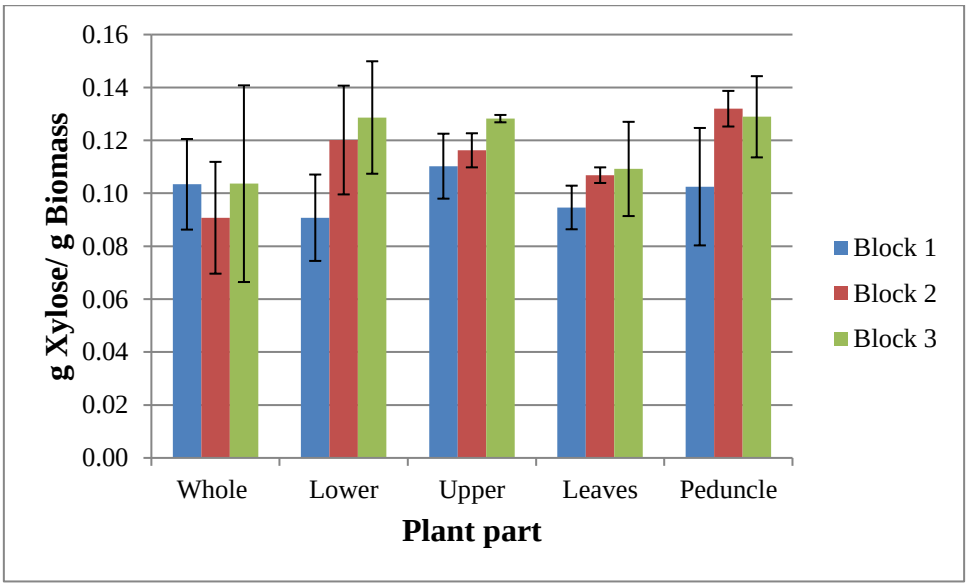


Figure 4.3.14: Effect of block on xylose content of the residue from pre-treated wheat straw tissues. Wheat was harvested and fractionated into leaf, peduncle, upper (stem above 10cm), and lower (stem below 10cm) fractions. Samples of "whole" un-fractionated and individual fractions were then milled to 2mm particle size, subjected to a Saemen's hydrolysis and sugar composition determined. Results shown are mean ± SD (n= 6).

Analysis of the data using ANOVA shows a significant difference for both glucose content and xylose content between the growing blocks (p<0.05). Post Hoc analysis showed the only difference seen for glucose is between blocks 2 and 3 (p<0.05) and for xylose between blocks 1 and 3 (p<0.01).

The effects of growing conditions on available glucose after a 72 hour enzyme hydrolysis can be seen (figure 4.3.15).

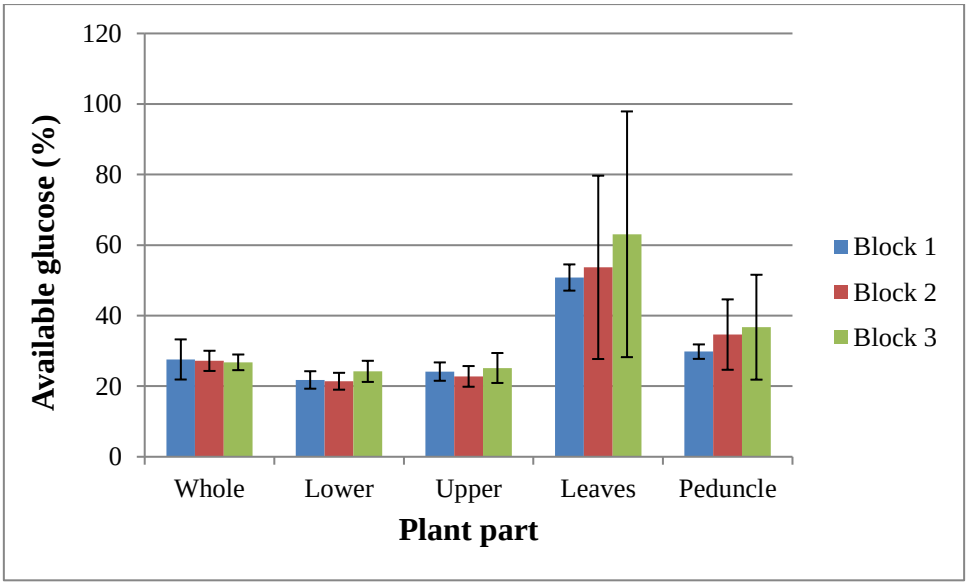


Figure 4.3.15: Available glucose (%) released from pre-treated wheat straw tissues after 72 hours. Wheat was harvested and fractionated leaf, peduncle, upper (stem above 10cm), and lower (stem below 10cm) fractions. Samples of "whole" un-fractionated and individual fractions were then milled to 2mm particle size, subjected to an acid pre-treatment, the solid residue was collected and digested using cellulase from *Trichoderma reesei* supplied by Sigma at 40 FPU per g/ biomass at 50ºC and sampled over 72 hours. Results shown are mean ± SD (n= 3).

Analysis of the data using ANOVA shows no significant difference between the three growing blocks (p>0.05) for the glucose % available after 72 hour enzyme digestion.

4.4: Effect of wheat variety on straw composition and digestibility.

Since the previous analysis (section 4.3) only demonstrated significant differences between leaf and stem tissues the effect of wheat variety was examined by comparing leaf, stem and "whole" tissues from the three commercial varieties; Cordiale, Grafton and Xi19. Cordiale and Grafton were grown in three blocks at the same time as Xi19 (see diagram 4.1). Given the impact of the block (growing position) seen in section 4.3 in this analysis, samples were only taken from block 1 (existing data for Xi19 was used from block 1 only).

Cordiale, Grafton and Xi19 samples were fractionated into leaf, stem and whole plant prior to knife milling to 2mm. They were then subjected to Saeman's hydrolysis to determine sugar composition of the raw straw; see figure 4.4.1 and 4.4.2 for glucose and xylose composition, respectively.

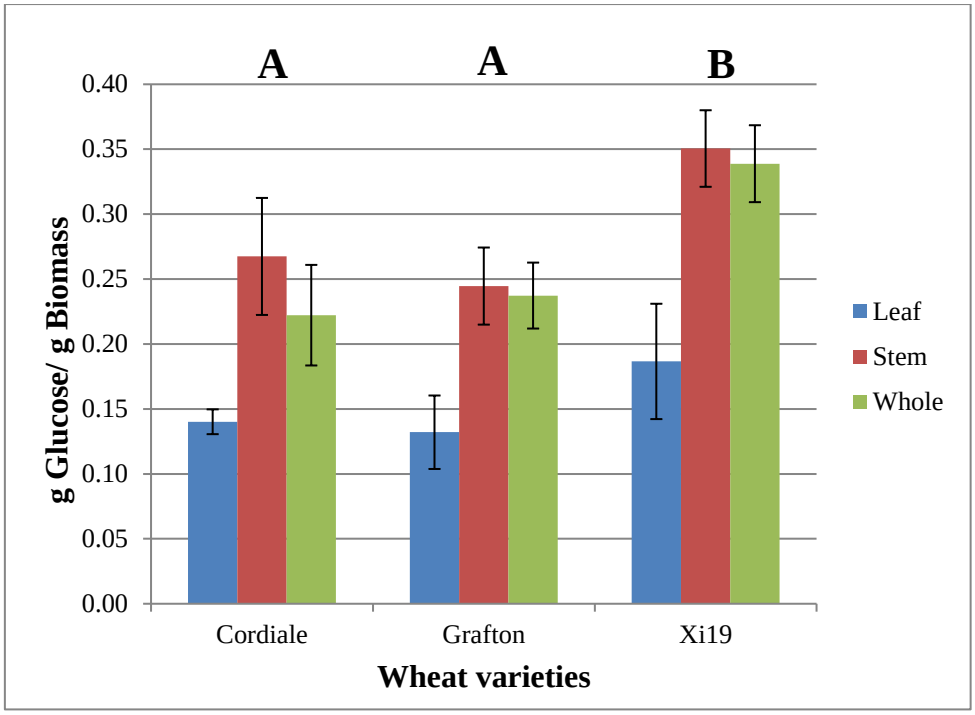


Figure 4.4.1: Glucose content of wheat straw tissues. Three wheat varieties were harvested and fractionated into Leaf, stem and samples of "whole" un-fractionated, individual fractions were then milled to 2mm particle size and sugar composition determined. Results shown are mean ± SD (n= 6), Varieties with different letters are significantly different (p<0.05)

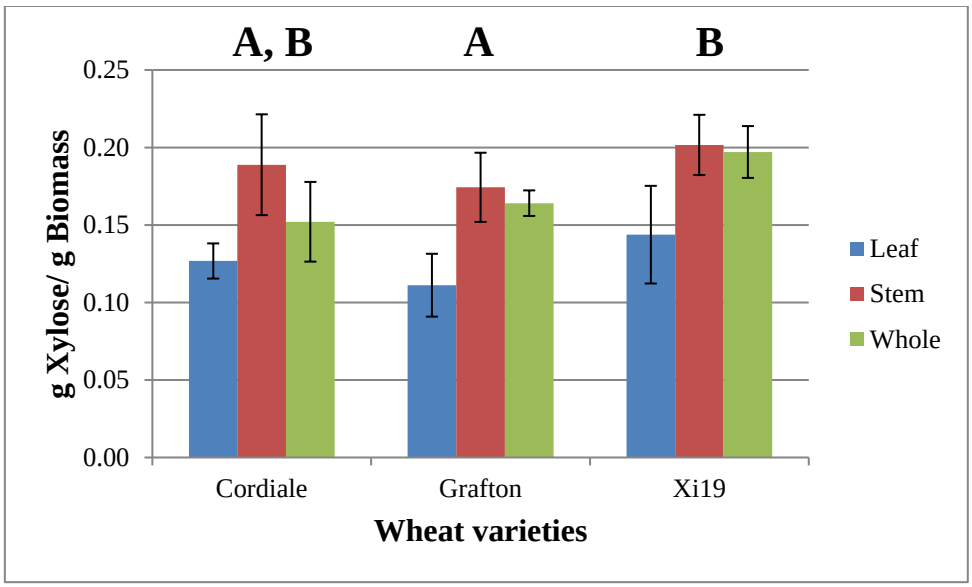


Figure 4.4.2: Xylose content of wheat straw tissues. Three wheat varieties were harvested and fractionated into Leaf, stem and samples of "whole" un-fractionated, individual fractions were then milled to 2mm particle size and sugar composition determined. Results shown are mean ± SD (n= 6), Varieties with different letters are significantly different (p<0.05)

ANOVA analysis of the glucose and xylose content showed that for glucose Cordiale and Grafton are identical to each other but both are significantly different to Xi19 ($p < 0.01$), and for xylose the only significant difference was seen between Grafton and Xi19 ($p < 0.05$).

The fractions were then subjected to a standard 1% H_2SO_4 pre-treatment as described in section 2.3 followed by a Saeman's hydrolysis. The glucose and xylose content of the pre-treated residues are shown in figures 4.4.3 and 4.4.4, respectively.

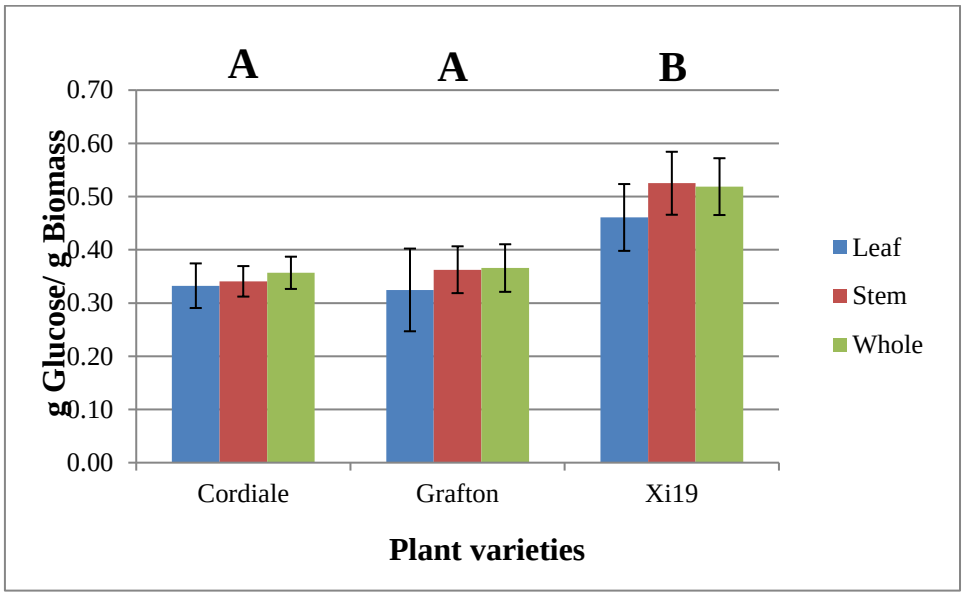


Figure 4.4.3: Glucose content of residue after acid pre-treatment. Three wheat straw varieties were harvested and fractionated into leaf, stem and whole plant; these were then milled to a 2mm particle size. These were then subjected to Saeman's hydrolysis to determine sugar composition. Results shown are mean ± SD (n= 6). Varieties with different letters show significant difference ($p<0.05$)

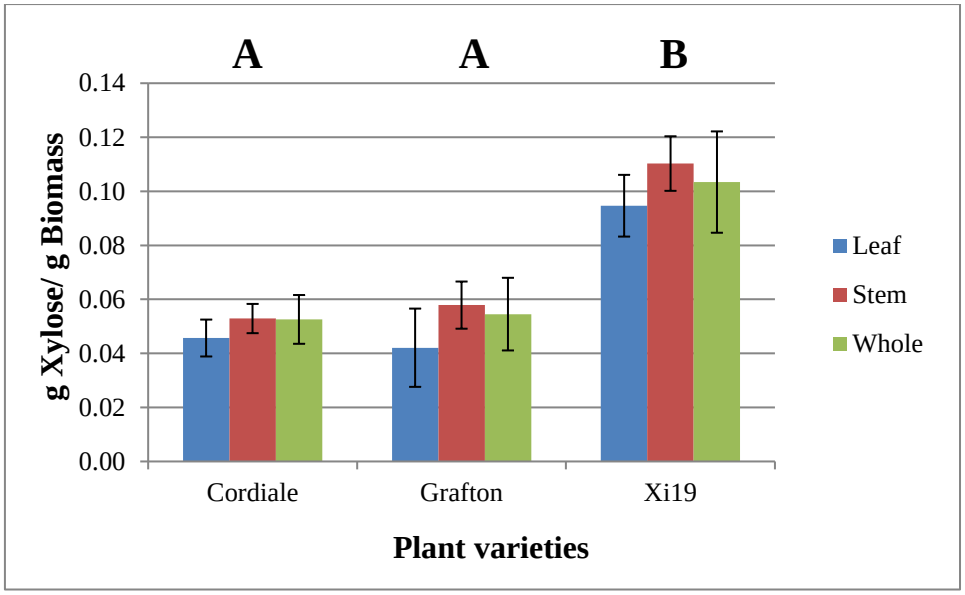


Figure 4.4.4: Xylose content of residue after acid pre-treatment. Three wheat straw varieties were harvested and fractionated into leaf, stem and whole plant; these were then milled to a 2mm particle size. These were then subjected to Saeman's hydrolysis to determine sugar composition. Results shown are mean ± SD (n= 6), Varieties with different letters show significant difference (p<0.05)

Analysis of data using ANOVA of the glucose and xylose content of the treated samples showed a significant difference (p<0.01). Post hoc analysis inferred that Coriale and Grafton are identical (p = 0.922 for glucose and p = 0.964 for xylose) but both were significantly different to Xi19.

The pre-treated residue for each plant variety tissue type was subjected to the standard digestion assay as described in 2.5 using cellulase from *Trichoderma reesei* supplied by Sigma and sampled after 72 hours to determine the glucose released, the results are shown in figure 4.4.5.

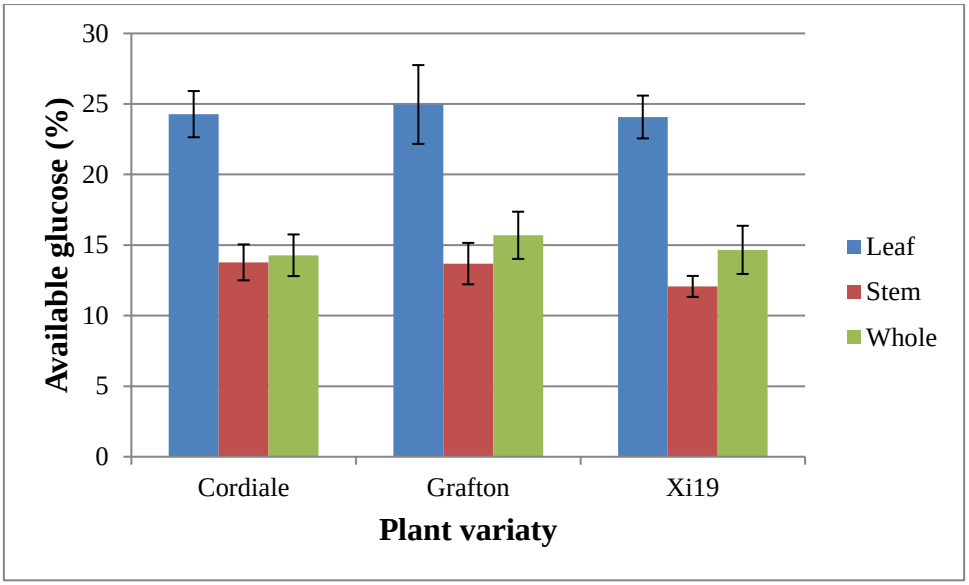


Figure 4.4.5: Glucose (%) released from pre-treated wheat straw. Wheat straw was harvested and fractionated into leaf, stem and whole plant prior to being milled to 2mm particle size, subjected to acid pre-treatment, the solid residue collected digested using cellulase from *Trichoderma reesei* supplied by Sigma at 40 FPU per g/ biomass at 50ºC and sampled after 72 hours. Results shown are mean ± SD (n= 6)

Analysis of data using ANOVA of the enzyme hydrolysis showed no significant differences between the three varieties of wheat (p>0.05) after a 72 hour hydrolysis.

4.5: Conclusions.

Results showed that for the raw Xi19 straw samples, all fractions of the stem contained the same levels of glucose with the leaf fraction containing significantly less, with all sample fractions containing similar levels of xylose. After a 1% H_2SO_4 pre-treatment both the glucose and xylose levels in the pre-treated residues were comparable between all fractions.

Results for the enzymatic hydrolysis show that leaf material digests quicker and releases a higher % of glucose. With no difference seen between the parts of the stem, there is no requirement for stem fractionation prior to pre-treatment. This is supported by work carried out by Zhang *et al*, 2013 who showed that the glucose content of wheat straw was only variable within the leaf fraction and that whilst this was lower it was far less recalcitrant. This was due to the leaves containing significantly less lignin in comparison to the stem fractions. This suggests that there is no need to fractionate the stem material as only the leaf fraction shows a difference; as a consequence, all future work will be carried out only fractionating the leaf and stem.

With no difference seen with regard to Nitrogen application or PGR application it seems that the addition of chemicals and fertilizers during the growth stage of the plant for bio-fuel purposes. Although it should be noted that the addition of nitrogen and PGR are still required for the prevention of lodging and for grain yield it has little to no effect on the recalcitrance of the straw. The differences seen between the three growing blocks could be due to a variety of external environmental effects such as edge effect, soil nurturance or field position. No further investigation into this was conducted due to time restraints.

Xi19 showed higher levels of glucose and xylose in the untreated samples compared to Grafton and Cordiale. The latter being comparable to each other. After a 1% H_2SO_4 pre-treatment Xi19 still showed the same higher levels, with Grafton and Cordiale again being comparable. However, all three varieties were comparable after a 72 hour enzyme digestion, suggesting that Xi19 is more recalcitrant. With Grafton and Cordiale being closely related (Cordiale X

CPBT W97 = Grafton) it is not surprising that they are more similar than Xi19, suggesting that there is a genetic link to glucose levels and recalcitrance.

Chapter 5: BC1 samples morphology, composition and digestibility.

5.1: Introduction.

As shown in section 4.4, wheat straws have a natural variation in sugar composition and levels of recalcitrance. To determine if these traits can be introduced or increased, back cross 1 (BC1) samples were created by crossing a range of elite wheat lines with the genome AABBDD that has had the Ph1/Ph1 locus deleted with a range of ancient varieties with the genome RR (tables 2.2 and 2.3). In wheat straw the Ph1 locus restricts recombination to identical homologous chromosomes from the same genome and requires removal to allow for homeologous recombination between the chromosomes of a wild relative and domesticated wheat (King *et al*, 2016). This allowed the creation of F1 hybrids (ABD R) that were then crossed again with an elite line (AABBDD) again with the Ph1/Ph1 deletion, and allowed to reach maturity before being allowed to senesce naturally prior to the heads being removed for seed collection. This created a substantial number of samples of which 48 were chosen at random from plants that had developed seeds.

5.2: Morphology of the BC1 wheat samples.

BC1 samples were harvested in June 2013; each plant sample was measured from base to the tip of the tallest tiller to determine height, prior to being cut at 100 mm above the base level and placed into individual bags for storage.

BC1 samples ranged in height from 600mm to 1490mm (figure 5.2.1 and 5.2.2) with a mean of 848.9mm. The total weight ranged from 10g to 65.6g (figures 5.2.3 and 5.2.4) with a mean of 28.82g. The % stem ranged from 53.77% to 80% (figures 5.2.5 and 5.2.6) with a mean of 63.41%. The BC1 samples showed a vast difference in morphological traits with the full details of height, weight and % stem biomass shown in table 5.1.



Figure 5.2.1: BC1 14 prior to being harvested.
BC1 14 was the tallest plant with a height of 1490mm, a stem weight of 26g, a leaf weight of 16g, and a leaf: stem ratio of 61.9%.

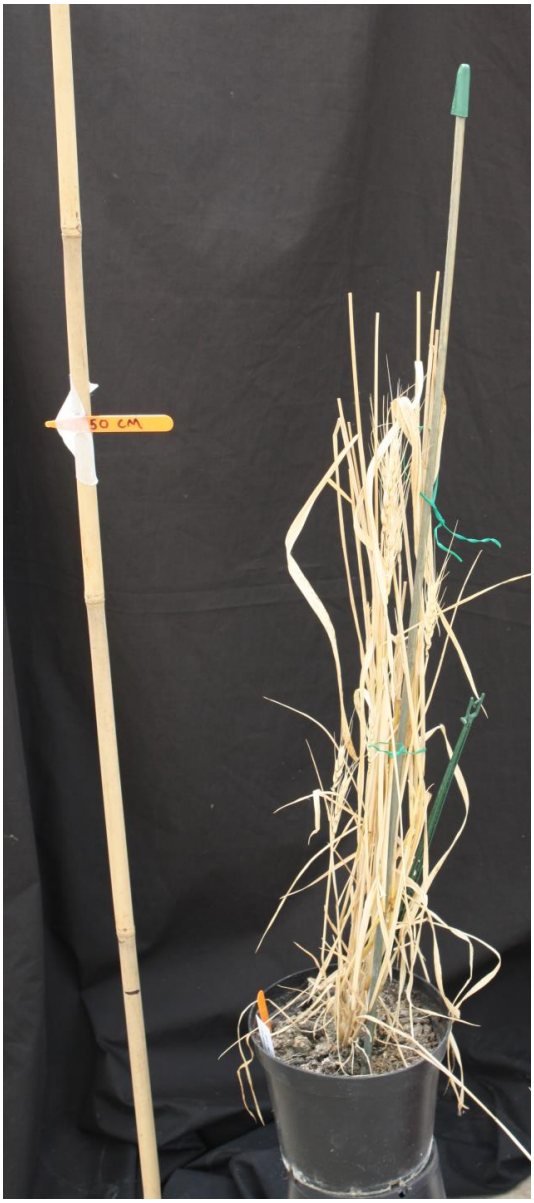


Figure 5.2.2: BC1 18 prior to being harvested.
BC1 18 was the smallest plant with a height of 610mm, a stem weight of 8g, a leaf weight of 4g, and a leaf: stem ratio of 66.6%.



Figure 5.2.3: BC1 40 prior to being harvested.
BC1 40 had the largest total biomass weighing 65.6g, with a height of 730mm and a leaf: stem of 68.9%.

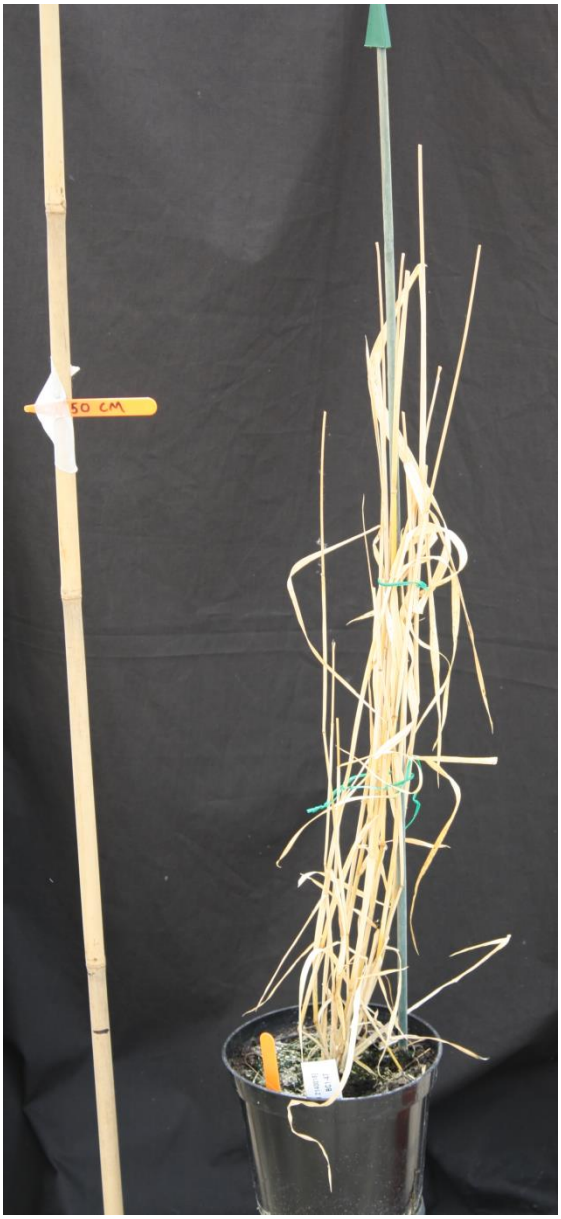


Figure 5.2.4: BC1 20 prior to being harvested.
BC1 20 had the lowest total biomass weighing 10g, with a height of 700mm and a leaf: stem of 80%.

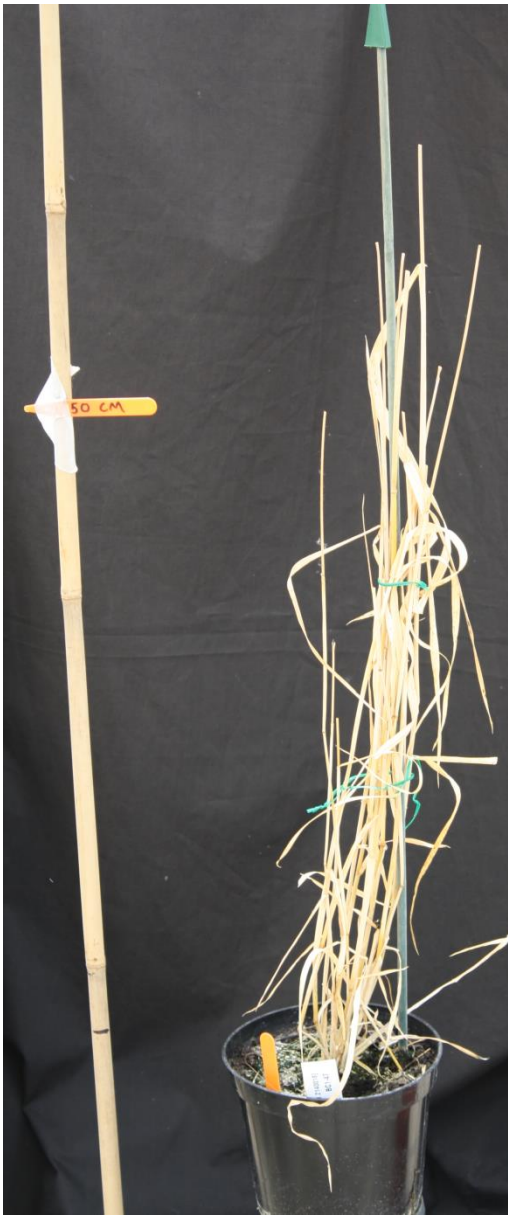


Figure 5.2.5: BC1 20 prior to being harvested.
BC1 20 had the highest leaf: stem ratio being 80%, weighing 10g, with a height of 700mm.



Figure 5.2.6: BC1 38 prior to being harvested.
BC1 38 had the lowest leaf: stem ratio being 53.7%, weighing 10g, with a height of 700mm.

BC1 sample	High (mm)	Total weight (g)	% stem biomass		BC1 sample	High (mm)	Total weight (g)	% stem biomass
1	800	12	66.6667		25	820	20	70
2	890	26	61.5385		26	840	22	54.5455
3	650	14	57.1429		27	990	18	55.5556
4	860	24	58.3333		28	1070	54	55.5556
5	820	16	62.5		29	760	32	62.5
6	1040	24	66.6667		30	740	22	72.7273
7	730	22	63.6364		31	1050	20	60
8	720	22	63.6364		32	750	32	62.5
9	740	22	54.5455		33	790	19.4	64.9485
10	890	26	61.5385		34	880	14.6	58.9041
11	960	32	68.75		35	860	20.2	62.3762
12	800	34	64.7059		36	1040	46.2	69.2641
13	970	22	63.6364		37	940	28.2	63.8298
14	1490	42	61.9048		38	700	21.2	53.7736
15	930	42	57.1429		39	840	23.8	56.3025
16	680	16	75		40	730	65.6	68.9024
17	810	26	61.5385		41	690	22.4	54.4643
18	610	12	66.6667		42	870	62.6	65.1757
19	820	34	64.7059		43	980	56	60.7143
20	700	10	80		44	850	54	62.963
21	600	20	70		45	1020	31.4	67.5159
22	850	30	73.3333		45	1018	64.6	63.4675
23	1200	42	61.9048		47	760	20.2	66.3366
24	800	14	57.1429		48	880	29	68.9655

Table 5.1: Morphological data for BC1 samples. BC1 samples were measured for height prior to being harvested from 100mm above ground level before being fractionated into leaf and stem prior to being weighed to determine biomass produced.

A Pearson's correlation coefficient was used to determine any correlations between the height of the plants and the total biomass (figure 5.2.7). This gave an r^2 value of 0.4279 inferring a weak positive correlation, suggesting that the levels of biomass is not simply due to plants being taller.

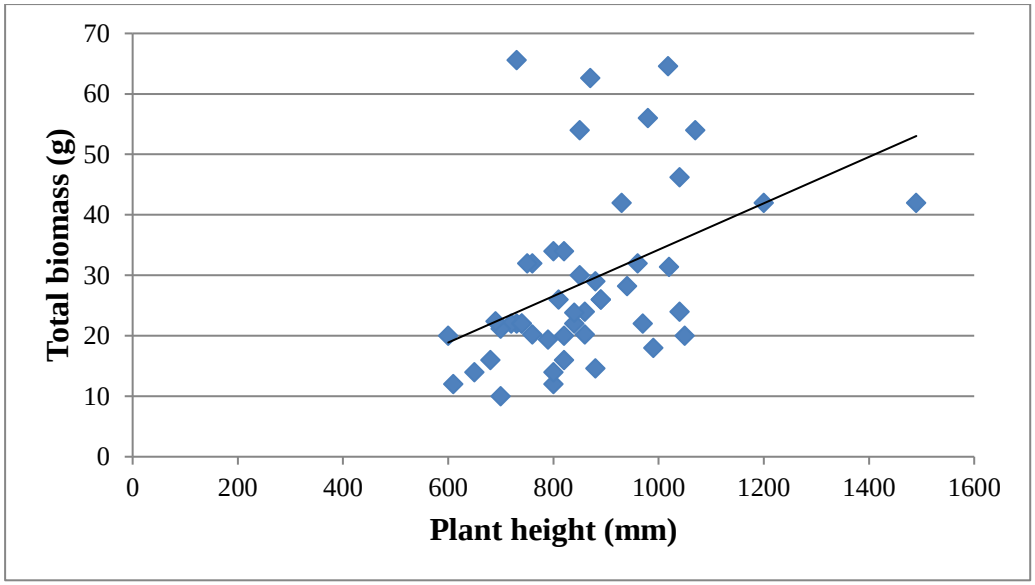


Figure 5.2.7: Scatter graph showing the relationship between BC1 plant height and total biomass produced. BC1 samples were measured for height prior to being harvested from 100mm above ground level before being weighed to determine biomass produced. A Pearson's correlation coefficient showed a weak positive correlation r^2= 0.4279 between height and biomass.

5.3: BC1 Compositional analysis.

The individual stem fractions were knife milled to a 2mm particle size and subjected to a Saeman's hydrolysis and analysed for monomer sugar composition by HPAEC for glucose (figure 5.3.1a), for xylose (figure 5.3.1b), and for arabinose (figure5.3.1c).

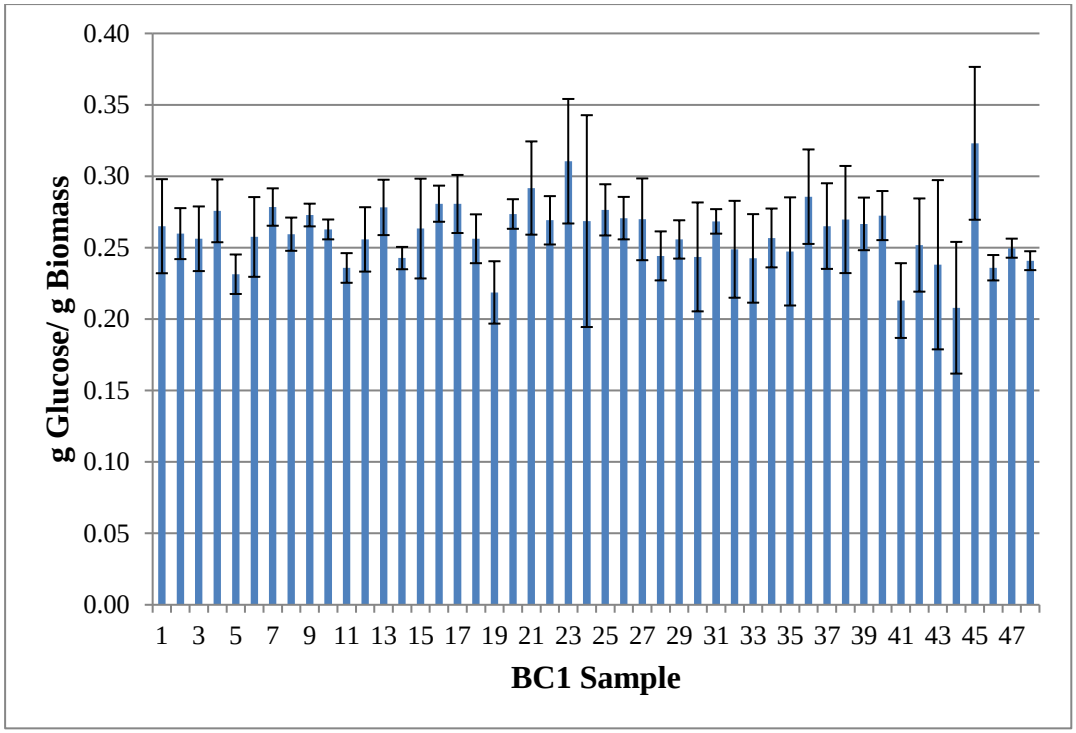


Figure 5.3.1a: Glucose content for stem tissue for BC1 samples. BC1 samples were harvested and fractionated into leaf and stem prior to being knife milled to a 2mm particle size. The stem fraction was then subjected to a Saemen's hydrolysis to determine sugar composition. Results shown are mean ± SD (n= 5).

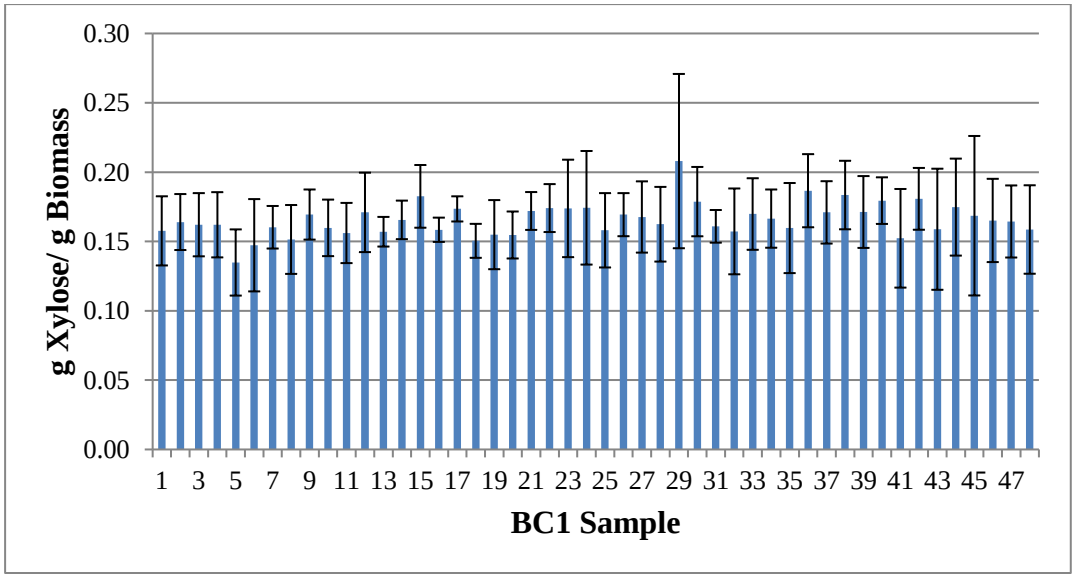


Figure 5.3.1b: Xylose content for stem tissue for BC1 samples. BC1 samples were harvested and fractionated into leaf and stem prior to being knife milled to a 2mm particle size. The stem fraction was then subjected to a Saemen's hydrolysis to determine sugar composition. Results shown are mean ± SD (n= 5).

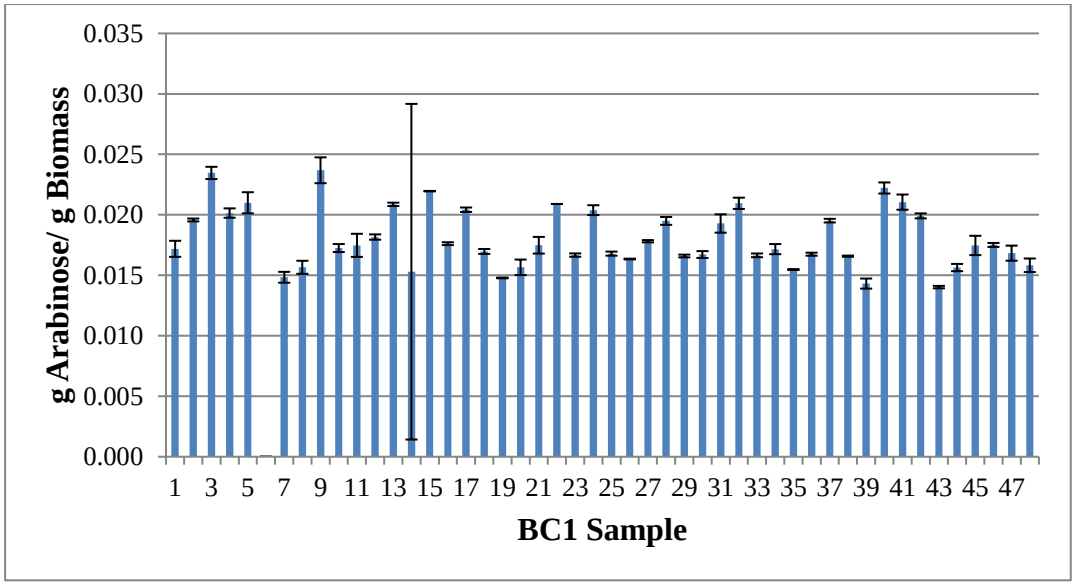


Figure 5.3.1c: Arabinose content for stem tissue for BC1 samples. BC1 samples were harvested and fractionated into leaf and stem prior to being knife milled to a 2mm particle size. The stem fraction was then subjected to a Saemen's hydrolysis to determine sugar composition. Results shown are mean ± SD (n= 2).

The stem fraction was then subjected to a standard 1% H_2SO_4 pre-treatment and the residue was washed and dried before being weighed to determine % biomass loss during the pre-treatment see figure 5.3.2.

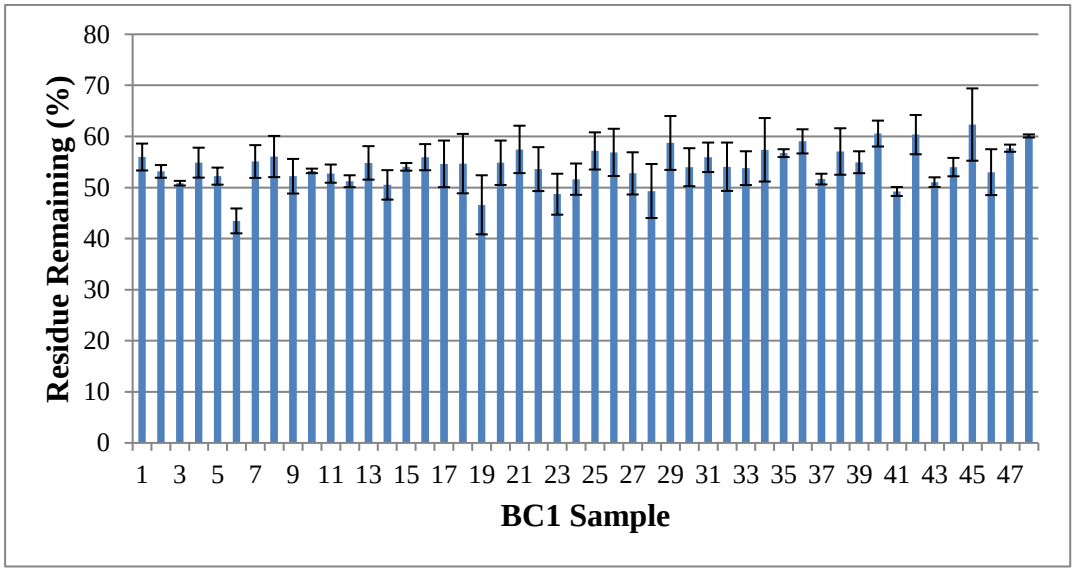


Figure 5.3.2: Residue remaining (%) following a 1% H_2SO_4 pre-treatment. BC1 stem tissue was subjected to a standard pre-treatment and allowed to air dry for 72 hours. Samples were then weighed to determine loss of mass during pre-treatment. Results shown are mean ± SD (n= 3)

The dried residue was then subjected to a Saemen's hydrolysis and analysed for monomeric sugar composition by HPAEC for glucose (figure 5.3.3a), and for xylose (figure 5.3.3b).

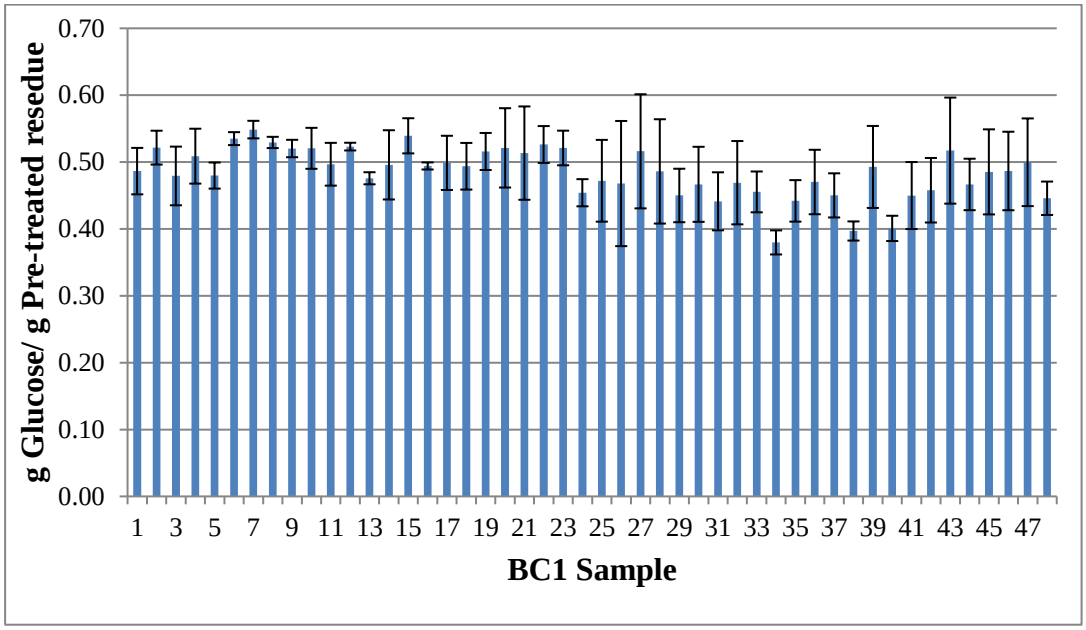


Figure 5.3.3a: Glucose content for pre-treated residue for BC1 samples. BC1 samples were subjected to a standard 1% H_2SO_4 pre-treatment. The residue fraction was air dried for 72 hours and then subjected to a Saemen's hydrolysis to determine sugar composition. Results shown are mean ± SD (n=4).

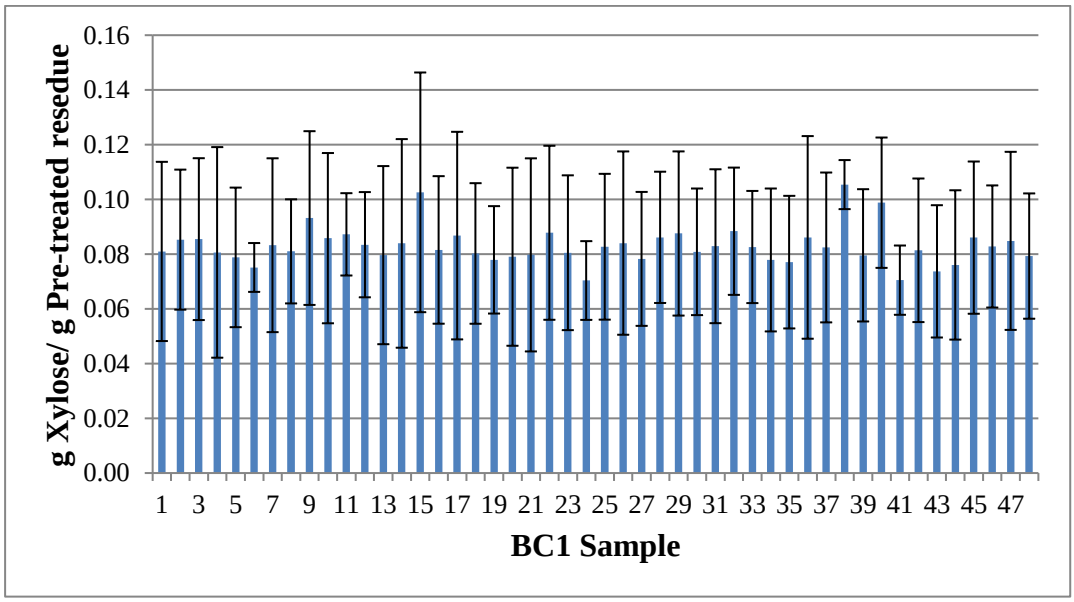


Figure 5.3.3b: Xylose content for pre-treated residue for BC1 samples. BC1 samples were subjected to a standard 1% H_2SO_4 pre-treatment. The residue fraction was air dried for 72 hours and then subjected to a Saemen's hydrolysis to determine sugar composition. Results shown are mean ± SD (n= 4).

The residue from the pre-treated BC1 samples were then subjected to the standard digestion assay and the results for the percentage of available glucose released (figure 5.3.4a), and for xylose (figure 5.3.4b).

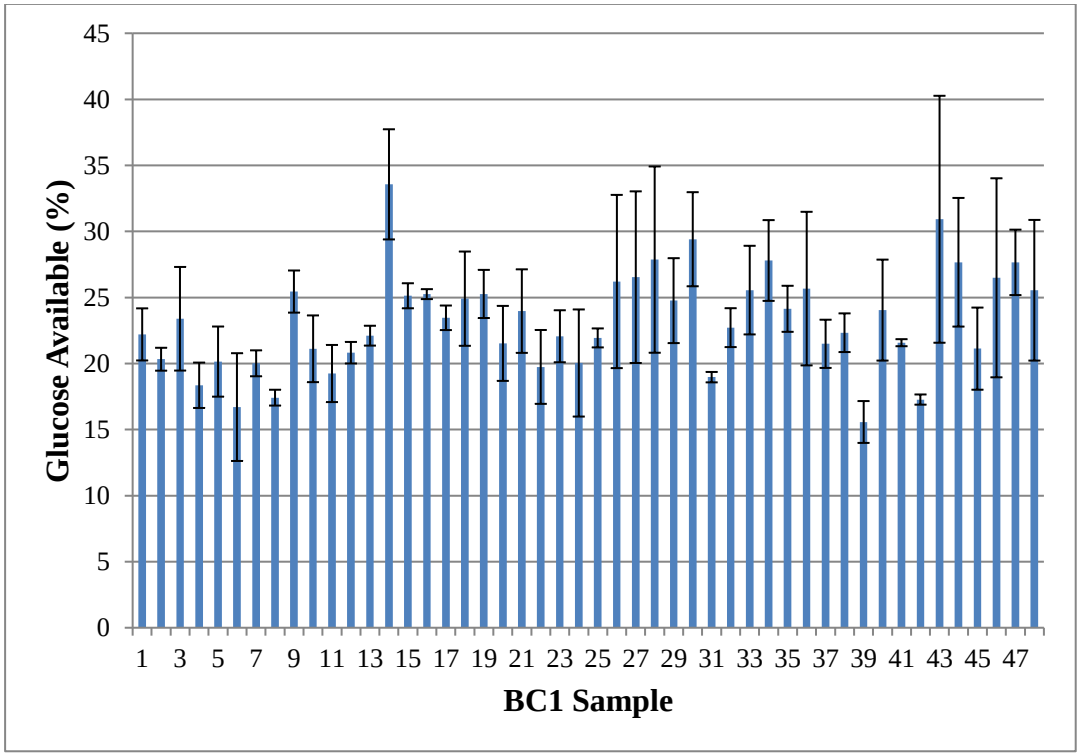


Figure 5.3.4a: Available glucose (%) released from pre-treated wheat straw tissues. BC1 samples were harvested and fractionated into leaf and stem. Fractions were then milled to 2mm particle size, subjected to an acid pre-treatment, the solid residue was collected and digested using cellulase from *Trichoderma reesei* supplied by Sigma at 40 FPU per g/ biomass at 50ºC and sampled over 72 hours. Results shown are mean ± SD (n= 3).

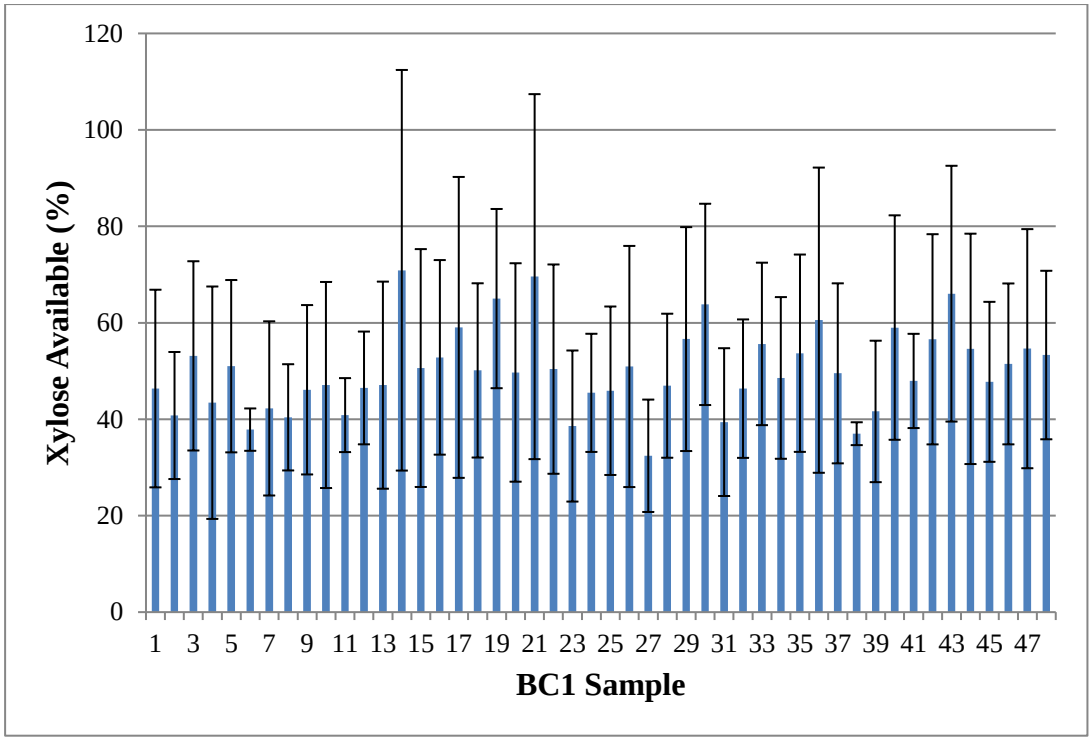


Figure 5.3.4b: Available xylose (%) released from pre-treated wheat straw tissues. BC1 samples were harvested and fractionated into leaf and stem. Fractions were then milled to 2mm particle size, subjected to an acid pre-treatment, the solid residue was collected and digested using cellulase from *Trichoderma reesei* supplied by Sigma at 40 FPU per g/ biomass at 50ºC and sampled over 72 hours. Results shown are mean ± SD (n= 3).

A Pearson's correlation showed no correlation between glucose recovered and plant height (r^2= 0.217), glucose recovered and weight of straw (r^2= 0.245) and no correlation between glucose recovered and % stem biomass (r^2= -0.030) (data not shown) suggesting that the morphology of the plant has no impact on digestibility of the straw.

5.4: Chemical pre-treatment and digestibility assay (York High throughput (HT)).

The stem fractions of the BC1 samples were knife milled to a size of 2mm and sent to Dr Leo Gomez at York University for analysis via robotic high throughput digestibility assay as per Gomez *et al* (2010) using both a 1% H_2SO_4 pre-treatment and a 0.5M NaOH pre-treatment, followed by an enzymatic digestion to determine reducing sugars (as described in 2.9) (figure 5.4.1).

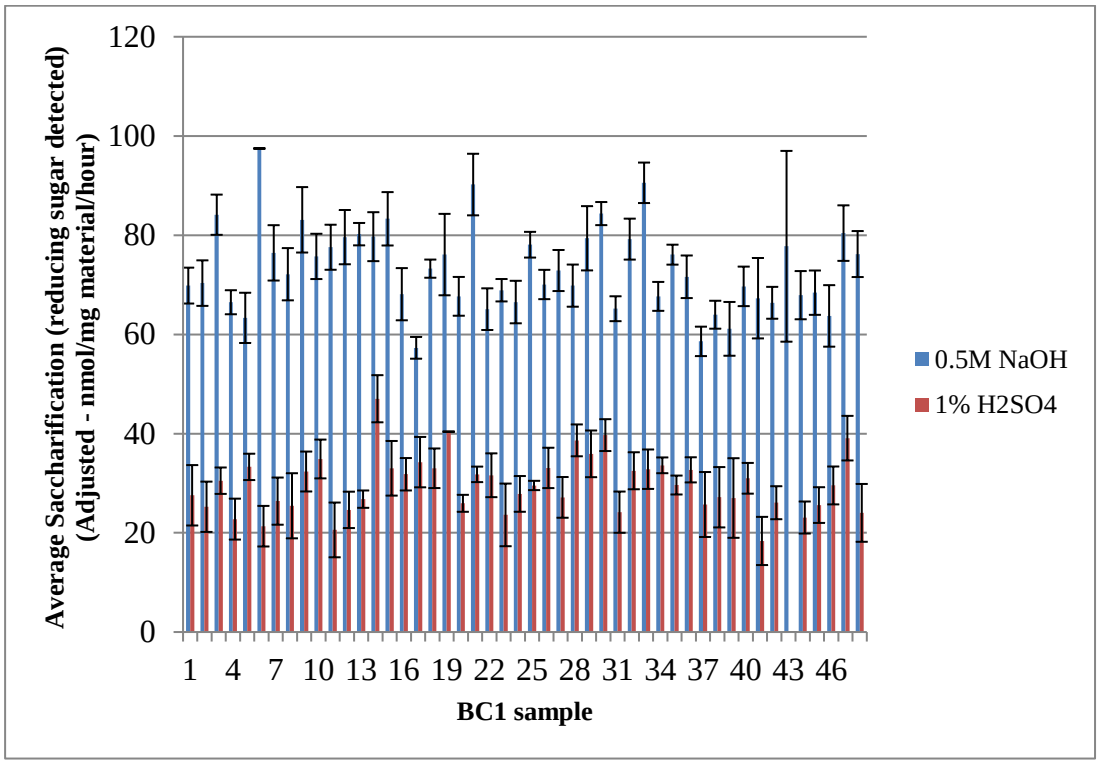


Figure 5.4.1: Reducing sugars following a high throughput assay of BC1 stem tissue. Knife milled BC1 samples were analysed using high throughput analysis. 4 mg of untreated straw was loaded into a 96-well plate and subjected to a 1% H_2SO_4 pre-treatment at 95^{O}C for 15 min prior to an enzyme hydrolysis and reducing sugars were determined. Results shown are mean ± SD (n= 4).

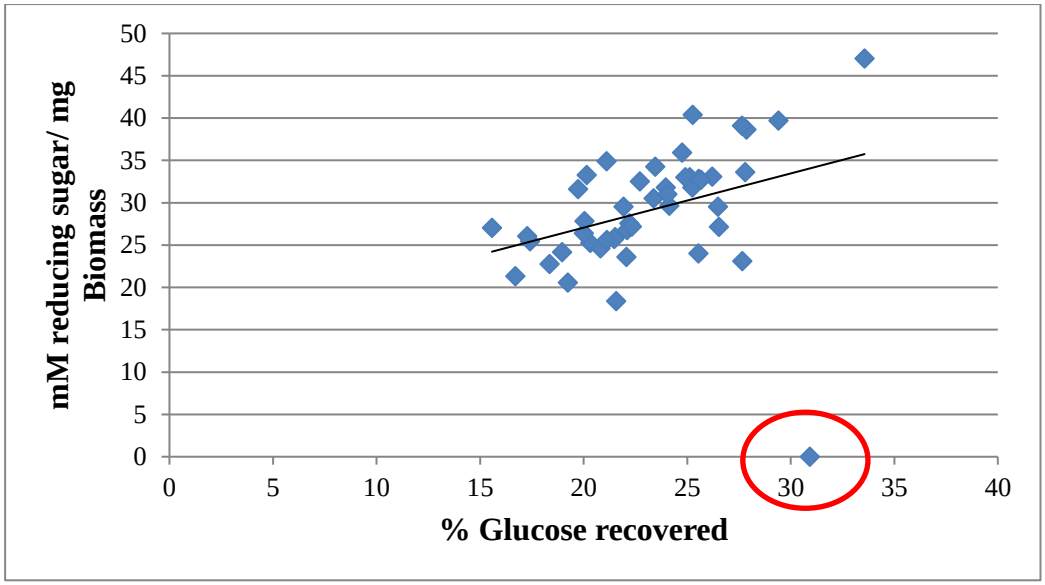


Figure 5.4.2: Scatter graph showing the relationship between glucose recovered and decreasing detected sugars. BC1 stem samples were treated with 1% H_2SO_4 and subjected to an enzymatic hydrolysis. The sample circled in red showed as a zero with the York method.

A Pearson's correlation coefficient was used to determine any correlations between the method used at Nottingham and the method used at York for the acid pre-treatments (figure 5.4.2). This showed a weak positive correlation (r^2= 0.338) between glucose recovered and the high throughput acid method. Although it should be noted that sample BC1 43 gave a zero response with the York assay, when this outliner is removed from the data the correlation increases to r^2 = 0.681, suggesting that both methods for determining digestibility yield similar results.

Analysis of the data using a Pearson's correlation coefficient showed there was no correlation between the amount of glucose released using the Nottingham acid pre-treatment and the total concentration of reducing sugars generated using the 0.5mM NaOH extraction method used at York (r^2= 0.225) (data not shown). Analysis of the data using a Pearson's correlation coefficient also showed no correlation between the total number of reducing sugars generated

following the York acid method and the York NaOH method (r^2=0.114) (data not shown).

5.5: Genetics analysis.

The BC1 samples generated at Nottingham were sent to Bristol University for genotyping. For illustration purposes the results returned for the *Aegilops speltoides* and *Triticum urartu* introgressions will be shown in this thesis, (Figures 5.5.1 and 5.5.2, respectively). In each case only a selection of the introgressions are shown, these being for the two BC1s with the highest digestibility and the two with the lowest digestibility.

The genotype for each of the seven chromosomes have been split into four columns, the two on the left of each chromosome have the highest digestibility and the two on the right have the lowest digestibility.

Blue segments show homogenous alleles for the elite wheat line parent, sections in red show one of the alleles is for the wild type parent, segments that are white are inconclusive.

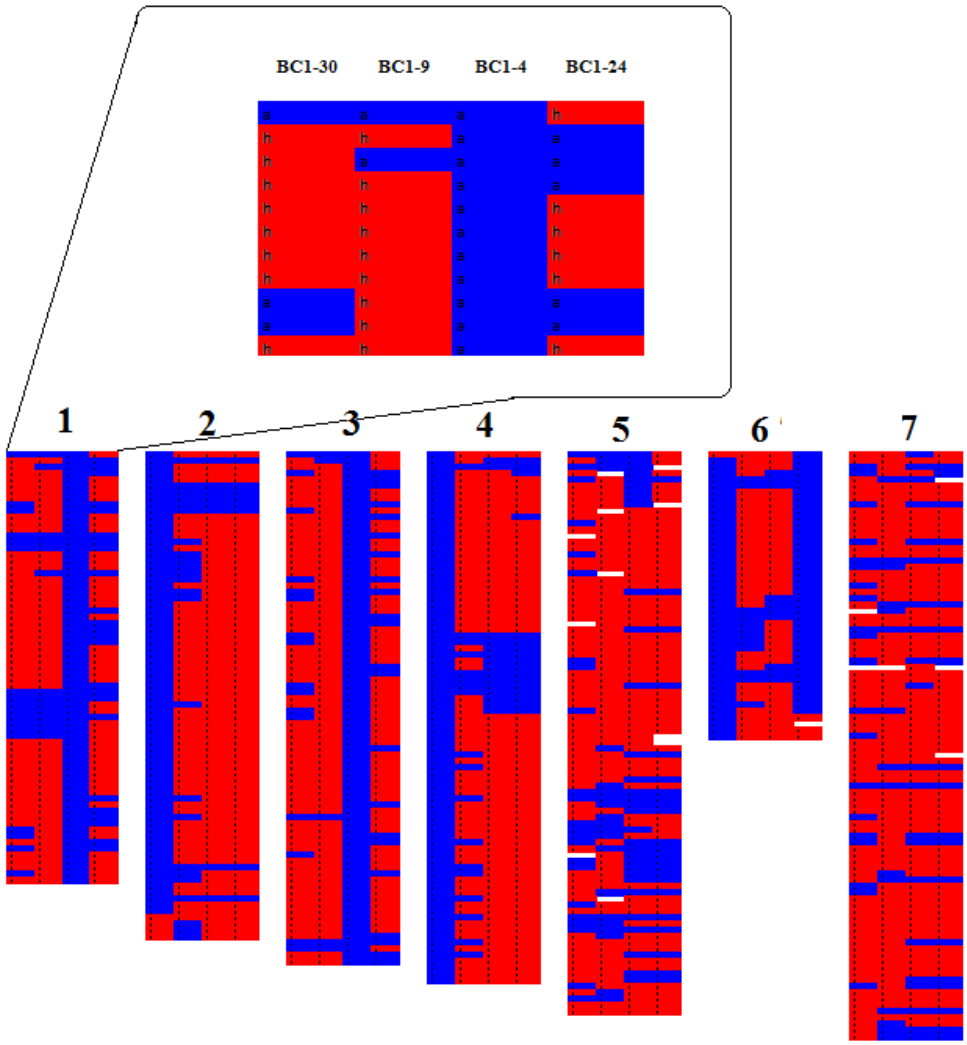


Figure 5.5.1: Genetic markers for *Aegilops speltoides* for the 7 chromosomes. BC1 samples were sent for analysis to determine the levels of genetic variation. The top 2 and bottom 2 samples for digestibility were then placed against each other for comparison; blue segments show homogenous alleles for the elite wheat line parent, sections in red show one of the alleles is for the wild type parent.

Aegilops speltoides shows large levels of recombination with the most digestible sample being BC1 30 that ranked as the 5th most digestible in comparison to BC1-24 that was ranked 43rd.

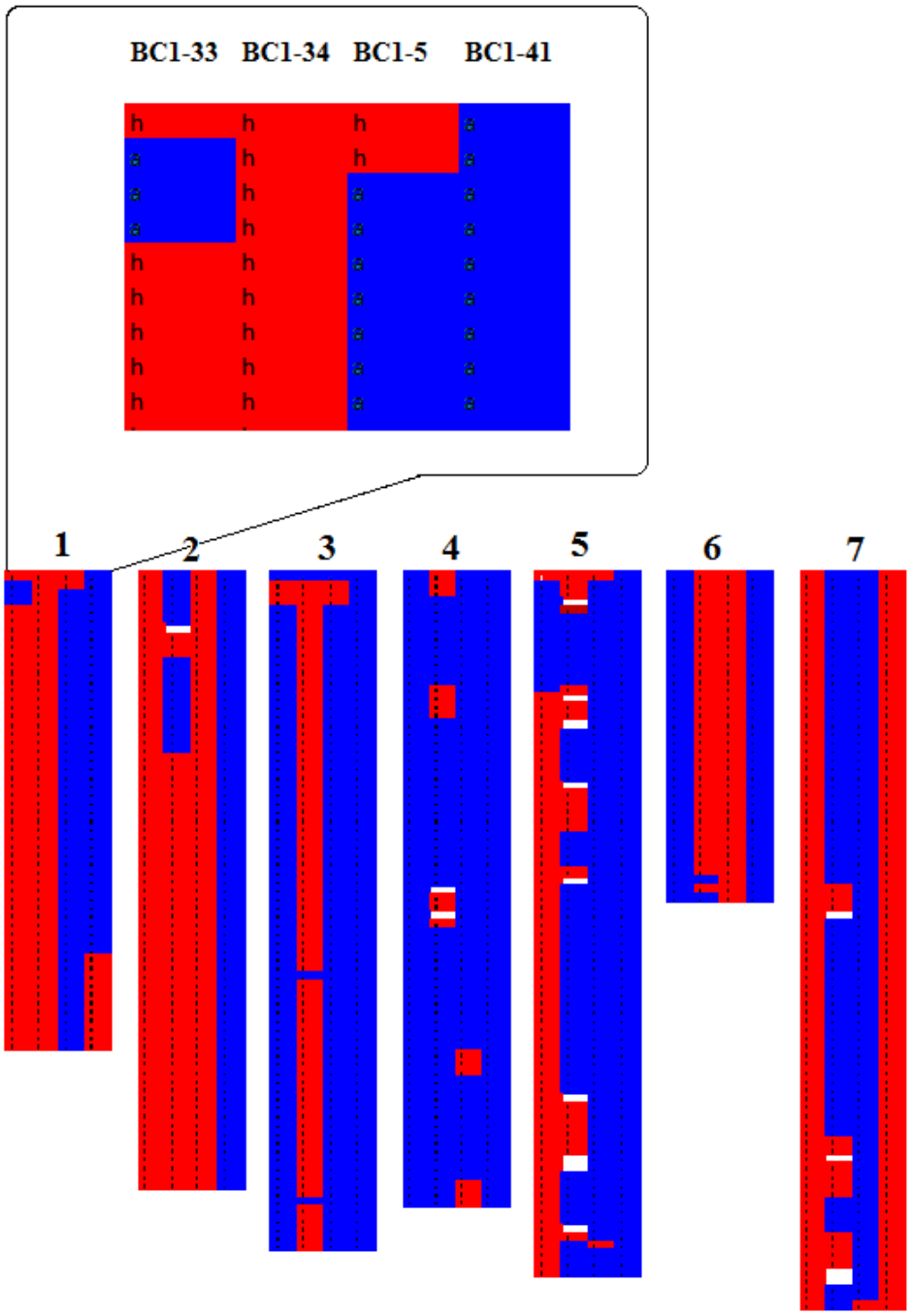


Figure 5.5.2: Genetic markers for *Triticum urartu* for the 7 chromosomes. BC1 samples were sent for analysis to determine the levels of genetic variation. The top 2 and bottom 2 samples for digestibility were then placed against each other for comparison; blue segments show homogenous alleles for the elite wheat line parent, sections in red show one of the alleles is for the wild type parent.

Triticum urartu shows slightly less recombination with the most digestible sample being BC1-33 that ranked as 17th compared to the least digestible BC1-41 that ranked 39th.

The results for the genetic mapping show there has been a larger than expected level of recombination. Whilst it is not possible to confirm what genome the recombination has occurred on, previous work using Fluorescence in situ hybridization (FISH) suggest that they have mainly taken place on the AA genome.

5.6: Conclusions.

BC1 samples showed a large degree of variation in morphology with regard to height/weight and leaf/stem ratios. Pearson's correlation coefficient suggests that the total biomass is not related to height. Whilst un-treated samples differ with regard to sugar composition, particularly for glucose and arabinose (BC1-6 not showing any arabinose), the glucose levels after a 1 % H_2SO_4 pre-treatment are comparable. As previously seen (chapter 3) alkaline is better at liberating sugars than acid during the pre-treatment stage. This said the method used for the York (HT) measures reducing sugars and will thus detect all sugars present including the hemicellulose fraction. The only sample not to give any results was sample BC1- 43 for the acid treatment, and when this is taken into account, it increases the Pearson's correlation coefficient to r^2=

0.681 making it a strong correlation. The enzymatic digestion after an acid pre-treatment for both the York and Nottingham data, showed that the best samples released nearly twice the amount of glucose/ reducing sugars compared the worst in each method. The genetic mapping shows that there has been a high level of recombination in the BC1 samples. This was actually far greater than anticipated and will make analysis of this data more complicated. On preliminary analysis however, it was possible to identify significant regions of the genome for both *Aegilops speltoides* and *Triticum urartu,* which would potentially be good sites for further analysis of later BC samples (BC3 or BC4) that would have a reduction of wild type alleles allowing for more specific regions to be identified that may impact upon digestion. This shows that the initial aims of this study, to determine if genetic variation can be increased in wheat, has shown that it is possible, and that "wild type" genes can be introduced to modern wheat cultivars.

6: Hemicellulose composition of BC1 samples.

6.1: Introduction.

It was shown in the previous chapter that the integration of ancient wheat genetic material may impact on the digestibility of the resultant BC1 plants. It has been suggested in the literature (Gao *et al*, 2013) that one of the major determinants of how recalcitrant a substrate is, is partly down to the hemicellulose structure. Hemicellulose is an amorphous branched structure that consists of repeating monomers that interact and connect the cellulose and lignin. Wheat straw hemicellulose primarily consists of xylose with trace amounts of glucose, arabinose and galactose (diagram 1.6.1.2). As seen in section 3.3 it may be possible to determine the hemicellulose composition by analysing the liquor fraction created following a standard 1% H_2SO_4 pre-treatment as described in section 2.3.

6.2: Analysis of hemicelluloses through xylose extraction and quantification.

To determine if the differences seen in the digestibility of the BC1 samples was related to the amount of hemicellulose found in each sample, a Pearson's correlation coefficient test was used to compare xylose and digestibility. The

correlation between the levels of xylose in the raw straw and digestibility was r^2=0.190 and for the arabinose in the raw straw and digestibility the r^2= 0.040. The xylose in the pre-treated residues with the digestibility was r^2=0.047. These results suggest that there is no correlation between the quantity of xylose in either the BC1 straw or the residues and their digestibility.

However, whilst xylose is the predominant sugar in hemicellulose it is not the only sugar present and this analysis only looked at the xylose levels present in the raw untreated samples and the samples following a treatment. Without looking at the xylose removed during treatment and the composition of the other sugars it would not be possible to determine how this may impact on digestion.

6.3: Hemicellulose composition and susceptibility to acid pre-treatment.

To determine if the BC1 samples had variable hemicellulose compositions it was necessary to hydrolyse and detect all the component sugars. The use of a Saemen's hydrolysis for this purpose is complicated as it is known that the individual sugars have variable stability to this process. This is demonstrated in figure 6.3.1 which shows the % sugars recovered after a Saemen's hydrolysis.

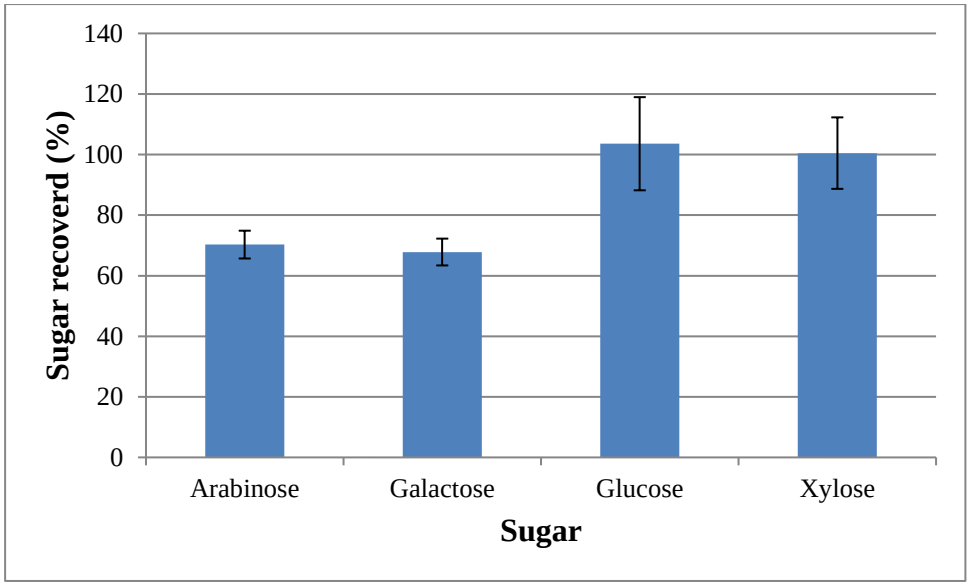


Figure 6.3.1: Sugar stability during Saemen's hydrolysis. 30mg of arabinose, galactose, glucose and xylose were dissolved in 12ml of water and analysed by HPAEC to determine sugar quantities, 30 mg of each was then subjected to a Saemen's hydrolysis followed by analysis by HPAEC to determine how stable sugars are under this condition. Results shown are mean ± SD (n= 6).

It can be seen that whilst glucose and xylose are stable in these conditions both arabinose and galactose degrade substantially. As the hemicellulose fraction of straw consists of arabinose, galactose, glucose and xylose, and as the BC1 samples gave only trace amounts of these sugars, the Saemen's hydrolysis is unsuitable as a means of determining differences in hemicellulose composition.

One of the main problems with the analysis of BC1 samples was the lack of sufficient amount of sample to work with, thus there was a need for a simple way to analyse the hemicellulose fraction for its sugar composition in a way that uses little substrate and allows for a high throughput. There are several methods in the literature for hemicelluloses analysis. One common method is the use of 2M TFA (Trifluoroacetic acid). However, this is both destructive

and a time consuming process. Previous work on pre-treatment (chapter 3) suggests that a milder acid pre-treatment might be used to obtain some limited compositional analysis of the hemicelluloses fraction whilst still allowing the pre-treated residue to be used for further analysis. Thus the liquor obtained from the pre-treatment of the BC1 straw with 1% H_2SO_4 was analysed directly for monomeric sugars. Whilst this is unlikely to provide a total composition (there was xylose left in the residue) it might be used to provide an indication of the relative compositions of hemicelluloses in the BC1 samples. Additionally it can provide further information on the susceptibility of the hemicelluloses to degradation during the acid pre-treatment.

As seen in figure 6.3.1 xylose is a stable sugar during a Saemen's hydrolysis, by utilising 2 commercial varieties of wheat straw it was possible to determine the levels of xylose removed during a standard 1 % H_2SO_4 pre-treatment (figure 6.3.2).

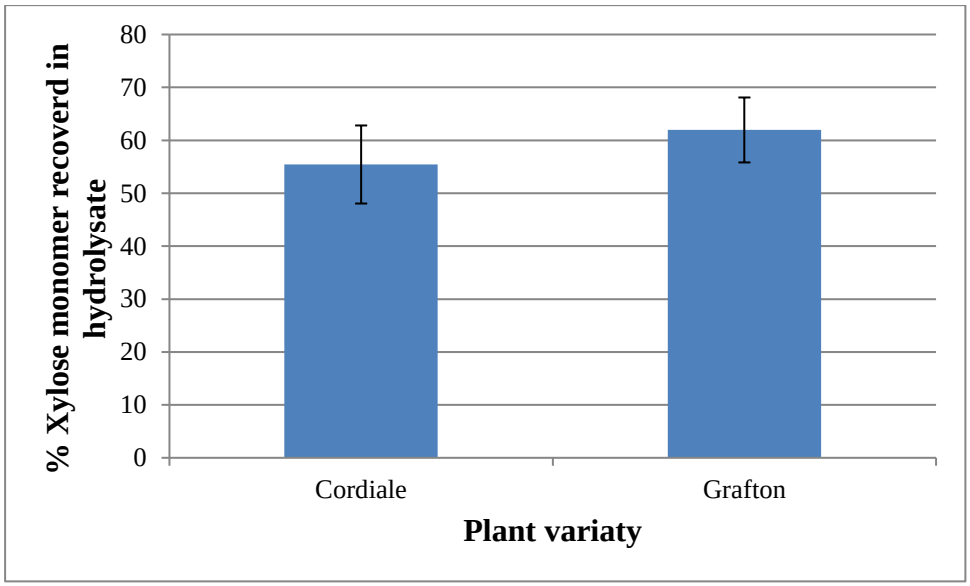


Figure 6.3.2: % Xylose removed during a standard 1% H_2SO_4 pre-treatment. Cordiale and Grafton wheat straw was knife milled to 2mm particle size and subjected to a Saemen's hydrolysis followed by analysis by HPAEC to determine raw xylose content, it was then subjected to a standard 1% H_2SO_4 pre-treatment followed by analysis by HPAEC to determine xylose in the liquor, the quantity of xylose removed was then calculated. Results shown are mean ± SD (n= 6).

Analysis of the data using ANOVA shows no differences (p>0.05) between the levels of xylose removed during a 1% H_2SO_4 pre-treatment for two commercial varieties. This suggests it should be possible to remove between 55% and 65% of the xylose from the BC1 samples using a standard 1% H_2SO_4 pre-treatment as described in 2.3 for possible analysis of the hemicelluloses fraction.

The liquor from the pre-treatment was analysed for monomer sugar composition by HPAEC for arabinose (figure 6.3.3), for galactose (figure 6.3.4), for glucose (figure 6.3.5), and for xylose (figure6.3.6).

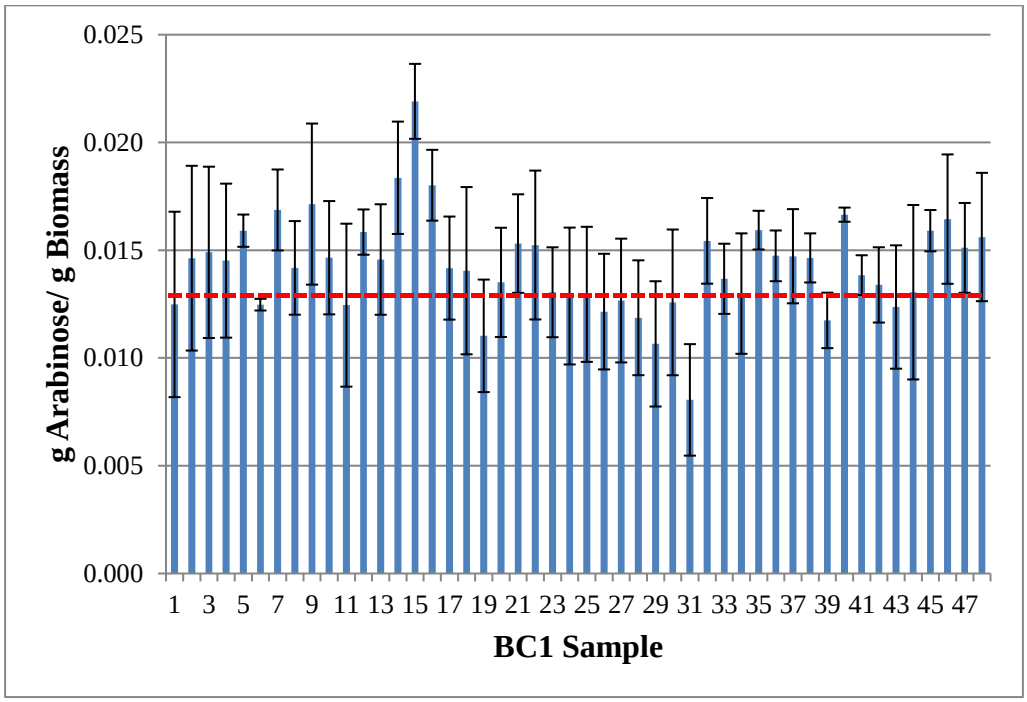


Figure 6.3.3: Arabinose recovered in the pre-treatment liquor. BC1 stem tissue was knife milled to a 2mm particle size and subjected to a standard 1% H2SO4 pre-treatment and the liquor fraction was analysed using HPAEC to determine monomeric sugar composition. Results shown are mean ± SD (n= 3).

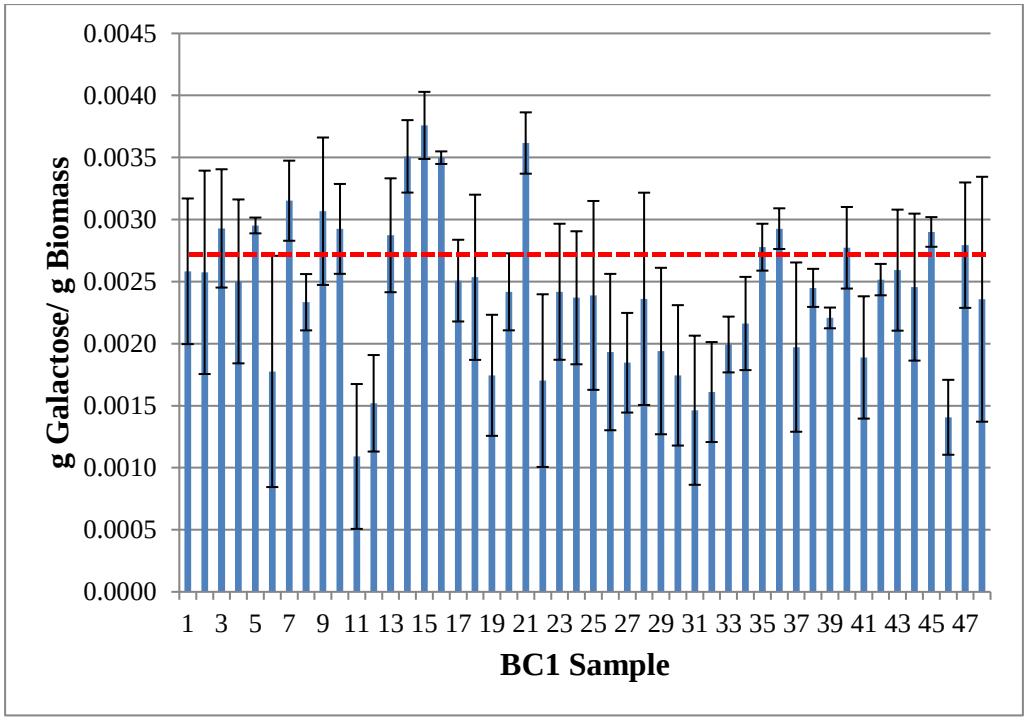


Figure 6.3.4: Galactose recovered in the pre-treatment liquor. BC1 stem tissue was knife milled to a 2mm particle size and subjected to a standard 1% H2SO4 pre-treatment and the liquor fraction was analysed using HPAEC to determine monomeric sugar composition. Results shown are mean ± SD (n= 3).

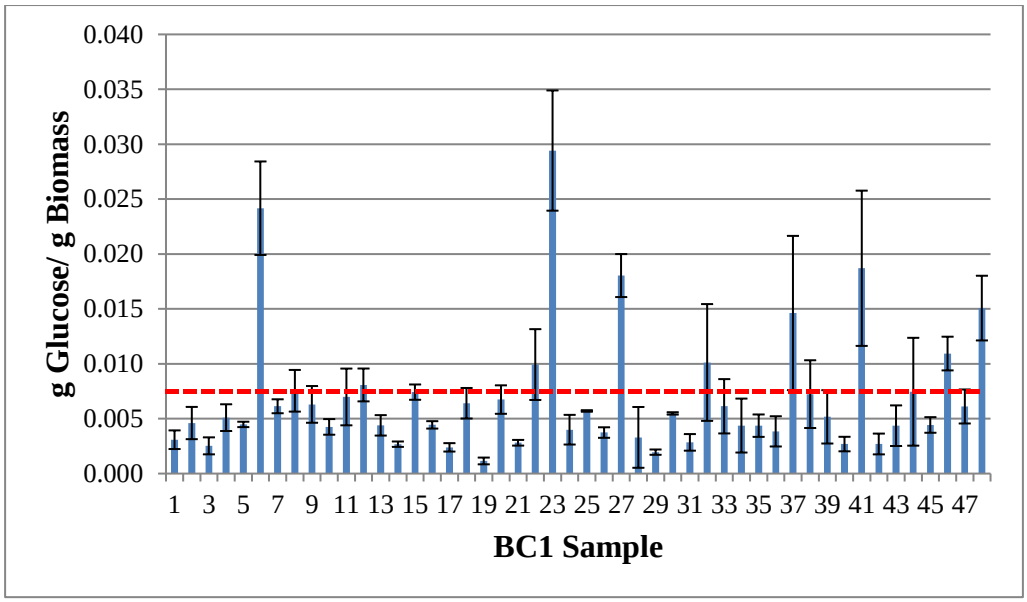


Figure 6.3.5: Glucose recovered in the pre-treatment liquor. BC1 stem tissue was knife milled to a 2mm particle size and subjected to a standard 1% H2SO4 pre-treatment and the liquor fraction was analysed using HPAEC to determine monomeric sugar composition. Results shown are mean ± SD (n= 3).

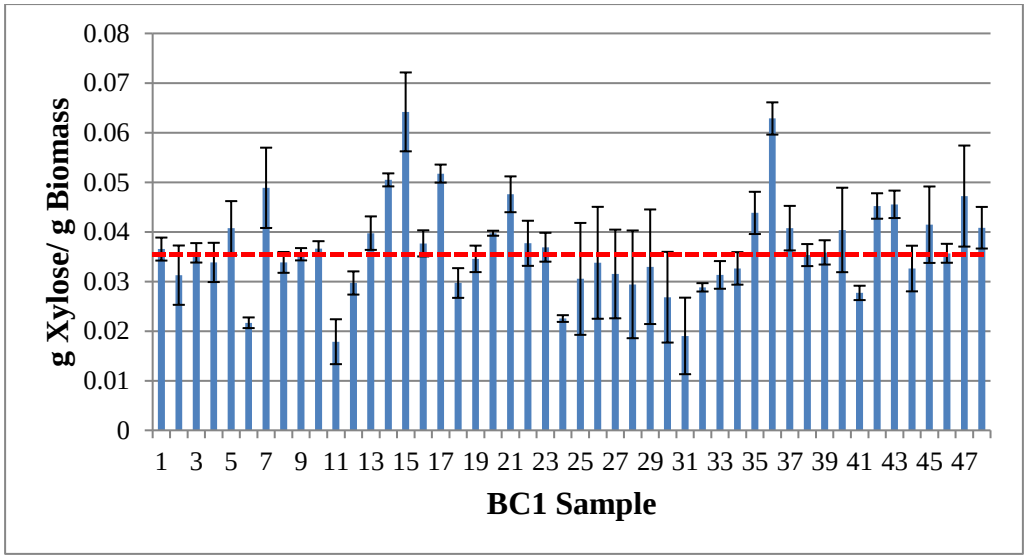


Figure 6.3.6: Xylose recovered in the pre-treatment liquor. BC1 stem tissue was knife milled to a 2mm particle size and subjected to a standard 1% H2SO4 pre-treatment and the liquor fraction was analysed using HPAEC to determine monomeric sugar composition. Results shown are mean ± SD (n= 3).

Analysis of the data using a Pearson's correlation coefficient was used to first determine correlations between the levels of the individual monomeric sugar detected in the pre-treated liquor (table 6.3.1).

	Arabinose	Galactose	Glucose	Xylose
Arabinose		0.6223	-0.002	0.6047
Galactose	0.6223		-0.305	0.7007
Glucose	-0.002	-0.305		-0.207
Xylose	0.6047	0.7007	-0.207	

Table 6.3.1: Correlations between the monomeric sugars detected in the pre-treated liquor. BC1 stem tissue was knife milled to a 2mm particle size and subjected to a standard 1% H_2SO_4 pre-treatment and the liquor fraction was analysed using HPAEC to determine monomeric sugar composition.

This showed that there were strong positive correlations between arabinose, galactose and xylose but glucose had a weak negative correlation with all the other sugars. This might imply that the relative composition of the hemicelluloses in the BC1 population has not been altered significantly. The higher levels of glucose seen in some of the samples could be due to an

increased level of amorphous regions of cellulose. However, confirmation of this would require a more thorough analysis of the composition.

A Pearson's correlation coefficient was then used to determine any correlations between the levels of the individual sugars detected in the pre-treated liquor and the digestibility of the residue and showed a weak positive correlation for arabinose (r^2=0.174), a weak positive correlation for galactose (r^2=0.210), a weak negative correlation for glucose (r^2=-0.166), and a weak positive correlation for xylose (r^2=0.278). The sum of all sugars detected in the residue show a weak positive correlation of r^2=0.187. A Pearson's correlation coefficient was also used to determine the correlation between the levels of xylose found in the pre-treated liquor and the level of xylose in the untreated BC1 samples, this showed a weak positive correlation (r^2=0.270).

Although weak, the positive correlations with the three sugars, especially xylose, might imply a link between hemicellulose degradation during the pre-treatment and digestibility.

As seen in figure 6.3.2, it should be expected to remove in the region of 55% - 65% of the total xylose in the BC1 samples. Figure 6.3.7 shows that only 11% - 35% is being degraded.

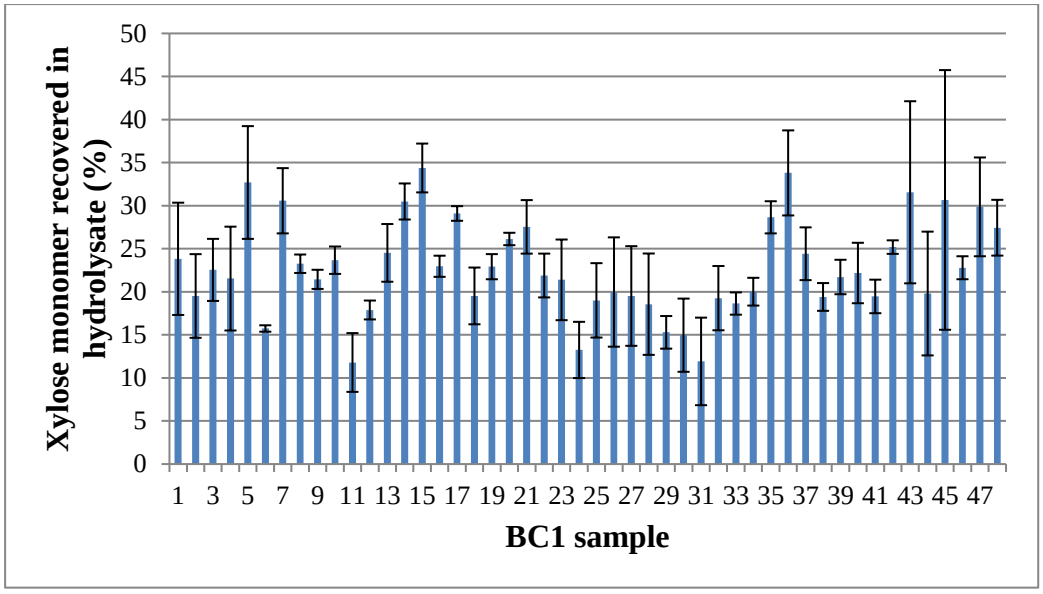


Figure 6.3.7: Xylose (%) removed during a standard 1% H_2SO_4 pre-treatment. BC1 stem tissue was knife milled to 2mm particle size and subjected to a Saemen's hydrolysis followed by analysis by HPAEC to determine raw xylose content; it was then subjected to a standard 1% H2SO4 pre-treatment followed by analysis by HPAEC to determine xylose in the liquor, the quantity of xylose removed was then calculated. Results shown are mean ± SD (n= 3).

A Pearson's correlation coefficient was then used to determine any correlations between the levels of the xylose removed during a 1% H_2SO_4 pre-treatment and digestibility, and again this shows a weak positive correlation r^2=0.227.

Whilst it appears that the total hemicellulose composition is not changing dramatically, the digestibility of the xylose fraction is varied and dramatically different to commercial wheat varieties suggesting that it could be down to hemicelluloses structure.

6.4: Differential scanning calorimetry (DSC) to assess hemicellulose degradation.

To determine which BC1 samples should be used for further in depth analysis, all the BC1s were ranked according to the two digestibility assays previously used (section 5.3 and 5.4). The 10 highest and 10 lowest in each assay are shown in table 6.4.1. It can be seen that BC1 14 is consistently the best for both assays and BC1 6 was one of the worse for both, BC1 3 was also chosen as it ranked mid-way for both Nottingham and York (Nottingham 25 and York 27).

Top 10 samples	York (HT) method (acid)	Standard method	Bottom 10 samples	York (HT) method (acid)	Standard method
1	14	14	39	2	24
2	19	43	40	12	7
3	30	30	41	31	22
4	47	28	42	48	11
5	28	34	43	23	31
6	29	44	44	44	4
7	10	47	45	4	8
8	17	27	46	6	42
9	34	46	47	11	6
10	5	26	48	41	39

Table 6.4.1: Ranking of the top 10 BC1 samples and the bottom 10 BC1 samples. BC1 samples were ranked in accordance of how digestible they were for both Nottingham and York to enable samples to be chosen for further examination, samples shown in red are in both Nottingham and York Top and Bottom 10.

These three were analysed by differential scanning calorimetry (DCS) to determine if there were any differences in their degradability. The results are

shown in figure 6.4.1. The peak at around 220ºC represents the degradation of the hemicellulose fraction and that around 280ºC the cellulose fraction. The cellulose trough appears to be identical in all three BC1 samples. However, for the hemicellulose trough there seems to be a trend towards a lower temperature as digestibility decreases but perhaps more significantly there is an apparent reduction in the magnitude of the trough as digestibility increases.

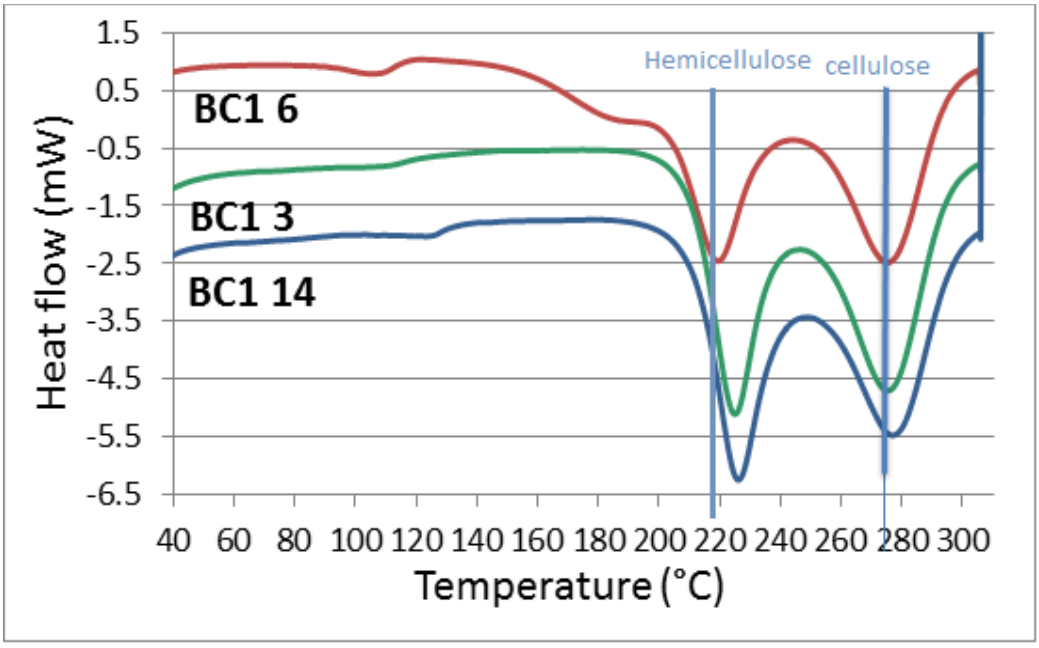


Figure 6.4.1: The heat flow and degradation of BC1 samples. Stem fractions were hammer milled to a 0.5mm size and mixed with de-mineralised water for a 1:1w/v ratio paste. 15mg was then placed into a gold DSC pan and a lid and cap secured these were then analysed using DSC (Mettler Toledo DSC 823^e with a 50801R0 Robot sample placing) the samples were run from +20ºC to +310ºC and sampled at 5 min intervals.

This may suggest that the BC1 samples have different hemicellulose structures; work on pre-treatments (see chapter 3) suggested that with the mild acid pre-treatment it was possible to break down the hemicelluloses structure into monomeric sugars without significant degradation of the sugars, allowing for a limited analysis of hemicellulose composition. The chromatograms from the HPAEC analysis of the pre-treatment liquors from BC6 and BC14 straws

were thus compared and are shown in figures 6.4.2, it can be seen that the profile for BC1 14 has 7 detectable peaks. The four major peaks being for xylose, arabinose, galactose and glucose. The other three peaks are minor and unknown. In contrast the profile for BC1 6 has a larger amount, 13 detectable peaks. Xylose, arabinose and galactose are clearly detectable but at lower intensities than for BC1 14. Glucose by comparison is increased in intensity. Some of the other peaks are of significant intensity but their identification remains unknown.

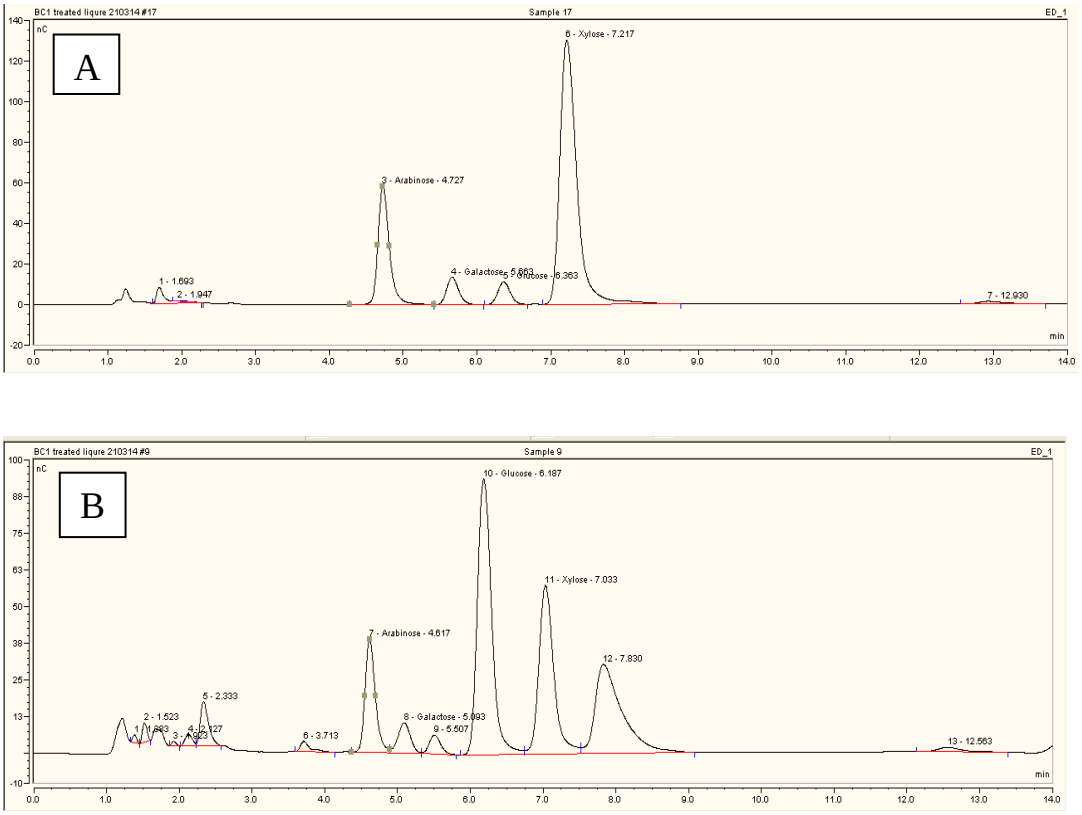


Figure 6.4.2: HPAEC chromatographs for the liquors from pre-treatment straw. Stem tissue was knife milled to a 2mm particle size and subjected to a standard 1% H_2SO_4 pre-treatment and the liquor fraction was analysed using HPAEC to determine monomeric sugar composition, (A) liquor from BC1 14 straw (B) liquor from BC1 6 straw

This may suggest that the hemicelluloses fractions of BC1 6 and 14 may be different to each other. This trend with unusual HPAEC chromatographs can also be seen in other BC1 samples, for example 11 and 41, with these having 9 detectable peaks each. The HPAEC profile of the liquor from an elite wheat straw undergoing the same pre-treatment is shown in figure 6.4.3. This shows only 5 detectable peaks and as such is much closer to the more digestible BC1 14.

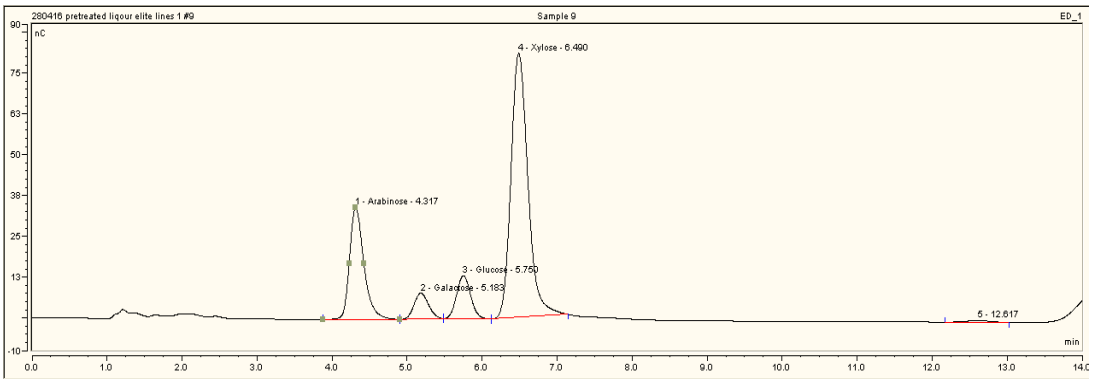


Figure 6.4.3: HPAEC chromatograph for the liquor for an elite line following a 1% H_2SO_4 pre-treatment. Stem tissue was knife milled to a 2mm particle size and subjected to a standard 1% H2SO4 pre-treatment and the liquor fraction was analysed using HPAEC to determine monomeric sugar composition.

6.5: Conclusions.

These results suggest that the hemicellulose fractions of the BC1 samples may differ in both composition and structure when compared to standard wheat varieties, as there was no significant correlations between any of the sugars from the hemicelluloses fraction and the overall digestibility it is not possible to use the hemicellulose fraction as an indicator for digestibility when analysed

in this way. However it should be noted that a 1% H_2SO_4 pre-treatment is capable of liberating monomeric sugars from hemicellulose without significant degradation. The differences seen in levels of sugar in the pre-treated liquor from the BC1 samples suggest that the hemicelluloses fraction is being degraded in different ways. As the levels of xylose that have been removed from each sample during the pre-treatment also differ, this suggests that the xylose fractions have differing levels of digestion. This can be seen when comparing the xylose recovered in the hydrolysate that shows a great level of variation to the xylose content for the untreated samples (section 5.3). The moderate to strong correlation in levels of arabinose, galactose and xylose suggest that the composition of the hemicelluloses is fairly consistent between the BC1 samples; this suggests that the hemicellulose matrix may have a different structure. Samples that showed a higher than average glucose content recovered in the pre-treated liquor correspond to the samples that showed some of the lowest glucose recovery following an enzyme hydrolysis. This could be due to them having an increased level of amorphous cellulose that is being degraded during the pre-treatment although if this was the case you would expect the samples with lower digestion to rank higher in the pre-treatment and digestibility assay conducted at York (section 5.4) as this method measures reducing groups and there is no separation of the pre-treated liquor and residue.

The DSC suggests that the hemicellulose is structurally different between BC1-6 and BC1-14 with regards to the energy requirement for a phase change in the hemicellulose fraction. This also occurred at a slightly lower temperature suggesting that the hemicelluloses is less stable in the BC1 samples with lower digestibility, suggesting a different hemicelluloses structure. The cellulose

fraction showed more consistency between the three samples with only minimal differences seen.

The chemical composition of the pre-treated liquor analysed via HPAEC show that many of the BC1 samples have considerably different profiles suggesting that some of the samples may have far more in the hemicellulose fraction than expected (for wheat straw mainly xylose with some arabinose, galactose, and trace levels of glucose). Whilst it was not possible to determine what these additional peaks were as a CarboPacTM PA 20 column (3 x 150 mm) was used for the separation it can be assumed that it was monomeric. Whilst the HPAEC profile for BC1 14 (figure 6.4.2 A) shows seven detectable peaks, only the four expected peaks are of quantifiable levels (arabinose, galactose, glucose and xylose) as seen in a standard elite cultivar (figure 6.4.3). The HPAEC profile for BC1 6 (figure 6.4.2 B) shows 13 peaks, of these, 3 additional peaks are of quantifiable levels in addition to those expected. Peak 5 is most likely a sugary alcohol whilst peak 8 is likely to be glucosamine as this is the only compound according to the Carbopac pa20 analytical column product manual that eludes between arabinose and galactose (Product manual, CarboPac PA20, Thermo Scientific, 2011). Peak 12 the largest of the additional peaks seen (figure 6.4.2B), this could possibly be fructose (a monosaccharide that is often bound to glucose) and could explain the higher levels of glucose seen in this sample but would suggest the presence of sucrose in the sample. This would need to be investigated running additional samples with the presence of fructose and the use of a column capable of detecting disaccharides (a carbopac PA 200) to detect sucrose. Although it should be noted that sucrose is not commonly associated with grass species. It is also possible that it could be due to the

hemicellulose containing Glucomannan (β-(1-4)-linked D-mannose and D-glucose in a ratio of 1.6:1). Both of these support the theory that it is the hemicellulose fraction that is increasing the glucose in the pre-treated liquor and not the levels of amorphous cellulose.

Chapter 7: Use of sea water for Bio-fuel production.

One of the problems associated with second generation bio-fuels is the high water usage. Excluding the water used to grow the plants there is still a high requirement as it is needed for both the pre-treatment stage and the enzyme hydrolysis stage, although this will vary depending upon what feed-stock is being used. Whilst fresh water is an abundant resource in certain parts of the world, many other parts have little fresh water available. The ability to utilise sea water as a replacement for fresh water could revolutionise the bio-fuel industry. Sea water consists of mainly water (96.5%) with the remaining 3.5% consisting of dissolved salts (table 7.1).

Chemical ion	concentration ppm	part of salinity %
Chloride (Cl)	19345	55.03
Sodium (Na)	10752	30.59
Sulfate (SO4)	2701	7.68
Magnesium (Mg)	1295	3.68
Calcium (Ca)	416	1.18
Potassium (K)	390	1.11
Bicarbonate (HCO3)	145	0.41
Bromide (Br)	66	0.19
Borate (BO3)	27	0.08
Strontium (Sr)	13	0.04
Fluoride (F)	1	0.003

Table 7.1: Sea water composition. Relative concentrations of chemical ions found in the North Sea.

7.1: Impact of sea water on pre-treatment and enzyme hydrolysis.

To determine if potable water can be substituted with sea water for the production of second generation bio ethanol, sea water was collected from Brancaster beach in Norfolk (52°58'29.2"N 0°38'13.9"E) in June 2015 and stored at 4ºC until needed. Revelation straw was milled to a 2mm particle size as described (section 2.1) and subjected to a standard pre-treatment as described (section 2.3) in 1 % H_2SO_4 made using sea water or a 1 % H_2SO_4 made using fresh water. The liquor was removed and the residue washed in either sea water or fresh water depending upon what they had been treated in, dried and weighed to determine the levels of biomass remaining after the pre-treatment (figure 7.1.1).

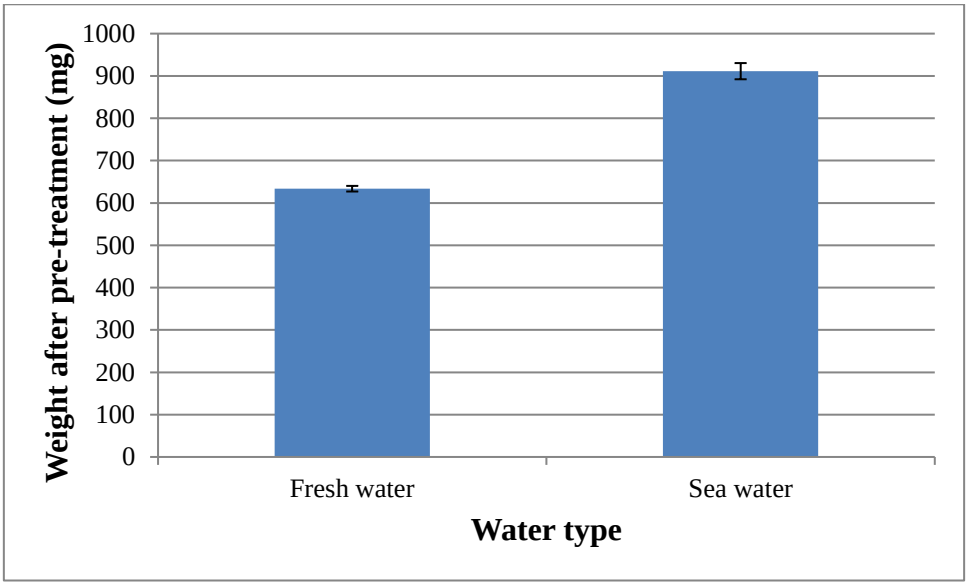
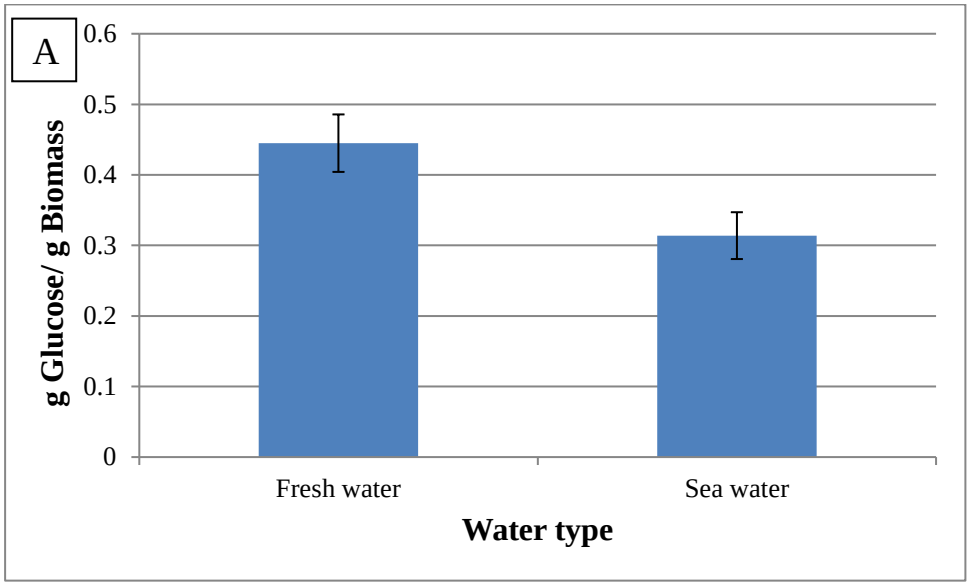


Figure 7.1.1: Effect of fresh or sea water on the weight of residue remaining after pre-treatment. 1 gram of Reverlation straw as bailed was knife milled to 2mm, this were then subjected to a acid pre-treatment in either fresh water or sea water and washed in fresh and sea water, respectively. The resulting residue was dried before being weighed. Results shown are mean ± SD (n= 10), bars with different letters are significantly different (p<0.05)

Analysis of the data using ANOVA for the residue remaining following a 1%H_2SO_4 pre-treatment shows a significant difference (p<0.005) between the fresh water pre-treatment and the sea water pre-treatment with the latter weighing significantly more following treatment. The residue following the pre-treatment was then subjected to a Saeman hydrolysis to determine sugar composition as per section 2.2, The results for glucose and xylose are shown in figures 7.1.2 A and 7.1.2 B, respectively.



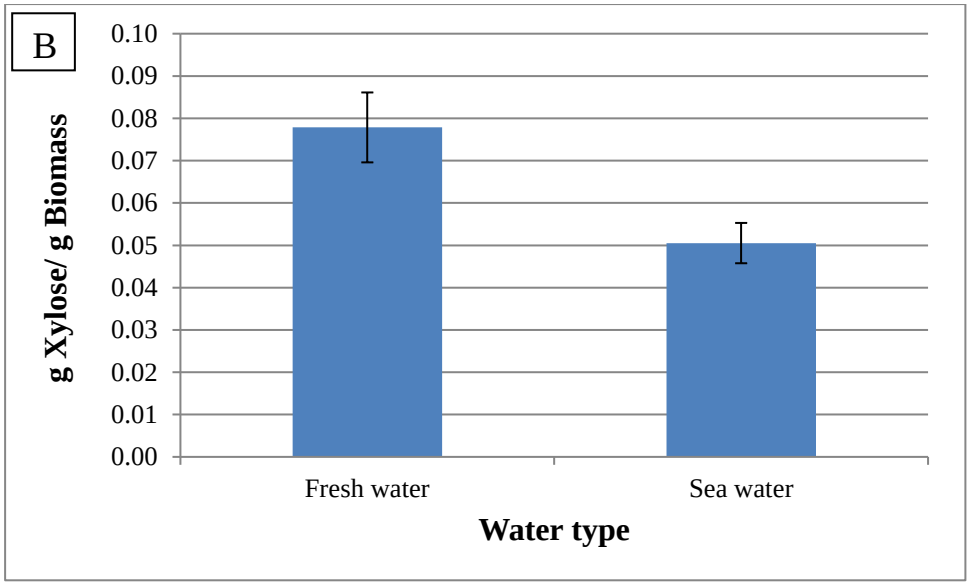


Figure 7.1.2: Sugar composition of residue from pre-treatment. Revelation wheat straw as bailed was milled to a 2mm particle size and subjected to an acid pre-treatment the residue was the subjected to a Saemen's hydrolysis to determine sugar composition, (A) g Glucose/ g Biomass, (B) g Xylose/ g Biomass. Results shown are mean ± SD (n= 20).

Analysis of the data using ANOVA again showed a significant difference (p<0.005) between fresh water and sea water with values in the fresh water having significantly more glucose and xylose.

As it was shown in section 3.3 that a weaker acid removes less hemicellulose during the pre-treatment, to determine if this decrease seen in the Saeman hydrolysis was due to a reduction of the action of the acid made using sea water the pre-treated liquor was then analysed for monomeric sugar composition using HPAEC see figure 7.1.3.

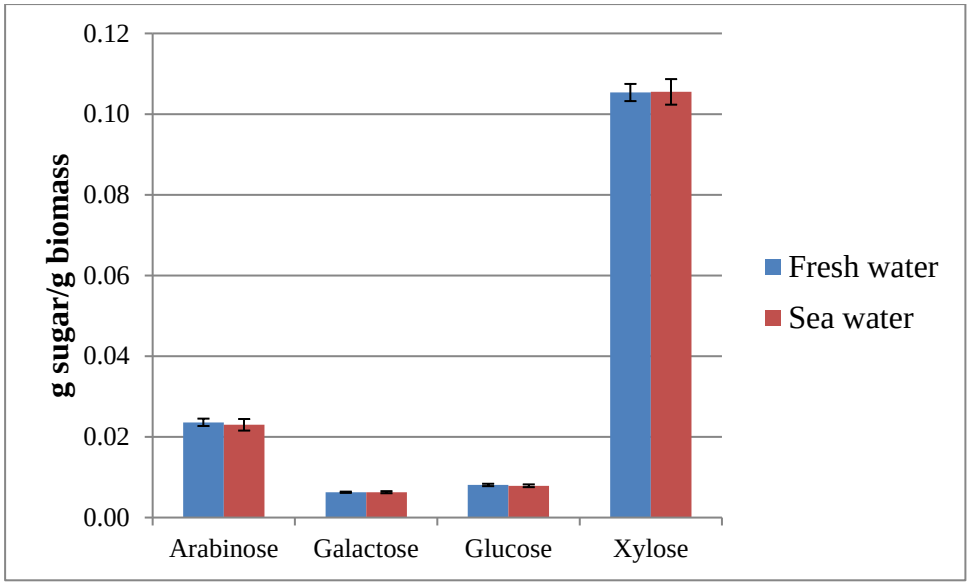


Figure 7.1.3: Sugar recovered in the pre-treated liquor (%), Revelation wheat straw as bailed was milled to a 2mm particle size and subjected to a acid pre-treatment the liquor fraction was analysed using HPAEC to determine monomeric sugar composition.. Results shown are mean ± SD (n= 20)

Analysis of the data using ANOVA showed that there were no significant differences (p<0.005) for any of the sugars detected in the pre-treated liquor.

The results for the pre-treated liquor analysis showed that the hemicelluloses fraction that is being removed is the same for both the fresh water treatment and the sea water treatment. To determine if the increase seen in biomass weight was due to salts and other impurities found in the sea water, 20ml was evaporated and the residue was weighed leaving a residue of 803mg.

This may simply account for why the residue from the sea water pre-treatment had a significantly greater mass. When this is taken into account the actual total content of the two sugars in the residues are identical at around 0.27g and 0,045g of glucose and xylose, respectively.

To determine if the salinity of the water had an impact on enzyme activity the dried residues were then subjected to a enzyme hydrolysis using Cellic CTec2 supplied by Novozymes as described (section 2.6). The samples that had been pre-treated in fresh water had the enzyme solution made with 50mM sodium citrate buffer as did one set of the samples pre-treated in sea water. The other set of samples pre-treated in sea water had the enzyme solution made with untreated, unsterilized sea water. In all cases 39.2 filter paper units (FPU) of cellulase was added and all were incubated at 50°C and sampled over 72 hours. The glucose released is shown (figure 7.1.4).

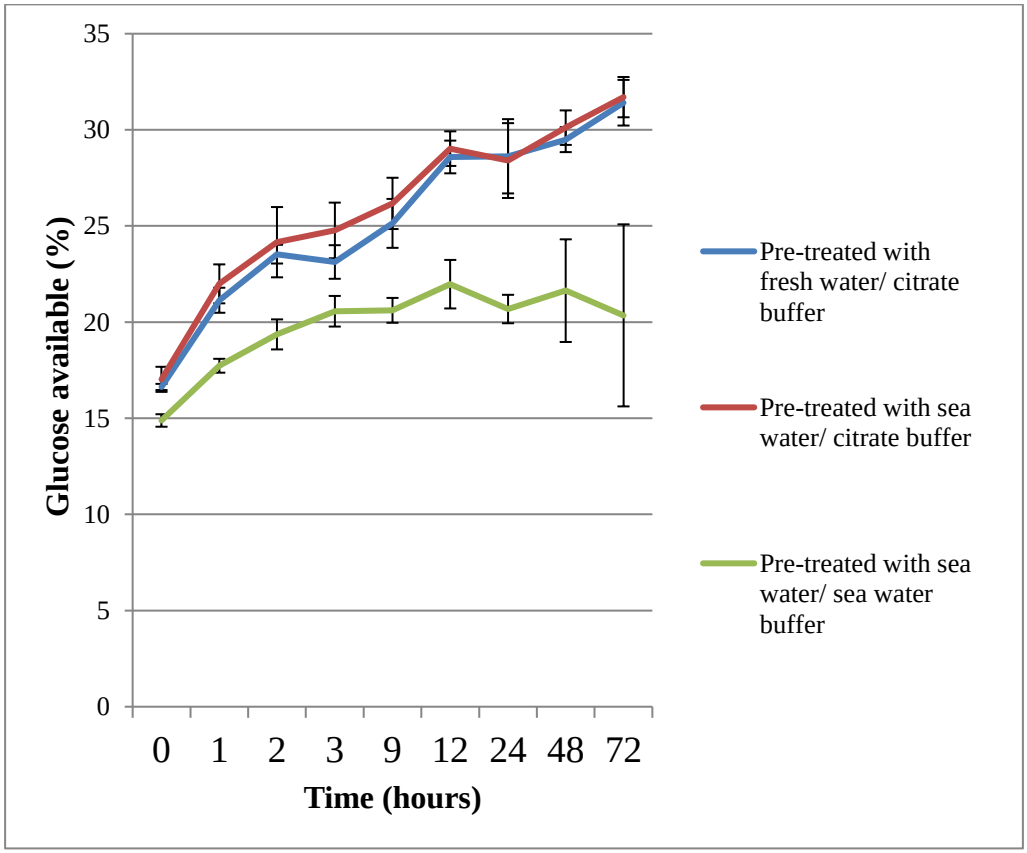


Figure 7.1.4: Effect of fresh and sea water on available glucose (%) released from revelation wheat straw. Revelation wheat straw was knife milled to a 2mm particle size and subjected to a standard 1% H_2SO_4 pre-treatment made with either fresh water or sea water, the residue was washed in either fresh or sea water as per it's pre-treatment prior to air drying, this was then subjected to an enzyme hydrolysis using 39.2 FPU of Cellic **CTec2** supplied by Novozymes, the samples pre-treated in fresh water had the enzyme solution made with 50mM sodium citrate buffer as did one set of the samples pre-treated in sea water, the other set of samples pre-treated in sea water has the enzyme solution made with untreated, unsterilized sea water with all samples were incubated at 50°C and sampled over 72 hours. Results shown are mean ± SD (n= 5)

Analysis of the data using ANOVA showed a significant difference (p = <0.005). Post hoc analysis inferred that the fresh water and sea water pre-treatment with the enzyme hydrolysis performed in a sodium citrate buffer were identical after 72 hours but the sea water pre-treatment with the enzyme hydrolysis performed in sea water was significantly different (p = <0.01).

7.2: Impact of hydrolysates on microbial growth.

The ability of microbes to grow is dependent on the level of nutrients (suitable sugar (most yeast convert C6 sugars) and nitrogen) available and the concentration of inhibitor compounds present. It was important to determine if either salinity itself or differential impact of inhibitors had an impact on the metabolic activity of yeast. To test this a phenotype array (Biolog) system was used to measure the metabolic activity of the yeast *Saccharomyces cerevisiae* strain NCYC2592 when incubated in either fresh or sea water with the addition of 6% glucose to determine the effects of salinity on metabolic activity. To determine if the liquid fraction from the enzymatic hydrolysis for the sea and fresh water contain differing levels of inhibitor compounds it was analysed for the inhibition of microbial growth with (Figure 7.2.1) and without (Figure 7.2.2) the addition of 6% glucose.

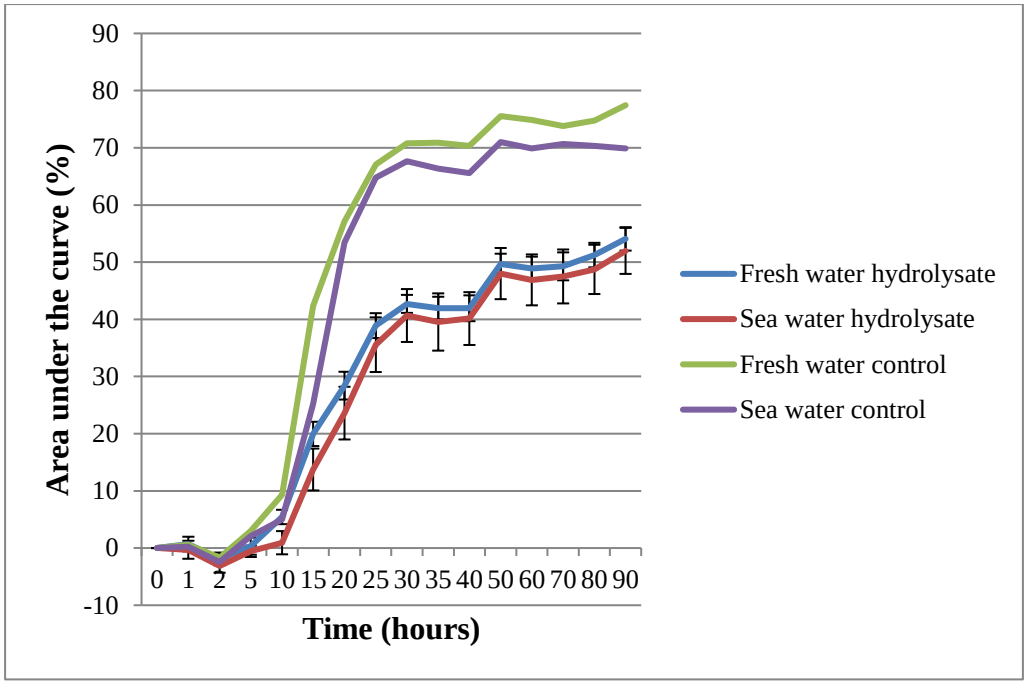


Figure 7.2.1: The ability of hydrolysate to support growth. Revelation wheat as bailed was milled to 2mm particle size, subjected to an acid pre-treatment made with either fresh water or sea water, the residue was then washed and dried prior to being subjected to an enzyme hydrolysis. The residual liquor was then neutralised to pH 4.5 and spiked with a 6% glucose solution and yeast species *Saccharomyces cerevisiae* NCYC2592 was used to determine microbial growth by measuring metabolic output over 72 hours. Results shown are mean ± SD (n= 10). For the two controls a single measurement was made.

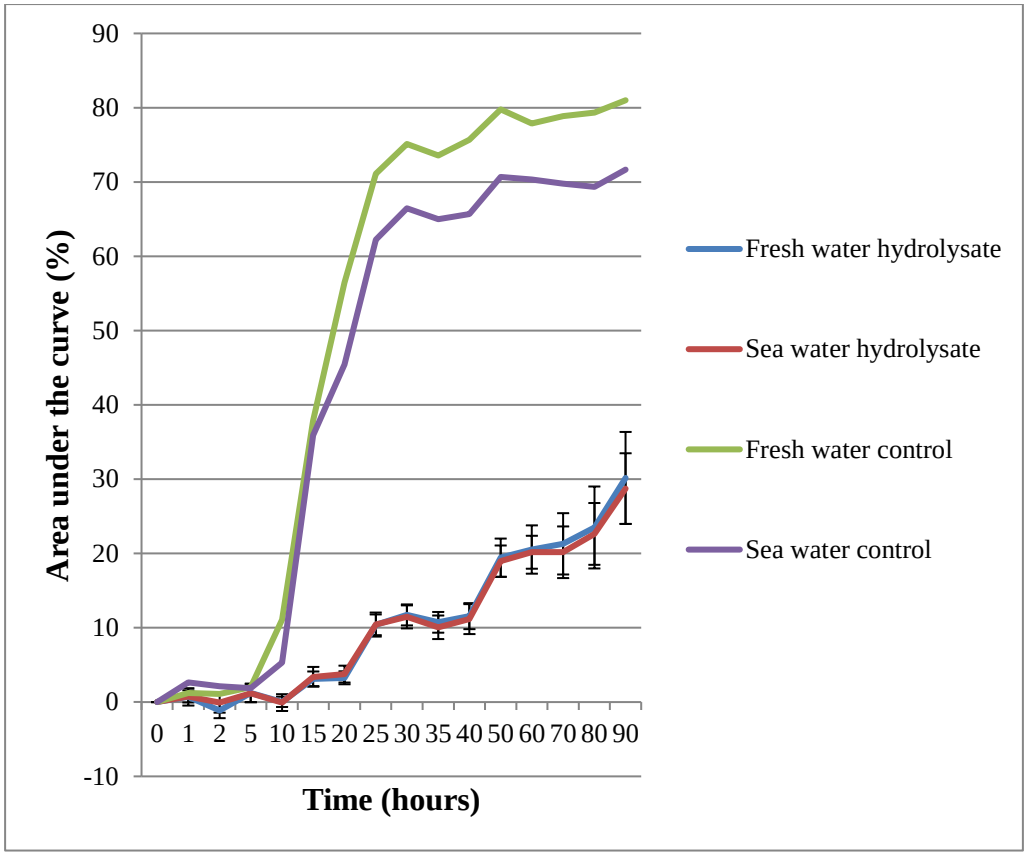


Figure 7.2.2: The ability of hydrolysate to support growth. Revelation wheat as bailed was milled to 2mm particle size, subjected to an acid pre-treatment made with either fresh water or sea water, the residue was then washed and dried prior to being subjected to an enzyme hydrolysis. The residual liquor was then neutralised to pH 4.5 and yeast species *Saccharomyces cerevisiae* NCYC2592 was used to determine microbial growth by measuring metabolic output over 72 hours. Results shown are mean ± SD (n= 10). For the two controls a single measurement was made.

In each case there was a slight reduction in metabolic activity when sea water was used as a control when compared to the fresh water control. When the hydrolysate was spiked with glucose there was a distinct reduction in both sea and freshwater samples, compared to the controls, but analysis of the data using ANOVA showed no significant difference between them after 72 hours (p = 0.158). Similar results were obtained without the glucose spike (p = 0.568) suggesting that the levels of inhibitor compounds are the same between fresh water and sea water.

7.3: One pot pre-treatment and hydrolysis to fermentation.

A leap forward in bio-ethanol production would be the removal of some of the energy intensive ant time consuming steeps such as washing and filtering the substrate after pre-treatment, this would allow for a SSCF method that would be more time and cost effective. Linking this with the need to reduce the water footprint of bio-fuel production a method was developed for the utilisation of sea water as this was shown not to impact on the separate steeps of pre-treatment and enzyme hydrolysis and only showed a slight reduction in fermentation.

Revelation wheat straw as bailed was knife milled to 0.5mm and subjected to a 1% H_2SO_4 pre-treatment made with either fresh water or sea water. In this instance the liquor and solid residues were both taken through to the subsequent enzyme hydrolysis as this more closely mimics actual commercial practice. The pre-treated sample was adjusted to pH 5.5 and subjected to an enzyme hydrolysis using Cellic CTec2 supplied by Novozymes at 400FPU for 24h at 50^{O}C. The hydrolysate was analysed for monomeric sugar composition using HPAEC (figure 7.3.2). The hydrolysate was then inoculated with the yeast species *Saccharomyces cerevisiae* strain NCYC2592 and incubated at 35^{O}C as described (section 2.7), during the fermentation the samples were weighed to determine weight loss (figure 7.3.2) and for CO_2 production (figure 7.3.3) and ethanol production (figure 7.3.4).

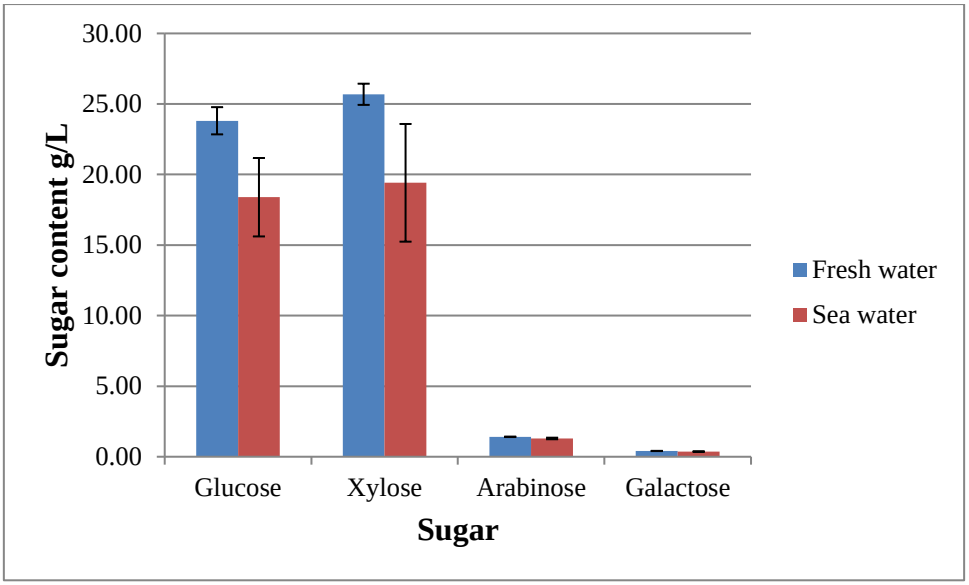


Figure 7.3.1: Sugar composition of the enzyme hydrolysate for revelation wheat straw. Revelation wheat straw was knife milled to a 0.5mm particle size and subjected to a standard 1% H_2SO_4 pre-treatment @120^{0}c made with either fresh water or sea water, the pH was adjusted to 5.5 and subjected to a enzyme hydrolysis using Cellic CTec2 supplied by Novozymes at 400fpu with a solid loading w/v of 10% for 24h at 50^{0}C. The sugar composition was analysed by HPAEC. Results shown are mean ± SD (n= 3)

Analysis of the data using ANOVA for the available sugars following 24 hour enzyme hydrolysis showed a significant difference (p<0.005). A post hoc analysis inferred a difference between fresh water and sea water for glucose (p=0.03) with fresh water being higher, a difference between fresh water and sea water for arabinose (p= 0.03) and a difference between fresh water and sea water for galactose (p= 0.02). ANOVA showed no significant difference between fresh water and sea water for xylose (p= 0.0623).

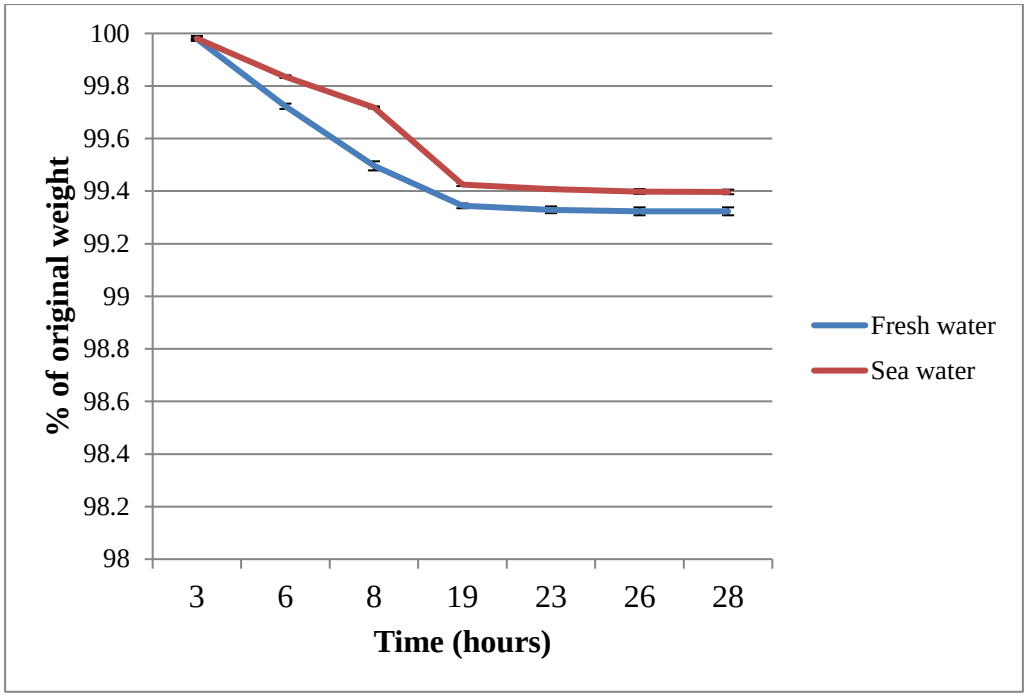


Figure 7.3.2: Weight loss during fermentation of wheat straw hydrolysate. Revelation wheat straw as bailed was knife milled to a 0.5mm particle size and subjected to a 1% H_2SO_4 pre-treatment made either with fresh water or sea water, the pH was adjusted to 5.5 and subjected to a enzyme hydrolysis Cellic CTec2 supplied by Novozymes at 400fpu for 24h at 50^{O}C. These were then inoculated with *Saccharomyces cerevisiae* NCYC2592 and incubated at 35^{O}C and weighed to determine weight loss. Results shown are mean ± SD (n= 3).

Analysis of the data using ANOVA showed a significant difference between the % of the original weight remaining for fresh water and sea water ($p < 0.01$) for all time points.

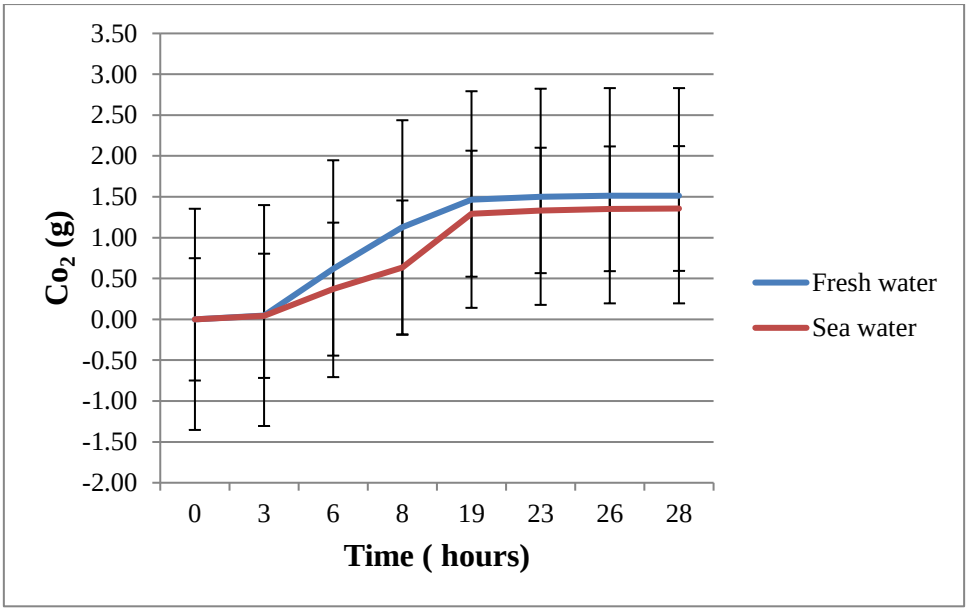


Figure 7.3.3: CO_2 production during fermentation of wheat straw hydrolysate. Revelation wheat straw as bailed was knife milled to a 0.5mm particle size and subjected to a 1% H_2SO_4 pre-treatment made either with fresh water or sea water, the pH was adjusted to 5.5 and subjected to a enzyme hydrolysis Cellic CTec2 supplied by Novozymes at 400fpu for 24h at $50^{O}C$. These were then inoculated with *Saccharomyces cerevisiae* NCYC2592 and incubated at $35^{O}C$ and weighed weight loss was used to determine CO_2. Results shown are mean ± SD (n= 3).

Analysis of the data using ANOVA showed no difference between fresh water and sea water for CO_2 production (p >0.05).

The fermentation was deemed to have stopped after CO_2 production stabilised (24 hours), however the fermentation was tracked for a further 96 hours to determine if further enzyme activity occurred and for the determination of ethanol production a sample was taken using a syringe at 24 hour time points.

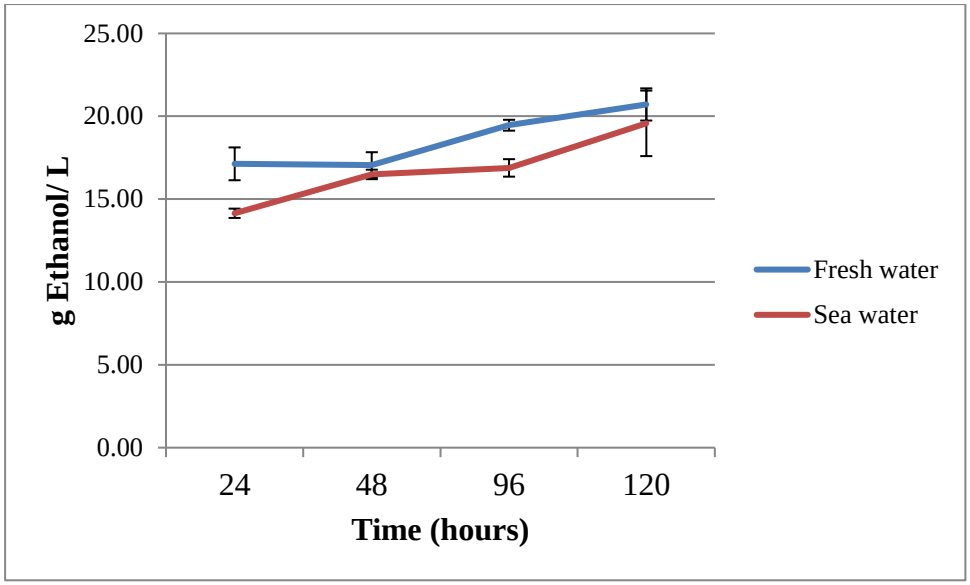


Figure 7.3.4: Ethanol production during fermentation of wheat straw hydrolysate. Revelation wheat straw as bailed was knife milled to a 0.5mm particle size and subjected to a 1% H_2SO_4 pre-treatment made either with fresh water or sea water, the pH was adjusted to 5.5 and subjected to a enzyme hydrolysis Cellic CTec2 supplied by Novozymes at 400fpu for 24h at 50^{O}C. These were then inoculated with *Saccharomyces cerevisiae* NCYC2592 and incubated at 35^{O}C samples were analysed via Jasco for ethanol. Results shown are mean ± SD (n= 3).

Analysis of the data using ANOVA showed a significant difference between fresh water and sea water at 24 hours and 96 hours (p <0.05) with fresh water being significantly higher but at time points 48 hours and 120 hours no difference was seen (p > 0.05).

7.3: Conclusions.

With regard to the pre-treatment of lignocellulosic biomass, it appears that sea water can be substituted for fresh water when a 1 % H_2SO_4 pre-treatment is used with little to no impact on hemicellulose breakdown, as shown with the

pre-treated liquor (figure 7.1.3). The glucose and xylose content following a Saemen's hydrolysis does appear to be affected, this is possibly due to less substrate being used for sea water when compared to fresh water as there is a weight gain from the sea water (20 ml of sea water when evaporated resulted in a residue that weighed 803mg) with some of this being left behind on the pre-treated residue (increasing the substrate weight by around 30%). When this is taken into account, both the fresh water and sea water pre-treatments contain identical levels of glucose and xylose.

The successful enzymatic hydrolysis showed that the pre-treatment stage, using 1% H_2SO_4 made using sea water and residual salts left behind had no impact on the enzyme activity. As a result the first stage of the bio-fuel process could be performed with the use of sea water when the enzyme cocktail is made using a sodium citrate buffer.

There was an apparent decrease in the glucose available for the enzyme hydrolysis performed in raw sea water. Whilst the enzyme hydrolysis performed in sea water gave a lower percentage glucose release, it should be noted that the sea water was un-filtered and un-sterilised and as the error bars are much broader, and increasing over the last two time points, this suggests that it could be due to microbial contamination which may be utilising the sugar. There are vast numbers of micro and macro-organisms in the sea, these include all bacteria, cyanobacteria (bacteria that contain chlorophyll) most protozoa and some fungi and algal species. Cyanobacteria are considered to be one of the main global primary producers supporting the food chain. Whilst

they are photosynthetic, several species such as *Prochlorococcus sp* and *Synechococcus sp* have been shown to utilise external glucose when it is available (Gomez-Baena *et al*, 2008). It may also be due to higher salinity levels, due to both the carryover of salts from the pre-treatment stage and the addition of salts in the sea water that could be impacting on enzyme efficiency.

This suggests that whilst it may not be feasible to perform the enzymatic hydrolysis stage in raw sea water at present, as there is a 10-12% reduction in glucose, it is possible. This shows a line of research that could be pursued to identify more saline tolerant enzymes or the sterilisation of sea water prior to this stage.

Whilst *Saccharomyces cerevisiae* is a model organism for the investigation of bio-fuels and is at present the most utilised in the bio-fuel industry, it is known not to have high osmotolerance (Li *et al*, 2014; Radecka *et al*, 2015). Organisms that grow in at high osmotic pressure accumulate substances with a low molecular mass to equilibrate the cytoplasm. Yeast that are osmotically stressed accumulate osmoregulatory solutes such as glycerol and arabitol (Hosono, 1991). This said, the salinity did not seem to have an impact on fermentation, or on yeast metabolic activity as both the Biolog plates with and without the addition of 6 % glucose showed no differences between sea water and fresh water samples, suggesting the possibility of being able to substitute sea water for fresh water for the fermentation stage of bio-fuel production.

The one pot treatment showed potential for batch fermentation without the need for a wash phase or to separate the pre-treated liquor and residue. Unfortunately, due to the high solid loading it was not possible to stir the samples in a way that allowed for the lower quantity of enzymes to mix thoroughly. Therefore, a high enzyme concentration was needed to allow the substrate to mix and for the enzyme to work effectively. As the one pot treatment method has each step performed in the same vessel (pre-treatment @ 121^{o}C for 15 minutes, followed by the enzymatic hydrolysis and fermentation), it suggests that the reduction seen in glucose during the enzymatic hydrolysis (section 7.1) is most likely down to the increase of salinity, as the same reduction is seen during the one pot treatment and this has been sterilised during the pre-treatment stage.

The ability to substitute sea water for fresh water, for all or part of the bio-fuel process has the potential to reduce the water footprint for second generation bio-fuel without significantly impacting on productivity.

Chapter 8: Discussion.

As previously stated, for all intents and purposes the planet we live on is a closed environment; we have limited recourses that will run out in the not so distant future and an ever increasing population to feed. As a species, we do not have the time to wait for them to replenish, for example millions of years for coal, oil and gas. On top of this problem is the need to reduce the CO_2 levels in the atmosphere that have increased to over 410 PPM at the start of 2017 (March 2017) (www.co2.earth, accessed 11/03/17). The utilisation of bio-fuels could mitigate some of these issues and help to provide a planet that is more sustainable for future generations.

With this in mind, the aim of this work was to determine if it could be possible to create a new food source that could also be used as a feed-stock for the generation of bio-fuels as a way to feed a growing population and also mitigate greenhouse gasses and climate change.

One of the main issues surrounding lignocellulosic biomass as stated (chapter 1) is the need for a pre-treatment stage in order to allow the enzymes to gain access to the cellulose structure. Commercially the pre-treatment used would be one that liberates the maximum sugars during enzyme hydrolysis whilst maintaining economic viability, without degrading the biomass significantly or producing high levels of inhibitory compounds that will impact on other processes further down the line like fermentation.

The overall aim of this study was to screen a selection of BC1 samples with unknown levels of cellulose, hemicelluloses and lignin. It was decided that a pre-treatment that would only liberate approximately 20-30% of the available glucose after enzyme hydrolysis from a commercial wheat variety was needed to allow for determining differences in levels of recalcitrance within the BC1 population.

As shown in chapter 5, many of the BC1 samples produced very little biomass to work with. With this in mind, a range of acid and base chemicals were trialled on a commercially available wheat straw variety (Orbit straw) for the purpose of developing a method that was suitable for analysing the BC1 population. It was seen that both the alkaline pre-treatments and the acid pre-treatments had the same outcome with regards to the levels of available glucose in the pre-treated residue but both the 1% KOH and1 % NaOH had more than twice the xylose levels compared to the acid treatments (chapter 3). It suggests that the acid treatments are removing significantly more of the hemicellulose fraction than the alkaline treatments. This is to be expected as it is known that acid hydrolysis results in the breakdown of the hemicellulose into smaller oligomemric fractions whilst alkali tends to solubilise intact hemicellulose polymers. The differences seen between the acid treatments were not as large with regards to levels of xylose in the pre-treated residue, although that said, the 1 % H_2SO_4 had twice the xylose as the 5 % H_2SO_4. This can also be seen in the analysis of the pre-treated liquor; all of the acid treatments showed good levels of arabinose, galactose, glucose and xylose when compared to the alkaline treatments. The latter showed no detectable sugars in the liquor which is in line with work carried out by Wilkinson *et al*

(2016) who found similar results with various lignocellulosic samples including wheat straw. It should be noted though that the analysis was carried out using a CarboPacTM PA 20 column (3 x 150 mm), and that this is only capable of detecting monomeric sugars and if the alkaline treatments degraded the hemicellulose fraction into oligomers they would not be detectable. That said, the major reactions that take place during alkaline pre-treatments include the dissolution of lignin and hemicellulose and the de-esterification of the intermolecular ester bonds (Kim *et al*, 2015) that generally leave the hemicelluloses fraction intact. To determine if any of the hemicellulose fraction is being degraded into oligomers, samples would need to be analysed using equipment for the detection of oligomers (CarboPacTM PA 200 column). The enzymatic hydrolysis showed that alkaline based treatments are significantly better at reducing the recalcitrance of the wheat straw as they increased the glucose released by almost double when compared to all the acid based treatments. The xylose released was almost five fold, as the enzyme used also had a xylanase action as well, and with the results from the Saemen's hydrolysis showing a high level of xylose in the pre-treated residue it suggests that there is less of the hemicelluloses fraction being removed when a alkaline treatment is used.

As all of the pre-treated samples had comparable weights after the pre-treatment it suggests that the alkaline pre-treatment is removing and possibly degrading some of the lignin from the samples. If this is the case, whilst it may appear that the alkaline treatments are better, there will be a significant increase in inhibitory products such as aromatic compounds like phenol, and acetyl derivatives (Toquero and Bolado, 2014). This will affect microbial

growth in later steps, thus possibly reducing ethanol production or increasing fermentation time and reducing the sustainability of the process.

As previously stated, the aim was to develop a pre-treatment that would allow for the release of approximately 20 – 30 % of glucose during the enzyme hydrolysis stage. Whilst the alkaline treatments were significantly more efficient, liberating between 50 and 60 % of glucose, the majority of commercially based processes for pre-treating straw seem at present to be based on either hydrothermal or acid pre-treatments (McIntosh *et al, 2016*; Larsen *et al*, 2008). Thus, it was decided in the first instance to use an acid based screening process, and since the differences seen between the acids were limited a 1% H_2SO_4 treatment was chosen. That said, as pre-treatment and fermentation technologies advance the use of alkaline treatments may increase. In the craft paper industry sodium hydroxide and sodium sulphide are used as the pulping reagent; the solution remaining following the pulping process has been shown to be highly selective at removing lignin whilst leaving the carbohydrate fraction intact in lignocellulosic material and has thus drawn considerable attention for possible use as a pre-treatment for lignocellulosic bio-fuel production (Wu *et al*, 2011). The use of a waste stream product for another industry could mitigate the issues previously stated regarding the inhibitor effects created by using an alkaline based pre-treatment. Whilst the fractionation of wheat straw would never be done in commercial practice as this would add additional costs both in terms of time and money, it was important to determine if there was a difference between plant fractions as this could possible skew the analysis of the BC1 samples due to the vast

morphological differences seen or suggest breeding plants with more or less of a certain trait.

In terms of the glucose content in the untreated samples, the only differences seen between the separate plant fractions leaf, peduncle, upper (stem above 10cm), and lower (stem below 10cm) fractions were between the leaf and all other fractions (chapter 4). The xylose content in the untreated samples showed no differences between the fractions suggesting that the hemicellulose fraction is consistent. As previously stated (chapter 1) wheat straw is made of three layers, the primary cell wall, the middle lamella, and the secondary cell wall (Popper, 2008) and whilst they have differing compositions, it appears that they do not impact upon recalcitrance. This could be due to them having undergone a physical pre-treatment (knife milling) prior to chemical treatment, or could be that all fractions of the stem have the same relative % of each of the three layers and only the leaf material is differing. This is supported by results from Zhang *et al* (2013) who analysed leaf and stem material from nine wheat varieties and found glucose to be lower in the leaf fraction compared to the stem, and xylose to be the same in both leaf and stem. It is also comparable with Collins *et al* (2014) who analysed different wheat straw (node, internode, leaf and ear) and found that the glucose was only lower in the leaf tissue, and xylose was approximately half that of glucose and was the same between the fractions.

Following a 1 % H_2SO_4 pre-treatment the glucose levels in the residue became more comparable with each other, but still showed a significant difference between leaves and the peduncle, upper (stem above 10cm) and samples of "whole" un-fractionated straw. The xylose content showed no differences between the fractions after a 1 % H_2SO_4 pre-treatment, but had reduced by approximately 1/3 of what was in the untreated samples; this is comparable with Carvalho *et al* (2015) who showed that the glucose content of wheat straw following an acid pre-treatment is around 50%, the xylose content for their residue was significantly lower around 2.8%. This is likely due to the more severe pre-treatment method used (4.5% H_2SO_4) suggesting that they have removed far more of the hemicellulose fraction, this is also comparable with the results seen for the 5 % H_2SO_4 pre-treatment (chapter 3).

Enzymatic hydrolysis shows that leaf tissue digests far easier than any of the stem fractions; this is also supported by results from Zhang *et al* (2013) who showed that the sugar composition of straw is only variable between the leaf and stem and that the pure leaf fraction digests significantly better. It should however, be noted though that Zhang *et al* (2013) only used the stem fraction as a whole and not as individual parts of the stem, and whilst leaf material digested quicker and released a higher % of available glucose the leaf fraction contained less glucose to start with. Collins *et al* (2014) showed the main components of leaf material to be hemicellulose, pectin and cellulose with reduced levels of lignin, this would account for the improved digestibility of leaf material and why there was less glucose present to begin with.

As the leaf: stem ratio for wheat straw is 20-40% and 60-80% respectively (Zhang *et al* 2013), and will have natural variation between variety and harvesting methods, the vast majority of glucose will be derived from the stem tissue. As many of the BC 1 samples had vastly different leaf stem ratios it was felt this could give inaccurate data on digestibility. Taking this into consideration, it was decided that analysis of the BC1 samples should be performed on the un-fractionated stem tissue only due to the fact that they are non-reproducible, and with only a limited amount of material it would not be possible to analyse as individual fractions and as a whole plant.

The use of PGR and nitrogen applications is widespread in agriculture; whilst this is needed for increased yield for food production, surprisingly there seems to be no noticeable effect with regards to sugar content of the straw or with regards to levels of recalcitrance. This could be because out of the five recognized groups of plant hormones: auxins, gibberellins, cytokinins, abscisic acid (ABA) and ethylene, only gibberellins act on stem elongation and auxins act on cell elongation, with all others acting upon ether cell division in the root system and regulating seed germination (Trigiano and Gray, 2005).

The most noticeable effect on sugar composition appears to be environmental as this was seen between the blocks that the plants were grown in. In contrast, Murozuka *et al* (2014) used winter wheat (*T. Aestivum*) grown in field blocks and analysed as a whole plan. They found that nitrogen can impact on enzymatic saccharification, but they concluded that this was by altering the levels of silicon and potassium, as they did point out that the levels of cellulose and hemicellulose in the untreated straw were not affected. This group used a different pre-treatment method (180^{o}C for 17.5 minutes without a chemical

catalyst) followed by an enzyme hydrolysis using CTec2. This may have a different efficiency to the process used in this thesis and thus, could also have contributed to the different results obtained.

As stated above, there did seem to be significant environmental impacts, which could be due to a number of factors including soil condition, drainage for water, or prevailing wind direction. Unfortunately with so many possible factors that could contribute to this "block effect" it was impossible to ascertain a reason for this. Murozuka *et al* (2015) also found location and environmental conditions effected the enzymatic saccharification when 20 winter wheat varieties were grown in two separate locations with a significant difference seen between both location and varieties.

The analysis of three different commercial cultivars (Cordiale, Grafton, and Xi19) showed again that the leaf and stem tissues were vastly different with regard to the glucose released. Xi 19 showed higher levels of sugars in the pre-treated stem residue when compared to Cordiale and Grafton, again as the latter two are closely related this suggests a possible genetic link to levels of recalcitrance. This is also in line with Collins *et al* (2014) who used FTIR and PLS modelling to compare 90 wheat cultivars and found that there was a significant natural variation with regards to chemistry and composition. Importantly, they found one of the main impactors to be the leaf/ stem ratio, and showed that the glucose, xylose and lignin positively correlated with stem tissue but negatively correlated with leaf tissue.

Tishler *et al* (2015) analysed and screened the upper three nodes and leaves of 48 wheat lines from six different genetic groups (Tetraploid wild emmer wheat (*Triticumturgidumvar diccocoides*, TTD), Tetraploid domesticated emmer wheat (*Triticumturgidum var diccocum*,TTC), Tetraploid Durum wheat (*Triticumturgidum var durum*, TTR) both landrace (traditional) and modern cultivars and Hexaploid bread wheat (*Triticum aestivum var. aestivum*, TAA) both landrace and modern cultivars), and concluded that after pre-treatment and enzyme hydrolysis there was a significant degree of variation between the samples; interestingly, the samples that performed best were from the wild type plants not the domesticated samples. This suggests that it might be possible to breed new varieties specifically for bio-fuel production. Whilst the wheat lines selected for this work are not the same varieties as used for the generation of BC1 samples, it does show that there is a huge, potentially untapped resource of genetic diversity that can be used to improve wheat varieties not only for better agronomic traits but also for improved bio-fuel production (chapter 5).

The aim with regard to the BC1 sample population was to introduce new genetic material into wheat, and this was very successful, if not too successful. The vast differences seen with the morphology of the BC1 samples show that they have a wide range of heights, total biomass and leaf/ stem ratios, these morphological traits however, showed little or no correlation with digestibility,

Current farming practice is to sow 325 to 400 seeds /m2 to achieve 275 plants per square metre, this said, the seed density will vary depending upon what variety is being cultivated, the location, and planed fertilising schedule (Theobald *et al*, 2006). Based on this, the differences seen with the BC1

population in regard to biomass produced per square metre varies from a low of 2.75kg to a high of 18.04kg, representing a 6.5-fold increase. Whilst this is speculative, as it is based on existing data for commercial varieties, it does suggest that it is possible to increase the quantities of available feed stocks for second generation bio-fuel production.

Bellucci *et al* (2015) analysed 100 hexaploid winter wheat line accessions (*T. aestivum L*) and found that whilst plant height negatively correlated with grain yield, there was a weak positive correlation for height and glucose released after pre-treatment and enzyme hydrolysis (r^2= 0.24), which is in line with the correlation found between the BC1 plant height and Glucose (r^2= 0.217). The stem fraction of the BC1 samples showed a slight variation in the levels of glucose, xylose and arabinose in the untreated samples, but levels of glucose and xylose were comparable in the residue remaining after a 1 % H_2SO_4 pre-treatment. The pre-treatment removed a substantial part of the hemicellulose fraction (40 – 50%) of the total weight. The % available glucose released following an enzyme hydrolysis varied between 15% and 33% suggesting that the BC1 samples have differing levels of recalcitrance. This was also seen with the York high throughput method, with both methods showing a correlation (r^2=0.681) and identifying 5 of the same samples in the top 10 and 4 samples in the bottom 10. As the two methods differ for the way sugar is analysed, it suggests that both methods are capable of identifying samples that are more or less recalcitrant and confirms the variation seen in the BC1 population. It may have been possible to increase the correlation if Celtec 2 (Novozymes) had

been used in the Nottingham study instead of Cellulase (SIGMA) as the latter is less effective.

Analysis of the liquor following the pre-treatment suggests that the hemicellulose fractions of the BC1s may differ in composition (chapter 6). The evidence for this lies in the fact that the various sugars in the liquor fraction show only moderate correlations suggesting that whilst the chemical composition is similar there may be some variation in composition. There is also some evidence to suggest that the hemicellulose matrix structure is possibly differing between the BC1 samples. This comes from the results from the DSC where the "melting curves" for the hemicellulosic fraction from the best and worst performing samples were different. Work carried out by Werner *et al*, 2014 suggest that the hemicellulose fraction in BC1 14 may contain higher levels of β-glucans,whilst BC1 6 may contain higher levels of glucomannan, arabinoxylan (AX) or glucuronoarabinoxylans (GAX). This is supported by Yang *et al*, 2006, who showed that the presence of glucuronic acid units in arabinoxylan (AX) and glucuronoarabinoxylans (GAX) have an increased impact upon heat flow as these carboxylic acids groups (-COOH) are prone to undergo decarboxylation and result in the production of CO_2 and exothermal enthalpy changes compared to β-glucans.

For commercial wheat varieties (Cordiale and Grafton), a 1% H_2SO_4 pre-treatment was sufficient at removing 50-60% of the xylose when compared to the BC1 samples; this is reduced to between 11 and 33 % suggesting that the

hemicellulose fraction is less degradable under the standard pre-treatment used in this thesis. This again suggests that the hemicellulose fraction may be structurally different in the BC1s than in commercial varieties. The chromatographs from the HPLC of the liquor fractions following the acid pre-treatment show some very interesting differences between samples of BC1s. Whilst most show the classic profile of the four major sugars- xylose, galactose, arabinose and glucose, there were some BC1 plants whose liquor showed more peaks being detected. These could be different sugars or other compounds like sugary acids. Collins *et al* (2014) analysed the variation in cell wall composition of six wheat cultivars and found it to consist primarily of glucose, xylose, arabinose, and uronic acid with trace amounts of galactose, mannose, fucose, and rhamnose,. It is not possible that any of these sugars could account for the peak seen after xylose as they all elute before it. That said the Carbopac PA20 column is designed for the separation of glycoprotein monosaccharides and the manufacturer gives a run order that suggest that the peak seen in BC1 6 at around 5 minutes may possibility be glucosamine, and at around 8 minutes could be fructose. If this were indeed the case, then the increase in glucose and appearance of fructose in the BC1 6 sample might imply the presence of sucrose with the pre-treatment breaking down the sucrose into glucose and fructose. However, sucrose is not normally found associated with senescent tissues, as used in this study, and this would require further investigation and is more likely that it is due to the presence of β-glucans in the hemicellulose. It is interesting to note that the appearance of these extra peaks may be associated with increased recalcitrance of the stem. However, how these may be causal is not clear, but could be linked to the

lignin fraction being more tightly bound to the cellulose. This could be tested by using an alkaline treatment as this has been shown (chapter 3) to remove lignin whilst leaving most of the carbohydrate fractions relatively intact.

The BC1 samples were genotyped by Bristol University during the course of the PhD. This demonstrated that the introgressions, as expected from the morphological variation, had been very successful. Preliminary visual analysis of the introgession patterns suggests possible regions of interest on chromosomes 3 and 6 for *Aegilops speltoides* and on chromosome 1 for *Triticum urartu.* This was to be expected as the original F1 hybrid contained 50% elite line genes and 50% wild type genes, with the subsequent BC1 population containing 75% elite line genes and 25% wild type genes, with each successive back cross generation having 50% less wild type genes thus, analysis of BC4 and BC5 samples would be required. After BC5, the average percentage of elite alleles will be almost 99%, the other 1% are alleles from the wild type line and would hopefully show better regions to identify genetic markers that may impact on digestibility.

The ability to substitute sea water for fresh water is worthy of further research. As fresh water becomes a more costly and scarce resource, the use of it for bio-fuel production could become as controversial as the food vs. fuel debate (chapter 7). This work has shown the ability to use sea water for at least part of the process with no detrimental effects on yield during the pre-treatment stage,

and with limited impact during enzymatic hydrolysis (a reduction of around 10 – 12 %).

This could possibly be mitigated with the development of more saline tolerant enzymes or by increasing enzyme loading, whilst the latter would increase operational costs, it could be cost effective due to the lower costs of sea water and less of a requirement for decontaminating when compared to fresh water.

If a batch process was used, the first two stages could easily be performed with sea water without any significant impact. During the fermentation stage, there was a reduction in metabolic output. This could be due to the salinity of the sea water but also could be due to the pH; it was not possible to adjust the pH of the sea water because when NaOH was added there was a precipitate drop out, this was assumed to be calcium and magnesium.

With the one pot pre-treatment, enzyme digestion followed by fermentation showed promising results with the most basic of equipment. The analysis of metabolic output using the Biolog suggests that the salinity has no impact; whilst this may be accurate, it does not take into account other inhibitory compounds that may impede metabolic output. As discussed in chapter 7, whilst *Saccharomyces cerevisiae* is a model organism for the investigation of bio-fuels, it is known not to have high osmotolerance (Li *et al*, 2014). Radecka *et al*, 2015 suggests that for bio-fuel production other yeasts, that are more osmotolerant such as *Zygosaccharomyces rouxii* could be used as a way to deal with the high sugar concentrations; this could also be applicable to fermentations carried out using sea water as the medium.

One of the main issues surrounding this was the inability to increase the solid loading ratio above 5% as this caused the samples to become too viscous, and without a suitable way to move the substrate around, the enzymes were unable to break down the majority of the straw. Whilst this was done using a very high enzyme loading, if the substrate was stirred it could be possible to reduce the enzyme loading to a more acceptable level without reducing the efficiency and fermentable sugar recovery.

If this was possible it could lead to a full SSCF (simultaneous saccharification and co-fermentation) or SSF (simultaneous saccharification and fermentation) system that could utilise sea water instead of fresh water.

8.1: Future work.

It appears that the University of Nottingham is potentially several years ahead of other institutes as the potential breading program has already started and is currently at BC4 and BC5. Analysis of these later samples could give a great insight into how well a full-scale breeding program for second generation bio-fuel could be. For bio-ethanol production BC4 and BC5 samples that are the progeny of the high performing BC1 samples should be analysed using a more state-of-the-art enzyme like Cellic CTec2 and HTec2 as this would be more in line with commercial practice. That said, the screening of later BC varieties should not only look at the progeny of the better BC1 samples, but look at

them all again. This would allow for the determination of hemicellulose and, if for example, as seen in BC1 6 the hemicellulose fraction is predominantly β-glucans, an alkaline treatment could be better as this would leave far more fermentable sugars in the form of cellulose and hemicellulose and possibly increase the total quantity of fermentable sugars above the best acid treated BC samples. Whilst this screen could be carried out using the Nottingham method developed in this thesis, it could also be incorporated with the chemical pre-treatment and digestibility assay (York High throughput (HT)) as a fast way to determine recalcitrance. As many of the poorer performing BC1 samples had many more peaks in the liquor from the 1% H_2SO_4 pre-treatment, the progeny of these should be evaluated for the possibility of producing other high value chemicals from the hemicelluloses fraction. a-As the BC1 samples appear to have differing composition and structure this could be performed Via HPLC to identify what compounds are present.

There has been little to no research on the substitution of sea water for fresh water for second generation bio-fuel production; sea water showed good results for the pre-treatment and the enzyme hydrolysis stages and could be improved with the use of an enzyme cocktail that is more tolerant to saline conditions. With regard to the breeding program it has been shown that the introduction of "wild type" alleles can be achieved making it possible to introduce other traits such as high salt tolerance from Pampas Grass (*Cortaderia selloeana*) that can survive in a high saline environment. This could provide a renewable bio-resource that could be utilised by large parts of the world that have access to costal water but little access to fresh water, and

could help provide a sustainable source of food and fuel for millions of people who live in arid parts of the world.

As the fermentation stage showed a slight reduction with sea water it should be followed by further work to determine if the loss of sugar seen was due to possible contamination or salinity affecting the yeasts. Whilst it looks as though it is the later, as the one pot system had been autoclaved and still showed the same reductions in glucose, possibly future work should look at this with a view to identifying more saline tolerant organisms for fermentation. These could be species that are known of but not traditionally used in the bio-fuel industry such as *Zygosaccharomyces rouxii* or possible new species could be found that originated in a saline environment.

With the one pot system, it was apparent that the main issue was the inability to mix the enzyme and substrate and should be followed up with equipment that is more suitable for the purpose. This would allow for the reduction in enzyme loading therefore making the process more cost effective and sustainable, This would require a specialist piece of equipment and should have careful consideration before any purchase is made however, it is possible that equipment used in the food manufacturing industry would be suitable and should be investigated.

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