OVERCOMING CHALLENGES FOR THE BIOREFINERIES OF THE FUTURE

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ABSTRACT

Non-food lignocellulosic biomass is the most abundantly available raw material. Utilising it for biorefinery purposes not only circumnavigates the food vs fuel debate, but is also important for a complete move to a fossil fuel free society. Some of the major challenges in using lignocellulosic feedstock includes its recalcitrance, potential shortage for year-round mono feedstock supply and economic viability with current technology.

The first part of this thesis explores the potential of overcoming recalcitrance of wheat straw by exploiting new wheat back cross (BC_1) plants, which were developed at Nottingham, using introgression of genes from wild relatives, with the aim to improve wheat genetic variation. The straw from a subset of 128 BC_1 were screened for sugar content and saccharification efficiency. Plants showed wide compositional and structural differences, reflecting a high degree of genetic diversity. The digestibility of the stem tissue was assessed following acid hydrothermal pre-treatment and a significant variation was detected. This demonstrated that this approach to wheat breeding was successful in introducing a wide range of phenotypic, compositional and structural changes and digestibility into the wheat straw.

The second part of the thesis aimed to address the potential bottle neck in mono-feedstock availability in a biorefinery, as most lignocellulosic feedstocks are likely to be seasonal. One way to overcome this may be by utilising a mixed feedstock to maintain a year-round supply. This study reports the impact of mixing three of the UK's most important feedstocks- wheat straw, willow and *Miscanthus* on two major performance indicators - sugar yield and fermentation inhibitor production. A hot

water pre-treatment regime of 200°C for 5 minutes was applied to each feedstock individually and to 1:1 (w/w) mixes and the predicted sugar yield in the mixes was compared to the observed values. All the mixes resulted in improved sugar yields with willow + *Miscanthus* and wheat + willow showing a statistically significant improvement over predicted values. Inhibitor production during the pre-treatment and its impact on yeast metabolism and growth were also compared and no adverse impacts of mixing observed. The use of mixed feedstocks in a hot water based commercial production of biofuels is unlikely to have any adverse effects on productivity and may indeed prove beneficial.

The final part of the thesis, explores the potential of combining extraction of a high value product (anthocyanin) from black rice - *Chakhao poireiton* straw, followed by use of the residual straw for saccharification for an economical biorefinery. The anthocyanin content of black rice straw, as determined by standard methanol extraction, was 62.8±4.2 mg/100g. Aqueous microwave treatment at 90°C for 5 minutes extracted 85.8% of this anthocyanin and importantly the extract then displayed higher antioxidant capacity compared to methanol extracts. Extracts showed negligible cytotoxicity, or induction of apoptosis, on Jurkat cell lines even at high concentrations (200-800µg/ml). The straw residue after anthocyanin extraction showed a glucose digestibility of 49.67±1.4% after a subsequent pre-treatment at 200 °C for 5 minutes. This study demonstrates the potential to develop a combined biorefinery process for anthocyanin and second-generation sugars using black rice straw as the feedstock.

PUBLICATIONS

Moirangthem K, Waldron P, Tucker G, King I, King J. Dual purpose wheat for a future that demands more food and energy. *Aspects of Applied Biology* 131. (2015), 163-167.

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IMPACT STATEMENT

Wheat wild relative introgression

Availability of suitable non-food feedstock would ultimately boost the bio-economy and pave the way for the economical production of bio alternatives of petrochemicals such as biofuels and bioplastics; creation of new direct and indirect job markets; and even secondary income for the farmer by selling the straw. The research could also improve wheat straw's animal feed value since straw digestibility is not only an important characteristic for second generation biofuel production but also for animal feed.

Although this research is focussed on biofuels, the main research project will contribute towards tackling the impending challenges of global food security by improving wheat's agronomic traits. Due to synteny of cereal genomes, plant breeders can use genetic markers, identified in this project, and translate these to other cereals such as rice and barley. More importantly, the germplasm developed here is free of IP and available to anyone who wishes to utilise the knowledge to advance wheat breeding.

Once such wheat varieties are developed, they must undergo all the statutory testing processes a new crop variety must go through before it can be placed on the market or register of approved varieties. Official trials must be conducted, in most cases for a minimum of two years, to test each candidate

variety for a range of characteristics which together determine its distinctness from other varieties, as well as its value to growers and end-users.

Economic analysis including gross margins, the value of the output less the variable costs, which provide an aid to farmers when making decisions about which crops to grow must be developed. Hence agronomic practicalities will also need to be considered: harvesting efficiencies; nutrient removal and fertilizer replacement values; and competing economic uses for harvested cereal straw.

The potential applications of alien introgression approach are immense. In future, the technology could be used for various purposes such as improving carbon sequestration, producing plantibodies, bio-fortification etc. In our current political environment, investment in such technology is of paramount important for our very survival.

Mixed Feedstock

Utilising a range of feedstocks for biorefinery will help circumnavigate the seasonal availability of many individual feedstocks and allow a year-round operation of future biorefineries. Since, the three-feedstocks used here are potentially local UK crops, it will also decrease our reliance on imported feedstocks and encourage the local economy. Utilising a diverse range of feedstock will also reduce pressure of potential mono culture of energy crops.

The improved saccharification after pre-treatment of mixed feedstock is also accompanied by reasonable inhibitor composition and impact on yeast growth. All this would serve to increasing the efficiency of the biorefinery and ultimately reduce its operational costs.

The mixed feedstock approach can also be extended from use in the biochemical route for liquid fuel production to use in anaerobic digesters for biogas production or for thermochemical approaches in furnaces.

Such an approach will allow for utilisation of waste streams which are not suitable in a biorefinery on its own such as municipal solid waste (MSW). Finding an eco-friendly way of utilising MSW instead of disposing it in landfill will derive new value from an unwanted resource.

Black rice straw

Anthocyanins are high value products with nutraceutical and medical applications. Hence, utilising anthocyanins from black rice straw (waste stream of agriculture) would provide a low-cost source of health-beneficial bioactive compounds.

Proving an additional source of health beneficial compounds that could contribute to fighting modern day medical challenges such as cancer would contribute to the general well-being of our society. Additionally, anthocyanin can also be used to fortify food products making them more nutritional. Both

society and industry would benefit from exploring the use of plant residues, in this instance -rice straw, as an alternate and cheaper source for beneficial high-value chemicals.

The residual straw can be used to get renewable sugars which can be fermented to get bio alternatives of petrochemical refinery and the return on the sale of the anthocyanin could offset the cost of bio product production. This would also result in reducing the burning of rice straw in Asian countries. This will be an excellent example of utilising skills gained in the UK to diversity the economy back in the homes of international researchers, in this case India. Depending on the techno economic feasibility of the project it could led to SMEs utilising Nottingham's research output in collaboration with the Indian Council of Scientific and Industrial Research (ICSIR).

Following the reduced usage of synthetic dyes due to adverse effects to human health, anthocyanin is greatly valued as an alternative natural food colourant. It is considered safe to use by the FDA (Food and Drug Administration) and does not require certification for usage. Thus, utilising the anthocyanin as food colourant would improve consumer health and image of the company.

Black rice cultivation is declining as farmers prefer to grow more high yielding non-coloured rice varieties. This project could indirectly incentivise research into developing high yielding varieties of black rice, revitalise black rice cultivation.

TABLE OF CONTENTS

ABSTRACT ..... **I**

PUBLICATIONS ..... **III**

ACKNOWLEDGEMENTS ..... **IV**

IMPACT STATEMENT ..... **V**

TABLE OF CONTENTS ..... **IX**

LIST OF FIGURES ..... **XIII**

LIST OF TABLES ..... **XV**

ABREVIATIONS ..... **XVI**

CHAPTER 1 THESIS OVERVIEW **1**

CHAPTER 2 GENERAL INTRODUCTION **3**

2.1. BIOFUELS 4

2.1.1. First Generation Biofuels 5

2.1.2. Second Generation Biofuels 6

2.2. LIGNOCELLULOSIC FEEDSTOCK – WHEAT STRAW 7

2.2.1. Cellulose 10

2.2.2. Hemicellulose 11

2.2.3. Lignin 13

2.3. CONVERSION OF PLANT BIOMASS TO BIOFUELS 14

2.3.1. Pre-treatment 15

2.3.1.1. Physical pre-treatment 18

2.3.1.2. Dilute acid pre-treatment 19

2.3.1.3. Hydrothermal pre-treatment 19

2.3.2. Enzymatic Hydrolysis 20

2.3.2.1. Cellulases 21

2.3.2.2. Hemicellulases 22

2.3.3. Fermentation and inhibitors 23

2.4. THESIS AIMS AND OBJECTVES 26

2.4.1. Improving wheat straw by introgression 26

2.4.2. Utilising mixed feedstock 27

2.4.3. Black rice straw as a feedstock for anthocyanin and sugars 28

CHAPTER 3 IMPROVING WHEAT STRAW BY INTROGRESSION **30**

3.2. INTRODUCTION 30
3.2.1. Evolution and genetic variation 31
3.2.2. Improving genetic variation - Introgression 33
3.2.3. Straw improvement for biorefinery 35
3.3. MATERIALS AND METHODS 39
3.3.1. Plant material 39
3.3.2. Sugar analysis 41
3.3.3. High Performance Anion Exchange Chromatography (HPAEC) 41
3.3.4. Digestibility – manual saccharification assay 42
3.3.5. Digestibility- automated saccharification assay 43
3.3.6. Determination of lignin content by spectrophotometry 44
3.3.7. Cellulose crystallinity analysis 46
3.3.8. Fourier transformed infra-red spectroscopy (FTIR) 47
3.3.9. Statistical analysis 48
3.4. RESULTS 49
3.4.1. Phenotypic Analysis 49
3.4.2. Sugar Composition of BC_1 stem 50
3.4.3. Digestibility of the BC1 stems 50
3.4.3.1. Acid pre-treatment 50
3.4.3.2. Digestibility assays 52
3.4.4. Lignin analysis by UV quantification 55
3.4.5. Determination of cellulose crystallinity by X-ray diffraction 56
3.4.6. Compositional analysis by Fourier Transformed Infrared Spectroscopy (FTIR) 58
3.5. DISCUSSION 62
CHAPTER 4 IMPACT OF MIXED FEEDSTOCK ON BIOREFINERY TRAITS 72
4.2. INTRODUCTION 72
4.2.1. Feedstocks 74
4.3. MATERIALS AND METHODS 77
4.3.1. Plant Material 77
4.3.2. Compositional analysis 77
4.3.2.1. Sugar analysis 77
4.3.2.2. High Performance Anion Exchange Chromatography (HPAEC) 77
4.3.3. Hydrothermal pre-treatment and enzymatic saccharification 77

4.3.3.1. Pre-treatment *77*
4.3.3.2. Enzymatic saccharification *79*
4.3.4. Determination of the effect inhibitory compounds on yeast metabolism 80
4.3.5. Determination of the effect of inhibitors on yeast growth 81
4.3.6. Analysis of inhibitor composition 81
4.3.7. Statistical analysis 82
4.4. RESULTS 83
4.4.1. Effect of mixed feedstocks on sugar composition of pre-treated residue 88
4.4.2. Enzyme saccharification 91
4.4.3. Analysis of saccharification kinetics 93
4.4.4. Effect of mixed feedstock on yeast metabolism and growth 94
4.4.5. Effect of mixed feedstock on pre-treatment energy requirement 96
4.5. DISCUSSION 97
CHAPTER 5 BLACK RICE STRAW FOR ANTHOCYANIN AND SUGARS 102
5.2. INTRODUCTION 102
5.2.1. Black Rice 103
5.2.2. Potential value of anthocyanins 105
5.2.3. Extraction of anthocyanin 106
5.3. MATERIALS AND METHODS 109
5.3.1. Feedstock 109
5.3.2. Extraction and pre-treatment 109
5.3.2.1. Anthocyanin extraction *109*
5.3.2.2. Microwave pre-treatment *110*
5.3.2.3. Anthocyanin measurement *110*
5.3.3. Microscopy 111
5.3.4. Analysis of Antioxidant capacity 111
5.3.5. Bioactivity assay: cytotoxicity and apoptosis assays 112
5.3.5.1. Preparation of extracts *112*
5.3.5.2. Preparation of cells *113*
5.3.5.3. Assay procedure *113*
5.3.6. Composition and saccharification 114
5.3.6.1. Sugar composition analysis *114*
5.3.6.2. High Performance Anion Exchange Chromatography (HPAEC) *114*

5.3.6.3. Digestibility assay *114*
5.3.6.4. Fourier Transformed Infrared Spectroscopy (FTIR) analysis *114*
5.3.7. Statistical analysis 114
5.4. RESULTS 116
5.4.1. Feedstock 116
5.4.2. Content and Distribution of Anthocyanin in black rice fractions 116
5.4.3. Hydrothermal Microwave Anthocyanin Extraction 117
5.4.4. Microscopy of the straw 118
5.4.5. Antioxidant Activity of Extracts 119
5.4.6. Cytotoxicity and Apoptosis 121
5.4.7. Analysis for sugar source 122
5.4.7.1. Sugar composition analysis *122*
5.4.7.2. Digestibility *125*
5.4.7.3. Fourier Transformed Infrared Spectroscopy *126*
5.5. DISCUSSION 129
CHAPTER 6 CONCLUSION AND FUTURE WORK 136
6.2. CONCLUSION 136
6.3. FUTURE WORK 139
6.3.1. Wheat wild relative Introgression 139
6.3.2. Mixed Feedstock work 141
6.3.3. Black rice straw 142
CHAPTER 7 REFERENCES 145
Appendix A Wheat introgression 159
Appendix B Mixed feedstock 166

LIST OF FIGURES

Figure 2.1 Overview of lignocellulose. 8

Figure 2.2 Hemicellulose structure 13

Figure 2.3 Different biochemical and thermochemical pathways and their products. 15

Figure 2.4 Structural profile of lignocellulose derived fermentation inhibitors. 25

Figure 3.1 Wheat evolution from prehistoric grasses to modern Durham and Bread wheat. 31

Figure 3.2 Wild relative introgression strategy. 35

Figure 3.3 Typical HPAEC standard curve for arabinose, galactose, glucose and xylose. 42

Figure 3.4 Typical lignin standard curve. 45

Figure 3.5 Total straw, and stem and leaf composition of 80 BC_1 plants. 49

Figure 3.6 Sugar composition of the stems from 80 BC_1 plants and 4 modern wheat lines. 50

Figure 3.7 Sugar composition of the pre-treated residue from 80 BC_1 stems and 4 modern lines. 51

Figure 3.8 Digestibility of BC_1 stems and 4 modern lines measured by HPAEC. 53

Figure 3.9 Digestibility of BC_1 stems as assessed by automated saccharification assay. 54

Figure 3.10 Lignin content of selected BC1 stems. 55

Figure 3.11 Cellulose crystallinity and fibril dimensions 57

Figure 3.12 PCA of FTIR spectrum of raw and pre-treated stem tissue from 80 BC_1 plants. 59

Figure 4.1 Effect of varying temperature/time during pre-treatments of wheat straw 84

Figure 4.2 Effect of varying temperature/time during pre-treatments of willow 85

Figure 4.3Effect of varying temperature/time during pre-treatments of *Miscanthus* 86

Figure 4.4 Effect of pre-treatment regime on final pH of the hydrolysate. 86

Figure 4.5 Effect of mixed feedstocks on sugar composition of pre-treatment residue 89

Figure 4.6 Sugar composition of the pre-treated liquor for individual and mixed feedstock. 90

Figure 4.7 Saccharification of pre-treated individual and mixed feedstocks 92

Figure.5.1 Different rice landraces from *Manipur* (India) and their grain morphology 104

Figure 5.2 Standard curve for the DPPH assay using Trolox at 0 µg/mL to 160 µg/mL. Values are mean ±SD n=2. 112

Figure 5.3 Different fractions of black rice and stereo microscope image of straw. 116

Figure 5.4 Anthocyanin content of various black rice fractions 117

Figure 5.5 Anthocyanin extraction at various hydrothermal treatment temperature. 118

Figure 5.6 Cross sectional view of the straw 119

Figure 5.7 Antioxidant activity of various black rice fractions extracted by methanol 120

Figure 5.8 Antioxidant activity of straw water and methanol extracts..... 121

Figure 5.9 Bioactivity of straw and bran extract 122

Figure 5.10 Digestibility of pre-treated black and white rice straw..... 126

Figure 5.11 ATR-FTIR spectra of rice straw..... 128

Figure A. 1 Genetic markers for *Aegilops speltoides* for the 7 chromosomes. 162

Figure A. 2 Genetic markers for *Aegilops mutica* for the 7 chromosomes. 163

Figure A. 3 Genetic markers for *Triticum timopheevii* for the 7 chromosomes. 164

Figure A. 4 Genetic markers for *Triticum urartu* for the 7 chromosomes. 165

Figure B. 5 Weight of pre-treated residue of individual and mixed feedstocks 166

Figure B. 6 pH of the pre-treated liquor for individual feedstocks and their 1:1 mixed ratios. .. 166

Figure B. 7 HPAEC profile of pre-treated liquor of wheat and willow, and the mixture. 167

Figure B. 8 HPAEC profile of pre-treated liquor of *Miscanthus* and wheat, and the mixture. ... 168

Figure B. 9 HPAEC profile of pre-treated liquor of willow and *Miscanthus*, and the mixture... 169

LIST OF TABLES

TABLE 2.1 COMPOSITION OF STRAW DERIVED FROM NINE DIFFERENT WHEAT CULTIVARS 9

TABLE 2.2 PRE-TREATMENT METHODS FOR LIGNOCELLULOSIC BIOMASS. 17

TABLE 2.3 SUGAR YIELD IN THE ENZYMATIC HYDROLYSIS OF WHEAT STRAW AFTER VARIOUS PRE-TREATMENTS. 21

TABLE 3.1 IN-VITRO ORGANIC MATTER DIGESTION OF WHEAT AND RICE STRAWS FRACTIONS. 37

TABLE 3.2 MODERN WHEAT CULTIVARS..... 39

TABLE 3.3 ANCIENT VARIETIES. 39

TABLE 3.4 CRYSTALLINITY AND DIGESTIBILITY OF PRE-TREATED BC_1. 58

TABLE 3.5 CRYSTALLINITY AND IR CRYSTALLINITY OF RAW BALL MILLED REVELATION STRAW..... 60

TABLE 3.6 CRYSTALLINITY AND IR CRYSTALLINITY OF PRE-TREATED BC_1 REVELATION STRAW 60

TABLE 3.7 DIFFERENCES BETWEEN THE MANUAL AND AUTOMATIC SACCHARIFICATION ASSAYS..... 66

TABLE 4.1 COMPOSITION OF WHEAT, WILLOW AND *MISCANTHUS*. 75

TABLE 4.2 PRE-TREATMENT SEVERITY FACTORS 83

TABLE 4.3 COMPARISON OF OBSERVED AND PREDICTED VALUES FOR GLUCOSE AND XYLOSE YIELDS AT 72HR. .. 92

TABLE 4.4 IMPACT OF MIXED FEEDSTOCKS ON SACCHARIFICATION KINETICS 94

TABLE 4.5 IMPACT OF MIXED FEEDSTOCKS ON YEAST METABOLISM, GROWTH AND INHIBITOR PRODUCTION..... 95

TABLE 4.6 IMPACT OF MIXED FEEDSTOCKS ON ENERGY REQUIREMENTS. 96

TABLE 5.1 COMPOSITION OF RICE AND WHEAT STRAW 102

TABLE 5.2 SUGAR COMPOSITION OF RAW BLACK AND WHITE RICE STRAW 123

TABLE 5.3 SUGAR COMPOSITION OF PRE-TREATED BLACK RICE AND WHITE RICE STRAW 124

TABLE 5.4 CHANGES IN BLACK RICE WEIGHT AND LIQUOR COMPOSITION WITH PRE-TREATMENT REGIMES. 124

TABLE 5.5 CHANGES IN WHITE RICE WEIGHT AND LIQUOR COMPOSITION WITH PRE-TREATMENT REGIMES..... 125

TABLE A. 1 GENETIC PROVIDENCE OF THE BC_1 PLANTS..... 159

ABREVIATIONS

BC_1	back cross 1	kj	kilo joule
$Ca(OH)_2$	calcium hydroxide	KWht-1	Kilo Watt hour/time
cN	centiNewton	mm	millimetre
crl	crystallinity	nm	nanometre
CO_2	carbon dioxide	ml	millilitre
DPPH	2-diphenyl-1-picrylhydrazyl	l	litre
		µg	microgram
DSC	differential scanning calorimetry	mM	millimole
		mg	milligram
FPU	filter paper unit	Mg	mega gram
FTIR	Fourier transformed infrared	g	gram
		kg	kilogram
GHG	greenhouse gas	min	minute
H_2O_2	hydrogen peroxide	h	hour
Ha	hectare	NaOH	sodium hydroxide
HCl	hydrochloric acid	QTL	quantitative trait loci
HMF	hydroxyl methyl-furfural	SNP	small nucleotide polymorphism
HPAEC	high performance anion exchange chromatography	w	watt
		XRD	X-ray diffraction
IR	infrared		

CHAPTER 1 THESIS OVERVIEW

The main PhD programme assessed the impact of breeding modern wheat with its wild relatives (introgression) to find the genetic or biochemical basis that can make non-edible parts of wheat (straw) suitable for biorefinery (including traits such as more biomass, more sugars and easy release of sugars). The breeding work was conducted by Prof. Ian King and Dr. Julie King's group with a general focus on wheat's agronomic traits including grain yield and photosynthesis efficiency. Their group have generated a number of backcrossed introgression plants. I have worked on the straw of 80 plants, resulting from the first backcross (BC_1s) into modern wheat varieties harvested in 2013 and examined the potential to improve the straw characteristics for sugar production in a biorefinery. This study was then extended to investigate potential impacts on cellulose and lignin components, while my colleague worked on a further 48 BC_1 plants harvested in 2012 and extended the study to examine potential impacts on hemicellulose. The final objective was to develop wheat varieties which provide both food and fuel from the same farm with less resource inputs – a dual purpose crop. The aim of this research was to examine a sub-set of BC_1 lines in order to determine the impact of this genetic variation on straw composition and digestibility. Most of the work has been focused around pre-treatment and enzymatic hydrolysis since sugar yields in these two steps is critical in determining the economics of biofuel production (Humbird et al., 2011) and hence its potential as feedstock.

The second part of this research project looked at one of the processing challenges of biorefineries – feedstock availability and the seasonal variability of its supply. Studies on the impact of mixing three of the UK's most important potential feedstocks -wheat straw, willow and *Miscanthus* on processing efficiency using a microwave hydrothermal pre-treatment and subsequent enzymatic hydrolysis to sugars were studied. The research explored the feasibility of using them in combinations to cater for processing demand while minimising any inhibitory effect on yeast metabolism.

The third part of this research project was focussed on black rice from my home town (Manipur, India) with the aim of extracting a high value product - anthocyanin (an antioxidant) prior to utilising the straw as a sugar source. In the biorefinery context, this may be able to make the process more economical by combining the production of a high value, low volume product-anthocyanin- with a low-value high-volume product - sugars. This potential was explored using microwave hydrothermal treatments to optimise anthocyanin extraction and sugar production compared for black and white rice straw. The extracted anthocyanin was tested for stability and potential bioactivity. This research is the first of its kind to extract anthocyanin from straw (not just rice straw).

CHAPTER 2 GENERAL INTRODUCTION

It is no longer up for debate that our planet is undergoing an unprecedented climate change. We must reduce our carbon emissions which have been fuelled by our unsustainable reliance on petrochemical products such as fossil fuel (Popp et al., 2014). However, fossil fuel still continues to be the major source of energy supply and world energy consumption is projected to increase 44% by 2030 (BP, 2017; Energy Information Administration, 2009; Khanal and Lamsal, 2010). Hence, Governments are increasingly promoting alternative fuels able to mitigate climate change and reduce the consumption of fossil resources (Balat, 2011).

While much of the discussion on alternative energy has focussed on wind and solar power, across Europe the biggest source of green energy is biomass which supplies around 65% of renewable power - usually electricity generated from burning wood pellets (McGrath, 2017). Hence, there is a need to look for local feedstock alternatives such as willow and *Miscanthus* to meet this supply. However, Duncan (Duncan, 2017), suggests that it may release more carbon than fossil fuels such as coal per unit of energy, over the full life cycle (Duncan, 2017).

Of the world's total delivered energy, almost 30% is used for transportation. Mostly in the form of liquid fuels (Jansen, 2012), this accounts for about a quarter of our energy related CO_2 emissions (International Energy Agency,

2016). On February 2017, the European Commission sent final warnings to Germany, France, Spain, Italy and the United Kingdom for failing to address repeated breaches of air pollution limits for nitrogen dioxide (European Commission, 2017). Nitrogen dioxide pollution is a serious health risk and most emissions result from road traffic.

Amongst the renewable energy alternatives for transportation fuels, biofuels rank as the most favourable and sustainable choice in the foreseeable future (Nigam and Singh, 2011; Tutt et al., 2012). Biofuels such as bio-methanol and bio-gas have also been highlighted to be the ideal fuel for marine and inland transport in the EU (Moirangthem, 2016). Biofuel such as bioethanol are already commercially available with Brazil and USA as the leading producers (International Energy Agency, 2008)

2.1. BIOFUELS

Biofuel refers to energy produced from biological materials. It can be solid, liquid or gaseous, and all three forms are considered sustainable and renewable because they are produced from plants and animals, and can be replaced in a short time span (Scragg, 2010). Apart from being renewable, biofuels replacing transportation fossil fuel is attractive in terms of carbon footprint. It is more difficult to capture CO_2 emissions from transportation than from other types of energy because of its highly-distributed nature (Carroll and Somerville, 2009). This is particularly true for long-distance travel and heavy-duty applications, such as aviation and long-haul trucking, (Laser and Lynd,

2014). To this extent, biofuels supplied in the UK during 2009/2010 and in the US during 2014 generated GHG emissions savings equivalent to taking half a million vehicles off the road (BBSRC, 2014a) and 8.4 million cars from the road for an entire year (Renewable Fuels Association, 2015), respectively. Environmental Protection Agency and major automobile manufacturers have also started including ethanol blend up to 15% in their automobile warranty (Renewable Fuels Association, 2015).

Depending on the feedstock used in the process, biofuels can be classified as either first or second generation.

2.1.1. First Generation Biofuels

First generation biofuels are obtained from the alcoholic fermentation of easily processed monosaccharides and starch from food crops (Wang et al., 2012). Almost 99% of biofuels currently used in EU road transport are first generation (Didier, 2015). They are associated with concerns such as: feasibility in terms of cultivation area (Ayrinhac et al., 2011), direct competition with food (Vermerris, 2008) and concerns over negative energy balance (Pimentel and Patzek, 2005; Wesseler, 2007). However, one of the purposes of producing first generation biofuels, from food crops, was to show that fossil fuels could be replaced. It was clear from the early days that any greater use of this would compromise food crops (Scragg, 2010). They paved the way for second-generation biofuels.

2.1.2. Second Generation Biofuels

Second generation biofuels are those which are made from non-edible fractions of plant or lignocellulosic biomass such as crop residues, pulpwood and forestry residues, dedicated energy crops and municipal solid waste (Tilman et al., 2009). Lignocellulose refers to plant dry matter or biomass and is the most abundant raw material on Earth, with an estimated global terrestrial availability of 220 billion tons annually (Khanal and Lamsal, 2010). They have the potential to meet most of the global transportation fuel needs without impacting negatively on food security (Abramson et al., 2010).

The major challenges in using straw, or indeed any lignocellulosic feedstock, compared to material used in 1st generation biofuel is that straw is difficult to digest. This is termed recalcitrance, and represents a major bottleneck for conversion (saccharification) of lignocellulosic biomass to the simple sugars required by microorganisms to be converted into ethanol or other products (Lionetti et al., 2010; Meng and Ragauskas, 2014; Sorek et al., 2014). With current technology, it is not yet economically viable to produce second generation biofuels without relying on government subsidiaries (Carroll and Somerville, 2009).

There are various routes to feasibility: i) Reducing production and especially conversion costs through plant genetic engineering to reduce feedstock recalcitrance (Gressel, 2008; Himmel et al., 2007); by making the carbohydrates more accessible for enzymes; or optimizing the composition for the

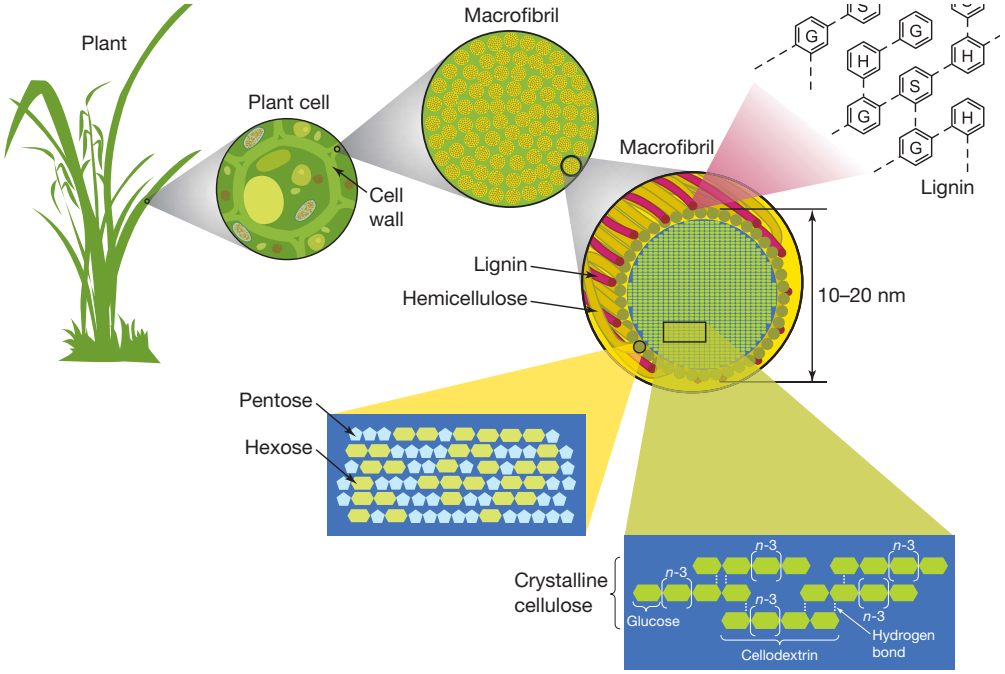
fermentation step (Meng and Ragauskas, 2014; Sorek et al., 2014). ii)Production of high value co-products to offset the cost of producing biofuels. This is desirable as well as feasible since biomass is the only source for bio alternatives to petrochemical products (Laser and Lynd, 2014). iii) Overcome the feedstock shortage that restricts current biorefineries to operate few a months per year due to lack of supplies (Carl, 2017), by utilising a mixed feedstock or by increasing the yield of lignocellulosic biomass/ha (Somerville et al., 2010).

In addition to lignocellulose, plant cell wall is also known to contain phenolic (Naczk and Shahidi, 2006). Plant biomass is also a good source of nutraceuticals such as anthocyanin a plant secondary metabolite present in tissues such as grape marce and black rice (Liang et al., 2008; Min et al., 2010). Value added products such as bioplastics, polyglutamic acid, butendiol and xylitol has been reported (Abraham et al., 2016). Combining the production of biofuels with such high value products could potentially offset the economics of second generation biofuel. This is discussed further in section 5.2, using black rice straw as an example.

2.2. LIGNOCELLULOSIC FEEDSTOCK – WHEAT STRAW

Plant biomass derived from wheat straw is mainly composed of secondary cell wall material formed after the primary cell wall is complete and the cell has stopped expanding (Chen, 2014). The primary cell wall, formed when the cell is growing, is generally a thin, flexible and extensible layer with cellulose,

hemicellulose (mainly arabinoxylans in wheat) and pectin as the major carbohydrates. In contrast, cellulose, hemicelluloses and lignin form the major constituents of secondary cell wall from which the generic term *lignocellulose* was coined (Kristensen et al., 2008). Lignocellulose has been widely investigated as a renewable substrate to produce biofuels, e.g., ethanol, methane, hydrogen, and butanol, as well as chemicals such as citric acid, lactic acid, and xanthan gum (Karimi and Taherzadeh, 2016). Figure 2.1 shows the commonly accepted structure of Lignocellulose in a typical plant (Rubin, 2008).



(Rubin, 2008)

Figure 2.1 Overview of lignocellulose.

Wheat straw is the agricultural residue obtained from different parts of the plant like stem and leaves after the grain has been harvested, and is composed mainly of cellulose, hemicellulose and lignin (Table 2.1). Global wheat

production for 2014 was 732 million tons (Taylor and Koo, 2015) and assuming a residue/crop ratio of between 1.3 (Talebnia et al., 2010) and 1.5 (Yuan and Sun, 2010), about 1000 million tonnes of wheat residues are produced annually. This makes wheat straw a promising lignocellulosic biomass for second-generation ethanol production. However, if crops such as wheat are to be used for multifunctional purposes, i.e. food and fuel, the assessment of lignocellulose composition and digestibility must be included as part of our crop breeding programmes (Ali et al., 2012).

Cultivar	Cellulose (weight fraction)	Hemicellulose (weight fraction)	Lignin (weight fraction)
Flame	0.3871 ± 0.0048	0.2962 ± 0.0066	0.1185 ± 0.0018
Longbow	0.3921 ± 0.0051	0.2782 ± 0.0097	0.1078 ± 0.0031
Baldus	0.4060 ± 0.0046	0.2371 ± 0.0064	0.1214 ± 0.0022
Trappe	0.3924 ± 0.0062	0.3544 ± 0.0164	0.1193 ± 0.0021
Riband	0.3939 ± 0.0104	0.3039 ± 0.0131	0.1085 ± 0.0027
Raffel	0.3873 ± 0.0139	0.3320 ± 0.0136	0.1190 ± 0.0031
Equation	0.3865 ± 0.0084	0.2965 ± 0.0111	0.1119 ± 0.0009
Byson	0.3854 ± 0.0116	0.3201 ± 0.0157	0.1239 ± 0.0026
Einstein	0.3846 ± 0.0111	0.2750 ± 0.0136	0.1318 ± 0.0049

(Ali et al., 2012)

Table 2.1 Composition of straw derived from nine different wheat cultivars

Improving wheat straw as a feedstock for biorefineries at a genetic level will include: a) Optimising quantitative agronomic traits for increased yield of straw

biomass/ha; b) Enhancing sugar yield; c) Lowering recalcitrance to digestion with hydrolytic enzymes to generate the sugars required for fermentation (Meng and Ragauskas, 2014; Sorek et al., 2014). All these tailored to specific environmental weather or growth conditions (Somerville et al., 2010). In addition, improvements specifically targeting cellulose yield and compositional characteristics such as reduced or altered lignin and hemicellulose contents, and even plants that produce cell wall degrading enzymes themselves have been reported (Chang, 2007; Furtado et al., 2014; Sticklen, 2006).

2.2.1. Cellulose

Cellulose is a homo polymer made up of D-glucose units which are linked by β-1,4-glucosidic bonds into a linear/parallel structure with a high degree of polymerisation (Michelin et al., 2013). These parallel cellulose chains are bound together by hydrogen bonds to form para-crystalline cellulose fibrils. The fibrils are 3-5 nm in diameter (Klemm et al., 2005) and adhere together to form macro fibrils up to 200nm wide (Ding and Himmel, 2006). It is synthesised at the plasma membrane and the cellulose microfibrils are deposited into the extracellular wall matrix at a location adjacent to the plasma membrane (Somerville, 2006). The extensive hydrogen bonding between cellulose chains coupled with β-linkages connecting glucan units (as opposed to α linkages found in starch) results in considerable recalcitrance to depolymerizing enzymes. Fibrils are also responsible for the great tensile strength (resistance to tension or stretching) of the cell wall (Carpita and Gibeaut, 1993).

Cellulase enzymes have been reported to degrade amorphous cellulose more efficiently (Laureano-Perez et al., 2005). The rate of hydrolysis of amorphous cellulose has been reported to be 3-30 times faster than that of high crystalline cellulose (Lynd et al., 2002). However, cellulose crystallinity is not the only factor affecting digestibility (Kumar and Wyman, 2009). These factors include surface area, porosity and the degree of polymerisation of cellulose, and the overall composition of the lignocellulosic material (presence of lignin and hemicellulose) (Hall et al., 2010).

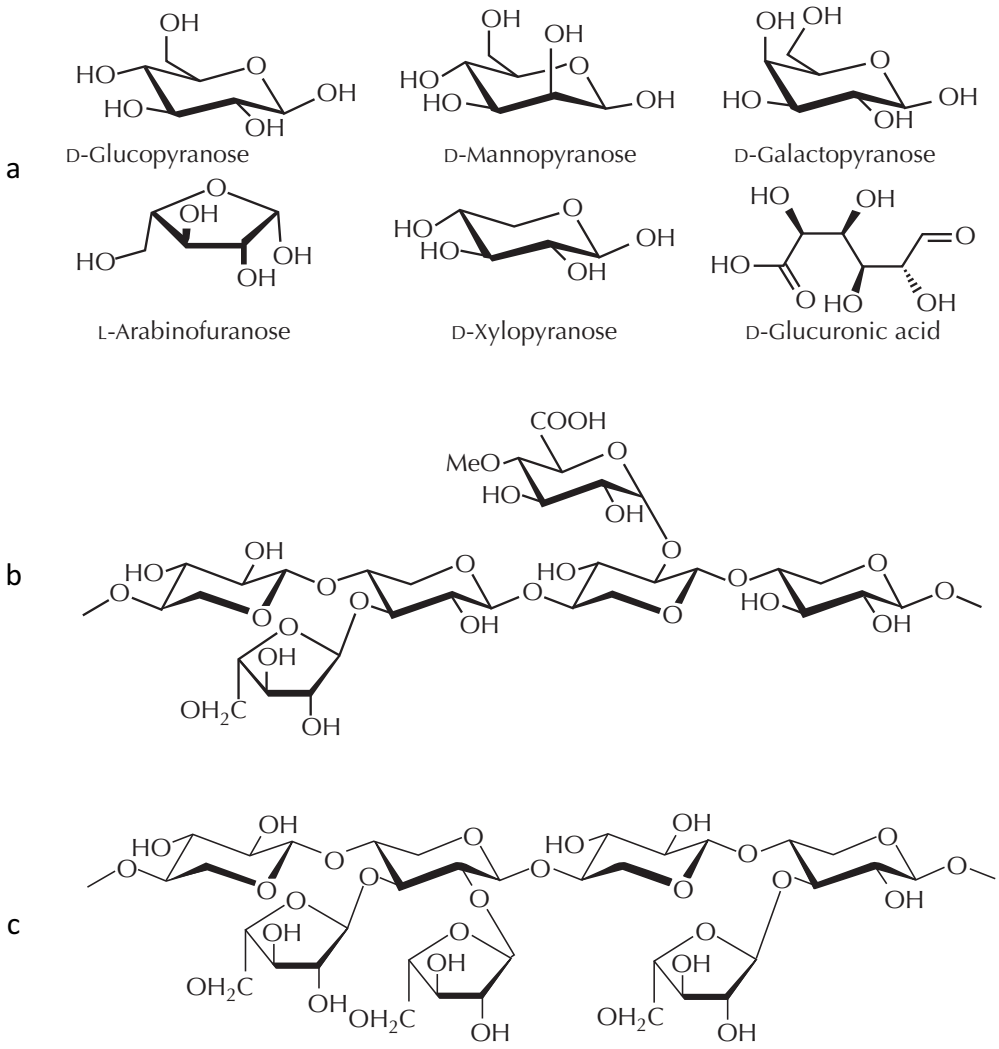
2.2.2. Hemicellulose

Hemicelluloses are 'cellulose-associated' polysaccharides, which can be extracted in alkaline solution (Kong et al., 1992). The specific structure and composition of hemicellulose largely depends on plant genus and cell type. These polymers are amorphous and easier to hydrolyse than cellulose (Chen, 2014). They are synthesised in the golgi apparatus (Himmel et al., 2007) and packaged into secretory vesicles before delivery to the cell surface and incorporated into the wall matrix. The major biological role of hemicelluloses is to act as cross-linkage between cellulose and lignin. Through this cross-linking it creates distance between cellulose fibrils, maintaining a certain degree of flexibility, and anchors the cell wall matrix together (Carpita and Gibeaut, 1993).

In mature wheat straw, D-xylose is the predominant sugar accounting for ~80% of hemicellulose and ~25% of dry straw. Besides D-xylose, wheat straw

hemicellulose also contains (albeit at individual levels below ~13%), L-arabinose, D-galactose, D-mannose, D-glucuronic acid or its 4-0-methyl ether and possibly D-glucose (Lawther et al., 1995; Menon and Rao, 2012). The xylan backbone is also partially acetylated at the C-2 and C-3 positions (Gröndahl and Gatenholm, 2004). Figure 2.2 shows the main constitutes of hemicellulose and primary structures of arabino-glucurono-xylan (common in coniferous species) and arabino-xylan (common in cereal) (Ren and Sun, 2010).

The arabinose substitution degree of xylan (i.e., the reverse xylose/arabinose ratio) has been reported as a major positive factor impacting on biomass saccharification in grass species (Wang et al., 2016). Li *et al.,* also reported xylan to have a positive effect on biomass enzymatic digestibility by reducing cellulose crystallinity (Li et al., 2014). Hence, increasing the hemicellulose represents another promising strategy for enhancing both biomass yield and lignocellulose enzymatic saccharification in bioenergy crops.



(Ren and Sun, 2010)

Figure 2.2 Hemicellulose structure

a) Main constituents of hemicellulose, b) Primary structure of L-arabino-4-O-methyl-D-glucurono-D-xylan, c) Primary structure of L-arabino-D-xylan.

2.2.3. Lignin

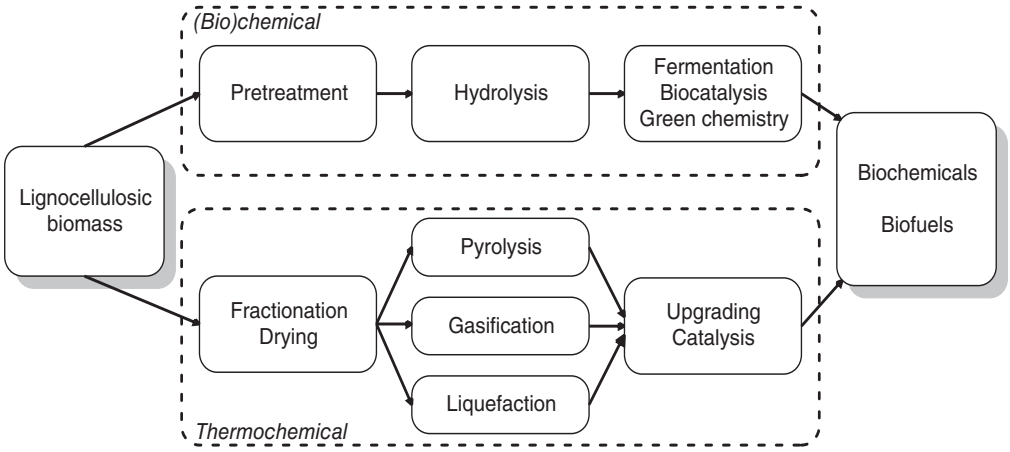
Lignin is the second most abundant biopolymer after cellulose. It is a hydrophobic and extremely complex three-dimensional polymer composed of p-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units (Loqué et al., 2015). Wheat straw lignin mainly consists of non-condensed guaiacyl units, non-condensed syringyl units, and other condensed units; the ratio of n (non-

condensed guaiacyl units) to n (non-condensed syringyl units) to n (condensed units) is 1.44:1:3.24 (Chen, 2014). In context of enzymatic hydrolysis, lignin is considered to exert two primary effects: i) Preventing the swelling of lignocellulosic material to reduce surface area access for cellulase enzymes (Maurya et al., 2015), and ii) Preventing cellulase action directly (Keating et al., 2006) by covalently bonding to and masking carbohydrates (Akin, 2007).

An enzyme-based strategy to reduce lignin content by half without compromising biomass yield has recently been reported (Eudes et al., 2015). The approach focused on the creation of a competitive metabolic pathway in cells producing secondary walls to reduce the availability of shikimate, which is necessary for the activity of hydroxyl-cinnamoyl-Coenzyme-A shikimate hydroxy-cinnamoyl-transferase, a key enzyme of the lignin synthesis pathway.

2.3. CONVERSION OF PLANT BIOMASS TO BIOFUELS

Biofuels and biochemical from lignocellulosic biomass can be produced using two major pathways: biochemical and thermochemical process (Figure 2.3). In the thermochemical process, biomass is heated to different temperatures, pressures and varying oxygen levels. For this thesis, the biochemical route, reported to be more cost effective compared to thermochemical conversion has been the focus (International Energy Agency, 2008; Khanal and Lamsal, 2010).



(Dobbelaere et al., 2014)

Figure 2.3 Different biochemical and thermochemical pathways and their products.

In the biochemical process, two stages are key: i) Pre-treatment and saccharification: the deconstruction of plant biomass to release fermentable sugars, and ii) Fermentation: biochemical conversion of the sugars by microbial action (yeast, bacteria or algae) to produce the required end-products (Furtado et al., 2014). These are also the two major cost intensive steps which dictate the final cost of fuel production for a given conversion process (Lynd et al., 2008).

2.3.1. Pre-treatment

Lignocelluloses such as wheat straw are highly resistant due to their structure which is capable of resisting microbial and enzymatic attacks for bioconversion (ElMekawy et al., 2013). This resistance or recalcitrance has been described as the greatest barrier to realising the potential of lignocellulose as an industrial feedstock (Lynd et al., 2008). Therefore, many physical, thermal, chemical, and biological pre-treatment methods or various combinations thereof have been

developed to open their structure (Dobbelaere et al., 2014; Karimi and Taherzadeh, 2016; Kumar and Wyman, 2009) - Table 2.2.

Most pre-treatments have been optimised for a specific model biomass, which has challenged the effective use of other biomass sources (Wi et al., 2015). During commercial operation, a biorefinery may be faced with the need to incorporate a range of unique optimised pre-treatments revolving around seasonal availability of different feedstocks. The ability to switch to a different feedstock mid production cycle potentially mitigate feedstock shortage. However, utilisation of lignocellulosic biomass materials from various plant sources require development of effective pre-treatment technologies for different feedstock combinations.

Pre-treatment method	Process and conditions	Possible changes in biomass	Disadvantages
Steam explosion	No agent, 160-260°C, 20-50 bar, 2-15 minutes	Dissolve hemicellulose, Low sugar degradation	Partially degrade hemicellulose
Ammonia fibre explosion	Ammonia as agent, 65- 90°C, 0.5-3 hours	Change biomass physical structure enhancing hemicelluloses hydrolysis	Limited effects on soft and hardwood
Dilute acid	H_2SO_4 as agent, over 160°C, 2-10 minutes	Effectively dissolve hemicelluloses	Needs neutralisation, formation of fermentation inhibitors, polysaccharide hydrolysis
Alkaline	NaOH/ $Ca(OH)_2$ /Ammonia as agent, 70-120°C, 20-60 min	Partial removal of lignin, Low hemicelluloses degradation	Costs of reagents and wastewater treatment are high
Organic solvent	Ethanol as agent, 140- 200°C, 30-150 minutes	Removal of lignin	Cost of solvent recovery is high
Ionic liquid	Ionic liquid as agent, 120 °C, 22 hours	Remove of lignin and hemicellulose	Costs of reagents and long treatment time

(Cheng and Wang, 2013)

Table 2.2 Pre-treatment methods for lignocellulosic biomass.

Of these only a small number of pre-treatment methods are cost effective at the current time: steam explosion, liquid hot water, dilute acid, lime, and ammonia pre-treatments (Dobbelaere et al., 2014). Kumar and Sharma provides detailed description and comparison of the various pre-treatment technologies (Kumar and Sharma, 2017). A few that are relevant to this research are explained below.

2.3.1.1. Physical pre-treatment

This involves combinations of chipping, grinding or milling depending on the final particle size of the material: 10–30 mm after chipping and 0.2–2 mm after milling or grinding (Tomás-Pejó et al., 2011). The objective is to reduce particle size and sometimes crystallinity (by ball milling) of cellulose to increase specific surface area and reduce the degree of polymerisation (Alvira et al., 2010; Lam et al., 2015).

To achieve an ethanol concentration of >4% (w/w), (benchmark for an efficient distillation) sugar levels of ≥ 8% (w/w) are needed. To get this sugar concentration most lignocellulosic biomass requires initial solid contents of >20% (w/w) (Larsen et al., 2008). However, cellulosic slurries become progressively more viscous, and difficult to handle at concentrations of solids >15% (w/w). This can be overcome by reducing particle size which has been reported to also result in reduced viscosity of the slurry (Viamajala et al., 2009).

However, energy consumption is a limiting factor for fine grinding biomass industrially (Silva et al., 2012). They also differ with biomass, for example specific energy consumptions for grinding wheat straw with a hammer mill and screen sizes of 0.8 and 3.2 mm were reported to be 51.6 and 11.4 kW ht^{-1} respectively, which were higher than corresponding values for corn stover at similar moisture content 22.07 and 6.96 kW ht^{-1} respectively (Mani et al., 2004).

2.3.1.2. Dilute acid pre-treatment

Pre-treatment with dilute acid is low-priced and effectively hydrolyses hemicelluloses into monomeric sugars, causing structural transformation of lignocellulosic matrix, thereby increasing enzyme accessibility and improving cellulose conversion (Cao et al., 2012). The efficiency of hemicellulose removal is approximately 90%, with sugar losses by degradation at around 1% (Moutta et al., 2012). A downside of the dilute acid pre-treatment method is the formation of by-products that may be inhibitory for the subsequent fermentation process. Sulphuric acid is the acid most often used in this category (Taherzadeh and Karimi, 2008). Hydrolysis yields as high as 74% have been shown when wheat straw is subjected to 0.75% v/v of H_2SO_4 at 121 °C for 1 h (Saha et al., 2005).

In this research, a mild pre-treatment of 1% dilute sulphuric acid at 121 °C for 15min was used to disrupt straw stems milled to 2mm. This was performed to screen wheat straw for digestibility after pre-treatment.

2.3.1.3. Hydrothermal pre-treatment

Hydrothermal pre-treatment is generally performed by pre-treating biomass at temperatures between 140 and 220°C under high pressure to maintain water in the liquid state (Kim et al., 2015). Above 150-180° C, parts of the lignocellulosic biomass (firstly the hemicelluloses and shortly after that lignin) start to solubilise (Bobleter, 1994; Garrote et al., 1999).

Hydrothermal treatment is mild or less severe compared to dilute acid pre-treatment, but has similar effects in terms of the chemical and physical changes that occur in the structure of lignocellulose (Pu et al., 2013). The reaction medium becomes acidic due to the release of acetic acid and other weak acids derived from hemicellulose, and to the auto-dissociation of water at elevated temperatures (hydronium ions) (Cantarella et al., 2004; Gurram et al., 2011).

2.3.2. Enzymatic Hydrolysis

Like pre-treatment, enzymatic hydrolysis is also considered to be an important step in biomass conversion process (Silva et al., 2012). The main factors that affect the rate of hydrolysis of cellulose are: accessibility of the fibres to enzymes, the structure of cellulose (Balat, 2011), crystallinity of cellulose, and the hemicellulose and lignin content (Sun and Cheng, 2002).

Hydrolysis of polysaccharides to soluble sugars is referred to as called "saccharification" (Dobbelaere et al., 2014). The goal of this process is the de-polymerisation of cellulose and hemicelluloses into soluble monomer sugars. The methods for hydrolysis can be grouped into two major classes: chemical and enzymatic hydrolysis. The latter process is particularly interesting because it is selective for the biomass degradation, can be operated at mild temperature and pH conditions (Dobbelaere et al., 2014) and does not create a corrosion problem (Duff and Murray, 1996). Typically, two classes of enzymes (cellulases in section 2.3.2.1 and hemicellulases in section 2.3.2.2) are employed together

and synergistically to attack cellulose and hemicellulose.

The sugar yield derived from wheat straw is depended on both the pre-treatment used and hydrolysis conditions. Table 2.3 provides an overview of typical sugar yields of wheat straw following different pre-treatment and enzyme hydrolysis techniques.

Pre-treatment	Enzymes mixture	Source of enzyme	Condi-tion	Sugar yield (mg/g dry mass)	% max. theo.
Dilute H_2SO_4 impregnation + SE	Cellulase β-Glucosidase	T. *reesei* A. *niger*	40 °C pH 5.0 96 h	612	99.6
0.75% (v/v) H_2SO_4, 121°C	Cellulase β -Glucosidase Xylanase	T. *reesei* A. *niger* T. *longibrachiatum*	45 °C pH 5.0 72 h	565	74
2.15% (v/v) H_2O_2, 35 °C	Cellulase β -Glucosidase xylanase	T. *reesei* A. *niger* T. *longibrachiatum*	45 °C pH 5.0 120 h	672	96.7
Fine grinding + wet oxidation	Cellulase β-Glucosidase	T. *reesei* A. *niger*	50 °C pH 5.0 24 h	638	92

(Talebnia et al., 2010)

Table 2.3 Sugar yield in the enzymatic hydrolysis of wheat straw after various pre-treatments.

2.3.2.1. Cellulases

A complex mixture of enzymes termed “cellulases” carry out degradation of cellulose (Bayer et al., 1998). They distinguish themselves from most other classes of enzymes by being able to hydrolyse insoluble substrate. Cellulases have been conventionally divided into three major groups (Bai et al., 2017):

i.) Endoglucanases, which cut at random internal amorphous sites in the cellulose polysaccharide chain, generating oligosaccharides of various lengths, and consequently shorter chains appear.

ii.) Cellobiohydrolases or exoglucanases, which act in a progressive manner on the reducing and non-reducing ends of the cellulose chains liberating either glucose or cellobiose as major products.

iii.) *β*- glucosidases, which hydrolyse soluble cellobiose to glucose and also cleave off glucose units from cello-oligosaccharides.

These three groups of enzymes work synergistically to degrade cellulose by creating new sites for each other and preventing product inhibition (Eriksson et al., 2002; Väljamäe et al., 2003). Cellulolytic cocktails are present in commercial preparations supplied by several biotechnological companies such as Novozymes (Denmark), Genecor (Palo Alto, CA) and Iogen (Canada) (Paola et al., 2011).

2.3.2.2. Hemicellulases

The hemicellulosic enzyme system is more complex than celluloases and involves, for example, glucuronidases, acetylesterase, xylanase, β-xylosidase, galactomannase and glucomannase (Sun and Cheng, 2002; Verardi et al., 2012). This is because unlike cellulose which is a homo-polymer, the hemicellulose is composed of diverse group of heterogeneous polymers with various side groups.

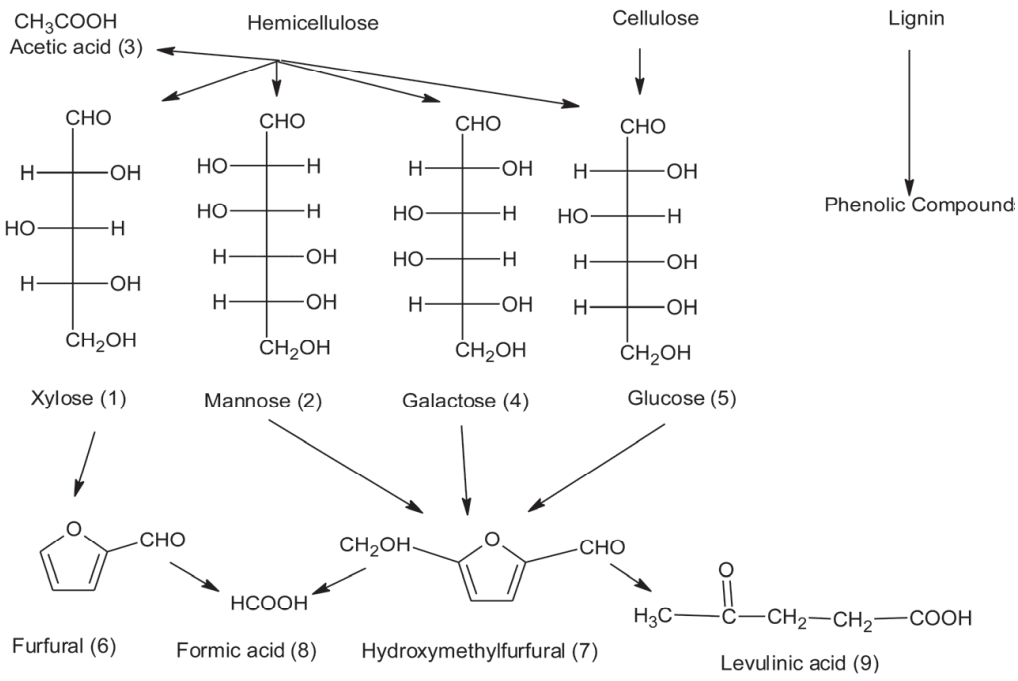
2.3.3. Fermentation and inhibitors

Following the chemical and/or enzymatic hydrolysis, the released sugars can be fermented to form ethanol, butanol, acetone, single cell protein and methane (Anderson et al., 2005). *Saccharomyces cerevisiae* is the most popular microorganism used for ethanol production due to its good fermentative capacity and ethanol tolerance, allowing it to produce up to 20% (v/v) ethanol; generally regarded as safe status (GRAS); and capacity to grow rapidly under anaerobic conditions (Guimarães et al., 2010). While fermentation of hexose sugars such as glucose, mannose and galactose is well established on large scale, the conversion of the pentose sugars (which can comprise up to 30% of plant biomass), namely xylose and arabinose, is more difficult (Paola et al., 2011).

Microbial inhibitors released during pre-treatment have been shown to affect the overall cell physiology of the fermenting organism, often resulting in decreased viability, ethanol yield, and productivity (Chandel et al., 2007). Hence, removal of inhibitors from hydrolysates is necessary to achieve an efficient fermentation (Paola et al., 2011).

Mussatto and Roberto classified these inhibitors into three major groups: i) Organic acids (acetic, formic and levulinic acids); ii) Furan derivatives (mainly constituted by 5- hydroxymethyl-2-furaldehyde (5-HMF) and 2-furaldehyde generated by dehydration of hexoses and pentoses, respectively); and iii) Phenolic compounds (Mussatto, 2004). Their inhibition mechanism is not only

based on the inhibitory effect caused by each compound individually, but also on their interaction and synergy (Tomás-Pejó et al., 2011). Among these, furfural and HMF are are more potent than the acetate and lignin degradation products (Yang, 2014). However, their effects on yeast are dose dependent (Liu et al., 2005). At low concentrations, furfural can be beneficial to fermentation since yeast can use it to regenerate nicotinamide adenine dinucleotide (NAD^+), thus reducing glycerol production (Sutton, 2011). At higher concentrations, cell replication is inhibited by furfural and 5-HMF, inducing an increased lag-phase in growth as well as halting anaerobic growth (Sutton, 2011). Hence, their concentration after pre-treatment is an essential parameter to be considered. Phenolic compounds such as vanillin have been reported to be usually no more than 0.1 mM and at this concentration vanillin has negligible effects on yeast and fermentation (Greetham et al., 2014).



(Palmqvist and Hähn-Hägerdal, 2000)

Figure 2.4 Structural profile of lignocellulose derived fermentation inhibitors.

Figure 2.4 shows the inhibitor profile of lignocellulose hydrolysates. The presence and quantity of such compounds depend on the raw material as well as on the severity of pre-treatment. Therefore, the associated problems of lignocellulose conversion can be addressed either by using feedstocks that are less recalcitrant or by developing efficient pre-treatment techniques optimizing pre-treatment conditions for each feedstock(Simms-Borre, 2011; Soudham, 2015). It is difficult to prevent the formation of weak acids since they are intrinsic to the biomass feedstock, however species that are created from sugar or lignin degradation can be reduced by optimizing pre-treatment conditions. Phenolic compounds, like vanillin, syringaldehyde, and ferulate, are a major constituent of lignin and are also linked to hemicellulose in some biomass substrates, for example grasses. To avoid these inhibitors, a process should be selected that either removes much of the lignin or leaves the lignin intact (Simms-Borre, 2011).

2.4. THESIS AIMS AND OBJECTVES

2.4.1. Improving wheat straw by introgression

Hypothesis 1: Wheat introgression will result in phenotypic variations or altered morphological make-up of the straw.

Objective: To screen BC_1 population for phenotypes such as biomass (leaf, stem and total), and measure sugar composition of the stems.

Hypothesis 2: The BC_1 stems will respond differently to acid pre-treatment, enzymatic saccharification and hence, possess a range of digestibility.

Objective: To measure sugar composition of the pre-treated BC_1 stem residue; measure the released monomeric glucose and xylose after enzymatic hydrolysis of pre-treated BC_1 stem. Digestibility is a complex trait and due to limited sample, the data was supplemented with a high throughput saccharification assay from York University.

Hypothesis 3: Differences in digestibility or the introgression effect could be accompanied by structural changes that are related to digestibility.

Objectives: Study the structural composition using techniques such as X-Ray Diffraction, Fourier Transformed Infra-Red Spectroscopy (FTIR) and check for correlation with cellulose and lignin.

Hypothesis 4: The genetic basis for digestibility traits can be linked to introgression segment(s)

Objectives: Compare BC_1 digestibility with SNP mapping data. Identify potential overlapping introgression segments that can be followed to the next cross carrying smaller segments.

2.4.2. Utilising mixed feedstock

Hypothesis 1: Mixing lignocellulosic feedstocks could result in improved pre-treatment

Objective: To compare the sugar composition of the pre-treated residue of mixed feedstocks against expected sugar composition values. Expected values were calculated by averaging sugar composition of pre-treated residues of the individual feedstocks.

Hypothesis 2: Mixing feedstocks will result in improved saccharification

Objective: To measure digestibility of the pre-treated mixed feedstock residue and compared against calculated values from the pre-treatment individual feedstock data.

Hypothesis 3: Mixing feedstocks will result in reduced inhibitor production during pre-treatment, accompanied by a resultant decrease in the inhibitory effect on yeast metabolism and growth.

Objective: To analyse the composition of the pre-treated liquor from the mixed feedstocks, its effect on yeast metabolism and growth, and to compare these values against their respective calculated values from the individual feedstocks.

2.4.3. Black rice straw as a feedstock for anthocyanin and sugars

Hypothesis 1: Black rice straw is a good source of anthocyanin.

Objective: To measure anthocyanin from black rice straw and compare this against anthocyanin content from its different fractions including bran, paddy rice and polished rice.

Hypothesis 2: A microwave hydrothermal method is a good alternative to organic solvent for anthocyanin extraction.

Objective: To compare values of anthocyanin extracted from black rice straw using hydrothermal pre-treatment at different temperature regimes against the value obtained from organic solvent extraction as a theoretical maximum.

Hypothesis 3: Hydrothermal hydrolysis results in anthocyanin extracts that are stable or at least comparable to methanol extracts.

Objectives: To compare antioxidant potential of straw hydrothermal extracts against straw methanol extract. To extend antioxidant study to extracts from different fractions of black rice and correlate with their anthocyanin contents.

Hypothesis 4: The hydrothermally extracted anthocyanins are bioactive.

Objectives: To expose mammalian cell lines with the extract and measure cytotoxicity, apoptosis and cell death using flow cytometry.

Hypothesis 5: The straw residue can be a good source of sugar comparable to that obtained from white rice.

Objectives: To compare digestibility of black and white rice residues after hydrothermal pre-treatments at high temperatures.

CHAPTER 3 IMPROVING WHEAT STRAW BY INTROGRESSION

3.2. INTRODUCTION

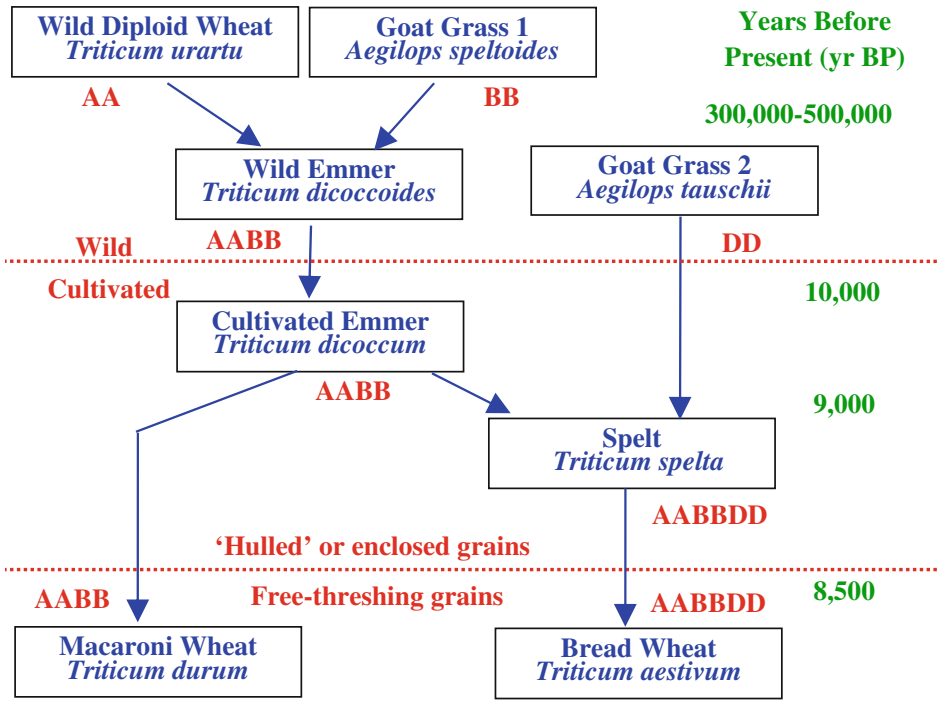
With a global production of 740 million tons in 2017, wheat is one of the most important global food crops (FAO, 2017) and the leading source of vegetable protein (BBSRC, 2014b). However, if the historic rates of improvement in the wheat yield per hectare of land are maintained through this century; then by 2050, there will be a shortfall of 30% in the amount of wheat produced globally (Ray et al., 2013). Traditionally, cereals such as wheat have been optimised for food and feed applications (Furtado et al., 2014) while non-edible parts of plants have evolved or breed to successfully resist microbial degradation (Gressel, 2008; Himmel et al., 2007). With the need to replace petroleum products, improving wheat straw as a biorefinery feedstock could be a part of the modern wheat breeding programme. Hence, it would be desirable to develop superior wheat which is adapted to the fluctuating environment and possesses straw suitable for the biorefinery.

Crossing modern wheat, which possess a relatively small range of genetic variability, with wild relatives could potentially provide a vast and largely untapped reservoir of genetic variation (King et al., 2013), potentially resulting in varieties that hold both desirable agronomic and biofuel traits. To do this, it is necessary to understand how current wheat varieties evolved through natural selection and human interventions, the factors causing reduction in its

genetic variation (required to breed better adapted varieties) and finally what strategy might improve genetic variation.

3.2.1. Evolution and genetic variation

None of the major food crops grown in Britain are native to this country. They originated in many different parts of the world and have all been adapted, through plant breeding, to thrive under UK growing conditions (BPSB, 2014). Originally from the Fertile Crescent region (Middle east), wheat has evolved (in part by nature and in part by domestication) from its primitive form (emmer wheat) into the presently cultivated varieties (Figure 3.1).



(Peng et al., 2011)

Figure 3.1 Wheat evolution from prehistoric grasses to modern Durham and Bread wheat.

Overall, wheat has evolved three new traits: the seeds grew larger; the “rachis” which binds the seeds together became less brittle so whole ears of grain, rather than individual seeds, could be gathered; and the leaf-like glumes that covered each seed loosened, thus making the grains “free-threshing”. Mutations for these changes have been located within the wheat plant's genome (Simonetti et al., 1999; Watanabe et al., 2002).

Despite its relatively recent origin, wheat has shown sufficient genetic diversity to allow the development of over 25,000 types that are adapted to a wide range of temperature and environments (Shewry, 2009). Breeders have had considerable success in producing higher yielding varieties with the limited genetic variation available (King et al., 2016) However, during agricultural development, early wheat was gradually replaced first by landraces and traditional varieties, and later by genetically less diverse modern cultivars. This was followed by a further reduction in the cultivated forms during domestication—by 69% in bread and 84% in durum wheat (Haudry et al., 2007).

This genetic reduction is alarming because genetic diversity is the basis of plant breeding and selection depends upon the existence of an appropriate diversity for the trait being selected for (Khayatnejad et al., 2010). For cereal, such as wheat they are critical for enhancing and maintaining the yield potential, as they provide new sources of resistance and tolerance to biotic and abiotic stresses (Fu and Somers, 2009; Moghaddam et al., 1997). There is also growing

evidence that wheat yields are plateauing as a direct result of the exhaustion of the available genetic variation compounded by environmental change (Charmet, 2011; Ray et al., 2013). Hence, it is important to find new sources of genetic variation that may enable breeders to develop the next generation of superior high yielding wheat varieties that are better adapted to biotic and abiotic stress.

3.2.2. Improving genetic variation - Introgression

Reduction in diversity caused by intensive selection can be counter balanced by introgression of novel germplasm (Reif et al., 2005). Introgression is a non-GM approach where small chromosomal segments of 'wild' and 'distant' grass varieties are transferred into 'modern wheat cultivars' through conventional breeding. Wheat is related to several species many of which are wild and uncultivated. These wild relatives, unlike modern wheat, provide a vast and untapped reservoir of genetic variation for potentially most, if not all, agronomically important traits (King et al., 2016). Hence, one of the best strategies for wheat improvement is to utilise the adaptive genetic resource of the wild progenitors, including wild emmer *T. dicoccoides* and other wheat relatives (Peng et al., 2011).

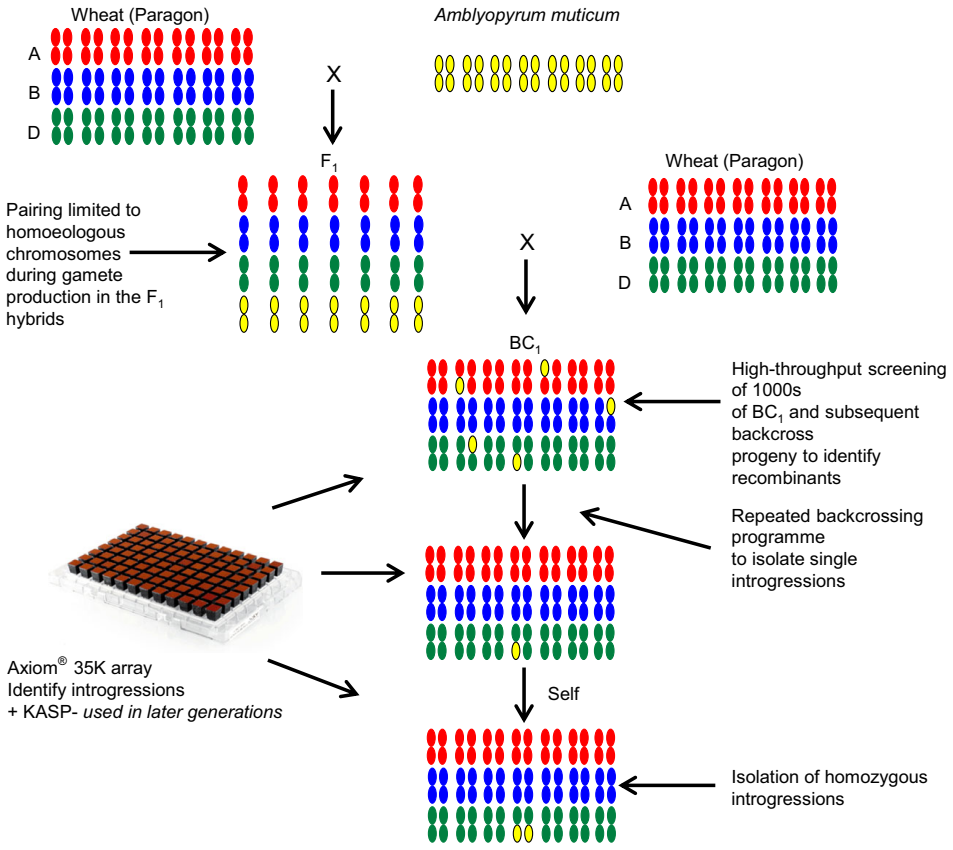
The technique of introgression was developed in 1956 by Sears (Sears, 1956) and showed that it is possible to isolate small alien chromosome segments carrying only the target gene(s). This strategy involved the inter-crossing of two lines with different but overlapping alien chromosome segments, that also

carry the same target gene. Because of recombination between the two overlapping alien segments, in the presence of pairing control genes, some of the progeny produced are recombinant, carrying reduced alien chromosome segments with the target gene, but lacking deleterious genes (Reynolds et al., 2012). Figure 3.2 provides an overview of the alien introgression used in this thesis.

In the past, attempts have been made to exploit the genetic variation from these wild species with limited success. The major blocks to the successful large-scale genome wide exploitation of genetic variation from wild relatives have been firstly the lack of high-throughput screening technology to quickly identify and characterize introgressions when they occur and secondly the apparent low frequency of recombination between the chromosomes of wheat and many of its wild relatives (King et al., 2016). Hence, although the value of genetic variation from wild species has been clearly demonstrated, only a fraction of their full potential in breeding has so far been exploited in breeding programmes (King et al., 2013) some specific examples are: *Aegilops umbellulata* - leaf rust resistance which is believed to have saved US wheat production from catastrophic failure in 1960 (Sears, 1972; 1956); and *Aegilops ventricosa* conferring resistance to eyespot has been exploited in breeding programmes (Garcia-Olmeda et al., 1984)

However, with the development of next-generation sequencing technologies and single nucleotide polymorphism (SNP) markers, mechanism for the

detection of introgressions into wheat from its wild relatives has been demonstrated (King et al., 2016; Tiwari et al., 2015; 2014).



(King et al., 2016)

Figure 3.2 Wild relative introgression strategy.

Wheat/wild relative introgression between modern wheat (Paragon) and wild relative (*Amblyopyrum muticum*).

3.2.3. Straw improvement for biorefinery

Genetic variation has been shown to not only confer agronomically important traits, but also traits that could be beneficial in biofuel processing. For example, Ali *et al.,* showed that wheat genotype affects the degradability and ethanol yield from straw (Ali et al., 2012). Jensen *et al.*, reported significant differences between the digestibility of 106 currently grown, or new wheat cultivars, with

sugar release per unit of material ranging from 258 to 407g kg-1 of dry matter (Jensen et al., 2011). Lindedam, *et al.*, reported up to 26% difference in sugar release between straw of 20 winter wheat cultivars collected in duplicates on two sites in Denmark; and no correlation between grain yield and digestibility (Lindedam et al., 2012). Collins *et al.,* investigated the variation in the chemical compounds of 6 wheat straw varieties under the same cultivation conditions and reported a considerable variation between the varieties (Collins et al., 2014). Additionally, Wu *et al.,* in assessing 115 wheat accessions, found that the plant dry matter consisted of 27.5% to 36.4% cellulose, 28.4% to 35.1% hemicellulose and 19.3% to 24.5% lignin (Wu et al., 2014).

Apart from varietal differences, digestibility also varies in different fractions of the wheat straw (Gill and Friebe, 2002; Pearce et al., 1988). These results are summarised in Table 3.1. For wheat, leaf material has been shown to be more digestible than stem resulting in overall digestibility varying with the leaf-to-stem ratio (Habib et al., 1995; Tolera et al., 2008; Zhang et al., 2014). Within the leaf fraction it has been shown that the blade has greater digestibility than the leaf sheath (Ohlde et al., 1992). Stem-leaf ratio of wheat straw has also been reported to be determinants of straw biochemistry (Collins et al., 2014). On the other hand, Lindedam *et al.*, found that wheat straw sugar yields were influenced more by cultivar than by Leaf/Stem ratio (Lindedam et al., 2012).

Fraction	In Vitro Organic Matter Digestion * %	
	Wheat	Rice Straw
Stem internode	21 – 35	42 – 77
Leaf sheath	45 – 63	38 – 56
Leaf blade	58 – 77	45 – 60
Whole straw	21-58	30 -62

(Gill and Friebe, 2002; Pearce et al., 1988)

Table 3.1 In-vitro organic matter digestion of wheat and rice straws fractions.

*In-vitro organic matter digestion can be correlated to digestibility and ethanol production from biomass (Weimer et al., 2005).

Only a few studies are available that have targeted the simultaneous improvement of both grain yield and biomass (straw) convertibility. Wu *et al*., showed that commercial cultivars of wheat with straws exhibiting improved sugar release can be developed without sacrificing grain yield (Wu et al., 2014). Traits such as grain yield have also been positively correlated to biological yield, straw yield and number of spike per plant (Hannachi et al., 2013; Mohamed, 1999). Hannachi *et al.,* also reported significant positive correlations between grain yield, biomass yield and straw yield, respectively (Hannachi et al., 2013). Lindedam *et al.,* on the other hand found that the ability of the cultivar to release sugar for ethanol production was not related to the grain yield (Lindedam et al., 2012).

The aim of this chapter was to assess the potential impact of the introgression of "alien" chromosomal segments on the composition and digestibility of wheat straw. To achieve this a number of BC_1 plants from a diverse range of

introgression events were analysed for their phenotype, straw composition and digestibility. The potential biochemical basis for any altered digestibility was then explored by analysis of the lignin and cellulose composition of the plants and the genetic basis explored by comparison of the genotype and digestibility of the plants. The BC_1 population was chosen for this preliminary investigation since this will have the greatest number of introgression events and hence the highest probability of identifying altered biochemical properties.

3.3. MATERIALS AND METHODS

3.3.1. Plant material

Back cross wheat (BC_1) plants were created by crossing modern wheat lines (Table 3.2) possessing genomes AABBDD and with the *Ph1* locus deleted, with wild relatives (Table 3.3) having genome RR to create F_1 hybrids (ABD R) and then back crossing with an elite line (AABBDD) without the *Ph1/Ph1* deletion. These BC_1 lines were generated by Professor Ian King and Dr. Julie King and grown in the glasshouses at The University of Nottingham's Sutton Bonington campus: 16hr light, 8hr dark and temperature between 20-35 °C.

Chinese Spring	Chinese Spring Mutant (*ph1ph1*)	Paragon mutant (*ph1ph1*)
Paragon	Creso mutant (*ph1ph1*)	Highbury
Pavon 76		

Table 3.2 Modern wheat cultivars.

Thinopyrum elongatum 401007	*Aegilops speltoides 2140008*	*Aegilops speltoides 2140007*
Secale iranicum P208/11	*Aegilops mutica 2130008*	*Aegilops mutica 2130004*
Aegilops mutica 2130012	*Secale cereale 390382*	*Secale cereale 428373*
Aegilops caudata 2090001	*Secale vavilovii 573649*	*Triticum macrochaetum*
Triticum urartu 1010001	*Triticum urartu 1010002*	*Triticum urartu 1010007*
Thinopyrum bessarabicum 531712	*Triticum triaristata P95-92.1-1*	*Triticum timopheevii P95-99.1-1*
Aegilops speltoides 2140066	*Secale anatolicum P208/141*	*Thinopyrum ponticum 531737*

Table 3.3 Ancient varieties.

In total 128 BC_1 plants, representing a diverse range of introgression events, were harvested- 48 in 2012 and 80 in 2013 (Appendix A – Table A.1). The 48 plants harvested in 2012 have been analysed previously (Waldron, 2017)- for phenotype, sugar composition and digestibility. The 80 BC_1 plants harvested in 2013 were used for this investigation. However, 20 of the plants from the 2012 harvest were also used in the lignin analysis and all 48 from that harvest were included in the FTIR analysis to increase the sample size.

Straw was harvested from senescent plants and fractionated into a leaf fraction- containing leaf sheath and leaf blade, and a stem fraction containing nodes and internodes. These were milled separately to 2mm mesh size using a Pulverisette 19 knife mill (FRITSCH, Germany). Orbit and Revelation straws were also milled and used as references. All samples were sealed and stored in the cold room (4°C) for further analysis.

Additionally, when required, straw was also milled with a Ball mill and a Hammer mill. Knife milled Revelation straw was milled with a Pulverisette 5 ball mill (FRITSCH, Germany) using ceramic pods, 8 ceramic balls (1.5cm dia.) in each. Milling was carried out at 400 rpm with actual milling times of 10, 20, 30, 40, 50, 60 and 70 min generating samples with reduced crystallinity. The milling was carried out in short bursts to avoid over heating of the sample. Hammer milling was carried out on knife-milled samples using a Lab Mill 3100 (Perten, Sweden) to 0.5mm particle size.

All chemicals and regents were obtained from Fisher Scientific (Loughborough, UK) unless otherwise stated.

3.3.2. Sugar analysis

Sugar composition of biomass was determined by Saeman hydrolysis (Saeman et al., 1945). 30 mg of biomass was weigh out and put into a 50 ml Teflon cap tube (Pyrex, UK). 1 ml of 12 M sulphuric acid was added and incubated at 37°C for 1 h. 11 ml of distilled water was added to dilute the acid solution to 1M and the hydrolysis continued at 100°C for 2 h. This was followed by analysis of the monomer sugars by high performance anion exchange chromatography (HPAEC).

3.3.3. High Performance Anion Exchange Chromatography (HPAEC)

Monosaccharides were analysed by Reagent-FreeTM Ion Chromatography using a Dionex ICS-3000 (ThermoFisher, USA), electrochemical detection using ED 40 and computer controller. The CarboPac^{TM} PA 20 column (3 x 150 mm/; Dionex, USA) was used and the mobile phase was 10 mM NaOH with a flow rate of 0.5 mL/min. The injection volume was 10 µl and the column temperature was 30°C. The sample analysis was completed in 14min. The column was flushed at 1.0 mL/min with 200 mM NaOH between runs for 10min to remove any carbonates bound to the column.

Standard sugar samples were prepared from a stock consisting of 1g of each sugar dissolved in 0.5L of deionised water, which was then diluted to create

standards of $2gL^{-}1$, $1gL^{-1}$, $0.5gL^{-1}$ and $0.25gL^{-1}$. Typical standard curves for arabinose, galactose, glucose and xylose are shown in Figure 3.3. Approximate retention times (min) for individual sugars were: L-arabinose, 4.1 min; D-galactose, 5.2 min; D-glucose, 5.8 min; and D-xylose, 6.5 min. All samples and standards were diluted 1:100 in 10mM NaOH.

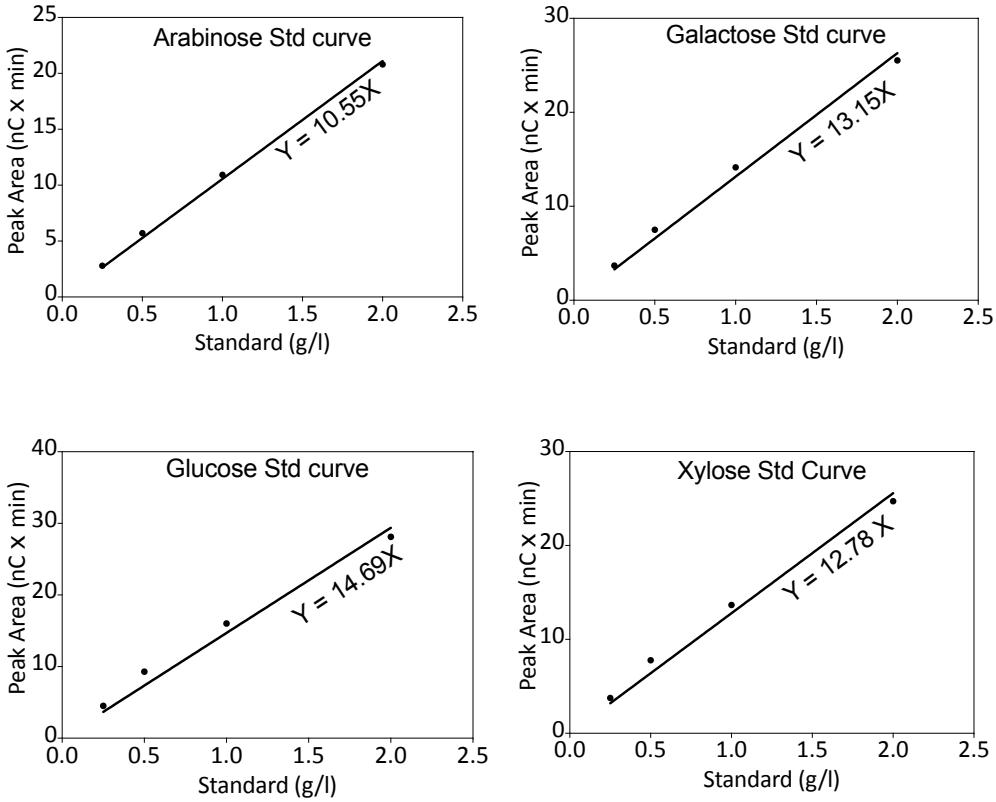


Figure 3.3 Typical HPAEC standard curve for arabinose, galactose, glucose and xylose.

3.3.4. Digestibility – manual saccharification assay

Straw (1g) was added to 20 mL 1% H_2SO_4 and autoclaved at 121°C for 15 min. The liquor (supernatant) was carefully removed after centrifuging for 5 min at 4472x*g* (Megafuge 16 centrifuge, Thermo Scientific, UK) and stored at -20^{o}C for future analysis. The residue was collected and rinsed 4 times with distilled

water after centrifuging at 4472xG for 5 min in between rinses, while discarding the subsequent supernatants. The final pellet was air dried for 96h and weighed. The sugar composition of the dried residue was assessed by Saeman hydrolysis and HPAEC (section 3.2.3).

Digestibility of the residue was assessed using a modification to the method described by the National Research Energy Laboratory (Selig et al., 2008). A stock of cellulase enzyme was prepared by dissolving 200mg of cellulase from *Trichoderma reesei* ATCC 20921 lot # 31M128V (Sigma, UK) into 20 ml of 50mM sodium citrate buffer (pH 4.8). This stock was made up as required on the day of use. 200mg of air-dried acid pre-treated biomass was placed into a 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 50ml Falcon tube to make 0.005 wt./wt. dispersion with cellulase activity of 40 FPU per g/ biomass. The samples were then incubated in a shaking incubator at 50°C and 150 rpm. Sugar release was monitored over 72h by HPAEC (0, 4, 24 and 72h) (section 3.3.3). Digestibility was expressed as glucose release at 72h/available glucose in the residue x 100 %.

3.3.5. Digestibility- automated saccharification assay

High throughput assays for biomass digestibility of the 80 BC_1 samples were carried out by Dr Leo Gomez at York University as described in (Gomez et al., 2010). The handling of the biomass for weighing into 96 wells and the processing of the reaction mixture was performed by a robotic station (Labman

Automation, UK), where the plant material was delivered to the desired well in the plates and weighed with a precision of ± 0.1 mg.

8x4 mg of each untreated knife milled straw samples were loaded into the 96-well plate. They were pre-treatment with 1% H_2SO_4 95^{O}C for 15 min, subsequently neutralised by adding an appropriate amount of alkali. 8 FPU of Celtec 2 (Novozymes, Denmark) was added and the plates incubated at 50^{o}C for 8 hrs. Aliquots from the hydrolysis were then analysed for the release of reducing sugar equivalents using Di-Nitro-Salicylic acid reagent.

3.3.6. Determination of lignin content by spectrophotometry

Lignin analysis was carried out as described by Hatfield & Fukushima using acetyl bromide (Hatfield and Fukushima, 2005) .

A lignin standard stock solution was prepared by dissolving 10 mg of lignin (Sigma Aldrich, UK) in 5 ml of 80% v/v dioxane (Simax, USA). Lignin standards were prepared by pipetting 0.2, 0.3, 0.4, 0.5 and 0.6 ml of the stock into 50 ml Teflon tubes (Pyrex, UK). Then 0.5 ml of 25%v/v acetyl bromide in glacial acetic acid was added to each tube and thoroughly mixed. The reaction tubes were incubated at 50°C for 30 min then cooled to room temperature. After cooling, 2.5 ml of glacial acetic acid, 1.5 ml of 0.3M NaOH (2.4 g NaOH dissolved in 100 ml of distilled water and made up to 200 ml) and 0.5 ml of 0.5M hydroxylamine HCl (6.95 g hydroxylamine HCl dissolved in 100 ml of distilled water and made up to 200 ml) were added. The final standard solutions were all made up to 10

ml with glacial acetic acid and absorbance was read at 280nm in a spectrophotometer-7315 (Jenway, UK) against a blank comprising of 0.2 ml dioxane with 0.5 ml acetyl bromide solution treated as above. This was performed to produce standard lignin concentrations of 400, 600, 800, 1000, 1200mg/l. A typical standard curve for the lignin assay is shown in Figure 3.4.

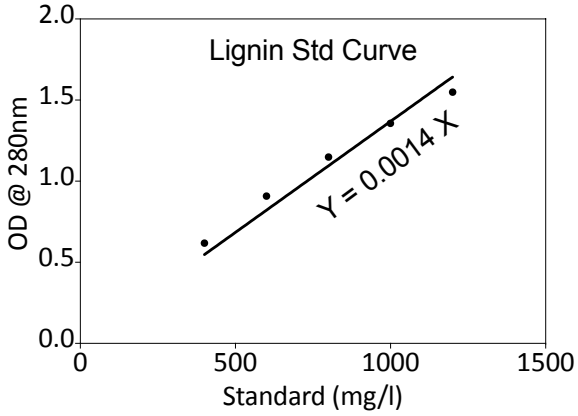


Figure 3.4 Typical lignin standard curve.

For analysis of samples, 25 mg of biomass was weighed out and put into a 50 ml teflon cap tube (Pyrex, UK). 1 ml of 25% v/v acetyl bromide in glacial acetic acid was added and incubated in a water bath at 50°C for 2 h with occasional mixing. The reaction tube was cooled to room temperature. Then 3 ml of glacial acetic acid was added to the reaction tube. The reaction solution was thoroughly mixed and centrifuged at 4,696 X *g* for 5 min. 0.5 ml of sample solution was then added to a fresh teflon tube (Pyrex, UK). 2.5 ml of glacial acetic acid, 1.5 ml of 0.3 M NaOH and 0.5 ml of 0.5 M hydroxylamine HCl were added and thoroughly mixed by vortex. The final solution was made up to 10 ml by adding glacial acetic acid and the absorbance was read at 280nm against

the blank comprising of 0.2 ml dioxane with 0.5 ml acetyl bromide solution treated as above.

3.3.7. Cellulose crystallinity analysis

The crystallinity of biomass was measured using X-ray diffraction (XRD) (D5000, Siemens/Bruker Gmbh) with a copper Kα X-ray irradiation source (λ = 1.541 Angstroms). Hammer milled fractions (~74 µm) were put into a circulator holder. The 2θ scans were performed from 5 to 50° with 0.05° increments. The baseline was adjusted by using ball milled wheat straw. Sample crystallinity was calculated based on the method proposed by Ibbett *et al.,* (Ibbett et al., 2013). The reflexed beam from the sample was measured as the function of incident angle. This reflexion profile was curved fitted with Gaussian shapes with linear baseline interpolation and calculated using data analysis by Excel (Microsoft, USA).

For determination of the total crystallinity, the amorphous profile was scaled to match the intensity of the experimental profile at the position of 19^{o} 2θ. The integral between 5 to 50^{o} 2θ was then subtracted from the total integral, to give the crystalline integral. The sample crystallinity was expressed in percentage terms by dividing the crystalline integral by the total integral.

An estimate of the fibril lateral dimension (d) was obtained by measurement of the width of the 200 reflections of Cellulose-I (2θ = 22.5^{o}), according to the Scherrer method, from equation (1). The integral breadth (IB) is defined as the

integrated intensity of the fitted peak divided by the peak height, where λ is the X-ray wavelength (0.1541 nm) and θ is the diffraction angle (Ibbett et al., 2013).

$$d = \frac{0.9.\lambda}{IB.\cos(\theta)} \qquad \text{----------1}$$

3.3.8. Fourier transformed infra-red spectroscopy (FTIR)

Fourier transform infrared measurements were performed using the method described by Sevenou *et al*., (Sevenou et al., 2002). FTIR spectra were obtained using a Bruker Tensor 27 spectrometer with a DTGS detector equipped with a ATR single reflectance cell with a diamond crystal (45° incidence-angle) (golden gate cell, Graseby-Specac Ltd, Orpington, UK). Five replicates of the powder from each milled sample were individually loaded on the ATR crystal and pressed down with a clamp. A uniform force of 50 cN.m was applied to the samples using a torque wrench during the readings. For each replicate, 128 scans with a 4 cm^{-1} resolution over the frequency range from 4000 to 650 cm^{-1} were averaged and referenced against a background spectrum of the empty crystal.

The five-replicate data were averaged and the following transformation was applied - standard normal variate (SNV), unit vector normalisation and baseline offset using a multivariate analysis software The Unscrambler X 10.3 (CAMO, Norway) with assistance from Bill Macnaughtan (School of Biosciences). Principal component analysis (PCA) to visualise relatedness or grouping of

among the samples was conducted in the region of 1200 nm- 800 nm which is defined as the fingerprint region for sugar composition.

3.3.9. Statistical analysis

The BC_1 plants were unique and as such there was limited biomass available. Thus, experiments in this chapter have been carried out at least in duplicate and with greater replication wherever possible. Data values are reported as the mean values with standard errors. The statistical analysis for correlation was carried out using Mac Numbers 4.0.5 using inbuilt function. R values ≥ 0.5 is considered a good correlation.

3.4. RESULTS

3.4.1. Phenotypic Analysis

Straw from 128 BC_1 wheat plants, representing a diverse range of introgression events, were analysed. The genetic providences (introgression event) for each of the 80 plants utilised in this study are shown in Appendix A (Table A.1). A phenotypic analysis of the 80 BC_1 plants was carried out to assess the impact of wheat wild relative introgression on straw biomass yield (Figure 3.5). Stem, leaf and total biomass (leaf + stem) of the BC_1 ranged from 2.6 to 22 g, 2.6 to 19g and 5 to 39g per plant, respectively. This gave leaf: stem ratios of between 0.5 to 1.4.

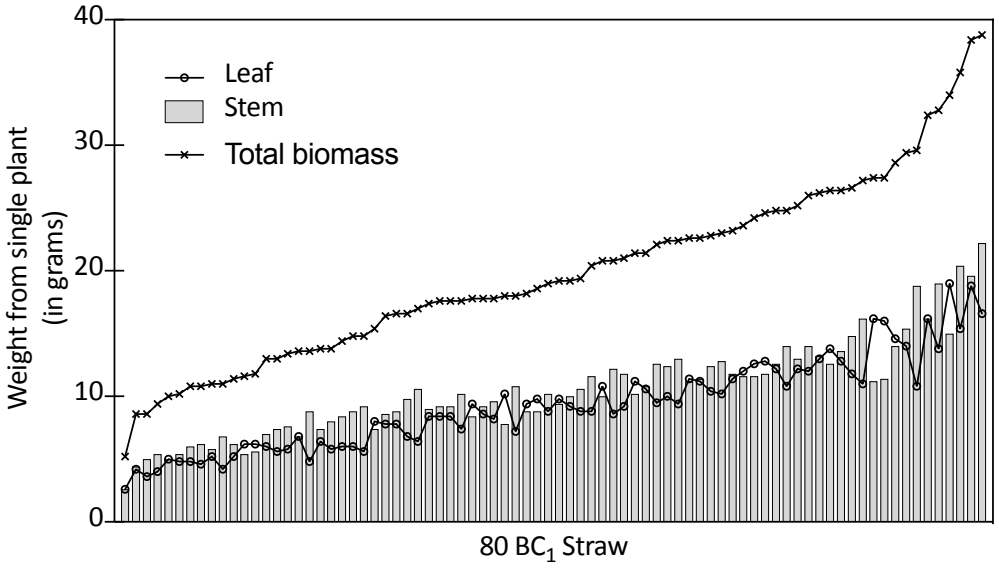


Figure 3.5 Total straw, and stem and leaf composition of 80 BC_1 plants.

3.4.2. Sugar Composition of BC_1 stem

Sugar composition of each milled BC_1 stem, along with that for 4 modern wheat lines stems and whole Orbit, was analysed using high performance anion exchange chromatography after Saeman hydrolysis. The glucose and xylose content of the BC_1 stem fractions ranged from 0.24 to 0.40 and 0.11 to 0.21 g/g biomass, respectively (Figure 3.6), giving a leaf: stem ratio of 0.5 to 1.4.

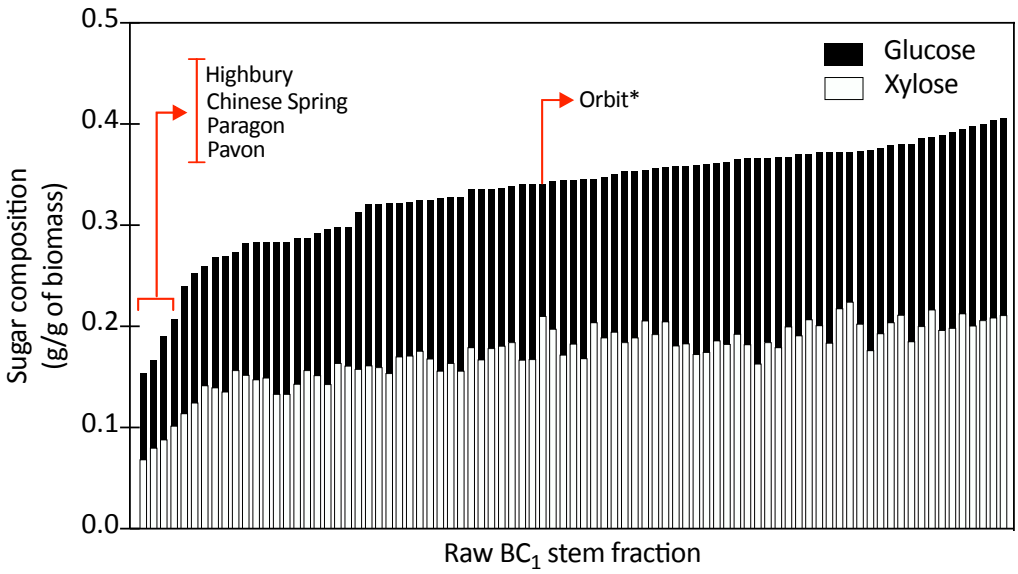


Figure 3.6 Sugar composition of the stems from 80 BC_1 plants and 4 modern wheat lines.

The stem was harvested and subjected to acid hydrolysis and sugars analysed by HPAEC. Results are expressed as g/g biomass. The results are the mean of technical replicates (n=2). * whole Orbit was used (leaf +stem).

3.4.3. Digestibility of the BC1 stems

3.4.3.1. Acid pre-treatment

The BC_1 stems were pre-treated with 1% H_2SO_4 at 121°C as described in section 3.2.4. The pre-treated liquor was removed after centrifugation. The pre-treated residue was washed with MilliQ water and air dried. The resultant dried pre-treated residue was then analysed for sugar composition by Saeman

hydrolysis (section 3.2.2) and HPAEC (section 3.2.3) the results are shown in Figure 3.7.

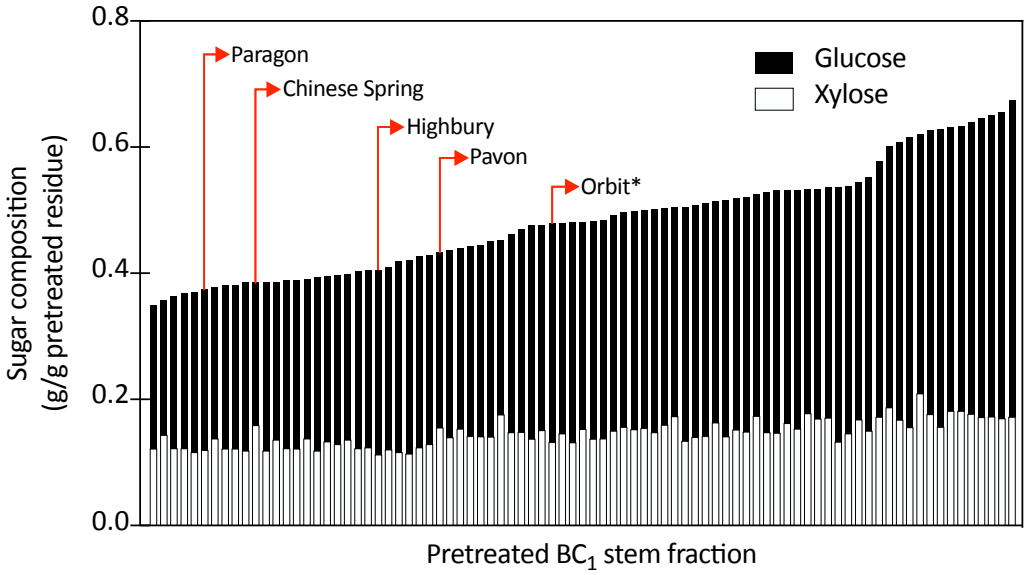


Figure 3.7 Sugar composition of the pre-treated residue from 80 BC_1 stems and 4 modern lines.

Pre-treated dried residue from stem tissue was subjected to acid hydrolysis and sugars analysed by HPAEC. Results are expressed as g/g residue. The results are the mean of technical replicates (n=2). * whole Orbit was used (leaf +stem).

The relative composition of glucose ranged from 0.35 to 0.67 and xylose from 0.12 to 0.17 g/g residue. These values will be used in the digestibility calculation in section 3.3.3,2. Pre-treatment resulted in a a relative reduction in xylose content but this was fairly constant among all the BC_1 plants. Glucose content was relatively increased in all cases based on visual analysis.

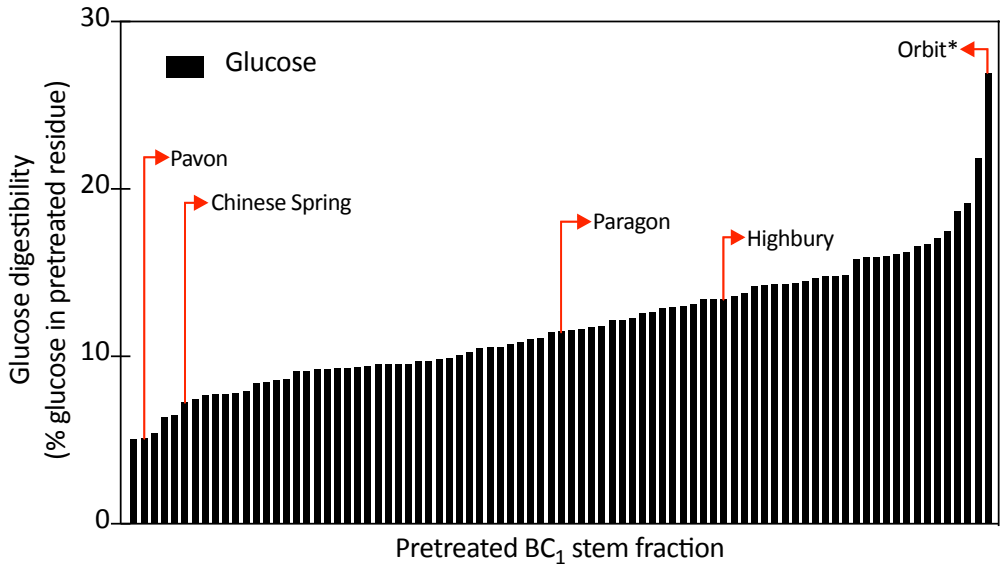
Even after the pre-treatment, almost half of the BC_1 residues still showed higher glucose contents than the Orbit straw. However, these were not just the same BC_1 plants that showed higher glucose than Orbit in the untreated stem (Figure 3.6), but a mix from all the BC_1 plants. There was no correlation found between the raw and pre-treated glucose compositions ($r = 0.012$), but a strong correlation of between the glucose and xylose content on the pre-treated residue ($r = 0.8$).

3.4.3.2. Digestibility assays

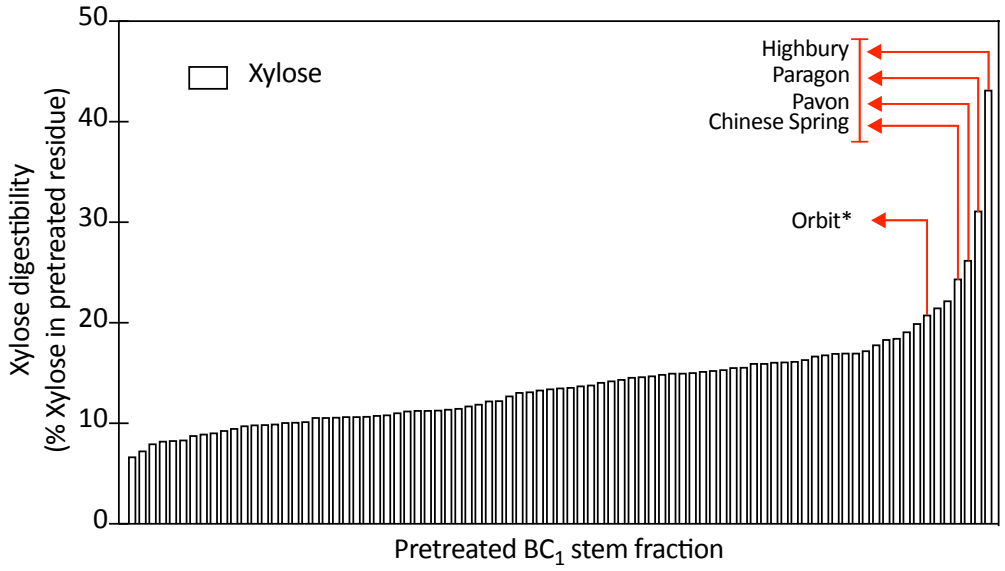
The effectiveness of enzymatic saccharification of the pre-treated residues was evaluated as the percentage of glucose and xylose released after 72h of enzyme hydrolysis. This was first carried out manually as described in section 3.3.4. The results are shown in Figure 3.8.

Glucose digestibility of the BC_1 residues ranged from 5 to 21.8%. Pavon (5%) and Chinese spring (7.2%) were at the lower end while Paragon (11.5%) and Highbury (13.4%) were in the middle range. Orbit which comprised of both stem and leaf fraction had the highest digestibility 26.9% (Figure 3.8a). Orbit's high digestibility could be due to presence of leaf fractions which are known to be more digestible than stem fractions.

The xylose release from the BC_1 residues ranged from 6.7 to 22%. The modern parental lines showed the highest digestibility with Chinese spring (24.4%), Pavon (26.3%), paragon (31.2%) and Highbury (43.2%). These were higher than Orbit (20.8%) which had leaf tissue as well (Figure 3.8b).



a



b

Figure 3.8 Digestibility of BC_1 stems and 4 modern lines measured by HPAEC.

Pre-treated dried residue from stem tissue was subjected to enzymatic saccharification and sugar release glucose (a) and xylose (b) released after 72h measured. Results are expressed as % available sugar in the pre-treated residue. The results are the mean of technical replicates (n=2). * whole Orbit was used (leaf +stem).

Digestibility is a complex trait to analyse and due to limitations on the amount of material available and time required for the manual saccharification assay, we were able to conduct this analysis only twice using this assay. For this

reason, the BC_1 stems were also sent for an independent high throughput automated saccharification assay at York University. Two BC_1 samples 5 and 43, and the parental lines were not used for this analysis.

The automated saccharification assay gave a digestibility ranged of 8 to 45 nmol/mg straw/hour (Figure 3.9). Here again Orbit gave the highest value. These values showed good correlations of r = 0.5 and 0.6 with the amount of monomeric glucose and combined glucose and xylose released (excluding Orbit), respectively from the manual digestibility assay.

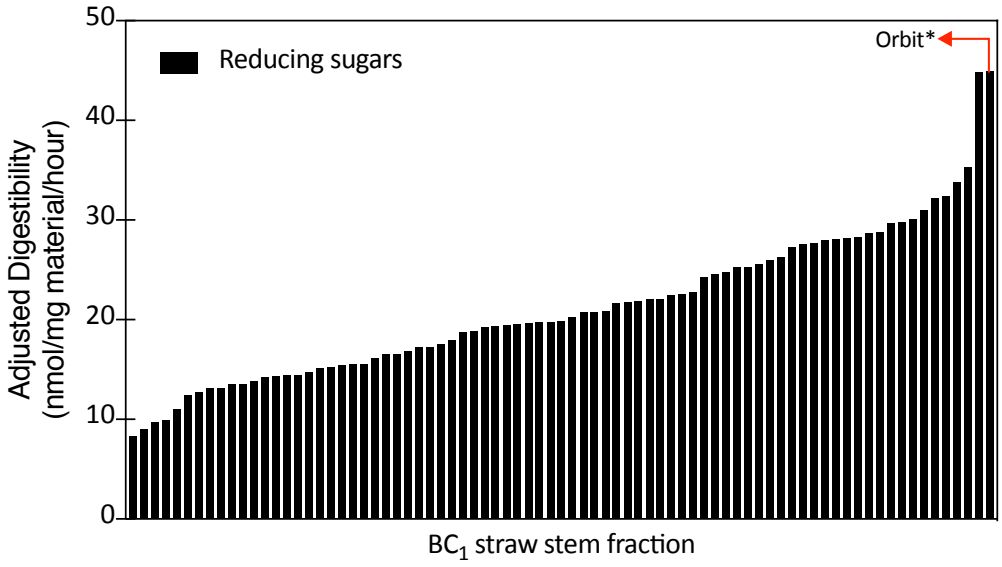


Figure 3.9 Digestibility of BC_1 stems as assessed by automated saccharification assay.

BC_1 stem tissue was acid pre-treated subjected to enzymatic saccharification for 8 hours using high throughput system. Digestibility was assessed as production of reducing groups (nmole/mgmaterial/hr). The results are the mean of technical replicate (n=8).

3.4.4. Lignin analysis by UV quantification

The results above demonstrated that the BC_1 plants had a very diverse range of digestibility. Lignin content in selected BC_1 plants was assessed as lignin has been known to influence digestibility. For this analysis, the BC_1 plants exhibiting a range of digestibility from both harvests -28 from 2012 and 20 from 2013 - were selected to assess differences in the two harvests.

To analyse the levels of lignin contained within the raw BC_1 stems, a direct UV quantification was undertaken on the milled stem as described in section 3.2.6, and expressed as a percentage of the stem biomass. Lignin content of 28 raw BC_1 harvested in 2013 ranged from 19.2 to 25.6% (Figure 3.10 a). Lignin content for the 20 raw BC_1 harvested in 2012 ranged from 17 to 23.7% (Figure 3.10 b).

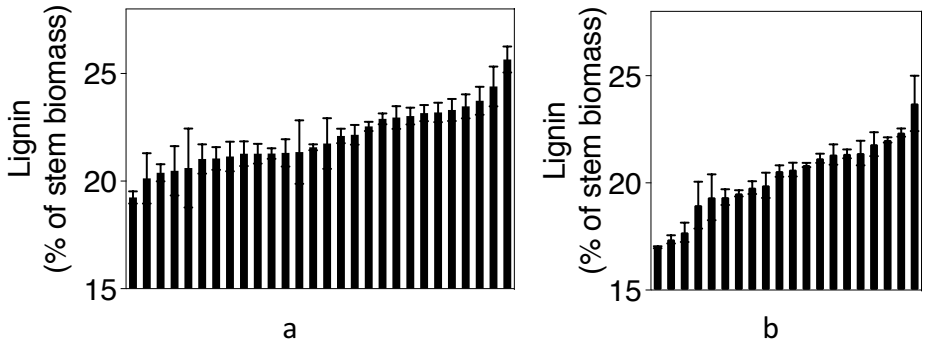


Figure 3.10 Lignin content of selected BC1 stems

Lignin content (a) 28 selected BC1 plants from 2013 harvest and b) 20 selected BC1 plants from 2012 harvest. Data represented is average of 3 technical replicates ± SD.

Lignin content of the combined 48 samples showed a strong negative correlation with both glucose and xylose digestibility: -0.68 and -0.55, respectively.

3.4.5. Determination of cellulose crystallinity by X-ray diffraction

With the observed variations in BC_1 phenotype, sugar composition, digestibility and lignin content, it was interesting to ask whether introgression had also resulted in any changes in the cellulose crystal structure. Crystallinity of selected stem and pre-treated residue samples was measured using X-ray diffraction (XRD). This was conducted for two reasons: to explore if XRD could detect any changes in the cellulose crystallinity of stem tissue caused by the pre-treatment, and to qualitatively confirm the crystallinity profile of ball milled Revelation straw, milled for a range of time, which will also be used in subsequent section 3.3.6. Following this the crystallinity of four more BC_1 pre-treated samples were measured, making a total of seven pre-treated BC_1.

Three samples were selected from the 2013 harvest for this preliminary study – one showing high digestibility (sample 22), one average digestibility (sample 7) and one low digestibility (sample 5). XRD was used to measure percentage crystallinity (CrI) and fibril dimension (nm). The stem samples were measured both in their raw state and after acid pre-treatment and hammer milling – Figure 3.11.

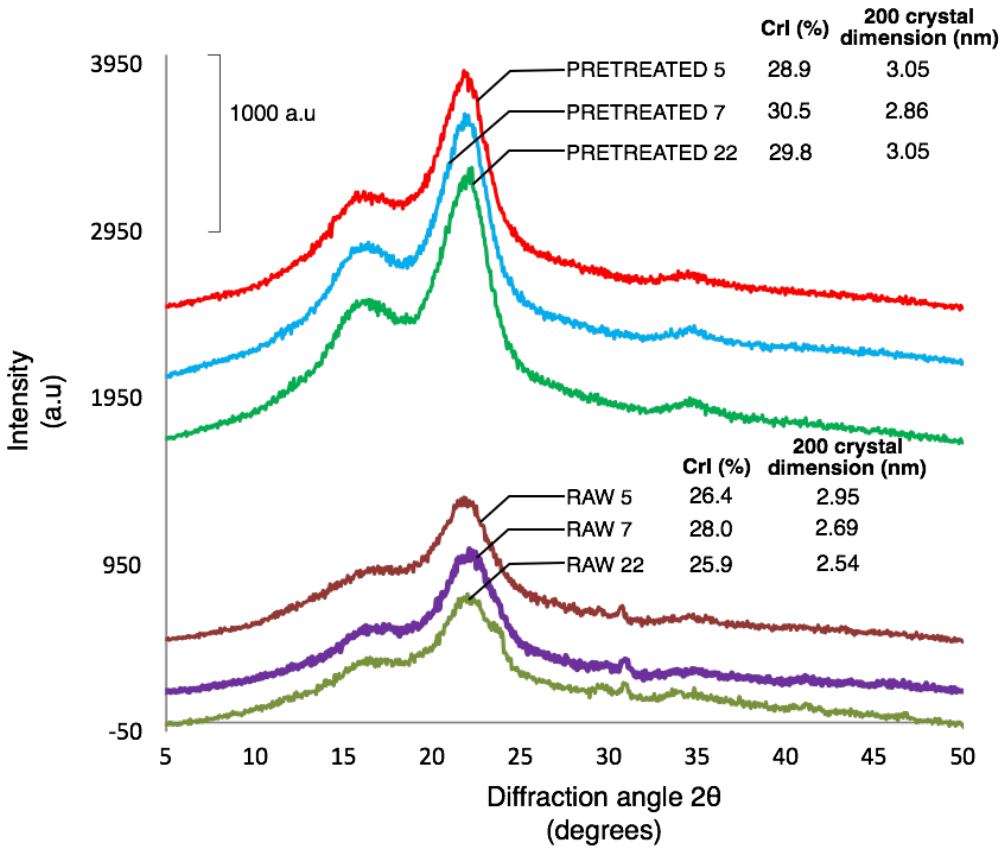


Figure 3.11 Cellulose crystallinity and fibril dimensions

Crystallinity measured by XRD of three BC_1 raw stems and pre-treated residues. Three plants were selected which showed low (5), average (7) or high (22) digestibility. Data has been offset for ease of visualisation.

The raw stems gave CrI values of between 25.9 to 28% but with no apparent correlation with digestibility. Similarly, crystal dimensions ranged from 2.54 to 2.95nm again with no apparent relationship to digestibility. Pre-treatment appears to have increased both the CrI and crystal dimension in all three cases but again with no apparent relationships with digestibility.

Following this, crystallinity of four more pre-treated BC_1 stems on the top and bottom end of the digestibility spectrum were analysed. Table 3.4 shows the CrI and crystal dimensions, along with their relative digestibility, for all 7 plants

analysed. Again, there was no apparent relationship between cellulose structure and digestibility of the residue (r = - 0.14).

Sample	Crystallinity (% CrI)	Crystal width (200) (nm)	Glucose digestibility (% glucose in pre-treated residue)
5	28.87	3.05	6.5107
7	30.45	2.86	11.6249
22	29.8	3.05	15.8914
33	28.79	3.05	5.0307
39	28.39	3.05	19.1564
71	27.63	2.86	13.0955
72	31.23	2.86	9.6866

Table 3.4 Crystallinity and digestibility of pre-treated BC_1.

Crystallinity (%) and crystal width (200) (nm) of the pre-treated residue from selected BC_1 plants from the 2013 harvest. Estimated error in %Cr = +/- 0.8%, estimated error in fibril width = +/- 0.2nm

3.4.6. Compositional analysis by Fourier Transformed Infrared Spectroscopy (FTIR)

FTIR was used as an independent non-destructive method of analysis to determine compositional changes in the BC_1. Analysis of 80 BC_1 samples in their raw and pre-treated states were analysed by FTIR and a principal component analysis (PCA) was carried out using multivariate analysis software (Unscrambler) to compare the two sets of spectra (Figure.3.12). This demonstrated that FTIR can clearly differentiate between raw and pre-treated samples, but it was not among the group samples.

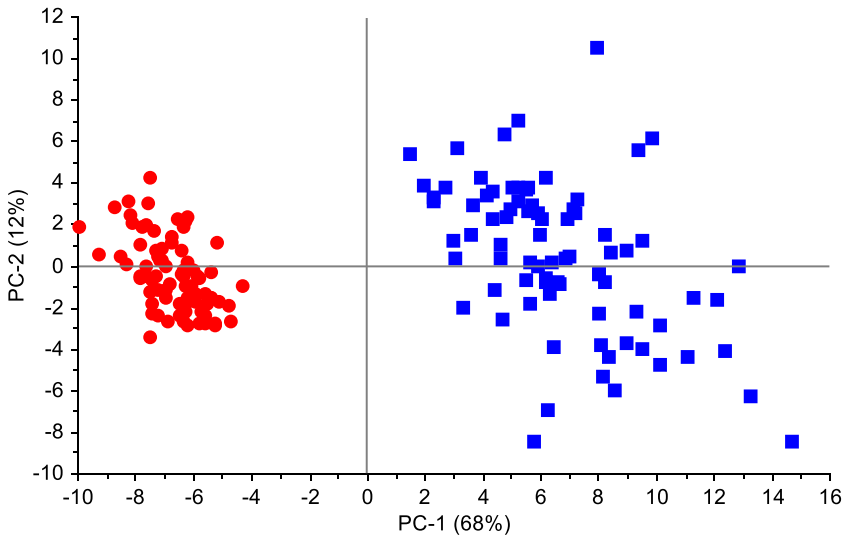


Figure 3.12 PCA of FTIR spectrum of raw and pre-treated stem tissue from 80 BC_1 plants.

FTIR spectra were obtained from the raw stems (Blue) and residues from the acid pre-treatment (Red) and subjected to spectral transformations in the polysaccharide region (800-1800 cm^{-1}). Data points represent the average of 5 replicates.

The potential to use this FTIR data to predict cellulose crystallinity was explored. Revelation straw was ball milled to give a range of crystallinities, these were measured by XRD (Table 3.5). The FTIR spectra from the ball milled sample was used to obtain potential IR crystallinity peak ratios 1429:897 (Kristensen et al., 2008) and 1430:898 (Ciolacu et al., 2011) (Table 3.5). These ratios were then correlated with the XRD values of the ball milled fractions. Correlation of the IR peaks with ball milled fractions' XRD values were 0.57 for 1429:897 and 0.599 for 1430:898.

Ball mill	Crystallinity (%CrI)	IR 1429/897	IR 1430/898
10 min	27.63	0.630150885	0.626586052
20 min	26.55	0.621144048	0.617852718
30 min	25.84	0.618828921	0.615692712
40 min	24.80	0.637574242	0.634196289
50 min	24.04	0.620217774	0.616560147
60 min	20.97	0.617228871	0.612764796
70 min	14.53	0.614057509	0.609833554

Table 3.5 Crystallinity and IR crystallinity of raw ball milled Revelation straw

Raw straw was ball milled for fixed times between 10 and 70min. Samples after milling were then assessed for crystallinity using XRD. The same samples were then subjected to FTIR and selected IR ratios determined.

A similar correlation analysis was carried out between the FTIR ratios and crystallinity (as measured by XRD) for the seven BC_1 samples (Table 3.6). These were 0.35 for 1429:897 and 0.36 for 1430:898 respectively.

Sample	Crystallinity (%CrI)	IR 1429/897	IR 1430/898
5	28.87	0.678090115	0.66992084
7	30.45	0.74811648	0.741007605
22	29.8	0.689243202	0.685117138
33	28.79	0.679655109	0.671534955
39	28.39	0.718994732	0.712285727
71	27.63	0.667715881	0.660916951
72	31.23	0.683439372	0.676409376

Table 3.6 Crystallinity and IR crystallinity of pre-treated BC_1 Revelation straw

Samples were assessed for crystallinity using XRD. The same samples were then subjected to FTIR and selected IR ratios determined.

These correlations were not particularly strong, but nonetheless, the FTIR ratios were used to generate an estimate of crystallinity of the pre-treated residues for all 80 BC1s. A correlation analysis between these ratios and digestibility of the samples gave values of r = 0.14 for peak ratio 1429:897 and 0.15 for peak ratio 1430:898.

3.5. DISCUSSION

The discussion of this chapter must be done in the light of the following limitations. Due to the very nature of introgression, viable seeds produced between modern and wild relatives are very limited. In the rare occasion where introgression results in more than one viable seed, each seed represents a unique recombination event giving raise to plants that are not biological replicates. Hence, the BC1 used in this research did not have biological replicates. However, higher back crosses are being made to generate plants which carry the majority of the genome of one species but also carry one or more chromosome segments from the other parental species. The plants can be self to produced stable homologous biological replicates produced.

First hypothesis was that wheat introgression will result in phenotypic variations or altered morphological make-up of the straw. This has been proven to be true by the observed phenotype and sugar composition of the straw stems. Phenotypic results demonstrated that the introgression events were successful in introducing a high degree of variation into the BC_1 plant straw biomass. This provided preliminary confidence to go forward with the exhaustive analysis of the impact of introgression on composition and digestibility. Studying the impact on biomass is important since pants with high biomass yield would allow for feedstock production on a smaller land footprint (Furtado et al., 2014). Identifying wheat straw leaf: stem ratio can impact compositional profiles and enzymatic digestion (Zhang et al., 2013). In general, pure leaf hydrolyses significantly better than pure stem as leaf tissues have

lower lignin content. Hence, breeding wheat with high biomass and high leaf: stem ratios is a strategy to improve saccharification yields (Townsend et al., 2017). However, leaf tissues also have lower cellulose content which could equate to a lower glucose yield (Collins et al., 2014).

The main objective of this thesis was to study the impact on digestibility and identify BC1s at both extremes of digestibility for identification of the genetic basis of this trait in future wheat breeding programme. Towards this goal, analysis of sugar composition and digestibility have been focussed on the more recalcitrant fraction of the straw – its stem. The high variance in leaf: stem (0.5 to 1.4) could have potentially interfered with digestibility purely based on differences of the more digestible leaf fraction in the straw, industrial digestibility. For this reason, in this thesis, stem digestibility has been assumed to be representative of the digestibility of the whole plant.

Overall, the raw stem glucose content was approximately twice that of the Xylose content. Similar sugar composition has been reported with 41% glucan and 17.7% xylan for wheat stem (Zhang et al., 2013). An interesting observation of the raw BC_1 sugar composition is that all the BC_1 stems had higher sugar content than the stems of the four modern parental lines used in the introgression. This observed range in BC_1 stem composition is a clear indication of introgression's positive impact on the sugar composition. Also, almost half of the BC_1 showed higher sugar composition than standard Orbit straw which was a mix of both stem and leaf. Hence, the effect of introgression has not been

limited to the phenotypic variation observed, but it extends to the stem cell wall components, such as cellulose and hemicellulose, as well.

The second hypothesis was that the BC_1 stems will respond differently to acid pre-treatment, enzymatic saccharification and hence, possess a range of digestibility. This has been proven to be true by the observed sugar composition after pre-treatment and range of digestibility shown by the pre-treated BC_1 stems. After the acid pre-treatment, the glucose content in the residue was increased. This is characteristic of an acid pre-treatment due to a reduction in hemicellulose (Barisik et al., 2016; Djioleu and Carrier, 2016; Kootstra et al., 2009). However, there was no correlation between the raw and pre-treated glucose compositions ($r = 0.012$). This was an early indication that BC_1 with similar high or low sugar composition may also possess different recalcitrance, as shown by their different responses to the acid pre-treatment.

Studying sugar composition is important because deconstruction of plants with high cellulose or glucose content per weight would translate to larger amounts of available glucose for subsequent conversion to biofuels or biomaterials (Furtado et al., 2014). However, xylose should not be neglected as this can contribute to fermentable sugar in the feedstocks (straw) and also gives a good indication of the hemicellulose content. Additionally, hemicellulose composition can play an important role in digestibility of lignocellulose by reducing the crystallinity of the feedstock.

More important than the sugar composition of the stem is the digestibility, as the amount of soluble carbohydrates produced per unit biomass can help identify highly reactive or recalcitrant feedstock samples, which could then be investigated in more detail. This was measured using two different independent assays. Irrespective of the measurement method the BC_1 samples showed a good variation in digestibility.

Overall, the mild acid pre-treatment and enzyme hydrolysis was shown to be suitable for screening for variations in digestibility. This mild assay along with potential hornification of the pre-treated residue due to air-drying could be responsible for the relatively low digestibility values obtained. A harsher assay could have enhanced the digestibility but at the expense of masking the variation. Commercial treatment would normally employ a much more severe process with a much higher glucose digestibility from wheat straw. For example, yields of 96% and 98% were observed after pre-treatment at 170 °C for 30 min with 50mM maleic and sulphuric acid respectively (Kootstra et al., 2009). Similarly, yield of 85% was observed after a steam explosion pre-treatment with 0.9% sulphuric acid at 180 °C for 10 minute (Ballesteros et al., 2006). There was also a clear contrast between the glucose and xylose digestibility of the modern parental lines, with the modern lines having highest xylose digestibility.

The full impact of introgression or parental lines on the convertibility of xylan and glucan, was revealed when the BC_1 digestibility values were studied against

the genetic background. For example, crosses with the parental variety paragon resulted in glucose digestibility of between 5% (sample 33) and 19% (sample 45) depending on the wild relatives it was crossed with. In the same way although the modern lines showed highest xylose digestibility, crossing with wild relatives resulted in a spectrum of xylose digestibility. This showed that the digestibility of the BC_1 had been impacted by the wild parental lines used in the crosses.

BC_1 stems were also sent for an independent high throughput automated assay at York University. We studied the correlation between the two digestibility assays to improve confidence in the digestibility raking of the BC_1s. For this, it was importance to understand how digestibility is measured in the two assays. The procedural differences are summarised in Table 3.7. Thus, whilst the two assays may not be directly comparable they both provide useful data.

	Pre-treatment	**Liquor**	**Neutralisation**	**Hydrolysis**	**Measureing**
Automatic saccharification	1%H_2SO_4 95 ° C, 15min	Carried over to hydrolysis	With alkali	8FPU, Celtec 2 (inc. Xylanase), 8hr	Reducing sugar/time (DNS assay)
Manual saccharification	1%H_2SO_4 121 ° C, 15min	Removed, residue washed	n.a	40FPU, Celluclast (no Xylanase), 72hr	Monomeric sugar (HPAEC)

Table 3.7 Differences between the manual and automatic saccharification assays.

The automated assay measured reducing groups and digestibility is expressed as reducing sugars released - dimers, trimers, oligomers. While it measures all

sugars not just glucose and xylose, it also provides a more accurate estimate of the amount of enzyme activity as a whole. On the other hand, the manual assay measured free glucose and xylose yields which are more suitable indicators for fermentation potential. This meant that correlation with the manual digestibility expressed as percentage of available sugars will not be ideal. Hence, correlation against manual digestibility expressed as monomeric sugars released was used instead. This gave a good correlation: r = 0.5 with just glucose release, and r= 0.6 with a combined glucose and xylose release (excluding Orbit). Next, we compared the top 10 and bottom 10 BC_1 plants from the two digestibility assays – automated and combined glucose and xylose manual assay. The two assays shared 6 BC_1 plants in the top 10 list and 3 BC_1 plants in the bottom 10 list, which is in line with their correlation value. More importantly, the top 10 and bottom 10 BC_1 plants were mutually exclusive in their respective categories, i.e. none of the top 10 samples from automated assay came up in the bottom 10 in the manual assay and vice versa. Hence, they highlight similar grouping or ranking of the BC_1 digestibility. Following this digestibility ranking of the BC_1 was based on glucose digestibility expressed as percentage of available glucose in the pre-treated stem.

The third hypothesis was that differences in digestibility or the introgression effect could be accompanied by structural changes that are related to digestibility. The results showed that digestibility was only correlated to lignin content. The hypothesis was a logical assumption as many characteristics determine digestibility of lignocellulosic material, such as lignin content and

cellulose crystallinity (Chang and Holtzapple, 2000). Hence, variability in digestibility could hint towards a possible structural basis - crystallinity of the sample and/or lignin content. Hemicellulose, which is also a major structural component could also play a role in straw digestibility. The hemicellulose degradation profile of select BC_1 stems using differential scanning calorimetry (DSC) has previously indicated that the hemicellulose content or composition may play a significant role in stem digestibility of the BC1 (Waldron, 2017).

The lignin content of the combined 48 samples showed a strong negative correlation with both glucose and xylose digestibility: -0.68 and -0.55, respectively. Such negative correlation between lignin content and digestibility has previously been reported (Lindedam et al., 2012; Wu et al., 2013). In addition to a direct structural impact on digestibility, it is known that lignin can irreversibly bind cellulase enzymes, thereby reducing their catalytic potential for sugar release and making its reduction highly desirable (Esteghlalian et al., 2000). The specific location of lignin and the mechanism by which the lignin interacts with other components of the cell wall, could also be responsible rather than the total amount of lignin alone. However, there are other reports that did not find any relationship between lignin content and digestibility (Habib et al., 1995). Regardless of whether lignin is the deciding factor in all lignocellulose digestibility, it can be seen here that introgression has impacted the lignin content of the BC_1 stem and at least for the studied BC_1, high lignin content appears to have a negative impact on digestibility.

It was evident for all samples that acid pre-treatment resulted in an increase in crystallinity. Increase in crystallinity is at least partly due to the removal of hemicellulose by the acid pre-treatment and preferential degradation of amorphous cellulose (Sannigrahi et al., 2008). There was also an increase in the 200 dimensions fibril that was probably due to an annealing effect known to occur with dilute acid pre-treatment of cellulosic materials (Sun et al., 2014). The 200-dimension also gives an indication of fibril lateral size and perfection. A main peak at 22.3°, a broad peak at 15.7° and a small peak at 34.8° indicate that cellulose is in cellulose I crystal form (Cheng and Wang, 2013). These peaks were observed in the XRD patterns of both the raw and pre-treated straw. This information is valuable for developing enzyme mixtures optimized for the hydrolysis (Thygesen et al., 2005): exo-glucanase for crystalline cellulose and endo-glucanase for amorphous cellulose. Hence, pre-treatment has resulted in increased crystallinity and crystal fibril dimensions without changing the crystal state. However, crystallinity of the pre-treated BC_1 had no correlation (r= -0.14) with digestibility. A similar observation was made with the crystallinity estimate from the FTIR data (on all 80 BC1 samples), a correlation of 0.14 for peak ratio 1429:897 and 0.15 for peak ratio 1430:898 with digestibility. However, the 2mm particle size of the stem sample could have imparted a degree of variation as the sample would be non-homogeneous. Due to the limited amount of sample available after the wet analysis, particle size of samples was limited to the 2mm.

Phenotypic data, such as stem biomass showed no correlation with glucose (r=-0.006) or xylose (r= -0.174) digestibility, showing that digestibility was not defined by or related to this morphological trait. Xylose digestibility showed no correlation with glucose (r= -0.07) and xylose (r= -0.13) content in pre-treated residue. However, glucose digestibility had a strong negative correlation with glucose (r= -0.57) and xylose (r= -0.48) content in pre-treated residues. Such negative influences of cellulose content on conversion has been reported by Habib *et al.,* for 15 wheat straw cultivars (Habib et al., 1995). Lindedam *et al.,* suggested this result may be due to the positive correlation seen between cellulose and hemicellulose contents and the negative impact hemicellulose has on sugar release (Lindedam et al., 2012). Hemicellulose has been reported to restrict enzymatic access to cellulose (Jeoh et al., 2007; Meng et al., 2013), and since extra xylanases were not added in the experiment, hemicellulose could indirectly cause the low sugar convertibility at high cellulose levels. This is also agreeable with the strong correlation between glucose and xylose content (r = 0.8) of the pre-treated BC_1 residue (section 3.3.3.1). Additionally, cellulose levels have been reported to have a negative impact on biomass enzymatic saccharification, likely due to the positive correlation with cellulose CrI (Wang et al., 2016). However, there are also studies indicating that cellulose content tends not to always determine differences in digestibility (Murozuka et al., 2015).

The fourth hypothesis was that the genetic basis for digestibility traits can be linked to introgression segment(s). The genetic analysis conducted by Bristol

showed that more at this stage of crossing, it was difficult to link the digestibility traits with introjection segments (as explained in appendeix A-Genetic analysis of BC_1 samples).

However, linking the improvement of straw biorefinery traits with the grain yield is very important since it could potentially move practices away from planting separate crops. Most breeding programs have historically focused on improving crops for food. Hence, this ideology could be important in combining energy and food security needs. Improving the digestibility could also improve the animal-feed value of straw (rumen digestibility) and in turn add value for the farmer.

Continuing research in this field could set the foundation for the development of superior feedstocks combined with efficient food production. Such research may move us from a food vs. fuel debate towards a food and fuel debate.

CHAPTER 4 IMPACT OF MIXED FEEDSTOCK ON BIOREFINERY TRAITS

4.2. INTRODUCTION

Lignocellulosic biomass, such as energy crops and agricultural residues represent a potential biorefinery feedstock to produce fuel or other chemicals. Some of the challenges to making this feasible and economical includes securing a year round sustainable supply of feedstock as they are likely to be seasonal with annual variability in yields (Yue et al., 2014) and may thus have limited availability and supply (Balat, 2011); overcoming recalcitrance of the feedstock in terms of saccharification to fermentable sugars and this requires pre-treatment to disrupt the lignocellulose matrix (Yue et al., 2014); and reducing inhibitory compound formation during pre-treatment that could interfere with the subsequent enzymatic hydrolysis and/or fermentation (Ko et al., 2015).

The pressing need for a better biomass feedstock system has been recently highlighted in the Request For Information (RFI) from the US Department of Energy (DOE, 2016). The RFI, predicts that existing feedstock supply systems may be unable to reliably provide high-quality feedstock at the increasing volumes required over the next few decades for a growing bio-economy. The report also suggests that new logistics and processing technologies, which may involve central processing and distribution centres such as feedstock depots, may be required. Additionally, biofuel is on the agenda of many Governments, especially in the light of the 2015 Paris submit and with the EU aiming to shift

more road transport to marine transport with a new renewable fuel mandate which includes marine transport (Moirangthem, 2016). This again may require improvements in feedstock delivery, availability and handling procedures. Hence, technologies to provide sustainable feedstock in large enough quantities to make a biorefinery process economical are desirable. One potential approach to overcoming the challenges associated with feedstock availability is to utilize mixed feedstocks within a biorefinery. In such cases, it is important to understand the impact of mixed substrates on process efficiency.

There have been limited studies on the effect of mixing feedstocks on process efficiency. One study has looked at the effect of mixing municipal solid waste paper and corn stover in a 1:1 ratio in condition with an ionic liquid pre-treatment (Sun et al., 2015). This study monitored the effect of mixing on saccharification and reported a slight improvement in glucose yield. Another study mixed eucalyptus residues, wheat straw and olive tree pruning at a 50:25:25 ratio using a hydrothermal pre-treatment and monitored sugar recovery in both the pre-treated biomass and liquor and inhibitor composition (Silva-Fernandes et al., 2015). This study showed that mixtures of lignocellulosic materials can be efficiently processed by auto hydrolysis and generate a consistent product composition that was independent of the different proportions of each feedstock. Concentration of inhibitors were also reported to be low for the three different mixes (furfural 0.33-0.49g/l and HMF 0.17-0.29g/l). A further study examined the effect of mixing aspen, balsam and

switch grass in 1:1 ratios using an acid pre-treatment and monitored saccharification and inhibitor composition (Brodeur-Campbell et al., 2012) and found glucose yields for the all mixed feedstocks were very similar to the predicted values. Oke *et al* provides a more in-depth study on the prospects and challenges of mixed feedstock biorefineries (Oke et al., 2017).

4.2.1. Feedstocks

Within the context of a UK or EU biorefinery, the major feedstocks are likely to include agricultural waste (wheat straw) mixed with dedicated energy crops such as willow and *Miscanthus*. Wheat straw is a promising feedstock for bioethanol production in Europe due to its large production and high carbohydrate content (Alvira et al., 2016). The majority of agricultural straw is derived from wheat in nearly all regions of the UK except Scotland where barley prevails (Di Maio and Turley, 2014). For bioenergy crops in Europe, research has focused on *Miscanthus* (Lewandowski et al., 2000) with the sterile hybrid *Miscanthus × giganteus* currently being the main commercially exploited species of this genus for biomass purposes due to its high yield potential. Willow has many desirable characteristics as a feedstock and for biomass production. With its coppicing ability and vigorous juvenile growth, it can produce high biomass yields (>11t (oven dried tones) ha^{-1} $year^{-1}$) on marginal land that is not suitable for conventional food crops (Volk et al., 2011). *Miscanthus* and short rotation coppice derived from willow have been the most widely planted species in the UK. Productivity of crops considered here ranged from 0.1 to 1.75 Mg/ha/yr. (dry basis) for wheat straw (crop residue), 14 to 16

Mg/ha/yr. (dry basis) for willow (woody crop), to as high as 1-44 Mg/ha/yr. (dry basis) for *Miscanthus (*Herbaceous crop) (Laser and Lynd, 2014).

	Cellulose	**Hemicellulose**	**Lignin**
Wheat	33-40	20-25	15-21
Willow	30	45	25
Miscanthus	31-55	25-38	6-13

(Di Maio and Turley, 2014)

Table 4.1 Composition of wheat, willow and *Miscanthus*.

In this research, we have utilised microwave hydrothermal pre-treatment. Some of the advantages of employing microwave heating over conventional heating include reduction of process energy requirements, uniform and selective processing, and the capacity to start and stop the process instantaneously (Keshwani and Cheng, 2010). The heating step is a faster process, since heat is generated internally via direct interaction between the electromagnetic field and components of the heated material. It selectively heats the more polar molecules and this unique heating feature results in an improved disruption of the recalcitrant structures of lignocellulose. The electromagnetic field also helps to accelerate the destruction of crystalline structures and changes the super molecular structure of lignocellulosic material improving its reactivity (Tomás-Pejó et al., 2011).

To the best of our knowledge there have been no reports in the literature regarding the potential impact of mixing wheat straw, willow and *Miscanthus*

on process efficiency. Thus, in this chapter, we have studied the impact of wheat straw, willow and *Miscanthus* in 2 components 1:1 (w/w) mixtures on saccharification yield and kinetics, inhibitor production and energy requirements using hydrothermal pre-treatment.

4.3. MATERIALS AND METHODS

4.3.1. Plant Material

Wheat straw – *var.* Revelation – harvested in 2015, was obtained from the Sutton Bonington farm (University of Nottingham, UK). Willow – *var.* tora and *Miscanthus* – *var.* goliath both harvested in 2016 were kindly provided by Prof Iain Donnison (Aberystwyth University, UK). Wheat straw was air-dried, while willow and *Miscanthus* were both oven dried at 60° C. The three feedstocks were milled separately to 2mm mesh size using a Pulverisette 19 knife mill (FRITSCH, Germany) and stored at 4° C in bags with airtight seals.

4.3.2. Compositional analysis

4.3.2.1. Sugar analysis

Sugar composition was measured by Saeman hydrolysis as described in section 3.2.2

4.3.2.2. High Performance Anion Exchange Chromatography (HPAEC)

Analysis of sugars was performed on a Dionex ICS-3000 (ThermoFisher, USA) as described in section 3.2.3

4.3.3. Hydrothermal pre-treatment and enzymatic saccharification

4.3.3.1. Pre-treatment

Pre-treatment was conducted in a Monowave 300 microwave generator (Anton Paar, Germany). 1 g of sample was mixed with 10 mL of MilliQ water, left at room temperature for 2 min and then heated at different combinations

of temperature and holding times ranging from 180-220° C in steps of 20° C and 5-20 min in steps of 5 min, respectively as shown in Table 4.2.

Temp (° C)	Holding time (minutes)		
180	5	10	20
200	5	10	20
220	5	10	20

Table 4.2 Microwave pre-treatment regimes for mixed feedstocks

The resultant pre-treated mixture was centrifuged for 10 min at 4472xG and the supernatant (hydrolysate) decanted. The hydrolysate composition was analysed for sugars using HPAEC (section 3.2.3) and after measuring pH to gve an indication of acids formation, was stored at -20° C. The pellet (residue) was air dried for 72 hr, weighed and stored at 4° C for further analysis.

Pre-treatment severity (log R_0) which provides an approximate indication of pre-treatment conditions was calculated using equation 1 (Overend and Chornet, 1987):

$R_0 = t.e\text{^}(\frac{T-100}{14.75})$---------- (1),

Where, *t* is the time (min) and T the temperature (°C).

Pre-treatment energy for the different regimes was calculated using power data obtained from the microwave generator (Anton Paar, Germany) using equation 2:

Power (W) = $\frac{Energy\ (j)}{Time\ (s)}$- ---------- (2);

The Monowave provides power per second measurements making, power = energy. Hence total pre-treatment energy was calculated as shown in equation 3:

$$\int_0^t energy\ (kj) = \int_0^t \frac{Power(w)}{1000} \text{ ---------- (3);}$$

where t = pre-treatment time (heating + holding time).

4.3.3.2. Enzymatic saccharification

Pre-treated biomass residue, was hydrolysed using Cellic® CTec3 (Novozyme A/S, Demark). This was carried out using a method based on that described by the National Research Energy Laboratory (Selig et al., 2008). Enzyme solution was made up using 5mL of Cellic® CTec3 in 1 L of 50mM sodium citrate buffer at pH 3.76 (FPU not calculated due to commercial reason). 40mL of this solution was added to 200mg of sample and incubated at 50° C for 72 hr at 150rpm (MaxQ 4358 shaking incubator, Thermo Scientific, UK). This concentration of enzyme was used to avoid the enzyme being a limiting factor in digestion. Aliquots were taken at 6 different time points – 15 min, 3 hr, 6 hr, 24 hr, 33 hr and 72 hr and monomer sugar composition assessed using HPAEC (section 3.2.3). The sugar concentration present in the enzyme solution was also analysed and subtracted to allow an accurate calculation of the % glucose released and latter was expressed as a % of available glucose.

Kinetic analysis of the enzyme hydrolysis step was carried out assuming the reaction followed first order behaviour. This was found to be valid for the data sets of this study, thus avoiding the need for more complex kinetic algorithms, and allowing simple comparison of kinetic parameters (Ibbett et al., 2013). A

non-linear optimization method was used to fit a simulated curve to the experimental points using the solver function in Microsoft Excel 2016. A function was used describing a single first-order exponential growth process, according to equation (4), where S is the glucose yield at time t, x is the rate constant and A is the final equilibrium glucose yield.

S=$A(1 - e^{-xt})$ ---------- (4)

4.3.4. Determination of the effect inhibitory compounds on yeast metabolism

Yeast metabolism was measured using a Phenotypic Microarray Omnilog reader (Biolog, USA) as described by (Greetham et al., 2014). A redox reporter within the assay (dye D) permits analysis of metabolic activity defined as redox signal intensity.

S. cerevisiae strain NCYC2592 NCYC (National Collection of Yeast Cultures, UK) was incubated at 30°C for ~48 hr in yeast peptone broth (YPD- 20 g/L glucose, 20 g/L bacto peptone, 10 g/L yeast extract). Cell density was adjusted using a transmittance meter to reduce 100% transmittance of MilliQ water to ~65%. 1 mL of this was mixed with IFY buffer™ (Biolog, USA) to produce the working cell suspension (~5 x 10^4 cells/mL). The composition of each well was as follows: 20.8 µL of hydrolysate, 9 µL of glucose (80% stock), 0.2 µL of dye D (Biolog, Hayward, CA, USA) and 90 µL of cell suspension. This gives a glucose concentration of 6% in each well. Control consisted of 2.8 µL YNB (28%), 18 µL

MilliQ water, 9 µL glucose (80% stock), 0.2 µL of dye D and 90 µL of cell suspension. The plates were then placed in the Omnilog reader and incubated for 48hr at 30°C under aerobic condition with readings every 15 min.

Yeast metabolism was represented as % inhibition according to equation (5)

Metabolic inhibition= $\int_0^t \frac{Redox\ intensity\ of\ sample}{Redox\ intensity\ of\ control} X100$---------- (5); t = 48 hr.

4.3.5. Determination of the effect of inhibitors on yeast growth

Yeast growth was measured using a FLUOstar OPTIMA (BMG LABTECH, Germany) at 600nm, under identical growth conditions as for phenotypic microarray assay (section 4.2.4), monitored for 48h at 30°C with a reading every 15 min.

4.3.6. Analysis of inhibitor composition

Inhibitors were quantified as described by (Wilkinson et al., 2014). Furfural and 5- hydroxyl methyl furfural in the hydrolysate were measured by HPLC using UV detection at 280 nm (2695 HPLC system and 996 Photodiode Array Detector, Waters, USA), with UV spectra for secondary confirmation. The chromatographic conditions were as follows: Techsphere ODS C18 column (5 µm, 4.6 mm × 250 mm; HPLC Technologies, UK) at ambient temperature. The mobile phase was a binary mixture of 1% acetic acid (solvent A) and methanol (solvent B) with an overall flow rate of 1.0mL.min^{-1} and compounds were detected at 280nm. The system was operated in gradient mode, ramping from 20% to 50% methanol over 30 min with a 100% methanol column cleaning

phase and a 9-min re-equilibration period. The sample injection volume was 10 µL. Data were recorded using Millennium Chromatography software (Waters, USA). Quantification was performed by comparison of peak areas of authentic standards (0.025–0.4 g/L concentration range) of HMF and Furfural.

4.3.7. Statistical analysis

The data were analysed by one-way Analysis of Variance (ANOVA) using Genstat 18.1 (VSN International Ltd). For the comparison of observed and expected values the three predefined individual feedstock values were included to directly compare each mixed feedstock to the average of its two components when they were used alone. The comparative ANOVA was conducted by Jim Craigon (School of Biosciences). A significant effect would indicate that the components were responding differently when mixed with other feedstocks. All experiments were carried out in triplicate.

4.4. RESULTS

Prior to this study, a preliminary scoping experiment was carried out using three-individual feedstocks to establish their individual responses to different pre-treatment regimes at 180, 200 and 220°C for 5, 10 and 20 min reaction time. The severity factors for this scoping work ranged from 3.05 - 4.83 (Table 4.3).

	5min	10min	20min
180 °C	3.05	3.36	3.66
200 °C	3.64	3.94	4.25
220 °C	4.23	4.53	4.83

Table 4.2 Pre-treatment severity factors

The extent of lignocellulose matrix disruption and polymer degradation were assessed from the liquor and pre-treated residue sugar composition (Figure 4.1a-b to 4.3a-b), while the effect on enzymatic digestibility was measured by enzyme hydrolysis (Figure 4.1c to 4.3c).

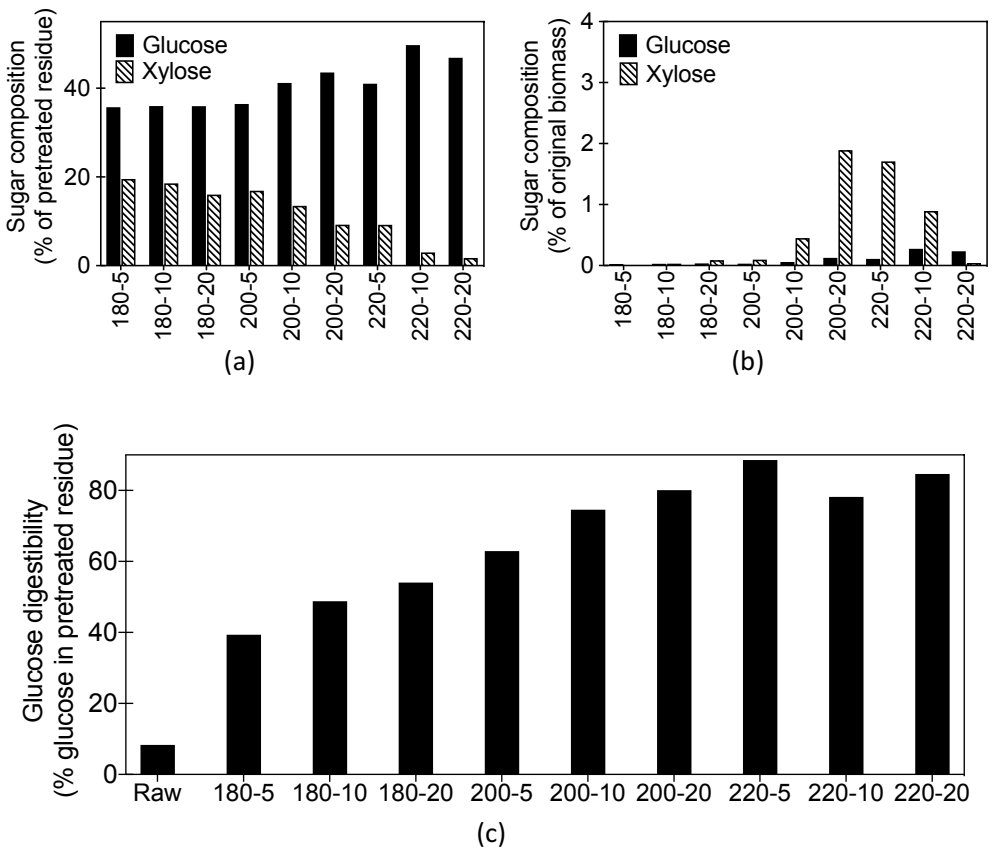


Figure 4.1 Effect of varying temperature/time during pre-treatments of wheat straw

(a) Sugar composition of pre-treated residue, (b) Sugar composition of hydrolysate liquor after pre-treatment and (c) Digestibility after 72 hours expressed as % of available glucose in the pre-treated residue (n=1).

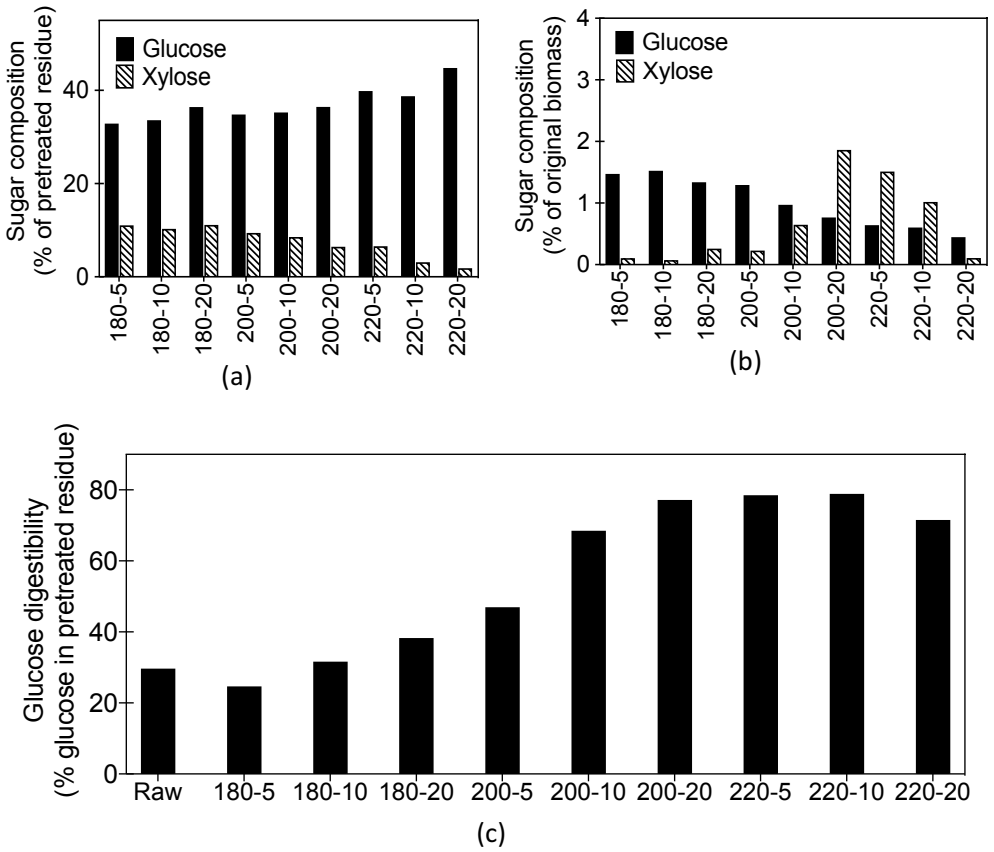


Figure 4.2 Effect of varying temperature/time during pre-treatments of willow

(a) Sugar composition of pre-treated residue, (b) Sugar composition of hydrolysate liquor after pre-treatment and (c) Digestibility after 72 hours expressed as % of available glucose in the pre-treated residue (n=1).

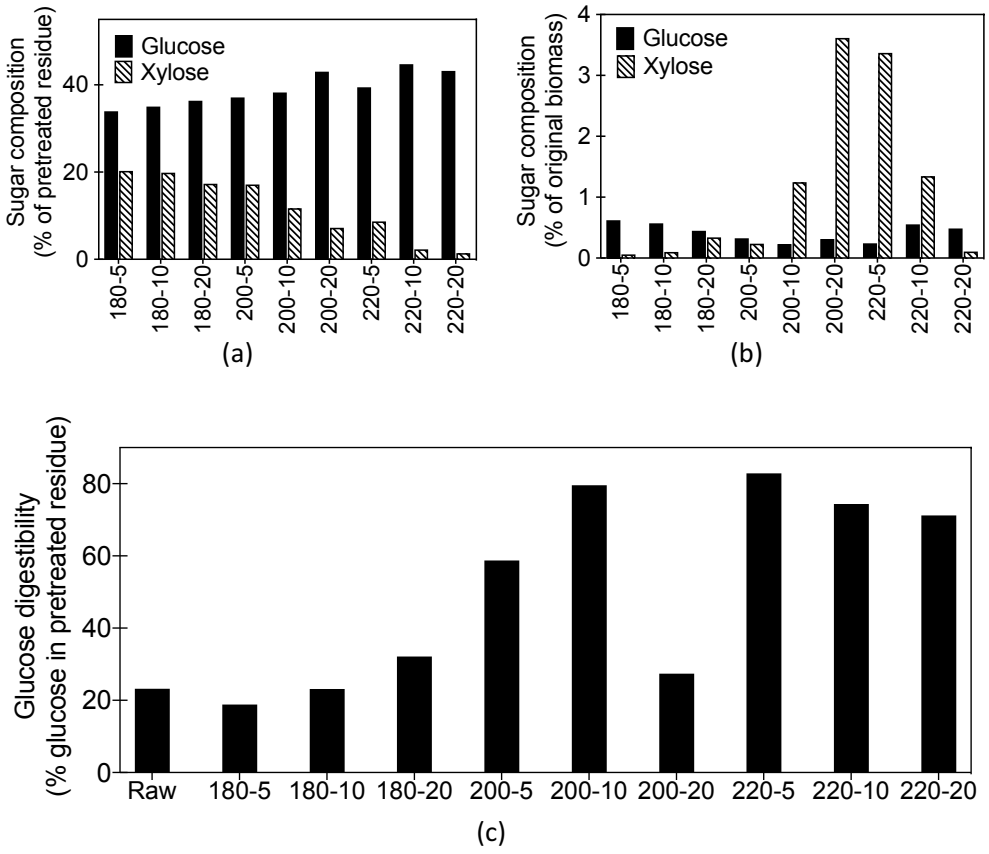


Figure 4.3Effect of varying temperature/time during pre-treatments of *Miscanthus*

(a) Sugar composition of pre-treated residue, (b) Sugar composition of hydrolysate liquor after pre-treatment and (c) Digestibility after 72 hours expressed as % of available glucose in the pre-treated residue (n=1).

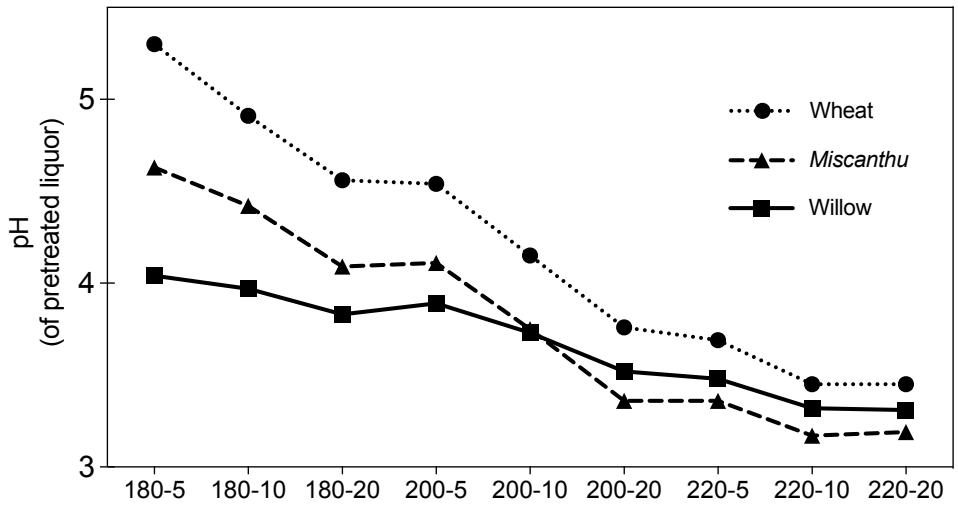


Figure 4.4 Effect of pre-treatment regime on final pH of the hydrolysate.

The release of sugars into the liquor can be taken as an indication of the breakdown of the hemicellulose fraction during the pre-treatment. For wheat straw, there was very little glucose released at any severity, whilst xylose release started to increase at around 200^{o}C for 5 minutes and peaked at 200^{o}C for 20 minutes. The levels of xylose in the liquor at higher severities then declined presumably due to its conversion to inhibitors. The profiles for *Miscanthus* were very similar to that for wheat except that there were slightly higher levels of both glucose and xylose detected. The profile for xylose release from willow was again very similar to that for wheat in terms of response to severity and levels of xylose released. However, for willow there was a much larger release of glucose detected, especially with the lower severity treatments and this declined as severity increased. For all the feedstocks, severity of pre-treatment was accompanied by a decrease in the pH of the pre-treated liquors (Figure 4.4).

These observations are reflected in those for the sugar composition of the pre-treated residue. In wheat, there was a gradual decline in the percentage of xylose in the residue, from an initial value of about 20%. This was accompanied by a gradual increase in the percentage of glucose up to around 40-50%. A similar profile was observed in both *Miscanthus* and willow except that in the case of willow the xylose percentage at the lower severities was about half that found in the other two feedstocks. The three feedstocks all responded very similarly to the pre-treatments in terms of enzyme digestibility, despite demonstrating quite marked differences in digestibility in the raw state. In each

case, there was a gradual increase in digestibility as severity increased up to 220^{o}C for 5 minutes at which point all three feedstocks were showing about 80% digestibility.

Following this screening of the impact of various pre-treatments on saccharification of wheat straw, willow and *Miscanthus* individually, a hot water pre-treatment regime utilising 200°C for 5 minutes was selected. This was a severity that resulted in around 50-60% glucose digestibility in all three feedstocks and as such would allow any synergistic enhancement of digestibility by mixing to be detected. The selected pre-treatment (200°C, 5min) was applied to each feedstock individually and to 1:1 (w/w) mixtures. In each case the impact on saccharification was determined by monitoring glucose and xylose release over 72 hours using a standard NREL digestion with Cellic® CTec3 (Novozyme, Denmark). Inhibitor composition of the resultant hydrolysates was measured and the effect of the hydrolysates on yeast metabolism and growth was monitored. Experimental values of the mixes were compared against theoretical expected values using a comparative ANOVA.

4.4.1. Effect of mixed feedstocks on sugar composition of pre-treated residue

Sugar composition of the residues from individual pre-treated feedstocks were measured (Figure 4.5). These values were then used to calculate expected compositions from 1:1 mixes and these were compared to those obtained experimentally (Figure 4.5). The total weight of the residues after pre-treatment was also measured (Appendix B - Figure B.1) and shown to be

relatively constant for all individual feedstocks and 1:1 mixes at 800mg. This represents an 80% recovery of material from the original 1g of biomass used in the pre-treatment.

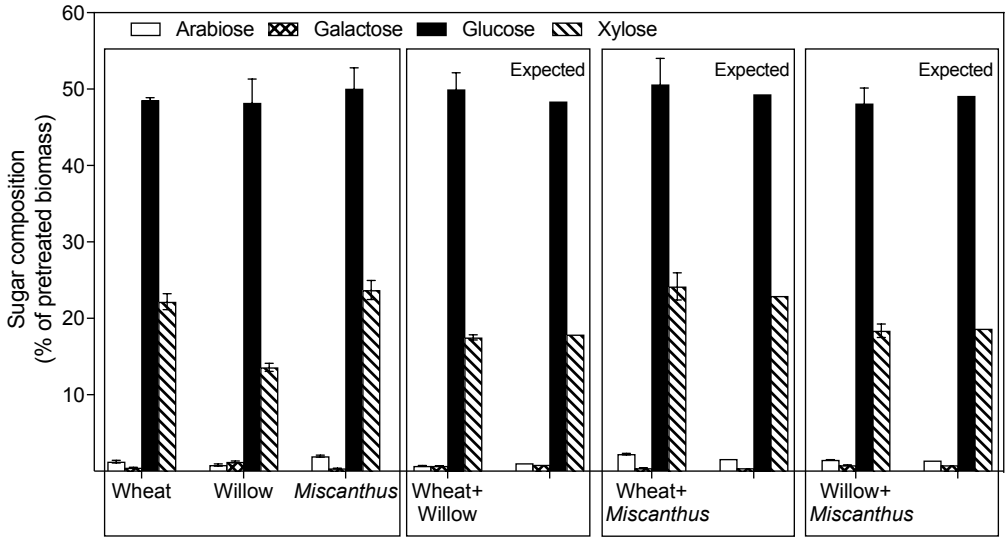


Figure 4.5 Effect of mixed feedstocks on sugar composition of pre-treatment residue

Individual feedstocks and 1:1 mixtures were subjected to a standard pre-treatment and the resultant residues analysed for sugar composition. Results are expressed as mean ± SD (n=3).

The sugar composition of the residue obtained after pre-treatment of the mixes was similar to their expected values in all cases, and in particular the glucose composition of all the 3 mixes was at a similar level.

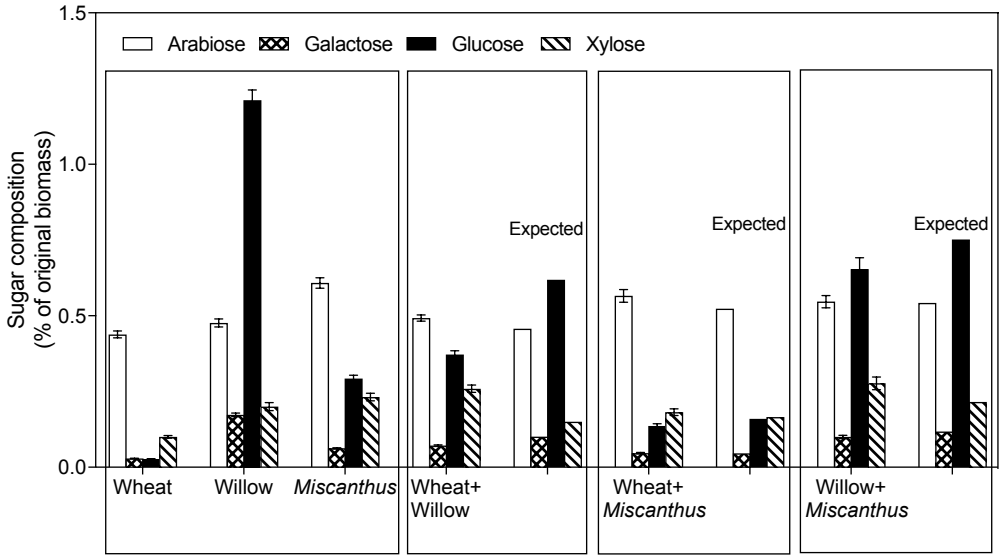


Figure 4.6 Sugar composition of the pre-treated liquor for individual and mixed feedstock.

Sugar composition measured through HPAEC. Sugar content is expressed as % original residue, mean ± SD (n =3).

The sugar composition of the liquor from the pre-treatments was also measured (Figure 4.6). For the two mixtures- willow + *Miscanthus* and wheat + *Miscanthus*- the sugar profiles observed were very similar to those predicted. In the case of the third mixture (wheat +willow), the profiles for arabinose, xylose and galactose are all as expected but the level of glucose is lower than expected. The pH of the liquor at the end of the pre-treatment was measured (Appendix B – Figure B.2) and found to be as expected for all the mixtures. Typical chromatograms from the HPAEC for the sugars from the individual pre-treated feedstocks and 1:1 mixes are also shown (Appendix B – Figure B3-B5). The profiles from wheat and *Miscanthus* are very similar with clear peaks for arabinose, galactose, xylose and glucose with several other minor unknown peaks. That for willow is again very similar except for the presence of a fifth major peak eluting before arabinose. The identity of the compound(s)

responsible for this peak is unknown. The chromatograms from the mixtures do not show any major additional peaks.

4.4.2. Enzyme saccharification

In each case the impact on enzymatic saccharification of the resultant residues was determined by monitoring glucose and xylose release over 72 hours. Glucose yields for the individual and mixed feedstocks over time are shown in Figure 4.7, expressed as g/g of the original biomass. Experimental glucose and xylose yields of the mixes were then compared against theoretical expected values (Table 4.3).

For the individual feedstocks, wheat straw was the least recalcitrant followed by willow and then *Miscanthus*. ANOVA test between the 3 individual feedstocks showed that their responses to the pre-treatment were statistically different for wheat vs willow ($p=0.0013$) and wheat vs *Miscanthus* ($p=0.0005$) but, not for willow vs *Miscanthus* ($p=0.0763$).

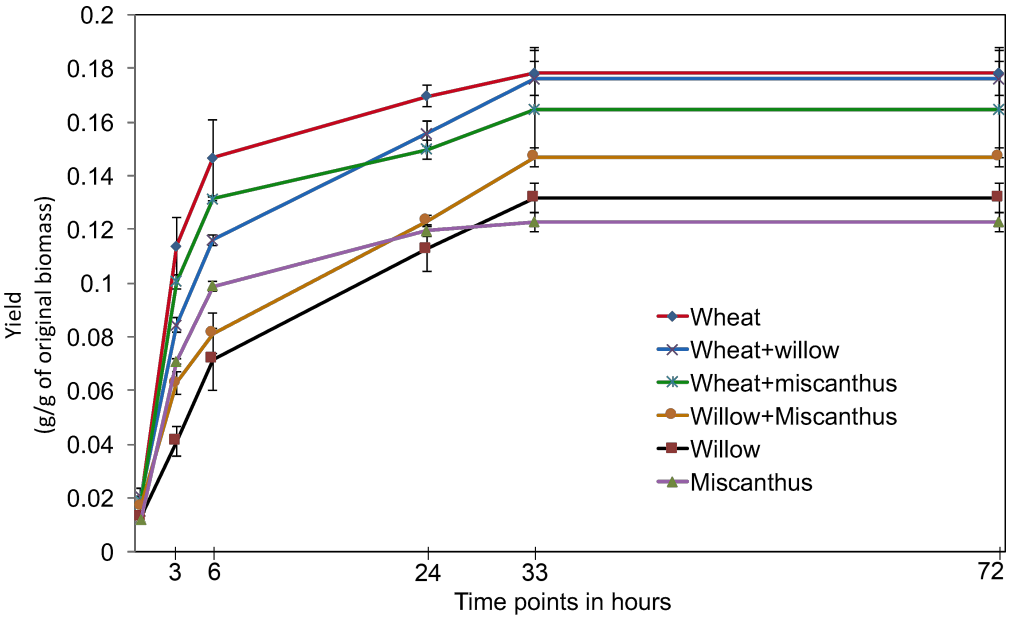


Figure 4.7 Saccharification of pre-treated individual and mixed feedstocks

Individual feedstocks and 1:1 mixtures were all subjected to a standard pre-treatment of 200°C hot water for 5min and saccharification potential assessed by following glucose release during digestion of the dried residue with Cellic® CTec3 enzyme cocktail between 0 to 72 hours. Results are expressed as g/g of original biomass and are the mean ± SD (n=3).

	Wheat	**Willow**	***Miscanthus***	**Wheat+ Willow**	**Wheat+ *Miscanthus***	**Willow+ *Miscanthus***
Glucose yield (g/g)	0.178 ±0.008	0.132 ±0.006	0.123 ±0.004	0.176 ±0.012*	0.165 ±0.018	0.147 ±0.004*
Theoretical	-	-	-	0.155	0.151	0.127
% yield gain	-	-	-	+13.6%	+9.4%	+15.5%
Xylose yield (g/g)	0.144 ±0.005	0.082 ±0.002	0.116 ±0.001	0.118 ±0.008	0.133 ±0.010	0.111 ±0.006*
Theoretical	-	-	-	0.113	0.130	0.099
% yield gain	-	-	-	+4.6%	+2%	+11.8%

Table 4.3 Comparison of observed and predicted values for glucose and xylose yields at 72hr.

The performance of the individual feedstocks was used to calculate an expected yield of glucose and xylose in the 1:1 mixed experiments. These were then compared to the observed yields. Results are expressed as g/g of original biomass and are the mean ± SD (n=3). Observed values marked with * are statistically significantly different from their equivalent expected values ($p<0.05$).

When the observed glucose and xylose yields after 72h of the 3 different mixes were compared with their respective theoretical yields (Table 4.4), the observed yields were found to be higher than expected in all cases. The *Miscanthus* + willow with an expected 0.127g glucose /g original biomass and observed 0.147g glucose /g original biomass; and wheat + willow mixes with an expected 0.155g glucose /g original biomass and observed 0.176g glucose /g original biomass, demonstrated the largest enhancement between expected and observed values these being 15.4% and 13.6% respectively - both being statistically significant (p=0.015 and 0.010, respectively). For the wheat + *Miscanthus* mix while the actual glucose yield of 0.165g glucose/g original biomass was still higher than expected the 0.151 g glucose/g original biomass, this was not statistically significant (p=0.065).

The observed xylose yields from all three mixes were again higher than expected, however in the case of the two mixtures: *Miscanthus* + wheat and willow + wheat, this was insignificant (p=0.499 and 0.255, respectively). Only the mixed *Miscanthus* and willow feedstock showed any significant enhancement in xylose yield (11.8 %, p=0.019).

4.4.3. Analysis of saccharification kinetics

The data shown in Figure 4.8 was used to calculate rate constants describing the kinetics of glucose release for either individual feedstocks or their 1:1 mixes (Table 4.4).

	Wheat	Willow	*Miscanthus*	Wheat+ Willow	Wheat+ *Miscanthus*	Willow+ *Miscanthus*
Rate Constant (hr^{-1})	0.34± 0.03	0.13± 0.03	0.29± 0.01	0.21± 0.03	0.32± 0.07	0.14±0.02

Table 4.4 Impact of mixed feedstocks on saccharification kinetics

Rate constants ($hour^{-1}$) were calculated from g/g glucose release from original biomass at the 6 time points in enzyme hydrolysis shown in figure 2.5. Results are expressed as mean ± SD (n=3).

For the individual feedstocks, wheat straw and *Miscanthus* showed the fastest saccharification rates and these were statistically identical (p=0.0549). In contrast, willow showed a significantly slower rate of glucose release than either of the other two feedstocks [wheat vs willow (p=0.001) and willow vs *Miscanthus* (p= 0.0006)].

4.4.4. Effect of mixed feedstock on yeast metabolism and growth

The impact of the pre-treated liquors (hydrolysates) on the metabolic activity and growth of yeast was assessed (Table 4.5).

	Wheat	**Willow**	***Miscanthus***	**Wheat+ Willow**	**Wheat+ *Miscanthus***	**Willow+ *Miscanthus***
Yeast metabolism (% of control)	84.05 ±1.49	111.03 ±5.46	69.86 ±3.59	109.34 ±0.90	70.91 ±6.45	68.29 ±5.35
Yeast growth (% of control)	33.06 ±3.22	41.12 ±1.07	37.9 ±1.7	35.63 ±0.35	31.88 ±2.96	44.05 ±3.38
HMF (mg/l)	13.55 ±0.96	167.63 ±1.6	42.44 ±1.73	55.51 ±0.90	18.40 ± 0.89	84.56 ±11.45
Furfural (mg/l)	59.57 ±0.03	105.16 ±8.08	134.54 ±3.41	118.29 ±6.72	109.77 ±4.21	131.29 ±11.93

Table 4.5 Impact of mixed feedstocks on yeast metabolism, growth and inhibitor production

Impact of pre-treatment liquor on yeast metabolism and growth are presented as % compared to the controls after 48 hours. Inhibitor composition of the liquor (HMF and Furfural) are presented as mg/ml of the pre-treated liquor. All results are expressed as mean ± SD (n=3).

For the individual feedstocks, the impact on yeast metabolism ranged from about 30% inhibition for *Miscanthus* to an apparent stimulation in the case of willow (Table 4.5). In terms of the mixed feedstocks the observed inhibitory responses were in the same range as for the individual feedstocks.

Following this, the impact of the liquor on yeast growth was assessed (Table 4.5). In this case wheat demonstrated the greatest inhibition and willow again had the least effect. In contrast to the effect on metabolic rate, the impact on growth was quite substantial ranging from 70 to 60% inhibition of growth as compared to the control. The range of the inhibitory effect of the liquors from the mixed feedstocks was similar to that for the individual feedstocks.

The concentrations of two major inhibitory compounds- furfural and HMF in the liquors were analysed (Table 4.5). *Miscanthus* had the highest level of furfural and wheat the lowest while willow had the highest level of HMF and wheat again the lowest. The only major difference exhibited between the observed and expected levels of these inhibitors in the mixed feedstocks was for the level of furfural in the wheat + willow mix which was higher than expected.

4.4.5. Effect of mixed feedstock on pre-treatment energy requirement

All the samples were pre-treated at 200° C for 5 mins. The energy required was calculated as described in section 4.2.3 and results are shown in Table 4.6.

	Wheat	**Willow**	***Miscanthus***	**Wheat+ Willow**	**Wheat+ *Miscanthus***	**Willow+ *Miscanthus***
Energy (kj)	32.15 ±2.9	29.89 ± 1.6	27.4 ±1.3	26.7 ± 0.5	28.7 ±1.9	32.3 ±1.5

Table 4.6 Impact of mixed feedstocks on energy requirements.

Pre-treatment energy required to heat the biomass at 200°C for 5minutes. All results are expressed as mean ± SD (n=3).

For the individual feedstocks wheat straw required the highest energy followed by willow and *Miscanthus*. However, there were no significant differences between the three. The energy requirements for the three mixes were all within the same range as observed for the individual feedstocks.

4.5. DISCUSSION

The impact of the various pre-treatment regimes on the sugar composition of the residue, and release of sugars into the hydrolysate liquor was similar in all three feedstocks. As expected there was an increased loss of xylose into the liquor as severity of treatment increased and in all cases, there was little impact on glucose retention in the residue. The pH of the liquor decreased with increasing severity of pre-treatment in all three cases (r=-0.98), with the total sugar in the liquor (r=-0.5).

Following a pre-treatment regime of 200 °C for 5min, the three feedstocks had varying levels of recalcitrance. Literature values of their glucose digestibility after higher severity pre-treatment were reported to be: Miscanthus 80% after alkaline hydrogen peroxide treatment of ball milled samples (Hideno et al., 2013); willow 94% after calcined Na_2SiO_3 and ionic liquid (1-butyl-3-methylimidazolium) pre-treated of ball milling samples (Sun et al., 2016); and wheat straw 76% after Alkaline extrusion (Coimbra et al., 2016).

The first hypothesis was that mixing lignocellulosic feedstocks could result in improved pre-treatment. This was unclear as pre-treatment of mixed feedstock resulted in a pre-treated residue with similar sugar composition, and these values were similar to the expected values. (Silva-Fernandes et al., 2015) reported similar findings: mixtures of lignocellulosic materials processed by auto hydrolysis generated consistent product composition independently of the different proportions of each feedstock. Hence, it is unlikely that

saccharification yields of the mixed feedstocks would be impacted by the pre-treated sugar composition. The improved glucose saccharification of 9.4% to 15.5%, may therefore be due to a reduction in recalcitrance of the residue possibly due to improved response to pre-treatment brought about by the mixed nature of the feedstock.

The second hypothesis was that mixing feedstocks will result in improved saccharification. The has been proven true as saccharification results of mixed feedstock demonstrated a significant increase in saccharification efficiency for some of the mixed feedstocks investigated which has not been reported in previous mixing studies with different feedstocks. Previous studies reported glucose yields of 84% from a municipal solid waste paper and corn stover mix after ionic liquid pre-treatment compared to a 82.2% expected, with a xylose yield of only 40% compared to an expected value of 59.3 % (Sun et al., 2015). Another study on mixes of aspen and balsam reported a glucose yield of 27.34% compared to the expected value of 24.5%, and a xylose yield of 8.16% compared to 2.2% expected (Brodeur-Campbell et al., 2012). However, aspen and switch grass in the same study gave a glucose yield of 52.51% compared to an expected value of 53%, with an observed xylose yield of 3.81% compared to the expected 4.8%. Hence, the feedstock used for mixing and the pre-treatment method employed could also impact the performance of the saccharification. This combination of feedstock and pre-treatment, could be optimised to utilise local feedstocks.

Among the individual feedstocks, the significantly slower release of glucose for willow could be due to differences in their cell wall compositions (Di Maio and Turley, 2014) (Table 4.1), or the higher bulk density of willow (210 kg/m^3) compared to *Miscanthus* (180kg/m^3) and wheat straw (20kg/m^3) (Carroll and Finnan, 2010) These factors could have impacted the enzymatic hydrolysis rate, but an in-depth study is required to identify and confirm the mechanism of the underlying factors. Mixing wheat and *Miscanthus* had no effect on the rate constant for glucose release. The rate of release for the wheat and willow mix was intermediate between that for the two feedstocks individually, while the rate for the willow and Miscanthus was similar to that for the willow alone. Thus, the enhancement in glucose yields observed when willow was mixed with either of the two "grasses" was not associated with an increased rate of initial release.

The third hypothesis was that mixing feedstocks will result in reduced inhibitor production during pre-treatment, accompanied by a resultant decrease in the inhibitory effect on yeast metabolism. This was important as inhibitory compounds in pre-treated liquor are believed to result in either a reduction in the conversion of sugars into biomass (growth) or a reduction in metabolic output, or both simultaneously (Greetham et al., 2014). Although not a reduction, inhibitor production and its effect on yeast growth and metabolism of the mixed feedstock have been shown to be similar to the average of their individual feedstock values.

A synergistic effect of inhibitors such as HMF and vanillin, or HMF and furfural have been reported on yeast performance, where they stopped or slowed yeast activity (Greetham et al., 2016). The presence of low concentrations of acetic acid has been shown to have a beneficial effect on fermentations and improved yeast tolerance to other inhibitory compounds (Greetham et al., 2016). However, at higher concentrations of acetic acid (>25 mM) a synergistic effect on yeast metabolism is observed with other inhibitory compounds (including furfural, vanillin and HMF) (Greetham et al., 2016). This could be one of the reason for the high yeast metabolism from willow hydrolysate which showed the highest overall inhibitor composition among the individual feedstocks. Hence, it could have been an interesting to measure the acetic acid concentration rather than the drop in pH of the hydrolysates in this research which would have been influence by other acids from sugar degradation such as formic acid and levulinic acid. Willow's pre-treated liquor's high inhibitor content could be attributed to the higher hemicellulose content of willow cell wall (Di Maio and Turley, 2014), which resulted in a larger amount of glucose/galactose and xylose/arabinose (Figure 4.7) for the production of these HMF and Furfural inhibitors respectively during pre-treatment at 200 °C 5min. Similar, in the mixed feedstock, the amount of HMF and furfurals could be attributed to the respective sugar monomers.

Given that the reported toxicity threshold for yeast is 2.4g/l of furfural (Allen et al., 2010), it is unlikely that the inhibitory effects on growth seen in the present experiment are directly due to furfural, but are more likely to have

arisen from additional or synergistic impacts of a wider range of inhibitory compounds. The synergistic effect of inhibitors such as HMF and vanillin or HMF and furfural have been reported on yeast performance, where it stopped or slowed yeast activity (Greetham et al., 2016).

In a study consisting of aspen, switch grass and balsam, furfural concentrations at the optimal pre-treatment conditions were 0.36g/l for the aspen: balsam blend(0.33g/l expected), and 0.67 for the aspen: switch- grass blend (1.125g/l expected) (Brodeur-Campbell et al., 2012). In comparison, a study with wheat straw, olive pruning and eucalyptus residues, indicated that furfural levels were 0.33-0.44g/l and HMF levels were 0.17 to 0.29g/l depending on the mixes (Silva-Fernandes et al., 2015). The inhibitor levels produced in this study were much lower when compared to those in previous studies. This may reflect the different solid loadings and pre-treatment regimes used in each study, or, may have occurred directly as a result of the different feedstocks used. The hydrothermal pre-treatment used in this study is known to solubilise hemicellulose mainly as oligomers and not monomers, which reduces the risk of degradation of the soluble hemicellulose fraction (Mosier et al., 2005) and the resultant inhibitors.

CHAPTER 5 BLACK RICE STRAW FOR ANTHOCYANIN AND SUGARS

5.2. INTRODUCTION

Rice is the main staple food for many Asian countries and with a global production of over 748 million tons in 2016, it is the third most grown cereal in the world (FAO, 2016). The major residues from rice cultivation are rice straw and rice husk. Rice straw has been traditionally used as animal feed, feedstock for the paper industry and as organic fertilizer (Abraham et al., 2016). However, due to the large quantities produced, farmers across south east asia burn tens of millions metric tons of leftover stalks (Bond et al., 2013). The released black carbon or soot, forms the second most important contributor to global warming, second only to carbon dioxide (Abraham et al., 2016). The incorporation of the straw into the soil, as compared to its removal from the fields, has also been reported to emit greenhouse gases, mainly methane(Alberto et al., 2015; Koga and Tajima, 2011). The straw is one of the most abundant agricultural byproducts and with composition comparable to wheat straw (Table 5.1), it is an attractive lignocellulosic feedstock for a biorefinery.

	Cellulose	**Hemicellulose**	**Lignin**
	(Expressed as % biomass)		
Rice straw [a,b]	32-39	26-28	9-19
Wheat straw [c]	38-40	23-35	10-13

[a] Shawky et al., 2011, [b]Rahnama et al., 2013, [c]Ali et al., 2012

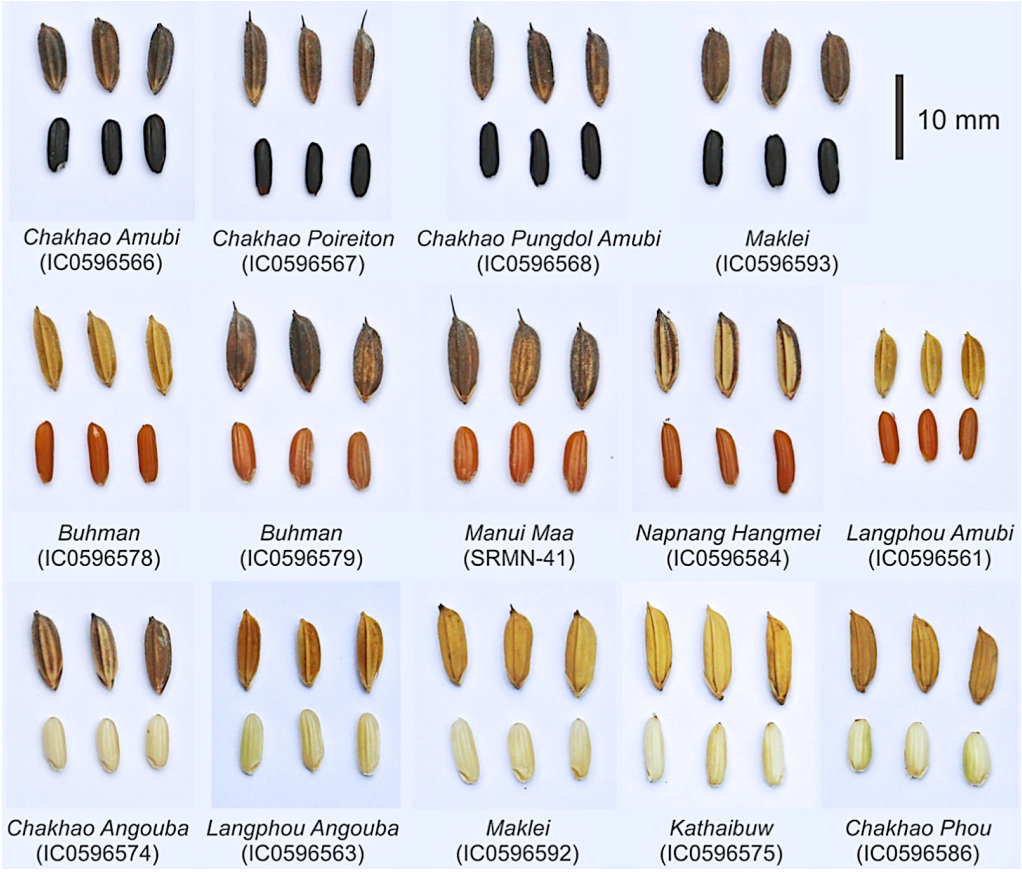
Table 5.1 Composition of rice and wheat straw

However, as with all lignocellulosic feedstocks, rice straw is complex compared to the starch used for first generation biofuels, and liberation of the sugars locked in this matrix in a biorefinery is uneconomical at present. Accessing or making the sugar available involves pre-treatments with expensive chemicals or enzymes and is an energy consuming processes which entails significant resource consumption (e.g. water) and generates many residual streams including fermentation inhibitors (Chuetor et al., 2015). To overcome this economic challenge, a biorefinery approach with a dual focus - low volume high value (LVHV) bioactive components in addition to high-volume low-value (HVLV) products like sugars for bioethanol is an appealing option. This approach is expected to improve the economics of the overall biomass conversion process as it generates a diverse product stream that offsets the overall production cost (Abraham et al., 2016).

5.2.1. Black Rice

In this study, we used straw from the black (dark purple) scented rice variety *Chakhao Poireiton (Oryza sativa Linnaeus)* from Manipur, a north-eastern state of India. The black scented rice (*Chakhao*) are used for the community feast as well as ceremonial purposes as a delicacy. Dishes made with black rice include desserts, flakes, bread, cakes, beverages and a special snack —*Utong Chak*, prepared within bamboo sticks. They are sold in the local markets at a premium rate (Asem et al., 2015). There are many varieties of *Chakhao* found in Manipur (Figure 5.1) and they are of increasing interest due to their medicinal value. The

literal meaning of *Chakhao* is delicious rice (*Chak*=rice; *hao*=delicious), hence you can have *Chakhao* which are non-coloured such as *Chakhao Angouba* – Figure 5.1.



(Roy et al., 2014)

Figure.5.1 Different rice landraces from *Manipur* (India) and their grain morphology

The dark purple colour is attributed to high levels of anthocyanins, a group of reddish-purple water-soluble pigments in the pericarp layers of the grain (Asem et al., 2015). Synthesis of anthocyanins occurs in the cytoplasm up to its precursor anthocyanidin, anthocyanidin is then tagged with glutathione by glutathione-S transferase, and is transported via a glutathione pump into the vacuole (Marrs et al., 1995). It is within the vacuole that anthocyanins take on

their species-specific coloration which results from hydroxylation, methylation, sugar addition, or acylation (Simoes et al., 2012).

Over 500 anthocyanin pigments have been described in the literature (Andersen and Jordhein, 2006) and up to 19 different naturally occurring anthocyanidins have been reported to be found in plant tissues (Iwashina, 2000), though the bronze-coloured cyanidin-3-glucoside is the most common form encountered in leaves (Lee and Gould, 2002).

As well as being pigments anthocyanins also exhibit antioxidant activity. Among the anthocyanins, delphinidin, cyanidin and cyanidin-3-glucoside exhibit the highest antioxidant activity. These antioxidant activities are significantly higher than other natural antioxidants such as alpha-tocopherol, butylated hydroxytoluene and ascorbic acid (Fukumoto and Mazza, 2000). In *Chakhao Poireiton* four main anthocyanins have been identified: delphinidin 3-galactoside, delphinidin 3-arabinoside, cyanidin 3-galactoside and cyanidin 3-glucoside (Asem et al., 2015).

5.2.2. Potential value of anthocyanins

Anthocyanins are known for their ability to protect mammalian cells from damage due to biotic and abiotic stresses and have also been considered as potential cancer chemo-preventative agents (Hou, 2003). As a dietary antioxidant, they can also help combat reactive oxygen species, free radicals and help decrease the risk of chronic diseases such as coronary heart disease

(Abdel-Aal et al., 2008). Additionally, they are approved for use as a food additive or colouring agent in the EU, Australia and New Zealand with the E number E163 (INS number 163) (FSA, 2016). Commercial sources of anthocyanin include grape Marc or grape skin with an anthocyanin content of ~100mg/100g (Liang et al., 2008).

5.2.3. Extraction of anthocyanin

The extraction method has a strong influence on the anti-oxidative activity and anthocyanins are usually extracted from plant materials using an acidified organic solvent, often methanol (Naczk and Shahidi, 2006). Total anthocyanin content in *Chakhao poireiton* grain after extraction into acidified methanol has been reported as being 740 mg/kg of powdered rice, with a strong antioxidant activity of 70.28% (Asem et al., 2015), and with acetone:water (75:25 v/v) anthocyanin content was 35.87mg/100g with 94.19% free radical scavenging activity (Saikia et al., 2012).

In terms of their stability on exposure to high temperature, anthocyanins tend to break down into smaller stable forms which may or may not show antioxidant activity. Although the precise reason is not currently known, extracts from *Chakhao poireiton* have been reported as being more thermo-stable and having a superior phytochemical content and antioxidant capacity compared to other black rice varieties such as *Amubi* (Saikia et al., 2012). This is important because temperature has been reported as an important factor in the efficiency of the extraction process (Bridgers et al., 2010;

Piyapanrungrueang et al., 2015).

Microwave hydrothermal pre-treatment has been chosen for this research because this process is cost-saving and environmentally benign due to no catalyst requirement. Furthermore, the potential release of mild acids during the process may aid in extraction. Water is utilised as the only reaction medium (no chemicals) which lowers the construction costs of pre-treatment reactors and is less hazardous to the environment (Alvira et al., 2010). In rice straw, hydrothermal treatment can dissolve part of the lignin and hemicellulose into the liquid fraction, thus facilitating enzyme accessibility to cellulose (Sasaki et al., 2015). Additionally, in a study that compared anthocyanin extraction using conventional heating, ultrasonic, microwave and ohmic heating techniques, microwave gave the highest extraction, shortest reaction time and lowest power consumption (Piyapanrungrueang et al., 2015).

Microwave pre-treatment has also been reported to be beneficial for biomass such as rice straw and husk that are rich in additional components such as silica (Bhatt and Shilpa, 2015). Other pre-treatment techniques reported for rice straw with their respective glucose digestibility include: steam explosion 45% (Wood et al., 2016); combined dilute sulphuric acid and aqueous ammonia - 86% (Kim et al., 2013), dilute sulphuric acid – 83% (Hsu et al., 2010) and microwave (2.45 GHz) 38% (Ma et al., 2009), alkali 60% and alkali microwave (2.4 GHz) 65%.

Chakhao cultivation is at risk in *Manipur*, as the farmers prefer to grow improved high yielding varieties. There are also no official records of the scale of *Chakhao* cultivation in Manipur. Therefore, to assure the future of this crop, research is needed to improve the yield of this indigenous variety, its value as a source of high value nutraceuticals and to find better use for its by-products.

So far, the anthocyanin from black rice has been studied from the grain but not the straw. To the best of our knowledge, this is the first and foremost study on the extraction and quantification of anthocyanin from the straw and the use of the straw residue for sugar production in a combined biorefinery process. The objective of the present research study was to optimize extraction and saccharification efficiency while maintaining the bioactivity of the extracted anthocyanin. Towards this, we applied a microwave hydrothermal pre-treatment, a cost-effective and environmentally friendly technique to aid anthocyanin extraction and improve the straw lignocellulose digestibility. Thus, the current study may develop a genuine interest in black scented rice of *Manipur*, the improvement of these cultivars and to thereby generate new employment in the state.

5.3. MATERIALS AND METHODS

5.3.1. Feedstock

Black rice straw – *Chakhao poireiton* harvested in 2015, along with its industrially milled fractions – husk, bran, polished rice and paddy rice, were imported from a rice field in Imphal (India). The straw was air dried and fractionated into leaf fraction- containing leaf sheath and leaf blade; and a stem fraction- containing nodes and internodes. The stem was further fractionated into stem top fraction – consisting of only the first internode connected to the rachis; and the stem lower fraction consisting of the remaining stem internodes and nodes. These were milled separately to 2mm mesh size using a Pulverisette 19 knife mill (FRITSCH, Germany) and stored along with the other fractions at 4°C in separate bags with airtight seals. Rice grain (paddy and polished) and husk were ground using a coffee grinder. White *Brojen* rice straw, also from Imphal (India), was used for comparison and was also treated as described above.

5.3.2. Extraction and pre-treatment

5.3.2.1. Anthocyanin extraction

Anthocyanin extraction was carried out using a method based on that described by Sutharut (Sutharut and Sudarat, 2012). 500mg of plant material was added to 10ml methanol and vortexed for 30s followed by incubation at 60°C for 20min with samples being vortexed twice more during the incubation. The samples were centrifuged at 4472x*g* for 10 min and the supernatant

collected. The pellet was mixed with another 5ml of methanol, vortexed, centrifuged and the supernatant collected. The pooled supernatants were centrifuged again, filtered through a 0.22µm syringe filter, stored at -20 °C and all analysis was conducted within a month.

5.3.2.2. Microwave pre-treatment

Microwave pre-treatment was conducted using a Monowave 300 microwave generator (Anton Paar, Germany). 500 mg of plant material was added to 10ml of water, steeped at room temperature for 2 min and then heated at different temperatures starting at room temperature (25°C) and ranging from 60-200°C for 5 min. The samples were then centrifuged at 4472x*g* for 10 min, the supernatant filtered through 0.22µm syringe filters. The residues were air dried for 72 h, weighed and stored at 4°C for further analysis.

5.3.2.3. Anthocyanin measurement

The total anthocyanin content (TAC) was determined using the pH-differential method (Bondet et al., 1997; Piyapanrungrueang et al., 2015; Sutharut and Sudarat, 2012). Briefly, two dilutions of 0.5ml of the extract was made - one with 9.5ml of 0.025M potassium chloride, pH1.0 and another with 9.5ml of 0.4M sodium acetate buffer, pH4.5. Absorbances at 510nm and 700nm was measured using Spectrophotometer-7315 (Jenway, UK). The TAC was calculated using Eq. (1) and expressed as milligrams of cyanindin-3- glucoside (cyn-3-glu) equivalents per 100 g dry sample (mg cyn-3-glu/100 g):

TAC (mg/100g) = $\frac{A \times MW \times DF \times V \times 100}{\varepsilon \times L \times m}$ ---------- (1);

where A= $(A_{510} - A_{700})_{pH\ 1.0} - (A_{510} - A_{700})_{pH\ 4.5}$; *MW* is the molecular weight of anthocyanin (449.2 g/mol), *DF* is the dilution factor, *V* is the extract volume (mL), ε is the cyn-3-glu molar absorbance (26,900 L/mol/cm), *L* is the cell path length (1 cm) and *m* is the sample weight (g).

5.3.3. Microscopy

Untreated black rice straw, and straw subjected to the aqueous pre-treatment, were taken to the Division of Plant sciences to visualise the anthocyanin presence. These tests were undertaken by Priya Ramakrishna (School of Biosciences). Dried samples were sectioned by hand were viewed on a Stemi SV 6 stereomicroscope (Zeiss, USA) with an Axiocam ERc 5S (Zeiss, USA), both before and after subjecting to an aqueous treatment of 100 °C for 5min. The cross sections were imaged as they were upon storage at ambient conditions while the pre-treated samples were blot dried to remove excess liquid and imaged.

NUTRACEUTICAL ANALYSIS

5.3.4. Analysis of Antioxidant capacity

Anthocyanin extracts were assessed for their antioxidant potential at the Division of Plant and Crop Sciences by Priya Ramakrishna (School of Biosciences). The diphenyl-1-picryl-hydrazyl-hydrate (DPPH) free radical scavenging method was adapted from Wong (Wong et al., 2006). DPPH is an organic chemical compound composed of stable free-radical molecules which

monitor chemical reactions involving free radicals in an antioxidant assay. Briefly, 0.1 mL of sample was pipetted into a test tube followed by 3 mL of 0.1 mM DPPH (Sigma Aldrich, UK). For blank, the sample was replaced with extraction solvent. The mixture was vortexed thoroughly and placed in the dark for 30 min. The absorbance was measured at 515nm using a Spectrophotometer-7315 (Jenway, UK). Figure 5.2, shows a standard calibration curve for Trolox, an antioxidant (analogue of vitamin E). Results were expressed as µg Trolox equivalent/ml of extract.

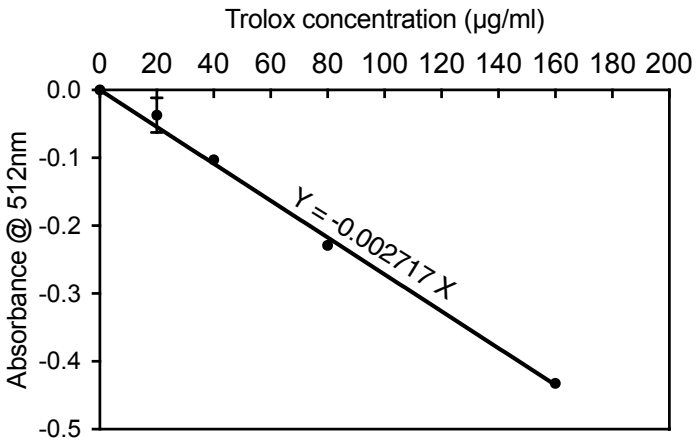


Figure 5.2 Standard curve for the DPPH assay using Trolox at 0 µg/mL to 160 µg/mL. Values are mean ±SD n=2.

5.3.5. Bioactivity assay: cytotoxicity and apoptosis assays

5.3.5.1. Preparation of extracts

Methanol extracts were concentrated using a rotary drum evaporator (Heidolph Rotary Evaporator, Germany) connected to a chiller unit (Julabo F250, Germany). The evaporator was set to 20°C and 30 rpm and the chiller unit was maintained at -9°C under vacuum. The methanol extracts were then subjected to drying over a flow of nitrogen until all the methanol had evaporated and a constant weight was achieved. Water extracts were freeze-

dried overnight using a freeze drier (Telstar Cryodos, Spain). Working solutions were made in MilliQ water.

5.3.5.2. Preparation of cells

The cytotoxicity and apoptosis tests were conducted at the Medical school by Ashfaq Ghumra (School of Biosciences). Jurkat (J.RT3-T3.5) cells (ATCC TIB-153) (which are human T lymphocyte cells) were grown in Roswell Park Memorial Institute (RPMI)-1640 medium containing 2mM L-glutamine, 10mM HEPES, 1mM sodium pyruvate, 4500mg/L glucose, 1500mg/L sodium bicarbonate and 10% Foetal calf serum (FCS) at 37°C with 5% CO_2. Cell density was maintained at 1 x 10^6 cells/ml by regular exchange of media as described in the manufacturers guidelines. Approximately 100,000 cells were used in each well for the assay.

5.3.5.3. Assay procedure

Jurkat cells (100,000 cells) were incubated with extracts at 100, 200, 400, 800 µg/ml in 100 µl total volume for 24 hours at 37°C with 5% CO_2. Cells incubated without the extract were used as positive controls and cells incubated with 3 µg/ml of cytotoxic ionophore (A23187, Sigma) in absence of extract were used as negative controls. After incubation, cells were washed with Annexin-V binding buffer (MACS, UK), stained with 5 µg/ml Annexin-V and 5 µg/ml of propidium iodide (PI) and incubated at 4°C for 30min. Cells were washed and re-suspended in 500µl binding buffer and analysed using an FC500 flow cytometer (Beckman Coulter) by Ashfaq Ghumra (School of Biosciences) and

David Onion (School of Life Sciences). Ten thousand events were acquired for each sample and data analysed using WEASEL 3.0 (Walter and Eliza Hall Institute of Medical Research). Cells were defined as: live Annexin-V^{-ve}/PI^{-ve}; Apoptotic Annexin-V^{+ve}/PI^{-ve} or dead Annexin-V^{+ve}/PI^{+ve}.

5.3.6. Composition and saccharification

5.3.6.1. Sugar composition analysis

Sugar composition was measured as described in section 3.2.2.

5.3.6.2. High Performance Anion Exchange Chromatography (HPAEC)

Analysis of sugars was performed on a Dionex ICS-3000 (ThermoFisher, USA) as described in section 3.2.3.

5.3.6.3. Digestibility assay

Enzymatic saccharification was carried out as described in section 4.2.4

5.3.6.4. Fourier Transformed Infrared Spectroscopy (FTIR) analysis

FTIR analysis was carried out as described in section 3.2.8.

5.3.7. Statistical analysis

All experiments were carried out in triplicate. Statistical analysis of variance (ANOVA) and standard deviations were calculated using Prism 7.0b for Mac (GraphPad Software, Inc) and Microsoft Excel 2016.

5.4. RESULTS

5.4.1. Feedstock

Different fractions of the black rice grain were imported from Manipur. These included intact paddy rice; the unpolished grain and husk obtained from milling the paddy rice; the polished rice and bran obtained from polishing the grain in a commercial mill in *Manipur*, India – Figure 5.3. The straw was also imported and was further fractionated into stem, stem lower internodes, stem top internode and leaf.

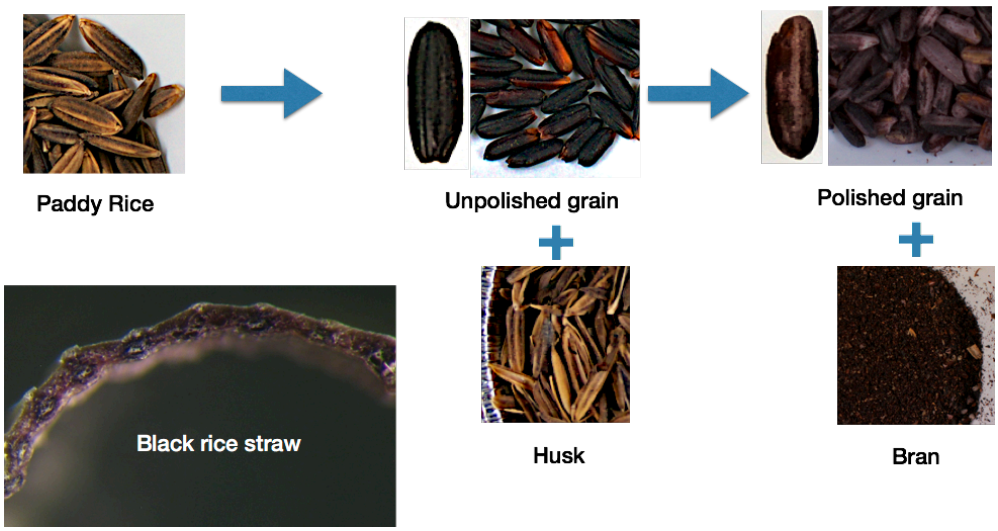


Figure 5.3 Different fractions of black rice and stereo microscope image of straw.

5.4.2. Content and Distribution of Anthocyanin in black rice fractions

Anthocyanin was extracted from the grain (paddy rice, polished rice, husk, bran) and straw (whole straw, whole stem, stem lower internodes, stem top internode and leaf) fractions using the standard methanol method (section 5.2.2.1) and quantified spectrophotometrically using the pH differential method (section 5.2.2.3) (Figure 5.4). Bran in the grain fraction followed by

stem lower internodes in the straw fraction had the highest anthocyanin content.

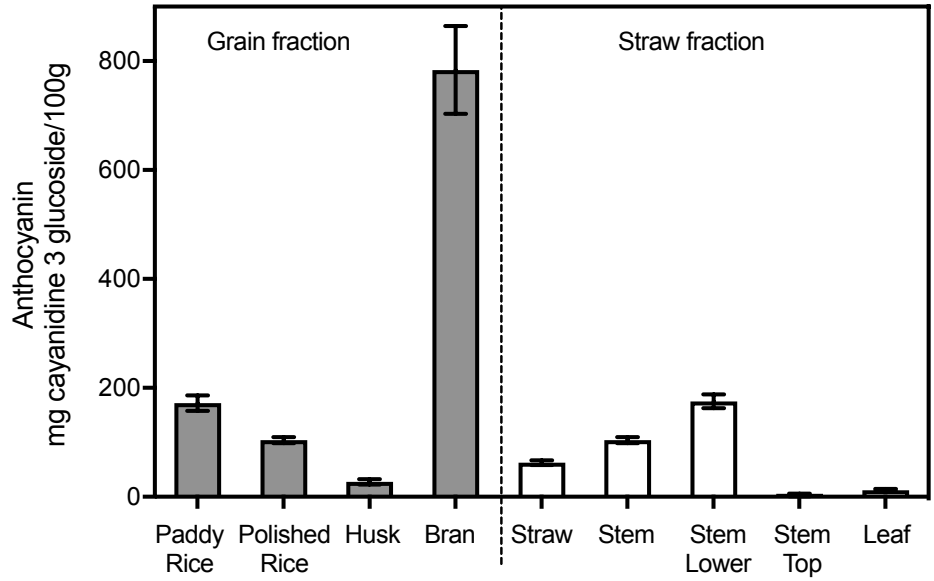


Figure 5.4 Anthocyanin content of various black rice fractions

Anthocyanin extracted by methanol and expressed as mg of cyanidin 3-glucoside/100g of dry biomass (mean ± SD, n=3)

5.4.3. Hydrothermal Microwave Anthocyanin Extraction

Microwave hydrothermal extraction of anthocyanin was explored as an "environmentally friendly" alternative to organic solvent extraction. Anthocyanin content, as extracted by the standard methanol extraction, was used as a baseline (called here the theoretical maximum) and was used to test the efficiency of the hydrothermal microwave extraction at a temperature range from 25 to 200°C for 5 min (Figure 5.5). The highest anthocyanin extraction from the straw was obtained at 90 °C (53.9 ±5.7 mg/100g), followed by extraction at 100° C (50.8±3.1mg/100g). The difference in extraction efficiency at 90 and 100°C was statistically insignificant (p=0.75).

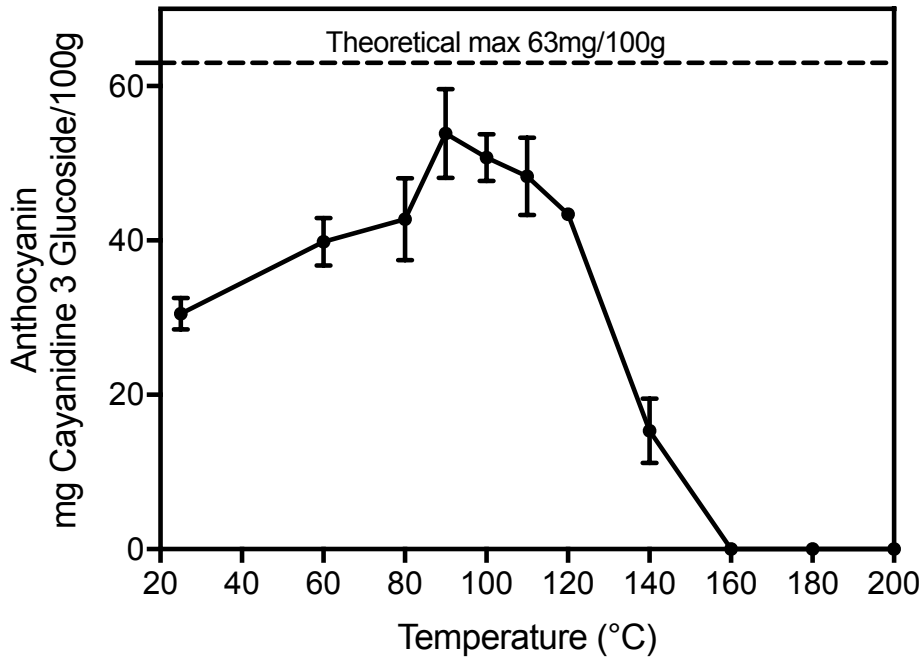


Figure 5.5 Anthocyanin extraction at various hydrothermal treatment temperature.

Anthocyanin from black rice straw at different hydrothermal temperatures compared against methanol extract and expressed as mg of cyanidin 3-glucoside/100g of dry biomass (mean ± SD, n=3)

5.4.4. Microscopy of the straw

Sections of the whole straw and stem and leaf fractions were observed under the disecting microscope before and after aqueous extraction at 100°C for 5min (Figure 5.6). The visual examination is shown in Figure 5.6: AB shows pigment located in stem while nothing in outer leaf sheaf and the loss of pigment after 100 °C extraction; CD shows pigment in stem fraction and a small amount left after 100 °C extraction; and EF shows lack of pigment in the leaf fraction. This confirms the extraction efficiency and localisation of the pigment demonstrated previously.

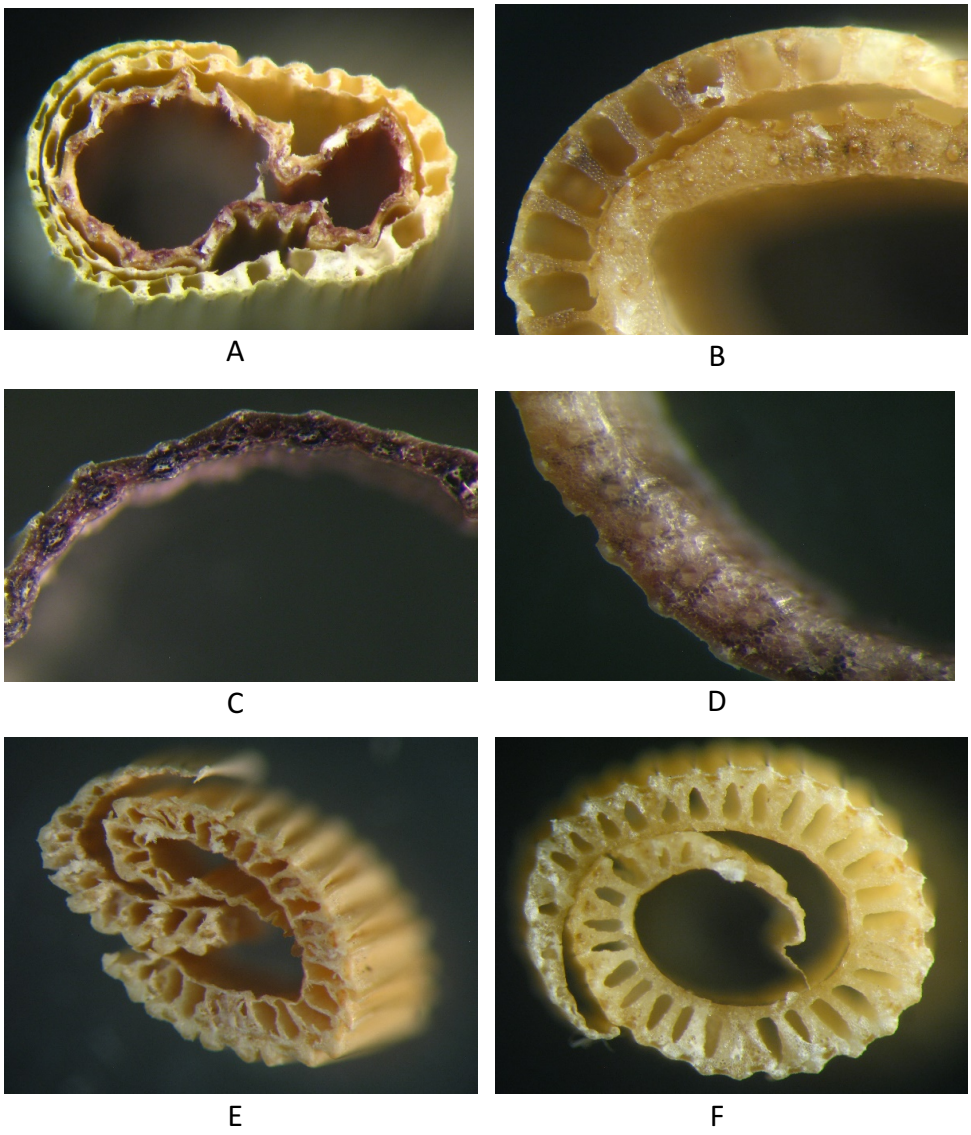


Figure 5.6 Cross sectional view of the straw

Before (Left) and after subjecting to 100 °C for 5min (Right). A,B – straw; C,D – stem; E,F – Leaf

5.4.5. Antioxidant Activity of Extracts

The antioxidant activity of the methanol extractions from the various rice fractions is shown in Figure 5.7. A comparison of the anthocyanin contents and respective antioxidant capacity for various extract and fractions showed a positive correlation (r = 0.86).

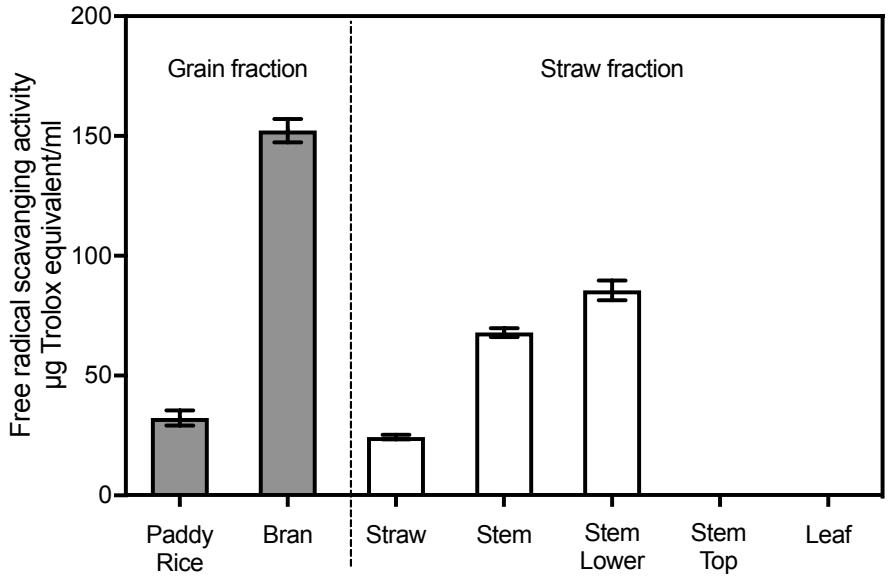


Figure 5.7 Antioxidant activity of various black rice fractions extracted by methanol

Antioxidant activity of the different extracts was measured using DPPH assay and expressed as free radical scavenging activity in µg Trolox equivalent/ml (mean ± SD, n =3).

The antioxidant activity for the methanol and hydrothermal anthocyanin extracts is shown in Figure 5.8. The 90 and 100°C hydrothermal extracts showed similar antioxidant activity and were significantly higher (almost double) compared to the methanol extract (p= 0.004 and 0.007 for water 90° and 100°C, respectively). Additionally, scavenging activity of water extracts at 100°C was higher than the 90° C extract but this was not statistically significant (p=0.75).

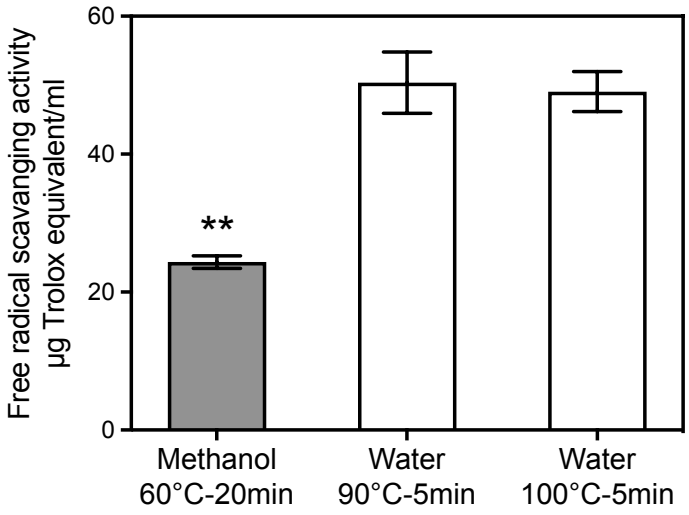


Figure 5.8 Antioxidant activity of straw water and methanol extracts

Antioxidant activity of the different extracts was measured using DPPH assay and expressed as free radical scavenging activity in µg Trolox equivalent/ml (mean ± SD, n =3).

5.4.6. Cytotoxicity and Apoptosis

Bioactivity of the extract was determined by measuring the viability of a Jurkat cells following exposure to 200-800µg/ml of the 100°C hydrothermal extract from straw. Hydrothermal bran extract was also included to assess if the two extracts induce similar responses to the cells. Apoptosis and cytotoxicity of Jurkat cells were detected by Annexin-V and propidium iodide (PI) staining (section 5.2.5.3), respectively followed by analysis with flow cytometry (Figure 5.9). The positive control shows typical result for cells under normal condition (only % live and % dead were measured for +ve control) and the negative controls show typical response of cells in presence of a cytotoxic agent. Results show that cell viability is not affected by the extracts and increasing extract from 200-800 µg/ml does not result in any further death of cells nor induction of apoptosis.

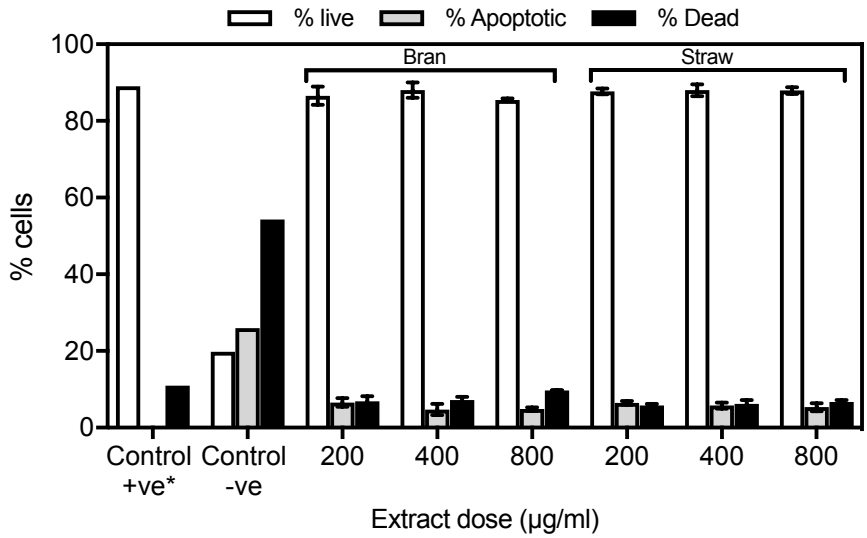


Figure 5.9 Bioactivity of straw and bran extract

Effect of straw and bran water extracts at 200-800µg/ml on cytotoxicity and apoptosis of jurkat cell lines after 24 hours of incubation measured with flow cytometry following staining with propidium iodide and annexin V (mean ± SD, n =3). Cells were defined as: live Annexin-V^{-ve}/PI^{-ve}; Apoptotic Annexin-V^{+ve}/PI^{-ve} or dead Annexin-V^{+ve}/PI^{+ve}. *only % live and % death was measured for +ve control.

5.4.7. Analysis for sugar source

5.4.7.1. Sugar composition analysis

There are two major waste fractions from rice farming: straw and husk. Preliminary experiments showed that the husk had a glucose content of 33.7 ±1.1% and xylose content of 16.1% ±0.5 (expressed as percentage of biomass). Pre-treatment of the husk at 200 °C for 5 min resulted in glucose and xylose composition of 41.3% ±3.4 and 14.3% ±1.3 respectively (expressed as percentage of pre-treated residue). However, husk digestibility after pre-treatment was only 15.5% ±1.6 and 11.9% ±0.5 for glucose and xylose respectability (expressed as percentage of available sugars). Given that the husk has minimal anthocyanin and was highly recalcitrant no further studies on

this fraction were performed.

The sugar composition of the raw total black straw was determined and is shown in Table 5.3. The straw was compared with “normal” white rice as a comparator. Black rice straw had a greater level of glucose than the white straw but xylose, arabinose and galactose levels in the two rice straws were comparable.

	Arabinose	**Galactose**	**Glucose**	**Xylose**
Black rice straw	4.0±0.4	1.4±0.2	40.1±1.4	20.4±1.2
White rice straw	3.3±0.3	1.1±0.1	30.7±1.0	16.7±1.6

Table 5.2 Sugar composition of raw black and white rice straw

Raw straw was subjected to Saeman hydrolysis and monomeric sugars measured by HPAEC. Results are expressed as percentage of original biomass (±SD, n =3)

The sugar composition of the dried solid residues, after pre-treatment at 100-220 °C for 5 minutes, from black and white rice straw were also assessed (Table 5.4). In this instance, the glucose contents of the residues between the two straws seemed to be fairly comparable and xylose whilst showing a higher level in the black rice residues at the lower treatment temperatures were fairly comparable after pre-treatment at 200 °C.

Sample	Temp (°C) (5 mins)	Black rice residue		White rice residue	
		Glucose	Xylose	Glucose	Xylose
Straw	100	43.7 ±1.4	33.9 ±1	40.4 ±1.9	22.6 ±0.8
	120	42.1 ±1.9	32.8 ±1.3	40.3 ±2.3	22.3 ±1.5
	140	43.6 ±1.4	34.2 ±0.8	38.8 ±1.8	21 ±1
	160	43.1 ±2.1	23.4 ±1.2	39.3 ±2.7	21.3 ±1.2
	180	42.8 ±1.9	23.1 ±1	40 ±1.3	21.3 ±1
	200	46 ±2.6	18.4 ±2.5	43 ±2.7	17.2 ±0.8

Table 5.3 Sugar composition of pre-treated black rice and white rice straw

Pre-treated straw residue was subjected to Saeman hydrolysis and monomeric sugars measured by HPAEC. Results are expressed as percentage of pre-treated residue (±SD, n =3)

Other changes associated with the pre-treatment such as weight loss, pH of the pre-treated liquor and the liquor sugar composition were also measured (Tables 5.5 and 5.6). In both cases an increase in pre-treatment severity was accompanied by a decrease in weight of recovered pre-treated residue and pH of the hydrolysate. In the hydrolysate, black rice straw had a greater level of glucose than the white straw but xylose, arabinose and galactose levels in the two rice straws were comparable.

Temp (°C) 5 min	Wt. (%)	pH	Sugar in pre-treated liquor (% of original biomass)			
			Arabinose	Galactose	Glucose	Xylose
100	86.4±1	6.1 ±0.01	0	0	1.82 ±0.06	0
120	84.7±0	6 ±0.01	0	0	1.74 ±0.1	0
140	83.5±1	5.7 ±0.03	0.03 ±0	0	1.77 ±0.08	0
160	82.5±1	5.2 ±0.03	0.08 ±0.01	0	1.75 ±0.07	0
180	80.4±1	4.7 ±0.02	0.4 ±0.06	0	1.57 ±0.05	0.01 ±0.02
200	67.5±2	4.1 ±0.02	0.96 ±0.03	0.08 ±0.01	1 ±0.08	0.21 ±0.09

Table 5.4 Changes in black rice weight and liquor composition with pre-treatment regimes.

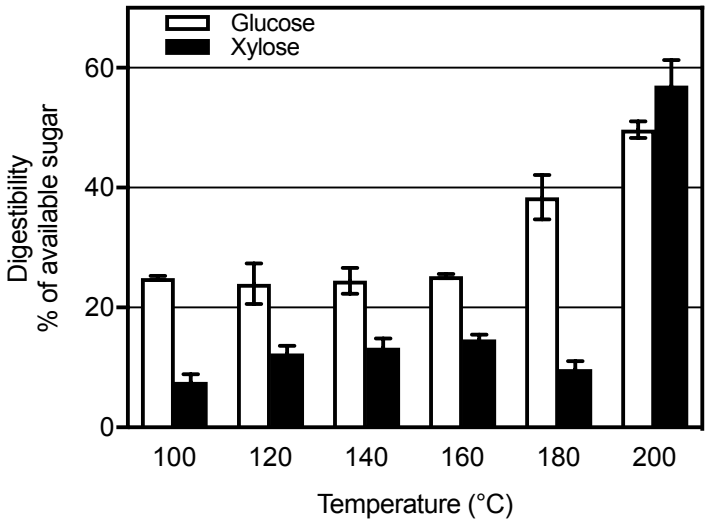
Temp (°C) 5 min	Wt. (%)	pH	Sugar in pre-treated liquor (% original biomass)			
			Arabinose	Galactose	Glucose	Xylose
100	90.3±0.6	6.7 ±0.01	0	0	0.14 ±0.01	0
120	89.3±1	6.5 ±0.1	0	0	0.11 ±0.01	0
140	88.3±1	6.2 ±0.06	0	0	0.12 ±0.01	0
160	88.9±0.7	5.7 ±0.03	0.04 ±0	0	0.11 ±0.01	0
180	84.1±1.4	5.1 ±0.03	0.3 ±0.01	0	0.07 ±0.01	0
200	73.1±1.4	4.6 ±0.04	0.6 ±0.01	0.06 ±0	0.0 4±0	0.13 ±0

Table 5.5 Changes in white rice weight and liquor composition with pre-treatment regimes.

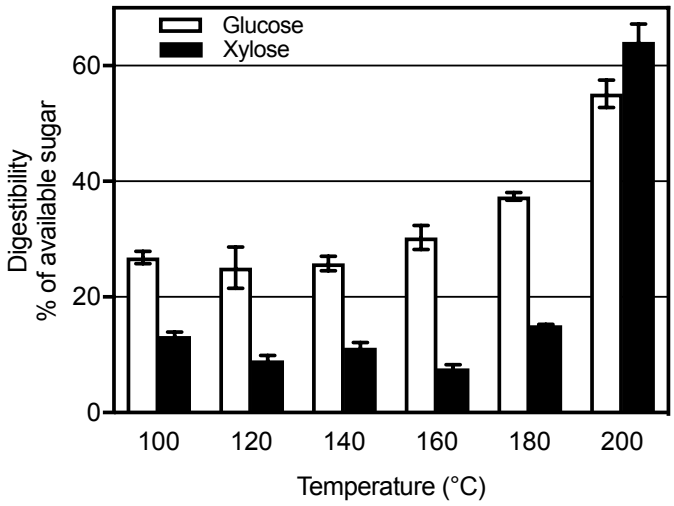
5.4.7.2. Digestibility

Pre-treated residues from the above experiment were hydrolysed using CTec 3 enzyme and saccharification efficiency quantified by measuring the glucose and xylose released at 72 hr using HPAEC as described in section3.2.3 (Figure 5.10). Digestibility was expressed as percentage of the available sugar in the pre-treated residue.

Digestibility of both the residues increased with the increase in the pre-treatment regime up to 200 °C used in the experiment. Glucose and xylose digestibility of both the straw was comparable, with white rice showing a slightly higher digestibility but these were statistically insignificant ($p = 0.05$ and $p= 0.11$ for glucose and xylose respectively).



a



b

Figure 5.10 Digestibility of pre-treated black and white rice straw

Biomass, (a) black rice straw and (b) white rice straw was pre-treated for 5min at the temperature shown. Digestibility of the washed and dried residues was assessed by measuring release of monomeric sugars after 72hr of enzymatic saccharification. Results are expressed as % available sugar in the residue ±SD, (n=3).

5.4.7.3. Fourier Transformed Infrared Spectroscopy

FTIR was used as an independent analysis to determine generic compositional differences between total black and white rice straw, changes following the pre-treatment of the two straw and changes between the different fractions of

the black rice straw (Figure.5.11). Spectra of both the black and white rice straw showed more prominent cellulose bands (3300 and 900 cm-1), lignin bands (1595 and 1510 cm-1) and wax bands (2850 and 2920 cm-1) in the pre-treated residue (Figure 5.11 a-b). Figure 5.11c, depicts the varying chemical composition of the different straw fractions as reflected by the distinct variations between the FTIR spectra of the different straw fractions. Here, the stem lower internodes showed the most distinctive lignocellulosic bands and the leaf showed the least.

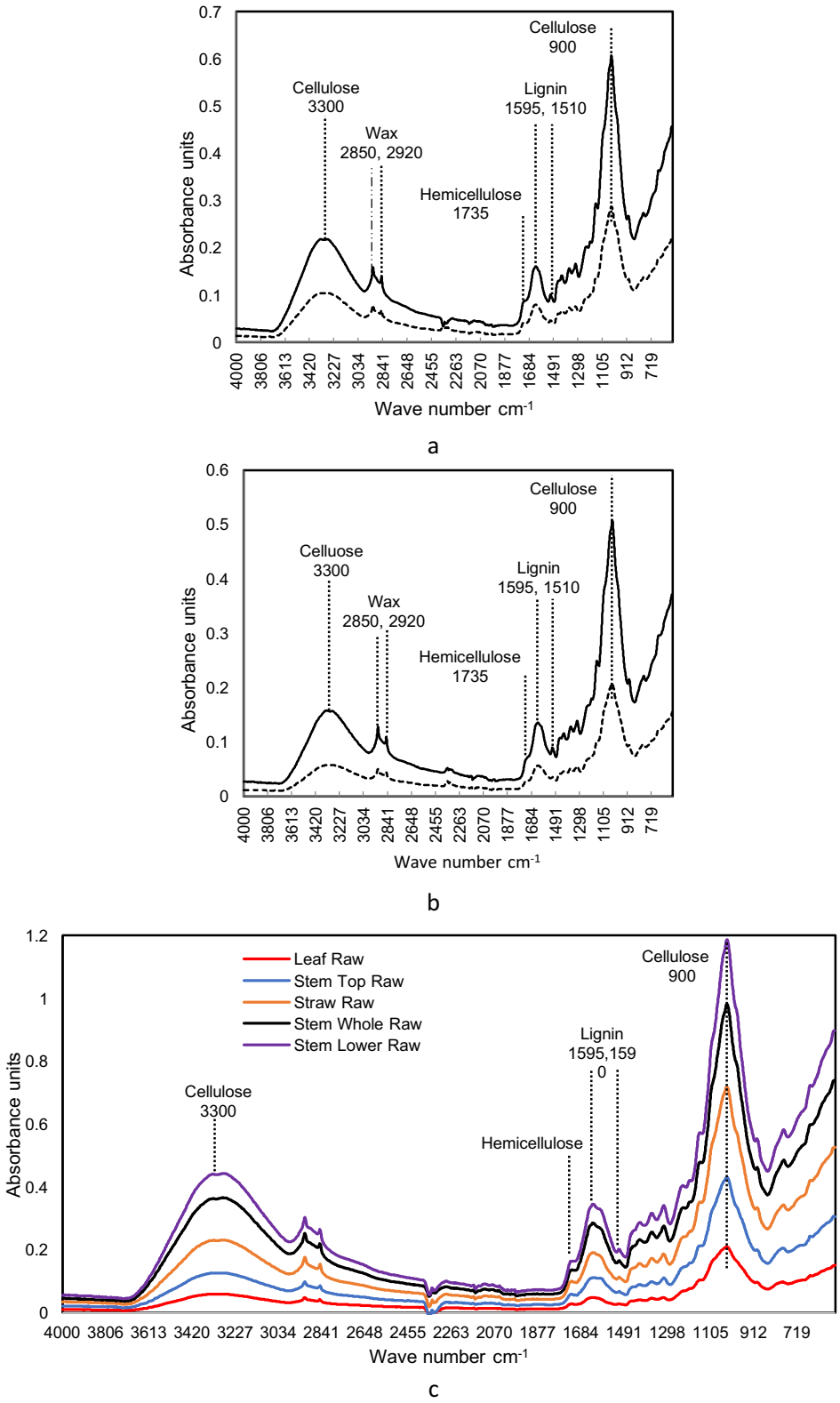


Figure 5.11 ATR-FTIR spectra of rice straw

Untreated straw (dotted line) and hydrothermally pre-treated (strong line)- a) black rice straw, b) white rice straw and c) different fractions of untreated black rice straw with leaf (red), stem top (blue), whole straw (green), whole stem (black) and stem lower (purple) with components approximate peak values from literature. All spectra are offset on y-axis for easy visualisation.

5.5. DISCUSSION

Measuring the anthocyanin content of the grain and straw fractions showed that the total paddy grain had a higher content than the total straw. In the grain fraction, as expected, the bran had the highest content. Polishing removes the bran and this led to almost half the anthocyanin in polished rice compared to paddy grain. The low levels of anthocyanin in the husk was probably associated with contamination with bran during industrial de-husking. In the straw, anthocyanin was present mainly in the stem compared to leaf. The little anthocyanin in the leaf was mainly from the leaf sheath. Chin *et al.,* have also reported low levels of anthocyanin in leaf of rice to be concentrated in leaf sheath - 1.2 µmole/g in leaf sheet compared to 0.3 µmole/g for leaf blade which was not visually detectable (Chin et al., 2016). The stem was divided into lower internodes and top most internode fractions. The anthocyanin was found to be concentrated in the lower internodes while the top most internode had negligible anthocyanin. Commercial sources of anthocyanin include grape Marc which contains ~100mg/100g (Liang et al., 2008), while whole grape has 27 mg/100g (Wu et al., 2006). Hence, the straw with 63mg/100g forms an attractive source for anthocyanin in Manipur where grape cultivation is rare. This proves the first hypothesis that Black rice straw is a good source of anthocyanin.

The straw anthocyanin content of 63mg/100g, measured after methanol extraction, was used as the theoretical maximum to compare the efficiency of the aqueous microwave extraction. The microwave extraction could extract

85.8% of the theoretical maximum anthocyanin from the straw after 5min at 90°C. Beyond this temperature, anthocyanin levels decreased and were undetectable from 160°C onwards possibly due to the heat labile nature of anthocyanins resulting in their degradation. However, for commercial purposes an extraction temperature of 100°C may be more suitable as it is easier to maintain water at 100°C and the difference in extraction efficiency at 90 and 100°C was statistically insignificant ($p=0.75$). For both the methanol and microwave hydrothermal extractions, a solid loading of 5% (w/v) was chosen as high loading has been reported to have negligible impact on anthocyanin extraction (Bridgers et al., 2010) and 5% represents a loading factor that may be commercially significant. This proves the second hypothesis that microwave hydrothermal method is a good alternative to organic solvent for anthocyanin extraction.

The antioxidant activity of the extract was measured via DPPH assay. The DPPH assay is a frequently employed method to measure ability of anthocyanin compounds to act as free radical scavengers (Szabo et al., 2007). In the first instance, a correlation was conducted between the anthocyanin content of the different fractions extracted by methanol together with straw water extract with their antioxidant values. This gave a correlation of 0.68, indicating that anthocyanin could be a major contributor in the observed antioxidant effect. The correlation value may also be indicative of the presence of other compounds in the extracts that contribute to antioxidant capacity in addition to the anthocyanin. This could explain the significantly higher (almost double)

antioxidant activity of the water compared to the methanol extracts, with 53.9mg/100g of anthocyanin compared to the methanol extract with 63mg/100g. The higher values of water extracts compared to methanol extracts could be potentially due to the release of additional antioxidant compounds such as bound phenolics during the higher temperature water extraction of or decrease in stability of the methanol extract due to the longer extraction time. This proves the third hypothesis that hydrothermal hydrolysis results in anthocyanin extracts that are stable or at least comparable to methanol extracts. In this case even better than methanol extract.

The measurement of antioxidant activity was crucial to demonstrate that the anthocyanin was not denatured during the extraction and thus warrants further study into the potential bioactivity. The potential bioactivity of the extract was assessed by measuring viability, apoptosis and death of Jurkat cell lines after exposure to 200-800µg/ml the straw water extract. Water bran extract was included for comparison. The results showed that both straw and water extracts up to 800800µg/ml have negligible effects on cell death or apoptosis. Anthocyanin are approved for use as a food additive or colouring agent in the EU, Australia and New Zealand with the E number E163 (INS number 163) (FSA, 2016). This could mean that the extract is suitable to fortify foods with anthocyanin, boosting nutraceutical value of the food while also acting as a natural colourant. This proves the fourth hypothesis that hydrothermally extracted anthocyanins are bioactive.

After confirming the potential for anthocyanin extraction, it was important to analyse the straw as a source of renewable sugar. Black rice straw had a greater level of glucose than the white straw but xylose, arabinose and galactose levels in the two rice straws were comparable. These values were similar to reported sugar composition of rice straw: arabinose 3.3 galactose 1.2 glucose 36-42 xylose 21-26 (Chen et al., 2012; Wi et al., 2013)..

Following this the solid residue from the water extraction at 100 °C to 200 °C of both black and white rice straws was further analysed for its sugar composition. In both the straw glucose content of the residue was constant, while there was a decline in the xylose content at higher temperatures. This was more evident in the black rice which had a higher xylose content than the white rice in the residue after pre-treatment at 100^{o}C. In both straws, the pre-treatment up to 180° C did not seem to have a drastic impact on the glucose and xylose composition. However, at 200°C there was a slight increase in glucose and decrease in xylose. This was accompanied by a sudden decrease in the percent recovered weight compared to 180°C with 80-67% recovered for black rice and 84-73% for the white rice. The decrease in recovered weight and liquor pH is due to a combination of solubilisation of cell wall components (predominantly hemicelluloses and salts), hydrolysis, breakdown, and some volatilisation of wall components (Wood et al., 2016). In both the straw, monomeric sugars arabinose, galactose, glucose and xylose in the liquor was minimal. As reported by Yu *et al.,* glucose detected in the liquors at lower temperatures may result mainly from easily solubilised polysaccharides in the

rice straw and hardly from crystalline cellulose, which is usually degraded at higher temperatures (Yu et al., 2010). In this regards the black rice liquor had almost 10 times more free glucose than the white rice straw. This could be due to incomplete translocation of soluble carbohydrates, such as starch, from the stem to the grain, making them available for utilisation in a biorefinery (Capper et al., 1992).

The dried residues from the pre-treatment were subjected to a standard digestion assay. Associated with the drastic loss of weight at 180°C, the residues showed reduced recalcitrance from 180°C as demonstrated by an increase glucose and xylose digestibility. For both the straw, pre-treatment from 100°C to 200°C resulted in a two-fold increase in digestibility of both straw samples – giving a glucose digestibility of 50 and 55% for black and white rice, respectively. These values equate to a glucose yield at 200°C of 147 and 173mg/g of original straw for black and white straw, respectively. The xylose yield at 200°C was 67 and 80.2mg/g of original straw for black and white straw, respectively. This proves the fifth hypothesis that the straw residue can be a good source of sugar comparable to that obtained from white rice.

Varying levels of rice straw glucose digestibility has been reported depending on the pre-treatment used: 45% after steam explosion (220°C for 10 min) (Wood et al., 2016); 38% after microwave (2.45 GHz) (Ma et al., 2009); 60% after alkali 60% and 65% after alkali microwave (700w) (Zhu et al., 2005). Hence, the efficiency of glucose digestibility of 50 % (black rice) and 60% (white

rice) observed with the microwave hydrothermal process used in this research is comparable to reported values or even better.

Qualitative compositional differences of the different straw fractions of the black and white rice were analysed using FTIR. There were clear differences in absorbance and shape of bands between the spectra of untreated and pre-treated rice straw. They show an increase of lignin and cellulose with distortion of hemicellulose structures. Bands at 2850 and 2920cm^{-1} (CH_2- stretching bands ascribed to wax) increased upon the pre-treatment. Bands at 1735cm^{-1} (carbonyl, ascribed to hemicellulose) (Kristensen et al., 2008), reflect the partial removal of hemicellulose after the pre-treatment. Lignin bands at approximately 1595cm^{-1} and, 1510cm-1 (aromatic ring stretch) (Stewart et al., 1995) were strongly enhanced in the hydrothermally pre-treated samples. One explanation for this could be a relative increase in the amount of lignin due to the removal of hemicelluloses. Another reason could be that lignin is released and re-deposited on the surface. Bands observed around 3300cm^{-1} and 900cm^{-1} are assigned to cellulose and were slightly increased after the pre-treatment (Swain and Krishnan, 2015). This is expected as hydrothermal pre-treatment has been reported to not degrade the fibrillar structure of cellulose but, causes strong lignin re-localisation and partial removal of hemicellulose (Kristensen et al., 2008).

The different factions of black rice straw for which the anthocyanin contents were assessed was also assed for their composition by FTIR, with particular

interest in cellulose. Like their anthocyanin contents, the leaf and stem top fractions also showed the least cellulose content. Hence, in a scenario where a fractionation of the black rice straw is needed, the lower half fraction would be a good source of anthocyanin and sugars and the top fractions for example as animal feed. However, lower stem has been reported to be less digestible than the upper stem (Ohlde et al., 1992; Summers et al., 2003).

CHAPTER 6 CONCLUSION AND FUTURE WORK

6.2. CONCLUSION

The thesis has provided some potential routes to a feasible lignocellulosic biorefinery. Wheat wild relative introgression results showed not only a range of variation in straw phenotype and sugar composition but also on digestibility of the BC_1 stems. Phenotypic data, such as stem biomass which ranged from 3-22g/plant showed no correlation with digestibility, showing digestibility was not defined by, or related to, this morphological trait. The introgression also influences the lignin contents of BC_1 stem and the lignin content of the BC_1 plants had a negative correlation with their respective digestibility. The available genetic map of the BC_1 showed that there has been a larger than expected level of recombination, meaning, identifying introgressions linked to our trait of interest and generating stable lines will be a significant undertaking. These findings are of interest for the biofuel industry because this mean that wheat lines can theoretically be developed possessing straw from which a significant amount of sugars could be extracted with reduced cost associated with the pre-treatment.

In work on mixing feedstocks of wheat straw, willow and *Miscanthus*; all the mixes gave higher than expected sugar yields with wheat + willow and willow + *Miscanthus* mixes in particular showing statistically significant improvements. Additionally, impact of mixing on inhibitor productions seems to be minimal and does not adversely impact on either metabolic activity or

growth of yeast. Improvement in saccharification has been suggested to be due to a reduction in recalcitrance of the residue possibly due to improved response to pre-treatment brought about by the mixed nature of the feedstock. Degradation products such as acetic acid from one feedstock could have impacted the overall pH of the reaction mixture and brought about an enhance pre-treatment. Since most lignocellulosic feedstocks are likely to be seasonal, mixing different feedstock could be used as an attempt the offset the feedstock demand and provide a steady stream of feedstock. This feedstock although mixed could have a controlled feedstock composition and a possible year-round availability depending on the crop in season/ local produce. However, it would require storage to house all the different feedstocks.

Analysis of black rice work also showed the potential to develop a combined biorefinery process for anthocyanin and second-generation sugars using black rice straw. The optimised microwave treatment was able to extract anthocyanin utilising only water without additional chemicals, organic solvents or harsh treatments. More importantly, the water extract showed twice the antioxidant activity in comparison to the methanol extract; while also not being cytotoxic to mammalian cell lines. Finally, the black straw residue showed a glucose digestibility of 49.6 ±1.4%. This was similar to the efficiency of 55.1 ± 2.4% observed with white rice straw. Since anthocyanin will start to degrade at temperatures ≥160°C, it is recommended to first extract anthocyanin at a lower temperature and subsequently treat the residue at high temperature to be used as a source of sugar for biorefining. Such complete and judicious use of

available agricultural by-products would play an important role in biorefineries of the future, especially in improving the economics of production.

6.3. FUTURE WORK

6.3.1. Wheat wild relative Introgression

Further work is needed to identify the genetic basis for the improved biorefinery traits and to implement the breeding towards improved wheat varieties. This might be achieved by:

i) Following on with the preliminary mining of the genotyping data to identify introgressed segments associated with those plants exhibiting the best and worst level of the desirable traits for all parental lines. This will hopefully identify commonly inherited sets of introgression segments for both the desirable and undesirable trait. Genotyping data from subsequent backcross populations (BC_2, BC_3 etc) could then be used to identify plants which have inherited fractions of these common segments. These plants could then be phenotyped for the desirable trait.

ii) One potential problem with the first approach is that the preliminary genotyping has shown that there are a very large number of introgressed segments associated with the BC_1 population. This may make mining the data difficult. Thus, a second approach could be carried out in parallel. This will use the BC_1 data as a proof-of-concept and start a fresh analysis using all the current crosses available – BC_2, BC_3 etc. In this instance as many plants as possible from these later back crosses (with fewer introgression events) will be screened for the desirable traits.

However, with each subsequent crossing the number of samples to analyse will be multiplied due to segregation of the interested introgression segments to multiple plants. Thus, analysis of digestibility and sugar composition will require high throughput assays. The robotic saccharification assay at York University which has shown good correlation with our more in-depth, but low throughput, manual digestibility assay at Nottingham, could be used to overcome the screening demands.

Preliminary analysis of lignin content of the BC_1 straw in this thesis showed a strong negative correlation with digestibility and no correlation with crystallinity. Additionally, results from the 2012 harvest suggested that hemicellulose composition may also be a crucial factor in reduced recalcitrance (Waldron, 2017). Studying how these relationships are effected in the BC_2, BC_3 and BC_4 populations as the crosses become closer to the modern wheat would also be very interesting.

Additional parameters reported in the literature regarding digestibility not covered in the thesis could also be included. These include: i) The arabinose substitution degree of xylan (i.e., the reverse xylose/arabinose ratio) reported as a positive factor on biomass saccharification in all grass species (Ohlde et al., 1992; Summers et al., 2003) could be conducted using 1M sulphuric acid hydrolysis of straw followed by high performance anion exchange chromatography; ii) Expansins, a group of proteins with the capacity of relaxing cell wall tension by disrupting the hydrogen bonds binding together cellulose

fibrils and cellulose and other polysaccharides through a non-enzymatic process, improving subsequent sugar releasing (Wang et al., 2016) could also be analysed; and iii) Explore other pre-treatment conditions such as using alkali and also look at taking forward the pre-treated residue to enzyme hydrolysis without drying as drying could impacted digestibility by collapsing the disrupted lignoclelluosic matrix. The information on introgressed segments could eventually be exploited for marker assisted breeding of dual purpose wheat lines. However, these would need to be tested to ensure that their agronomic traits have not been adversely effected and would obviously need to undergo the required statutory testing.

6.3.2. Mixed Feedstock work

Further work is needed to confirm that the improvements seen in this thesis are also applicable on a larger scale and to investigate the basis of the improvements. If a larger scale pressurised microwave reactor is not commercially available, the pre-treatment conditions could be replaced with ones that are available such as steam explosion or conventional hydrothermal pre-treatment. The ratio of Wheat: Willow; Wheat: *Miscanthus*, Willow: *Miscanthus* could be optimised or the impact of mixing all three feedstocks examined. This could be carried out in the lab-scale microwave reactor used for the work conducted in this project.

The current experiments carried out used high enzyme loading and low solid loading. For commercial significance, the performance at lower enzyme loading

and higher solid loading should be explored. Actual fermentation yields will also need to be examined for it be of commercial significance. Microbial inhibitors study could be extended to lignin degradation products such as phenolics and organic acids which were not covered in the thesis. This could provide some more light on the yeast physiological responses to the pre-treated liquor.

The approach can be extended to include municipal solid waste (MSW) and waste from food industries. Using different lignocellulosic feedstock with MSW would result in contributing towards the handling and conversion of MSW in a sustainable way while expanding the biomass feedstock. A full economic and lifecycle analysis including supply chain logistics of the multiple feedstock and storage conditions will need to be assessed and accounted for.

6.3.3. Black rice straw

Further work is needed to confirm the anthocyanin extraction and bioactivity and saccharification of the pre-treated residue on a larger scale.

Anthocyanin extraction could be tested on a larger scale using conventional hydrothermal pre-treatment with water at 100 °C. This could be conducted with collaboration with Council for Scientific and Industrial Research (CSIR) (Govt. of India) at the local production site with the aim to empower local farmers. This is also important as importing large quantity of black rice straw to the UK will involve significant cost and administrative procedures. CSIR encourages such technology transfer, their involvement will also help promote

revitalisation of the black rice cultivation and the work will contribute to UK's OECED support.

The extracts have been shown to be safe on mammalian cell lines. Additional bioactivity studies can be conducted on the anthocyanin extract for its suitability in medical applications. One such work is currently on track where the extract is being studied for potential anti-cancer effects. This will be essential to improve the economic value of the extract.

A life cycle and techno-economic analysis of the whole process needs to be carried out to assess the feasibility of the process in a biorefinery context – suitable pre-treatment, enzyme and solid loading, fermentation yields etc.

Transforming the black rice to a high yielding variety adapted to changing biotic and abiotic conditions while also increasing the expression of anthocyanin in its straw is not far from the realm of possibility. Since anthocyanin also contribute to the plant's biotic and abiotic resistance, developing hybrid varieties could be a way forward to transfer this adaptive trait to normal white rice. This could be a localised expression to the plant straw (to get white rice kernels) or a systemic expression, in which case it will also improve the nutritional and nutraceutical value of the rice. Unlike golden rice which involved expression of β-carotene (precursor of Vitamin A) gene from daffodil and bacterium using GM techniques, the transfer of nutraceutical genes in the same species (rice) could be potentially feasible with non-GM techniques,

advance breeding techniques or even Crisper Cas9 techniques. This will also act as bio-fortification since black rice has been found to contain higher levels of Oryzanol, tocotrienol, tocopherol, Vit. E, Polyphenol (bound and soluble), and antioxidant capacity compared to traditional white rice variety in India such as Indian rice (IR) 64, Jyothi and *Nirjavara* (unpublished data).

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APPENDIX A WHEAT INTROGRESSION

Table A. 1 Genetic providence of the BC_1 plants

Sample	BC1 Code	Species	Female	Male
1	112	*Aegilops mutica*	CS 94 x *Ae mutica* 2130004	Paragon
2	234	*Triticum urartu*	CS mutant 84 x *T urartu* B14	Highbury
3	115B	*Triticum timopheevii*	Paragon mut 13 x *T timopheevii* P95-99.1-1	Paragon
4	228	*Triticum urartu*	CS mutant 84 x *T urartu* A6	Highbury
5	110B	*Aegilops mutica*	CS 94 x *Ae mutica* 2130004	Paragon
6	226	*Aegilops speltoides*	n.d	n.d
7	114B	*Triticum timopheevii*	Paragon mut 13 x *T timopheevii* P95-99.1-1	Paragon
8	106	*Aegilops caudata*	Pavon 76 x *Ae caudata* 2090001	Highbury
9	113B	*Triticum timopheevii*	Paragon mut 13 x *T timopheevii* P95-99.1-1	Highbury
10	124C	*Aegilops speltoides*	Highbury x *Ae speltoides* 2140008	Paragon
11	192B	*Triticum timopheevii*	CS mut 84 x *T timopheevii* P95-99.1-1	Paragon
12	285	*Triticum urartu*	CS mutant 84 x *T urartu* B30	Highbury
13	170B	*Seclae cereale*	CS 94	CS mutant 84 x *S cereale* 390382
14	35A	*Aegilops speltoides*	CS 94 x *Ae speltoides* 2140008	Paragon
15	156A	*Seclae cereale*	CS mutant 84 x *S cereale* 390382	Paragon
16	154	*Seclae cereale*	CS mutant 84 x *S cereale* 390382	CS 94
17	157	*Seclae cereale*	CS mutant 84 x *S cereale* 390382	CS 94
18	155	*Seclae cereale*	CS mutant 84 x *S cereale* 390382	Paragon
19	262	*Aegilops speltoides*	Highbury x *Ae speltoides* 2140008	Highbury
20	104	*Aegilops caudata*	CS mut 84 *x Ae caudata* 2090001	Paragon
21	300B	*Triticum timopheevii*	Paragon mut 13 x *T timopheevii* P95-99.1-1	Paragon
22	282F	*Thinopyrum bessarabicum*	Paragon	Creso phph x *Th bessarabicum* S63 (1)
23	227	*Triticum urartu*	CS mutant 84 x *T urartu* A6	Highbury
24	171A	*Secale cereale*	CS 94	CS mutant 84 x *S cereale* 390382
25	203	*Secale cereale*	CS 94	CS mutant 84 x *S cereale* 390382
26	141	*Thinopyrum bessarabicum*	CS mut 84 x *Th bessarabicum* 531712	Pavon 76
27	131	*Triticum urartu*	CS mutant 84 x *T urartu* A6	Highbury
28	100	*Aegilops caudata*	CS mut 84 x *Ae caudata* 2090001	Paragon
29	117	*Aegilops triaristata*	CS mut 84 x *T triaristata* P95-92.1-1	Paragon
30	127	*Aegilops speltoides*	CS 94 x *Ae speltoides* 2140007	Pavon 76
31	119	*Secale anatolicum*	CS mut 84 x *S anatolicum* P208/141	Paragon

32	129	*Aegilops speltoides*	CS 94 x *Ae speltoides* 2140007	Paragon
33	146	*Secale cereale*	CS mutant P208/511 x *S cereale* 428373	Paragon
34	187	*Aegilops mutica*	CS 94 x *Ae mutica* 2130008	Paragon
35	121	*Secale vavilovii*	CS 94 x *S vavilovii* 573649	Paragon
36	293	*Triticum urartu*	CS mutant 84 x *T urartu* G65	Highbury
37	268B	*Secale cereale*	CS 94	CS mutant 84 x *S cereale* 390382
38	170D	*Secale cereale*	CS 94	CS mutant 84 x *S cereale* 390382
39	114A	*Triticum timopheevii*	Paragon mut 13 x *T timopheevii* P95-99.1-1	Paragon
40	172B	*Aegilops speltoides*	Highbury *x Ae speltoides* 2140008	Highbury
41	160	*Secale cereale*	CS mutant 84 x *S cereale* 390382	SELF
42	115A	*Triticum timopheevii*	Paragon mut 13 x *T timopheevii* P95-99.1-1	Paragon
43	107	*Aegilops caudata*	Pavon 76 *x Ae caudata* 2090001	Highbury
44	254B	*Aegilops speltoides*	CS 94 x *Ae speltoides* 2140007	Pavon 76
45	105	*Aegilops caudata*	Pavon 76 x *Ae caudata* 2090001	Paragon
46	125	*Aegilops speltoides*	Highbury x *Ae speltoides* 2140008	Paragon
47	195	*Triticum timopheevii*	CS mut 84 x *T timopheevii* P95-99.1-1	Highbury
48	170A	*Secale cereale*	CS 94	CS mutant 84 x *S cereale* 390382
49	170C	*Secale cereale*	CS 94	CS mutant 84 x *S cereale* 390382
50	171B	*Secale cereale*	CS 94	CS mutant 84 x *S cereale* 390382
51	304	*Triticum timopheevii*	CS mut 84 x *T timopheevii* P95-99.1-1	Paragon
52	300C	*Triticum timopheevii*	Paragon mut 13 x *T timopheevii* P95-99.1-1	Paragon
53	124A	*Aegilops speltoides*	Highbury x *Ae speltoides* 2140008	Paragon
54	153	*Secale cereale*	CS mutant 84 x *S cereale* 390382	Paragon
55	130A	*Aegilops speltoides*	CS 94 x *Ae speltoides* 2140007	Paragon
56	103	*Aegilops caudata*	CS mut P208/511 x *Ae caudata* 2090001	Paragon
57	253	*Aegilops mutica*	CS 94 x *Ae mutica* 2130012	CS 94
58	224	*Triticum urartu*	CS mutant 84 x *T urartu* A6	Paragon
59	172A	*Aegilops speltoides*	Highbury x *Ae speltoides* 2140008	Highbury
60	284	*Triticum urartu*	CS mutant 84 x *T urartu* B30	Paragon mutant P208/372
61	188B	*Aegilops mutica*	Pavon 76 x *Ae mutica* 2130012	Paragon
62	219A	*Triticum urartu*	CS mutant 84 x *T urartu B*	Paragon
63	113C	*Triticum timopheevii*	Paragon mut 13 x *T timopheevii* P95-99.1-1	Highbury
64	259	*Aegilops speltoides*	CS 94 x *Ae speltoides* 2140007	Pavon 76
65	302	*Triticum timopheevii*	Paragon mut 13 x *T timopheevii* P95-99.1-1	Highbury
66	265	*Aegilops speltoides*	CS 94 x *Ae speltoides* 2140018	Paragon

67	225	*Triticum urartu*	CS mutant 84 x *T urartu* A6	Paragon mutant P208/372
68	194	*Triticum timopheevii*	Paragon mut 13 x *T timopheevii* P95-99.1-1	Highbury
69	128	*Aegilops speltoides*	CS 94 x *Ae speltoides* 2140007	Pavon 76
70	124B	*Aegilops speltoides*	Highbury x *Ae speltoides* 2140008	Paragon
71	184	*Aegilops mutica*	CS mutant 84 x *Ae mutica* 2130012	Paragon
72	139	*Thinopyrum bessarabicum*	CS mut P208/535 x *Th bessarabicum* 531712	CS 94
73	117	*Aegilops triaristata*	CS mut 84 x *T triaristat*a P95-92.1-1	Paragon
74	246	*Aegilops mutica*	CS 94 x *Ae mutica* 2130012	Pavon 76
75	179	*Aegilops speltoides*	Highbury x *Ae speltoides* 2140008	Paragon
76	118	*Aegilops triaristata*	CS mut 84 x *T triaristata* P95-92.1-1	Paragon
77	256	*Aegilops speltoides*	CS 94 x *Ae speltoides* 2140007	Pavon 76
78	180B	*Aegilops speltoides*	Pavon 76 x *Ae speltoides* 2140007	Paragon
79	249	*Aegilops mutica*	Pavon 76 x *Ae mutica* 2130012	Pavon 76
80	299	*Triticum urartu*	CS mutant 84 x *T urartu* G27	CS 94

Genetic analysis of BC_1 samples.

The BC_1 samples generated at Nottingham were all sent to Bristol University for genotyping. The results returned for the *Aegilops speltoides*, *Aegilops mutica, Triticum timopheevii* and *Triticum urartu* introgresions will be shown in this thesis – Figure A.1-4. In each case only a selection of the introgressions are shown, these being for the two BC_1 plants with the lowest digestibility and the two with the highest digestibility in each group. The genotype for each of the seven chromosomes has been split into four columns, the two on the left of each chromosome have the lowest digestibility and the two on the right have the highest digestibility. The blue segments are where there is a homogenous allele for the elite wheat line parent, sections in red show where one of the alleles is from the wild type parent and yellow show non-readable segments.

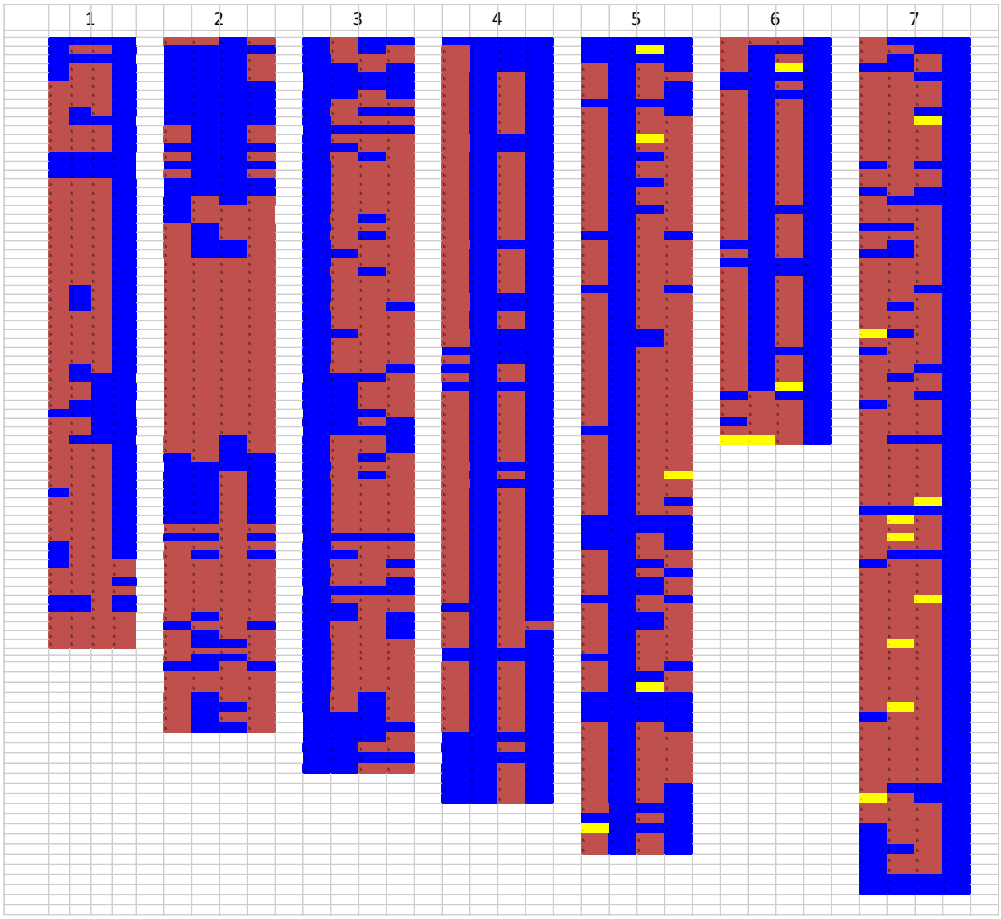


Figure A. 1 Genetic markers for *Aegilops speltoides* for the 7 chromosomes.

BC_1 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 and yellow show non-readable segments. Glucose digestibility: 17.5, 16.6, 8.4 and 7%.

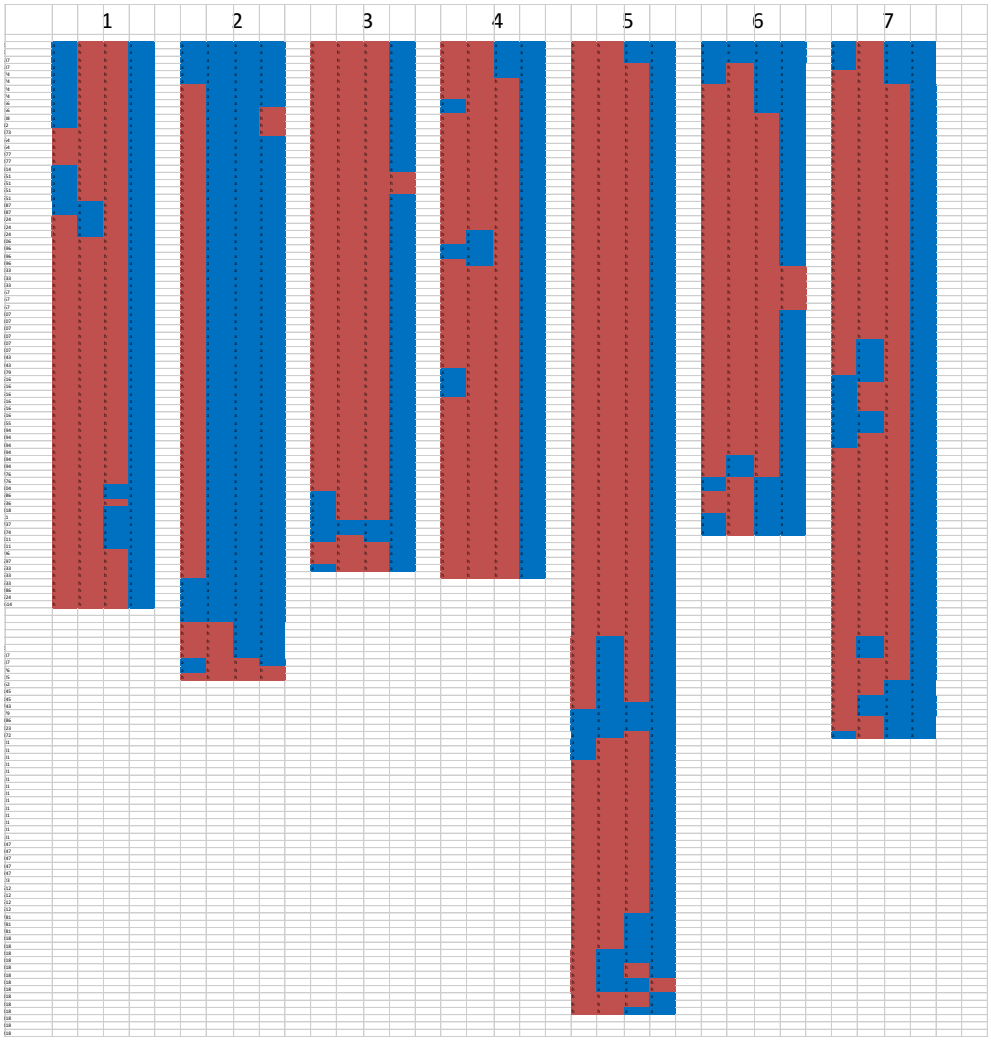


Figure A. 2 Genetic markers for *Aegilops mutica* for the 7 chromosomes.

BC_1 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 and yellow show non-readable segments. Glucose digestibility: 14.8, 13.1, 6.5 and 5.4%.

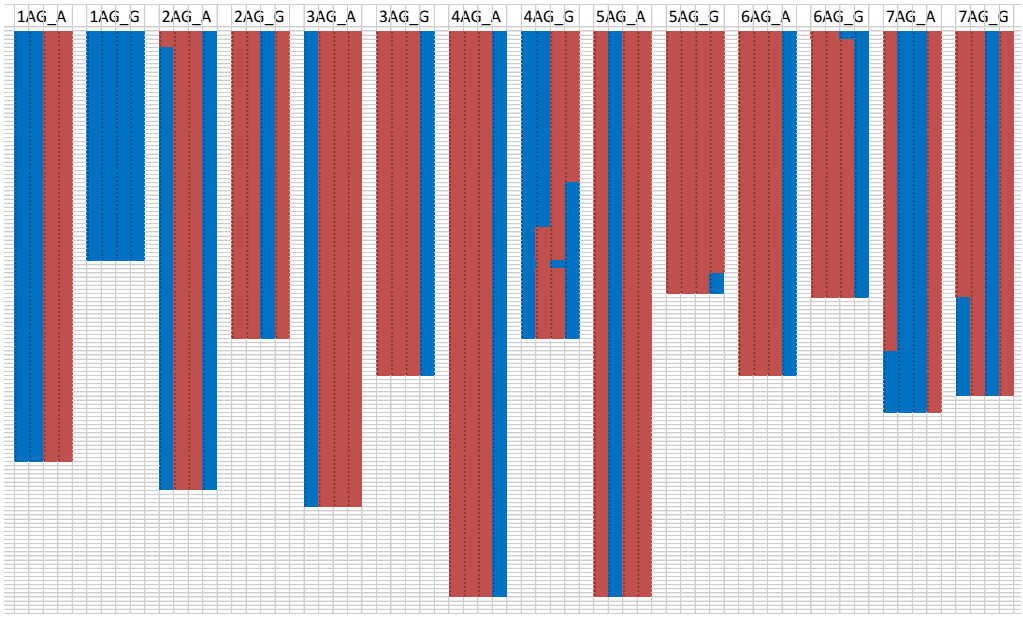


Figure A. 3 Genetic markers for *Triticum timopheevii* for the 7 chromosomes.

BC_1 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 and yellow show non-readable segments. Glucose digestibility: 21.8, 19.2, 9.2 and 7.7%.

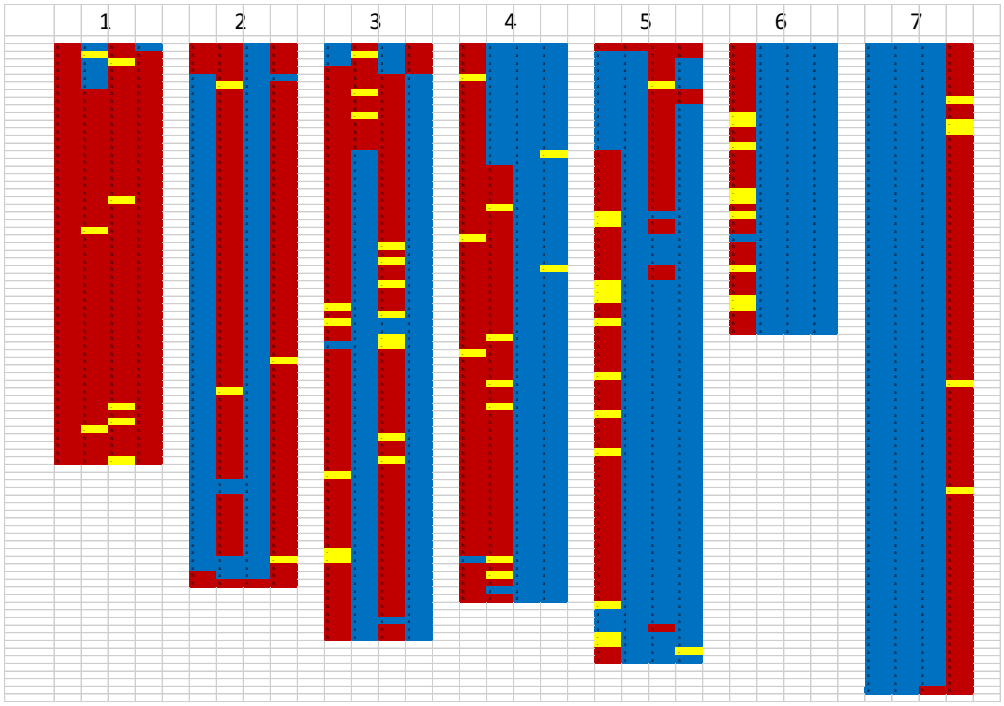


Figure A. 4 Genetic markers for *Triticum urartu* for the 7 chromosomes.

BC_1 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 and yellow show non-readable segments. Glucose digestibility: 16.2, 15.8, 8.4 and 6.3%.

APPENDIX B MIXED FEEDSTOCK

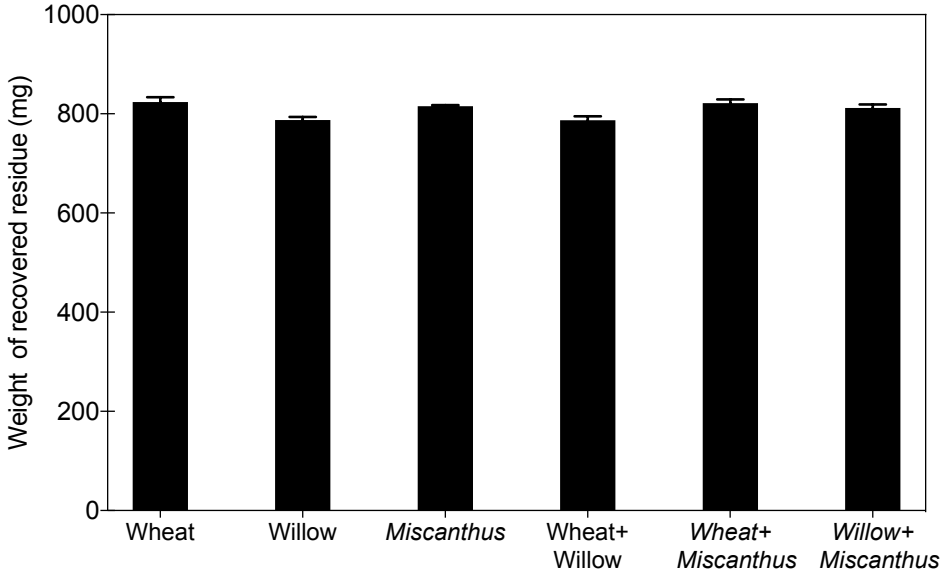


Figure B. 5 Weight of pre-treated residue of individual and mixed feedstocks

Dried weight recovered after pre-treatment of 1 gram of individual feedstocks and their 1:1 mixtures, mean ± SD (n=3).

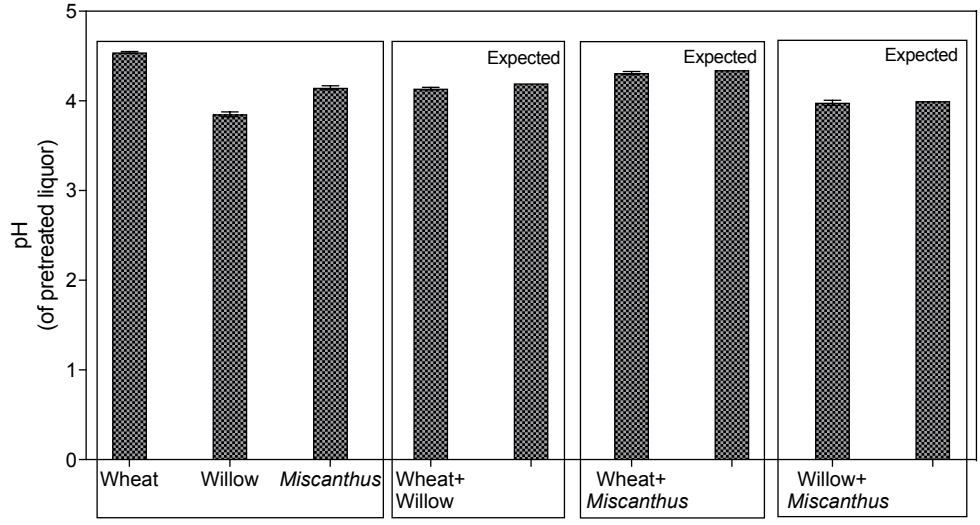


Figure B. 6 pH of the pre-treated liquor for individual feedstocks and their 1:1 mixed ratios.

Mean + SD (n =3)

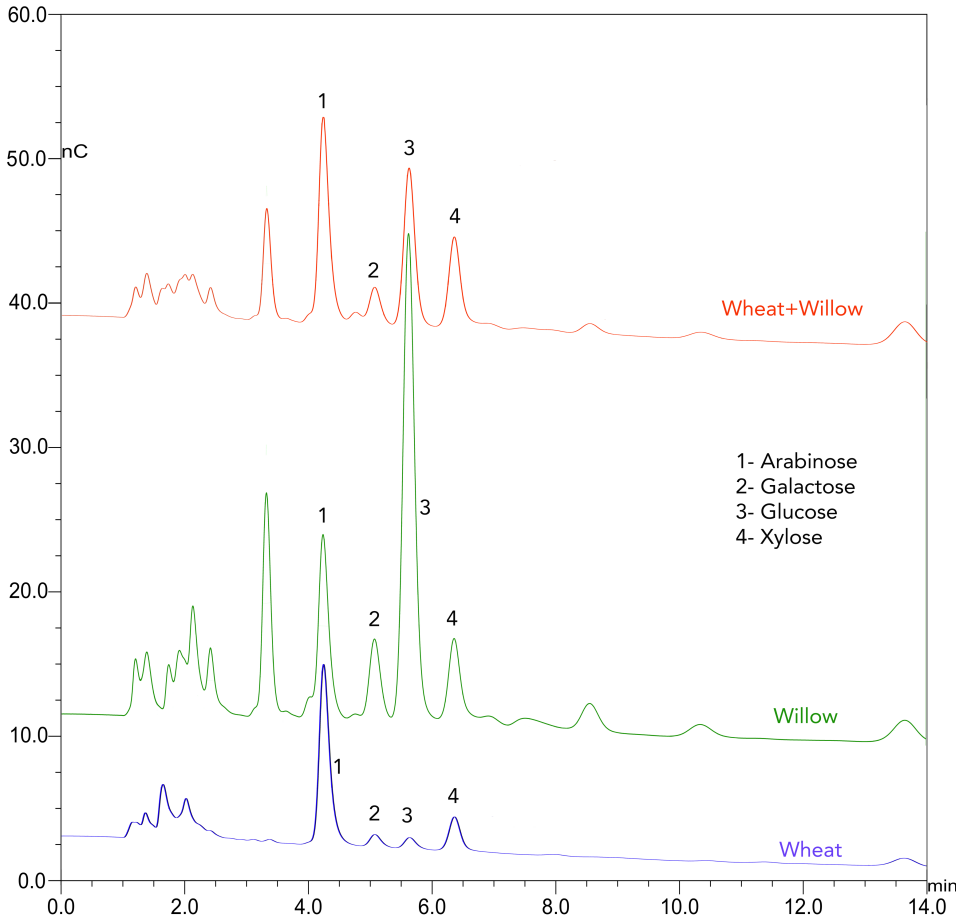


Figure B. 7 HPAEC profile of pre-treated liquor of wheat and willow, and the mixture.

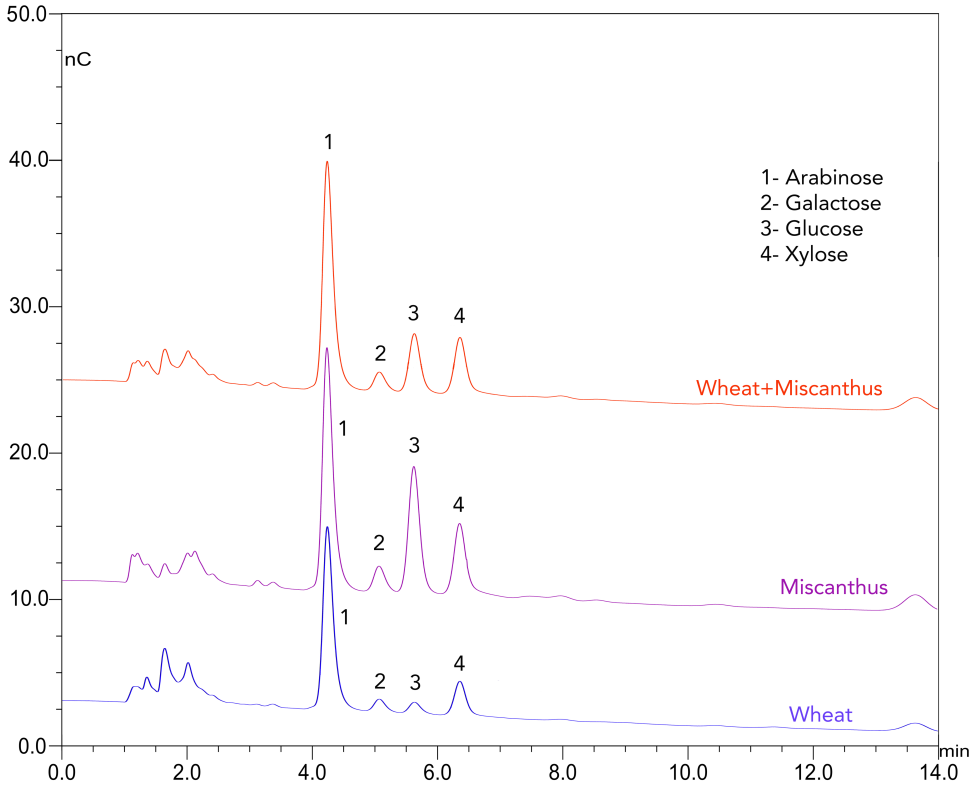


Figure B. 8 HPAEC profile of pre-treated liquor of *Miscanthus* and wheat, and the mixture.

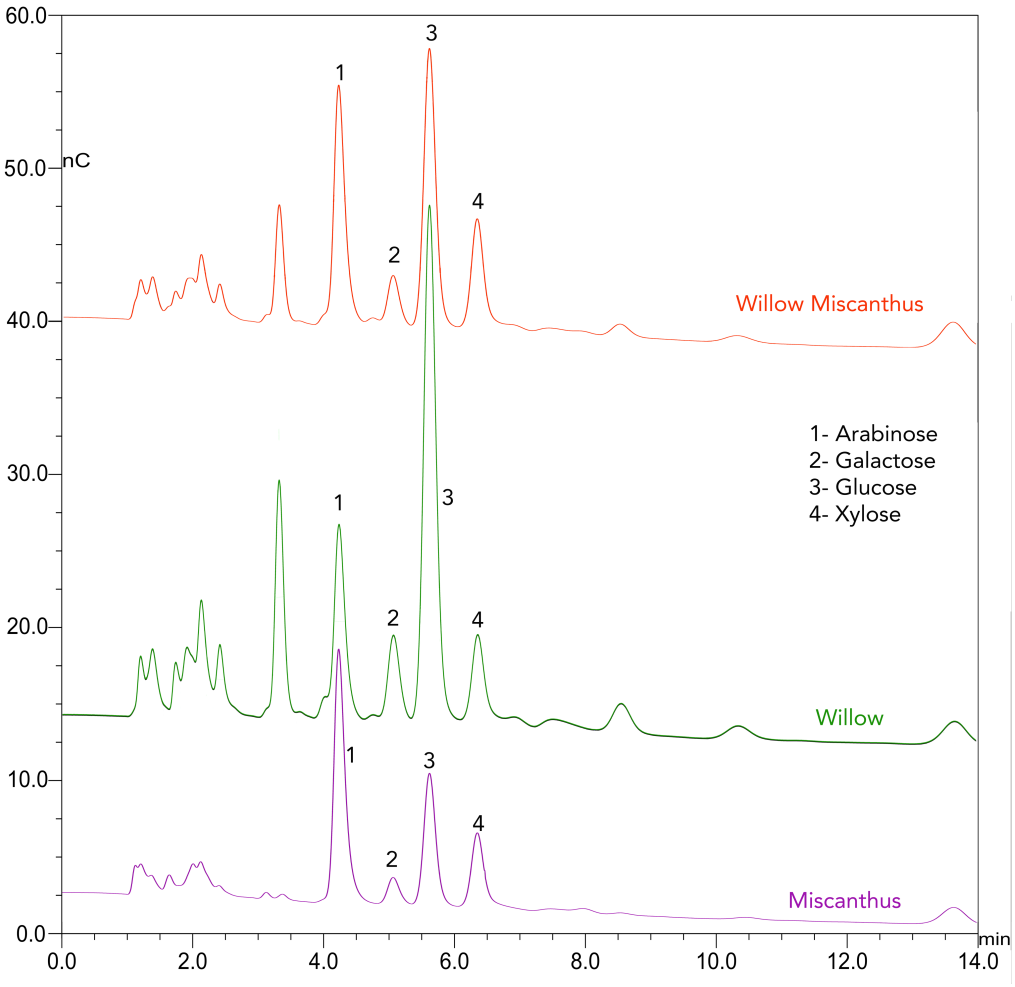


Figure B. 9 HPAEC profile of pre-treated liquor of willow and *Miscanthus*, and the mixture.