Exploiting yeast diversity to produce renewable chemicals from rice straw and husk

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Declaration

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I certify that the work contained in this thesis is entirely the result of my own work, except where due reference is made to other authors as part of a joint piece of work. It has not previously been submitted in any form to the University of East Anglia or any other University.

Jia Wu

Abstract

Exploiting organic lignocellulosic wastes via bio-refining processes has been widely accepted as one of the renewable, environmentally friendly solutions to producing platform chemicals and liquid fuels. Pre-treatment serves as an initial step to improve the accessibility of lignocellulosic polysaccharides to enzymes, and fermentation is a core step to obtain a range of products from the sugars. However, inhibitors of enzymatic saccharification and fermentation are unavoidably generated during hydrothermal pre-treatment. Therefore, the aim of this study has been to assess the associations and possibly correlations between severities of pre-treatment, yield of fermentable sugars and formation of inhibitors, and to evaluate the potential of 11 yeast diverse yeast strains for the potential to produce not only ethanol but also some highly-sought-after platform chemicals.

Air dried rice husk (RH) and rice straw (RS) from the same rice cultivar (*Oryza sativa*, cv. KhangDan18) were used as substrates. Carbohydrate compositions of each were similar whereas lignin contents differed significantly. Using complementary analytical approaches including a new, rapid NMR screening method, 40 compounds including carbohydrates, organic acids, phenolics and furans were identified from the solids and liquors of pre-treated RH and RS. However, the quantities of compounds differed between the two substrates. Fermentation inhibitors included 5-HMF, 2-FA and phenolic acids such as *para*-couamric acid (pCA) and *trans*-ferulic acid (tFA). Differences in lignin, tFA, diferulic acids (DiFA) and pCA between RS and RH reflect differences in cell wall physiology and are probably responsible for the higher recalcitrance of RH. After pre-treatment at a severity of 3.65, ethanol was produced from RS with a yield double that from RH. Above a severity of 5, fermentation was completely inhibited in both RH and RS. More careful control of pre-treatment may be sufficient to reduce the levels of fermentation inhibitors. Such inhibition was found to occur with a range of genetically diverse yeast strains which differed considerably in their metabolic capabilities and production of ethanol. A number could produce significant amounts of ethyl acetate, arabinitol, glycerol and acetate in addition to ethanol, including from hitherto unreported carbon sources. Moreover, a new catabolic property of *Rhodotorula mucilaginosa* (NCYC 65) was discovered in which sucrose is cleaved into glucose and fructose but they are not metabolised. Engineering some of

properties discovered in this study and transferring such properties to conventional industrial yeast strains could greatly expand their biotechnological utility.

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Abbreviations

2-FA: Furfural

2-DOG: 2-Deoxy glucose

5-HMF: hydroxymethyl furfural

ΔOD: Efficiency of yeast growth

AFEX: Ammonia fibre/freeze explosion

ARP: Ammonia recycle percolation

CCD: The Core Collection of Diverse taxa software

COSY: Correlation spectroscopy

CO_2: Carbon dioxide

CA: Coumaric acid

cFA: cis-ferulic acid

$Ca(OH)_2$: Calcium hydroxide

CBH: Exoglycanases

CBP: Consolidated bioprocessing

CTRLs: Controls

CTRL: Control

DOE: US Department of Energy

DNS: Dinitrosalicylic acid method

DiFA: Diferulic acid

DAD: Diode Array Detection

DMC: Direct microbial conversion

DCM: Dichloromethane

DT: Doubling time

D_2O: Deuterium oxide

EG: Endo-β-glucanases

EX: Endo-β-xylanases

FTIR-ATR: Fourier Transform Infrared–Attenuated Total Reflectance

FA: Ferulic acid

FPU: Filter paper unit per millilitre enzyme

GOPOD: D-Glucose Assay Kit

GC: Gas Chromatography

Glucose-6-P: Glucose-6-phosphate

Fructose-6-P: Fructose-6-phosphate

H_2SO_4: Sulfuric acid

HCl: Hydrogen chloride

HPLC: High performance liquid chromatography

HSQC: Heteronuclear single quantum coherence

HMBC: Heteronuclear multiple bond coherence

KOH: Potassium hydroxide

K_2HPO_4: Potassium hydrogen phosphate

LP: Lag phase

NH_3: Ammonia

NCYC: National Collection of Yeast Cultures

NMR: Nuclear magnetic resonance

NaOH: Sodium hydroxide

$NaBH_4$: Sodium borohydride

$NaH_2PO_4.H_2O$: Sodium dihydrogen phosphate

NaN_3: Sodium azide

PCA: Principal component analysis

PVDF: Polyvinylidene difluoride

PT: Pre-treated

PTRH: Pre-treated rice husk

PTRS: Pre-treated rice straw

PCR: Polymerase chain reaction

pCA: para-coumaric acid

pCald: Protocatechuic aldehyde

p-OH-B: p-OH-benzoic acid

p-OH-Bzald: p-OH-benzaldehyde

p-OH-PAA: p-OH-phenyl acetic acid

PA: Protocatechuic aldehyde

RH: Rice husk

RS: Rice straw

SSF: Simultaneous saccharification and fermentation

SHF: Separate hydrolysis and fermentation

STD: Standard

STDs: Standards

TSP: sodium 3-(Trimethylsilyl)-propionate-d4

tFA: trans-ferulic acid

UV: Ultraviolet radiation

VA: vanillic acid

YNB: Yeast nitrogen base

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Chapter 1：

General Introduction

1.1 Opportunities and challenges of bio-refining

Fossil derived fuels are a major resource consumed globally in abundance. With the rapid increase in energy demand, the depletion of fossil derived resources, and impact on climate, a search for a solution has been long debated, involving a range of topics from the "peak oil" to the improvements in green technologies. Unfortunately, over 80% of our energy and 90% of the demand for organic chemicals are still supplied by the products derived from petroleum refining which accounted for 75% of the anthropogenic emission of the carbon dioxides (Binder & Raines, 2009; Bozell, 2001; Houghton et al., 2001). Since the concerns of environment are increasing, low-carbon electrical energy, wind power, solar energy, nuclear fission/fusion and biomass have been frequently pursued and developed (Barnham et al., 2006; Blair, 1976; Ellabban et al., 2014; FitzPatrick et al., 2010). Some categories of renewable energy are shown in Figure 1.1. It has been suggested that renewable energy could potentially provide over 3000 times the current global energy demands (Ellabban et al., 2014).

Biomass energy is considered to be potentially renewable and sustainable because it may be sourced from many organic raw materials and wastes such as cereal crops, lumbering and food industry/chain wastes (Reddy & Srinivas, 2013; Srirangan et al., 2012). However, energy obtained from animal fats and oil crops such as palm, and carbohydrate crops such as sugar beet, wheat, barley and maize are likely to create conflicts with the increasing global demand for food (Nigam & Singh, 2011; Singh et al., 2011). In this case, the challenge is to exploit non edible biomass by converting it into storable and transportable bio-fuels (including gas fuels and liquid bio-fuels) which are suitable for heating, power generation, transportation fuels and gas turbine via bio-refining processes (Ellabban et al., 2014; Gupta et al., 2010).

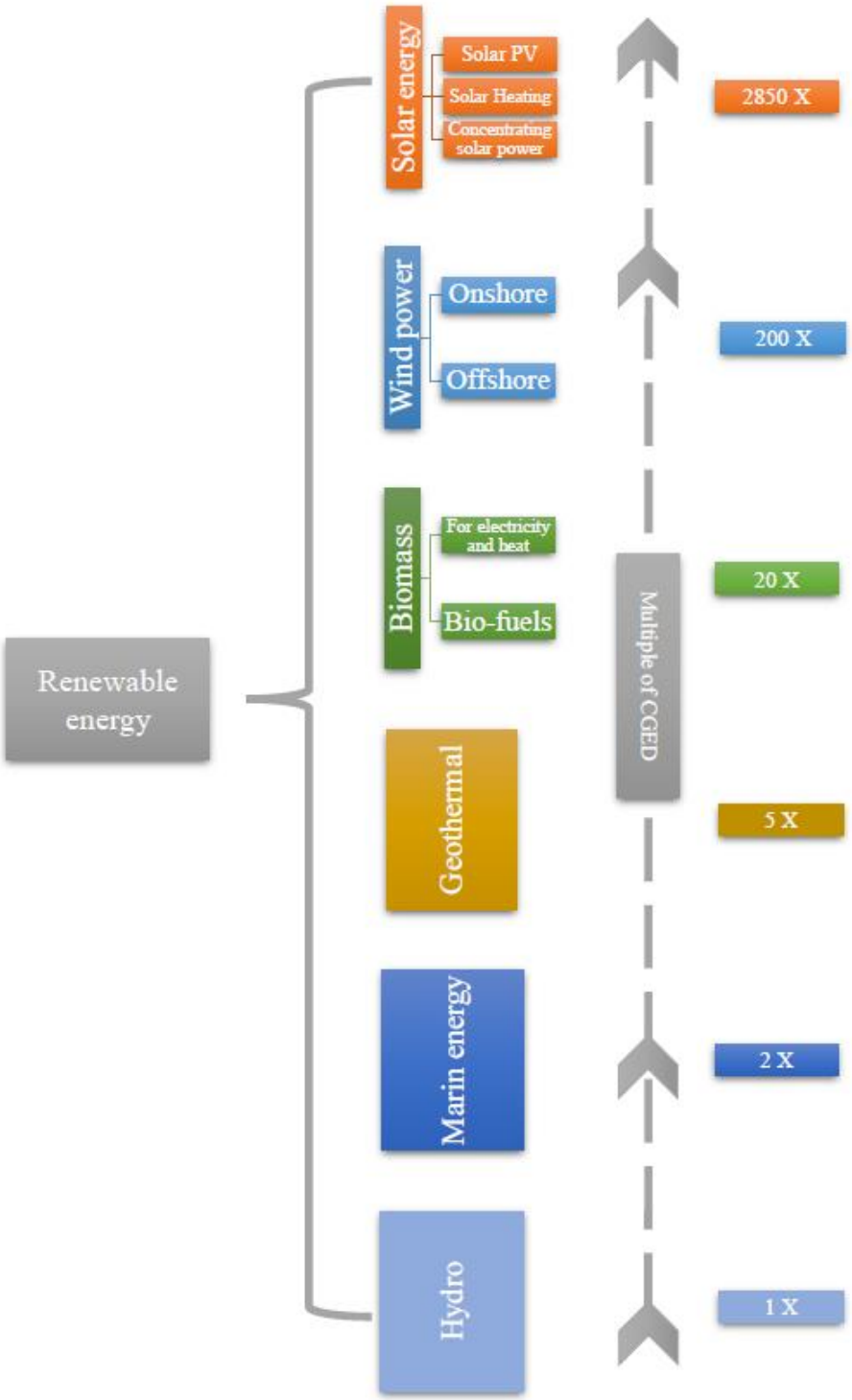


Figure 1.1. Categories of renewable energy (theoretical primary energy) and multiples of the current global energy demands (CGED) in quantities (Ellabban et al., 2014).

A range of bio-fuels have been commercialised including alcoholic bio-fuels, bio-diesel, bio-crude and synthetic oils and categorised as 4 different generations on the basis of varying feedstocks and technologies introduced (Liew et al., 2014). The first generation of bio-fuels are mainly produced from plants or food containing high levels of sugars or oil such as soybean, rapeseeds, corn and sugarcane (Hayashida et al., 1982; Leung et al., 2010). For example, in the study of Leung et al. (2010), 20 different feedstocks including 12 food plants have been used for the production of bio-diesel leading to an competition between food and fuels in land use (Singh et al., 2011). With the concerns of land use, the later generations of bio-fuels which require either non-food biomass or no extra land use have been developed and intensively researched. The second generation of bio-fuels tend to use the wasted organic biomass such as cereal straws, sugar cane bagasse, forest residues and energy crops which are identified as lignocellulosic feedstocks (Sims et al., 2010). For the third and fourth generation of bio-fuels, hydrophytic microalgae is the considered the best candidate of the feedstock. The potential to genetically modify algae for higher CO_2 capture or lipid production is currently seen to be a priority for future fourth generation technology (Dutta et al., 2014). However, those newer (2^{nd} – 4^{th}) generations of bio-fuels also present significant disadvantages and drawbacks. For example, complex and costly processes are required to hydrolyse lignocellulosic polysaccharides to fermentable sugars for producing the second generation of bio-fuels (Lattanzio et al., 2006; Liew et al., 2014; Lin et al., 2010); the main issue of the third and fourth generations is presently their undeveloped technologies requiring significant research to establish advanced processes (Dutta et al., 2014; Liew et al., 2014). In addition, and common to many examples of "science-push" innovation, aspects such as the downstream logistics of biomass recovery and processing have been ignored in the race to create the new biotechnology.

1.2 Chemical composition of lignocellulosic plant cell wall

Second generation bio-fuels produced from lignocellulosic feedstocks, such as cellulosic bio-ethanol, are of predominant interest due to the recognition that lignocellulosic biomass is the most abundant resource available now, which contains

a large amount of plant cell wall derived polysaccharides such as cellulose and hemicellulose. The world annual production of lignocellulosic biomass has been reported to be over 2000 million tons from cereal crops, 160 million tons from pulse crops, 15 million tons from oil seed crops and 540 million tons from plantation crops (Kuhad & Singh, 1993; Rajaram & Varma, 1990). The multilayer lignocellulosic plant cell wall consists of two main phases: 1) the microfibrillar phase which is constructed from microfibrils formed from parallel chains of cellulose. 2) the matrix phase which is generally more complex and mainly formed of pectin, hemicellulose, proteins, phenolic compounds and lignin (Brett & Waldron, 1996a). A schematic diagram of lignocellulose is shown in Figure 1.2.

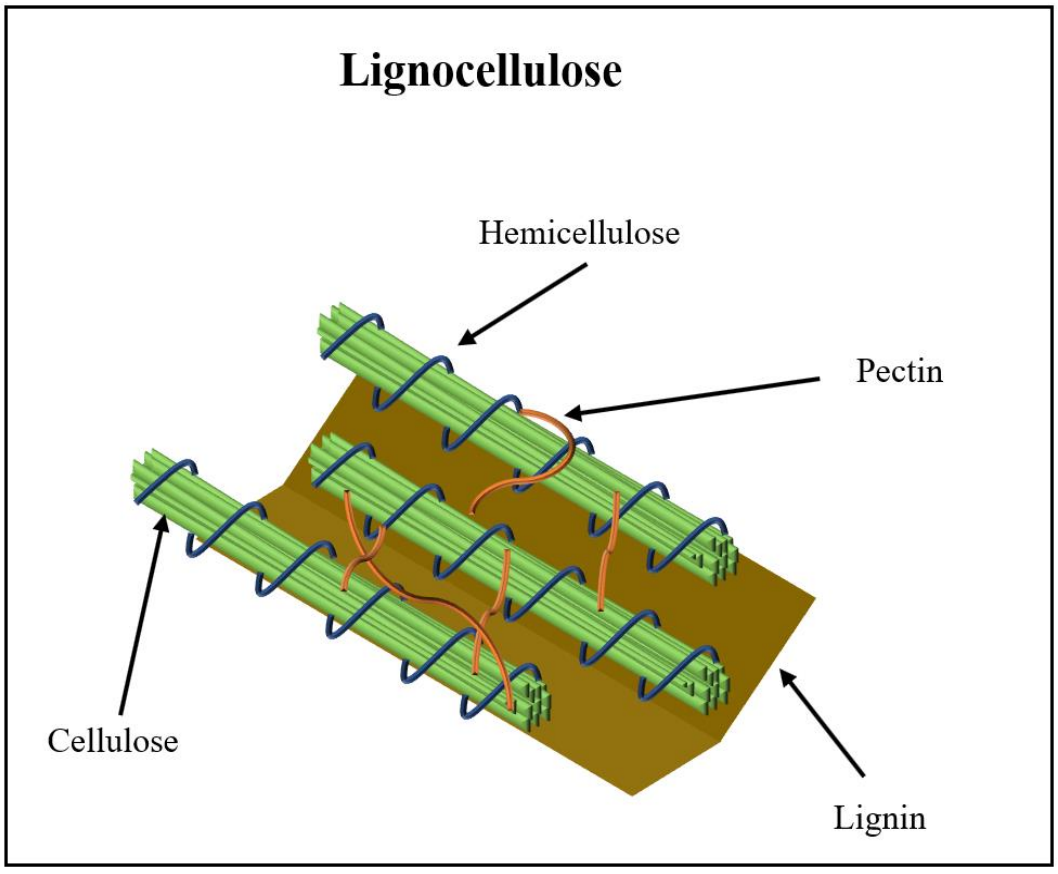


Figure 1.2. Main structure of lignocellulose from rice husk and straw. Phenolics and protein are not shown on this figure.

Cellulose is the highly crystallised long chain polysaccharide in which the monosaccharides are linked together by O-glycosidic bonds, and large groups of individual cellulose polysaccharides (approximately 30-100 units) connected to each other forming an extremely long and thin structures as known as the microfibrils (Brett

& Waldron, 1996a). Cellulose is also described as a β-1,4-glucan since carbon atoms 1 and 4 within each β-D-glucose are linked to other glucose residues via O-glycosidic bonds. Hence cellulose is an important major potential source of fermentable glucose for bio-refining and can be found at up to 50% (w/w) in lignocellulosic biomass (Brett & Waldron, 1996a; Hendriks & Zeeman, 2009; Lee, 1997; McKendry, 2002; Yang et al., 2007). The chemical structure of cellulose is shown in Figure 1.3.

Hemicellulose (Figure 1.3) is the second most abundant component in lignocellulosic biomass and is found at a ratio to cellulose generally of between 2:1 to 1:1 (Hoch, 2007), and takes up approximately 20-35% (w/w) of the dry biomass (Pérez et al., 2002; Saha, 2003). Unlike the chemically homogeneous cellulose, a range of polysaccharides are contained in hemicellulose such as xylans, glucomannan, mannan, galactomannan and arabinogalactan II (Brett & Waldron, 1996a). Moreover, the chemical compositions differ significantly in the cell walls of different plants. For example, all higher plants contain xyloglucans which are tightly bound to cellulose in primary cell walls, and xylans, which are the most abundant polysaccharides in the secondary cell wall of hardwoods and herbaceous plants (Puls, 1993; Saha, 2003). Xylans are polymers containing mainly xylose which commonly take up to 20-30% of the biomass and even up to 50% of biomass in some tissues of grasses and cereal plants (Ebringerová et al., 2005; Gírio et al., 2010). In the secondary cell walls of conifers and seeds of *Leguminosae*, the most abundant polysaccharides are mannan, glucomannan and galactomannan (Schädel et al., 2010). Hemicellulose can also be converted into substantial amounts of sugar monomers for potential bio-conversion.

The pectic polysaccharides (Figure 1.3) are common components of primary cell walls, formed mainly from α-galacturonic acid with varying displays of methyl ester groups (Liu et al., 2006) in addition to several polysaccharides such as rhamnogalacturonan I, arabinan, galactan, arabinogalactan I and homogalacturonan (Brett & Waldron, 1996a). Pectic polysaccharides are found in abundance in many edible fruits such as citrus and apple. Indeed, the proportion of pectin can reach approximately 50% of the polymeric content of the cell wall (Brummell, 2006). Some industrial applications of pectin include their use as a thickener, texturiser, emulsifier, stabilizer or fat replacer in spreads and salad dressings (Hawthorne et al., 2000; Liu et al., 2006). Extracted pectins are generally water soluble and are also sensitive to thermodynamic degradation so

that their extraction might be affected by microwave pre-treatment (Brett & Waldron, 1996a; Liu et al., 2006). Although rich in galacturonic acid, pectins might potentially contribute to the production of bio-ethanol and other bio-products from plant cell walls (Doran et al., 2000; Hutnan et al., 2000).

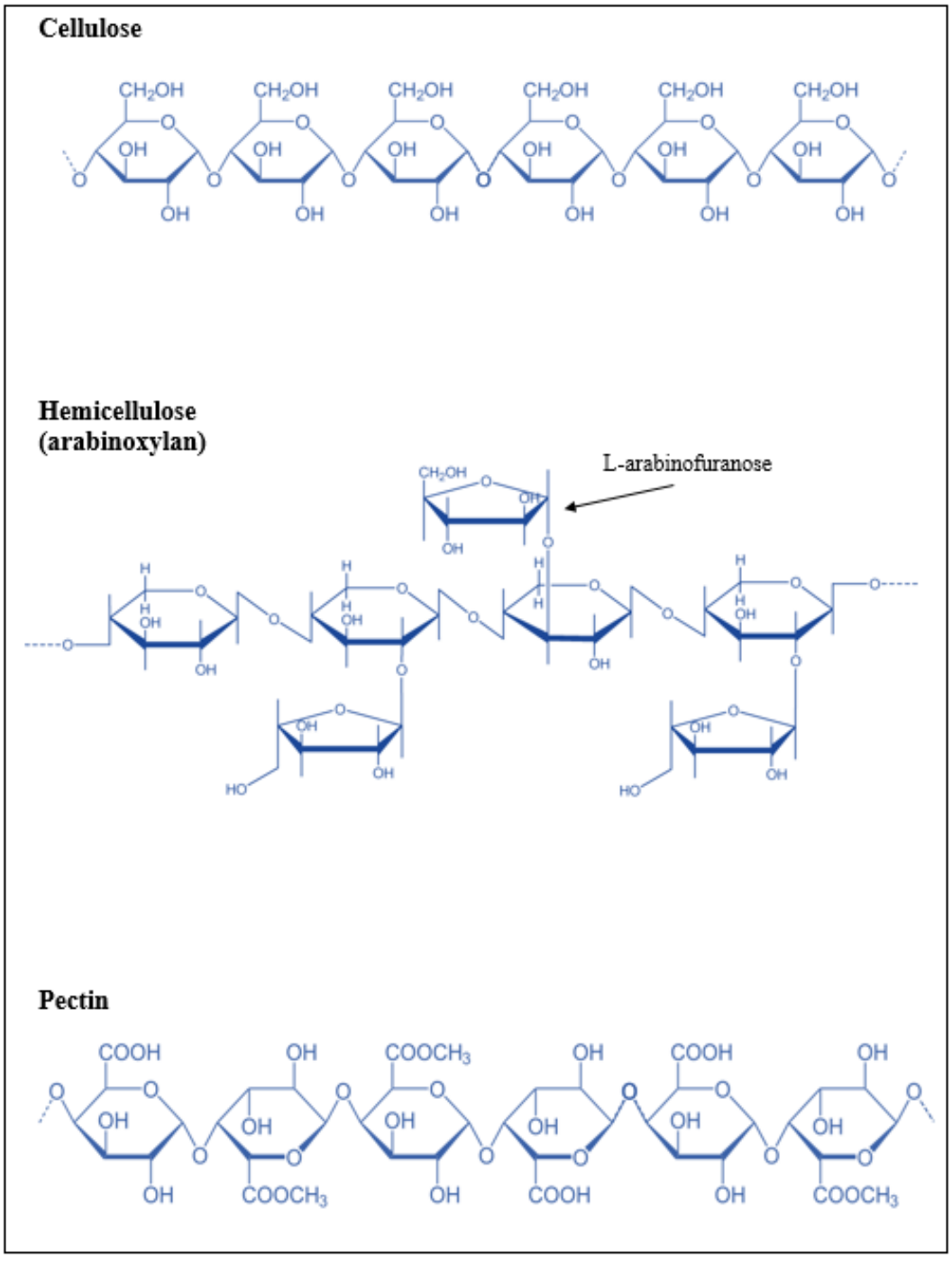


Figure 1.3. Structures of cellulose, hemicellulose and pectin. Because hemicelluloses are heterogeneous, , arabinoxylan is shown as an example (Miguel et al., 2013).

A very significant component in plant cell wall is lignin, a polymer of phenolics making up to 10-25% (w/w) of the lignocellulose biomass and containing almost no carbohydrates and being very resistant to enzymatic degradation (Brett & Waldron, 1996a; Kumar et al., 2008). Three precursors of lignin, p-coumaryl, guiacyl and sinapyl alcohols link to the final polymer by different bonds including several types of β-o-4, α-o-4, 4-o-5 linkages and carbon-carbon bonds (Brett & Waldron, 1996a; Mansouri & Salvadó, 2006). Lignin is closely bound to a variety of components of lignocellulose such as cellulose and hemicellulose. It therefore creates a barrier that reduces the accessibility of the carbohydrate-microfibrils to enzymatic saccharification necessary for accumulating microorganism-fermentable sugars (Avgerinos & Wang, 1983; Fu et al., 2011). In addition to lignin-derived phenolics, other phenolic compounds might also be present in the plant cell wall such as hydroxycinnamic acids (ferulic acids and p-coumaric acid), phenylacetic acids, hydroxybenzoic acids, flavonoids and tannins (Balasundram et al., 2006; Brett & Waldron, 1996a). Those phenolic compounds are produced in plants via the pentose, phosphate, shikimate and phenylpropanoid metabolic pathways (Randhir et al., 2004). Such phenolics have been found to have a number of physiological functions such as anti-allergenic, anti-atherogenic, anti-inflammatory and anti-oxidant (Balasundram et al., 2006; Benavente-García et al., 2000; Manach et al., 2005; Middleton et al., 2000; Puupponen-Pimiä et al., 2001). Phenolic acids are regarded as one of the most common dietary phenolic compounds comprising a wide variety of compounds, but being particularly rich in the hydroxybenzoic and hydroxycinnamic acids (King & Young, 1999) (Figure 1.4). The role of phenolic compounds in human health suggests that these plant cell wall phenolics might be potentially important as value-adding coproducts during the production of bio-ethanol or other bio-products from lignocellulosic biomass.

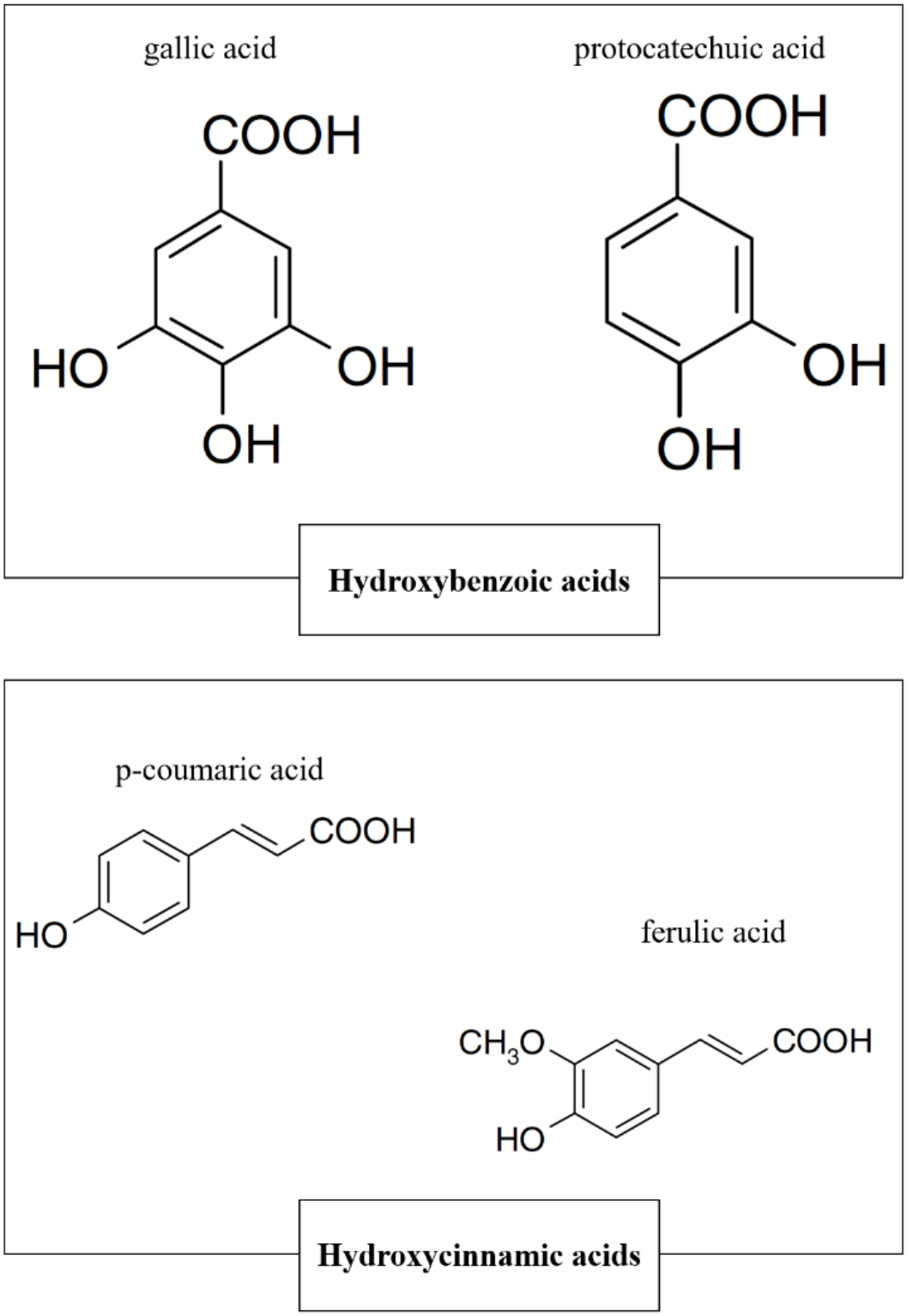


Figure 1.4. Main groups of phenolics (hydroxybenzoic acids and hydroxycinnamic acids) derived from lignocellulosic plant cell wall.

1.3 Processes of bio-refining

Lignocellulosic biomass contains substantial quantities of polysaccharides. However, in contrast to the exploitation of starches and sucrose sources that can be readily converted to C_6 monomeric sugars for fermentation, exploitation of sugars in lignocellulosic polysaccharides is more complicated due to the natural recalcitrance of lignocellulosic biomass. This reflects the important structural features of the cell wall which the plant needs to protect (Himmel et al., 2007). This concept underlies the so called substrate-related factors of enzymatic digestibility (Mansfield et al., 1999; Waldron, 2010; Zhang & Lynd, 2004) which include the biomass particle size, the porosity of plant cell wall, lignin type and cross-linking phenolics, degree of cellulosic crystallization and polymerization, and the side-chain branching of hemicelluloses (Besle et al., 1994; Chang & Holtzapple, 2000; Chundawat et al., 2007; Fan et al., 1981; Ishizawa et al., 2007; Laureano-Perez et al., 2005; Liu et al., 2013; Ramos et al., 1993; Stålbrand et al., 1998; Waldron, 2010; Zadrazil & Puniya, 1995). Similarly, the activities of enzyme, enzyme synergy, enzyme inactivation during hydrolysis and inhibitors produced during pre-treatment (Bhat & Hazlewood, 2000; Eriksson et al., 2002a; Eriksson et al., 2002b; Percival Zhang et al., 2006; Rosgaard et al., 2007; Selig et al., 2007; Yang et al., 2006) are categorised as the enzyme-related factors (Mansfield et al., 1999; Waldron, 2010; Zhang & Lynd, 2004) that affect enzyme digestibility of lignocellulosic biomass. The complex progresses of converting lignocellulosic polysaccharides into fermentable sugars for producing bio-ethanol and other bio-products can be simplified as four main steps (Figure 1.5): pre-treatment (increasing accessibility of enzyme to polysaccharides), saccharification (converting carbohydrate polymers into fermentable sugars), fermentation (accumulating target chemicals via microbial metabolism), purification (concentrating and isolating target compounds for final bio-products) (Balat et al., 2008; Hahn-Hägerdal et al., 2006).

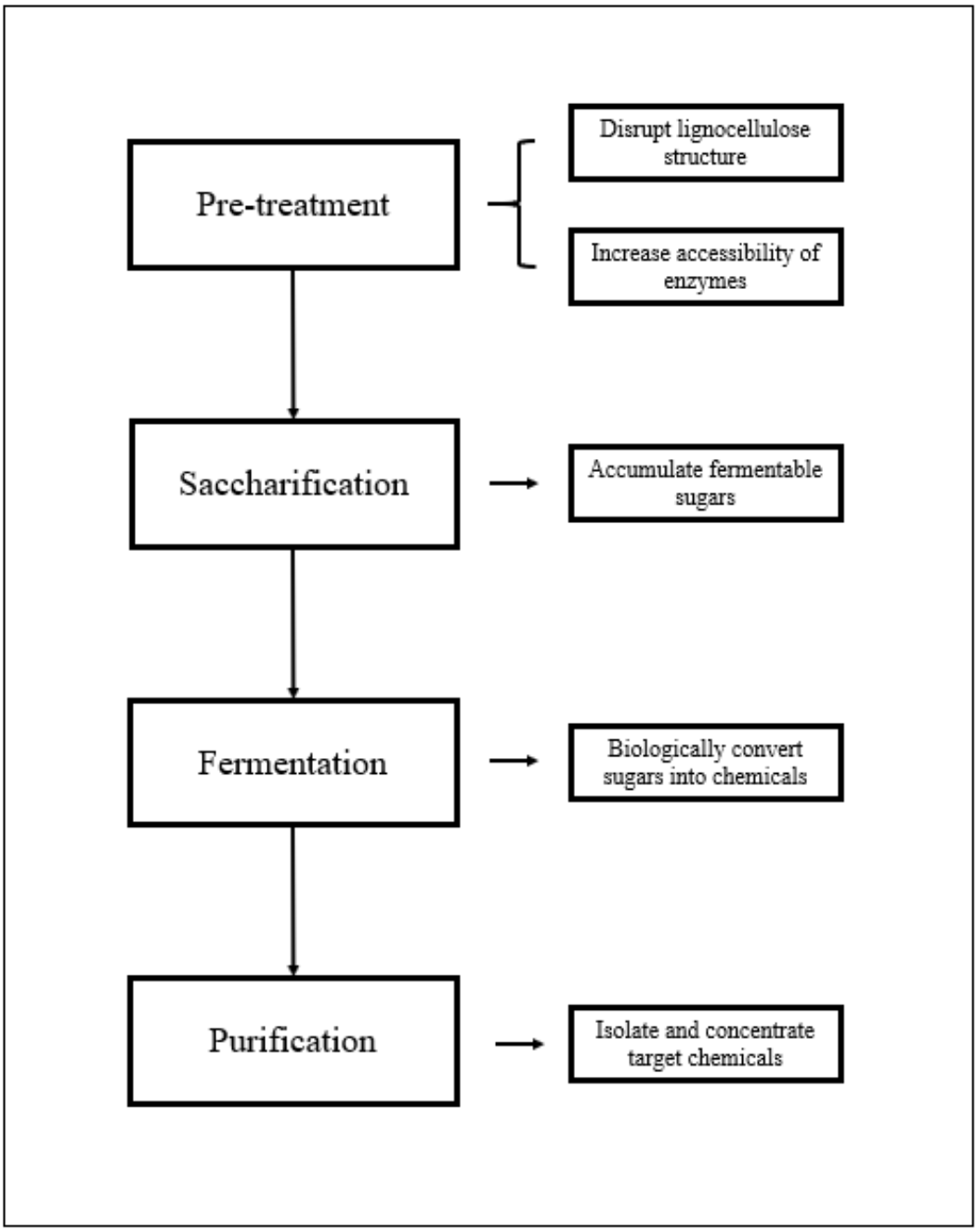


Figure 1.5. Diagram of bio-refining process. The process has been divided into four main steps including pre-treatment, saccharification, fermentation and purification.

1.3.1 Pre-treatments and pre-treatment related inhibitory compounds

Lignin, cellulose and hemicellulose are naturally bound together to form a complex matrix making the structure of lignocellulosic plant cell walls highly recalcitrant to enzymatic degradation by microorganisms and disruption of cells (Brett & Waldron,

1996a; Waldron, 2010). Pre-treatments are required prior to further conversions to reduce the lignocellulose recalcitrance by separating lignin, cellulose and hemicellulose, reducing the degree of cellulose crystallization and the length of cellulose chain, and further increasing the surface areas of polysaccharides to relevant enzymes (such as cellulase) (Cheng & Stomp, 2009b; Donohoe et al., 2008; Kumar et al., 2009; Mosier et al., 2005). A very simple diagram of lignocellulose disruption after pre-treatment is shown as Figure 1.6.

The ideal pre-treatment would be able to improve the saccharification yield, minimise the loss of carbohydrates and production of inhibitory compounds, and be economical (Balat et al., 2008). A range of pre-treatment methods have therefore been developed to reflect the properties of different feedstocks which can be categorised in several ways. They can be considered in relation to the pH of pre-treatment conditions: pre-treatment methods can be considered as alkaline, acidic and neutral methods (Galbe & Zacchi, 2007; Kumar et al., 2009; Saha et al., 2005; Sun & Cheng, 2005). They can alternatively be classified as chemical, physical, biological and multiple pre-treatments (Agbor et al., 2011; Harmsen et al., 2010; Kumar et al., 2009; Octave & Thomas, 2009; Sindhu et al., 2016).

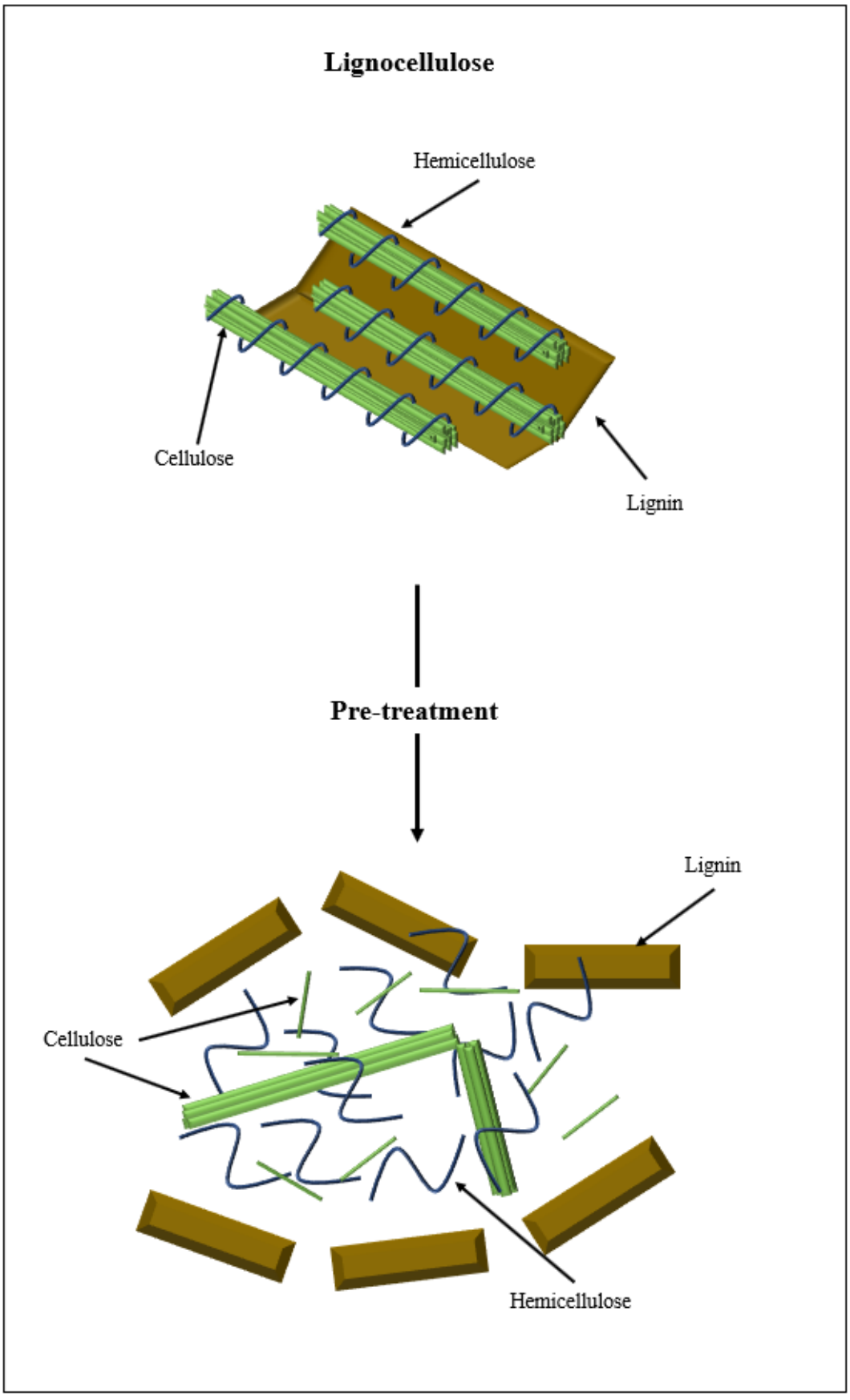


Figure 1.6. Pre-treatment related structure interruption of lignocellulose.

1.3.2 Physical pre-treatments

Milling, grinding, shredding, chipping and irradiation are all categorised as physical pre-treatments which either can significantly increase the surface area available to enzymes and size of pores or reduce the degree of polymerization and cellulose crystallinity of lignocellulosic biomass (irradiation) (Palmowski & Müller, 2000; Taherzadeh & Karimi, 2008). For instance, milling or grinding process by using ball milling significantly reduces the size of materials (e.g. from 10-30 mm to 0.2-2 mm) increasing surface area. Many studies have suggested that the influence of particle size on cellulose digestibility is largely depending on the type of lignocellulosic biomass (Vidal et al., 2011). In the studies of carboard and newspaper, the digestibility of cellulose was not noticeable increased by reducing the particle size (Rivers & Emert, 1988a; Rivers & Emert, 1988b; Rivers & Emert, 1987). Nevertheless, cellulose conversion to fermentable sugars was increased up to 50% by ball milling polar wood for 8 days (Chang & Holtzapple, 2000). In addition, irradiation processing using γ-rays can directly cleave the β-1,4-glycosidic bond and will result in reduced crystallinity of cellulose (Sun & Cheng, 2002; Takács et al., 2000). However, such physical pre-treatments are not feasible at industrial scale since the energy input is higher than the energy content of most biomass and therefore too expensive to be used in a full-scale process (Cadoche & López, 1989; Galbe & Zacchi, 2007; Hendriks & Zeeman, 2009; Kumar et al., 2009).

1.3.3 Chemical pre-treatments

The aim of chemical pre-treatments is to break down the structure of plant cell walls and lignocellulose by adding chemicals such as alkalis, acids, organic solvents and ionic liquids, usually at high temperature and pressure. Alkalis such as NaOH (sodium hydroxide), KOH (potassium hydroxide), $Ca(OH)_2$ (calcium hydroxide), hydrazine and anhydrous ammonia are commonly used in alkaline pre-treatment which can cause solvation and saphonication. These reactions are responsible for the swelling of pre-treated substrates which increase the surface area making the substrates more accessible for enzymes (Hendriks & Zeeman, 2009). In alkaline condition, polysaccharides such as xylan and glucomannans can be degraded to low molecular

compounds by hydrolytic reactions which potentially benefit to further conversion (David & Shiraishi, 2001). Alkalis can also disrupt the both lignin structures and linkages between lignin and other compounds leading to the solubilization, redistribution and condensation of lignin (Fengel & Wegener, 1984; Hendriks & Zeeman, 2009). Moreover, alkaline pre-treatments can remove acetyl or uronic acid that potentially lower the activities of cell wall degrading enzymes and then further improve the efficiency of enzymatic hydrolysis of carbohydrate polymers (Chandra et al., 2007). Sulfuric acid is one of the most commonly-used acids for both concentrated acid pre-treatment and dilute acid pre-treatment (Agbor et al., 2011; Kumar et al., 2009). Acid pre-treatment generally can be carried out with diluted acids or concentrated acids. Unlike the alkaline methods, xylan and glucomannan is relatively more stable in acidic conditions (Hendriks & Zeeman, 2009). Therefore, the main reaction that can improve the accessibility of cellulose to enzyme is the hydrolysis of hemicellulose which result in the formation of furfural, HMF and other monomers (Fengel & Wegener, 1984; Ramos, 2003). During acid pre-treatment, lignin can be firstly solubilised and later precipitated leading to removal and relocation of lignin which also benefit to further hydrolysis of polysaccharides (Liu & Wyman, 2003; Shevchenko et al., 1999; Xiao & Clarkson, 1997). The concentrated acid pre-treatment can significantly improve the yield of fermentable sugars but since the remaining acids after hydrolysis are still concentrated, toxic, corrosive and hazardous, extra steps for removing or collecting those acids are required which increase the overall costs (Sun & Cheng, 2002; von Sivers & Zacchi, 1995). Therefore, dilute acid pre-treatment has been developed and can be applied with other pre-treatment methods such as steam explosion for better performance. However, the cost of diluted acid pre-treatment is still higher than most of the physicochemical pre-treatments such as ammonia fibre/freeze explosion (AFEX) due to the required neutralisation prior to downstream fermentation which gives extra costs (Agbor et al., 2011; Kumar et al., 2009). More importantly, several fermentation inhibitors are unavoidable with this pre-treatment method, which makes the hydrolysates difficult to ferment (Palmqvist & Hahn-Hägerdal, 2000a). The diluted acid pre-treatment has, instead, been suggested for industrial furfural production from lignocellulosic biomass (Zeitsch, 2000). There are also some other methods such as the ozone pre-treatment that can sufficiently increase

the yield and does not generate toxic compounds but is limited by cost (Vidal & Molinier, 1988).

Pre-treatment can be also processed with organic solvent such as alcohols (ethanol, methanol, ethylene glycol and glycerol) and organic acids (acetic acid and formic acid) (Zhang et al., 2016). Organic solvent breaks the internal bonds of lignin and hemicellulose to dissolve both into solvent, therefore lignin and hemicellulose are effectively separated from cellulose in consequence the surface area and pore volume of cellulose is increased and further lead to the increase of enzymatic accessibility (Holtzapple & Humphrey, 1984; Koo et al., 2011). Cellulose can be then separated by filtration for further fermentation to produce bio-ethanol and other chemicals. Such a separation allows the recovery of organic solvent (e.g. alcohols by distillation) and lignin by adding acids for precipitation (Zhang et al., 2016). However, the drawbacks of this pre-treatment method are obvious. For example, to improve pre-treatment result and precipitation of lignin, extra catalyst such as acids or alkalis are generally required which potentially increases the overall cost; solvents easy to recover such as ethanol and methanol require the pre-treatment process to be carried out at high temperature and lower process temperature demanded alcohols such as ethylene glycol and glycerol require more energy input to be recovered; organic acids such as acetic acid and formic acid are suggested as low cost for recovery but organic acids have been reported that can lead to corrosion and cellulose acetylation (Espinoza-Acosta et al., 2014; Tian et al., 2018; Zhang et al., 2016). Therefore, more research required to optimise this method for industrial utilisation.

Ionic liquid pre-treatment has been suggested as the replacement of organic solvent pre-treatment due to some features such as relatively low melt point (<100 ^{o}C), chemical and thermal stability, non-flammable and non-volatile (Cull et al., 2000; Mäki-Arvela et al., 2010; Mallakpour & Kolahdoozan, 2008; Zhu et al., 2006). Ionic liquid formed by organic cations and inorganic anions that can be used to dissolve lignin and cellulose, separate lignin and hemicellulose, disrupt structure of cellulose then enhance downstream saccharification and fermentation (Anugwom et al., 2012; Liu et al., 2012; Zhu et al., 2013). This pre-treatment method is relatively new and interesting to academia and industry, therefore a number of research have been carried out to investigate ionic liquids for pre-treating lignocellulosic biomass (D'andola et al.,

2011). For example, 1-butyl-3-methylimidazolium chloride ([BMIM]Cl) and 1-allyl-3-methylimidazolium chloride ([AMIM]Cl) have been reported that disrupt the crystalline structure of cellulose and dissolved cellulose (Zhu et al., 2006). This due to the chloride ions (Cl^-) can disrupt the hydrogen bonds of cellulose (Mäki-Arvela et al., 2010). The pre-treatment condition of ionic liquid 1-butyl-3-methylimidazolium acetate ([BMIM]Ac) for degrading wheat straw has been reported at a temperature 158 ^oC with ionic liquid concentration of 49.5% for 3.6 hours. this study presented a compromise between fermentable sugar recovery and polysaccharides digestibility (Fu & Mazza, 2011). However, there are also some limitations of ionic liquid pre-treatment. Ionic liquids can be very expensive as they sometimes need to be mixed with water or other organic solvent to avoid getting viscous during pre-treatment and this lead to an energy-intensive recovering process of ionic liquids (Espinoza-Acosta et al., 2014). More importantly, ionic liquids have been reported as potential risk for environment due to they are poorly biodegradable and might be toxic to some microorganisms and plants (Cvjetko Bubalo et al., 2014; Espinoza-Acosta et al., 2014; Liu et al., 2015).

1.3.4 Biological pre-treatment

Biological methods aim to remove lignin and hemicellulose from cellulose to overcome cellulose resistance to enzymoloysis (Taherzadeh & Karimi, 2008). Some microorganisms such as the brown (Ray et al., 2010), white and soft rot fungi can produce specific enzymes to degrade lignin, hemicellulose (Chen et al., 2010; Octave & Thomas, 2009; Sun & Cheng, 2002). In the study of Suhara et al. (2012), 50% lignin of bamboo culms was removed by using *Punctualaria* sp. TUFC20056. For corn stalks, 82% of hydrolysis yields was achieved after 28 hours biological pre-treatment (Du et al., 2011). Unlike the chemical pre-treatment, biological pre-treatment does not require neutralisation of pre-treated hydrolysates and recycling of chemicals, also it is free of toxic compounds and relatively more eco-friendly than other pre-treatment methods (Sindhu et al., 2016). However, the drawbacks of this method are also significant such as a long residence time and requiring highly controlled conditions for growing microorganisms (Agbor et al., 2011; Chaturvedi & Verma, 2013; Kumar et al., 2009; Octave & Thomas, 2009). Also, the cellulose may be degraded and utilised by the microorganisms.

1.3.5 Physicochemical pre-treatment

Physicochemical pre-treatment covers the multi-pre-treatment methods that combine both physical and chemical methods, including steam explosion, liquid hot water pre-treatment, ammonia fibre explosion (AFEX), ammonia recycle percolation (ARP), organosolv pre-treatment and wet oxidation pre-treatment (Agbor et al., 2011; Chandra et al., 2007; Kumar et al., 2009; Octave & Thomas, 2009). For example, steam explosion is the most common method which has been used to process a range of lignocellulosic biomass (McMillan, 1994). Raw materials are treated with high pressure saturated steam for a short period (from seconds to minutes) followed by a sudden pressure reduction thus "exploding" the plant material. This leads to the disruption of the lignin, hemicellulose and cellulose (Kumar et al., 2009). Steam explosion typically operates at temperatures from 160-260^{o}C and pressures from 0.69-4.83 MPa, Such severe conditions can increase the efficiency cellulose enzymatic hydrolysis and yields of fermentable sugars (Sun & Cheng, 2002; Wood et al., 2014) by increasing the cellulose fibre reactivity as lignin and hemicellulose are significantly removed from the cellulose (Laser et al., 2002). However, steam explosion of lignocellulosic biomass leads to the formation of some inhibitory compounds that can affect the downstream enzymatic hydrolysis and fermentation (Cantarella et al., 2004; García-Aparicio et al., 2006; Mackie et al., 1985). The mechanism of hot water pre-treatment is very similar to steam explosion but instead of using steam, large quantities of water are required in this method. The formation of inhibitory compounds is relatively low compared with the steam explosion (Yang & Wyman, 2004; Yang & Wyman, 2008). AFEX is a dry to dry process which also requires high pressure with the addition of concentrated ammonia (0.3-2 kg ammonia per kg dry weight material) but is carried out at much lower temperatures (60-140^{o}C) (Agbor et al., 2011; Jönsson & Martín, 2016; Kumar et al., 2009; Waldron, 2010). Due to the lack of water, the lignin and hemicellulose are not significantly solubilized and removed from the lignocellulose. However, approximately 90% hydrolysis of cellulose and hemicellulose was achieved after AFEX pre-treatment of bermudagrass (Holtzapple et al., 1991). The reasons for the impact of AFEX are due to the deacetylation and cleavage of the lignin-carbohydrate complexes, and degradation of hemicelluloses to oligomeric sugars in addition to deacetylation (Gollapalli et al., 2002; Laureano-Perez

et al., 2006). Therefore, in AFEX operation, it is the structure changes of lignin and hemicellulose that improve the digestibility of sugar polymers (Galbe & Zacchi, 2007). The drawback of AFEX is that this method could not sufficiently process some high lignin content biomass (above 25%) such as newspaper and aspen, and the cost of chemicals or the extra chemical recovery step is also an expensive limitation of this method (Jönsson & Martín, 2016; Kumar et al., 2009; McMillan, 1994).

1.3.6 Inhibitory components generated during pre-treatment

Several pre-treatment methods mentioned above have been shown to increase the digestibility of lignocellulosic carbohydrate polymers, such as the very promising steam explosion pre-treatment. However, a range of inhibitors to either enzymatic or microbial are generated in significant quantities during hydrothermal pre-treatments, including furans (furfural and 5-hydroxymethylfurfural), organic acids (acetic acid, formic acid and levulinic acid) and phenolic compounds (lignin-derived phenolics and non-lignin-derived phenolics) (Jönsson et al., 2013; Jönsson & Martín, 2016; Palmqvist & Hahn-Hägerdal, 2000b). The formation of furans and organic acid is unavoidable due to the degradation of lignocellulosic carbohydrates during hydrothermal pre-treatment. For example (Figure 1.7), the 5-hydroxymethylfurfural (5-HMF) is produced by degradation of hexoses (cellulose and hemicellulose derived glucose, mannose and galactose) and furfural (2-FA) is produced by the degradation of pentose (hemicellulose derived xylose and arabinose), and acetic acid is primarily generated from the degradation of hemicellulose related acetyl groups whilst the furans are further degraded to formic acid and levulinic acid (Jönsson et al., 2013; Palmqvist & Hahn-Hägerdal, 2000b).

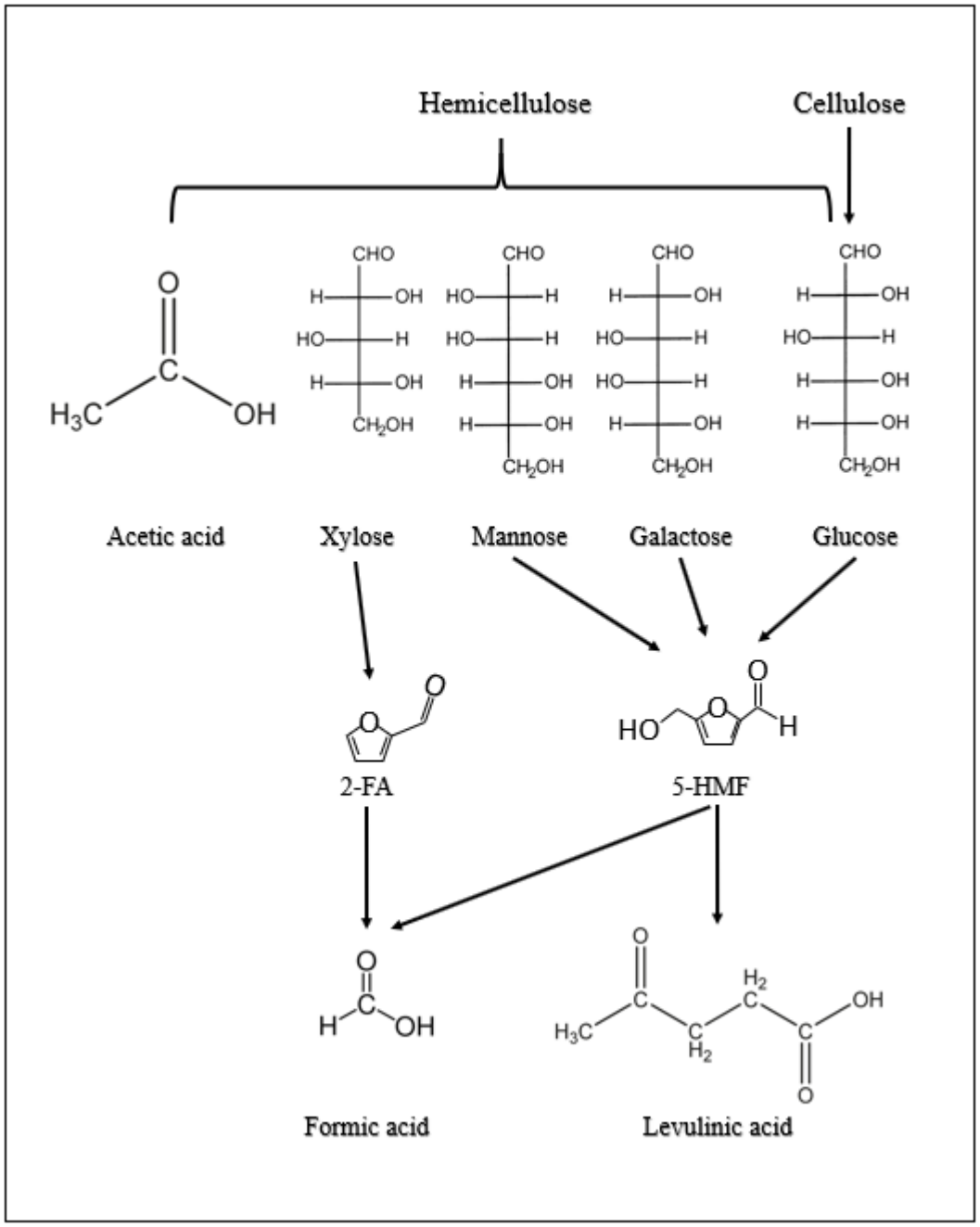


Figure 1.7. Formation of furans and organic acids from lignocellulosic biomass during hydrothermal pre-treatment.

Inhibition to the growth of fermenting yeasts occurs when the concentration of those organic acids reach up to approximately 100 mmol/l in the extracellular environment (Larsson et al., 1999). Inhibitory activities of those organic acid are explained as being due to the change of intracellular pH. Organic acids can diffuse through the microbial

cell membrane and then dissociate in the natural cytosolic environment which then led to the decrease of intracellular pH, and this change of pH causes the death of microorganisms (Imai & Ohno, 1995; Pampulha & Loureiro-Dias, 1989). In contrast, organic acids might improve the ethanol production when the concentration of them is lower than 100 mmol/l (Horváth et al., 2005) because the low concentration of acids causes more protons to be expelled from the intracellular environment to maintain the consistent pH (Verduyn et al., 1992; Verduyn et al., 1990; Viegas & Sá-Correia, 1991). Similarly, furfural and 5-HMF have been reported to inhibit the growth of yeasts by increasing the lag phase (Chung & Lee, 1985; Jönsson et al., 2013; Liu et al., 2004). Some researchers reported that the anaerobic growth of *S. cereviseae* (a common yeast strain) was insignificantly affected by furfural and 5-HMF (Jönsson et al., 2013; Palmqvist et al., 1999) suggesting the toxicity of those compounds to yeasts fermentation was relatively low (Jönsson & Martín, 2016). However, furfural and 5-HMF can be produced in very high concentrations in hydrolysates of specific biomass and eventually lead to the death of yeast cells (Jönsson & Martín, 2016; Palmqvist et al., 1999; Palmqvist & Hahn-Hägerdal, 2000b). Moreover, phenolic compounds can inhibit both growth of yeasts and ethanol production (Jönsson et al., 2013). For example, ferulic acid has been reported to be inhibitory to *S. cerevisiase* at a concentration of 1 mmol/l (Larsson et al., 2000). However, the mechanism of phenolics-related inhibition on yeasts growth has not been sufficiently researched and elucidated. Keweloh et al. (1990) suggested a possible mechanism is that phenolics might affect the function of cell membrane and change the ratio of protein to lipid.

1.4 Enzymatic hydrolysis of lignocellulosic polysaccharides

Pre-treated biomass hydrolysates can be converted into fermentable sugars by using suitable enzymes such as cellulase for cellulose (Chandel et al., 2012). Similarly, specific enzymes are required to hydrolyse other polysaccharides such as hemicellulose and pectin.

1.4.1 Enzymatic hydrolysis of cellulose

Enzymes for industrial hydrolysis of cellulose are usually developed as enzyme cocktails essentially containing three different enzymes: endo-β-glucanases (EG), exoglycanases (cellobiohydrolases, CBH) and β-glucosidase (Hasunuma et al., 2013; Waldron, 2010). The function of EG is to cleave interior β-1,4-glucan linkage of the cellulose chains and release smaller units with more accessible 'ends' which can be then hydrolysed by CBH to release cellobiose units. In the very last step of cellulase catalysis, β-glucosidases break those cellobiose units into glucose monomers (Chandel et al., 2012; Kumar et al., 2008; Waldron, 2010). The cellulase catalysis progresses as shown in Figure 1.8.

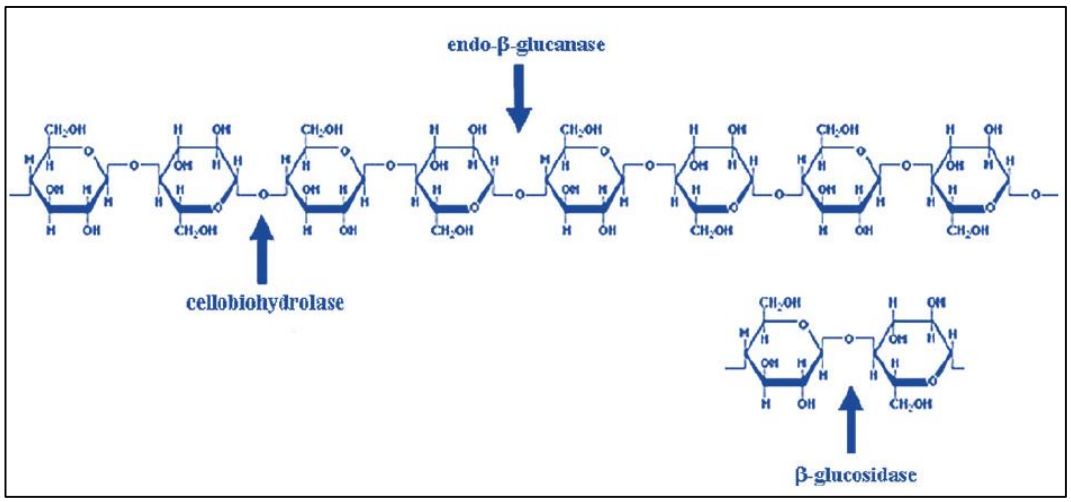


Figure 1.8. hydrolysis of cellulose by using cellulase cocktail containing EG (endo-β-glucanase), CBH (cellobiohydrolases) and β-glucosidase (Kumar et al., 2008).

1.4.2 Enzymatic hydrolysis of hemicellulose

As described above, there is a variation between plants in the component hemicelluloses. Therefore, enzyme cocktails necessary for the quantitative hydrolyse of hemicelluloses are more complicated than those for cellulose (Kumar et al., 2008). Enzyme cocktails for complete hydrolysis of hemicellulose generally contain endo-β-xylanases (EX), β-xylosidase, α-arabinofuranosidase, α-glucuronidase, acetyl xylan esterases and phenolic acid esterases (Bhat & Hazlewood, 2000; Saha, 2003). Like cellulases, each of the enzyme used to hydrolyse hemicellulose has unique function.

For example, the endo-β-xylanases are used to cleave interior β-1,4-xylosidic linkages of the xylan back bone; β-xylosidase can disrupt xylobiose units and short chain xyloogomers to release xylose; α-arabinofuranosidase is responsible for the cleavage of non-reducing α-arabinofuranose from the side chain of arabinoxylan, α-glucuronidase results in the release of glucuronic acid from the side chain of glucuronoxylan; acetyl xylan esterases and phenolic acid esterases can respectively hydrolyse the linkages of acetylated esters in the xylan backbone and feruloyl and p-coumaroyl esters in lignin-hemicellulose complexes (Bhat & Hazlewood, 2000; Kumar et al., 2008; Saha, 2003; Waldron, 2010). The diagram of enzymatic hydrolysis of arabinoxylan hemicellulose is summarised (Figure 1.9).

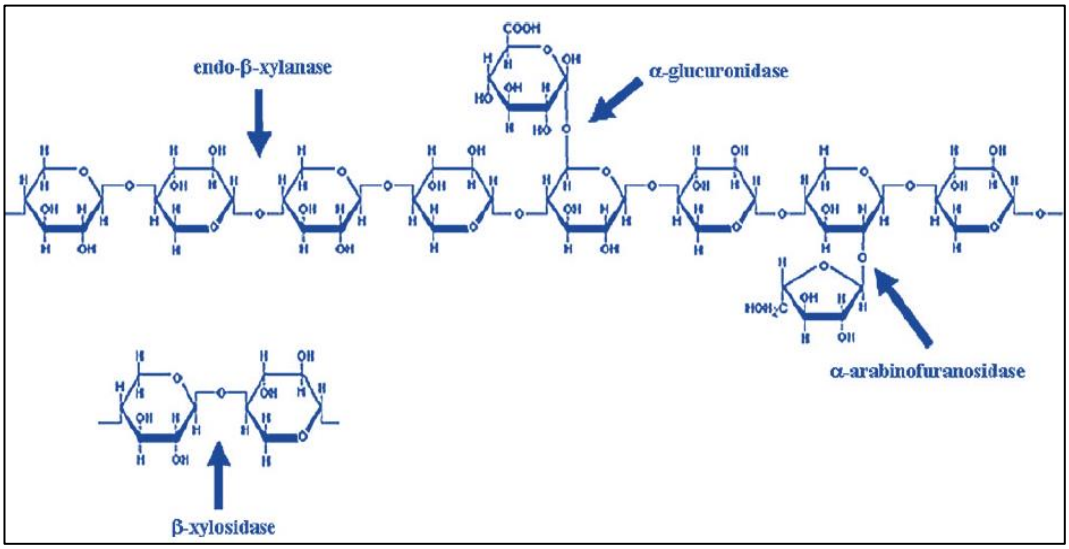


Figure 1.9. Enzymatic hydrolysis of arabinoxylan hemicellulose. The catalytic function of endo-β-xylanases, α-glucuronidase, α-Arabinofuranosidase and β-Xylosidase (Kumar et al., 2008).

1.4.3 Enzymatic hydrolysis of pectin

The hydrolysis of pectin includes a range of enzymes performing different actions such as polymethylgalacturonase, endo-polygalacturonase pectin depolymerase, pectinase, exopolygalacturonase and exopolygalacturanosidate (Kumar et al., 2009). These enzymes can hydrolyse the polygalacturonic acid chain of the pectin polymers (Jayani et al., 2005). Polymethylgalacturonate lyase (pectin lyase), polygalacturonate lyase (pectate lyase) and exopolygalacturonate lyase (pectate disaccharide lyase) can cleave

the galacturonic acid polymers by β-elimination (Kumar et al., 2009). The hydrolysis of rhamnogalacturonan that in the pectic backbone is executed by α-L-rhamnosidases and the side-chains of L-arabinose is hydrolysed by α-arabinofuranosidases, and resulting in the release of L-arabinose (Valášková & Baldrian, 2006). A simplified schematic diagram of hydrolysis of pectin is shown in Figure 1.10.

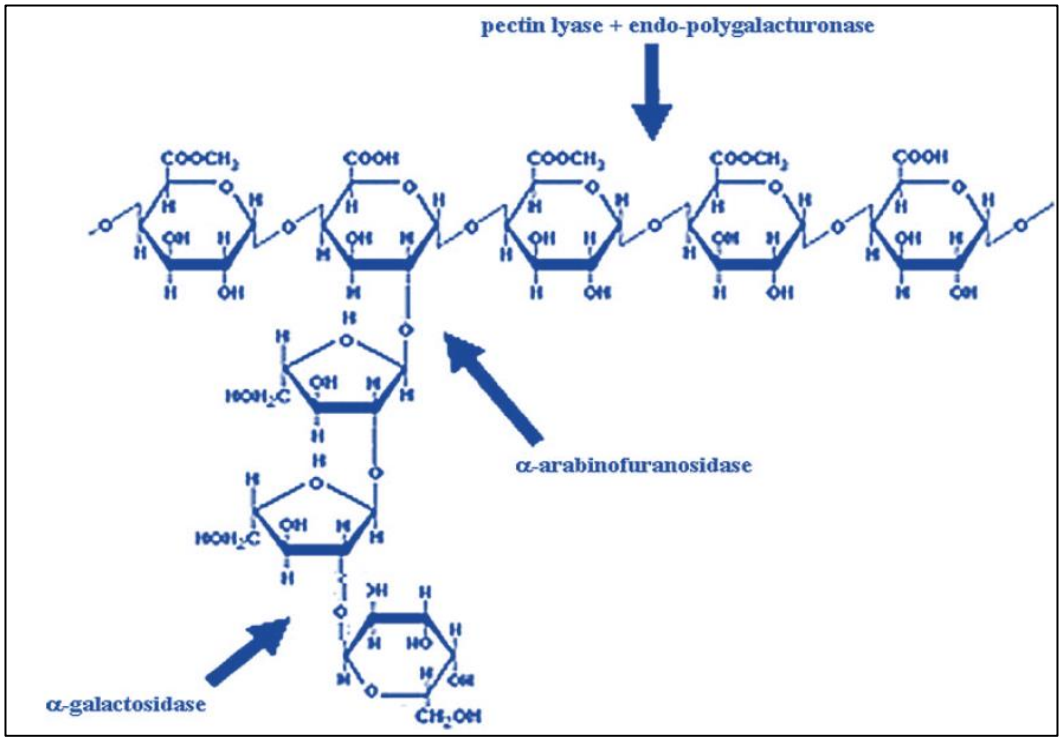


Figure 1.10. Enzymatic hydrolysis of pectin by pectin lyase, endo-polygalacturonase, α Arabinofuranosidases and α-galactosidase (Kumar et al., 2008). As this figure shows, pectin lyase: cleaves α-D-galacturonan methyl esters to release oligosaccharides by elimination. Endo-polygalacturonase: hydrolyses the α-1,4-glycosidic bonds between glacturonic acid units.

1.5 Fermentation

Fermentation is vital to the biological conversion of lignocellulose to bio-products. Pre-treated lignocellulosic biomass can be carried out by three different downstream fermentation methods: 1) separate hydrolysis and fermentation (SHF), 2) simultaneous saccharification and fermentation (SSF), 3) consolidated bioprocessing (CBP) (Waldron, 2010) (Figure 1.11).

When enzymatic hydrolysis is carried out separately followed by the fermentation for the SHF method, the optimised conditions such as temperatures for each step can be easily applied to enhance the performance of either enzymes (50^{o}C) and fermenting microorganisms (32-35^{o}C). Moreover, SHF makes the yeast recycling steps much easier (Dahnum et al., 2015; Öhgren et al., 2007a; Waldron, 2010).

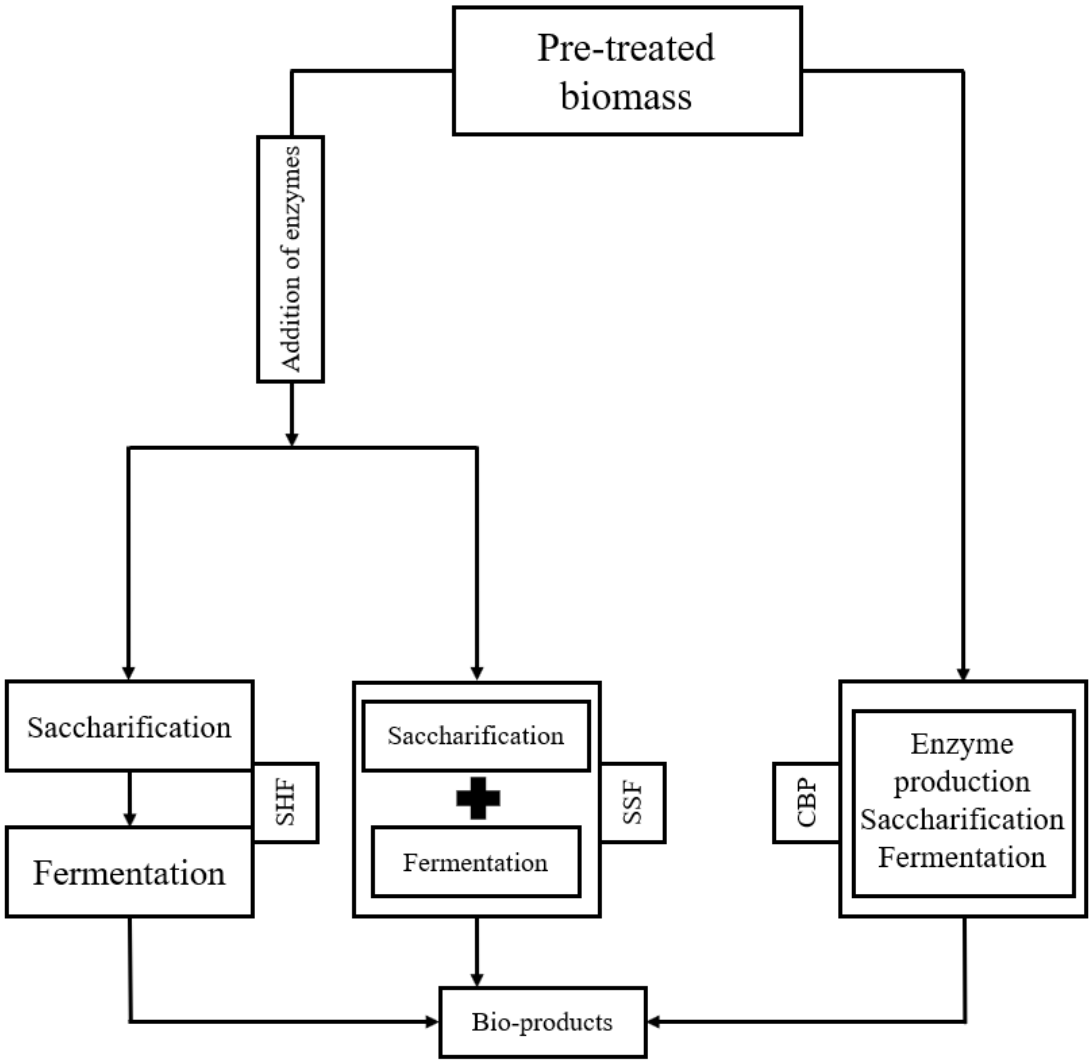


Figure 1.11. Three mainly fermentation methods in enzyme-based bio-products conversion from lignocellulosic biomass.

Simultaneous saccharification and fermentation (SSF) combines the enzymatic hydrolysis step and fermentation step by processing these two steps at the same time and in the same bio-reactor (Dahnum et al., 2015; Öhgren et al., 2007a; Waldron, 2010). This method had been extensively applied to the bioconversion of

lignocellulosic biomass since it was first reported in the research of Takagi et al. (1977). With the presence of both cellulolytic enzyme complex and fermenting yeasts, the accumulation of sugars within the reactor can be then reduced and this eventually increases the yield of fermentable sugars and the rate of enzymatic hydrolysis (Wyman & Hinman, 1990). Moreover, advantages described above can potentially reduce the numbers of bio-reactor required and enzyme loading-thereby further reducing the overall costs (Kádár et al., 2004; Waldron, 2010). However, the very significant drawback of SSF is that the difference in temperature required for hydrolysis and fermentation respectively. Cellulolytic enzymes generally operate optimally at approximately 50^{o}C but many common yeast strains are unable to sufficiently grow at such high temperatures (Ballesteros et al., 2004). Developing novel thermotolerant strains might help to solve the problems (Ballesteros et al., 1991; Szczodrak & Targoński, 1988).

Similar to SSF, CBP also known as direct microbial conversion (DMC) which ideally allows the cellulose degradation (saccharification) and fermentation to be carried out in the same step. Several studies claimed that CBP has the potential to significantly reduce the costs of bio-conversion process as the process for production of cellulase is not required. In this case, it only employs a microbial community for producing capable enzymes and for fermentation (Lynd et al., 2005; Waldron, 2010). For example, the natural fungal *Aspergillus* strains have been reported that either capable to produce β-glucosidase, xylanase and cellulase or to produce lipids (André et al., 2010; te Biesebeke et al., 2002). In the study presented by Hui et al. (2010), the fungal strain *Aspergillus oryzae* successfully produced cellulase with a activity of 1.82 FPU and produced liquids with a yield of 62.87 mg per g substrates. However, more studies are still needed to face the challenges in achieving high selectivity and yields (Lynd et al., 2005; Yang & Wyman, 2007).

1.6 Purification

The step of purification was not considered in this study; therefore, only a brief introduction to common separation methods is presented. Distillation is the most commonly used method for liquid mixture separation, which can be categorised to

three main methods: ordinary distillation, azeotropic distillation and extractive distillation (Huang et al., 2008; Waldron, 2010). As Huang et al. (2008) reported, three methods above can be used for the separation of ethanol but ethanol cannot be separated by any single distillation method due to the formation of an azeotrope during the distillation process. Pure ethanol can be extracted by combining different distillation methods: ordinary distillation can achieve the concentration to 92.4% (w/w) of ethanol, and the downstream separation methods can be azeotropic distillation, extractive distillation, liquid-liquid extraction or adsorption for further dehydration (Huang et al., 2008; Waldron, 2010). For separation of other bio-products, there are more methods that can be applied according to the target chemical. For example, steam distillation and molecular distillation (Huang & Ramaswamy, 2013). Modern methods of membrane separation are also becoming available.

1.7 Overall aims and objectives.

Considering the background study of lignocellulosic biomass, converting such biomass into bio-fuels and platform chemicals is vitally important for producing renewable energy and resources. However, the limits and challenges still outstanding include costly pre-treatments and the production of inhibitory compounds formed during pre-treatment (Wu et al., 2018b). Furthermore, there are opportunities such as the production of valuable by-products from bio-conversion processes and the potential to produce platform chemicals produced by a range of yeast strains (Wu et al., 2017). Therefore, the optimisation of pre-treatment, production of high value platform chemicals and value adding by-products might significantly improve the financial availability of the bio-conversion process.

To explore these hypotheses, several main objectives have been proposed as follows:

1) selection of raw lignocellulosic biomass: the importance of this is crucial in relation to the content of lignin and polysaccharides which can affect the final products. Ideal candidates should contain a low content of lignin that requires lower severity pre-treatment, and a high content of polysaccharides for higher yields of fermentable sugars.

2) identification of inhibitory components produced during pre-treatment and investigation of the relationship/correlation between the formation of inhibitors and conditions of pre-treatment. This knowledge could potentially help to optimise conditions of pre-treatment to minimise the formation of inhibitors therefore reducing the cost of both energy input and detoxification of biomass hydrolysates.

3) identification of (i) potential by-products generated or released during bio-conversion process and (ii) yeast strains that have potential for high value containing chemicals.

To achieve these aims, the most readily available and abundant sources of lignocellulose – rice husk and rice straw – have been used as raw materials in this study. The contents of lignin and sugars (using GC, gas chromatography) of both rice husk and rice straw had been analysed. The rice husk contained a significantly higher proportion of lignin compared with rice straw, but the contents and categories of sugars are very similar. Microwave hot water was applied as for a hydrothermal pre-treatment as this method involves an enclosed environment which prevents the loss of both materials and volatile components such as furfural thus enabling their quantification. This helped the investigations into the correlations between pre-treatment conditions and inhibitor formation. Moreover, by varying the parameter of "pre-treatment severity" (Chapter 3, Methodology), a series of pre-treatment conditions were tested on both rice husk and rice straw for a detailed understanding of the effects applied by pre-treatment on the results of both enzymatic hydrolysis (yields of fermentable sugars) and yeast fermentation (ethanol production). NMR (nuclear magnetic resonance) was used to identify and quantify components either released or produced during pre-treatment of rice husk and rice straw. Examples of inhibitors identified included 2-FA, 5-HMF, organic acids and other interesting compounds (for more details see Chapter 3). According to the study in Chapter 3, optimised pre-treatment conditions specific for rice straw have been presented which allowed for high yields of fermentable sugars and low concentrations of inhibitors for both enzymes and yeasts at a mild condition of pre-treatment (lower energy input).

Lignin related phenolics and their dimers have been reported that can cause significant inhibition activities on both enzymatic hydrolysis and yeast fermentation. The study presented in Chapter 4 had been carried out for investigating in depth of phenolics and

chemical compounds dissolved into liquor environment, and the correlation between those and pre-treatment severities. Chemical components of pre-treated rice husk and rice straw had been firstly investigated by using FTIR-ATR (Fourier transform infrared-attenuated total reflectance) and the removal of hemicellulose in pre-treatment samples has been clearly presented. Changes in lignin and degradation of phenolics were demonstrated using fluorescence microscopy. These gave rough indications of the effects cause by different pre-treatment conditions on lignocellulosic biomass related lignin, hemicellulose and phenolic compounds. A detailed identification and quantification of phenolics was then carried out by using HPLC (high performance liquid chromatography). HPLC results exposed the variation of phenolic compounds formed/released under different pre-treatment conditions, and the difference between rice husk and rice straw in the formation/release of phenolics. Moreover, the saponification of both raw and pre-treated samples indicated the very mild pre-treatment can significantly improve the extraction of phenolics. Investigations of pre-treated liquors and solids showed significant quantities of phenolics remained in the solids after pre-treatment, suggesting the hydrolysed or fermentation residues might be collected for phenolic extraction by an addition extraction step.

A set of genetically diverse yeast strains (10 non-*S. cerevisiae* strains plus one type strain of *S. cerevisiae*) was used to investigate the potential of diverse yeast strains for producing non-ethanol bio-products. An initial understanding of the growth and fermentation was achieved by incubating those selected yeast strains with 13 commercially pure sugars (Chapter 5) under either aerobic or anaerobic condition. Different behaviours of yeasts were observed on different sugar substrates. By using a new rapid NMR screening methodology, a range of metabolites were identified and quantified after the fermentation using 11 yeast strains on 13 sugars. Some compounds were produced by several strains in significant quantities in addition to ethanol, such as ethyl-acetate and arabinitol. These indicated the potential of diverse strains in producing novel platform chemicals. Lastly the 11 selected yeasts were further investigated for their proliferation and fermentation on pre-treated rice straw hydrolysates either containing fermentation inhibitors or inhibitors-free. Fermentation activities of those yeasts were significantly suppressed by high concentration of

inhibitors. Therefore, a theoretical optimised bio-conversion process can be presented as: Pre-treating RS at optimised conditions generating low levels of inhibitors followed by enzymatic hydrolysis for accumulating high levels of fermentable sugars (mainly glucose and xylose). Then the capable yeasts strains are used to high value content chemicals in addition to ethanol or increasing total yields of ethanol from both glucose and xylose. Finally, fermented RS residual solids containing phenolics can be then used to extract phenolic acids as value adding by-products.

Chapter 2：

General Materials and Methodology

2.1 Raw lignocellulosic biomass

The cultivar (*Oryza sativa*, cv. KhangDan18) of rice for provision of raw husk (RH) and rice straw (RS) was grown in a rice paddy field at the Ba Vi national park, Hanoi, Vietnam. After mature straws were harvested in spring 2012, the biomass was further fumigated and air-dried under ambient conditions (approximately 34^{o}C, 84% RH) at the Agricultural Genetics Institute, Hanoi, Vietnam. Moisture contents of RH and RS have been previously reported to be 9.98% (RH) and 9.01% (RS) (w/w) (Wood et al., 2016b). Air dried RH and RS have been well packaged with cardboard boxes and stored at room temperature (18 ^{o}C to 25 ^{o}C) in the Bio-refinery Centre (Norwich, Norfolk, UK).

2.2 Milling and freeze mill of air dried raw materials

Raw materials (RH and RS, air dried) were milled by using a RETSCH cyclone mill with a 0.5 mm mesh. Rice straw was pre-chopped into about 2 cm lengths prior to milling. Some of the milled (≤ 0.5 mm) RH and RS samples were then placed into freeze mill tubes and pre-frozen for 10 minutes. using liquid nitrogen, followed by a freeze mill process carried out using a 6700EFM Freezer/Mill. Milled and freeze milled samples were separately collected into plastic samples pots and stored under laboratory conditions (approximately 25^{o}C, on the dry bench) for less than 6 months. As mentioned in introduction, particle size influences the result of saccharification. In this study, the effect of particle size on saccharification and fermentation was not studied in details, and milling and freeze mill was aimed to reduce the size of samples for small-scale SSF.

2.3 Sugar analysis by using Gas Chromatography (GC)

Milled RH and RS triplicate samples (circa 5 mg) were weighed accurately and placed into Sovirel screw-cap glass reaction tubes. Sulfuric acid (72%, 200 µl) and 2 glass beads (to aid the mixing) were added into each tube for dissolution of plant cell walls. After mixing homogenization, the tubes were incubated at room temperature (25^{o}C) for 3 hours during which they were remixed every 30 minutes. Distilled water (2.2 ml)

was then added into each tube to bring the concentration of H_2SO_4 down to 1 mol/l and then mixed prior to a second incubation at 100^oC for 2.5 hours for further degradation of polysaccharides. After cooling, a pre-prepared internal standard (2-deoxy glucose, 2-DOG, 1 mg/ml) was added into each sample and each sugar mixture (1 mg/ml) (Figure 2.1) for making sugar standards (STDs, Table 2.1). 1 ml of each sugar standard and sample was then transferred into fresh tubes after mixing and centrifuged. Ammonia (25% w/w, 300 µl) was added into each sample and STD (for creating alkaline conditions), then 100 µl 3 mol/l NH_3 (Containing 150 mg/ml sodium borohydride-$NaBH_4$) was added followed by incubation at 30^oC for 1hour for further reduction of monosaccharides. Afterwards, tubes were all cooled on ice, 200 µl glacial acetic acid was then added to each tube to neutralise the pH of solution. Fresh tubes had been prepared into which 300 µl of each sample were transferred, the remaining samples were stored at -20^oC for less than 2 weeks in case insufficient results occurred. After the addition of 1-methylimidazole (450 µl) and 3.0 ml acetic anhydride, samples were incubated at 30 ^oC for 30 minutes for acetylation. (described in the research of Blakeney et al. (1983)). Whilst cooling those tubes on ice, 3.5 ml distilled water and 3.0 ml dichloromethane (DCM) were added for liquid-liquid extraction. After the processes of mixing and centrifugation (3000 rpm for 3 minutes), the upper layer was removed, and lower organic layer was transferred into new tubes. The same procedure was carried out two more times using 2.0 ml of DCM; all DCM extracts were combined into the same tube. The latter organic layers were washed again by adding 3.0 ml distilled water, and upper aqueous layers were removed (the washing processes was carried out three times) followed by the evaporation of DCM at 40^oC. Dried samples were re-dissolved in 1 ml acetone and then 200 µl of each sample was transferred into GC tubes and analysed using Gas Chromatography with an RTX-255 column to examine the alditol acetates produced from the monosaccharides. All samples were prepared in triplicate, STDs curves were shown in Figure ATC3.1, Appendix 3. Standard operating procedure (SOP) shown in Appendix 6.1.

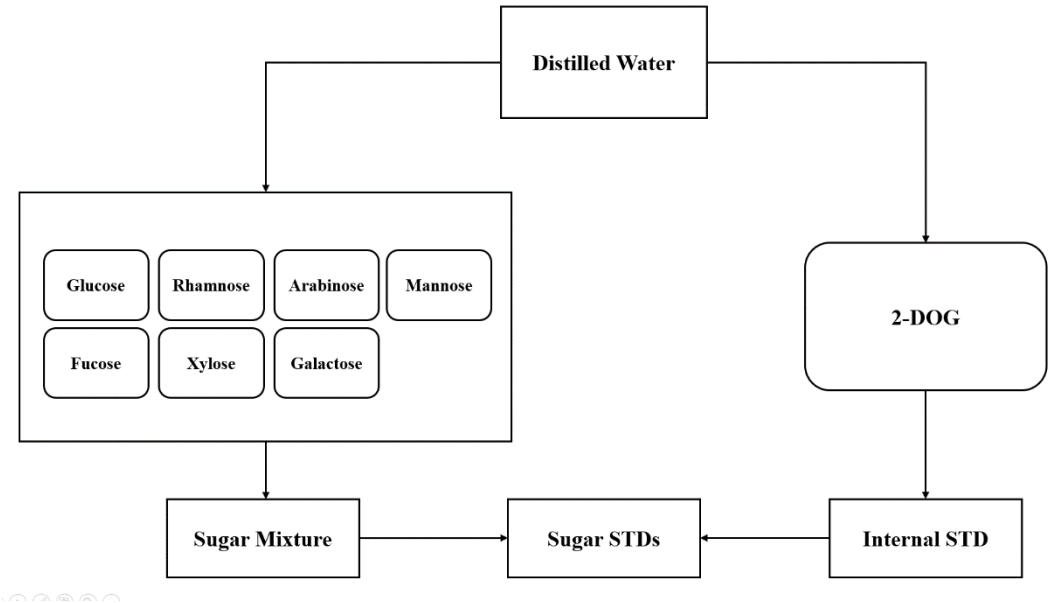


Figure 2.1. Diagram of making sugar standards. Mixture of sugars and 2-deoxy glucose (2-DOG). Seven different sugars have been dissolved into distilled water for making 1mg/ml (each sugar) sugar mixture solution. The internal STD (1 mg/ml 2-DOG) has been made in the same way. Sugar Standards (STDs) have been then made by mix the sugar mixture solutions with internal STD (show as Table 2.1).

Table 2.1. Compositions of sugar standards.

Compositions (µl)	**Sugar STDs (mg/ml)**				
	STD 1 (0)	**STD 2 (0.04)**	**STD 3 (0.08)**	**STD 4 (0.12)**	**STD 5 (0.16)**
Mixture sugars	0	100	200	300	400
Distilled water	2200	2100	2000	1900	1800
72% H_2SO_4	200	200	200	200	200
2-DOG	200	200	200	200	200

2.4 Hydrothermal pre-treatment of RH and RS

Freeze milled RH and RS were pre-treated by using a BIOTAGE® Initiator+ reactor (Figure 2.2) which worked as a microwave generator (SOP shown in Appendix 6.2). Samples were heated by microwave due to the rotation of dipolar molecules and vibrations of ions in an electromagnetic field. The hydrothermal microwave method has been reported that could reduce residence times, increase reaction rate and allow

more accurate control of reaction conditions (Biller et al., 2013). Five percent (w/w) suspensions of substrate were created by loading 750 mg of RH and RS and 14.25 ml distilled water into BIOTAGE® tubes (20 ml) respectively. Those tubes were then capped and treated at a series of pre-designed pre-treatment severities (Table 2.2). Pre-treatment severities were calculated from duration (time) and temperature, and the equation for calculation was adapted from the study of Overend et al. (1987):

$$\text{Severity } (Ro) = log_{10}(t.exp^{\frac{T-100}{14.75}}) \qquad \text{(Equation 2.1)}$$

Ro is the severity parameter, "t" is time and "T" is temperature. Table 2.2 shows times and temperatures to give the 26 differently severities for hydrothermal pre-treatment. Those cells labelled as N/A were not assessed due to the coverage of their severities by other conditions within the table. Those cells marked in red are severities of pre-treatments used in producing samples for further experiments such as fermentation and phenolic analysis in Chapters 4 and 5. Individual tubes containing 5% (w/w) suspensions of substrates were heated up to a required temperature for a required duration and then cooled with compressed air to room temperature. After pre-treatment, the tubes were then stored at -20ºC for less than 6 months.

Figure 2.2. The pictures of a BIOTAGE® Initiator+ reactor used to pre-treat rice husk and rice straw samples.

Table 2.2. Pre-designed 25 pre-treatment severities for thermodynamic pre-treatment of rice husk and rice straw.

Pre-treatment severities								
Time (minutes)	**Temp (°C)**							
	140	**150**	**160**	**170**	**180**	**190**	**200**	**210**
2.5	1.57	1.87	2.16	2.46	2.75	N/A	N/A	N/A
10	2.18	2.47	2.77	3.06	3.35	3.65	3.94	4.24
40	2.78	3.07	3.37	3.66	3.96	4.25	4.55	4.84
160	N/A	N/A	N/A	4.27	4.56	4.85	5.15	5.44

2.5 Klason lignin analysis of raw and PT (pre-treated) lignocellulosic biomass

Supernatants of PT RH and RS were separated from residues and stored at -20ºC for further investigations (less than 2 weeks). Residues and the Sintered glass funnels (porosity 4) were oven-dried at 60ºC overnight, and the weight of the funnels were recorded. The raw milled samples and oven dried residues of PT RH and RS were transferred to Sovirel culture tubes (25 ml) respectively, followed by the addition of 1.5 ml 72% H_2SO_4 after which hydrolysis was carried out by incubating the tubes at 25ºC for 3 hours. After the addition of 18 ml distilled water to dilute the 72% (w/w) H_2SO_4 to 1 mol/l H_2SO_4, the incubation was continued for a further 2.5 hours at 100 ºC. Acid digestion was applied to precipitate lignin and dissolve polysaccharides and water soluble components Afterwards, the tubes were cooled on ice and the hydrolysed slurries of RH and RS were filtered through the pre-weighed funnels and washed using distilled water to remove the acid. The funnels containing the washed hydrolysates

were oven-dried at 60ºC until a constant weight was obtained (completely dried). Funnels containing dried hydrolysates were then incinerated using a Vulcan PD Furnace 3-550 with a programmed incineration process: Temperature was increased 5ºC per minute from room temperature to 200ºC and 2ºC per minute from 200ºC to 500ºC, then the temperature was maintained at 500ºC for up to 48 hours until residues were completely burned to ash (white dust). The funnels were then allowed to slowly cool (naturally) to room temperature after which the weight of funnels containing ash were recorded. Samples were prepared as triplicates for data collection (N=3). The weight of lignin (g) can be calculated following the equation showed as below (the final results of PT samples were calculated in basis of raw materials to be consistent with the calculation of raw samples, mg/g substrate):

$$\mathbf{L\ (g) = W1\ (g) - W2\ (g)} \qquad \text{(Equation 2.2)}$$

L= Lignin

W1= Weight of funnels containing hydrolysates (oven-dried)

W2= Weight of funnels containing ash (incinerated)

SOP shown in Appendix 6.3.

2.6 Enzymatic hydrolysis of pre-treated RH and RS

Cellic® CTec-2, the cellulase complex (containing hemicellulose and high level of β-glucosidases) for degradation of cellulose to fermentable sugar was used for enzymatic hydrolysis in this study. Saccharification of all 26 pre-treated samples was carried out directly in the BIOTAGE® pre-treatment tubes (after they were completely defrosted). Prior to the additions of enzyme (Cellic® CTec-2, 187.5µL, 144 FPU), 5 ml of the acetate acetic acid buffer (0.4 mol/l, pH 5.0 and containing 0.04% v/v thimerosal to inhibit the growth of microbes) was loaded into each sample to buffer the pH of the solutions created by pre-treatment. The tubes were then re-capped and incubated at 50ºC in the orbital shaker (120 rpm) for 96 hours. After the deactivation of enzymes by heating the tubes in a water bath (100ºC for 10 minutes), samples were then cooled on ice and frozen for further analysis of glucose (by using GOPOD Format method)

and reducing sugars (by using Dinitrosalicylic acid (DNS) method). Experiment had been repeated in full two times.

2.7 Glucose analysis of PT RH and RS by using GOPOD Format (D-Glucose Assay Kit)

Defrosted samples were centrifuged at 3000 rpm for 3 minutes, and 5 µl liquor of each sample was transferred in to 96 well reader plates which contained a number of wells pre-loaded with D-glucose STDs (Table ATC2.1, Appendix 2). Then, 195 µl pre-made Glucose determination reagent (containing GOPOD Reagent enzymes and GOPOD Reagent buffer, following the instructions for the preparation of GOPOD DETERMINATION REAGENT on the website of Megazyme: https://secure.megazyme.com/files/Booklet/R-GLC4_DATA.pdf) was added into each sample well and D-glucose STD well. The GOPOD Reagent enzymes containing glucose oxidase, peroxidase and 4-aminoantipyrine that can be used for the measurement of D-glucose in hydrolysed samples. GOPOD reagent buffer is added to establish proper environment of solution for enzymatic hydrolysis. The plates were then incubated at 50^{o}C for 20 minutes (colour changing, principle is shown as Figure ATC2.1, Appendix 2) using an orbital shaker and the absorbance of each cell was recorded by using a microplate spectrophotometer at 510 nm. Experiments were repeated in full three times respectively and results were calculated against the D-glucose STDs curve (Figure ATC2.2, Appendix 2). SOP shown as Appendix 6.4.

2.8 Quantification of reducing sugars by using Dinitrosalicylic acid method (DNS)

After defrosting and centrifuging samples (3000 rpm for 3 minutes), 9 µl liquor of each sample was transferred into 96 well PCR plates containing Sugar STDs (Table ATC2.2, Appendix 2). The pre-made DNS Reagent (171 µl, contains 1% w/v 3,5-dinitrosalicylic acid and 30% w/v sodium potassium tartrate and 0.4 mol/l NaOH) was added into each cell of those plates which were then sealed with TPE PCR sealing mats. The 3,5-dinitrosalicylic acid contained in DNF Reagent and reducing sugars can

form 3-amino-5-nitrosalicylic acid which strongly absorbs light at 540 nm. In this case, the sealed plates were heated in a thermocycler at 100^{o}C for 3 minutes and then 150 µl of each sample was immediately transferred into 96 well reader plates by using multi-pipettes, then analysed using a microplate spectrophotometer at 540 nm. The method was adapted from the study of Wood et al. (2012). Samples were all made in triplicates and results were calculated against the Sugar STDs curves (Figure ATC2.3).

2.9 Selection of genetically diverse yeast strains

Ten genetically diverse yeast strains (Table ATC5.2, Appendix 5) were selected from a set of 96 diverse yeast strains supplied by the National Collection of Yeast Cultures (NCYC), and their genomes were paired-end sequenced at the Earlham Institute, Norwich (formerly the Genome Analysis Centre and carried out by Dr Jo Dicks) (Wu et al., 2017). A phylogenetic tree was kindly prepared by Dr Jo Dicks, (Figure 2.3) to enable selection of the 10 strains. Yeast strains were selected according to their distance to each other. For examples, the NCYC 16 has the longest distance from the root of phylogenetic tree, therefore it was first selected and the then the strain NCYC 49 has the longest distance from NCYC 16 was selected. By that analogy, there were 10 non-*saccharomyces cerevisiae* strains selected. In addition, a strain of *Saccharomyces cerevisiae* (NCYC 2728, Table ATC5.2, Appendix 5) was used as a cross-experiment standard. Yeast strains were originally stored in glycerol stocks (can be stored for 3 months to 6 months) so that a step of pre-growing those yeasts was required to remove glycerol. Before further use, yeast strains were transferred from glycerol stocks into agar plates and then grown in yeast nitrogen base (YNB) containing 1% glucose (YNB was pre-made and had been autoclaved) at 25^{o}C for 72 hours. Pre-grown yeast strains were then placed into fridge (-4^{o}C) until required for further experiment.

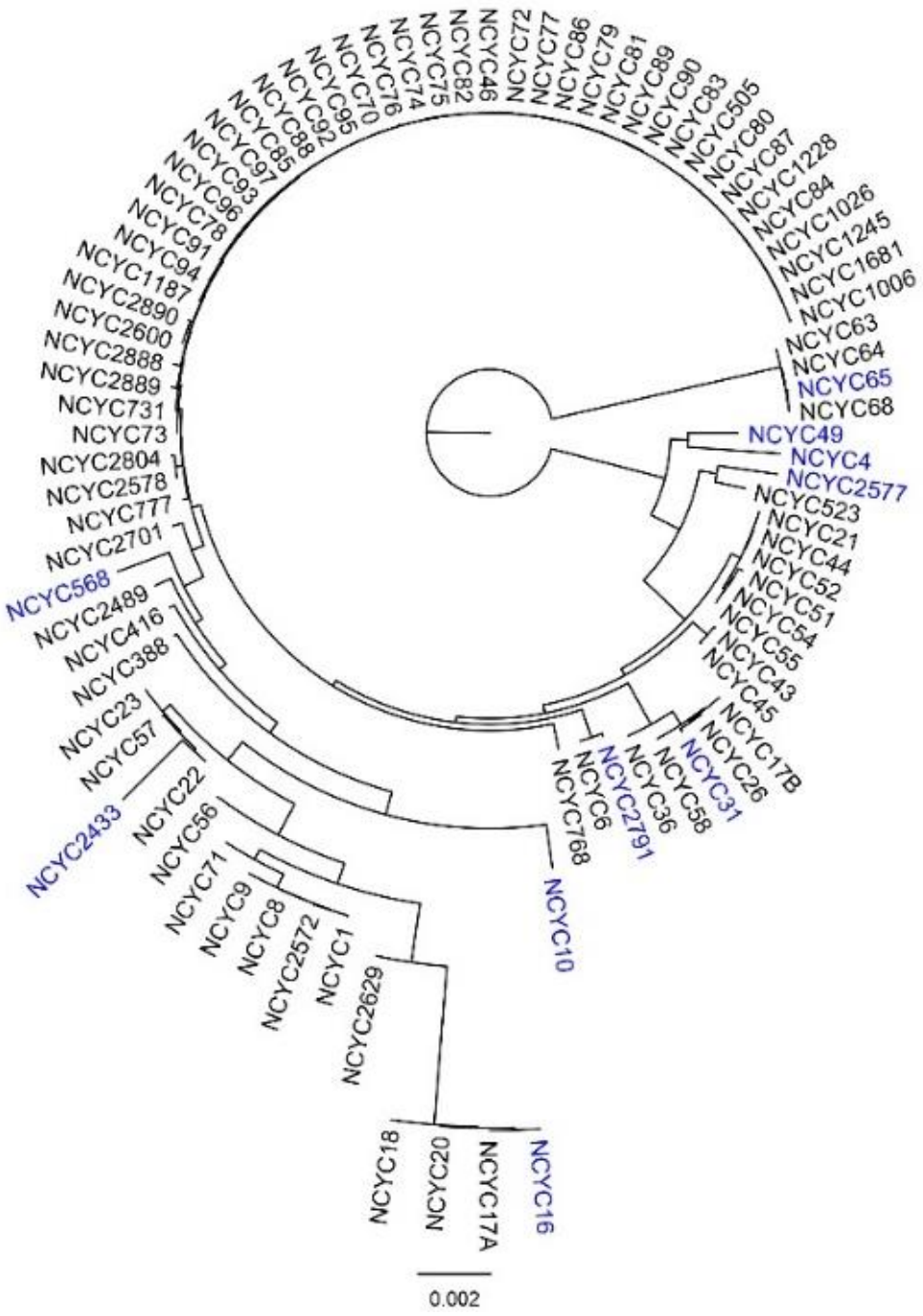


Figure 2.3. Phylogenetic tree of 10 selected genetically most diverse yeast strains. Those strains were shown as NCYC numbers, name and raw information were shown in Table ATC5.2 (Wu et al., 2017).

2.10 Growth and fermentation of 11 diverse yeast strains on 13 lab purified sugars

The 13 sugars (purity ⩾ 99.5%, Table ATC5.1, Appendix 5) were selected for the investigation of yeast growth and fermentation of all 11 diverse yeast strains on the basis that they were naturally available and/or are widespread in plants. The sugars were respectively dissolved into yeast nitrogen base (YNB) to establish a concentration of 10 mg/ml, then sterilized by autoclaving after which they were stored in sterile conditions at room temperature (less than 2 weeks) prior to use.

2.10.1 Aerobic growth

Aerobic growth of yeasts was carried out in 96 well reader plates containing 180 µl of each pre-made commercially pure sugar solution. After 20 µl of each pre-grown strain (number of cells was not counted) was added into each well containing different sugar solutions, the reader plates were capped loosely and incubated at 25^{o}C for 72 hours in a VersaMax ELISA Microplate Reader. Turbidities of plates were recorded every 30 minutes after shaking the plates (150 rpm) for 30 seconds during the incubation. Three parameters calculated based on turbidities were used to describe the growth of yeasts: The Lag phase (LP), Doubling time (DT) and Efficiency (ΔOD) (shown as the descriptive FigureATC5.7 adapted from Wu et al. (2017)). The software PRECOG was used for calculating the parameters and the method was adapted from Fernandez-Ricaud et al. (2016). This experiment was repeated in full three times.

2.10.2 Anaerobic fermentation

Fermentation of selected yeast strains was also carried out on solutions (10 mg/ml) of each commercially pure sugar. Each sugar solution (980 µl) was transferred into a set of 11 wells in 96 deep well plates (1 ml well volumes) followed by the additions of each individual pre-grown yeast strain (20 µl). The plates were then sealed with clear polypropylene PCR seals and placed on an orbital shaker for incubation (25 ^{o}C, 135 rpm, 72 hours). After incubation, deactivation of yeasts was conducted by placing the plates into boiling water (100^{o}C, using water bath) for 10 minutes, after which they

were cooled on ice. Supernatants of fermented sugar solutions were filtered through 0.2 µm filter plates (centrifuge at 3000 rpm for 5 minutes) and transferred into 96 well plates sealed with clear polypropylene PCR seals for ethanol analysis. Ethanol analysis and quantification was carried out by HPLC with a Series 200 LC equipped with a refractive index detector and photodiode array detector. Separations were performed on a BIO-RAD Aminex® HPX-87H organic acid analysis column (300 x 7.8mm; BIORAD Cat # 1250140), protected by a matching guard column, eluting with 0.004 mol/l H_2SO_4 mobile phase at a flow rate of 0.6 ml/minutes, column temperature 65 °C. Injection volume was 25 µl. Standard curves of sugars and ethanol were shown in the Appendix 5, Figure ATC5.1 to Figure ATC5.5. This experiment had been repeated in full three times for data collection.

2.10.3 Partial anaerobic fermentation

According to the results presented by NCYC 16 of producing ethyl-acetate, three controlled growth experiments were carried out to briefly investigate the effects of air (oxygen) on the production of ethyl-acetate. For more details of the conditions used please see Methodology of Chapter 3.

2.10.4 Small-scale simultaneous saccharification and fermentation (SSF)

Pre-treated RH and RS samples (severities 1.57, 3.65, 5.15 and 5.45) prepared in previous experiments were investigated for SSF. After PT, samples were defrosted, suspensions of their slurries were stirred rapidly to enable quantitative transfer of 937 µl in to Matrix tubes (1 ml tube). Then, to each tube, 12.5 µl Cellic® CTec-2 and 50 µl each of one of 11 selected yeast strains was added. Capped Matrix tubes were then placed into Matrix plates and incubated at 25^{o}C for 72 hours. Deactivation of both enzymes and yeasts involved heating the plates at 100^{o}C (water bath) for 10 minutes. The plates were then cooled with ice and centrifuged at 3000 rpm for 10 minutes to avoid transferring solids into 0.2 µm filter plates. Filtered supernatants (400 µl of SSF samples were then further transferred into 96 wells plates sealed with clear polypropylene PCR seals and then ethanol produced by yeast fermentation was

analysed and quantified by HPLC using a Series 200 LC equipped with a refractive index detector and photodiode array detector. Separations were performed on a BIO-RAD Aminex® HPX-87H organic acid analysis column (300 x 7.8mm; BIORAD Cat # 1250140), protected by a matching guard column, eluting with 0.004 mol/l H_2SO_4 mobile phase at a flow rate of 0.6 ml/minutes, column temperature 65 °C. Injection volume was 25 µl.

SSF was similarly carried out on washed PT samples for identifying the impacts resulting from soluble carbohydrates and fermentation / enzyme inhibitors produced during hydrothermal pre-treatments. Supernatants of PT samples were transferred into fresh tubes and stored at -20^{o}C (less than 2 weeks) for further experiment, and residues were washed with distilled water 3 times to remove soluble inhibitors and then volume was brought up to 15 ml with distilled water (to be consistent with 5% suspension). The following steps were executed as same as SSF for unwashed samples.

All experiments were repeated in full times and ethanol were calculated on basis of raw materials (w/w). Ethanol Standards and standard curve were applied for calculation (Ethanol STDs and STD curves were made for each individual experiment; see in the Methodology section of each Chapter). Differences in samples and yeast strains used for result Chapter are presented in the Methodology section of each Chapter.

2.11 Chemical compounds and yeast metabolites analysis of liquid samples by using ^{1}H NMR

Chemical components in the liquors of PT samples (RH and RS) (Chapter 3) and metabolites produced by yeast fermentation on lab purified sugars (Chapter 5) were analysed and quantified by using ^{1}H NMR. In general, supernatants of each sample (300 µl to 400 µl) were transferred into 96 well plates after centrifuging at 3000 rpm for 5 minutes, and then the same volume (v/v) of pre-made NMR buffer was added (buffer generated by mixing 8.4 g $NaH_2PO_4.H_2O$ (sodium dihydrogen phosphate), 3.3 g K_2HPO_4 (potassium hydrogen phosphate), 17.2 mg of sodium 3-(Trimethylsilyl)-propionate-d4 (TSP), 40 mg sodium azide (NaN_3) and 200 ml deuterium oxide (D_2O), pH 6.4) to establish 1:1 (v/v) mixture solutions. Then, 500 µl aliquots of each mixture

solution were transferred in to a 5-mm NMR tube for spectral acquisition. The ^{1}H NMR spectra of samples were recorded at 600 MHz on a Bruker Avance spectrometer running Topspin 3.2 software and fitted with a cryoprobe and a 60-slot auto sampler. Each spectrum was scanned for 64 times with a spectral width of 12500 Hz and an acquisition time of 2.62 seconds. The "noesygppr1d" pre-saturation sequence was used to suppress the residual water signal with a low-power selective irradiation at the water frequency during the recycle delay and a mixing time of 10 milliseconds. Spectra were transformed with a 0.3-Hz line broadening, manually phased, baseline corrected, and referenced by setting the TSP methyl signal to 0 ppm. The chemical compounds and metabolites were identified using information found either in the literature on the web (Human Metabolome Database, http://www.hmdb.ca/) in the Chenomx standards library or by use of the 2D-NMR methods, COSY, HSQC, and HMBC. Some additional spectra of standards were run in-house to supplement those available in the Chenomx library. Those identified chemical compounds and metabolites were then quantified using the software Chenomx NMR suite 7.6™, with quantification calculated relative to TSP. Data for chemical analysis (Chapter 3) and metabolites analysis (Chapter 5) were presented differently in Chapters (see the Methodology of each chapter). Sample preparation SOP shown in Appendix 6.5.

2.12 Fluorescence Microscopy of raw and PT lignocellulosic materials

Small aliquots of supernatant containing proportionate quantities of particulate residues (wet) of PT samples and raw samples (freeze milled) were suspended in distilled water to establish a condition of pH 7. Similarly, another set of those samples were treated with 1% NaOH (w/v) to create an alkali condition (pH 10-11). The UV (ultraviolet radiation)-auto-fluorescence of those samples were then respectively assessed by using an Olympus BX 60 Light microscope with Progress $C10^{plus}$ camera and software. Then, the auto fluorescence of those samples was recorded 3 times with a UV filter cube U-MWU, exciter filter BP330-385, barrier filter BA420.

2.13 Fourier transform infrared-attenuated total reflectance (FTIR-ATR) of raw and PT lignocellulosic materials

Cell wall components of dried residues (oven-dried at 65ºC overnight) and raw samples of RH and RS were assessed using FTIR. Small aliquots of each sample were loaded in a Golden Gate^{TM} diamond attenuated total reflectance (ATR) accessory and scanned 100 times at a resolution of 2 cm^{-1} and recorded in the region of 800-4000 cm^{-1} by using a BioRad FTS 175C Fourier transform infrared spectrometer. All samples were prepared as triplicates for data collection and spectra were averaged and referenced against a spectrum of the empty crystal and presented as area normalised figures.

2.14 Analysis of phenolic compounds in liquors and residues (dry) of PT and raw lignocellulosic materials

2.14.1 Phenolics analysis of raw and PT RH & RS solids (dry)

Pre-treated samples were defrosted and centrifuged at 3000 rpm for 5min, then the supernatant was removed from each sample. Residues was then dried in the oven at 65ºC overnight. 5 mg of each sample (both of raw and PT) was placed into a sovirel tube and saponification was carried out with the addition of 4 ml 1 mol/l NaOH (pre-deoxygenated by flushing nitrogen). Deoxygenation had also been done to all individual tubes by over-flushing nitrogen, then those tubes were then capped with screw-caps, wrapped with aluminium foil and placed in the dark on a mix wheel (30 rpm) for 21 hours. The solution of each tube was acidified by adding 1.5 ml distilled water and 0.5 ml of concentrated HCl (37%, v/v). Pre-made internal standard (trans-cinnamic acid, 0.2 mg/ml, dissolved in 1:1 v/v Methanol-water mixture) was added (50 µl) into each tube. Phenolic compounds were then extracted from the acidified solutions by partitioning into ethyl-acetate. The extraction was carried out 3 times and followed by the evaporation of ethyl-acetate (containing phenolics) by heating at 40ºC and flushing with nitrogen. 1 ml Methanol-water mixture was added into each dried tube to re-dissolve phenolics. A more intense saponification (by using 4 mol/l NaOH) was done with dried solids of un-treated and samples pre-treated at severity 1.57. Steps were same as described above. Each of all re-dissolved samples (200 µl) was filtered

with 0.2 µm filter plates (centrifuge at 3000 rpm for 5 min) and transferred into GC vials. Then, phenolics were analysed and quantified by using HPLC with a Perkin-Elmer series 200 LC Pump, Perkin-Elmer advanced LC Processor ISS200, Phenomenex Column Luna 5 µ C18 (2), 250*40 mm with pre-column and Perkin Elmer Diode Array UV Detector (Merali et al., 2013; Waldron et al., 1996). Phenolic compounds were identified against the chromatography spectrum of each phenolic and the relative retention time to the trans-cinnamic acid internal standard (see Appendix 2, Figure ATC2.4 – 2.7). Experiment had been repeated in full three time.

2.14.2 Phenolics analysis of liquors collected from PT RH and RS samples

As the suspension of pre-treated samples was 5% (w/w) substrates, 95 µl liquors (prepared in previous experiment) of each pre-treated sample were used for quantification of phenolics. Lignin derived phenolics might dissolve considerably in the liquors after pre-treatment according to the results of florescence microscopy. In order to investigate if dissolved phenolics were degraded to monomers or remained within polymers, three different methods (the direct method, the liquid-liquid extraction method and the saponification + liquid-liquid extraction method) had been tried to prepare the liquors (liquors of PT RS were used for method development).

For the direct method (method A), 95 µl of each liquor sample was transferred into wells in 96 well plates, each well containing 855 µl of the Methanol-water mixture. 50 µl of internal standard was added into each well to volume up to 1 ml which was consistent with the analysis of solids.

The liquid-liquid extraction (method B) was done 3 times by using ethyl-acetate. Acidification of each sample was carried out prior to extraction. 95 µl liquors of each sample were volume up to 1 ml by adding 50 µl of internal standard and 855 µl of distilled water. Then, 15 µl of HCl (37%) was added into each of all sample to establish an acidic condition (pH 1) and followed by the extraction. Ethyl-acetate was dried out from each tube by heating at 40^{o}C and flushing with nitrogen, then 1 ml Methanol-water mixture was added into each dried tube to re-dissolve phenolics.

The 3rd method (method C) included both saponification and liquid-liquid extraction. 95 µl liquors of each sample were transferred into Sovirel tubes and steps followed the

method for analysing phenolics of pre-treated solids. The same HPLC and method were used to analyse and quantify the phenolics (Waldron et al., 1996). Experiment had been repeated in full three times.

2.14.3 Phenolics analysis of liquors with additions of enzymes

The results of phenolics analysis of liquors showed that phenolics released were in esterified form rather than free as phenolics. To assess if the carbohydrate-esterified phenolics could be converted to free phenolics, commercially available enzymes used for saccharification of lignocellulose (CTec-2 and HTec-2) were loaded into liquors of each pre-treated sample. The RS samples pre-treated at severity 5.15 was used for developing this method. Liquors were firstly centrifuged and filtered through 0.2 µm filter plates to remove potential residuals. Then, 470 µl of filtered liquors were transferred into 96 well plates and 15 µl of both CTec-2 and HTec-2 were loaded into each sample respectively. Plates were then sealed with polypropylene PCR seals and placed into shaker (125 rpm) at 50^{o}C for 24 hours followed by deactivation of enzymes by placing the plates into 100^{o}C water bath for 10 min. After cooling on ice, each sample was brought up to 2 ml by adding 1.5 ml distilled water. Acidification was carried out by adding 15 µl of HCl (37%) to establish an acidic condition of pH 1. The subsequent liquid-liquid extraction and other steps were performed according to previous methods for phenolic analysis of solids. Analysis by HPLC also used the same method used for analysis and quantification of phenolics. Three different controls (CTRLs) were made to account for any background interference from enzyme preparations, including water-enzyme CTRL containing equal concentration of enzymes and brought to volume with distilled water; Pure PTRS CTRL containing only liquor of pre-treated (severity 5.15) RS; Time zero enzyme CTRL containing pre-treated liquors and enzyme (heating up liquors to over 80^{o}C priory to additions of enzymes). Experiment had been repeated in full three times.

SOP of phenolics extraction and HPLC analysis methods shown in Appendix 6.6.

2.15 Effects of enzymes on esterified phenolics in liquors from PT samples

Phenolics in PT liquors were assumed to be present as carbohydrate esterified forms (see rationale in Chapter 4). Enzymes such as CTec-2 and HTec-2 might potentially hydrolyse the carbohydrate-esterified phenolics. To investigate this, CTec-2 and HTec-2 was added into liquors of PT samples (RS, severity 5.15), followed by an incubation at 50^{o}C for 24 hours. Then, deactivation of enzymes was carried out by heating at 100^{o}C in water bath for 10 minutes and the cooled (room temperature) solution was acidified with HCl (37%) and liquid-liquid extracted with ethyl-acetate. After the evaporation of ethyl-acetate, re-dissolved phenolic residues (using Methanol-water mixture) were filtered through a 0.2 µm filter plates. 200 µl of each sample was then assessed for phenolics analysis and quantification using HPLC (More details of the experiment see the Methodology of Chapter 4).

For all phenolic analysis mentioned above, the phenolic compounds were identified against both the chromatography spectrum of each phenolic (Appendix 2, Figure ATC2.3 to ATC2.6) and the relative retention time to the trans-cinnamic acid internal standard (RRT, Appendix 2, Figure ATC2.7). The identification methods were generally adapted from the study of Waldron (1996).

Chapter 3:

Comparison of RH and RS as substrates for industrial bio-technology

3.1 Introduction

Technologies to produce renewable and low-carbon electrical energy have been rapidly developed including nuclear, wind or photovoltaic (Barnham et al., 2006; Blair, 1976). Nevertheless, liquid fuels are required in different fields such as road vehicles and aviation, and this has led to global interests and programmes for producing renewable second-generation bio-fuels such as cellulosic bio-ethanol that have the potential to be sustainable, and emit minimal levels of greenhouse gases (Nigam & Singh, 2011; Sims et al., 2010). Cellulose is a natural polymer of glucose, and is abundant in lignocellulosic biomass including agricultural residues such as forestry residues, pulping wastes, cereal straws, and threshing husks, as well as food processing by-products such as brewers spent grain (Hasunuma et al., 2013; Singh et al., 2011).

Rice is grown widely in over 100 countries and consumed by half of the world's population (Muthayya et al., 2014). Approximately 712 million tons of paddy rice is produced worldwide annually which means at least 712 million tons of rice straw (RS) and 178 million tons of rice husk (RH) will be potentially produced through the process of harvest and rice milling (Abbas & Ansumali, 2010; Binod et al., 2010; Muthayya et al., 2014). Since 90% of rice is harvested from Asian countries, in 2004, 667.59 million tons of RS from agricultural wastes were produced in Asia that could be theoretically converted to 281.72 billion litres of ethanol (Kim & Dale, 2004). More importantly, RH and RS each exhibit a high content of cellulose and hemicellulose that can be potentially hydrolysed into fermentable sugars. The husks contain approximately 29%-36% of cellulose and 12%-25% of hemicellulose which is very similar to the contents of cellulose and hemicellulose in RS (approximately 32%-47% of cellulose and 19%-27% of hemicellulose) (Abbas & Ansumali, 2010; Binod et al., 2010). These indicate that half of the weight of RH and RS can theoretically be hydrolysed into fermentable sugars for producing bio-ethanol or other bio-products (Figure 3.1).

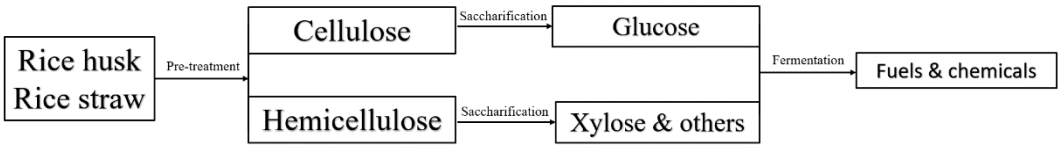


Figure 3.1. Brief path of converting RH and RS into bio-products.

However, it is highly challenging to convert cell wall sugars to bio-products due to the protective bio-chemicals (e.g. lignin, phenolics, silica) and the structural nature of the lignocellulosic biomass (Brett & Waldron, 1996b) which hinders the saccharification of the polysaccharides (Lattanzio et al., 2006; Lin et al., 2010). In general, the bio-refining processes involve four main steps: hydrothermal pre-treatment, enzymatic or chemical saccharification, fermentation and purification. Each step has its own purpose, for example, pre-treatment is to remove the lignin from the cellulose, reduce the structural barriers created by hemicelluloses, reduce cellulosic crystallinity to increase the surface areas and improve the accessibility of cellulose to cellulases (Cheng & Stomp, 2009a; Kumar et al., 2009). The fermentable sugars released after the step of saccharification can be then converted to products by microbes such as bacteria and yeasts (Carlozzi et al., 2010; Nuwamanya et al., 2012). Then, the product of interest can be finally recovered from the fermentation liquor with purification technics such as distillation. Varying results can be achieved with the different combinations of those four steps since each of those steps has a range of options.

RS and RH have been previously demonstrated to exhibit very different propensities for enzymatic saccharification and fermentation behaviour in response to steam explosion pre-treatment (Wood et al., 2016a). This Chapter describes investigation to evaluate the differences in the composition of these lignocellulosic materials, and the changes that occur in them during hydrothermal pre-treatments relevant to their bio-refining potential, with special reference to the release of potential fermentation inhibitors and related chemicals. This has been executed by using enclosed hydrothermal pre-treatment conditions to retain volatile substances that might be created and lost during steam explosion. Moreover, a much higher range of pre-treatment severities have been investigated by using different combinations of time and temperatures to assess effects of pre-treatment severities on formation of inhibitors, yields of fermentable sugars and production of ethanol for both rice husk and rice straw.

Conditions conducive to optimal simultaneous saccharification and fermentation (SSF) have also been explored.

3.2 Methodology

3.2.1 Small-scale simultaneous saccharification and fermentations (SSF) of pre-treated RH and RS

RH and RS pre-treated at 4 severities (pre-treatment conditions are in red-coloured fonts in Table 2.2 of General Materials and Methodology, those severities were selected as they were light pre-treatment, mild pre-treatment, strong and strongest pre-treatment conditions) were assessed. SSF was carried out in 1 ml Matrix tubes by loading 937.7 µl of slurry of each all pre-treated samples (each sample were prepared in triplicates), 12.5 µl of Cellic® CTec-2 and 50 µl of pre-grown yeast strains *S. cerevisiae* (in this Chapter, only one strain had been used for fermentation, more details of pre-grown strains see Chapter 2, General Materials and Methodology). Capped Matrix tubes were loaded into Matrix plates and then placed on shaker (135 rpm) at 25^{o}C for 72 hours incubation. By the end of incubation, enzyme and yeasts were deactivated by placing those Matrix plates into 100^{o}C water bath for 10 minutes. Supernatants of each sample were filtered using 0.2 µm filter plates. 200 µl of filtered supernatant of each sample were then transferred into a 96 well deep-well plate (1 ml round bottom) and analysed by using HPLC. Ethanol products from yeast fermentation were calculated against pre-made ethanol standards (see Figure ATC3.2, Appendix 3). Experiment had been repeated in full three times for data collection.

3.2.2 Hydrothermal pre-treatment of RH and RS

Hydrothermal pre-treatment was carried out by using a BIOTAGE® Initiator+ reactor. 750 mg of milled RH and RS were loaded respectively into 25 ml microwave pressure tubes containing 14.25 ml distilled water to give a 5% (w/w) suspension. Severities of pre-treatment consisted of temperature and durations given in Chapter 2 (General Materials and Methodology). In this Chapter, 4 severities were further expanded to 25 severities showing as Table 2.2 (see Chapter 2, General Materials and Methodology).

3.2.3 Principal components analysis (PCA) of chemical compounds from the liquors of PT RH and RS

To present visualised associations of those chemical compounds with severities and with each other. The PCA figure was generated by using Multi Variate Statistical Package version 3.22. (Kovak Computing Services, Anglesey, UK).

3.3 Results and discussion

3.3.1 Sugar and Klason lignin analysis of raw RH and RS

After milling, RH and RS samples were hydrolysed and saccharified by using H_2SO_4. Supernatants were used for sugar analysis and quantification executed by GC and data was calculated as a proportion of the weight of dry materials (~%DW). Hydrolysates were collected for lignin analysis. Uronic acid was not quantified. In both RH and RS, sugar compositions comprised arabinose, fucose, rhamnose, mannose, xylose, glucose and galactose (Figure 3.2). These are in keeping with previous studies of Abbas and Ansumali (2010), Park et al. (2009), Lim et al. (2012) and Ludueña et al. (2011). Glucose was the most abundant sugar in both RH and RS followed by hemicellulosic xylose with more in RS compared with RH. The content of Klason lignin was much higher in RH (35%, w/w) than in RS (21%, w/w). Content of lignin was reported as 22.3% (w/w) in RS and 24.4% (w/w) in RH by Lim et al. (2012). Ludueña et al. (2011) mentioned that the contents of lignin in RH can range from 26% to 31% (w/w). This evaluating difference might due to the genetic variation in lignin between rice cultivars (Penning et al., 2014). The very high content of lignin in RH will physically hamper the enzymatic saccharification (Öhgren et al., 2007b), and will also increase physical surface onto which cellulase may bind strongly to further reduce the availability of the dissociated enzyme (Wood et al., 2014).

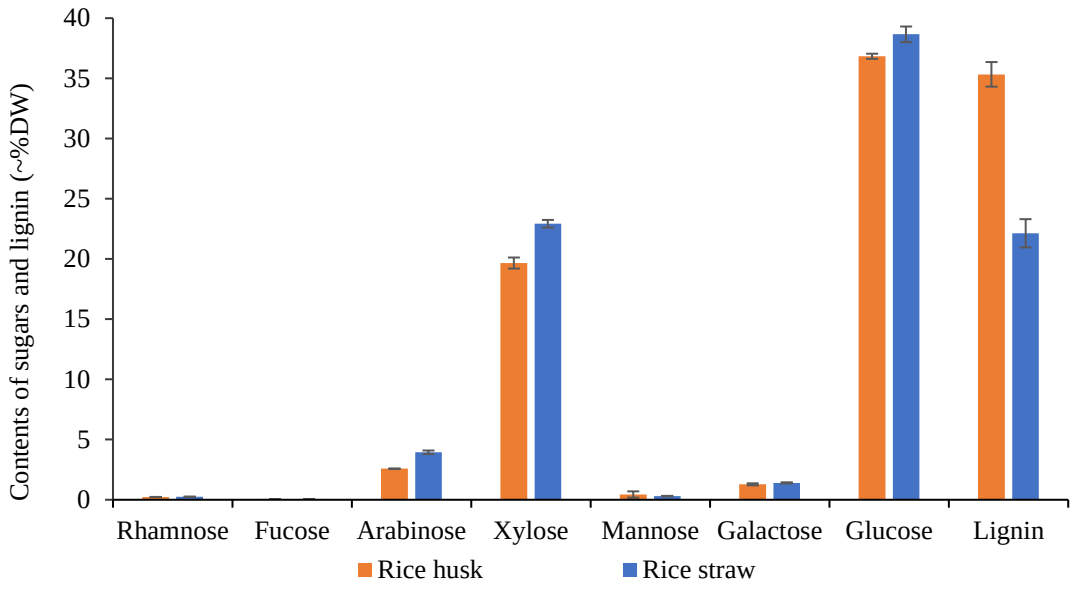


Figure 3.2. Contents of sugars and Klason lignin in milled raw RH and RS. The contents of compounds have been calculated to the proportion of the weight of dry raw materials (DW). Error bars are standard deviation (SD).

3.3.2 Saccharification of PT RH and RS with enzymes

Saccharification of pre-treated RH and RS was carried out in 25 ml microwave pressure tubes at 50°C for 96 hours. Figure 3.3 shows the reducing sugar and free glucose yields as a function of pre-treatment severities. As it shows, the yields of both reducing sugar and glucose increased with increasing severity. Comparable results were reported by Wood et al. (2016a): enzymatic hydrolysis of steam exploded RS released much higher quantities of glucose (max 43.6%, severity 5.15) and reducing sugars (max 66.1%, severity 4.27) compared with RH (max 16.3% of glucose, severity 5.44; max 35.3% reducing sugars, severity 4.55). In pre-treated RH samples, yield of reducing sugars increased steadily with increasing severity up to 4.5 then decreased whilst glucose yield continued to increase at above severity 4.5. In contrast to RH samples, the glucose yield from RS peaked at a severity of 4.8 then decreased at higher severities. Moreover, the yield of reducing sugars reached a peak at severity 4.3 and then dropped rapidly at higher pre-treatment severities in pre-treated samples of straw. These results indicated that significantly higher sugars yields were achieved from RS compared with RH via pre-treatment and enzymatic hydrolysis with similar conditions. The decreasing of reducing sugars and glucose at high severities may be ascribed to

the significant conversion of sugars into fermentation inhibitors such as furfural (2-FA), 5-hydroxymethylfurfural (5-HMF), formic acid and levulinic acid. Furfural and 5-HMF were generated from hexoses (mainly from glucose) and pentose (mainly from xylose) respectively and those were then further degraded into formic acid and levulinic acid (Pedersen & Meyer, 2010)

RS samples pre-treated at between severities of 3.65 and 4.25 presented a high yield of both glucose and reducing sugars after enzymatic hydrolysis which is approximately 10% lower than the maximum yield (Figure 3.3). However, further development of pre-treatment methods is required to address the challenge of economically and sufficiently converting RH into fermentable sugars. A wealth of studies have implicated that very harsh chemicals could be possibly used to surmount the recalcitrance of husk by removing or extracting lignin and other structural barriers to enzymolysis (Wood et al., 2016a). For instance, a range of additions of chemicals had been compared in the study of Ang et al. (2013) who reported a yield of 22.3% (w/w) of total sugar was achieved after pre-treating RH with only HCl. Furthermore, alkaline peroxide was used in the study of Saha and Cotta (2007) and achieved a saccharification yield of 42.8% (w/w). However, large quantities of chemicals (often, the same order of magnitude as the biomass being treated) are generally required to carry out such treatments. This will be costly both financially and environmentally (Harmsen et al., 2010; Sun & Cheng, 2005).

Another factor that is responsible for stronger recalcitrance of RH is possibly the very high content of silica which is present in both RH and RS at higher levels compared with other cereal-derived lignocellulosic biomass, and it is much higher in RH than in RS (Van Soest, 2006). However, this was not assessed in this study. In the study of Van Soest (2006), a severe impact of silica on ruminant digestibility of RH and straw was reported and it might also be expected to have an impact on the enzymatic digestibility of pre-treated rice biomass during saccharification. Khaleghian et al. (2017) recently considerably enhanced saccharification by chemically removing silica after previously removing lignin.

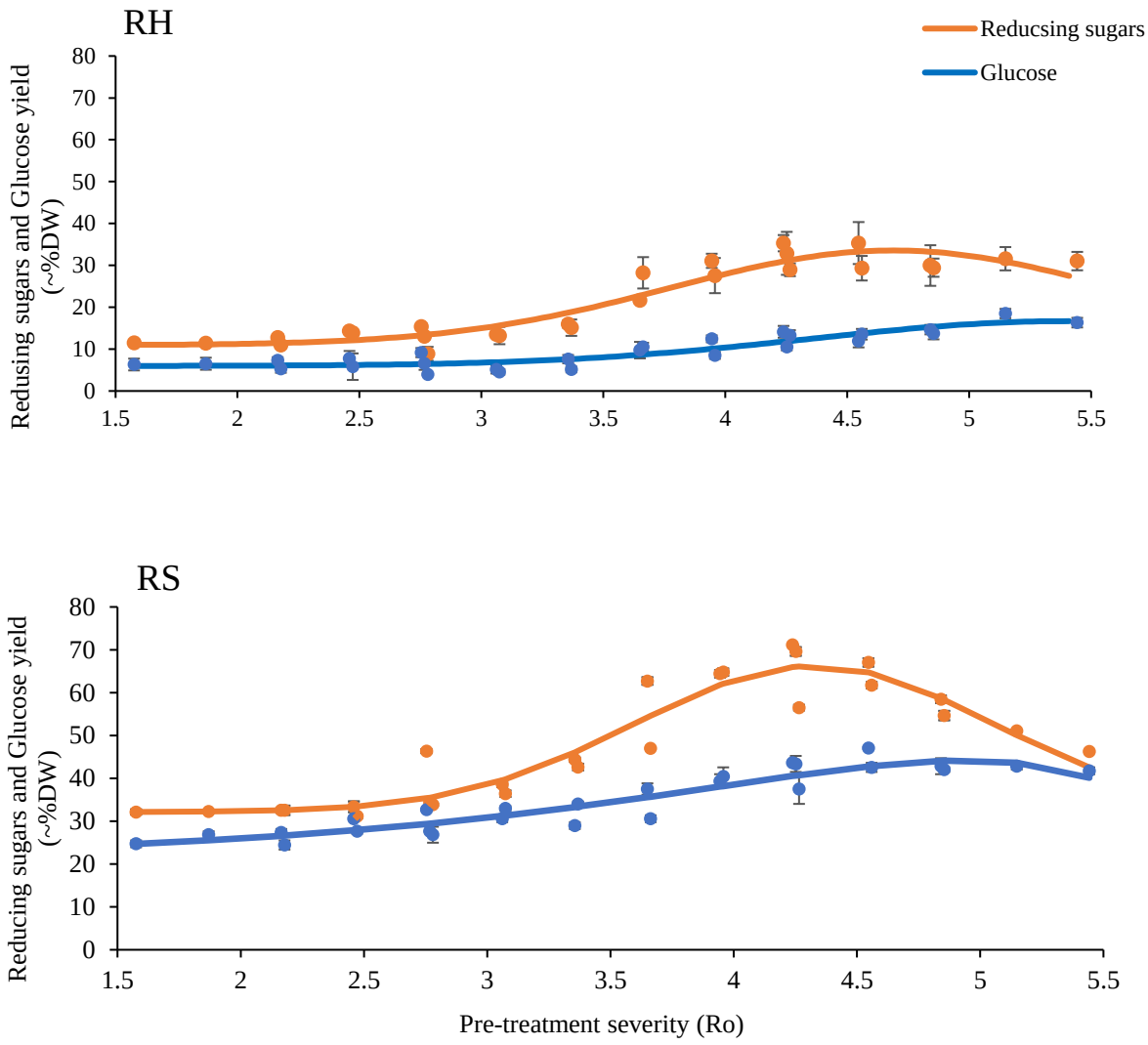


Figure 3.3. Yields of both glucose and reducing sugars of RH and RS (after pre-treatment over a range of severities) after saccharification using cellulase (CTec-2) for 96 h at 50°C. The orange curve represents the proportion of total reducing sugars in air dried biomass material and the blue curve represents the proportion of glucose in air dried biomass material. Data was processed using Genstart (Edition 18th) and error bars are SD (Wu et al., 2018b).

3.3.3 Small-scale simultaneous saccharification and fermentation (SSF) of PT RH and RS

RH and RS samples were pre-treated at four severities (1.57, 3.65, 5.15 and 5.45) spanning the range used earlier from low to very high. SSF was carried out at 25°C by adding CTec-2 (cellulase) and a pre-grown yeast strain (*Saccharomyces cerevisiae NCYC 2826*) to each of all pre-treated samples. Figure 3.4 illustrates that ethanol was

produced significantly more from RS (3.71 mg/ml at severity 1.57, 7.11 mg/ml at severity 3.65) than RH (1.79 mg/ml at severity 1.57, 3.77 mg/ml at severity 3.65) after pre-treatment at severities 1.57 and 3.65 reflecting that yeast behaviours differed on different hydrolysates. Very small quantities of ethanol were produced in both RH and RS samples pre-treated at severities 5.15 and 5.45 suggesting that fermentation activity of the yeast strains were supressed by inhibitors generated by high severity pre-treatment (Palmqvist & Hahn-Hägerdal, 2000b). The research reported in Chapter 5 and by Wu et al. (2017) showed that washing pre-treated RS prior to SSF significantly reduced the severity-related decline in SSF efficiency exposing the impact of fermentation inhibitors.

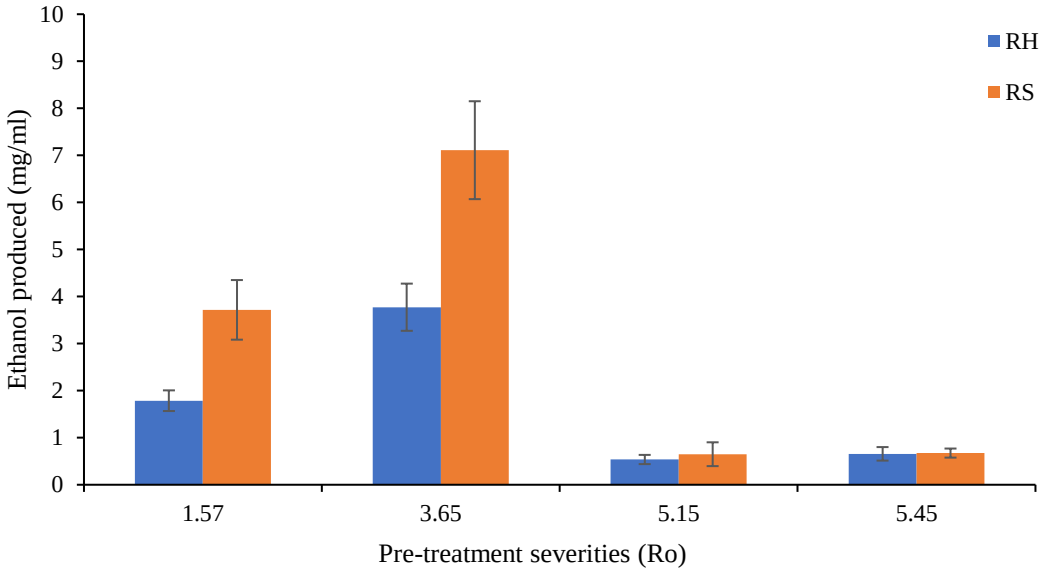


Figure 3.4. Ethanol produced from RH and RS pre-treated at 4 different severities (5% w/w of substrates in 15 mL slurry). Pre-treated samples were hydrolysed by CTec-2 and fermented by Saccharomyces cerevisiae (NCYC 2826). Bars show the concentration of ethanol and error bars are SD.

3.3.4 Characterisation and analysis of chemical compounds in the liquors from pre-treated RH and RS by using ^{1}H Nuclear Magnetic Resonance (NMR)

Rapid NMR (for more details see Chapter 2, General Materials and Methodology) was used to achieve more integrated understandings of the range of solubilised components generated from the RH and RS during hydrothermal pre-treatment. Figure 3.5 (part A

and part B) shows the diagnostic spectrum of the compounds detected from liquors of RH and RS pre-treated at severities 1.57, 3.65, 5.15 and 5.45. Different regions were scaled to visualise variation in concentration. The results showed that 25 different compounds (acetaldehyde and acetaldehyde hydrate were quantified as one compound) were detected in measurable quantities. Compounds changed in quantities whilst increasing pre-treatment severities. For example, 5-HMF and 2-FA increased noticeably in RH and RS samples pre-treated at severities 5.15 and 5.45 compared with those pre-treated at severities 1.57 and 3.65.

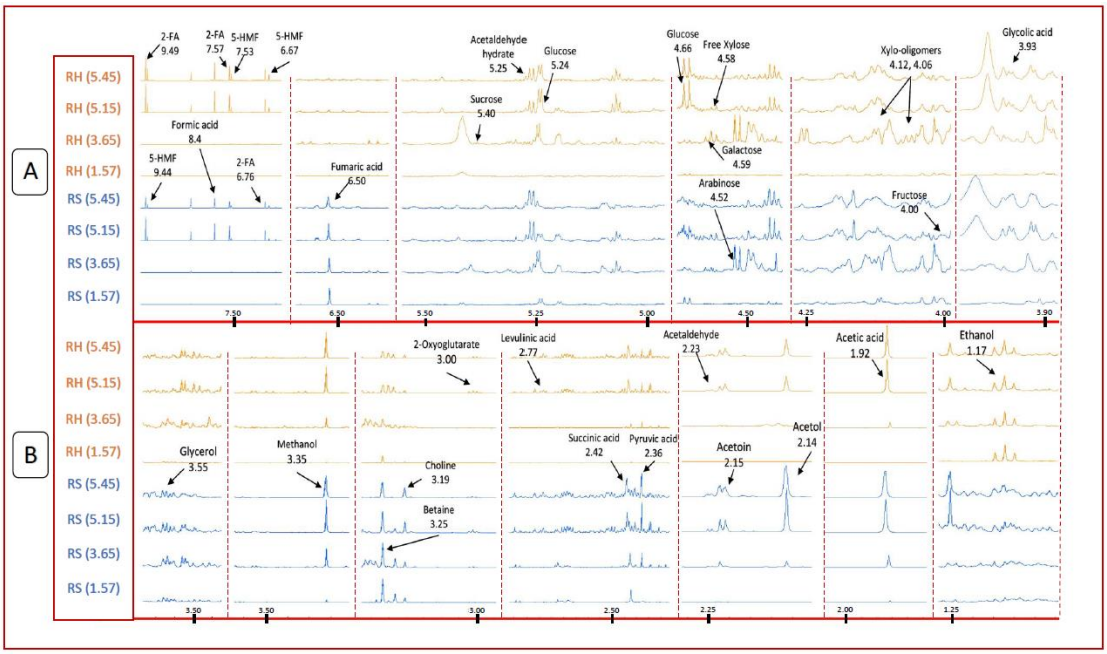


Figure 3.5. The ^{1}H NMR spectra of the liquors of RH and RS samples pre-treated at severity 1.57, 3.65, 5.15 and 5.45. Part A (10 ppm to 3.9 ppm) and Part B (3.9 ppm to 0 ppm) Different regions were scaled differently to visualise compounds present in small quantities (Wu et al., 2018b).

Principal component analysis (PCA) is a very useful tool in chemometrics, which can extract the important information from data containing several inter-correlated variables and then visualise the information by presenting a set of new orthogonal variables named the principal components (Abdi & Williams, 2010; Bro & Smilde, 2014). As Figure 3.6 shows, PCA was established to present the associations of those detected compounds (coloured dots) with pre-treatment severities (arrows). Those compounds were categorised as 9 previously unidentified compounds (green), 9

established fermentation inhibitors (red) and 7 sugars (Orange). Green dots are mostly located around low severities (the bottom left). In contrast, inhibitors (red dots) are generally positioned to the right region associated with the higher severities. Most of sugars (Orange dots) are positioned adjacent to moderate severity arrows.

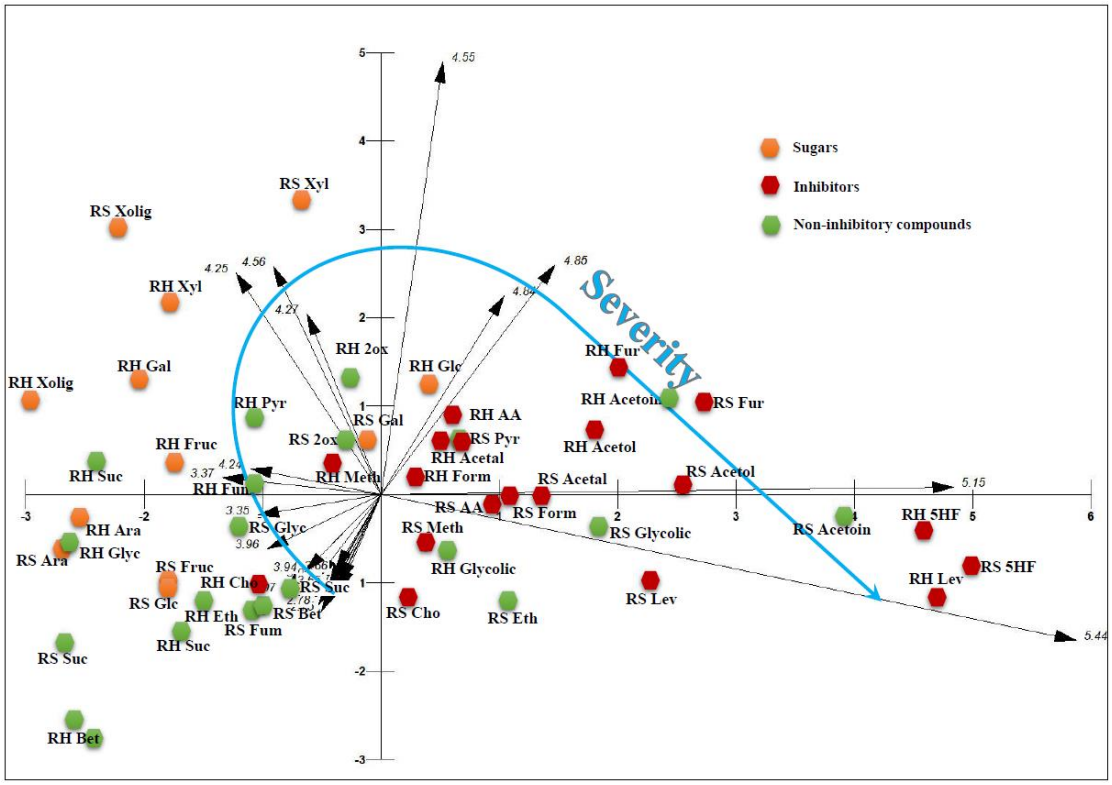


Figure 3.6. Principal component analysis (PCA) of all 25 compounds detected from the liquors of pre-treated RH and RS samples. The compounds were categorised and coloured differently. Red represents inhibitors, orange represents carbohydrates and green represents non-inhibitory compounds (Wu et al., 2018b).

A more comprehensive change in quantities of these compounds, as affected by severity of pre-treatment are shown graphically in Figure 3.7, Figure 3.8 and Figure 3.9. Chemical compounds mainly generated and (or) released from samples during low severity pre-treatment are shown in the Figure 3.7. Compounds of pyruvic, succinic, fumaric and 2-oxoglutaric acids are organic acids typically found in intermediary metabolism. Furthermore, acetoin, glycolic acid and glycerol were found additionally. Higher quantities of succinate, fumarate and pyruvate were detected in samples pre-treated at higher severities, particularly in pre-treated samples of straw. Consistent

increases of acetoin and glycolic acid from low severities to high severities were presented. However, glycerol, pyruvate and 2-oxoglutarate peaked at mid severities, then declined and this indicated that degradation occurred during high severity pre-treatment. Moreover, ethanol was detected in small quantities from the liquors of both RH and RS samples pre-treated at higher severities. Interestingly, the levels of betaine were different between pre-treated RH and pre-treated RS.

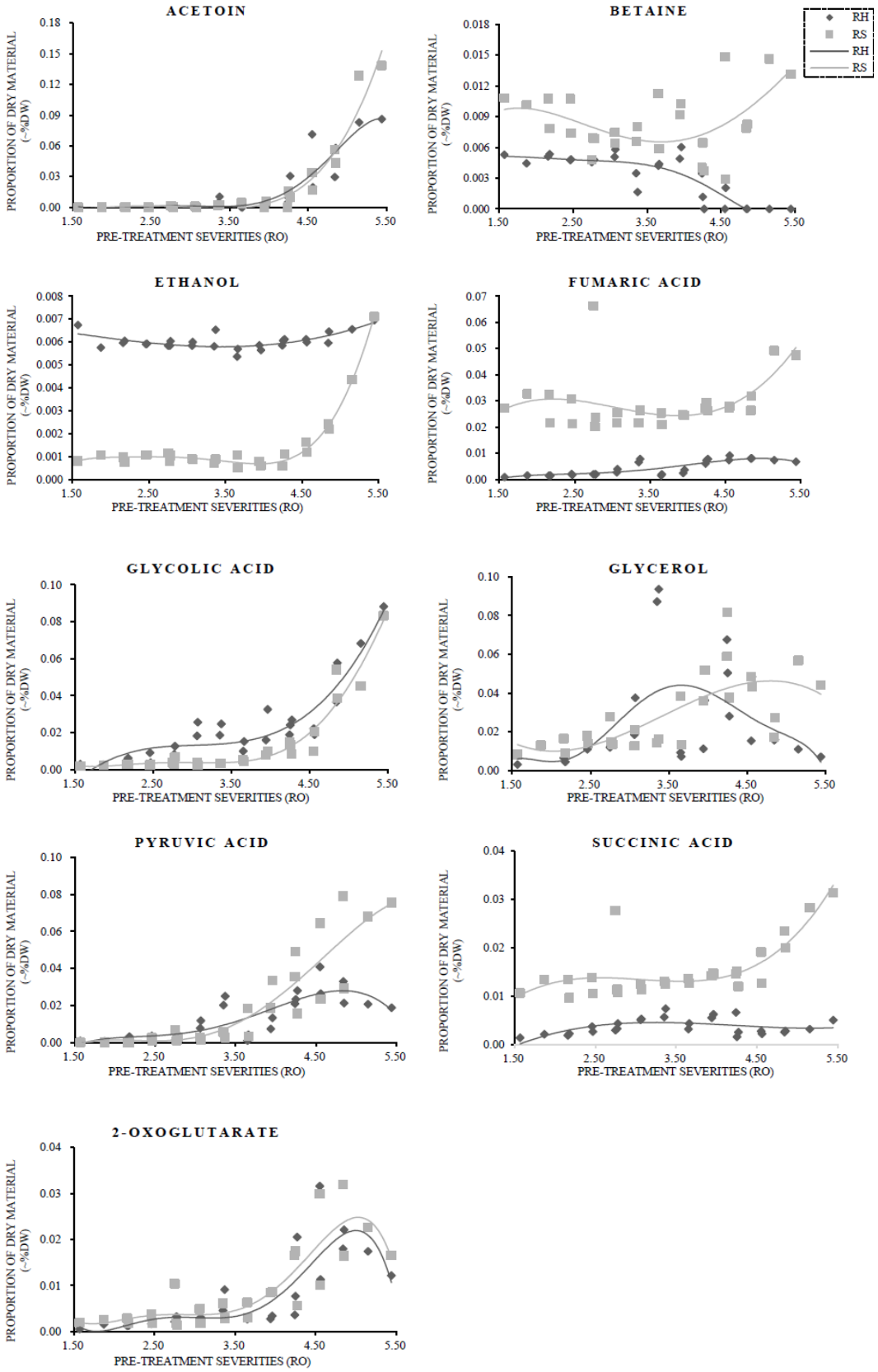


Figure 3.7. The trends of non-inhibitory compounds released/formed from pre-treated RH and RS samples (Wu et al., 2018b). Trends lines are purely as a visual aid to improve clarity of data.

Figure 3.8 shows that sugars and oligosaccharides were released into liquors of samples pre-treated at moderate severities. All sugars showed very similar trends: after they reached their peaks at around a severity of 4.5 and then declined suggesting degradation. These results are consistent with the trends of sugars shown in Figure 3.3 and concomitant with the increase in fermentation inhibitors shown in Figure 3.9. During pre-treatment RH released higher yields of sugars compared with RS. The presence of galactose presumably indicates that small quantities of pectic polymers were hydrolysed. The hydrolysis of hemicellulosic xylans and arabinoxylan may have led to the presence of xylose, xylo-oligomers and arabinose. Xylose and xylo-oligomers were released more significantly in quantities from pre-treated husk compared with pre-treated straw, with a total concentration of over 1 mg/ml (concentration was calculated from Figure 3.8) which have been shown to severely inhibit enzyme activities (cellulase) (Qing et al., 2010). Such notable concentrations of cellulase-inhibitory compounds might also contribute to the poor saccharification results of pre-treated RH samples (Figure 3.3).

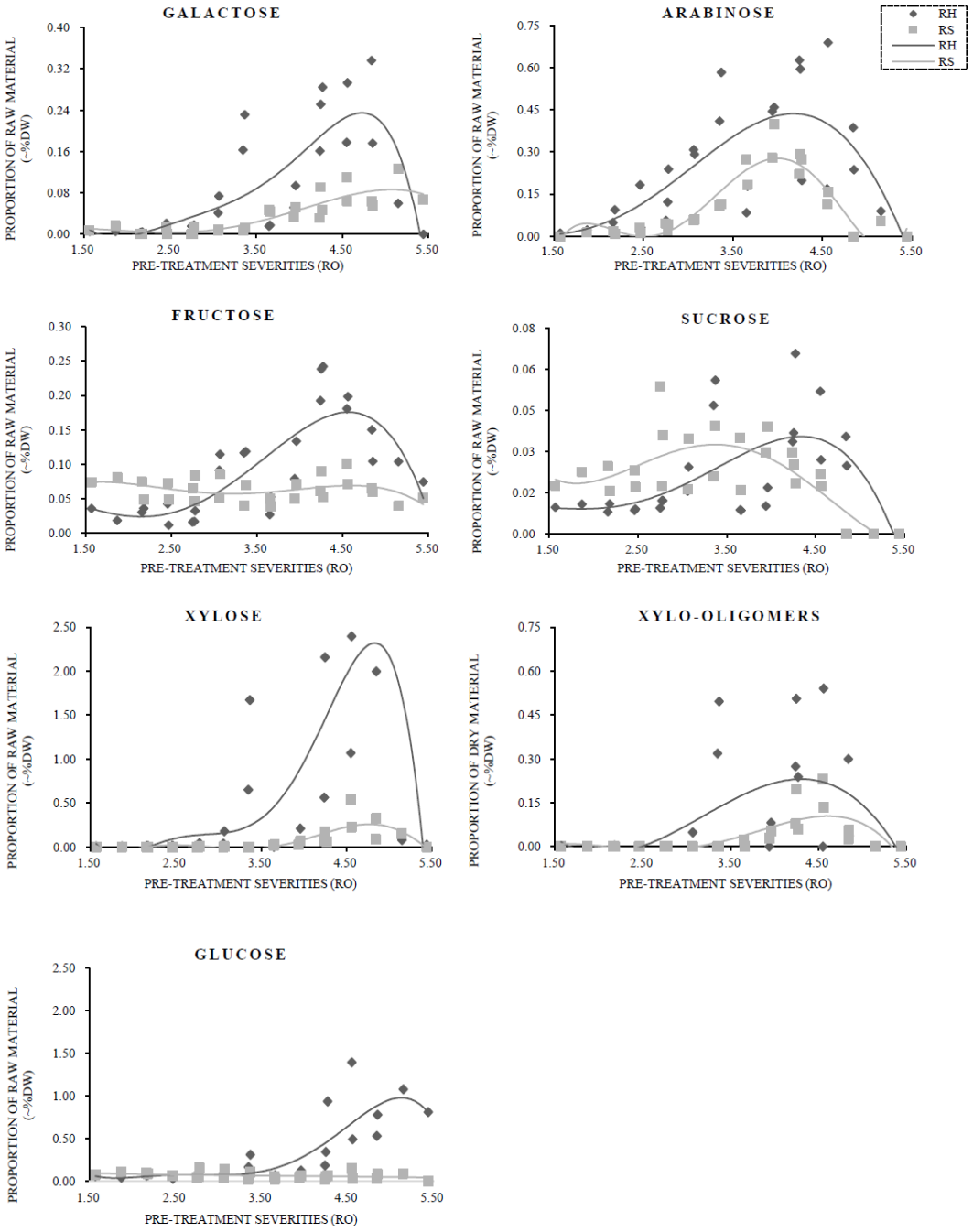


Figure 3.8. The trends of sugars released from pre-treated RH and RS (Wu et al., 2018b). Trends lines are purely as a visual aid to improve clarity of data.

Known inhibitory compounds to both saccharification and fermentation were released/generated at higher severities (shown as Figure 3.9). 5-HMF (hydroxymethylfurfrual), 2-FA (Furfural) and acetic acid were the most abundant compounds generated from pre-treated RH and RS. These highly deleterious inhibitory compounds would significantly impact on fermentation (Palmqvist & Hahn-Hägerdal,

2000a) (Palmqvist & Hahn-Hägerdal, 2000b). Most of the inhibitors increased with increasing severities and this is consistent with the study of Wood et al. (2016a). Higher levels of all the inhibitors were produced from RH samples pre-treated at higher severities compared with RS. This in keeping with results shown in Figure 3.8 which showed that higher levels of sugars were released and degraded from RH pre-treated at high severities. In this study, the levels of 5-HMF, 2-FA and acetic acid were formed at the much higher severities and were very much higher than those reported in the research of Wood et al. (2016a). This was additionally confirmed by HPLC and examples of data are shown in the Appendix 3. This might be explained in two ways: 1) the Maximum pre-treatment severity applied in Wood et al. (2016a) was a severity of 4.8 whilst in this study the pre-treatment went to much higher severity levels. 2) During the steam explosion process, it is very possible that considerable quantities of volatile compounds including 5-HMF, 2-FA and acetic acid were lost into vented steam. This would not occur during hydrothermal pre-treatment. Formic acid, acetol, acetaldehyde and methanol were also produced in significant quantities from the pre-treated samples. Inhibitory compounds such as choline and levulinc acid were also detected but were produced in much lower quantities compared with other inhibitors. Interestingly, there were higher levels of choline and levulinic acid formed in pre-treated RS compared with pre-treated RH.

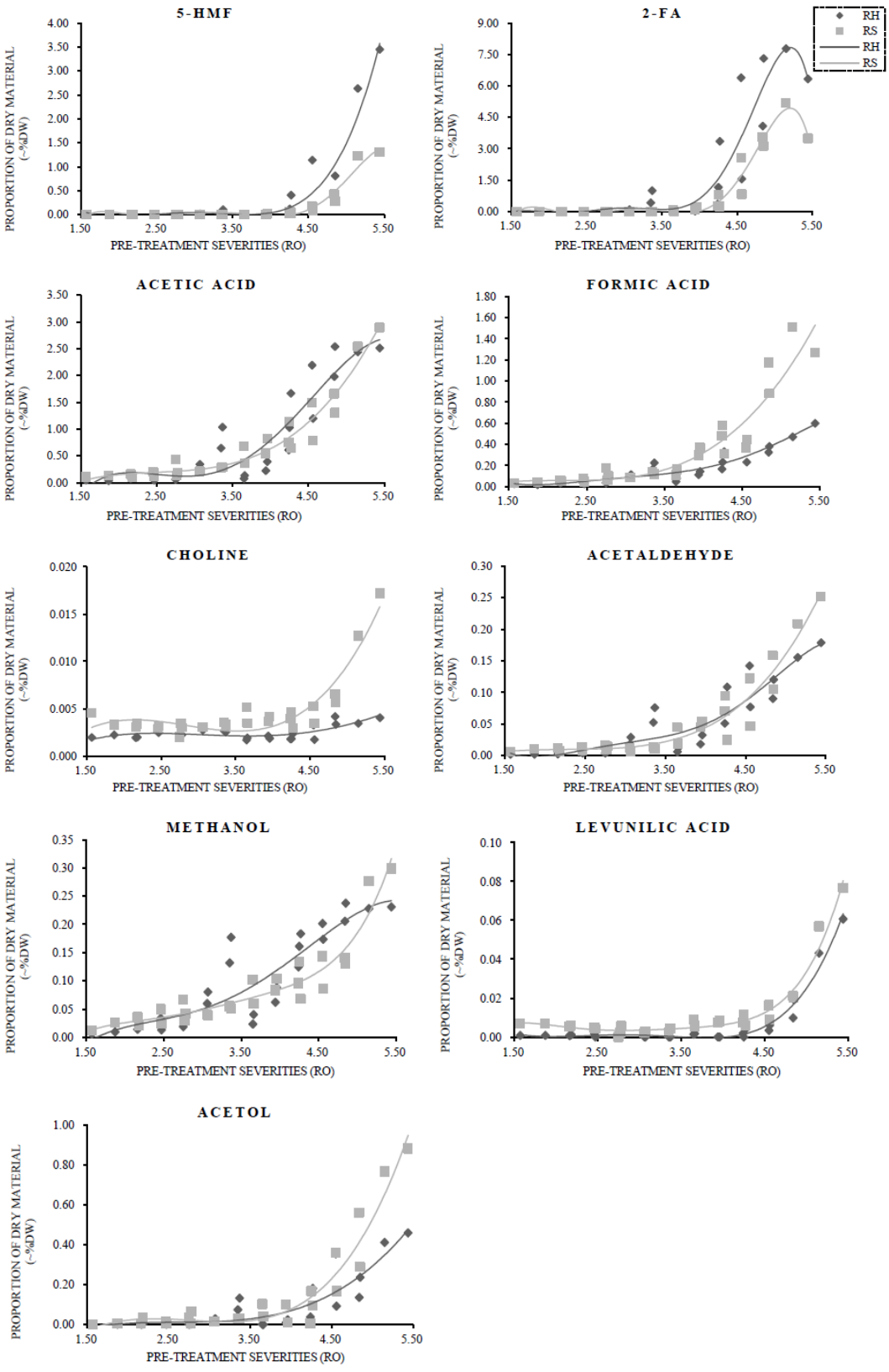


Figure 3.9. The trends of inhibitory compounds produced from pre-treated RH and RS (Wu et al., 2018b). Trends lines are purely as a visual aid to improve clarity of data.

These results indicated that large amounts of commonly known inhibitory compounds were generated during hydrothermal pre-treatment (hot water treatment in this study). An extra washing step of pre-treated biomass could largely remove those inhibitors and minimise their impact on fermentation (Wood et al., 2016a). However, the drawback of this extra washing step is that it would mean removing much of the solubilised sugars (e.g. xylose) which may otherwise be exploited and processed into a range of bio-products by using natural or modified pentoses-fermenting organisms. Interestingly, varying functionality of compounds have been reported. For example, acetaldehyde behaves as promoter enhancing fermentation by reducing the lag phase of yeast growth at a concentration of 0.01 mg/ml (Barber et al., 2002). In contrast, acetaldehyde can inhibit yeast growth while the concentration is higher than 0.1 mg/ml (Stanley et al., 1993).

RS has exhibited the potential to be one of the readily available candidates for bio-conversion as it could be converted into fermentable sugars sufficiently after being pre-treated at mild conditions whilst released ignorable concentration of inhibitory compounds. However, RH could not be effectively converted into fermentable sugars by hydrothermal pre-treatment. Further exploitation of pre-treating RH is therefore required.

3.4 Conclusion

RH and RS are abundant sources of agricultural wasted biomass and important for bio-refining. Compared with RS, RH was much more resistant to hydrothermal pre-treatment due to the higher contents of lignin, silica and inhibitors (involving both saccharification and fermentation inhibitory compounds) produced during pre-treatment. A wide range of chemical compounds were produced from rice biomass during hydrothermal pre-treatment with progressively higher severities and composition of those compounds changed throughout the severity range. Components such as organic acids are extracted from samples at low severities and broken down when pre-treatment severities went up to over 4. It is possible to ascribe the presence of sugars and oligosaccharides in samples pre-treated at mid-range severities to hydrolysis of cell wall polysaccharides. At the highest severities, many of those are lost and degraded into well-established fermentation inhibitors. Those volatile

inhibitory compounds might be reduced by steam explosion rather than additional washing steps. Silica known as a digestion inhibitor has not been investigated in details in this study. As many researchers presented, RH and RS contained relatively high content of silica than other cereal-derived lignocellulosic biomass. In this case, silica may have potential effects on the enzyme activity, fermentation result and chemical compounds in the PT liquors. Therefore, further research of silica is required.

Chapter 4：

Investigation of esterified phenolics in RH and RS and their release during hydrothermal pre-treatment

4.1 Introduction

Before conversion of lignocellulose to ethanol, the step of pre-treatment is essential as it serves to remove lignin and hemicellulose barriers from cellulose and interrupt the chain of crystallised cellulose thus increasing the surface for cellulases to act, which enhances the efficiency of saccharification (Arshadi et al., 2016; Merali et al., 2016). For example, the study of Auxenfans et al. (2017) summarises how the cell wall bonds and cell wall polymer network are disrupted by the hydrothermal, thermochemical and solvation pre-treatment processes. However, many researchers have demonstrated that solubilisation of chemical moieties released/generated from lignocellulosic biomass during pre-treatment can be inhibitory to either enzymatic hydrolysis or downstream organism fermentation (Palmqvist & Hahn-Hagerdal, 2000; Palmqvist & Hahn-Hägerdal, 2000b). Pre-treating lignocellulosic materials at high temperature leads to the formation of sugar derived inhibitors containing furans such as 5-HMF (hydromethylfurfural) and 2-FA (furfural). Moreover, some of phenolic compounds which are inhibitory to cellulases and xylanases also have been released in significant quantities during severe pre-treatments, including lignin derived phenolics such as coumaric acid and hemicellulose derived phenolics such as cinnamic acid esters including ferulic acid (González-Bautista et al., 2017; Hou et al., 2017; Kellock et al., 2017).

Lignin derived phenolics and furan-containing inhibitors have been investigated to a greater extent compared with other phenolic compounds such as relatively smaller contents of cinnamic acid related phenolics which have only been recently exposed as the effective microbial inhibitors. Some microorganisms (e.g. yeast strains and bacteria) have been developed in attempts to overcome the inhibitions caused by such phenolic compounds (Sato et al., 2014; Soares et al., 2016; Zhang et al., 2017). The study of Hou et al. (2017) demonstrated that ferulic acids were released at significant levels that were capable of inhibiting microbial activity after alkali pre-treatment of lignocellulosic biomass. Furthermore, recent hydrothermal pre-treatment studies have exhibited the capability of improving the degradation and solubilisation of arabinoxylans and reducing the levels of cell wall phenolic esters which resulted in the loss of alkaline UV turquoise fluorescence of pre-treated residuals, suggesting that such simple phenolics were presumably released from biomass by the pre-treatment (Merali et al., 2013; Merali et al., 2016). However, the yields of ferulic acids and their

derivatives from hydrothermally pre-treated rice husk and rice straw have not been systematically investigated.

In this Chapter, RH and RS have been pre-treated hydrothermally at several different severities. The effects of hydrothermal pre-treatment on the release of phenolics esters such as ferulic esters, diferulic acids and related phenolic compounds from RH and RS, and on the accumulation of phenolics in the pre-treated liquors have been investigated by using HPLC with Diode Array Detection (DAD) (see Methodology).

4.2 Methodology

4.2.1 Pre-treatment of RH and RS

Freeze milled RH and RS (750 mg air-dried weight of each sample respectively) were loaded into 25 ml microwave pressure tubes containing 14.25 ml distilled water to establish a 5% (w/w) suspension. Those tubes were then capped and placed into a BIOTAGE® Initiator+ reactor and pre-treated respectively at severities 1.57, 3.65, 5.15 and 5.45 (selected from the experimental design of Chapter 3). After cooling the tubes with compressed air to room temperature, pre-treated samples were stored at -20ºC for less than 6 months.

4.3 Results and discussion

4.3.1 FTIR-ATR of raw and pre-treated RH and RS solids

The gross cell wall composition of the dried solids (including raw materials) were evaluated by FTIR-ATR. The results are presented as spectra from wavelength 800 cm^{-1} to 1800 cm^{-1} (Figure 4.1A and 4.1B). Significant changes of spectra between each sample caused by pre-treatments were highlighted with vertical dashed lines containing inserted numbers (wavelength cm^{-1}). As both Figure 4.1A (RH) and Figure 4.1B (RS) illustrate, the intensity of peaks at wavelength around 1740 cm^{-1}, 1630 cm^{-1} and 1235 cm^{-1} are decreased whilst the pre-treatment severities increased. This indicates the hemicellulosic polysaccharides are hydrolysed and released from the residues due to those bands relate to C-O stretching and O-H bending of hemicellulose. The results are consistent with results reported in previous studies on a range of

biomass types such as oilseed rape straw (Ryden et al., 2014), wheat straw (Auxenfans et al., 2017; Collins et al., 2014; Merali et al., 2013), and rice wastes (husk and straw) (Wood et al., 2016b). The loss of hemicellulose is also responsible for the increasing sharpness of peaks corresponding to the C-O/C-H bond stretching in cellulose, and C-O-C stretching of β-(1-4) linkages at wavelengths around 1034 cm^{-1}, 1100 cm^{-1} and 1160 cm^{-1}, indicating the proportion of cellulose was increased in pre-treated solids as pre-treatment severities increased (Schwanninger et al., 2004). Moreover, the ratio of bands at 895 cm^{-1} and 1420-1430 cm^{-1} indicated the residual cellulose was more crystalline in nature (Auxenfans et al., 2017) and this was presumably ascribed to the degrading non-crystalline cellulose (amorphous) during hydrothermal pre-treatment. The range between wavelength 1600 cm^{-1} to 1300 cm^{-1} were related to lignin, particularly the peaks at wavelengths 1420 cm^{-1}, 1505 cm^{-1} and 1600 cm^{-1} corresponding to the C=O and C=C bonds becoming more pronounced (Schwanninger et al., 2004). These results indicated relative increases in the amount of lignin in pre-treated residues which is consistent with the results of following lignin analysis of PT RS and RH (Section 4.3.3, Chapter 4).

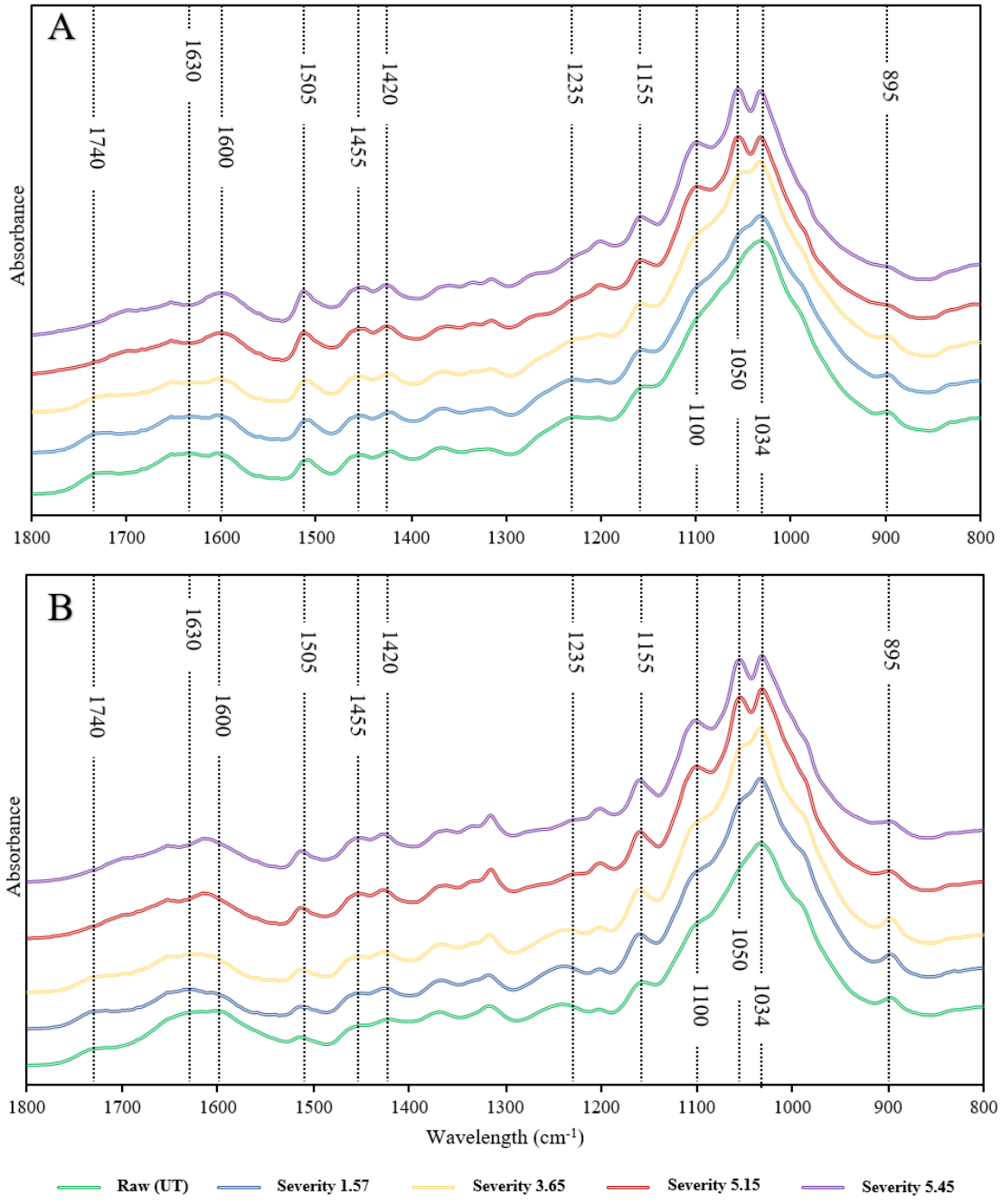


Figure 4.1. FTIR-ATR spectra of raw and pre-treated RH & RS solids (dried). Figure 4.1A is the spectra for RH and Figure 4.1B is for RS.

4.3.2 Fluorescence microscopy of raw and PT RH & RS

The visual appearance of both lignin and phenolic acids in the raw and PT samples (RH and RS) was provided by UV-autofluorescence under neutral conditions (Figure 4.2A). Under such conditions, lignin and cinnamic acid derivatives such as ferulic acid (tFA) fluoresce blue in colour. It is obvious that the lignin and phenolics lose fluorescence whilst increasing pre-treatment severities and this was for both RH and RS (Figure 4.2A (i) and (ii)). In order to distinguish lignin and esterified phenolics respectively, NaOH was added into samples to establish alkali conditions (shown as Figure 4.2B). In this case, lignin was shown in blue colour and tFA was green. As Figure 4.2B shows, raw RH is predominantly blue where the raw RS is mostly green or turquoise indicating the ratio of lignin to tFA in RH is higher than in RS. This is a preliminary suggestion that the contents of lignin and/or phenolics may differ between RH and RS. Also, the decreased levels of fluorescence in both PT RH and PT RS indicated the removal of lignin and phenolic acids (Wu et al., 2018a). However, the result of FTIR (Figure 4.1) and Klason lignin (Table 4.1A) showed the proportion of lignin increased in the residuals of PT samples. This may be presumably explained as some of lignin would not be detected in pre-treated samples due to the structure of lignin was changed during pre-treatment and lost the fluorescent moieties (Holopainen-Mantila et al., 2013; Wu et al., 2018a). Therefore, the lignin detected by UV is not precisely related to Klason lignin and the loss of fluorescence in pre-treated samples is not emblematic of the changes of lignin contents.

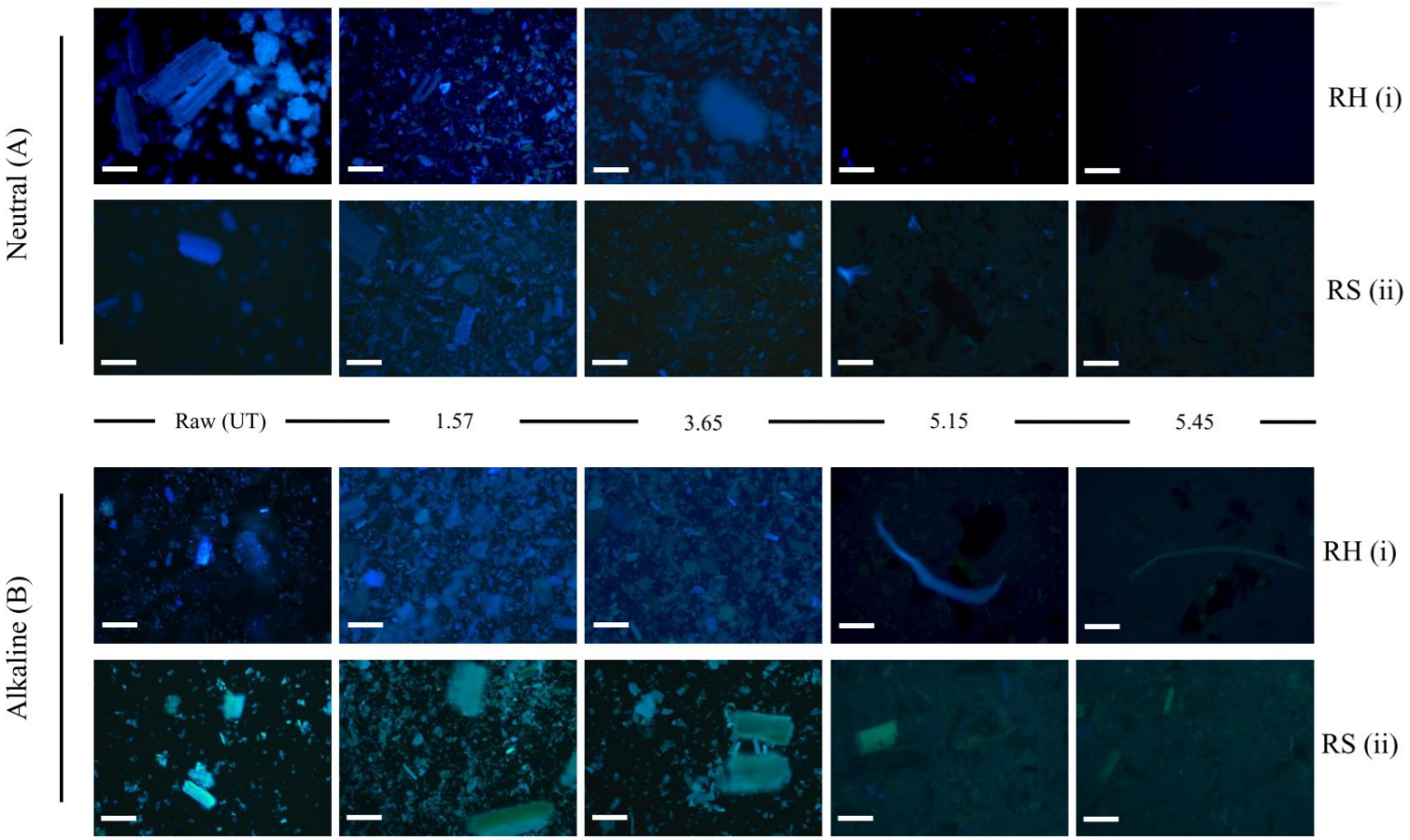


Figure 4.2. UV-Fluorescence micrographs of RH and RS (materials including raw and pre-treated at several different severities) under both neutral (A) and alkaline (B) conditions. For the Figure 4.2A, blue colour reflects of either lignin or ferulates (mainly ferulic acids). For the Figure 4.2B, blue colour reflects lignin and turquoise/green reflects ferulates. The scale bar is 100 μm.

4.3.3 Klason lignin in raw and pre-treated RH & RS

Quantification of Klason lignin in both raw and pre-treated samples had been carried out for both RH and RS. As Table 4.1 shows, significantly higher levels of lignin were found in the raw (UT) RH compared with raw RS sample, and this extended the results of the Fluorescence Microscopy (Figure 4.2) that lignin contents might differ between RH and RS (Figure 4.2B). Table 4.1A shows the significant increases the lignin proportion in both RH and RS pre-treated residues at progressively increased severities when the proportion of lignin were calculated on the basis of the weight of residuals after pre-treatment (w/w). Table 4.1B shows the levels of lignin in both RH and RS were negligibly changed in pre-treated samples after the proportion of lignin were calculated on the basis of the weight of raw materials (w/w) (biomass recover rate is shown as Appendix 4, Figure ATC4.1). This can be assumed to be due to the loss of water soluble and volatile compounds released during hydrothermal pre-treatment such as hemicellulosic polysaccharides and furfural as found in many other pre-treatment studies (Jönsson et al., 2013; Kristensen et al., 2008; Wu et al., 2018a). the negligible changes of lignin contents suggest the lignin was mainly separated from cellulose and relocated in solution instead of being chemically degraded.

Table 4.1. Contents of Klason lignin in raw and PT RH & RS. For Table 4.1A, data of raw materials was calculated on the basis of raw materials and data of PT residuals was calculated on the basis of the weight of PT residuals actually loaded. For Table 4.1B, data had been presented as mg/g raw materials according to the biomass recover rate (Appendix 4, Figure ATC 4.1). symbol "±" is adapted in the table to present SD.

Table 4.1A:

	Lignin content (mg/g)	
Severity	**Rice husk**	**Rice straw**
Raw	35.25 ± 1.23	22.01 ± 1.37
1.57	36.18 ± 1.83	24.08 ± 0.82
3.65	38.89 ± 1.90	26.48 ± 2.38
5.15	45.57 ± 1.46	34.86 ± 2.92
5.45	46.22 ± 0.85	36.73 ± 2.35

Table 4.1B:

	Lignin content (mg/g)	
Severity	**Rice husk**	**Rice straw**
Raw	35.25 ± 1.23	22.10 ± 1.37
1.57	34.89 ± 1.77	21.95 ± 0.76
3.65	31.44 ± 1.59	22.17 ± 2.05
5.15	34.36 ± 1.17	24.89 ± 2.63
5.45	32.80 ± 0.67	23.61 ± 0.69

4.3.4 Assessment of phenolics in the liquors of PT RH and RS

Liquors derived from PT substrates (severity 5.15) were analysed by HPLC to assess and quantify tFA, diferulates (DiFA) and other related phenolic compounds potentially released during pre-treatment. This included direct analysis of the pre-treatment liquors by filtration and injection (Figure 4.3A), liquid-liquid extraction of the otherwise unmodified pre-treatment liquors (Figure 4.3B) and both of saponification and liquid-liquid extraction of the pre-treatment liquors (Figure 4.3C). As Figure 4.3A and B show, only pCald (protocatechuic aldehyde), p-OH-Bzald (p-OH-benzaldehyde) and vanillin were identified (on the basis of their retention times and diode-array recorded spectra) from the liquors prepared via the direct method (method A) and the liquid-liquid extraction method (method B). A very significant UV-absorbing peak C was found in the liquor prepared by method A only, compared with method B and C. One may speculate that the unknown peak C consisted of mainly carbohydrates with esterified phenolics that could not be extracted by ethyl acetate. Liquors extracted by method C involved saponification followed by acidification and liquid-liquid extraction, then analysis by HPLC. As Figure 4.3 C shows, the level of early-running unidentified moieties was reduced by saponification leading to a further range of phenolics identified in addition to three phenolics, such as p-OH-B (p-OH-benzoic acid), VA (vanillic acid), pCA (p-coumaric acid), tFA and 8-0-4'-DiFA. The results show, therefore, that no free phenolic acids such as tFA, DiFA were released during hydrothermal pre-treatment, indicating most of phenolics presented in the pre-treated liquors were possibly esterified to fragments of plant cell wall derived polysaccharides which had been released from pre-treated substrates.

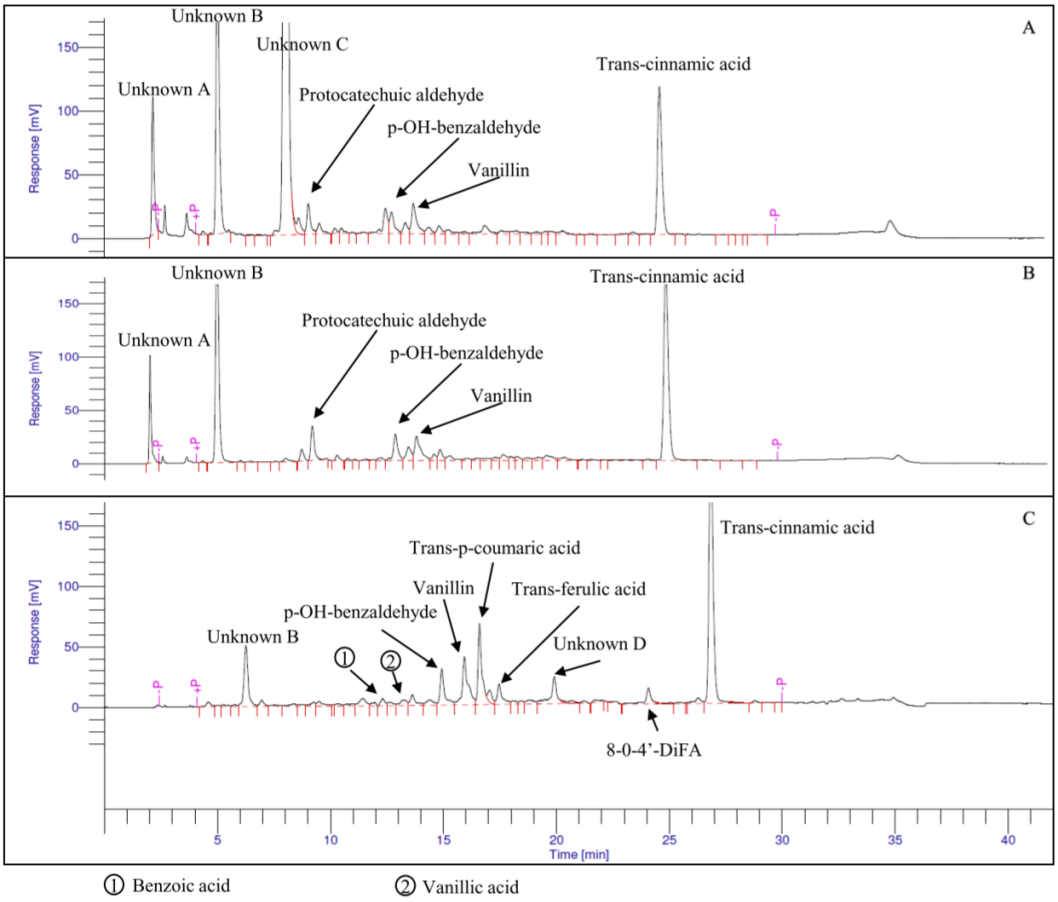


Figure 4.3. HPLC chromatogram of phenolic compounds in RS liquor produced at a severity of 5.15. Figure 4.3 (A): direct injection of liquor showing no identifiable phenolics; Figure 4.3 (B): HPLC of Liquid-liquid extracted moieties from liquor; Figure 4.3 (C): HPLC of moieties recovered by liquid-liquid extraction after saponification (showing identified phenolics).

4.3.5 Total phenolics analysis of raw and PT RH & RS

The saponification method was also used to extract and investigate the levels of phenolics esterified to the recalcitrant residues. Preliminary studies showed that for raw RH and RS residues, higher levels of phenolic compounds were extracted after saponification with 4 mol/l NaOH for 17 hours compared with 1 mol/l NaOH (Table 4.2). However, 1 mol/l NaOH was as effective as 4 mol/l NaOH for extracting phenolics from pre-treated samples so that unnecessary alkaline-degradation was prevented by using 1 mol/l NaOH for following saponification rather than 4 mol/l NaOH.

Table 4.2. Total phenolic compounds extracted from raw and pre-treated solids (RH and RS, severity 1.57). Saponification was carried out with either 1 mol/l NaOH and 4 mol/l NaOH respectively to both raw and pre-treated solids. Results were presented as mg/g of raw materials.

	Total Phenolics (mg/g of raw materials)			
	Rice Husk		**Rice Straw**	
Severity	**1 mol/l NaOH**	**4 mol/l NaOH**	**1 mol/l NaOH**	**4 mol/l NaOH**
Raw	14.57 ±0.57	15.82 ±1.41	14.37 ±0.45	17.20 ±1.57
1.57	15.90 ±0.44	15.96 ±1.21	16.23 ±0.78	14.24 ±0.83

4.3.6 Quantification of phenolics in the solids and liquors of raw and PT RH & RS

Table 4.3. Total phenolics in the solids and liquors of either raw and PT RH & RS. Results were presented as mg/g of raw materials.

	Phenolic compounds (mg/g raw materials)					
	RH			**RS**		
Severity	**Solids**	**Liquors**	**Total**	**Solids**	**Liquors**	**Total**
UT (4 mol/l)	15.82 (±1.27)	N/A	15.82	17.20 (±1.57)	N/A	17.20
1.57	15.90 (±0.44)	2.59 (±0.60)	18.43	16.23 (±0.78)	1.56 (±0.20)	17.93
3.65	13.13 (±0.28)	5.14 (±0.15)	18.28	13.73 (±0.65)	0.91 (±0.05)	14.93
5.15	6.50 (±0.50)	4.02 (±0.11)	10.52	3.37 (±0.21)	2.46 (±0.44)	5.83
5.45	5.07 (±0.43)	2.94 (±0.24)	8.01	2.27 (±0.20)	2.22 (±0.09)	4.49

Slightly higher levels of total phenolics were extracted from raw RS samples (17.20 mg/g) compared with raw RH samples (15.82 mg/g). However, the contents of phenolic compounds remaining in RH was more than in RS after pre-treatment, and the levels of total phenolics decreased in both PT RH and RH as pre-treatment severities increased. This can be attributed to the degradation of some phenolics during severer pre-treatments. For example, the study of Kucner et al. (2014) presented a 23.1% degradation of total polyphenols by treating blueberry fruits at 115 ^{o}C for 20 seconds. In this study, the decrease of total phenolics after pre-treatment mainly due to the degradation of p-coumaric acid and ferulic acid (Figure 4.4 and 4.5). Comparing the contents of total phenolics in raw and PT (1.57) samples, an initial increase was observed. This might due to enhancement of saponified by pre-treatment, enabling the release of phenolics that would otherwise be unextracted by either 1 mol/l or 4 mol/l NaOH.

As Figure 4.4-4.6 shows, 15 different phenolic compounds (12 phenolic acids, 2 aldehydes and vanillin) were identified and quantified in PT liquors and residues of RH and RS. In Figure 4.4, tFA, cFA (cis-ferulic acid) and diferulics were found in RH and RS. The level of tFA was considerately higher in untreated RS compared with untreated RH. However, less than 15% (w/w) of tFA remained in solids of RH and RS pre-treated at severities 5.15 and 5.45 whilst relatively low quantities of tFA were detected in the liquors. cFA was detected in much smaller quantities compared with tFA in all samples and showed a decrease in solids but an increase in liquors with increasing pre-treatment severity. In both RH and RS samples, 8,5'-DiFA, 8-O-4'-DiFA and 5,5'-DiFA were detected for the first time. 8-O-4'-DiFA was detected in the highest levels among those 3 diferulic acid moieties, followed by 5,5'-DiFA then 8,5'-DiFA. Higher levels of those diferulic acid moieties were found in the RS samples than in RH especially for those solids of samples. Like the cFA, quantities of those diferulic acids decreased in solids but increased in liquors as severity of pre-treatment increased. This indicated that cFA and diferulic acids moieties were more resisting to hydrothermal pre-treatment.

As Figure 4.5 and Figure 4.6 show, larger quantities of 10 different phenolic compounds were detected in RH rather than RS samples for both solids and liquors, except for truxillic acid (FA-derived). Significantly higher levels of para-coumaric acids (pCA; Figure 4.5) compared with tFA were present in RH and RS, and the

hydrothermal pre-treatment also effectively decreased the levels of pCA in the pre-treated residual biomass and liquors. P-OH-phenyl acetic acid (p-OH-PAA) showed a similar trend to cFA, a decrease was detected in pre-treated residues but an increase in pre-treated liquors. In contrast, the quantities of vanillic acid (VA) was increased in PT solids but decreased in PT liquors. The levels of p-OH-benzoic acid (p-OH-B) decreased in both PT solids and liquors. The other phenolic compounds comprised truxillates (FA and CA derived) (Figure 4.5), p-OH-benzaldehyde (p-OH-Bzald), protocatechuic aldehyde (PA) and vanillin. The levels of those phenolic compounds were interesting increased in both PT solids and liquors of RH and RS, and this might due to degradation of other lignin derived wall phenolics during hydrothermal pre-treatment hence they were detected in higher levels in RH samples compared to RS samples.

Interestingly, those phenolic compounds show different trends during pre-treatment and this mainly attributes to different thermal stabilities of phenolics. For instance, Cheng et al. (2014) treated ferulic acid with high temperature water (200 ^{o}C, 60 minutes) which lead to 60% ferulic acid wad degraded, and a completely decomposition occurred after increasing temperature to 250 ^{o}C. In the study of Volf et al. (2014), vanillic acid had been firstly dissolved in distilled water, then being treated at 80 ^{o}C by using water bath and observed a 25% degradation of vanilic acid. Phenolics such as diferulics, aldehydes and vanillin have been reported that exhibited much higher degree of thermal stability (Amen-Chen et al., 2001; Cheng et al., 2014; Parker et al., 2003b; Wu et al., 2018a). Therefore, increases of those phenolics might due to severer pre-treatment improved the release of them from lignin without being further degraded. Moreover, Rasmussen et al. (2017) suggested that hydrothermal pre-treatments can create numbers of oligophenolic compounds from wheat straw. This may be one of the reasons that causes increases of some phenolics from pre-treated rice husk and straw.

A range of lignocellulosic plant wall derived compounds are inhibitory to either enzyme saccharification and yeast fermentation such as lignin, phenolic compounds and saccharide breakdown products (Cho et al., 2009; Taherzadeh & Karimi, 2008; Zeng et al., 2014). Many other researchers have focused on the free phenolic acids released after alkali pre-treatments but the role of phenolic esters such as tFA and pCA was only recently put onto focus, and the levels of free tFA and pCA at approximately

2.5 mmol/l could inhibit the growth of *E.coli* (Hou et al., 2017). Moreover, pCA had been reported that could be inhibitory to microbial digesting activities of carbohydrates and implicated as a toxic barrier to digest materials during simulated rumen fermentation (Taboada et al., 2010; Theodorou et al., 1987). In this study, the results in Figure 4.3 illustrated that no free tFA and pCA was present in PT liquors but the esterified pCA and tFA could reach 1 mmol/l and 0.13 mmol/l in the liquors of PT RH respectively. If these esterified phenolics were freed, they would be significantly inhibitory to microbial fermenting organisms (Hou et al., 2017). However, there has not yet been any research to assess the inhibitory characteristics of such phenolic esters, and further studies are therefore required.

Simple phenolics remained esterified at significant levels in residues pre-treated at lower severities as cross-linking cell wall polymers (Figure 4.2). Such polymers might not directly inhibit the microbial fermenting activities but might additionally inhibit alcohol production by cross-linking polysaccharides with lignin which can potentially attenuate hemicellulose disassembly and solubility such as the diferulates established interpolymeric cross-links between arabinoxylan hemicelluloses (Bunzel et al., 2004; Ralph et al., 1995). Moreover, the rate and extent of cell separation in residues during pre-treatment might be affected by some diferulates which have been implicated as responsible for cell adhesion (Merali et al., 2013; Parker et al., 2003a; Parker & Waldron, 1995; Waldron et al., 1997). This may potentially prevent the increase of surface-area created by pre-treatment-induced disruption of cereal residues.

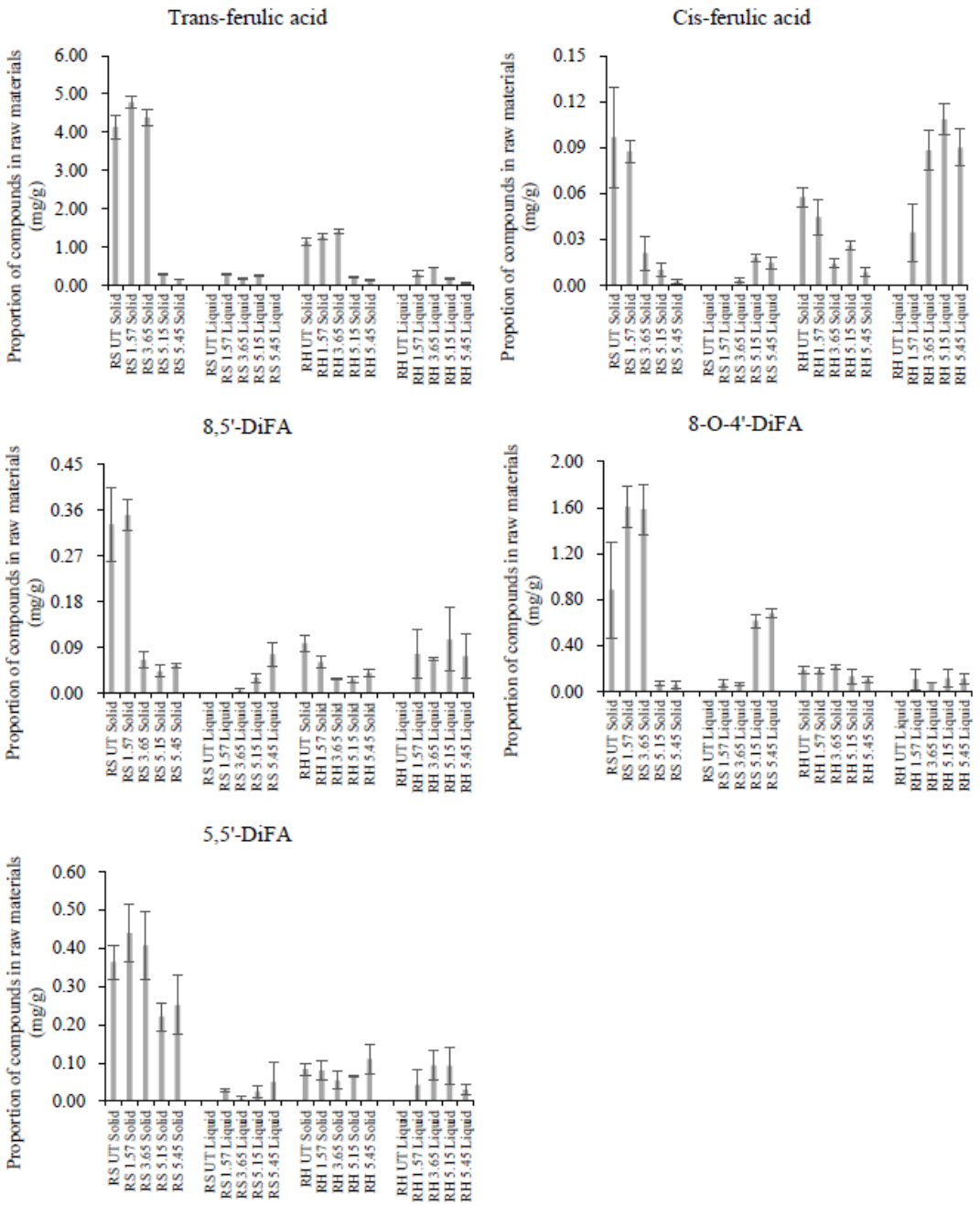


Figure 4.4. Quantification of ferulic acids in solids and liquors of RH and RS samples (raw and PT). Results had been calculated in basis of raw materials. N=3.

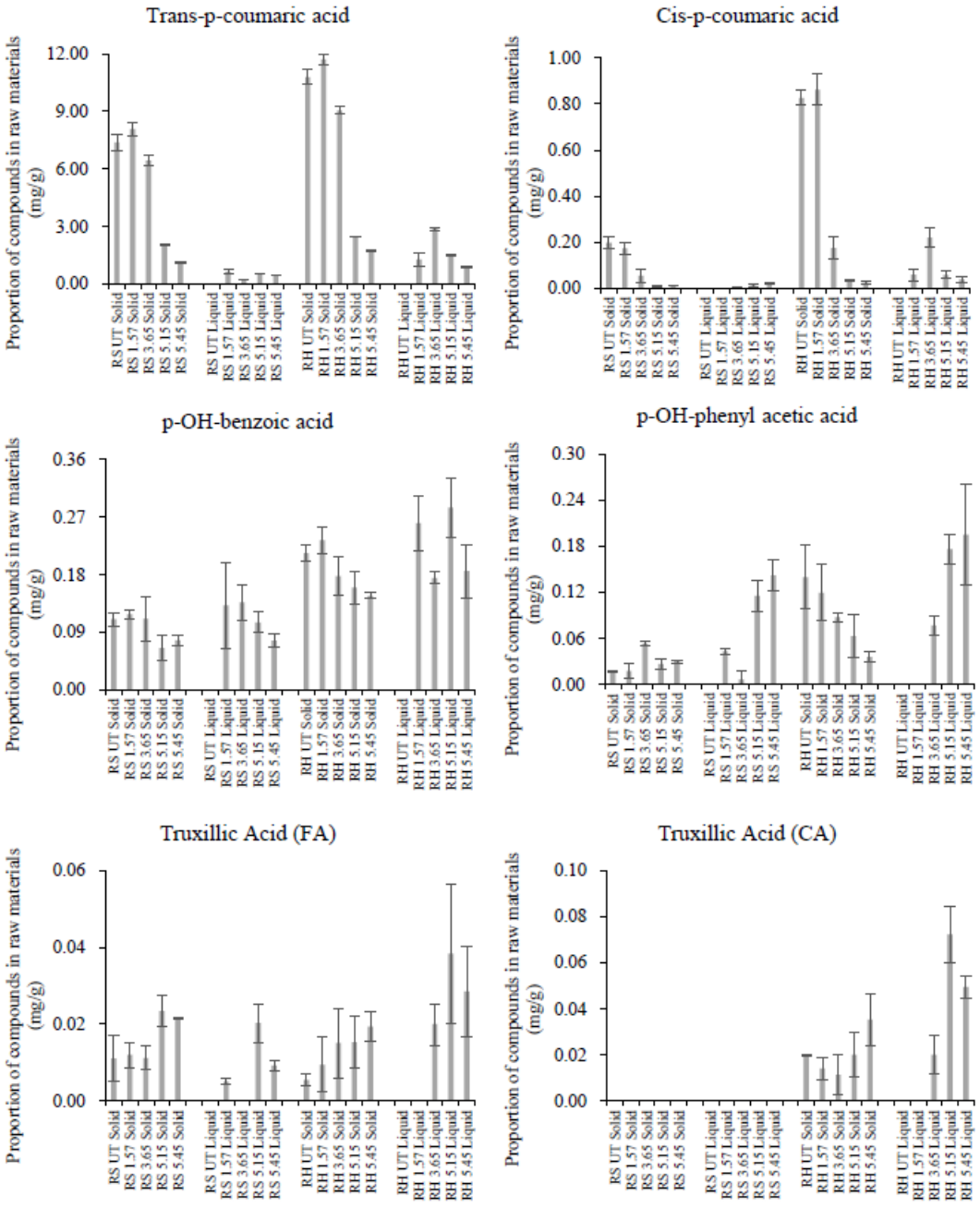


Figure 4.5. Quantification of phenolic compounds in solids and liquors of RH and RS samples (raw and PT). Results had been calculated in basis of raw materials. N=3.

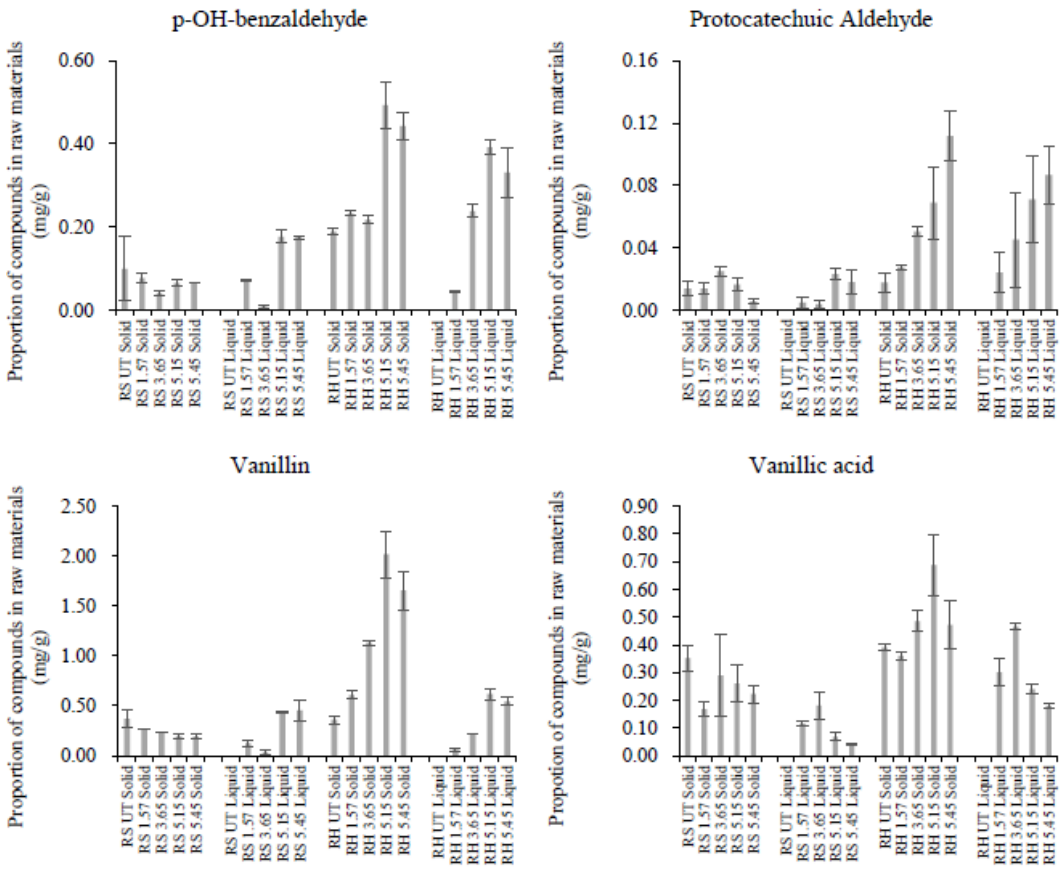


Figure 4.6. Quantification of phenolic compounds in solids and liquors of RH and RS samples (raw and PT). Results had been calculated in basis of raw materials. N=3.

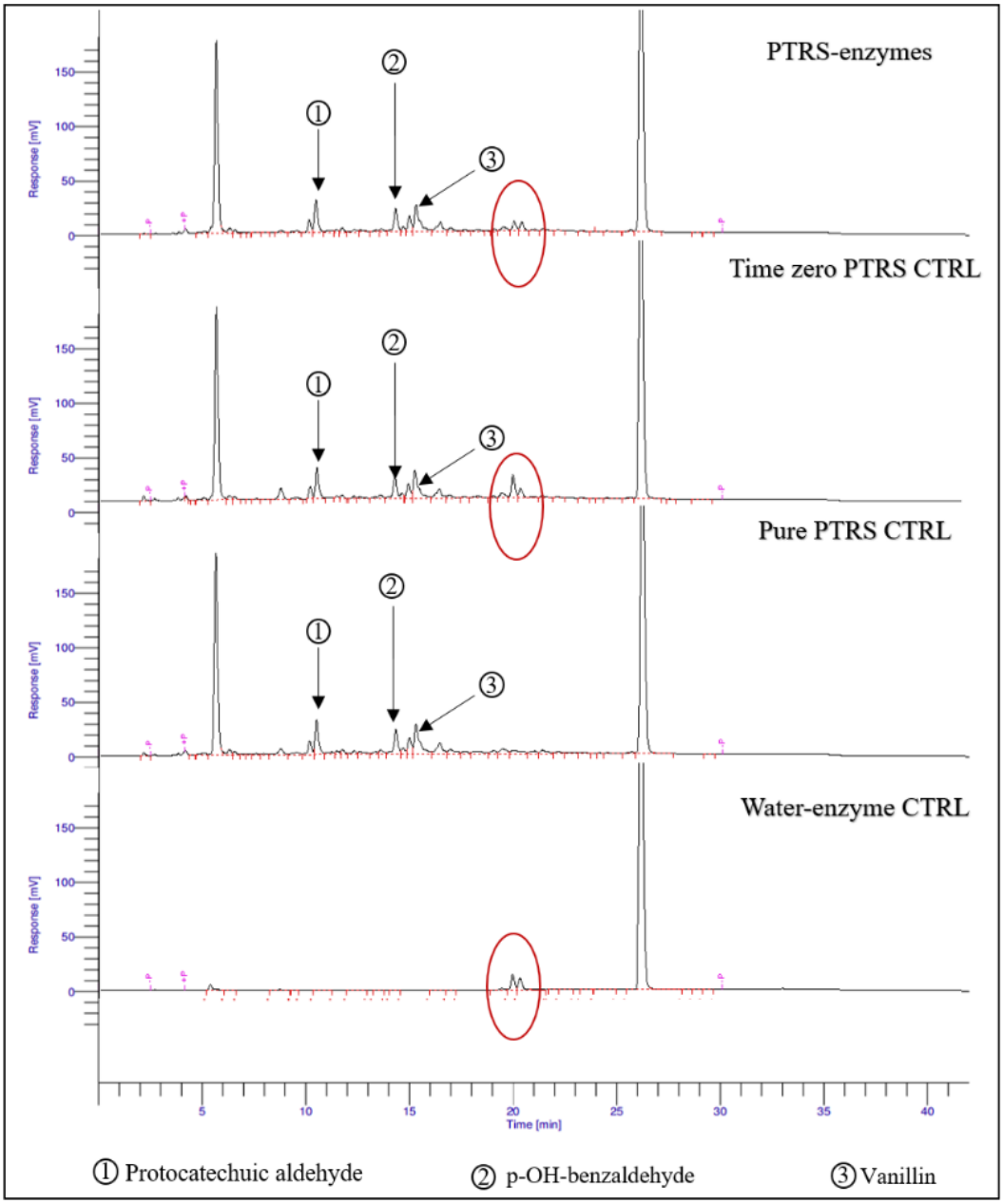


Figure 4.7. HPLC chromatogram of phenolic compounds in RS liquor (severity 5.15) after 24 hours' incubation with CTec-2 and HTec-2. Top figure shows the actual sample and the others are all different controls (see Methodology).

In the study of Lesage-Meessen et al. (2002), free ferulic acid was successfully released from autoclaved (thermodynamic treated) maize bran by using the filamentous fungi *Aspergillus niger* which could produce enzymes degrading polysaccharides and feruloyl esterases cleaving the ester linkages between hydroxycinnamic acids and carbohydrates. Therefore, the pre-treatment solubilised

phenolic esters might be hydrolysed during saccharification by esterases in enzyme cocktails. Enzymatic hydrolysis was carried out by adding CTec-2 and HTec-2 into pre-treated (severity 5.15) liquors of RS. Different controls were made to minimise any background signals from enzyme cocktails (details see Methodology). As Figure 4.7 shows, after 24 hours' incubation with CTec-2 and HTec-2, no significant quantities of extra free phenolic compounds were released in addition to PA, p-OH-Bzald and vanillin. This is consistent to the results shown in Figure 4.2, indicating most of phenolic compounds remained as esterified polymers after either hydrothermal pre-treatment or enzymatic hydrolysis. This might be assumed as the CTec-2 has no activities of esterases (Morrison et al., 2016; Watanabe et al., 2015) and there is lack of evidence that esterases contained in the HTec-2 could help releases of free phenolics from esterified forms. Even though, those free PA, p-OH-Bzald and vanillin may also play significant role of influencing total ethanol production from lignocellulosic biomass. For example, vanillin had been confirmed that can cause inhibitory effects on cellulase activities (Kim et al., 2011; Ximenes et al., 2011; Ximenes et al., 2010), and phenolic aldehydes can supress the growth of microbes (Figueiredo et al., 2008).

4.4 Conclusion

Hydrothermal pre-treatment effectively reduced the quantities of residual hemicelluloses in RH and RS and led to the loss of UV-fluorescence of both lignin and ferulic acids. The contents of lignin were then quantified and showed no significant decreases in RH and RS after pre-treatment, indicating that lignin remained in the lignocellulose, even though it was no longer inhibiting saccharification of the cellulose. Significant decreases of total phenolic compounds were detected in the PT solids. Simple phenolics such as tFA, diferulates and pCA were present in RH and RS. However, except for PA, p-OH-Bzald and vanillin, most of phenolics released into liquors were esterified rather than free phenolic compounds. These were not released to monomeric phenolics by additions of CTec-2 and HTec-2. Phenolics remained as cross-linking polymers in the solids after pre-treatment, and hydrothermal pre-treatment removed large quantities of tFA and pCA. According to the results above, lignin, tFA, DiFAs and pCA differed significantly between RH and RS, indicating the different physiology between the plant cell walls of RH and RS. This presumably

resulted in the higher recalcitrance of RH to pre-treatment. Although the severe pre-treatments could significantly reduce the total levels of phenolics (presumably by degradation), they also led to the increase in soluble levels of some phenolic such as DiFAs and vanillin. However, there is currently lack of information on the possible inhibitory nature of such soluble esterified phenolics to fermenting microbes. Therefore, further research is needed to clarify their effects.

Chapter 5:

Growth and metabolite profiling of genetically diverse yeast strains

5.1 Introduction

Bio-refining provides many potential advantages as a possible alternative technology to petroleum refining such as improving the quality of soil, water and air (Muller et al., 2007). Bio-refining technologies are ideally needed to convert different feedstocks consisting of organic biomass from plant residues, industrial wastes, municipal waste and agricultural waste to liquid fuels and/or platform chemicals (Ward et al., 2008). A range of platform chemicals that might be derived from biomass through microorganic fermentation, were listed by The US Department of Energy (DOE). Many of them are presently key renewable chemicals such as arabinitol, ethanol, succinic acid, lactic acid and levulinic acid (Bozell & Petersen, 2010; FitzPatrick et al., 2010).

As one kind of facultative anaerobes, yeast strains exhibit the capability of growing aerobically and anaerobically. Under the aerobic condition, yeasts tend to make ATP (adenosine triphosphate) by aerobic respiration which lead to a fast growth of cells and less metabolisms (e.g. ethanol and carbon dioxide). In contrast, without oxygen (anaerobic) yeasts obtain ATP by degrading organic substrates (e.g. carbohydrates) and efficiently convert substrates into metabolisms such as ethanol, but the replication of cells is not efficient comparing with the presence of oxygen. Therefore, yeasts are widely used in the food industry for the production of daily food products by fermentation such as the production of bread and wine (Kurtzman & Fell, 2006; Legras et al., 2007; Octave & Thomas, 2009). A range of metabolites can be created from sugars by yeasts, such a feature determines the category of products derivable from biomass. A very common species of yeast - Saccharomyces *cerevisiae* - can simply grow and ferment sugars such as glucose and sucrose and it has been generally considered as the preferred yeast for producing ethanol. Nevertheless, many of highly-sought-after platform chemicals have been reported as being produced by a range of non-*S. cerevisiae* yeast species (Lin & Tanaka, 2006). This indicates that fermentation of diverse yeast strains might have the potential to produce various valuable bio-products from different substrates.

In this Chapter, a number of genetically highly diverse yeast strains were investigated and compared to inform future yeast screening studies, and to explore the untapped potential of yeasts by comparing their natural abilities to ferment a range of carbon sources and to produce metabolites that could potentially benefit the renewable

chemicals industry. Furthermore, the ability of those selected yeasts to convert glucose from pre-treated rice straw under complex bio-refining conditions have been assessed to elucidate the potential of such yeast strains in industrial bio-refining.

5.2 Methodology

5.2.1 Diverse yeast strains and carbohydrates

Eleven specially selected genetically diverse yeast strains were supplied by the National collection of Yeast Cultures (NCYC) (see Chapter 2). All the yeasts were transferred from glycerol stocks into agar plates and then pre-grown in yeast nitrogen base (YNB) containing 1% glucose at 25^{o}C for 72 hours, then stored at 4^{o}C for less than 2 weeks. The diverse range of carbohydrates used in this Chapter consisted of 13 laboratory-purified sugars. These were individually prepared as 10 mg/ml concentrations with addition of YNB. Solutions of sugars were then autoclaved for sterilisation (Appendix 5, Table ATC5.1 and Table ATC5.2).

5.2.2 Fermentation of NCYC 16 on glucose with different oxygen availabilities

Fermentation (3 sets of conditions) was carried out with the same method explained as above. Set A was carried out with 1 ml matrix tubes (filled up) capped with screw caps so as to create a highly anaerobic condition. Set B was exactly following the same steps described above by using 96 deep well plates (2 ml well volumes) sealed with clear polypropylene PCR seals to establish a semi-anaerobic condition with limited air (liquid: air = 1: 1, v/v). For the Set C, 96 well plates were sealed with breathable seals to give unlimited oxygen and air. Data was calculated as mg/ml of fermented supernatants. Each sample had been prepared as nine replicates.

5.2.3 Analysis of metabolites using ^{1}H NMR

Sample preparation see in Chapter 2. Concentrations of metabolites were calculated as mg/ml of fermented liquors and the calculated results were then processed by using R (https://www.r-project.org/) and presented as Heat-maps (package “pheat-map”) with

colour coding from white (compounds yield from 0 mg/ml to 0.002 mg/ml which could not be confidently distinguished from baseline noise) through orange and red to Navy (5 mg/ml). To visualize some compounds produced in trace quantities, the colour scale was enhanced by setting BAIS to 5.65 and length to 17000 (2000 for 0 mg/ml to 1 mg/ml; 15000 for 1 mg/ml to 5 mg/ml). Therefore, yellow and light orange represented compounds that were produced in trace quantities (Figure 5.3). Each sample had been prepared in triplicates.

5.2.4 Pre-treatment of freeze milled RS

Freeze milled rice straw samples were transferred into microwave tubes, (750 mg for each tube) distilled water (14.25 ml) was then added into each tube to give a 5% (w/w) suspension. The tubes were then capped and pre-treated at severities 1.57, 3.65, 5.15 and 5.45 (selected from experiment design of Chapter 3) by using a BIOTAGE® Initiator+ reactor. Pre-treated samples were then cooled with compressed air to room temperature and stored at -20^{o}C for less than 6 months.

5.2.5 Simultaneous saccharification and fermentation (SSF) of 11 selected yeasts on RS

Pre-treated samples were defrosted and transferred (937.5 µl) by pipetting into 1 ml Matrix tubes respectively whilst mixing with small magnetic stirrer bars. SSF was carried out after the additions of 12.5 µl (144 FPU) of Cellic® CTec-2 and 50 µl of pre-grown yeast strains. The tubes were capped with screw caps and set into Matrix plates and then placed on a shaker (135 rpm) in a 25^{o}C incubation room for 72 hours. Fermentation was then terminated by heating at 100^{o}C in water bath for 10 minutes. After plates were cooled on ice and centrifuged (3000 rpm for 10 minutes), 400 µl of supernatants of each sample were filtered using 0.2 µm filter plates and centrifugation, then transferred in to 96 wells plates for HPLC analysis. Experiment had been repeated in full for three times.

SSF of pre-treated and washed rice straw was processed in the same way. The washing process included removal of supernatant by decanting followed by resuspension of the pellet in distilled water to volume up to 15 ml and then centrifuged to sediment the

residue. This was repeated 3 times. Ethanol standards were made for quantifying ethanol produced via fermentation (Appendix 5, Figure ATC5.6). The maximum theoretical yield of ethanol was calculated by the following equation:

$$\mathbf{C_6H_{12}O_6\ (glucose) \rightarrow 2C_2H_5OH\ (ethanol) + 2CO_2.}$$

The initial substrate loading was 5% w/w (50 mg/ml). 50 mg/ml raw rice straw containing 38.66% glucose (results from Chapter 3) would give a maximum concentration of glucose of 19.33 mg/ml. Therefore, the maximum yield of ethanol that could be produced theoretically, should be 9.67 mg/ml.

5.3 Results and discussion

5.3.1 Growth of diverse yeasts on different carbon sources

Aerobic growth at 25 ^{o}C of the 11 diverse yeast strains on 13 different lab-purified sugars (including pentoses (C5), hexoses (C6) and disaccharides (DIS) derived from plant tissues and microbial fermentation products) was monitored for 72 hours by recording the turbidity every 30 minutes and presented in Table 5.1 as three calculated parameters – the lag phase (LP), doubling time (DT) and efficiency (ΔOD) (see Methodology). The Colour coding of red-amber-green was introduced in Figure 5.1 to represent the extensive variation in LP, DT and ΔOD. The darker green represents the shorter times taken for LP and DT, and the more significant changes of turbidity for ΔOD indicating stronger growth of yeasts. The red colour indicates longer times taken for LP and DT, and weaker growth for ΔOD.

The variation in colours in the Table 5.1 (Wu et al., 2017) reflects the different LP, DT and ΔODs between strains. Among all 11 strains, there were only two strains - the NCYC 2577 and NCYC 10 could grow significantly on all 13 sugars. NCYC 49, NCYC 4 and NCYC 16 presented shorter DT or LP indicating faster growth, but on fewer sugars. Fucose was not an ideal carbon source for strains NCYC 2791, NCYC 65 and NCYC 2826. Additionally, both NCYC 2791 and NCYC 65 failed to grow on either rhamnose or maltose. NCYC 65 and NCYC 2826 could not effectively grow on

cellobiose, xylose, lactose, and respectively on lactose and ribose. Similarly, NCYC 568 and NCYC 31 could efficiently grow on glucose, fructose and mannose but insignificantly on sucrose. A short LP and DT of NCYC 2433 was detected on maltose, galactose and mannose but the growth (ΔOD) was jointly insignificant. According to the result shown above, both sugar utilization and growth were significantly varied between these genetic diverse yeast strains

Species and NCYC Numbers

Parameter	Carbon source	*Kluyveromyces marxianus* NCYC2791		*Rhodotorula mucilaginosa* NCYC65		*Hanseniaspora osmophila* NCYC31		*Debaryomyces hansenii* NCYC10		*Zygosaccharomyces rouxii* NCYC568		*Wickerhamomyces anomalus* NCYC16		*Lachancea thermotolerans* NCYC2433		*Candida tropicalis* NCYC4		*Kazachstania servazzii* NCYC2577		*Galactomyces candidus* NCYC49		*Saccharomyces cerevisiae* NCYC2826	
[a]LP (h)	Ribose	17.63	±1.39	13.84	±2.34	72.00	±0.00	17.97	±4.07	72.00	±0.00	11.90	±3.91	72.00	±0.00	6.42	±2.43	12.31	±1.12	20.50	±4.58	72.00	±0.00
	Cellobiose	7.22	±2.26	72.00	±0.00	7.55	±2.10	15.44	±3.99	72.00	±0.00	17.37	±1.42	72.00	±0.00	4.92	±0.17	18.69	±0.59	22.67	±6.82	72.00	±0.00
	Glucose	5.60	±1.91	8.98	±4.26	6.99	±2.06	10.36	±2.86	16.10	±3.93	9.72	±3.85	7.35	±3.51	3.10	±0.73	12.09	±2.76	13.88	±2.46	4.55	±3.53
	Maltose	72.00	±0.00	1.92	±0.11	12.05	±3.96	12.90	±3.75	35.32	±4.35	12.13	±4.64	7.83	±1.75	5.15	±2.28	11.41	±0.68	35.31	±6.47	5.01	±2.64
	Fructose	5.24	±2.45	9.34	±1.18	6.66	±3.42	11.21	±0.34	16.97	±6.52	7.23	±2.43	6.84	±2.86	4.30	±0.53	11.05	±3.45	12.10	±2.96	4.80	±2.86
	Xylose	17.10	±3.63	72.00	±0.00	72.00	±0.00	26.01	±2.00	72.00	±0.00	19.45	±1.86	72.00	±0.00	12.23	±3.96	18.16	±2.50	10.98	±1.78	72.00	±0.00
	Rhamnose	72.00	±0.00	72.00	±0.00	72.00	±0.00	19.43	±2.55	72.00	±0.00	18.15	±2.46	18.67	±1.51	4.98	±2.32	13.60	±2.02	19.34	±2.30	72.00	±0.00
	Mannose	4.63	±1.85	7.32	±3.07	19.73	±1.63	17.78	±4.71	10.01	±4.16	12.55	±2.86	7.87	±1.42	3.45	±2.78	10.12	±3.49	14.34	±0.23	5.40	±4.46
	Galactose	14.01	±1.80	8.99	±4.05	72.00	±0.00	10.46	±1.78	72.00	±0.00	23.77	±1.84	16.93	±4.28	8.51	±3.68	12.78	±3.02	15.31	±1.00	13.43	±1.71
	Arabinose	15.54	±2.06	11.23	±4.15	72.00	±0.00	15.27	±1.46	72.00	±0.00	20.54	±2.38	72.00	±0.00	11.17	±3.00	12.52	±1.38	14.00	1.73±	29.07	±4.82
	Sucrose	5.56	±2.27	10.32	±4.95	10.41	±4.27	8.64	±2.83	24.06	±3.57	11.56	±4.40	6.82	±2.41	4.32	±1.84	13.61	±0.16	11.78	±2.21	6.24	±3.61
	Fucose	72.00	±0.00	72.00	±0.00	72.00	±0.00	12.65	±0.41	72.00	±0.00	19.66	±2.99	8.44	±1.84	8.71	±1.12	7.50	±1.42	29.22	±2.92	22.28	±1.68
	Lactose	6.02	±3.23	72.00	±0.00	72.00	±0.00	12.17	±1.24	72.00	±0.00	20.36	±3.78	7.90	±1.05	5.81	±1.19	10.87	±4.79	23.00	±4.32	22.82	±2.42
[b]DT (h)	Ribose	12.58	±2.33	11.52	±3.88	72.00	±0.00	6.97	±1.75	72.00	±0.00	6.38	±1.53	72.00	±0.00	6.44	±2.03	12.60	±2.56	2.78	±2.04	72.00	±0.01
	Cellobiose	9.22	±2.30	72.00	±0.00	5.74	±0.15	9.69	±4.02	72.00	±0.00	2.72	±0.19	72.00	±0.00	3.90	±0.66	6.01	±2.12	2.24	±0.34	72.00	±0.00
	Glucose	2.42	±0.82	24.34	±2.78	1.85	±0.30	4.64	±2.34	1.69	±0.35	2.14	±0.06	2.28	±0.85	2.11	±0.18	5.54	±3.71	1.89	±0.65	1.79	±0.92
	Maltose	72.00	±0.00	7.81	±0.05	4.70	±1.68	5.10	±2.19	3.14	±1.67	2.67	±0.76	7.47	±0.66	2.86	±0.07	5.36	±1.35	2.93	±1.39	3.54	±2.12
	Fructose	2.88	±0.35	14.90	±0.14	2.84	±0.22	4.68	±1.52	2.53	±1.20	3.17	±0.72	2.94	±0.65	2.64	±0.28	6.74	±3.77	1.83	±0.47	1.83	±0.85
	Xylose	12.44	±3.59	72.00	±0.00	72.00	±0.00	5.01	±1.14	72.00	±0.00	3.92	±1.25	72.00	±0.00	2.96	±0.25	6.51	±1.55	1.69	±0.54	72.00	±0.00
	Rhamnose	72.00	±0.00	72.00	±0.00	72.00	±0.00	5.52	±3.96	72.00	±0.00	2.50	±0.04	19.93	±0.46	3.43	±1.17	5.37	±1.43	2.66	±0.70	72.00	±0.00
	Mannose	2.96	±0.72	24.56	±3.74	3.42	±1.76	4.28	±0.67	2.04	±0.25	2.74	±0.14	3.50	±1.18	2.46	±0.48	5.75	±2.07	2.51	±1.99	1.64	±0.71
	Galactose	6.98	±1.28	9.50	±3.87	72.00	±0.00	4.29	±1.74	72.00	±0.00	7.86	±1.88	14.41	±0.76	3.36	±1.20	5.89	±1.55	2.18	±0.28	1.85	±0.23
	Arabinose	15.40	±2.68	9.19	±0.25	72.00	±0.00	7.80	±3.80	72.00	±0.00	3.79	±0.78	72.00	±0.00	5.07	±1.32	6.06	±1.33	2.54	±1.12	2.30	±0.13
	Sucrose	2.61	±0.89	12.30	±1.13	3.81	±1.12	4.50	±2.32	4.82	±2.77	2.45	±0.71	3.03	±1.22	2.30	±0.51	3.49	±0.79	2.07	±0.33	1.40	±0.39
	Fucose	72.00	±0.00	72.00	±0.00	72.00	±0.00	8.10	±2.01	72.00	±0.00	4.24	±2.82	35.11	±5.11	4.32	±0.33	5.32	±1.86	2.68	±0.52	2.05	±0.46
	Lactose	7.61	±2.13	72.00	±0.00	72.00	±0.00	10.84	±3.62	72.00	±0.00	7.79	±3.04	18.04	±4.62	7.08	±5.26	3.55	±0.92	2.55	±0.28	2.34	±0.78
[c]ΔOD	Ribose	0.83	±0.28	0.60	±0.22	0.00	±0.00	2.10	±0.25	0.00	±0.00	1.61	±0.50	0.00	±0.00	1.10	±0.64	1.69	±0.55	1.12	±0.64	0.00	±0.02
	Cellobiose	1.14	±0.31	0.00	±0.00	0.41	±0.10	3.55	±0.44	0.00	±0.00	3.79	±0.72	0.00	±0.00	2.44	±0.55	2.56	±0.42	1.58	±0.49	0.00	±0.00
	Glucose	2.24	±0.40	1.13	±0.29	2.81	±0.34	4.81	±0.10	4.01	±0.05	3.51	±0.35	1.08	±0.08	3.60	±0.77	5.35	±0.05	7.62	±0.26	2.00	±0.61
	Maltose	0.00	±0.00	0.59	±0.23	0.40	±0.10	4.66	±0.03	1.93	±0.63	3.85	±0.31	0.47	±1.24	3.67	±0.62	5.41	±0.00	0.83	±0.21	3.02	±0.64
	Fructose	1.91	±0.62	0.97	±0.41	2.36	±0.45	4.99	±0.27	3.95	±0.22	3.62	±0.48	1.19	±0.23	3.60	±0.54	5.86	±0.01	9.76	±2.09	2.47	±0.34
	Xylose	1.11	±0.50	0.00	±0.00	0.00	±0.00	3.14	±0.70	0.00	±0.00	3.57	±0.77	0.00	±0.00	3.69	±0.42	1.97	±0.49	10.81	±2.42	0.00	±0.00
	Rhamnose	0.00	±0.00	0.00	±0.00	0.00	±0.00	4.71	±0.05	0.00	±0.00	3.93	±0.40	0.23	±0.05	2.43	±0.37	3.13	±0.18	1.44	±0.17	0.10	±0.00
	Mannose	1.28	±0.12	1.84	±0.71	1.47	±0.06	4.38	±0.70	3.80	±0.23	3.03	±0.83	0.84	±0.02	3.39	±0.57	4.99	±0.04	8.27	±4.06	2.77	±0.49
	Galactose	1.18	±0.33	0.92	±0.28	0.00	±0.00	4.44	±0.27	0.00	±0.00	0.61	±0.04	0.56	±0.11	2.95	±0.95	5.32	±0.00	7.95	±2.34	2.81	±0.17
	Arabinose	0.57	±0.03	1.00	±0.37	0.00	±0.00	3.49	±0.93	0.00	±0.00	1.18	±0.25	0.00	±0.00	2.02	±0.68	4.30	±0.85	1.12	±0.04	1.65	±0.52
	Sucrose	2.18	±0.25	1.87	±0.73	0.26	±0.11	5.14	±0.01	0.19	±0.16	3.22	±0.51	0.95	±0.47	3.51	±0.79	5.68	±0.11	4.18	±0.74	2.47	±0.28
	Fucose	0.00	±0.00	0.00	±0.00	0.00	±0.00	1.47	±0.04	0.00	±0.00	1.29	±0.39	0.27	±0.21	1.60	±0.58	1.30	±0.38	1.39	±0.30	1.74	±0.62
	Lactose	0.93	±0.33	0.00	±0.00	0.00	±0.00	5.37	±0.42	0.00	±0.00	0.55	±0.14	0.18	±0.02	0.50	±0.32	3.73	±0.49	0.49	±0.10	0.61	±0.34

[a]Lag phase (h), [b]Doubling time (h) and [c]Efficiency (ΔOD). Colours from green to red for lag phase and doubling time represent short time to longer time. Colours from green to red for efficiency represent good to bad growth. N=3.

Figure 5.1. Aerobic growth of diverse yeast strains on 13 different sugars adapted from Wu et al. (2017).

5.3.2 Fermentation of 11 yeasts on 13 sugars and the production of ethanol

Anaerobic fermentation of 11 yeasts on 13 sugars was carried out at 25°C for 72 hours. The supernatants were then collected and analysed by using HPLC for the levels of unmetabolized sugars substrates (Figure 5.2A) and ethanol production (Figure 5.2B). Ribose, rhamnose, arabinose and fucose were barely consumed (less than 5%) by each of the strains but the other sugars were significantly or completely consumed by at least one strain. Hence, fermentation of 11 yeast strains on those 4 sugars were not

further assessed. The readily-fermented sugars such as glucose, fructose and mannose were utilised to produce good amounts of ethanol by most strains except NCYC 65, NCYC 10 and NCYC 49. Galactose and sucrose were similarly consumed for producing ethanol but by fewer strains. None of the strains could significantly convert xylose into substantial quantities of ethanol including the NCYC 49 which was the only strain which could consume xylose (over 50%) well. Since xylose is the second most abundant fermentable sugar in lignocellulosic materials, producing bio-fuels by converting xylose from lignocellulosic hemicelluloses is considerably interesting. A range of microorganisms including bacteria, fungi and certain yeast strains were implicated that could ferment pentose and lactose (Delgenes et al., 1996; Guimarães et al., 2010; Zhang et al., 2015). *Pichia (Scheffersomyces) stipites*; *Candida shehatae* were highlighted in studies Hughes et al. (2012), Urbina and Blackwell (2012) and Martiniano et al. (2013) as the naturally-occurring pentose-fermenting yeast strains. However, the pentose fermenters are much less common than hexose fermenters, especially fermenters for xylose and arabinose (Martiniano et al., 2013). To address this, genetically modified yeasts have been developed to enhance the capability of converting pentose whilst also improving their tolerance to inhibitors from pre-treated lignocellulosic biomass (Senatham et al., 2016).

Interestingly, stoichiometric levels of ethanol were not produced by NCYC 16 even though it consumed a number of sugars. NCYC 31 completely consumed fructose and produced only 4 mg/ml of ethanol whilst NCYC 16 produced only 1 mg/ml of ethanol with a 100% consumption of the same sugar. Similarly, a small amount of ethanol was produced by NCYC 65 whilst sucrose was consumed completely. These varying fermentation behaviours of diverse yeast strains indicated that other metabolic products might be produced in addition to ethanol.

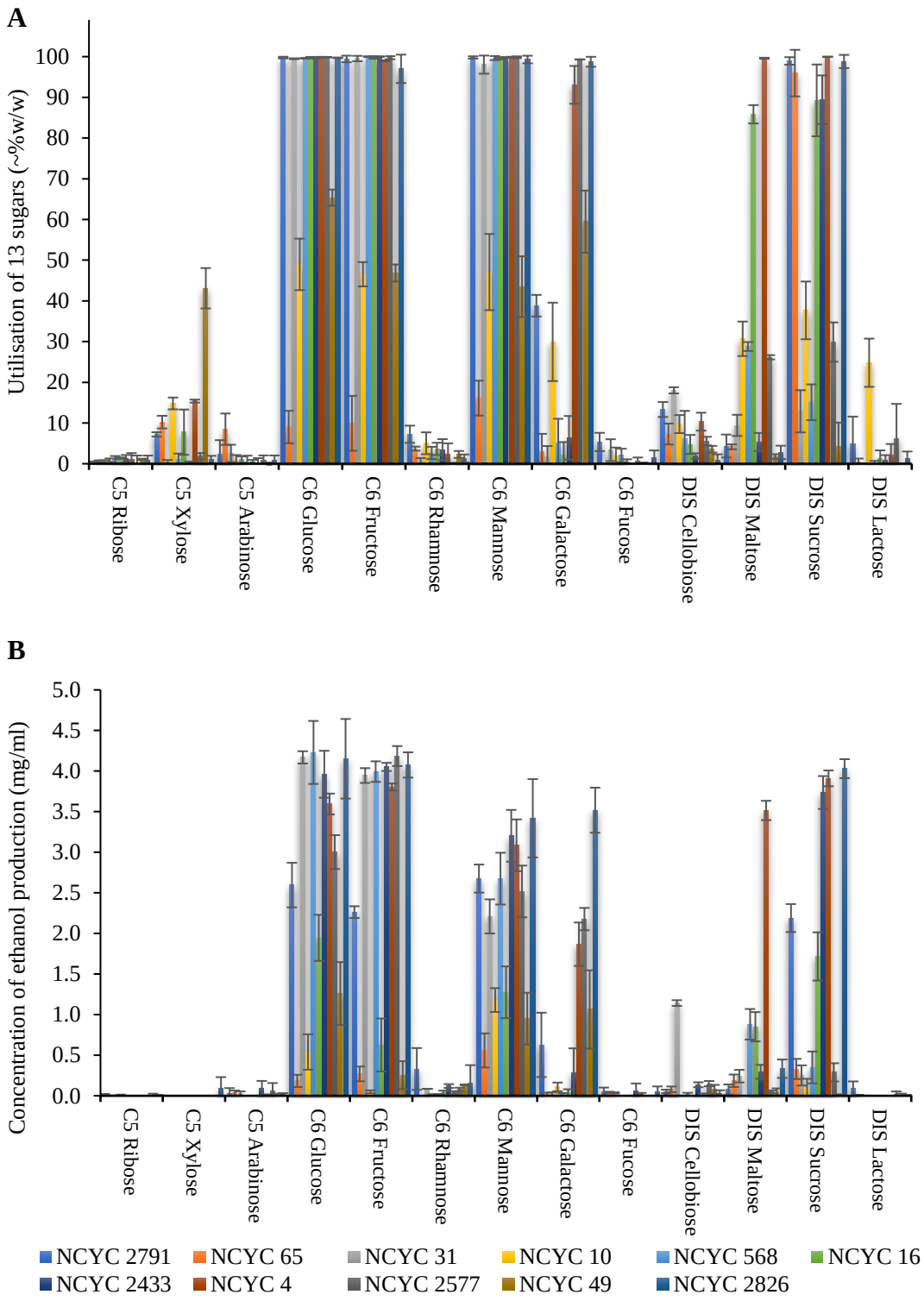


Figure 5.2. Utilisation of 13 sugars and ethanol production by 11 genetically diverse yeast strains. Figure 5.2A shows the utilisation of sugars by each strain and results have been calculated as the percentage of sugar substrates (~%w/w). Figure 5.2B shows the concentration ethanol in fermented sugar solutions. Each sugar was given as 10 mg/ml; therefore, the max theoretical yield of ethanol was up to 5.11 mg/ml. N=3.

5.3.3 Metabolic compounds Analysis by using ^{1}H NMR

A high throughput NMR method was used to analyse metabolites contained in fermented liquors which might potentially be utilised by bio-refining industries. Since ribose, rhamnose, arabinose and fucose had been eliminated from experiments, metabolites of 11 yeasts fermented on the remaining 9 sugars were investigated. Data was processed by using software "R" and presented as a group of heatmaps (Figure 5.3) (see Methodology). As Figure 5.3 shows, a total of 16 metabolites were detected in either significant or noticeable quantities and each graph represents 16 metabolites produced by 11 strains from one of those assessed sugars. A variety of chemicals were produced by different strains. Similarly, the variation of metabolites was also affected by the sugar substrates.

Some of the 16 chemicals were substantially produced from at least one sugar substrate such as 2,3-butanediol, acetic acid, arabinitol, citric acid, ethanol, glycerol, lactic acid, pyruvic acid, succinic acid, ethyl-acetate and malic acid. In contrast, the others were only produced in trace quantities. Acetic acid and ethanol were very commonly produced from all sugars but differed in quantities between the yeasts. Except lactose, arabinitol, glycerol, succinic acid and ethyl-acetate were detected in the liquors of 8 other sugars after fermentation. Lactic acid was detected in the liquors of all sugar but at considerably lower levels. Similarly, 2,3-butanediol was detected in fermented liquors of glucose, fructose, mannose, maltose and galactose but in low quantities. Citric acid was produced in significant quantities from only galactose. However, it had been widely detected in the liquors of a range of sugar substrates. Pyruvic acid was produced in higher levels from glucose, fructose, mannose and sucrose compared to the other sugars. Interestingly, malic acid was exclusively produced by NCYC 16 from only fructose and sucrose.

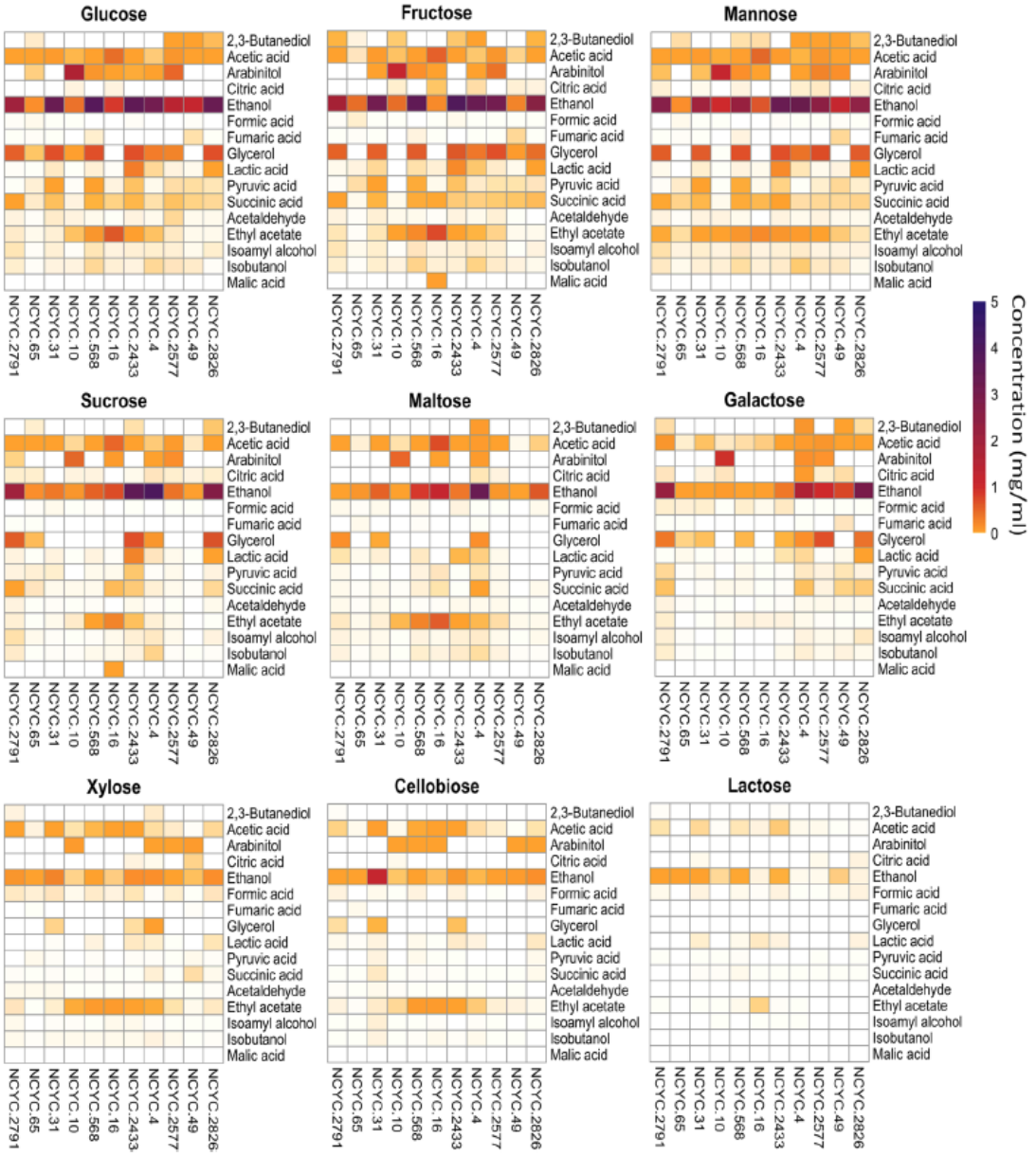


Figure 5.3. Quantities of 16 chemicals produced by 11 yeast strains from 13 different sugars. Results are presented as heatmaps generated by using software "R". Different concentrations are represented with the changes of colours from white (0 mg/ml) to dark purple (5 mg/ml). Figure is adapted from Wu et al. (2017). N=4. Supplementary data shown in Appendix 5, Table 5.3.

The significance of ethyl-acetate for industrial utilisation has been highlighted as it is an important platform chemical that can be used in the manufacture of food, glues, inks and perfumes as a naturally degradable and environmental friendly solvent. As Löser et al. (2015) reported in 2015, ethyl acetate was annually produced in the

quantity of 1.7 million tons which would not be able to satisfy the world's demand of 2.5 million tons (Nielsen et al., 2012). In this Chapter, two strains (NCYC 568 (*Zygosaccharomyces rouxii*) and NCYC 16 (*Wickerhamomyces anomalus)*) could produce ethyl acetate from a range of sugar substrates and the highest levels of ethyl acetate were produced by NCYC 16. These results extended the study of Walker (2011) which claimed *P. anomala* (recently renamed as *Wickerhamomyces anomalus*) was an outstanding producer for ethyl acetate production, and the studies of Kurita (2008) and Rojas et al. (2003) which investigated the fermentation of *P. anomala* on malt agar medium and glucose respectively.

The levels of ethyl acetate produced by the same strain were noticeably different between different sugar substrates. For instance, a higher level of ethyl acetate was produced by NCYC 16 from fructose compared with production from glucose. The different metabolic pathway of fructose and glucose might be one of the main reasons (Appendix 5, Figure ATC5.8). As Figure ATC5.8 shows, glucose is primarily converted to glucose-6-phosphate (glucose-6-P) for growing cells or then converted to fructose-6-phosphate (fructose-6-P) (Fredlund et al., 2004; Passoth et al., 2006) whilst fructose has been directly converted to fructose-6-P and then further metabolised to fructose-1,6-bisphosphate (Rodicio & Heinisch, 2009). Nevertheless, the levels of oxygen might also affect the formation of ethyl acetate in either way of being promoter or inhibitor (Davies, 1951; Fredlund et al., 2004; Tabachnick, 1953). The role of oxygen for the formation of ethyl acetate by fermentation of NCYC 16 on glucose was further analysed. Three sets of experiments were designed (Set A, anaerobic condition; Set B, semi-anaerobic condition; Set C, aerobic condition) and the results are shown in Figure 5.4. The highest level of ethyl acetate was produced from Set B indicating that the formation of ethyl acetate might be significantly enhanced by semi-anaerobic fermentation with intermediate levels of air or oxygen. However, further research is required to uncover more details and optimise the conditions for enhancing ethyl acetate production using NCYC 568 and 16.

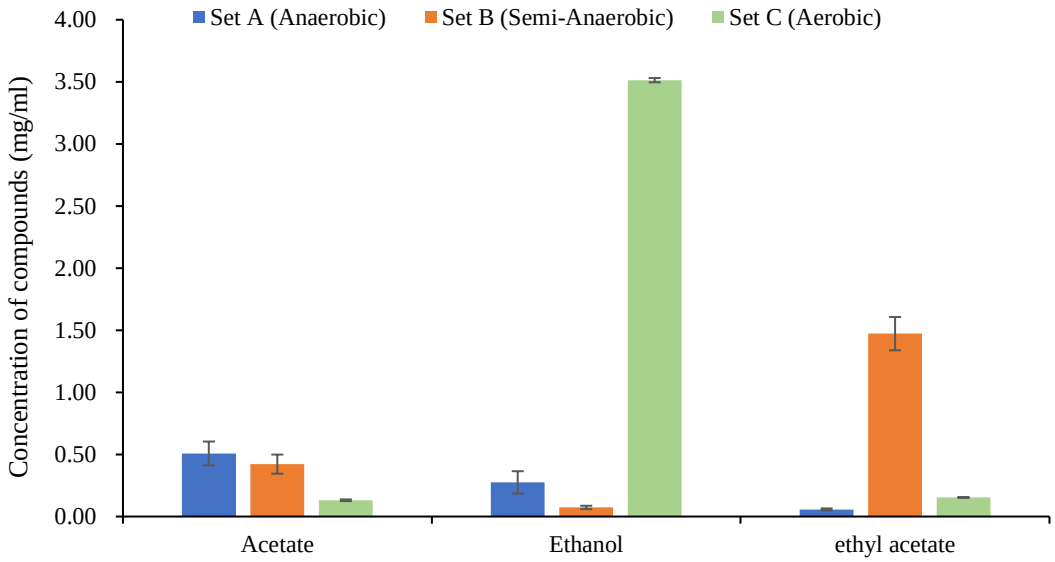


Figure 5.4. Concentration of acetate, ethanol and ethyl acetate produced by NCYC 16 fermented on glucose in the conditions of different air controls. Set A, B and C represent the condition of anaerobic, semi-anaerobic and aerobic respectively. N=9.

Arabinitol can be potentially used as a non-nutritive sweetener as well as xylitol and is an interesting platform chemical for producing ethylene glycol, propylene, enantiopure compounds, arabinoic and xylonic acids. However, it is currently synthesised via a chemical reaction requiring catalysis at high temperature (Kordowska-Wiater, 2015; Kumdam et al., 2013; Werpy et al., 2004b). In this Chapter, NCYC 568 (*Zygosaccharomyces rouxii*), NCYC 2577 (*Kazachstania servazzii*) and NCYC49 (*Galactomyces candidus*) have, for the first time, been shown to produce significant amounts of arabinitol from several sugar substrates (both hexoses and pentoses). In previous studies it was reported that the yeast strain *Debaryomyces hansenii* could accumulate arabinitol from a range of carbon sources (Koganti & Ju, 2013; Nobre & Costa, 1985). These studies have been confirmed and extended this by showing that NCYC 10 (*Debaryomyces hansenii*) produced arabinitol in very high quantities from different sugars and even higher than the production of ethanol, especially from the glucose, fructose, mannose, sucrose, maltose and galactose. Interestingly, NCYC 568 and NCYC 2577 produced multiple metabolites from some of the selected sugars in addition to ethanol: glycerol and ethyl acetate were additionally produced from several sugars by NCYC 568; NCYC 2577 produced

acetic acid from sucrose, and both acetic acid and glycerol from glucose, fructose, mannose, galactose in addition to ethanol production. Surprisingly, ethanol was produced significantly from cellobiose only by NCYC 31. This has been previously reported as due to its ability to produce β-glucosidase (Pavlova et al., 2002).

Sucrose was almost completely consumed by NCYC 65 (*Rhodotorula mucilaginosa*). However, none of the 16 chemicals were produced in a reasonable quantity (Figure 5.2 and Figure 5.3). Therefore, a closer investigation by using NMR was carried out and the NMR spectra are shown in Figure 5.5. The NCYC 65 (Figure 5.5A) fermented liquor showed a loss of peaks associated with sucrose (Figure 5.5B) whilst the peaks associated with glucose (Figure 5.5C) and fructose (Figure 5.5D) appeared. Quantification of glucose and fructose from fermented liquor also confirmed that sucrose was simply cleaved into glucose and fructose rather than utilised in fermentation. Nevertheless, neither glucose nor fructose were utilised as well as sucrose. This might due to the activity of invertase. Hence, NCYC 65 could be a potential enzyme producer and a direct biological route applying the function of invertase for industry. Interestingly, this strain could grow more rapidly on sucrose compared with on glucose. However, further investigation of this strain is required to explain questions such as how NCYC 65 obtains energy from sucrose without consuming significant amount of glucose and fructose. The present study indicated the strains of NCYC 65 could not ferment any of those sugars. Previous studies reported different activities of *R. mucilaginosa.* For example, a strain of *R. mucilaginosa* was identified as the producer of acetylxylan esterase (Lee et al., 1987) which could potentially enhance the activity of xylanases (Biely, 1985; Biely et al., 1985; Lee et al., 1987). The research of Li et al. (2010) mentioned a strain of *R. mucilaginosa* accumulating fatty acids that could be used for producing bio-diesel.

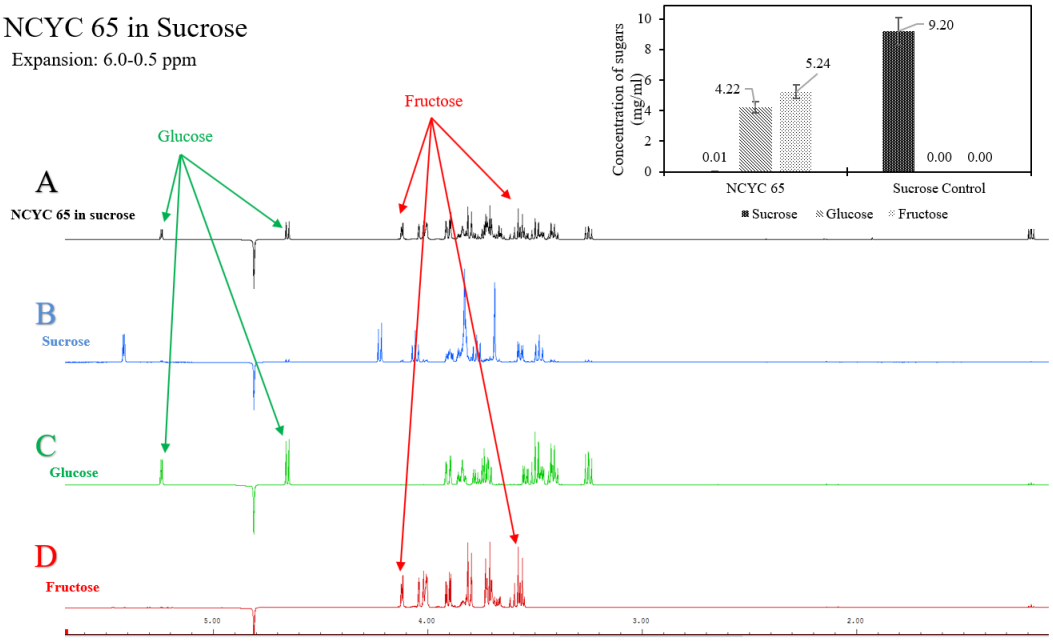


Figure 5.5. Comparison of the NMR spectra of NCYC 65 fermented sucrose (a), sucrose control (b), glucose control (c) and fructose control (d). The graph on the top right shows the quantification of glucose, fructose and sucrose from the liquor of NCYC 65 fermented sucrose and sucrose control Wu et al. (2017). N=3.

5.3.4 SSF of diverse yeasts on PT RS

Hydrothermally pre-treated rice straw was used to evaluate the ability of 11 diverse yeasts to utilise an industrially relevant lignocellulosic biomass – derived sugar source presenting a much more complex bio-chemical and chemical environment compared with the purified sugars. Rice straw was pre-treated at severities 1.57, 3.65, 5.15 and 5.45 and then used for SSF of 11 diverse yeast for 72 hours (see Methodology). Figure 5.6A shows the results of fermenting 11 yeasts on slurries of pre-treated rice straw. Ethanol was substantially produced from samples pre-treated at severities 1.57 and 3.65. However, none of 11 yeasts strains could produce ethanol in significant quantities from samples pre-treated at severities 5.15 and 5.45. This was thought to be due to sugar derived fermentation inhibitors previously described in the research of Wood et al. (2016a) such as furfural (2-FA), 5-hydroxymethylfurfural (5-HMF), formic acid and levulinic acid. Therefore, the strains were further evaluated by using pre-treated rice straw samples washed with distilled water 3 times prior to resuspension and SSF. As Figure 5.6B shows, the highest levels of ethanol were detected from

samples pre-treated at a severity of 5.15 and slightly lower levels were detected at a severity of 5.45. Except the strains of NCYC 65, NCYC 10, NCYC 49 and NCYC 2577, of the remaining strains could produce ethanol in substantial quantities by converting the sugars enzymatically released from the cell walls of rice straw. Regarding the 3 strains of NCYC 65, NCYC 10, NCYC 49, they have been shown not to convert sugars into ethanol significantly (Figure 5.2). Surprisingly, NCYC 2577 failed to produce ethanol in significant quantities from pre-treated samples but it was previously shown that it could produce ethanol (Figure 5.2 and Figure 5.3) effectively from a range of sugars. It is possible that NCYC 2577 is particularly sensitive to low levels of inhibitors remaining after washing. Different yeast strains can respond to those inhibitors differently (Field et al., 2015b). Fermentation inhibitors are unavoidably generated from lignocellulosic biomass during pre-treatment. A range of methods have been reported that could considerably impair the inhibition by developing and using inhibitor resistant strains (Chandel et al., 2011; Field et al., 2015b; Huang et al., 2009; Larsson et al., 2001), or introducing pre-treatment with either optimised condition (Chapter 3) or fine-tuning process (Pedersen & Meyer, 2010).

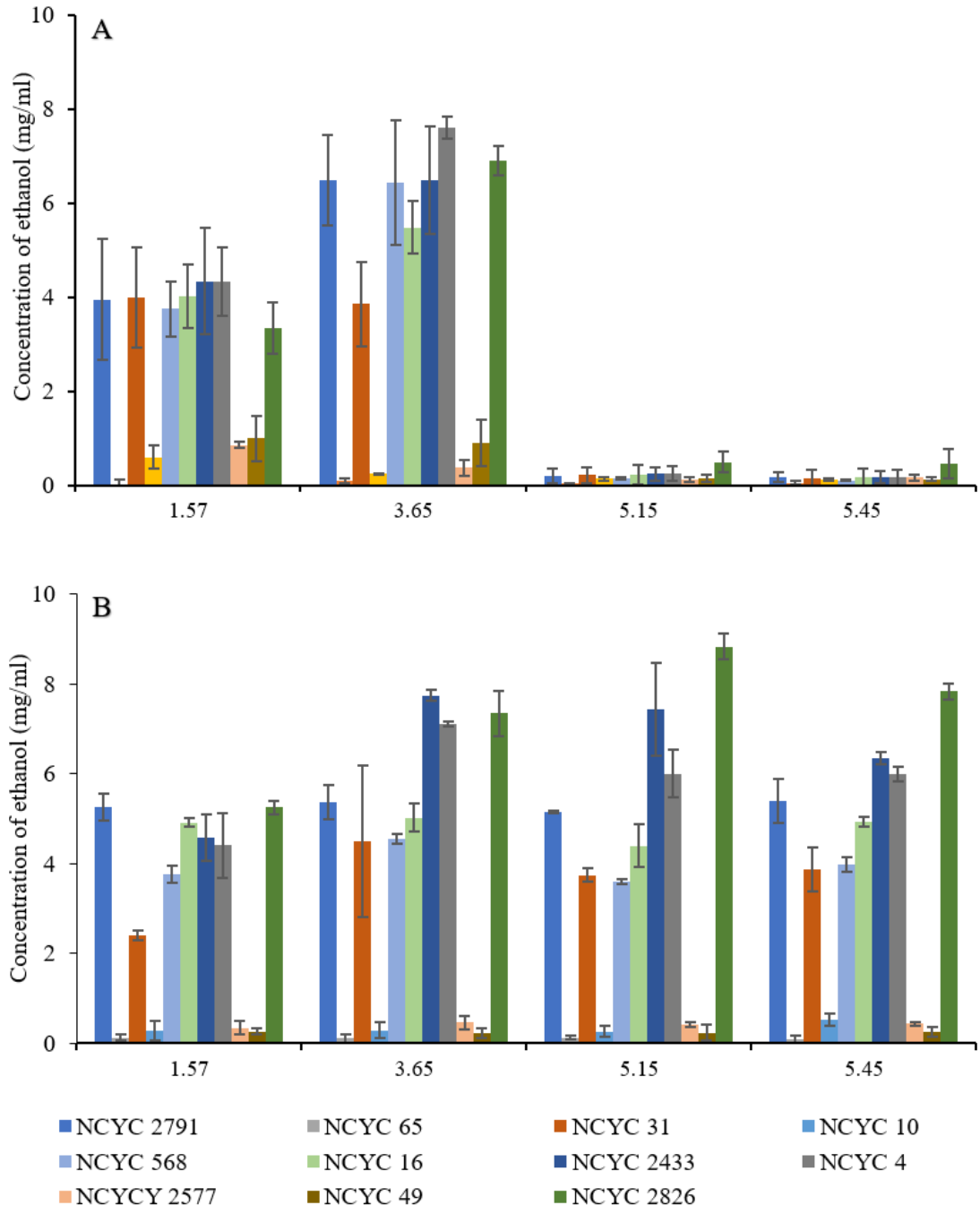


Figure 5.6. Quantification of ethanol produced by 11 yeast strains from RS pre-treated with 4 different severities. Figure 5.6A presents the concentration of ethanol in RS which contained the pre-treatment liquor (inhibitors included). Figure 5.6B presents the concentration of ethanol in washed (inhibitor free) RS (re-suspended in distilled water). The original Concentration of substrate in fermentation slurry was 5% which can be theoretically converted into a maxim ethanol yield of 9.89 mg/ml. result was calculated as mg per ml of fermented liquor (w/v). N=2.

5.4 Conclusion

This Chapter presented information on yeast behaviors when grown on a range of pure carbon sources, identifying the chemicals produced by some of strains across the different sugar substrates. It showed the chosen phylogenetic diversity of the strain set was matched by phenotype diversity, highlighting the importance of screening widely across the vast yeast taxonomy for key bio-industrial traits. Furthermore, by evaluating the yeast strains to ferment rice straw hydrolysates and comparing those data with fermentation on purified sugars, this study highlighted the challenges that need to be addressed when attempting to exploit yeasts industrially. Some interesting gaps were identified in knowledge that would be worth investigating by further research, such as optimising the condition of enhancing formation of ethyl-acetate or D-arabinitol and even trying to produce those chemicals from raw materials. Utilization of carbon sources and the range of metabolites produce by 11 genetically most diverse yeast strains were studied. There was considerable variation in the degree of fermentation, yeast behaviour, and ethanol production. Several strains consumed certain sugars but produced very low yields of ethanol (NCYC 16, *P. anomala*; NCYC 65, *R. musilaginosa*; and NCYC 10. *D. hansenii*). NMR screening of their fermentation liquors demonstrated that some produced relatively large amounts of ethyl-acetate, arabinitol and acetate rather than ethanol alone and interesting strains (NCYC 568 and NCYC 2577) which could produce several chemicals (arabinitol, ethanol, glycerol, acetate or ethyl acetate) from a unique carbon source. These yeast strains were also evaluated for their ability to ferment sugars derived from pre-treated rice straw biomass and the impact of fermentation inhibitors created during high severity pretreatment. All the yeasts suffered from inhibition in fermentation after substrate pre-treatment at high severities. Except for strain NCYC 2577 (*K. servazzii)* the impact of severe pretreatment- derived inhibitors could be avoided by washing the pre-treated biomass which will remove such inhibitors.

Chapter 6:

General discussion and conclusion

6.1 Discussion

Rice husk (RH) and rice straw (RS) were the raw materials. Key criteria relevant to their suitability as feedstocks for bio-conversion to ethanol were then compared. These included the cell wall sugar and lignin content, inhibitor formation during hydrothermal (hot water) pre-treatment, sugars yield during saccharification, and ethanol production during SSF. RH and RS contain large quantities of polysaccharides including cellulose (RS, 38.7% w/w; RH, 36.8% w/w) and hemicellulose (RS, 22.9%, w/w; RH, 19.7%, w/w) which have the potential to be enzymatically hydrolysed to fermentable sugars. However, the results of SSF in the presence of excess enzymes showed that ethanol production from RS was double that from RH after pre-treatment at severities 1.57 and 3.65. The reasons for these differences were considered to be:

1) RH contains much higher levels of lignin (35.3%) than RS (22.1%). Lignin acts as a strong barrier to disruption of cell structure and enzymatic hydrolysis of polysaccharides and provides a large, hydrophobic physical surface onto which cellulases may bind strongly, leading to removal and/or deactivation, and reducing the rate of enzymatic hydrolysis (Öhgren et al., 2007b; Wood et al., 2014).

2) higher levels of potential chemical inhibitors of enzymes and fermentation were generated from RH during pre-treatment. Some soluble carbohydrates which can inhibit enzymolysis such as xylose and xylo-oligomers were produced at over 1 mg/ml in liquors of pre-treatment RH samples which are known to significantly reduce cellulase activity (Qing et al., 2010). Similarly, lignin-derived phenolic compounds were detected that are known to significantly reduce the efficiency of producing cellulosic bio-ethanol by inhibiting the activity of cellulase and fermenting microorganisms (Hou et al., 2017; Jönsson & Martín, 2016; Kim et al., 2011; Ximenes

et al., 2010). For example, vanillin, syringaldehyde, trans-cinnamic acid and hydroxybenzoic acid (p-OH-benzoic acid) inhibit the hydrolysis activity of β-glucosidase, and endo- and exo-cellulases. Vanillin, particularly, produces the most severe inhibition (Ximenes et al., 2010). The less-well known inhibitory function of tFA and pCA has also been highlighted in the study of Hou et al. (2017). pCA reduces the digestibility of cell wall carbohydrates and is a toxin to microorganisms (Taboada et al., 2010; Theodorou et al., 1987). In this study, phenolic compounds such as vanillin, pCA, p-OH-benzoic acid have been found at significantly higher levels in the liquors of PT RH compared with PT RS (Chapter 4, Figure 4.5-4.6). Augmenting the effects of lignin and other well-established pre-treatment generated inhibitors, these phenolics are also likely to have contributed to the much greater inhibition of the fermentable sugar accumulation and ethanol production from RH, leading to the poor results of both hydrolysis and fermentation compared with RS. However, the results have shown that most of phenolic compounds solubilised by pre-treatment remain in carbohydrate-esterified forms. There is currently a lack of evidence regarding the efficacy of such esterified, but soluble phenolics and future work is required to investigate their inhibitory characteristics. Some researchers indicated that phenolics such as p-coumaric acid and ferulic acid can be released from their esterified forms by using enzyme cocktails containing both carbohydrates degrading enzymes and esterases (Benoit et al., 2006; Lesage-Meessen et al., 2002). However, Chapter 3 has indicated that free phenolics could not be releases by adding CTec-2 and HTec-2. The reason might be the lack of esterases activities in CTec-2 (Morrison et al., 2016; Watanabe et al., 2015) and lack of evidences to clarify esterases in HTec-2 to release free phenolic acids. Therefore, significant research efforts are still required to undercover more details. Furthermore, well-established fermentation inhibitors produced from PT RH

and RS such as 5-HMF, 2-FA, acetic acid and formic acid were (except for formic acid) produced at higher levels from RH samples compared with RS (Chapter 3, Figure 3.9), thus contributing further to the severe inhibition of SSF of PT RH (Wu et al., 2018b).

The results of SSF studies (Chapter 3, Figure 3.4) also showed that production of ethanol was almost completely inhibited from both RS and RH in samples pre-treated at severities 5.15 and 5.45. The explanation for this is the high concentration of soluble inhibitors including 5-HMF, 2-FA, acetic acid and formic (and all the others described above) which clearly supressed saccharification and/or yeast activities (Wu et al., 2018b). SSF could be successfully achieved by washing out the inhibitors. However, careful evaluation of saccharification across all the severities tested have shown that RS pre-treated at severity 3.65 released 37.5% (w/w dry materials) glucose which was about 80-90% of total glucose content in RS. Furthermore, at this severity, inhibitors were not concentrated enough to cause any significant inhibition on producing bio-ethanol. Therefore, pre-treating RS at severity 3.65 could benefit by decreasing the cost of detoxification and energy input whilst enabling a suitable yield of sugars for generating bio-products.

Although phenolic compounds are inhibitory to cellulase, there are some physiological functions such as anti-allergenic, anti-atherogenic, anti-inflammatory and anti-oxidant characteristics which make phenolics especially interesting as value-adding by-products of the bio-conversion process (Balasundram et al., 2006; Benavente-García et al., 2000; King & Young, 1999; Manach et al., 2005; Middleton et al., 2000; Puupponen-Pimiä et al., 2001). In this study, the extraction of phenolics from RH and RS were significantly enhanced by hydrothermal pre-treatments under relatively mild condition (severity 1.57) (Figure 4.2). Nevertheless, most of phenolic acids would

remain largely in the pre-treated solids rather than in the liquors (Figure 4.4-4.5) after being pre-treated at mild condition such as severity 3.65, suggesting that an extra step might be considered to extract phenolic acids from saccharified or fermented residual solids for producing the value adding phenolic acids products.

The studies above focused on understanding the differences in suitability of RH and RS as sources of lignocellulose for bio-ethanol production. Since a number of sugars may be created from biomass saccharification, and since yeasts have the potential to produce products in addition to or other than ethanol, eleven genetically diverse yeast strains were screened by growing and fermenting them on 13 commercially-purified sugars for identification of uncommonly used yeasts and potentially high value containing metabolites. The results showed (Chapter 5) that the yeasts differed considerably in their ability to consume a range of different sugars, and in their production of ethanol. However, pentoses such as xylose were barely fermented by any of the selected diverse yeasts. Although pentoses and lactose can be fermented by a range of microorganisms such as bacteria, fungi and yeasts, the naturally-occurring pentose-fermenting yeasts are very limited (Hughes et al., 2012; Martiniano et al., 2013; Urbina & Blackwell, 2012). Several strains had consumed sugars but had produced a low yield of ethanol such as NCYC 16 (*W. anomala*), NCYC 65 (*R. musilaginosa*) and NCYC 10 (*D. hansenii*). This indicated that those strains might have produced other metabolic products. A number were found to produce significant amounts of ethyl-acetate, arabinitol, glycerol and acetate in addition to ethanol (NCYC16, *W. anomala*; NCYC 10, *D. hansenii*; NCYC 568, *Z. rouxii*; and NCYC 2577, *K. servazzii)* from several different carbon sources.

Ethyl acetate can be used as an environmentally friendly solvent in the manufacture of food, glues, inks and perfumes, and it is currently produced via chemical processes

(Löser et al., 2015; Nielsen et al., 2012). The yeast strain NCYC 16 (*W. anomala*) is capable to produced high levels of ethyl acetate from a range of sugars, extending the previous research of Walker (2011) which identified the yeast strain *Pichia anomala* (*W. anomala*) as a ethyl acetate producer. However, the levels of ethyl acetate vary significantly between different sugar substrates used for fermentation. This might due to the bifurcated metabolic pathways for different sugars (Fredlund et al., 2004; Passoth et al., 2006; Rodicio & Heinisch, 2009). Moreover, the yields of ethyl acetate may also be influenced by the levels of oxygen during fermentation (Davies, 1951; Fredlund et al., 2004; Tabachnick, 1953). The present study (Chapter 5, Figure 5.4) achieved high level ethyl acetate yields by controlling the initial ratio of sugar solution and air as 1:1 (v/v). Further investigations are needed to give a better understanding of mechanisms and more accurate controlling of oxygen or air.

Arabinitol is a potential non-nutritive sweetener and feedstock in producing ethylene glycol, propylene, enantiopure compounds; arabinoic and xylonic acid, which are currently produced via chemical reactions require high temperature for catalysis (Kordowska-Wiater, 2015; Kumdam et al., 2013; Werpy et al., 2004a). In this study, several strains have been firstly identified as potential arabinitol producers such as NCYC 568 (*Z. rouxii*), NCYC 2577 (*K, servazzii*) and NCYC 49 (*G. candidus*) (Chapter 3, Figure 5.4). The previous studies of Nobre and Costa (1985) and Koganti and Ju (2013) have reported the yeast strain *Debaryomyces hansenii* can produce a significant quantities of arabinitol, and the present study reported NCYC 10 (a strain of *D. hansenii*) is capable of producing arabinitol from a number of sugars in large quantities even higher than ethanol. Those findings highlight the important potential of uncommonly used yeasts for producing high value platform chemicals from lignocellulosic biomass (Bozell & Petersen, 2010).

The present studies also assessed how the genetically diverse yeast reacted to the complex conditions created by lignocellulosic hydrolysate. They were then used to ferment pre-treated RS. The results showed that most strains behave as they did when used for the fermentation on pure glucose as none could ferment xylose. However, fermentation activities of all strains are largely inhibited on RS samples pre-treated at higher severities (severities 5.15 and 5.45). The reason was hypothesised to be due to the inhibitory compounds that reduced the ethanol production by suppressing yeast behaviours. This was confirmed by following the fermentation of the 11 yeast strains on washed RS hydrolysates. Therefore, detoxification of hydrolysates and the use of inhibitor-resistant yeast strains can be applied to minimise the effect of inhibition (Chandel et al., 2011; Huang et al., 2009; Larsson et al., 2001). Genetically modifying yeasts may significantly contribute to enhancing their capabilities for both fermenting pentoses and inhibitor tolerance (Field et al., 2015a; Senatham et al., 2016). Also, fine-tuning of the pre-treatment (as discussed above) process may be a reasonable way of reducing the effects caused by inhibitors (Pedersen & Meyer, 2010).

Therefore, an ideal process of bio-conversion especially for RS can be considered as: pre-treating RS at severity 3.65 by using hot water pre-treatment to establish the environment containing relatively low quantities of inhibitors, then addition of enzymes and selected yeasts to perform SSF to produce high value products in addition to ethanol such as arabinitol and ethyl acetate. Finally, the fermented residual solids could be used for phenolic acid extraction to produce by-products which further increases the economic value of the overall products. The whole process is shown diagrammatically in Figure 6.1. To achieve this, future investigation is required.

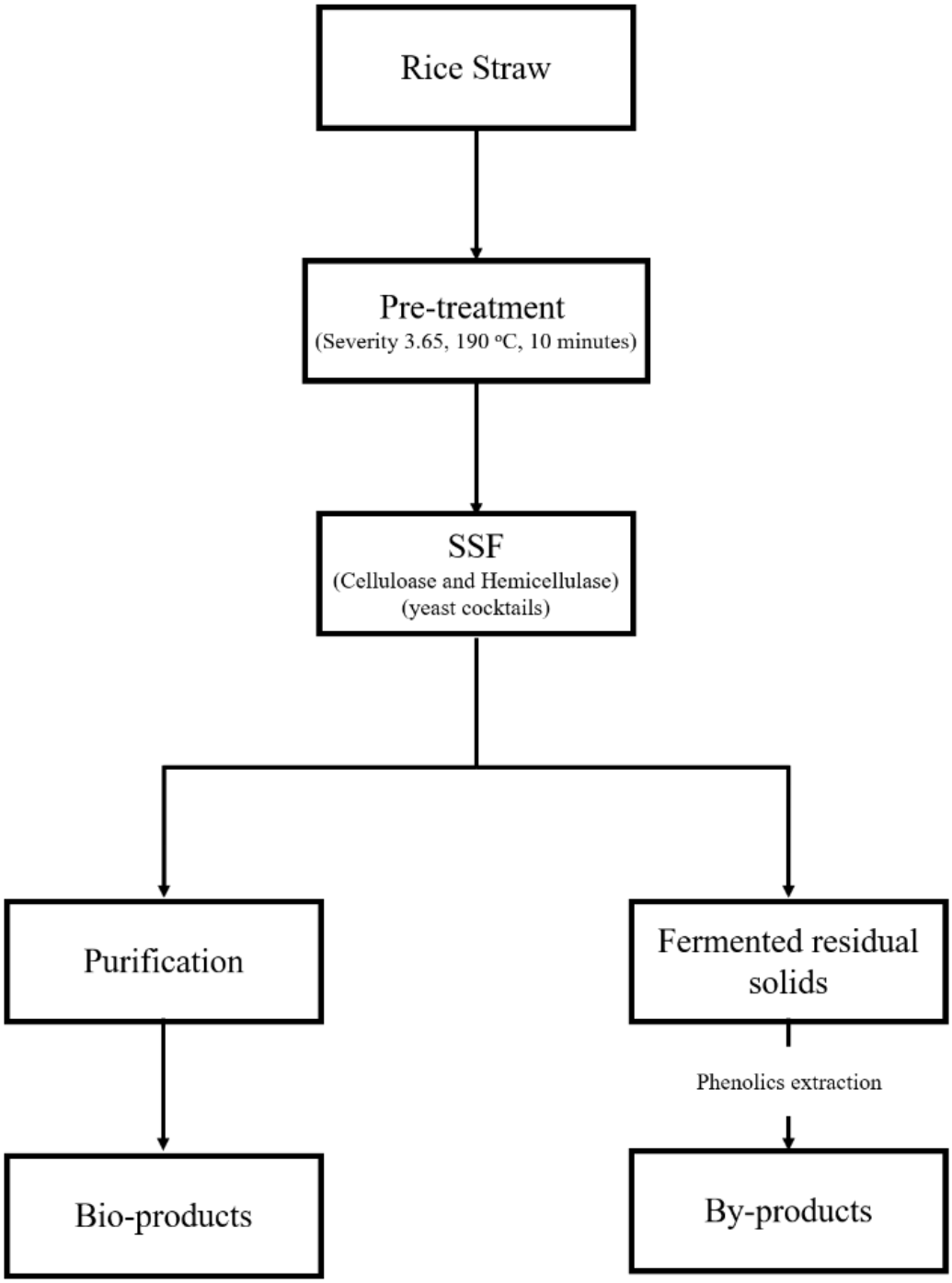


Figure 6.1. Diagram of theoretical advanced processes of bio-converting RS into bio-products and by-products.

6.2 Future work

This study highlights that RS is suitable as a feedstock for bio-conversion to bio-products, and optimised pre-treatment condition tailored for RS occurred at severity 3.65. The advantages of pre-treating RS by hydrothermal methods at such mild severities are the reduction in energy input, high yields of fermentable sugars (the enclosed conditions allow the presence of both glucose and xylose) and very low yields of inhibitors of either enzymolysis or yeast fermentation. However, due to the significant amount of water required, the hot water pre-treatment method may not be very cost effective. Future research might explore the transfer of this idea to optimise the conditions for steam explosion which will have two additional advantages: less water required and lower volatile inhibitors retained in the pre-treated materials. The presence of xylose in addition to glucose increases the potential quantities of final bio-products. Therefore, a combined fermentation of both glucose and xylose could be further assessed by using yeast cocktails containing glucose fermenting yeasts and xylose fermenting yeasts. Moreover, the ethyl acetate and arabinitol producers identified in this study can be further assessed with RS hydrolysates for metabolites in addition to ethanol. Investigation of the extraction method to separate phenolic acids from fermented RS residual solids is also important and interesting since the by-products could add extra value to the final products and enhance the financial availability of the bio-refining processes. Silica had been previously reported that it is responsible for severely inhibiting ruminant digestion of RH and RS as it is contained in significantly quantities in RH and RS, especially in RH. Therefore, to investigate the content of silica and its potential inhibitory effects on the digestibility of pre-treated RH and RS will be able to fill the gap of left in this study. Furthermore, the effect of fermenting yeasts on the contents of phenolics in fermented residual solids is unknown,

and this would be an interesting area to explore.

6.3 Conclusion

In summary, this research has focused on contributing to bio-refining technology by analysing the suitability of rice residual feedstocks, optimising conditions of pre-treatments and investigating the potential for producing novel products. Chapter 3 uncovered the optimised pre-treatment conditions specific for pre-treatment RS. By careful control of pre-treatment, the optimised conditions (between severity 3.65 and 4.25) may be sufficient to reduce the levels of fermentation inhibitors. Pre-treating RS with severity between 3.65 and 4.25 achieved a glucose yield of between 37.5% and 40% (w/DW of raw materials) which close to the theoretical glucose yield of 44.1%. These results indicate that RS is a readily available candidate for bio-refining, but RH requires more research.

Phenolic esters such as tFA and p-CA have been recently considered as serious inhibitors to fermenting microorganisms. The study of Chapter 4 extensively investigated the correlation between a range of hydrothermal severities and yields of phenolic esters by extracting those phenolic esters and quantifying their free form after saponification. This study provided new information on the fate of diferulic acids. The results indicated phenolic esters were also the major compounds released into liquor of lignocellulosic biomass during hydrothermal pre-treatment. Therefore, further research of their inhibition activities is crucial to bio-converting as there is currently lack of information about the inhibitory functionality of soluble phenolic esters.

Phenolic compounds especially the phenolic acids are potential value adding by-products. The quantification of total phenolics indicated that phenolic compounds mainly remained in the solids even after pre-treatment. This gives the possibility to extract phenolic acids from fermented RS residual solids. As reported in Chapter 5, yeast strains such as NCYC 10 (*D. hansenii*) and NCYC 16 (*W. anomala*) can produce high value bio-products such as arabinitol and ethyl acetate in a quantity higher than ethanol production. Those can be potentially used to convert glucose instead of typical *S. cerevisiae*. However, some further studies are required for reducing the water

demand, increasing ethanol yield by fermenting both glucose and xylose by using yeast cocktails, optimising the fermentation conditions for producing ethyl acetate and arabinitol with NCYC 16 and NCYC 10 and investigating the methods for extracting phenolic acids from fermented residues.

Reference

Abbas, A., Ansumali, S. 2010. Global Potential of Rice Husk as a Renewable Feedstock for Ethanol Biofuel Production. *BioEnergy Research*, **3**(4), 328-334.

Abdi, H., Williams, L.J. 2010. Principal component analysis. *Wiley interdisciplinary reviews: computational statistics*, **2**(4), 433-459.

Agbor, V.B., Cicek, N., Sparling, R., Berlin, A., Levin, D.B. 2011. Biomass pretreatment: Fundamentals toward application. *Biotechnology Advances*, **29**(6), 675-685.

Amen-Chen, C., Pakdel, H., Roy, C. 2001. Production of monomeric phenols by thermochemical conversion of biomass: a review. *Bioresource Technology*, **79**(3), 277-299.

André, A., Diamantopoulou, P., Philippoussis, A., Sarris, D., Komaitis, M., Papanikolaou, S. 2010. Biotechnological conversions of bio-diesel derived waste glycerol into added-value compounds by higher fungi: production of biomass, single cell oil and oxalic acid. *Industrial Crops and Products*, **31**(2), 407-416.

Ang, T.N., Ngoh, G.C., Chua, A.S.M. 2013. Comparative study of various pretreatment reagents on rice husk and structural changes assessment of the optimized pretreated rice husk. *Bioresource Technology*, **135**, 116-119.

Anugwom, I., Mäki-Arvela, P., Virtanen, P., Willför, S., Sjöholm, R., Mikkola, J.P. 2012. Selective extraction of hemicelluloses from spruce using switchable ionic liquids. *Carbohydrate Polymers*, **87**(3), 2005-2011.

Arshadi, M., Attard, T.M., Lukasik, R.M., Brncic, M., Lopes, A.M.D., Finell, M., Geladi, P., Gerschenson, L.N., Gogus, F., Herrero, M., Hunt, A.J., Ibanez, E., Kamm, B., Mateos-Aparicio, I., Matias, A., Mavroudis, N.E., Montoneri, E., Morais, A.R.C., Nilsson, C., Papaioannou, E.H., Richel, A., Ruperez, P., Skrbic, B., Solarov, M.B., Svarc-Gajic, J., Waldron, K.W., Yuste-Cordoba, F.J. 2016. Pre-treatment and extraction techniques for recovery of added value compounds from wastes throughout the agri-food chain. *Green Chemistry*, **18**(23), 6160-6204.

Auxenfans, T., Cronier, D., Chabbert, B., Paes, G. 2017. Understanding the structural and chemical changes of plant biomass following steam explosion pretreatment. *Biotechnology for Biofuels*, **10**.

Avgerinos, G.C., Wang, D.I. 1983. Selective solvent delignification for fermentation enhancement. *Biotechnology and bioengineering*, **25**(1), 67-83.

Balasundram, N., Sundram, K., Samman, S. 2006. Phenolic compounds in plants and agri-industrial by-products: Antioxidant activity, occurrence, and potential uses. *Food Chemistry*, **99**(1), 191-203.

Balat, M., Balat, H., Öz, C. 2008. Progress in bioethanol processing. *Progress in Energy and Combustion Science*, **34**(5), 551-573.

Ballesteros, I., Ballesteros, M., CabaÑas, A., Carrasco, J., MartÍn, C., Negro, M.J., Saez, F., Saez, R. 1991. Selection of thermotolerant yeasts for simultaneous saccharification and fermentation (SSF) of cellulose to ethanol. *Applied Biochemistry and Biotechnology*, **28**(1), 307.

Ballesteros, M., Oliva, J.M., Negro, M.J., Manzanares, P., Ballesteros, I. 2004. Ethanol from lignocellulosic materials by a simultaneous saccharification and fermentation process (SFS) with Kluyveromyces marxianus CECT 10875. *Process Biochemistry*, **39**(12), 1843-1848.

Barber, A.R., Vriesekoop, F., Pamment, N.B. 2002. Effects of acetaldehyde on Saccharomyces cerevisiae exposed to a range of chemical and environmental stresses. *Enzyme and Microbial Technology*, **30**(2), 240-250.

Barnham, K.W.J., Mazzer, M., Clive, B. 2006. Resolving the energy crisis: nuclear or photovoltaics? *Nat Mater*, **5**(3), 161-164.

Benavente-García, O., Castillo, J., Lorente, J., Ortuño, A., Del Rio, J.A. 2000. Antioxidant activity of phenolics extracted from Olea europaea L. leaves. *Food Chemistry*, **68**(4), 457-462.

Benoit, I., Navarro, D., Marnet, N., Rakotomanomana, N., Lesage-Meessen, L., Sigoillot, J.-C., Asther, M., Asther, M. 2006. Feruloyl esterases as a tool for the release of phenolic compounds from agro-industrial by-products. *Carbohydrate Research*, **341**(11), 1820-1827.

Besle, J.-M., Cornu, A., Jouany, J.-P. 1994. Roles of structural phenylpropanoids in forage cell wall digestion. *Journal of the Science of Food and Agriculture*, **64**(2), 171-190.

Bhat, M.K., Hazlewood, G.P. 2000. Enzymology and other characteristics of cellulases and xylanases, CABI Publishing. Wallingford, pp. 11-60.

Biely, P. 1985. Microbial xylanolytic systems. *Trends in Biotechnology*, **3**(11), 286-290.

Biely, P., Puls, J., Schneider, H. 1985. Acetyl xylan esterases in fungal cellulolytic systems. *FEBS Letters*, **186**(1), 80-84.

Biller, P., Friedman, C., Ross, A.B. 2013. Hydrothermal microwave processing of microalgae as a pre-treatment and extraction technique for bio-fuels and bio-products. *Bioresource Technology*, **136**, 188-195.

Binder, J.B., Raines, R.T. 2009. Simple chemical transformation of lignocellulosic biomass into furans for fuels and chemicals. *Journal of the American Chemical Society*, **131**(5), 1979-1985.

Binod, P., Sindhu, R., Singhania, R.R., Vikram, S., Devi, L., Nagalakshmi, S., Kurien, N., Sukumaran, R.K., Pandey, A. 2010. Bioethanol production from rice straw: An overview. *Bioresource Technology*, **101**(13), 4767-4774.

Blair, J.M. 1976. Control of oil.

Blakeney, A.B., Harris, P.J., Henry, R.J., Stone, B.A. 1983. A simple and rapid preparation of alditol acetates for monosaccharide analysis. *Carbohydrate Research*, **113**(2), 291-299.

Bozell, J.J. 2001. Chemicals and Materials from Renewable Resources. in: *Chemicals and Materials from Renewable Resources*, Vol. 784, American Chemical Society, pp. 1-9.

Bozell, J.J., Petersen, G.R. 2010. Technology development for the production of biobased products from biorefinery carbohydrates-the US Department of Energy's "Top 10" revisited. *Green Chemistry*, **12**(4), 539-554.

Brett, C.T., Waldron, K.W. 1996a. *Physiology and biochemistry of plant cell walls*. Chapman & Hall Ltd, London.

Brett, C.T., Waldron, K.W. 1996b. *Physiology and biochemistry of plant cell walls*. Springer Science & Business Media.

Bro, R., Smilde, A.K. 2014. Principal component analysis. *Analytical Methods*, **6**(9), 2812-2831.

Brummell, D.A. 2006. Cell wall disassembly in ripening fruit. *Functional Plant Biology*, **33**(2), 103-119.

Bunzel, M., Ralph, J., Lu, F., Hatfield, R.D., Steinhart, H. 2004. Lignins and ferulate-coniferyl alcohol cross-coupling products in cereal grains. *Journal of Agricultural and Food Chemistry*, **52**(21), 6496-6502.

Cadoche, L., López, G.D. 1989. Assessment of size reduction as a preliminary step in the production of ethanol from lignocellulosic wastes. *Biological Wastes*, **30**(2), 153-157.

Cantarella, M., Cantarella, L., Gallifuoco, A., Spera, A., Alfani, F. 2004. Effect of Inhibitors Released during Steam-Explosion Treatment of Poplar Wood on Subsequent Enzymatic Hydrolysis and SSF. *Biotechnology Progress*, **20**(1), 200-206.

Carlozzi, P., Buccioni, A., Minieri, S., Pushparaj, B., Piccardi, R., Ena, A., Pintucci, C. 2010. Production of bio-fuels (hydrogen and lipids) through a photofermentation process. *Bioresource Technology*, **101**(9), 3115-3120.

Chandel, A.K., Chandrasekhar, G., Silva, M.B., Silvério da Silva, S. 2012. The realm of cellulases in biorefinery development. *Critical Reviews in Biotechnology*, **32**(3), 187-202.

Chandel, A.K., Singh, O.V., da Silva, S.S. 2011. Detoxification of lignocellulosic hydrolysates for improved bioethanol production. in: *Biofuel Production-Recent Developments and Prospects*, (Ed.) M.A.D.S. Bernardes, InTech.

Chandra, R.P., Bura, R., Mabee, W.E., Berlin, A., Pan, X., Saddler, J.N. 2007. Substrate Pretreatment: The Key to Effective Enzymatic Hydrolysis of Lignocellulosics? in: *Biofuels*, (Ed.) L. Olsson, Springer Berlin Heidelberg. Berlin, Heidelberg, pp. 67-93.

Chang, V.S., Holtzapple, M.T. 2000. Fundamental Factors Affecting Biomass Enzymatic Reactivity. in: *Twenty-First Symposium on Biotechnology for Fuels and Chemicals: Proceedings of the Twenty-First Symposium on Biotechnology for Fuels and Chemicals Held May 2–6, 1999, in Fort Collins, Colorado*, (Eds.) M. Finkelstein, B.H. Davison, Humana Press. Totowa, NJ, pp. 5-37.

Chaturvedi, V., Verma, P. 2013. An overview of key pretreatment processes employed for bioconversion of lignocellulosic biomass into biofuels and value added products. *3 Biotech*, **3**(5), 415-431.

Chen, S., Zhang, X., Deepak, S., Yu, H., Yang, X. 2010. Biological pretreatment of lignocellulosics: potential, progress and challenges. *Biofuels*, **1**(1), 177-199.

Cheng, J.J., Stomp, A.M. 2009a. Growing duckweed to recover nutrients from wastewaters and for production of fuel ethanol and animal feed. *Clean–Soil, Air, Water*, **37**(1), 17-26.

Cheng, J.J., Stomp, A.M. 2009b. Growing duckweed to recover nutrients from wastewaters and for production of fuel ethanol and animal feed. *CLEAN - Soil, Air, Water*, **37**(1), 17-26.

Cheng, Y., Xu, Q., Liu, J., Zhao, C., Xue, F., Zhao, Y. 2014. Decomposition of five phenolic compounds in high temperature water. *Journal of the Brazilian Chemical Society*, **25**(11), 2102-2107.

Cho, D.H., Lee, Y.J., Um, Y., Sang, B.-I., Kim, Y.H. 2009. Detoxification of model phenolic compounds in lignocellulosic hydrolysates with peroxidase for butanol production from Clostridium beijerinckii. *Applied Microbiology and Biotechnology*, **83**(6), 1035-1043.

Chundawat, S.P.S., Venkatesh, B., Dale, B.E. 2007. Effect of particle size based separation of milled corn stover on AFEX pretreatment and enzymatic digestibility. *Biotechnology and Bioengineering*, **96**(2), 219-231.

Chung, I.S., Lee, Y.Y. 1985. Ethanol fermentation of crude acid hydrolyzate of cellulose using high-level yeast inocula. *Biotechnology and Bioengineering*, **27**(3), 308-315.

Collins, S.R.A., Wellner, N., Bordonado, I.M., Harper, A.L., Miller, C.N., Bancroft, I., Waldron, K.W. 2014. Variation in the chemical composition of wheat straw: the role of tissue ratio and composition. *Biotechnology for Biofuels*, **7**.

Cull, S.G., Holbrey, J.D., Vargas-Mora, V., Seddon, K.R., Lye, G.J. 2000. Room-temperature ionic liquids as replacements for organic solvents in multiphase bioprocess operations. *Biotechnology and Bioengineering*, **69**(2), 227-233.

Cvjetko Bubalo, M., Radošević, K., Radojčić Redovniković, I., Halambek, J., Gaurina Srček, V. 2014. A brief overview of the potential environmental hazards of ionic liquids. *Ecotoxicology and Environmental Safety*, **99**, 1-12.

D'andola, G., Szarvas, L., Massonne, K., Stegmann, V. 2011. Ionic liquids for solubilizing polymers, Google Patents.

Dahnum, D., Tasum, S.O., Triwahyuni, E., Nurdin, M., Abimanyu, H. 2015. Comparison of SHF and SSF Processes Using Enzyme and Dry Yeast for

Optimization of Bioethanol Production from Empty Fruit Bunch. *Energy Procedia*, **68**, 107-116.

David, N., Shiraishi, N. 2001. Wood and cellulosic chemistry. *New York and Basel*.

Davies, R., Falkiner, E.A., Wilkinson, J.F. and Peel, J.L. 1951. Ester formation by yeast. 1. Ethyl acetate formation by Hansenula species. *Biochemcal Journal*, **49**(1), 58-61.

Delgenes, J.P., Moletta, R., Navarro, J.M. 1996. Effects of lignocellulose degradation products on ethanol fermentations of glucose and xylose by Saccharomyces cerevisiae, Zymomonas mobilis, Pichia stipitis, and Candida shehatae. *Enzyme and Microbial Technology*, **19**(3), 220-225.

Donohoe, B.S., Decker, S.R., Tucker, M.P., Himmel, M.E., Vinzant, T.B. 2008. Visualizing lignin coalescence and migration through maize cell walls following thermochemical pretreatment. *Biotechnology and Bioengineering*, **101**(5), 913-925.

Doran, J.B., Cripe, J., Sutton, M., Foster, B. 2000. Fermentations of Pectin-Rich Biomass with Recombinant Bacteria to Produce Fuel Ethanol. in: *Twenty-First Symposium on Biotechnology for Fuels and Chemicals: Proceedings of the Twenty-First Symposium on Biotechnology for Fuels and Chemicals Held May 2–6, 1999, in Fort Collins, Colorado*, (Eds.) M. Finkelstein, B.H. Davison, Humana Press. Totowa, NJ, pp. 141-152.

Du, W., Yu, H., Song, L., Zhang, J., Weng, C., Ma, F., Zhang, X. 2011. The promoting effect of byproducts from Irpex lacteus on subsequent enzymatic hydrolysis of bio-pretreated cornstalks. *Biotechnology for Biofuels*, **4**(1), 37.

Dutta, K., Daverey, A., Lin, J.-G. 2014. Evolution retrospective for alternative fuels: First to fourth generation. *Renewable Energy*, **69**, 114-122.

Ebringerová, A., Hromádková, Z., Heinze, T. 2005. Hemicellulose. in: *Polysaccharides I: Structure, Characterization and Use*, (Ed.) T. Heinze, Springer Berlin Heidelberg. Berlin, Heidelberg, pp. 1-67.

Ellabban, O., Abu-Rub, H., Blaabjerg, F. 2014. Renewable energy resources: Current status, future prospects and their enabling technology. *Renewable and Sustainable Energy Reviews*, **39**, 748-764.

Eriksson, T., Börjesson, J., Tjerneld, F. 2002a. Mechanism of surfactant effect in enzymatic hydrolysis of lignocellulose. *Enzyme and Microbial Technology*, **31**(3), 353-364.

Eriksson, T., Karlsson, J., Tjerneld, F. 2002b. A model explaining declining rate in hydrolysis of lignocellulose substrates with cellobiohydrolase I (Cel7A) and endoglucanase I (Cel7B) of Trichoderma reesei. *Applied Biochemistry and Biotechnology*, **101**(1), 41-60.

Espinoza-Acosta, J.L., Torres-Chávez, P.I., Carvajal-Millán, E., Ramírez-Wong, B., Bello-Pérez, L.A., Montaño-Leyva, B. 2014. *Ionic Liquids and Organic Solvents for Recovering Lignin from Lignocellulosic Biomass*.

Fan, L.T., Lee, Y.H., Beardmore, D.R. 1981. The influence of major structural features of cellulose on rate of enzymatic hydrolysis. *Biotechnology and Bioengineering*, **23**(2), 419-424.

Fengel, D., Wegener, G. 1984. *Wood: chemistry, ultrastructure, reactions*. Walter de Gruyter.

Fernandez-Ricaud, L., Kourtchenko, O., Zackrisson, M., Warringer, J., Blomberg, A. 2016. PRECOG: a tool for automated extraction and visualization of fitness components in microbial growth phenomics. *BMC Bioinformatics*, **17**(1), 249.

Field, S.J., Ryden, P., Wilson, D., James, S.A., Roberts, I.N., Richardson, D.J., Waldron, K.W., Clarke, T.A. 2015a. Identification of furfural resistant strains of Saccharomyces cerevisiae and Saccharomyces paradoxus from a collection of environmental and industrial isolates. *Biotechnology for Biofuels*, **8**.

Field, S.J., Ryden, P., Wilson, D., James, S.A., Roberts, I.N., Richardson, D.J., Waldron, K.W., Clarke, T.A. 2015b. Identification of furfural resistant strains of Saccharomyces cerevisiae and Saccharomyces paradoxus from a collection of environmental and industrial isolates. *Biotechnology for Biofuels*, **8**(1), 33.

Figueiredo, A.R., Campos, F., de Freitas, V., Hogg, T., Couto, J.A. 2008. Effect of phenolic aldehydes and flavonoids on growth and inactivation of Oenococcus oeni and Lactobacillus hilgardii. *Food Microbiology*, **25**(1), 105-112.

FitzPatrick, M., Champagne, P., Cunningham, M.F., Whitney, R.A. 2010. A biorefinery processing perspective: treatment of lignocellulosic materials for the production of value-added products. *Bioresource technology*, **101**(23), 8915-8922.

Fredlund, E., Blank, L.M., Schnürer, J., Sauer, U., Passoth, V. 2004. Oxygen- and glucose-dependent regulation of central carbon metabolism in Pichia anomala. *Applied and environmental microbiology*, **70**(10), 5905-5911.

Fu, C., Mielenz, J.R., Xiao, X., Ge, Y., Hamilton, C.Y., Rodriguez, M., Chen, F., Foston, M., Ragauskas, A., Bouton, J., Dixon, R.A., Wang, Z.-Y. 2011. Genetic manipulation of lignin reduces recalcitrance and improves ethanol production from switchgrass. *Proceedings of the National Academy of Sciences*, **108**(9), 3803-3808.

Fu, D., Mazza, G. 2011. Optimization of processing conditions for the pretreatment of wheat straw using aqueous ionic liquid. *Bioresource Technology*, **102**(17), 8003-8010.

Galbe, M., Zacchi, G. 2007. Pretreatment of Lignocellulosic Materials for Efficient Bioethanol Production. in: *Biofuels*, (Ed.) L. Olsson, Springer Berlin Heidelberg. Berlin, Heidelberg, pp. 41-65.

García-Aparicio, M.P., Ballesteros, I., González, A., Oliva, J.M., Ballesteros, M., Negro, M.J. 2006. Effect of inhibitors released during steam-explosion pretreatment of barley straw on enzymatic hydrolysis. *Applied Biochemistry and Biotechnology*, **129**(1), 278-288.

Gírio, F.M., Fonseca, C., Carvalheiro, F., Duarte, L.C., Marques, S., Bogel-Łukasik, R. 2010. Hemicelluloses for fuel ethanol: A review. *Bioresource Technology*, **101**(13), 4775-4800.

Gollapalli, L.E., Dale, B.E., Rivers, D.M. 2002. Predicting digestibility of ammonia fiber explosion (AFEX)-treated rice straw. *Applied Biochemistry and Biotechnology*, **98**(1), 23-35.

González-Bautista, E., Santana-Morales, J.C., Ríos-Fránquez, F.J., Poggi-Varaldo, H.M., Ramos-Valdivia, A.C., Cristiani-Urbina, E., Ponce-Noyola, T. 2017. Phenolic compounds inhibit cellulase and xylanase activities of Cellulomonas flavigena PR-22 during saccharification of sugarcane bagasse. *Fuel*, **196**(Supplement C), 32-35.

Guimarães, P.M.R., Teixeira, J.A., Domingues, L. 2010. Fermentation of lactose to bio-ethanol by yeasts as part of integrated solutions for the valorisation of cheese whey. *Biotechnology Advances*, **28**(3), 375-384.

Gupta, K.K., Rehman, A., Sarviya, R.M. 2010. Bio-fuels for the gas turbine: A review. *Renewable and Sustainable Energy Reviews*, **14**(9), 2946-2955.

Hahn-Hägerdal, B., Galbe, M., Gorwa-Grauslund, M.F., Lidén, G., Zacchi, G. 2006. Bio-ethanol – the fuel of tomorrow from the residues of today. *Trends in Biotechnology*, **24**(12), 549-556.

Harmsen, P., Huijgen, W., Bermudez, L., Bakker, R. 2010. Literature review of physical and chemical pretreatment processes for lignocellulosic biomass. Wageningen UR Food & Biobased Research. 9085857570.

Hasunuma, T., Okazaki, F., Okai, N., Hara, K.Y., Ishii, J., Kondo, A. 2013. A review of enzymes and microbes for lignocellulosic biorefinery and the possibility of their application to consolidated bioprocessing technology. *Bioresource technology*, **135**, 513-522.

Hawthorne, S.B., Grabanski, C.B., Martin, E., Miller, D.J. 2000. Comparisons of Soxhlet extraction, pressurized liquid extraction, supercritical fluid extraction and subcritical water extraction for environmental solids: recovery, selectivity and effects on sample matrix. *Journal of Chromatography A*, **892**(1), 421-433.

Hayashida, S., Ohta, K., Flor, P.Q., Nanri, N., Miyahara, I. 1982. High Concentration—Ethanol Fermentation of Raw Ground Corn. *Agricultural and Biological Chemistry*, **46**(7), 1947-1950.

Hendriks, A.T.W.M., Zeeman, G. 2009. Pretreatments to enhance the digestibility of lignocellulosic biomass. *Bioresource Technology*, **100**(1), 10-18.

Himmel, M.E., Ding, S.-Y., Johnson, D.K., Adney, W.S., Nimlos, M.R., Brady, J.W., Foust, T.D. 2007. Biomass Recalcitrance: Engineering Plants and Enzymes for Biofuels Production. *Science*, **315**(5813), 804-807.

Hoch, G. 2007. Cell wall hemicelluloses as mobile carbon stores in non-reproductive plant tissues. *Functional Ecology*, **21**(5), 823-834.

Holopainen-Mantila, U., Marjamaa, K., Merali, Z., Käsper, A., de Bot, P., Jääskeläinen, A.-S., Waldron, K., Kruus, K., Tamminen, T. 2013. Impact of hydrothermal pre-treatment to chemical composition, enzymatic digestibility and spatial distribution of cell wall polymers. *Bioresource Technology*, **138**(Supplement C), 156-162.

Holtzapple, M.T., Humphrey, A.E. 1984. The effect of organosolv pretreatment on the enzymatic hydrolysis of poplar. *Biotechnology and Bioengineering*, **26**(7), 670-676.

Holtzapple, M.T., Jun, J.-H., Ashok, G., Patibandla, S.L., Dale, B.E. 1991. The ammonia freeze explosion (AFEX) process. *Applied Biochemistry and Biotechnology*, **28**(1), 59-74.

Horváth, I.S., Sjöde, A., Alriksson, B., Jönsson, L.J., Nilvebrant, N.-O. 2005. Critical Conditions for Improved Fermentability During Overliming of Acid Hydrolysates from Spruce. in: *Twenty-Sixth Symposium on Biotechnology for Fuels and Chemicals*, (Eds.) B.H. Davison, B.R. Evans, M. Finkelstein, J.D. McMillan, Humana Press. Totowa, NJ, pp. 1031-1044.

Hou, J.J., Ding, C., Qiu, Z., Zhang, Q.Z., Xiang, W.N. 2017. Inhibition efficiency evaluation of lignocellulose-derived compounds for bioethanol production. *Journal of Cleaner Production*, **165**, 1107-1114.

Houghton, J.T., Ding, Y., Griggs, D.J., Noguer, M., van der Linden, P.J., Dai, X., Maskell, K., Johnson, C. 2001. *Climate change 2001: the scientific basis*. The Press Syndicate of the University of Cambridge.

Huang, C.-F., Lin, T.-H., Guo, G.-L., Hwang, W.-S. 2009. Enhanced ethanol production by fermentation of rice straw hydrolysate without detoxification using a newly adapted strain of Pichia stipitis. *Bioresource Technology*, **100**(17), 3914-3920.

Huang, H.-J., Ramaswamy, S., Tschirner, U.W., Ramarao, B.V. 2008. A review of separation technologies in current and future biorefineries. *Separation and Purification Technology*, **62**(1), 1-21.

Huang, H.J., Ramaswamy, S. 2013. Overview of biomass conversion processes and separation and purification technologies in biorefineries. in: *Separation and Purification Technologies in Biorefineries*, John Wiley and Sons.

Hughes, S.R., Gibbons, W.R., Bang, S.S., Pinkelman, R., Bischoff, K.M., Slininger, P.J., Qureshi, N., Kurtzman, C.P., Liu, S.Q., Saha, B.C., Jackson, J.S., Cotta, M.A., Rich, J.O., Javers, J.E. 2012. Random UV-C mutagenesis of Scheffersomyces (formerly Pichia) stipitis NRRL Y-7124 to improve anaerobic growth on lignocellulosic sugars. *Journal of Industrial Microbiology & Biotechnology*, **39**(1), 163-173.

Hui, L., Wan, C., Hai-tao, D., Xue-jiao, C., Qi-fa, Z., Yu-hua, Z. 2010. Direct microbial conversion of wheat straw into lipid by a cellulolytic fungus of Aspergillus oryzae A-4 in solid-state fermentation. *Bioresource Technology*, **101**(19), 7556-7562.

Hutnan, M., Drtil, M., Mrafkova, L. 2000. Anaerobic biodegradation of sugar beet pulp. *Biodegradation*, **11**(4), 203-211.

Imai, T., Ohno, T. 1995. The relationship between viability and intracellular pH in the yeast Saccharomyces cerevisiae. *Applied and Environmental Microbiology*, **61**(10), 3604-3608.

Ishizawa, C.I., Davis, M.F., Schell, D.F., Johnson, D.K. 2007. Porosity and Its Effect on the Digestibility of Dilute Sulfuric Acid Pretreated Corn Stover. *Journal of Agricultural and Food Chemistry*, **55**(7), 2575-2581.

Jayani, R.S., Saxena, S., Gupta, R. 2005. Microbial pectinolytic enzymes: A review. *Process Biochemistry*, **40**(9), 2931-2944.

Jönsson, L.J., Alriksson, B., Nilvebrant, N.-O. 2013. Bioconversion of lignocellulose: inhibitors and detoxification. *Biotechnology for Biofuels*, **6**(1), 16.

Jönsson, L.J., Martín, C. 2016. Pretreatment of lignocellulose: Formation of inhibitory by-products and strategies for minimizing their effects. *Bioresource Technology*, **199**, 103-112.

Kádár, Z., Szengyel, Z., Réczey, K. 2004. Simultaneous saccharification and fermentation (SSF) of industrial wastes for the production of ethanol. *Industrial Crops and Products*, **20**(1), 103-110.

Kellock, M., Rahikainen, J., Marjamaa, K., Kruus, K. 2017. Lignin-derived inhibition of monocomponent cellulases and a xylanase in the hydrolysis of lignocellulosics. *Bioresource Technology*, **232**(Supplement C), 183-191.

Keweloh, H., Weyrauch, G., Rehm, H.-J. 1990. Phenol-induced membrane changes in free and immobilized Escherichia coli. *Applied Microbiology and Biotechnology*, **33**(1), 66-71.

Khaleghian, H., Molaverdi, M., Karimi, K. 2017. Silica Removal from Rice Straw To Improve its Hydrolysis and Ethanol Production. *Industrial & Engineering Chemistry Research*, **56**(35), 9793-9798.

Kim, S., Dale, B.E. 2004. Global potential bioethanol production from wasted crops and crop residues. *Biomass and Bioenergy*, **26**(4), 361-375.

Kim, Y., Ximenes, E., Mosier, N.S., Ladisch, M.R. 2011. Soluble inhibitors/deactivators of cellulase enzymes from lignocellulosic biomass. *Enzyme and Microbial Technology*, **48**(4), 408-415.

King, A.M.Y., Young, G. 1999. Characteristics and Occurrence of Phenolic Phytochemicals. *Journal of the American Dietetic Association*, **99**(2), 213-218.

Koganti, S., Ju, L.-K. 2013. Debaryomyces hansenii fermentation for arabitol production. *Biochemical Engineering Journal*, **79**, 112-119.

Koo, B.-W., Kim, H.-Y., Park, N., Lee, S.-M., Yeo, H., Choi, I.-G. 2011. Organosolv pretreatment of Liriodendron tulipifera and simultaneous saccharification and fermentation for bioethanol production. *Biomass and Bioenergy*, **35**(5), 1833-1840.

Kordowska-Wiater, M. 2015. Production of arabitol by yeasts: current status and future prospects. *Journal of applied microbiology*, **119**(2), 303-314.

Kristensen, J.B., Thygesen, L.G., Felby, C., Jørgensen, H., Elder, T. 2008. Cell-wall structural changes in wheat straw pretreated for bioethanol production. *Biotechnology for Biofuels*, **1**(1), 5.

Kucner, A., Papiewska, A., Klewicki, R., Sójka, M., Klewicka, E. 2014. Infl uence of thermal treatment on the stability of phenolic compounds and the microbiological quality of sucrose solution following osmotic dehydration of highbush blueberry fruits. *Acta Scientiarum Polonorum Technologia Alimentaria*, **13**(1), 79-88.

Kuhad, R.C., Singh, A. 1993. Lignocellulose Biotechnology: Current and Future Prospects. *Critical Reviews in Biotechnology*, **13**(2), 151-172.

Kumar, P., Barrett, D.M., Delwiche, M.J., Stroeve, P. 2009. Methods for pretreatment of lignocellulosic biomass for efficient hydrolysis and biofuel production. *Industrial & engineering chemistry research*, **48**(8), 3713-3729.

Kumar, R., Singh, S., Singh, O.V. 2008. Bioconversion of lignocellulosic biomass: biochemical and molecular perspectives. *Journal of Industrial Microbiology & Biotechnology*, **35**(5), 377-391.

Kumdam, H., Narayana Murthy, S., Gummadi, S.N. 2013. Production of ethanol and arabitol by Debaryomyces nepalensis: influence of process parameters. *AMB Express*, **3**(1), 23.

Kurita, O. 2008. Increase of acetate ester-hydrolysing esterase activity in mixed cultures of Saccharomyces cerevisiae and Pichia anomala. *Journal of Applied Microbiology*, **104**(4), 1051-1058.

Kurtzman, C.P., Fell, J.W. 2006. Yeast Systematics and Phylogeny — Implications of Molecular Identification Methods for Studies in Ecology. in: *Biodiversity and Ecophysiology of Yeasts*, (Eds.) G. Péter, C. Rosa, Springer Berlin Heidelberg. Berlin, Heidelberg, pp. 11-30.

Larsson, S., Cassland, P., Jönsson, L.J. 2001. Development of a Saccharomyces cerevisiae strain with enhanced resistance to phenolic fermentation inhibitors in lignocellulose hydrolysates by heterologous expression of laccase. *Applied and environmental microbiology*, **67**(3), 1163-1170.

Larsson, S., Palmqvist, E., Hahn-Hägerdal, B., Tengborg, C., Stenberg, K., Zacchi, G., Nilvebrant, N.-O. 1999. The generation of fermentation inhibitors during dilute acid hydrolysis of softwood. *Enzyme and Microbial Technology*, **24**(3), 151-159.

Larsson, S., Quintana-Sáinz, A., Reimann, A., Nilvebrant, N.-O., Jönsson, L.J. 2000. Influence of Lignocellulose-Derived Aromatic Compounds on Oxygen-Limited Growth and Ethanolic Fermentation by Saccharomyces cerevisiae. in: *Twenty-First Symposium on Biotechnology for Fuels and Chemicals: Proceedings of the Twenty-First Symposium on Biotechnology for Fuels and Chemicals Held May 2–6, 1999, in Fort Collins, Colorado*, (Eds.) M. Finkelstein, B.H. Davison, Humana Press. Totowa, NJ, pp. 617-632.

Laser, M., Schulman, D., Allen, S.G., Lichwa, J., Antal, M.J., Lynd, L.R. 2002. A comparison of liquid hot water and steam pretreatments of sugar cane bagasse for bioconversion to ethanol. *Bioresource Technology*, **81**(1), 33-44.

Lattanzio, V., Lattanzio, V.M., Cardinali, A. 2006. Role of phenolics in the resistance mechanisms of plants against fungal pathogens and insects. *Phytochemistry: Advances in research*, **661**, 23-67.

Laureano-Perez, L., Dale, B.E., O'Dwyer, J.P., Holtzapple, M. 2006. Statistical Correlation of Spectroscopic Analysis and Enzymatic Hydrolysis of Poplar Samples. *Biotechnology Progress*, **22**(3), 835-841.

Laureano-Perez, L., Teymouri, F., Alizadeh, H., Dale, B.E. 2005. Understanding factors that limit enzymatic hydrolysis of biomass. *Applied Biochemistry and Biotechnology*, **124**(1), 1081-1099.

Lee, H., To, R.J., Latta, R.K., Biely, P., Schneider, H. 1987. Some Properties of Extracellular Acetylxylan Esterase Produced by the Yeast Rhodotorula mucilaginosa. *Applied and environmental microbiology*, **53**(12), 2831-2834.

Lee, J. 1997. Biological conversion of lignocellulosic biomass to ethanol. *Journal of Biotechnology*, **56**(1), 1-24.

Legras, J.L., Merdinoglu, D., Cornuet, J.M., Karst, F. 2007. Bread, beer and wine: Saccharomyces cerevisiae diversity reflects human history. *Mol Ecol*, **16**(10), 2091-102.

Lesage-Meessen, L., Lomascolo, A., Bonnin, E., Thibault, J.F., Buleon, A., Roller, M., Record, E., Ceccaldi, B.C., Asther, M. 2002. A biotechnological process involving filamentous fungi to produce natural crystalline vanillin from maize bran. *Applied Biochemistry and Biotechnology - Part A Enzyme Engineering and Biotechnology*, **102-103**, 141-153.

Leung, D.Y.C., Wu, X., Leung, M.K.H. 2010. A review on biodiesel production using catalyzed transesterification. *Applied Energy*, **87**(4), 1083-1095.

Li, M., Liu, G.-L., Chi, Z., Chi, Z.-M. 2010. Single cell oil production from hydrolysate of cassava starch by marine-derived yeast Rhodotorula mucilaginosa TJY15a. *Biomass and Bioenergy*, **34**(1), 101-107.

Liew, W.H., Hassim, M.H., Ng, D.K.S. 2014. Review of evolution, technology and sustainability assessments of biofuel production. *Journal of Cleaner Production*, **71**, 11-29.

Lim, J.S., Abdul Manan, Z., Wan Alwi, S.R., Hashim, H. 2012. A review on utilisation of biomass from rice industry as a source of renewable energy. *Renewable and Sustainable Energy Reviews*, **16**(5), 3084-3094.

Lin, L., Yan, R., Liu, Y., Jiang, W. 2010. In-depth investigation of enzymatic hydrolysis of biomass wastes based on three major components: Cellulose, hemicellulose and lignin. *Bioresource Technology*, **101**(21), 8217-8223.

Lin, Y., Tanaka, S. 2006. Ethanol fermentation from biomass resources: current state and prospects. *Applied Microbiology and Biotechnology*, **69**(6), 627-642.

Liu, C.-Z., Wang, F., Stiles, A.R., Guo, C. 2012. Ionic liquids for biofuel production: Opportunities and challenges. *Applied Energy*, **92**, 406-414.

Liu, C., Wyman, C.E. 2003. The Effect of Flow Rate of Compressed Hot Water on Xylan, Lignin, and Total Mass Removal from Corn Stover. *Industrial & Engineering Chemistry Research*, **42**(21), 5409-5416.

Liu, H., Zhang, S., Zhang, X., Chen, C. 2015. Growth inhibition and effect on photosystem by three imidazolium chloride ionic liquids in rice seedlings. *Journal of Hazardous Materials*, **286**, 440-448.

Liu, Y., Shi, J., Langrish, T.A.G. 2006. Water-based extraction of pectin from flavedo and albedo of orange peels. *Chemical Engineering Journal*, **120**(3), 203-209.

Liu, Z.-H., Qin, L., Pang, F., Jin, M.-J., Li, B.-Z., Kang, Y., Dale, B.E., Yuan, Y.-J. 2013. Effects of biomass particle size on steam explosion pretreatment performance for improving the enzyme digestibility of corn stover. *Industrial Crops and Products*, **44**, 176-184.

Liu, Z.L., Slininger, P.J., Dien, B.S., Berhow, M.A., Kurtzman, C.P., Gorsich, S.W. 2004. Adaptive response of yeasts to furfural and 5-hydroxymethylfurfural and new chemical evidence for HMF conversion to 2,5-bis-hydroxymethylfuran. *Journal of Industrial Microbiology and Biotechnology*, **31**(8), 345-352.

Löser, C., Urit, T., Keil, P., Bley, T. 2015. Studies on the mechanism of synthesis of ethyl acetate in Kluyveromyces marxianus DSM 5422. *Applied Microbiology and Biotechnology*, **99**(3), 1131-1144.

Ludueña, L., Fasce, D., Alvarez, V.A., Stefani, P.M. 2011. Nanocellulose from rice husk following alkaline treatment to remove silica. *BioResources*, **6**(2), 1440-1453.

Lynd, L.R., Zyl, W.H.v., McBride, J.E., Laser, M. 2005. Consolidated bioprocessing of cellulosic biomass: an update. *Current Opinion in Biotechnology*, **16**(5), 577-583.

Mackie, K.L., Brownell, H.H., West, K.L., Saddler, J.N. 1985. Effect of Sulphur Dioxide and Sulphuric Acid on Steam Explosion of Aspenwood. *Journal of Wood Chemistry and Technology*, **5**(3), 405-425.

Mäki-Arvela, P., Anugwom, I., Virtanen, P., Sjöholm, R., Mikkola, J.P. 2010. Dissolution of lignocellulosic materials and its constituents using ionic liquids—A review. *Industrial Crops and Products*, **32**(3), 175-201.

Mallakpour, S., Kolahdoozan, M. 2008. Room Temperature Ionic Liquids as Replacements for Organic Solvents: Direct Preparation of Wholly Aromatic Polyamides Containing Phthalimide and S-valine Moieties. *Polymer Journal*, **40**, 513.

Manach, C., Mazur, A., Scalbert, A. 2005. Polyphenols and prevention of cardiovascular diseases. *Current Opinion in Lipidology*, **16**(1), 77-84.

Mansfield, S.D., Mooney, C., Saddler, J.N. 1999. Substrate and Enzyme Characteristics that Limit Cellulose Hydrolysis. *Biotechnology Progress*, **15**(5), 804-816.

Mansouri, N.-E.E., Salvadó, J. 2006. Structural characterization of technical lignins for the production of adhesives: Application to lignosulfonate, kraft, soda-anthraquinone, organosolv and ethanol process lignins. *Industrial Crops and Products*, **24**(1), 8-16.

Martiniano, S.E., Chandel, A.K., Soares, L.C.S.R., Pagnocca, F.C., da Silva, S.S. 2013. Evaluation of novel xylose-fermenting yeast strains from Brazilian forests for hemicellulosic ethanol production from sugarcane bagasse. *3 Biotech*, **3**(5), 345-352.

McKendry, P. 2002. Energy production from biomass (part 1): overview of biomass. *Bioresource Technology*, **83**(1), 37-46.

McMillan, J.D. 1994. Pretreatment of Lignocellulosic Biomass. in: *Enzymatic Conversion of Biomass for Fuels Production*, Vol. 566, American Chemical Society, pp. 292-324.

Merali, Z., Ho, J.D., Collins, S.R.A., Le Gall, G., Elliston, A., Kasper, A., Waldron, K.W. 2013. Characterization of cell wall components of wheat straw following hydrothermal pretreatment and fractionation. *Bioresource Technology*, **131**, 226-234.

Merali, Z., Marjamaa, K., Kasper, A., Kruus, K., Gunning, A.P., Morris, V.J., Waldron, K.W. 2016. Chemical characterization of hydrothermally pretreated and enzyme-digested wheat straw: An evaluation of recalcitrance. *Food Chemistry*, **198**, 132-140.

Middleton, E., Kandaswami, C., Theoharides, T.C. 2000. The Effects of Plant Flavonoids on Mammalian Cells:Implications for Inflammation, Heart Disease, and Cancer. *Pharmacological Reviews*, **52**(4), 673-751.

Miguel, A.n.S.M., Martins-Meyer, T.S., Figueiredo, E.r.V.s.d.C., Lobo, B.W.P., Dellamora-Ortiz, G.M. 2013. Enzymes in Bakery: Current and Future Trends. in: *Food Industry*, (Ed.) I. Muzzalupo, InTech. Rijeka, pp. Ch. 14.

Morrison, J.M., Elshahed, M.S., Youssef, N.H. 2016. Defined enzyme cocktail from the anaerobic fungus Orpinomyces sp. strain C1A effectively releases sugars from pretreated corn stover and switchgrass. *Scientific Reports*, **6**, 29217.

Mosier, N., Wyman, C., Dale, B., Elander, R., Lee, Y.Y., Holtzapple, M., Ladisch, M. 2005. Features of promising technologies for pretreatment of lignocellulosic biomass. *Bioresource Technology*, **96**(6), 673-686.

Muller, M., Yelden, T., Schoonover, H. 2007. Food versus fuel in the United States: can both win in the era of ethanol? *Institute for Agriculture and Trade Policy*. .

Muthayya, S., Sugimoto, J.D., Montgomery, S., Maberly, G.F. 2014. An overview of global rice production, supply, trade, and consumption. *Annals of the New York Academy of Sciences*, **1324**(1), 7-14.

Nielsen, M., Junge, H., Kammer, A., Beller, M. 2012. Towards a green process for bulk-scale synthesis of ethyl acetate: efficient acceptorless dehydrogenation of ethanol. *Angewandte Chemie (International ed. in English)*, **51**(23), 5711-5713.

Nigam, P.S., Singh, A. 2011. Production of liquid biofuels from renewable resources. *Progress in energy and combustion science*, **37**(1), 52-68.

Nobre, M.F., Costa, M.S.d. 1985. Factors favouring the accumulation of arabinitol in the yeast Debaryomyces hansenii. *Canadian journal of microbiology*, **31**(5), 467-471.

Nuwamanya, E., Chiwona-Karltun, L., Kawuki, R.S., Baguma, Y. 2012. Bio-Ethanol Production from Non-Food Parts of Cassava (Manihot esculenta Crantz). *AMBIO*, **41**(3), 262-270.

Octave, S., Thomas, D. 2009. Biorefinery: Toward an industrial metabolism. *Biochimie*, **91**(6), 659-664.

Öhgren, K., Bura, R., Lesnicki, G., Saddler, J., Zacchi, G. 2007a. A comparison between simultaneous saccharification and fermentation and separate hydrolysis and fermentation using steam-pretreated corn stover. *Process Biochemistry*, **42**(5), 834-839.

Öhgren, K., Bura, R., Saddler, J., Zacchi, G. 2007b. Effect of hemicellulose and lignin removal on enzymatic hydrolysis of steam pretreated corn stover. *Bioresource Technology*, **98**(13), 2503-2510.

Overend, R.P., Chornet, E., Gascoigne, J.A. 1987. Fractionation of Lignocellulosics by Steam-Aqueous Pretreatments [and Discussion]. *Philosophical Transactions of the Royal Society of London. Series A, Mathematical and Physical Sciences*, **321**(1561), 523-536.

Palmowski, L.M., Müller, J.A. 2000. Influence of the size reduction of organic waste on their anaerobic digestion. *Water Science and Technology*, **41**(3), 155-162.

Palmqvist, E., Almeida, J.S., Hahn-Hägerdal, B. 1999. Influence of furfural on anaerobic glycolytic kinetics of Saccharomyces cerevisiae in batch culture. *Biotechnology and bioengineering*, **62**(4), 447-454.

Palmqvist, E., Hahn-Hagerdal, B. 2000. Fermentation of lignocellulosic hydrolysates. I: inhibition and detoxification. *Bioresource Technology*, **74**(1), 17-24.

Palmqvist, E., Hahn-Hägerdal, B. 2000a. Fermentation of lignocellulosic hydrolysates. I: inhibition and detoxification. *Bioresource Technology*, **74**(1), 17-24.

Palmqvist, E., Hahn-Hägerdal, B. 2000b. Fermentation of lignocellulosic hydrolysates. II: inhibitors and mechanisms of inhibition. *Bioresource Technology*, **74**(1), 25-33.

Pampulha, M.E., Loureiro-Dias, M.C. 1989. Combined effect of acetic acid, pH and ethanol on intracellular pH of fermenting yeast. *Applied Microbiology and Biotechnology*, **31**(5), 547-550.

Park, J.-y., Seyama, T., Shiroma, R., Ike, M., Srichuwong, S., Nagata, K., Arai-Sanoh, Y., Kondo, M., Tokuyasu, K. 2009. Efficient Recovery of Glucose and Fructose via Enzymatic Saccharification of Rice Straw with Soft Carbohydrates. *Bioscience, Biotechnology, and Biochemistry*, **73**(5), 1072-1077.

Parker, C.C., Parker, M.L., Smith, A.C., Waldron, K.W. 2003a. Thermal stability of texture in chinese water chestnut may be dependent on 8,8 '-diferulic acid (aryltetralyn form). *Journal of Agricultural and Food Chemistry*, **51**(7), 2034-2039.

Parker, C.C., Parker, M.L., Smith, A.C., Waldron, K.W. 2003b. Thermal Stability of Texture in Chinese Water Chestnut May Be Dependent on 8,8‘-Diferulic Acid

(Aryltetralyn Form). *Journal of Agricultural and Food Chemistry*, **51**(7), 2034-2039.

Parker, M.L., Waldron, K.W. 1995. Texture of Chinese Water Chestnut - Involvement of Cell-Wall Phenolics. *Journal of the Science of Food and Agriculture*, **68**(3), 337-346.

Passoth, V., Fredlund, E., Druvefors, U.Ä., Schnürer, J. 2006. Biotechnology, physiology and genetics of the yeast Pichia anomala. *FEMS Yeast Research*, **6**(1), 3-13.

Pavlova, K., Angelova, G., Savova, I., Grigorova, D., Kupenov, L. 2002. Studies of Antarctic yeast for β-glucosidase production. *World Journal of Microbiology and Biotechnology*, **18**(6), 569-573.

Pedersen, M., Meyer, A.S. 2010. Lignocellulose pretreatment severity – relating pH to biomatrix opening. *New Biotechnology*, **27**(6), 739-750.

Penning, B.W., Sykes, R.W., Babcock, N.C., Dugard, C.K., Held, M.A., Klimek, J.F., Shreve, J.T., Fowler, M., Ziebell, A., Davis, M.F., Decker, S.R., Turner, G.B., Mosier, N.S., Springer, N.M., Thimmapuram, J., Weil, C.F., McCann, M.C., Carpita, N.C. 2014. Genetic Determinants for Enzymatic Digestion of Lignocellulosic Biomass Are Independent of Those for Lignin Abundance in a Maize Recombinant Inbred Population. *Plant Physiology*, **165**(4), 1475-1487.

Percival Zhang, Y.H., Himmel, M.E., Mielenz, J.R. 2006. Outlook for cellulase improvement: Screening and selection strategies. *Biotechnology Advances*, **24**(5), 452-481.

Pérez, J., Muñoz-Dorado, J., de la Rubia, T., Martínez, J. 2002. Biodegradation and biological treatments of cellulose, hemicellulose and lignin: an overview. *International Microbiology*, **5**(2), 53-63.

Puls, J. 1993. Chemistry of hemicelluloses : relationship between hemicellulose structure and enzymes required for hydrolysis. *Hemicellulose and hemicellulase*.

Puupponen-Pimiä, R., Nohynek, L., Meier, C., Kähkönen, M., Heinonen, M., Hopia, A., Oksman-Caldentey, K.M. 2001. Antimicrobial properties of phenolic compounds from berries. *Journal of Applied Microbiology*, **90**(4), 494-507.

Qing, Q., Yang, B., Wyman, C.E. 2010. Xylooligomers are strong inhibitors of cellulose hydrolysis by enzymes. *Bioresource Technology*, **101**(24), 9624-9630.

Rajaram, S., Varma, A. 1990. Production and characterization of xylanase from Bacillus thermoalkalophilus grown on agricultural wastes. *Applied Microbiology and Biotechnology*, **34**(1), 141-144.

Ralph, J., Grabber, J.H., Hatfield, R.D. 1995. Lignin-Ferulate Cross-Links in Grasses - Active Incorporation of Ferulate Polysaccharide Esters into Ryegrass Lignins. *Carbohydrate Research*, **275**(1), 167-178.

Ramos, L.P. 2003. The chemistry involved in the steam treatment of lignocellulosic materials. *Química Nova*, **26**(6), 863-871.

Ramos, L.P., Nazhad, M.M., Saddler, J.N. 1993. Effect of enzymatic hydrolysis on the morphology and fine structure of pretreated cellulosic residues. *Enzyme and Microbial Technology*, **15**(10), 821-831.

Randhir, R., Lin, Y.-T., Shetty, K. 2004. Phenolics, their antioxidant and antimicrobial activity in dark germinated fenugreek sprouts in response to peptide and phytochemical elicitors. *Asia Pacific journal of clinical nutrition*, **13**(3), 295-307.

Rasmussen, H., Tanner, D., Sorensen, H.R., Meyer, A.S. 2017. New degradation compounds from lignocellulosic biomass pretreatment: routes for formation of potent oligophenolic enzyme inhibitors. *Green Chemistry*, **19**(2), 464-473.

Ray, M.J., Leak, D.J., Spanu, P.D., Murphy, R.J. 2010. Brown rot fungal early stage decay mechanism as a biological pretreatment for softwood biomass in biofuel production. *Biomass and Bioenergy*, **34**(8), 1257-1262.

Reddy, B.V., Srinivas, T. 2013. Biomass based energy systems to meet the growing energy demand with reduced global warming: Role of energy and exergy analyses. *2013 International Conference on Energy Efficient Technologies for Sustainability*, 10-12 April 2013. pp. 18-23.

Rivers, D.B., Emert, G.H. 1988a. Factors affecting the enzymatic hydrolysis of bagasse and rice straw. *Biological Wastes*, **26**(2), 85-95.

Rivers, D.B., Emert, G.H. 1988b. Factors affecting the enzymatic hydrolysis of municipal-solid-waste components. *Biotechnology and Bioengineering*, **31**(3), 278-281.

Rivers, D.B., Emert, G.H. 1987. Lignocellulose pretreatment: A comparison of wet and dry ball attrition. *Biotechnology Letters*, **9**(5), 365-368.

Rodicio, R., Heinisch, J.J. 2009. Sugar Metabolism by Saccharomyces and non-Saccharomyces Yeasts. in: *Biology of Microorganisms on Grapes, in Must and in Wine*, (Eds.) H. König, G. Unden, J. Fröhlich, Springer Berlin Heidelberg. Berlin, Heidelberg, pp. 113-134.

Rojas, V., Gil, J.V., Piñaga, F., Manzanares, P. 2003. Acetate ester formation in wine by mixed cultures in laboratory fermentations. *International Journal of Food Microbiology*, **86**(1–2), 181-188.

Rosgaard, L., Pedersen, S., Langston, J., Akerhielm, D., Cherry, J.R., Meyer, A.S. 2007. Evaluation of Minimal Trichoderma reesei Cellulase Mixtures on Differently Pretreated Barley Straw Substrates. *Biotechnology Progress*, **23**(6), 1270-1276.

Ryden, P., Gautier, A., Wellner, N., Tapp, H.S., Horn, S.J., Eijsink, V.G.H., Waldron, K.W. 2014. Changes in the composition of the main polysaccharide groups of oil seed rape straw following steam explosion and saccharification. *Biomass & Bioenergy*, **61**, 121-130.

Saha, B., Iten, L., Cotta, M., Wu, V. 2005. Dilute acid pretreatment, enzymatic saccharification, and fermentation of rice hulls to ethanol. *Biotechnol Progr*, **21**.

Saha, B.C. 2003. Hemicellulose bioconversion. *Journal of Industrial Microbiology and Biotechnology*, **30**(5), 279-291.

Saha, B.C., Cotta, M.A. 2007. Enzymatic saccharification and fermentation of alkaline peroxide pretreated rice hulls to ethanol. *Enzyme and Microbial Technology*, **41**(4), 528-532.

Sato, T.K., Liu, T.J., Parreiras, L.S., Williams, D.L., Wohlbach, D.J., Bice, B.D., Ong, I.M., Breuer, R.J., Qin, L., Busalacchi, D., Deshpande, S., Daum, C., Gasch, A.P., Hodge, D.B. 2014. Harnessing Genetic Diversity in Saccharomyces cerevisiae for Fermentation of Xylose in Hydrolysates of Alkaline Hydrogen Peroxide-Pretreated Biomass. *Applied and Environmental Microbiology*, **80**(2), 540-554.

Schädel, C., Blöchl, A., Richter, A., Hoch, G. 2010. Quantification and monosaccharide composition of hemicelluloses from different plant functional types. *Plant Physiology and Biochemistry*, **48**(1), 1-8.

Schwanninger, M., Rodrigues, J.C., Pereira, H., Hinterstoisser, B. 2004. Effects of short-time vibratory ball milling on the shape of FT-IR spectra of wood and cellulose. *Vibrational Spectroscopy*, **36**(1), 23-40.

Selig, M.J., Viamajala, S., Decker, S.R., Tucker, M.P., Himmel, M.E., Vinzant, T.B. 2007. Deposition of Lignin Droplets Produced During Dilute Acid Pretreatment of Maize Stems Retards Enzymatic Hydrolysis of Cellulose. *Biotechnology Progress*, **23**(6), 1333-1339.

Senatham, S., Chamduang, T., Kaewchingduang, Y., Thammasittirong, A., Srisodsuk, M., Elliston, A., Roberts, I.N., Waldron, K.W., Thammasittirong, S.N.R. 2016. Enhanced xylose fermentation and hydrolysate inhibitor tolerance of Scheffersomyces shehatae for efficient ethanol production from non-detoxified lignocellulosic hydrolysate. *Springerplus*, **5**.

Shevchenko, S.M., Beatson, R.P., Saddler, J.N. 1999. The nature of lignin from steam explosion/enzymatic hydrolysis of softwood. *Twentieth Symposium on Biotechnology for Fuels and Chemicals*. Springer. pp. 867-876.

Sims, R.E.H., Mabee, W., Saddler, J.N., Taylor, M. 2010. An overview of second generation biofuel technologies. *Bioresource Technology*, **101**(6), 1570-1580.

Sindhu, R., Binod, P., Pandey, A. 2016. Biological pretreatment of lignocellulosic biomass – An overview. *Bioresource Technology*, **199**, 76-82.

Singh, A., Nigam, P.S., Murphy, J.D. 2011. Renewable fuels from algae: An answer to debatable land based fuels. *Bioresource Technology*, **102**(1), 10-16.

Soares, L.C.S.R., Chandel, A.K., Pagnocca, F.C., Gaikwad, S.C., Rai, M., da Silva, S.S. 2016. Screening of Yeasts for Selection of Potential Strains and Their Utilization for In Situ Microbial Detoxification (ISMD) of Sugarcane Bagasse Hemicellulosic Hydrolysate. *Indian Journal of Microbiology*, **56**(2), 172-181.

Srirangan, K., Akawi, L., Moo-Young, M., Chou, C.P. 2012. Towards sustainable production of clean energy carriers from biomass resources. *Applied Energy*, **100**, 172-186.

Stålbrand, H., Mansfield, S.D., Saddler, J.N., Kilburn, D.G., Warren, R.A., Gilkes, N.R. 1998. Analysis of molecular size distributions of cellulose molecules

during hydrolysis of cellulose by recombinant Cellulomonas fimi beta-1,4-glucanases. *Applied and environmental microbiology*, **64**(7), 2374-2379.

Stanley, G.A., Douglas, N.G., Every, E.J., Tzanatos, T., Pamment, N.B. 1993. Inhibition and stimulation of yeast growth by acetaldehyde. *Biotechnology Letters*, **15**(12), 1199-1204.

Suhara, H., Kodama, S., Kamei, I., Maekawa, N., Meguro, S. 2012. Screening of selective lignin-degrading basidiomycetes and biological pretreatment for enzymatic hydrolysis of bamboo culms. *International Biodeterioration & Biodegradation*, **75**, 176-180.

Sun, Y., Cheng, J. 2002. Hydrolysis of lignocellulosic materials for ethanol production: a review. *Bioresource Technology*, **83**(1), 1-11.

Sun, Y., Cheng, J.J. 2005. Dilute acid pretreatment of rye straw and bermudagrass for ethanol production. *Bioresource Technology*, **96**(14), 1599-1606.

Szczodrak, J., Targoński, Z. 1988. Selection of thermotolerant yeast strains for simultaneous saccharification and fermentation of cellulose. *Biotechnology and Bioengineering*, **31**(4), 300-303.

Tabachnick, J., and Joslyn, M. A. 1953. Formation of esters by yeast I. The production of ethyl acetate by standing surface cultures of Hansenula Anomala. *Journal of Bacteriology*, **65**(1), 1-9.

Taboada, A., Novo-Uzal, E., Flores, G., Loureda, M., Ros Barceló, A., Masa, A., Pomar, F. 2010. Digestibility of silages in relation to their hydroxycinnamic acid content and lignin composition. *Journal of the Science of Food and Agriculture*, **90**(7), 1155-1162.

Taherzadeh, M., Karimi, K. 2008. Pretreatment of Lignocellulosic Wastes to Improve Ethanol and Biogas Production: A Review. *International Journal of Molecular Sciences*, **9**(9), 1621.

Takács, E., Wojnárovits, L., Földváry, C., Hargittai, P., Borsa, J., Sajó, I. 2000. Effect of combined gamma-irradiation and alkali treatment on cotton–cellulose. *Radiation Physics and Chemistry*, **57**(3), 399-403.

Takagi, M., Abe, S., Suzuki, S., Emert, G.H., Yata, N. 1977. A method for production of alcohol directly from cellulose using cellulase and yeast. *Bioconversion Symposium; New Dehli, India*. pp. 551-571.

te Biesebeke, R., Ruijter, G., Rahardjo, Y.S.P., Hoogschagen, M.J., Heerikhuisen, M., Levin, A., van Driel, K.G.A., Schutyser, M.A.I., Dijksterhuis, J., Zhu, Y., Weber, F.J., de Vos, W.M., van den Hondel, K.A.M.J.J., Rinzema, A., Punt, P.J. 2002. Aspergillus oryzae in solid-state and submerged fermentationsProgress report on a multi-disciplinary project. *FEMS Yeast Research*, **2**(2), 245-248.

Theodorou, M.K., Gascoyne, D.J., Akin, D.E., Hartley, R.D. 1987. Effect of phenolic acids and phenolics from plant cell walls on rumenlike fermentation in consecutive batch culture. *Applied and Environmental Microbiology*, **53**(5), 1046-1050.

Tian, S.-Q., Zhao, R.-Y., Chen, Z.-C. 2018. Review of the pretreatment and bioconversion of lignocellulosic biomass from wheat straw materials. *Renewable and Sustainable Energy Reviews*, **91**, 483-489.

Urbina, H., Blackwell, M. 2012. Multilocus Phylogenetic Study of the Scheffersomyces Yeast Clade and Characterization of the N-Terminal Region of Xylose Reductase Gene. *Plos One*, **7**(6).

Valášková, V., Baldrian, P. 2006. Degradation of cellulose and hemicelluloses by the brown rot fungus Piptoporus betulinus – production of extracellular enzymes and characterization of the major cellulases. *Microbiology*, **152**(12), 3613-3622.

Van Soest, P.J. 2006. Rice straw, the role of silica and treatments to improve quality. *Animal Feed Science and Technology*, **130**(3-4), 137-171.

Verduyn, C., Postma, E., Scheffers, W.A., Van Dijken, J.P. 1992. Effect of benzoic acid on metabolic fluxes in yeasts: A continuous-culture study on the regulation of respiration and alcoholic fermentation. *Yeast*, **8**(7), 501-517.

Verduyn, C., Postma, E., Scheffers, W.A., van Dijken, J.P. 1990. Physiology of Saccharomyces Cerevisiae in Anaerobic Glucose-Limited Chemostat Culturesx. *Microbiology*, **136**(3), 395-403.

Vidal, B.C., Dien, B.S., Ting, K.C., Singh, V. 2011. Influence of Feedstock Particle Size on Lignocellulose Conversion—A Review. *Applied Biochemistry and Biotechnology*, **164**(8), 1405-1421.

Vidal, P.F., Molinier, J. 1988. Ozonolysis of lignin — Improvement of in vitro digestibility of poplar sawdust. *Biomass*, **16**(1), 1-17.

Viegas, C.A., Sá-Correia, I. 1991. Activation of plasma membrane ATPase of Saccharomyces cerevisiae by octanoic acid. *Microbiology*, **137**(3), 645-651.

Volf, I., Ignat, I., Neamtu, M., Popa, V.I. 2014. Thermal stability, antioxidant activity, and photo-oxidation of natural polyphenols. *Chemical Papers*, **68**(1), 121-129.

von Sivers, M., Zacchi, G. 1995. A techno-economical comparison of three processes for the production of ethanol from pine. *Bioresource Technology*, **51**(1), 43-52.

Waldron, K.W. 2010. *Bioalcohol Production: Biochemical Conversion of Lignocellulosic Biomass*. Woodhead, Oxford.

Waldron, K.W. 1996. Cell wall esterified phenolic dimers : Identification and quantification by reverse phase high performance liquid chromatography and diode array detection. *Phytochem. Anal.*, **7**, 305-312.

Waldron, K.W., Parr, A.J., Ng, A., Ralph, J. 1996. Cell wall esterified phenolic dimers: Identification and quantification by reverse phase high performance liquid chromatography and diode array detection. *Phytochemical Analysis*, **7**(6), 305-312.

Waldron, K.W., Smith, A.C., Parr, A.J., Ng, A., Parker, M.L. 1997. New approaches to understanding and controlling cell separation in relation to fruit and vegetable texture. *Trends in Food Science & Technology*, **8**(7), 213-221.

Walker, G.M. 2011. Pichia anomala: cell physiology and biotechnology relative to other yeasts. *Antonie van Leeuwenhoek*, **99**(1), 25-34.

Ward, A.J., Hobbs, P.J., Holliman, P.J., Jones, D.L. 2008. Optimisation of the anaerobic digestion of agricultural resources. *Bioresource Technology*, **99**(17), 7928-7940.

Watanabe, M., Yoshida, E., Fukada, H., Inoue, H., Tokura, M., Ishikawa, K. 2015. Characterization of a feruloyl esterase B from Talaromyces cellulolyticus. *Bioscience, Biotechnology, and Biochemistry*, **79**(11), 1845-1851.

Werpy, T., Petersen, G., Aden, A., Bozell, J., Holladay, J., White, J., Manheim, A., Eliot, D., Lasure, L., Jones, S. 2004a. Top value added chemicals from biomass. Volume 1-Results of screening for potential candidates from sugars and synthesis gas. DTIC Document.

Werpy, T.A., Holladay, J.E., White, J.F. 2004b. Top Value Added Chemicals From Biomass: I. Results of Screening for Potential Candidates from Sugars and Synthesis Gas.

Wood, I.P., Cao, H.-G., Tran, L., Cook, N., Ryden, P., Wilson, D.R., Moates, G.K., Collins, S.R.A., Elliston, A., Waldron, K.W. 2016a. Comparison of saccharification and fermentation of steam exploded rice straw and rice husk. *Biotechnology for Biofuels*, **9**(1), 193.

Wood, I.P., Cao, H.G., Tran, L., Cook, N., Ryden, P., Wilson, D.R., Moates, G.K., Collins, S.R.A., Elliston, A., Waldron, K.W. 2016b. Comparison of saccharification and fermentation of steam exploded rice straw and rice husk. *Biotechnology for Biofuels*, **9**.

Wood, I.P., Elliston, A., Collins, S.R.A., Wilson, D., Bancroft, I., Waldron, K.W. 2014. Steam explosion of oilseed rape straw: Establishing key determinants of saccharification efficiency. *Bioresource Technology*, **162**, 175-183.

Wood, I.P., Elliston, A., Ryden, P., Bancroft, I., Roberts, I.N., Waldron, K.W. 2012. Rapid quantification of reducing sugars in biomass hydrolysates: Improving the speed and precision of the dinitrosalicylic acid assay. *Biomass and Bioenergy*, **44**(Supplement C), 117-121.

Wu, J., Collins, S.R.A., Elliston, A., Wellner, N., Dicks, J., Roberts, I.N., Waldron, K.W. 2018a. Release of cell wall phenolic esters during hydrothermal pretreatment of rice husk and rice straw. *Biotechnology for Biofuels*, **11**(1), 162.

Wu, J., Elliston, A., Le Gall, G., Colquhoun, I.J., Collins, S.R.A., Dicks, J., Roberts, I.N., Waldron, K.W. 2017. Yeast diversity in relation to the production of fuels and chemicals. *Scientific Reports*, **7**(1), 14259.

Wu, J., Elliston, A., Le Gall, G., Colquhoun, I.J., Collins, S.R.A., Wood, I.P., Dicks, J., Roberts, I.N., Waldron, K.W. 2018b. Optimising conditions for bioethanol production from rice husk and rice straw: effects of pre-treatment on liquor composition and fermentation inhibitors. *Biotechnology for Biofuels*, **11**(1), 62.

Wyman, C.E., Hinman, N.D. 1990. Ethanol: fundamentals of production from renewable feedstocks and use as a transportation fuel. *Applied Biochemistry and Biotechnology*, **24**(1), 735-753.

Xiao, W., Clarkson, W.W. 1997. Acid solubilization of lignin and bioconversion of treated newsprint to methane. *Biodegradation*, **8**(1), 61-66.

Ximenes, E., Kim, Y., Mosier, N., Dien, B., Ladisch, M. 2011. Deactivation of cellulases by phenols. *Enzyme and Microbial Technology*, **48**(1), 54-60.

Ximenes, E., Kim, Y., Mosier, N., Dien, B., Ladisch, M. 2010. Inhibition of cellulases by phenols. *Enzyme and Microbial Technology*, **46**(3), 170-176.

Yang, B., Willies, D.M., Wyman, C.E. 2006. Changes in the enzymatic hydrolysis rate of Avicel cellulose with conversion. *Biotechnology and Bioengineering*, **94**(6), 1122-1128.

Yang, B., Wyman, C.E. 2007. Biotechnology for Cellulosic Ethanol. *Asia pacific Biotech News*, **11**, 9.

Yang, B., Wyman, C.E. 2004. Effect of xylan and lignin removal by batch and flowthrough pretreatment on the enzymatic digestibility of corn stover cellulose. *Biotechnology and Bioengineering*, **86**(1), 88-98.

Yang, B., Wyman, C.E. 2008. Pretreatment: the key to unlocking low-cost cellulosic ethanol. *Biofuels, Bioproducts and Biorefining*, **2**(1), 26-40.

Yang, H., Yan, R., Chen, H., Lee, D.H., Zheng, C. 2007. Characteristics of hemicellulose, cellulose and lignin pyrolysis. *Fuel*, **86**(12), 1781-1788.

Zadrazil, F., Puniya, A.K. 1995. Studies on the effect of particle size on solid-state fermentation of sugarcane bagasse into animal feed using white-rot fungi. *Bioresource Technology*, **54**(1), 85-87.

Zeitsch, K.J. 2000. The chemistry and technology of furfural and its many by-products.

Zeng, Y., Zhao, S., Yang, S., Ding, S.-Y. 2014. Lignin plays a negative role in the biochemical process for producing lignocellulosic biofuels. *Current Opinion in Biotechnology*, **27**(Supplement C), 38-45.

Zhang, G.-C., Liu, J.-J., Kong, I.I., Kwak, S., Jin, Y.-S. 2015. Combining C6 and C5 sugar metabolism for enhancing microbial bioconversion. *Current Opinion in Chemical Biology*, **29**, 49-57.

Zhang, K., Pei, Z., Wang, D. 2016. Organic solvent pretreatment of lignocellulosic biomass for biofuels and biochemicals: A review. *Bioresource Technology*, **199**, 21-33.

Zhang, Q.Z., Huang, H.Q., Han, H., Qiu, Z., Achal, V. 2017. Stimulatory effect of in-situ detoxification on bioethanol production by rice straw. *Energy*, **135**, 32-39.

Zhang, Y.-H.P., Lynd, L.R. 2004. Toward an aggregated understanding of enzymatic hydrolysis of cellulose: Noncomplexed cellulase systems. *Biotechnology and Bioengineering*, **88**(7), 797-824.

Zhu, S., Wu, Y., Chen, Q., Yu, Z., Wang, C., Jin, S., Ding, Y., Wu, G. 2006. Dissolution of cellulose with ionic liquids and its application: a mini-review. *Green Chemistry*, **8**(4), 325-327.

Zhu, S., Yu, P., Wang, Q., Cheng, B., Chen, J., Wu, Y. 2013. *Breaking the Barriers of Lignocellulosic Ethanol Production using Ionic Liquid Technology*.

Appendices

Appendix 1

Suppliers of experimental equipment and chemicals:

0.2 µm filter plates: Pall Corporation, World Headquarters, Washington, USA.

96 well reader plates (1 ml): Thermo Fisher Scientific, Waltham, MA, USA.

96 deep well plates (2 ml): Geriner Bio-One Ltd, Brunel Way, UK.

96 well PCR plates: Fisherbrand®, UK.

1-methylimidazole: Sigma-Aldrich, Gillingham, Dorset, UK.

6700EFM Freezer/Mill: Spex Sample Prep, Stanmore, UK.

Ammonia (NH_3): Sigma-Aldrich, Gillingham, Dorset, UK.

Acetic anhydride: Sigma-Aldrich, Gillingham, Dorset, UK.

BIOTAGE® Initiator+ reactor: Biotage AB, Box 8, 751 03, Uppsala, Sweden.

BioRad FTS 175C Fourier transform infrared spectrometer: BioRad, Cambridge, MA, USA.

Bruker Avance spectrometer: Bruker BioSpin GmbH, Rheinstetten, Germany.

Cellic® CTec-2 and HTec-2: Novozymes, Denmark.

Clear polypropylene PCR seal: STARLAB international GmbH, 22143 Hamburg, Germany.

Dichloromethane (DCM): Sigma-Aldrich, Gillingham, Dorset, UK.

Deuterium oxide (D_2O): Sigma-Aldrich, Gillingham, Dorset, UK.

Gas Chromatography (GC): Perkin-Elmer Autosystem XL, Perkin Elmer, Seer Green, UK.

GOPOD Format: D-Glucose Assay Kit, Megazyme, USA.

Golden Gate™ diamond attenuated total reflectance (ATR) accessory: Specac, Slough, UK.

High performance liquid chromatography (HPLC) - a Series 200 LC instrument: Perkin Elmer, Seer Green, UK.

Hydrogen chloride (HCl): Sigma-Aldrich, Gillingham, Dorset, UK.

Laboratory-purified 13 sugars: Sigma-Aldrich, Gillingham, Dorset, UK.

Microplate spectrophotometer: Biometra® T-Gradient, Germany.

Matrix Tubes and Matrix plates: Thermo Fisher Scientific, Waltham, MA, USA.

Multi Variate Statistical Package version 3.22: Kovach Computing Services, Anglesey, UK.

Olympus BX 60 Light microscope: Olympus, Tokyo, Japan.

Polypropylene PCR seal: STARLAB international GmbH, 22143 Hamburg, Germany.

Potassium hydrogen phosphate (K_2HPO_4): Sigma-Aldrich, Gillingham, Dorset, UK.

Phenomenex Column Luna 5 µ C18 (2), 250*40 mm with pre-column and Perkin Elmer Diode Array UV Detector: Waltham, Massachusetts, USA.

RTX-225 column: Restek, Bellefonte, USA,

RETSCH cyclone mill: Retsch Limited, Hope Valley, UK.

Sovirel culture tubes: The Science Company, 7625 W Hampden Ave, Unit 14, Lakewood, Colorado, USA.

Sodium borohydride ($NaBH_4$): Sigma-Aldrich, Gillingham, Dorset, UK.

Sintered glass funnels: VWR International Ltd, 1151 Budapest, Szövőgyár utca 11-13, Hungary.

Sodium dihydrogen phosphate ($NaH_2PO_4.H_2O$): Sigma-Aldrich, Gillingham, Dorset, UK.

Sodium 3-(Trimethylsilyl)-propionate-d4 (TSP): Sigma-Aldrich, Gillingham, Dorset, UK.

Sodium azide (NaN_3): Sigma-Aldrich, Gillingham, Dorset, UK.

Sodium hydroxide (NaOH):

Sulfuric acid (H_2SO_4): Sigma-Aldrich, Gillingham, Dorset, UK.

Thimerosal Orbital shaker: Thermo Fisher Scientific, Waltham, MA, USA.

TPE PCR sealing mats: BRAND, at Fisher, UK.

Thermocycler: Biometra T-Gradient, Germany.

Vulcan PD Furnace 3-550: Dentsply Sirona Global Headquarters, Susquehanna Commerce Center. 221 West Philadephia Street, Suite 60W, York PA, USA.

VersaMax ELISA Microplate Reader: Molecular Devices, Sunnyvale, CA, USA.

Yeast nitrogen base (YNB): Formedium, Hunstanton, Norfolk, UK.

Appendix 2

2.1Principle of GOPOD reactions and sugar STDs of glucose for GOPOD and DNS

2.1.1 principle and glucose STD of GOPOD

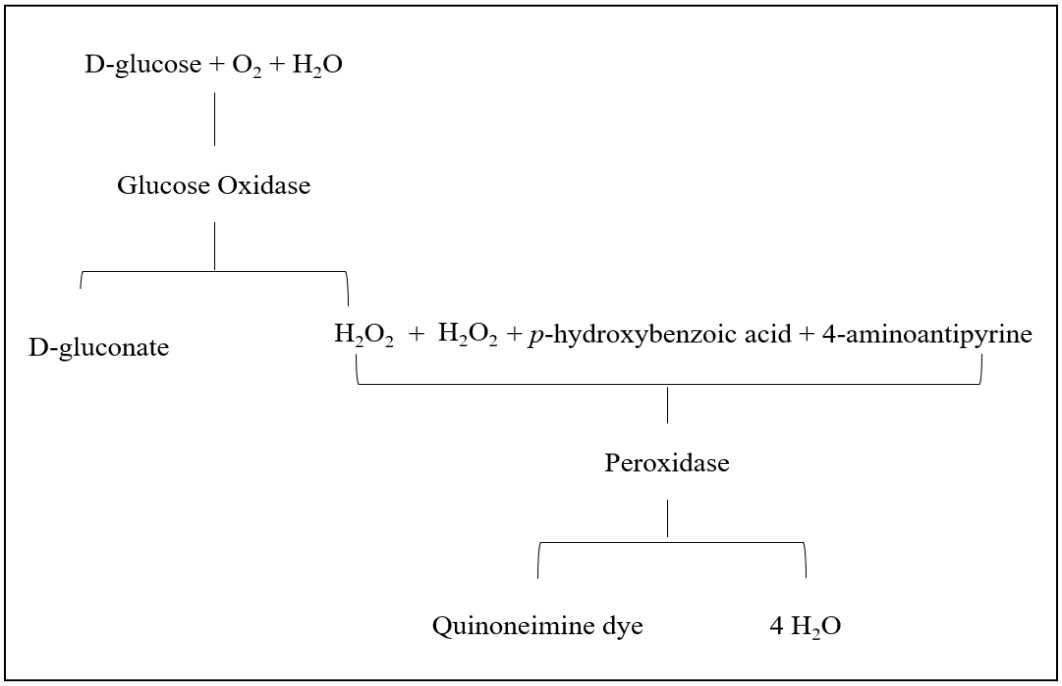


Figure ATC2.1. Diagram of GOPOD reactions.

Table ATC2.1. The series of D-glucose STDs.

Compositions (µl)	D-glucose STDs (mg/ml)				
	STD 1 (0)	**STD 2 (0.15)**	**STD 3 (0.5)**	**STD 4 (0.75)**	**STD 5 (1.00)**
Distilled water	1000	997	990	985	980
D-glucose solution*	0	3	10	15	20

**The concentration of D-glucose solution used to make up STDs was 50 mg/ml.*

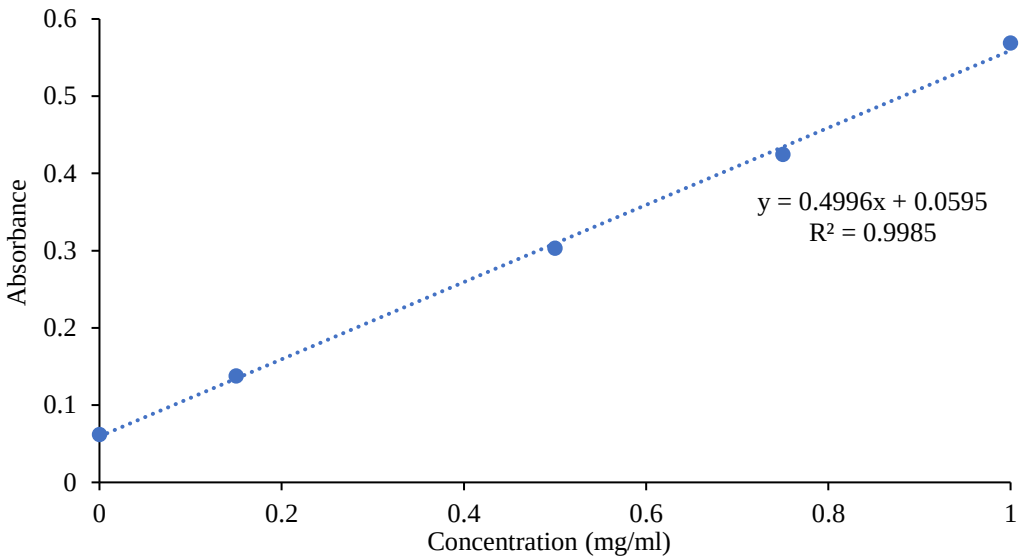


Figure ATC2.2. STDs curve of D-glucose for GOPOD.

2.1.2 Glucose STDs for DNS

Table ACT2.2. Sugar STDs for DNS method.

Compositions (ml)	**Sugar STDs (mg/ml)**				
	STD 1 (10)	**STD 2 (20)**	**STD 3 (30)**	**STD 4 (40)**	**STD 5 (50)**
Distilled water	0.8	0.6	0.4	0.2	0.0
D-glucose solution*	0.2	0.4	0.6	0.8	1.0

**The concentration of D-glucose solution used to make up STDs was 50 mg/ml.*

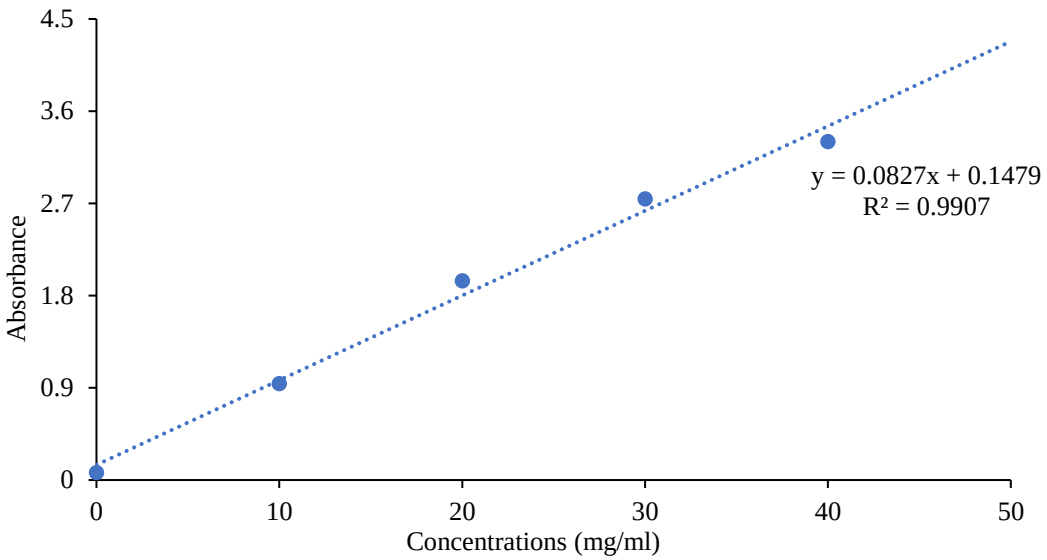


Figure ATC2.3. STDs curves of D-glucose for DNS.

2.2Chromatography spectra of phenolic compounds

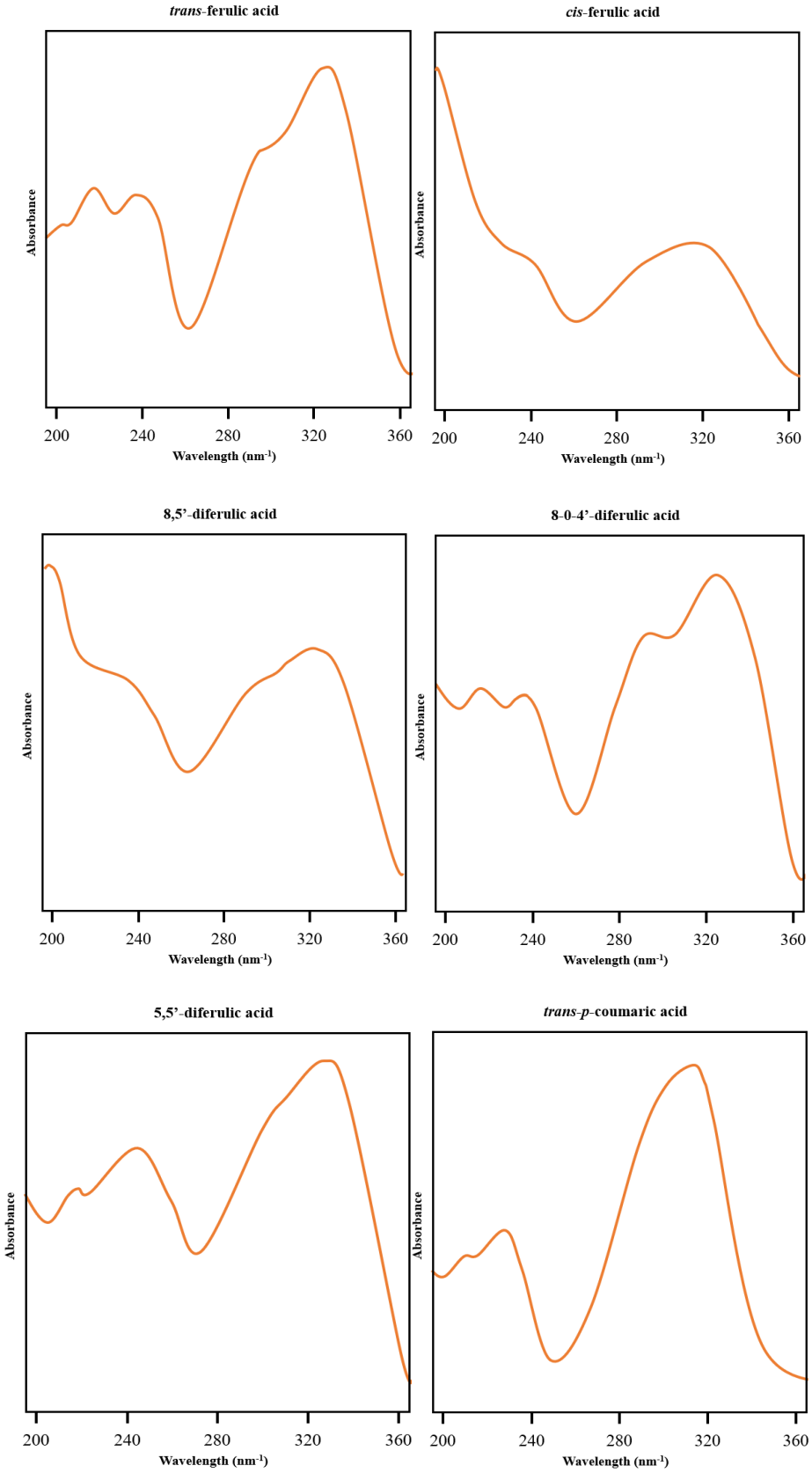


Figure ATC2.4. Spectra of phenolics (adapted from Waldron (1996))

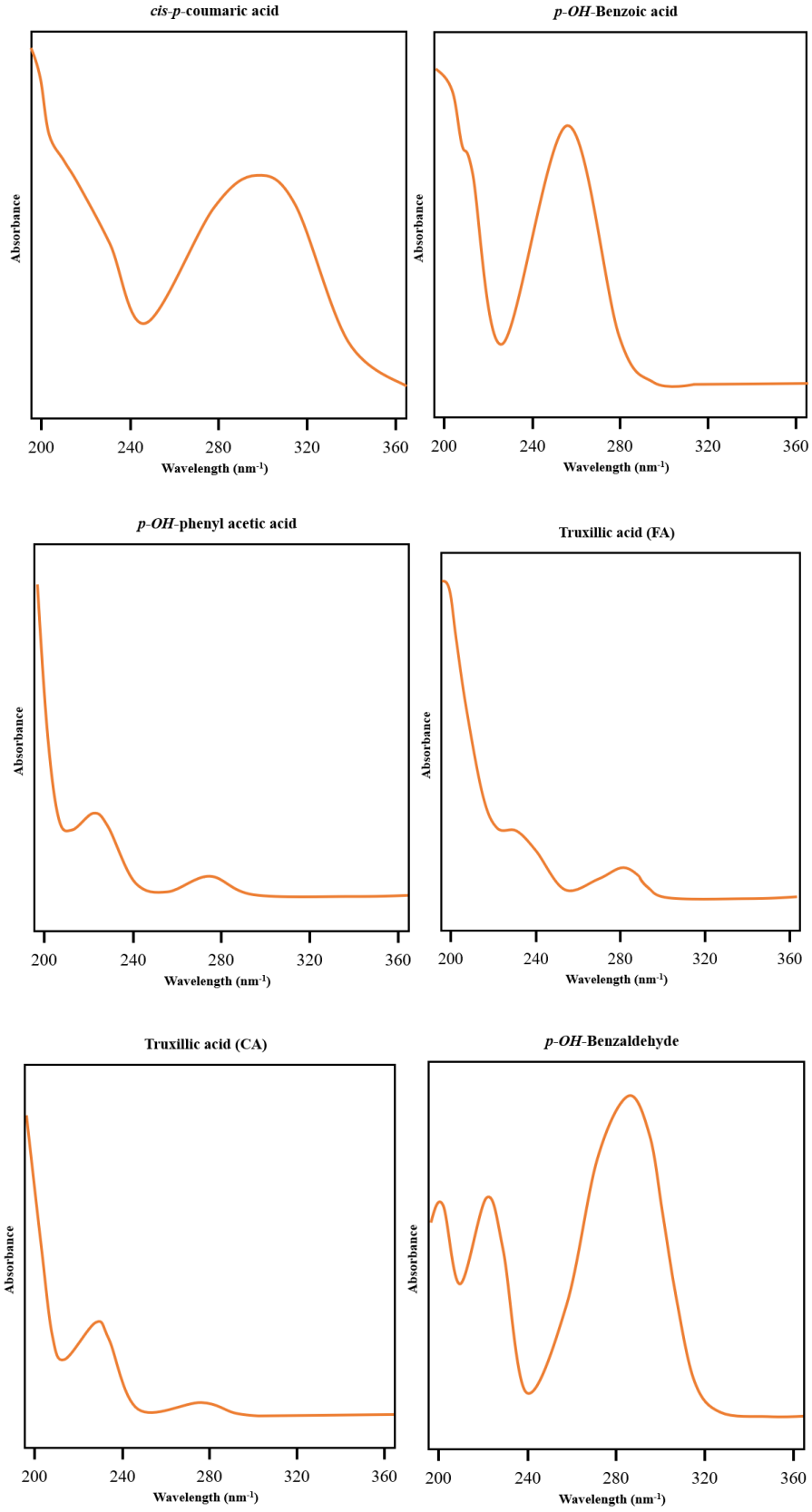


Figure ATC2.5. Spectra of phenolics (adapted from Waldron (1996)).

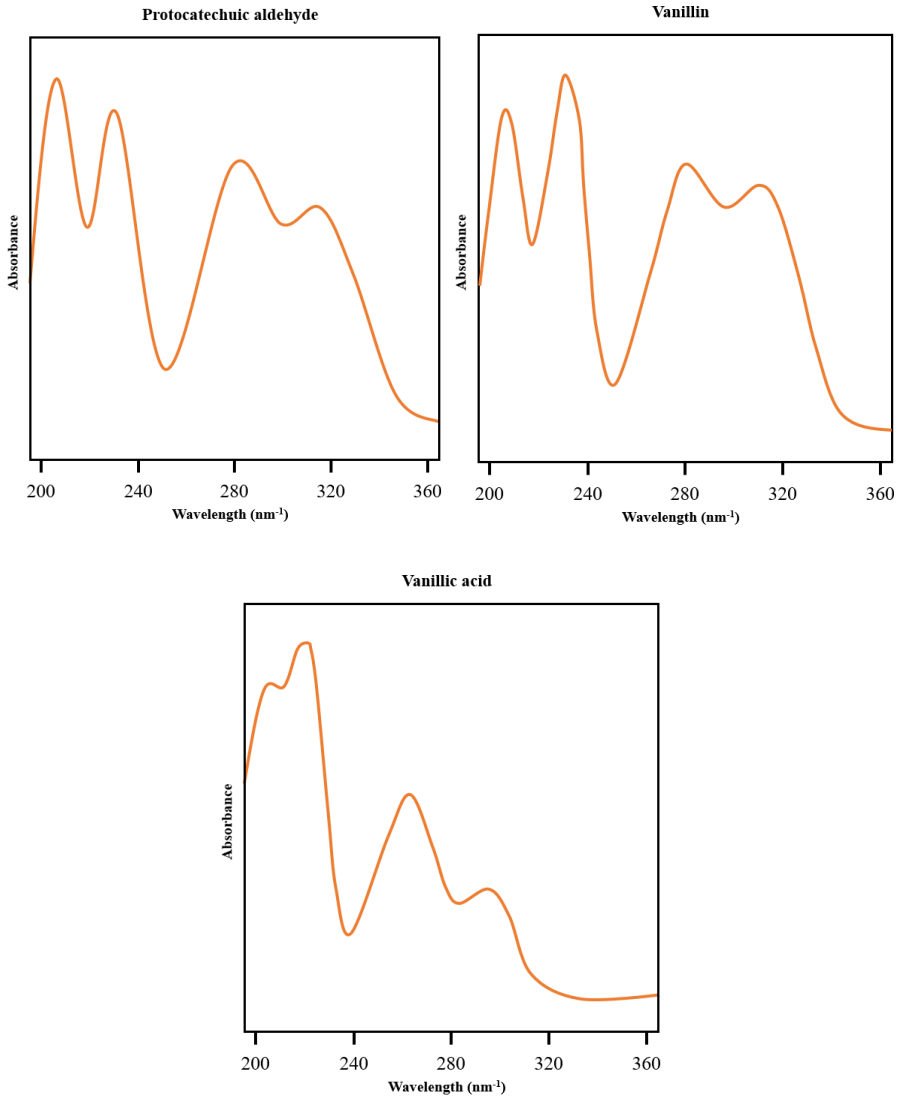
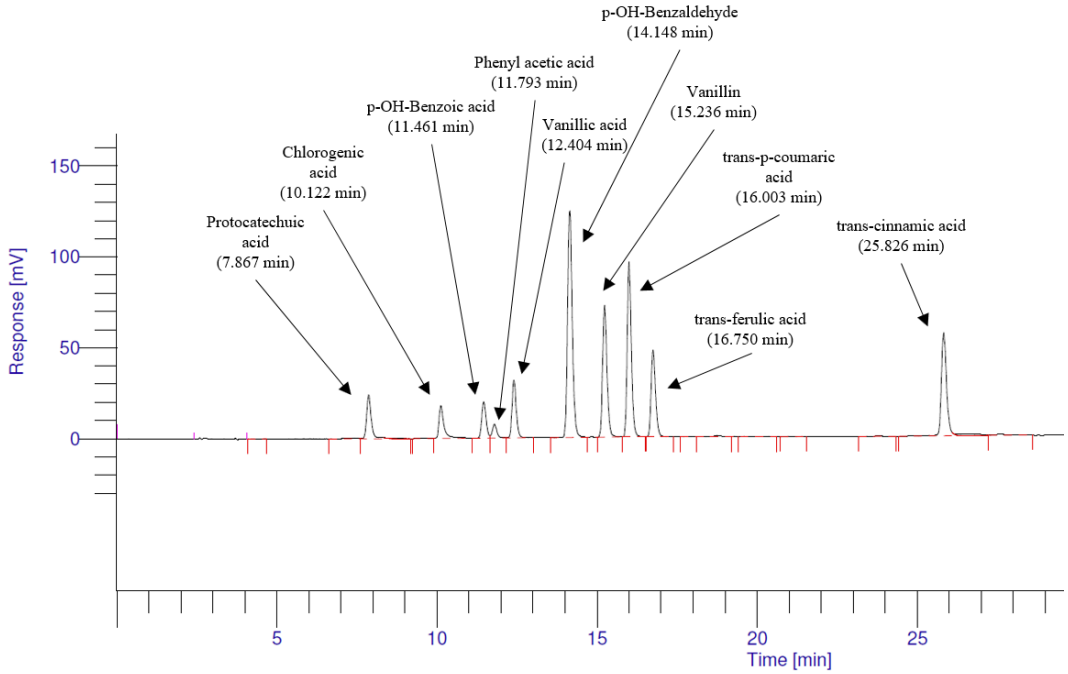


Figure ATC2.6. Spectra of phenolics (adapted from Waldron (1996)).



*PS: RRT = RT / RTIS
RRT = Relative retention time
RT = Retention time of individual phenolic compound
RTIS = Retention time of trans-cinnamic acid

Figure ATC2.7. Retention time of phenolic compounds and explanation of Relative retention time.

Appendix 3

3.1 Standard curves

3.1.1 standard curves of GC

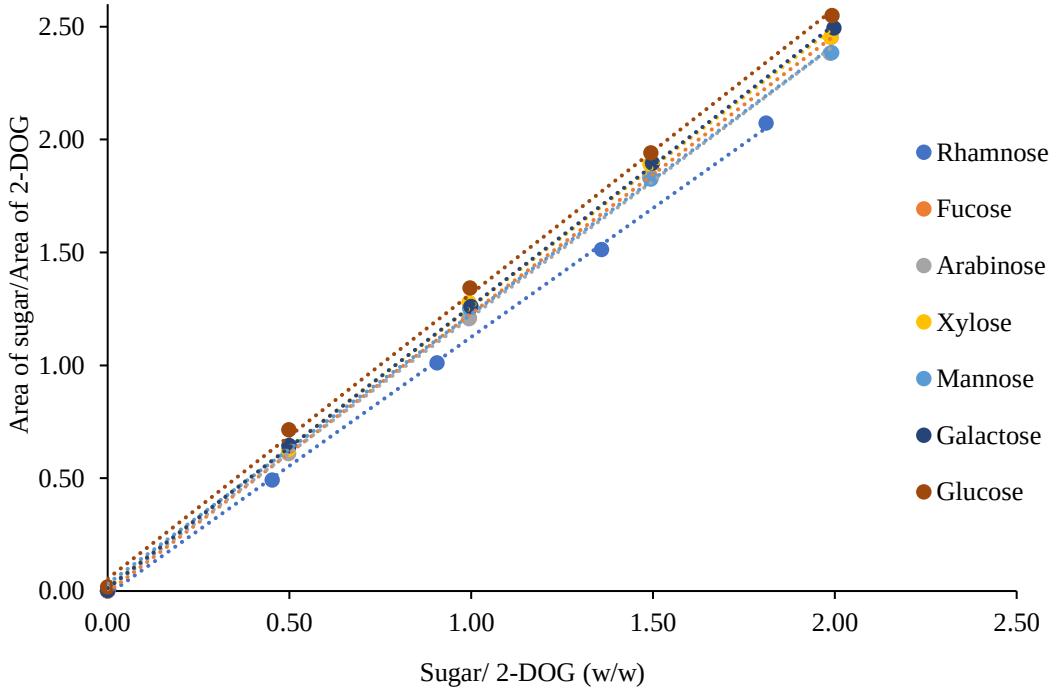


Figure ATC3.1. Standard curves of sugar analysis established by GC. Each of all sugars is presented with different colours and shapes. The X axis is the weight of sugar divided by the weight of internal standard (2-DOG, 2-Dimethyl oxide glucose) and the Y axis is the area of sugars divided by the area of 2-DOG.

3.1.2 Standard curves of ethanol (HPLC)

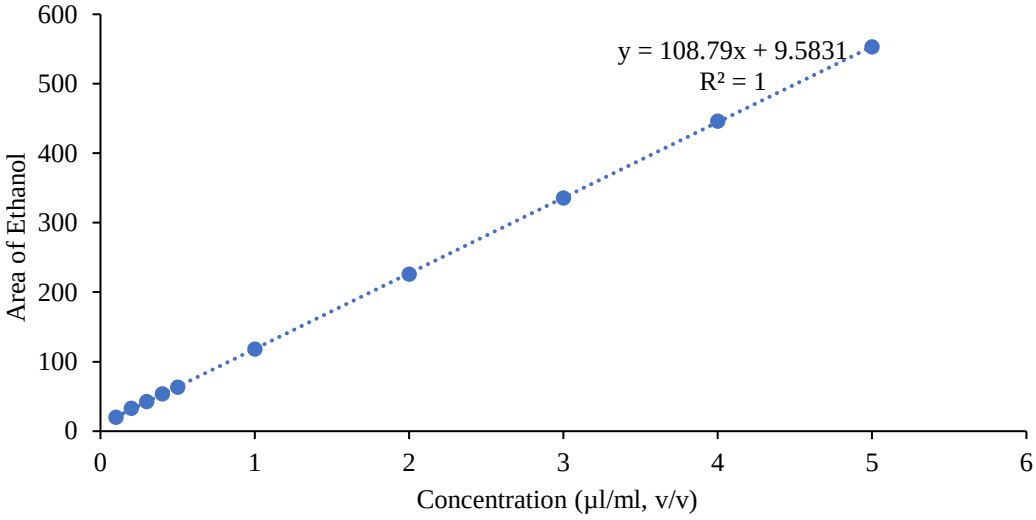
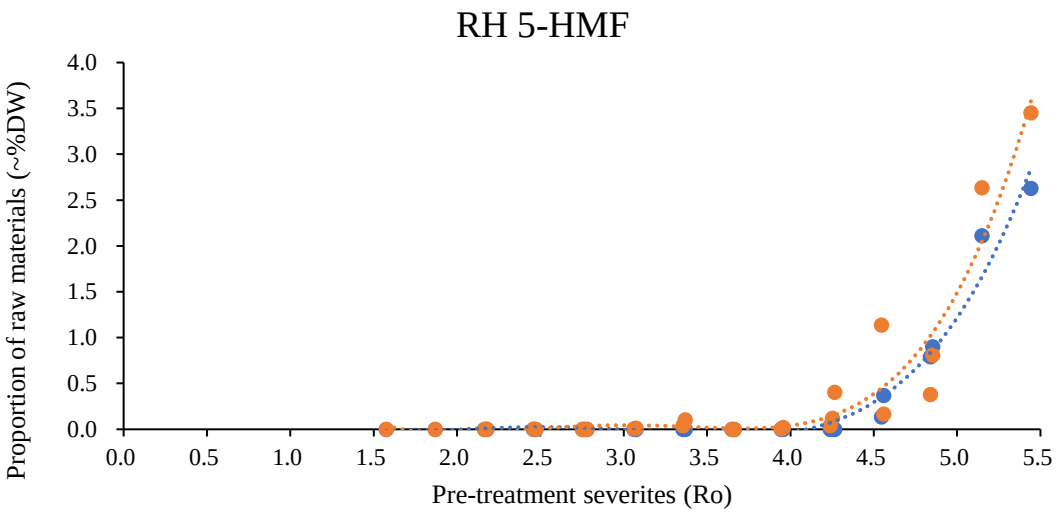
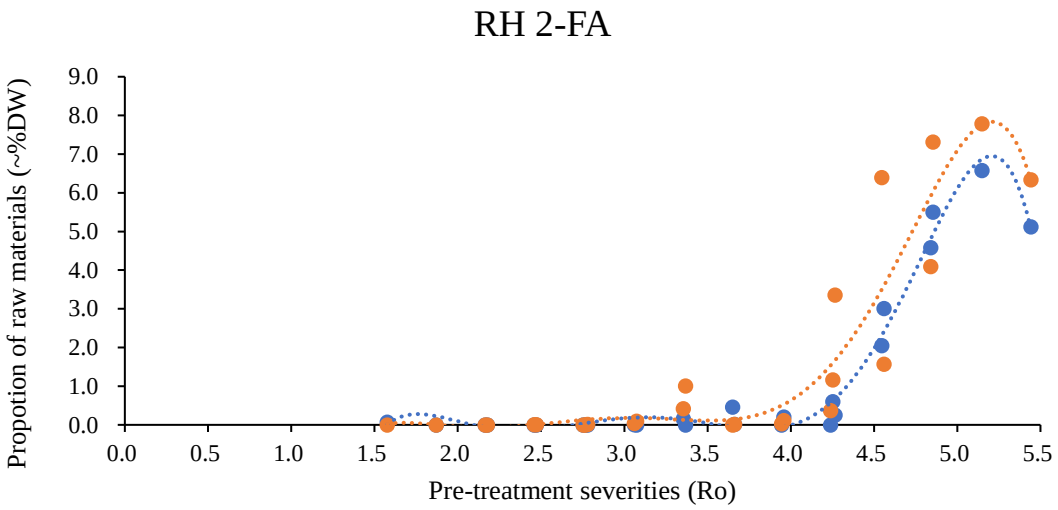


Figure ATC3.2. Standard curves of ethanol established by HPLC. The X axis is the concentration of pre-made ethanol and the Y axis is the area of ethanol.

3.3 Quantities of 5-HMF, 2-FA and acetic acid produced from pre-treated samples of husk (quantified by using HPLC)





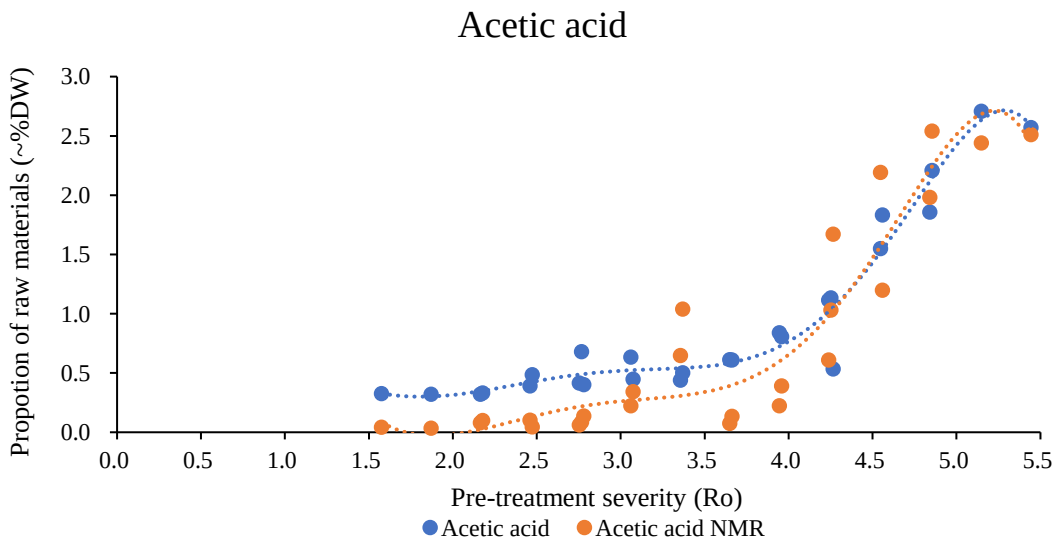


Figure ATC3.3. Comparison of quantities of 5-HMF, 2-FA and acetic acid produced from pre-treated RH samples by using NMR and HPLC. Curves and circles in blue represents results collected by using HPLC. Those in orange colour are results collected by using NMR.

Appendix 4

4.1 Biomass recover rate of PT RH and RS

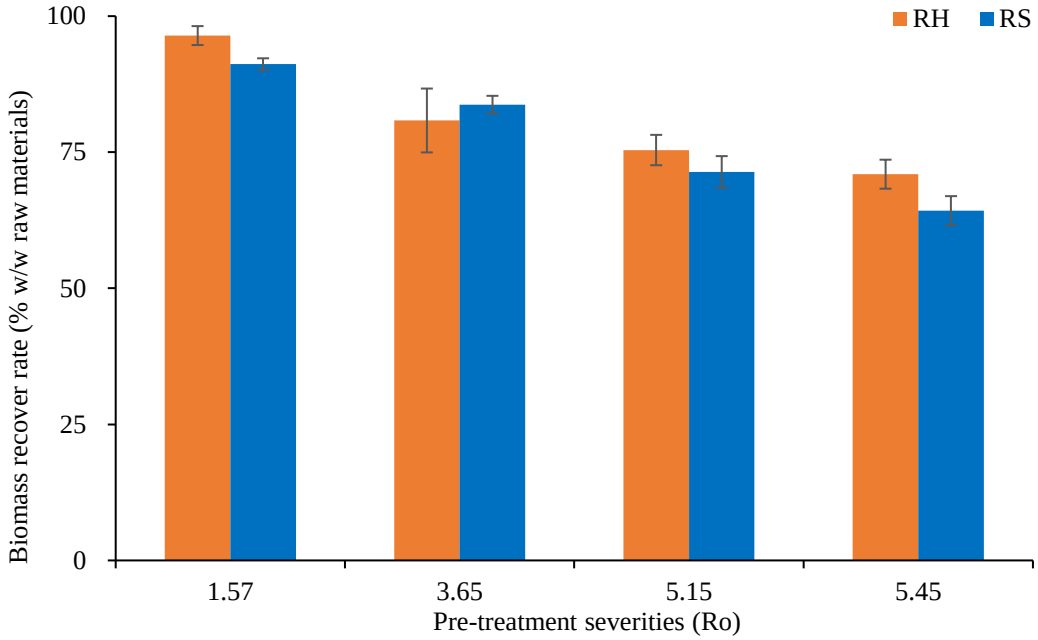


Figure ATC4.1. Biomass recover rate of PT RH and RS. Data has been calculated as the percentage of raw materials (w/w). N=3.

Appendix 5

5.1Suppliers of experimental equipment and chemicals.

Yeast nitrogen base (YNB), Formedium, Hunstanton, Norfolk, United Kingdom; laboratory-purified sugars, Sigma-Aldrich, Gillingham, Dorset, United Kingdom; 96 well reader plates, Thermo Fisher Scientific, Waltham, MA USA; VersaMax ELISA Microplate Reader, Molecular Devices, Sunnyvale, CA USA; 96 deep well plates (2 ml), Geriner Bio-One Ltd, Brunel Way, UK; polypropylene PCR seal, STARLAB international GmbH, 22143 Hamburg, Germany; 0.2 µm filter plates, Pall Corporation, World Headquarters, Washington USA; BIOTAGE® Initiator+ reactor, Biotage AB, Uppsala, Sweden; Matrix tubes, Thermo Fisher Scientific, Waltham, MA USA.

5.2Table of 11 selected yeasts and 13 lab-purified sugars

Table ATC5.1. Name and molecular formula of 13 lab-purified sugars

NUMBER	NAME	MOLECULAR FORMULA
1	Ribose	$C_5H_{10}O_5$
2	Cellobiose	$C_{12}H_{22}O_{11}$
3	Glucose	$C_6H_{12}O_6$
4	Maltose	$C_{12}H_{22}O_{11}$
5	Fructose	$C_6H_{12}O_6$
6	Rhamnose	$C_6H_{12}O_5$
7	Xylose	$C_5H_{10}O_5$
8	Mannose	$C_6H_{12}O_6$
9	Galactose	$C_6H_{12}O_6$
10	Arabinose	$C_5H_{10}O_5$
11	Sucrose	$C_{12}H_{22}O_{11}$
12	Fucose	$C_6H_{12}O_5$
13	Lactose	$C_{12}H_{22}O_{11}$

Table ATC5.2. Information of selected diverse yeast strains.

NUMBER	NCYC NUMBER	NAME	NCYC URL
1	NCYC 2791	*Kluyveromyces marxianus*	https://catalogue.ncyc.co.uk/kluyveromyces-marxianus-2791
2	NCYC 65	*Rhodotorula mucilaginosa*	https://catalogue.ncyc.co.uk/rhodotorulamucilaginosa-65
3	NCYC 31	*Hanseniaspora osmophila*	https://catalogue.ncyc.co.uk/hanseniaspora-osmophila-31
4	NCYC 10	*Debaryomyces hansenii*	https://catalogue.ncyc.co.uk/debaryomyces-hansenii-10
5	NCYC 568	*Zygosaccharomyces rouxii*	https://catalogue.ncyc.co.uk/zygosaccharomyces-rouxii-568
6	NCYC 16	*Wickerhamomyces anomalus*	https://catalogue.ncyc.co.uk/wickerhamomyces-anomalus-16
7	NCYC 2433	*Zygosaccharomyces thermotolerans*	https://catalogue.ncyc.co.uk/lachanceathermotolerans-2433
8	NCYC 4	*Candida tropicalis*	https://catalogue.ncyc.co.uk/candidatropicalis-4
9	NCYC 2577	*Kazachstania servazii*	https://catalogue.ncyc.co.uk/kazachstaniaservazzii-2577
10	NCYC 49	*Galactomycers candidus*	https://catalogue.ncyc.co.uk/galactomycescandidus-49
11	NCYC 2826	*Saccharomyces cerevisiae*	https://catalogue.ncyc.co.uk/saccharomyces-cerevisiae-2826

5.3Standard curves of sugars and ethanol (HPLC)

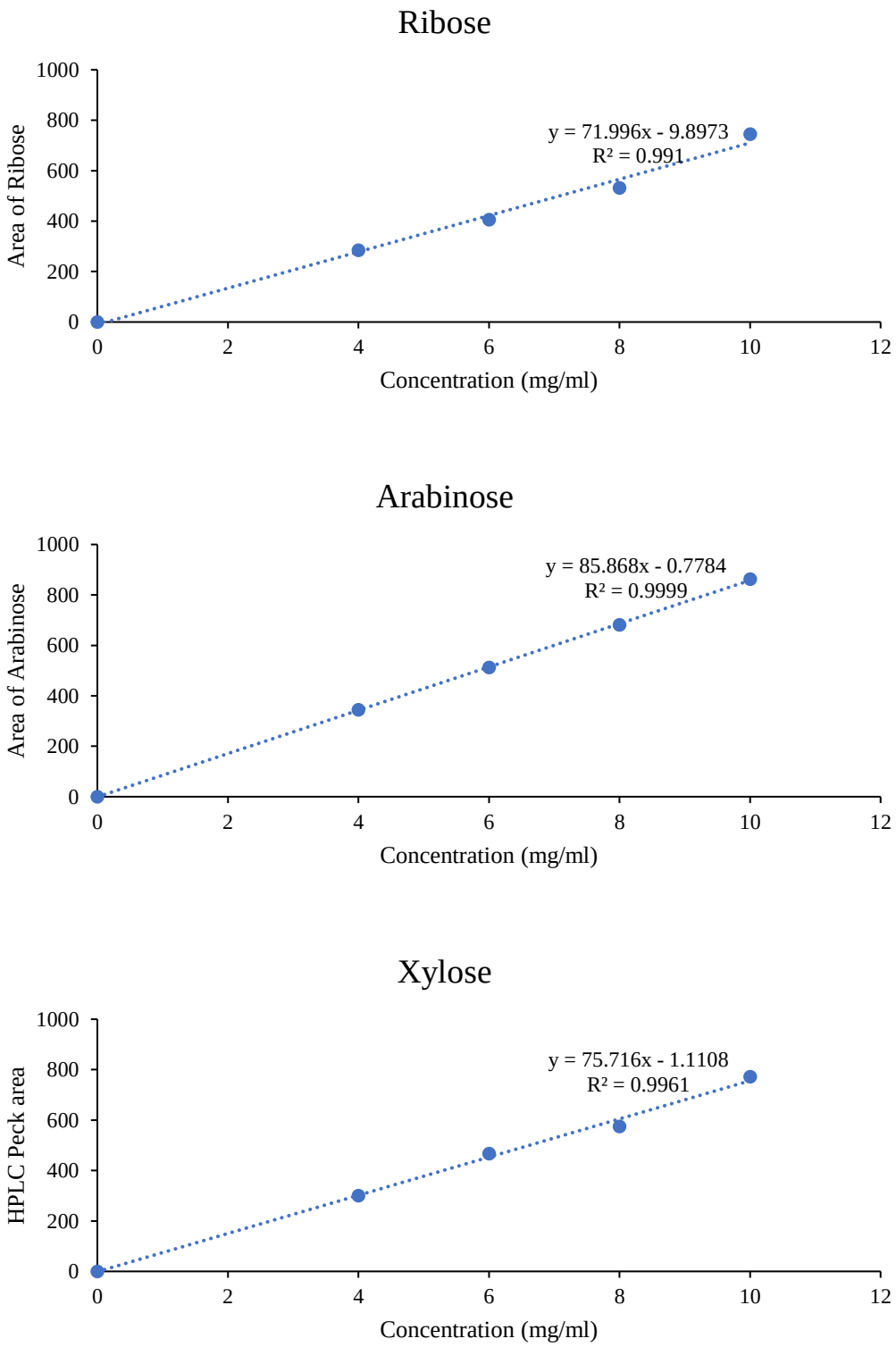


Figure ATC5.1. Standard curves of pentose (ribose, arabinose and xylose).

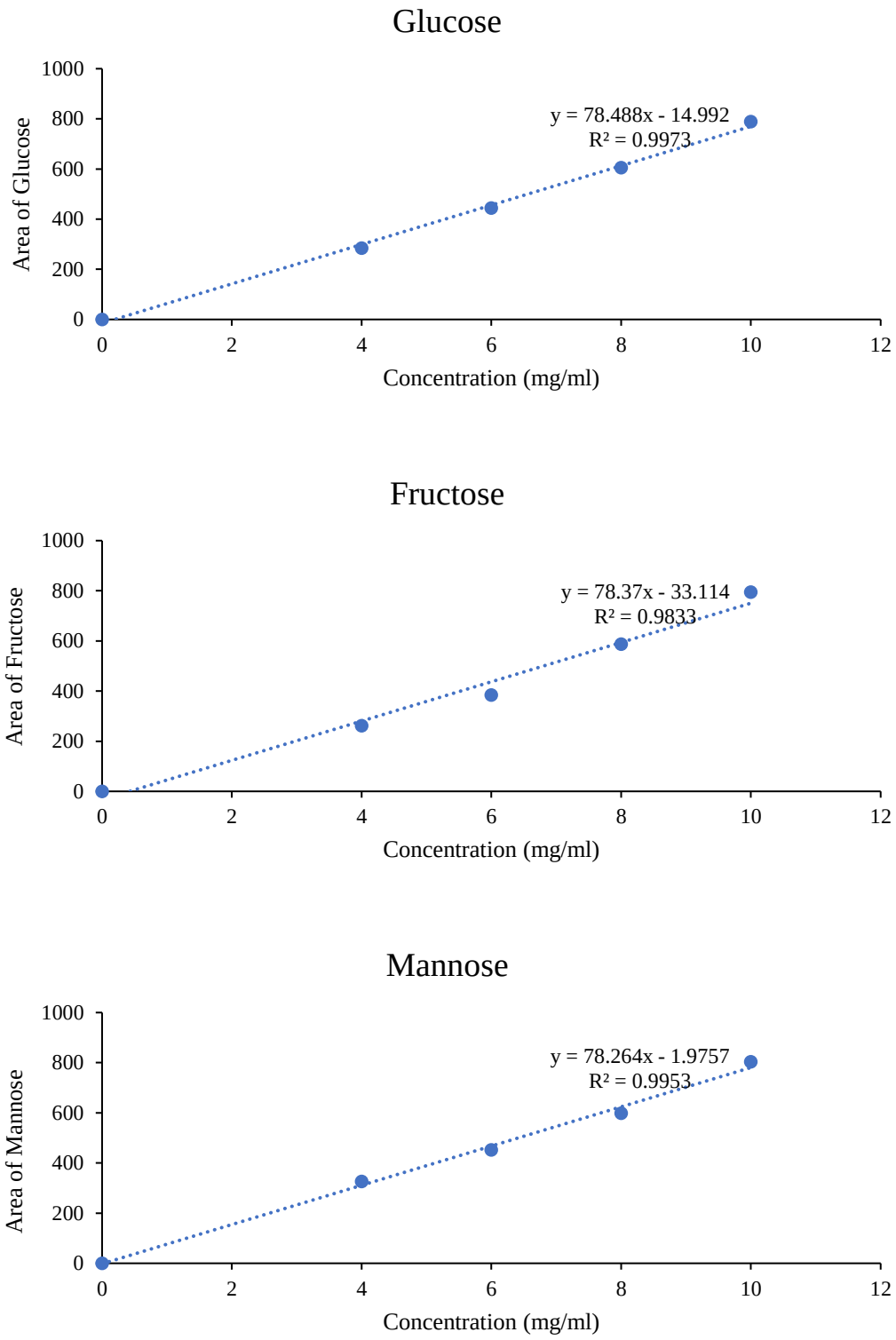


Figure ATC5.2. Standard curves of hexose (part 1: glucose, fructose and mannose).

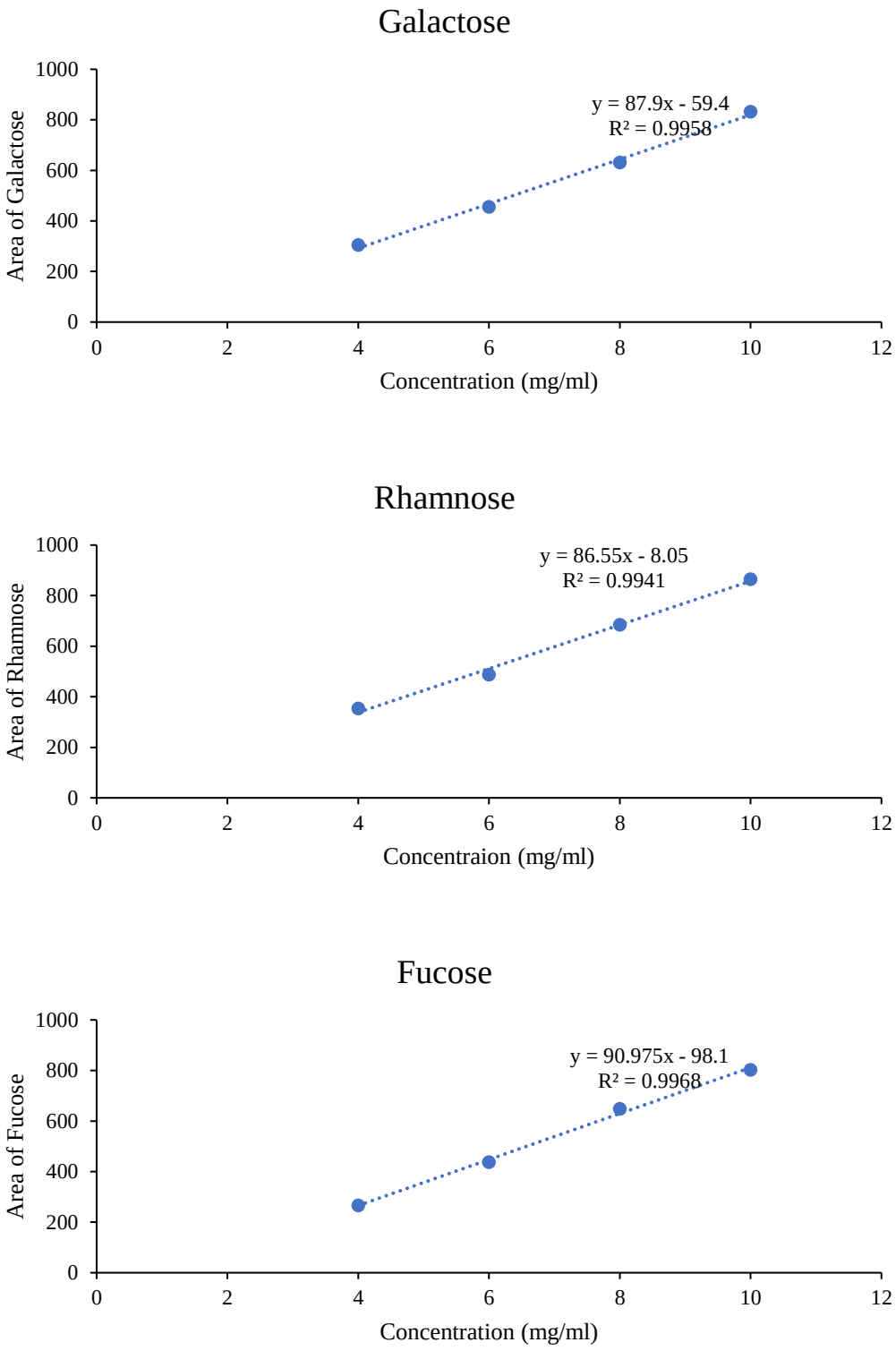


Figure ATC5.3. Standard curves of hexose (part 2: galactose, rhamnose and fucose).

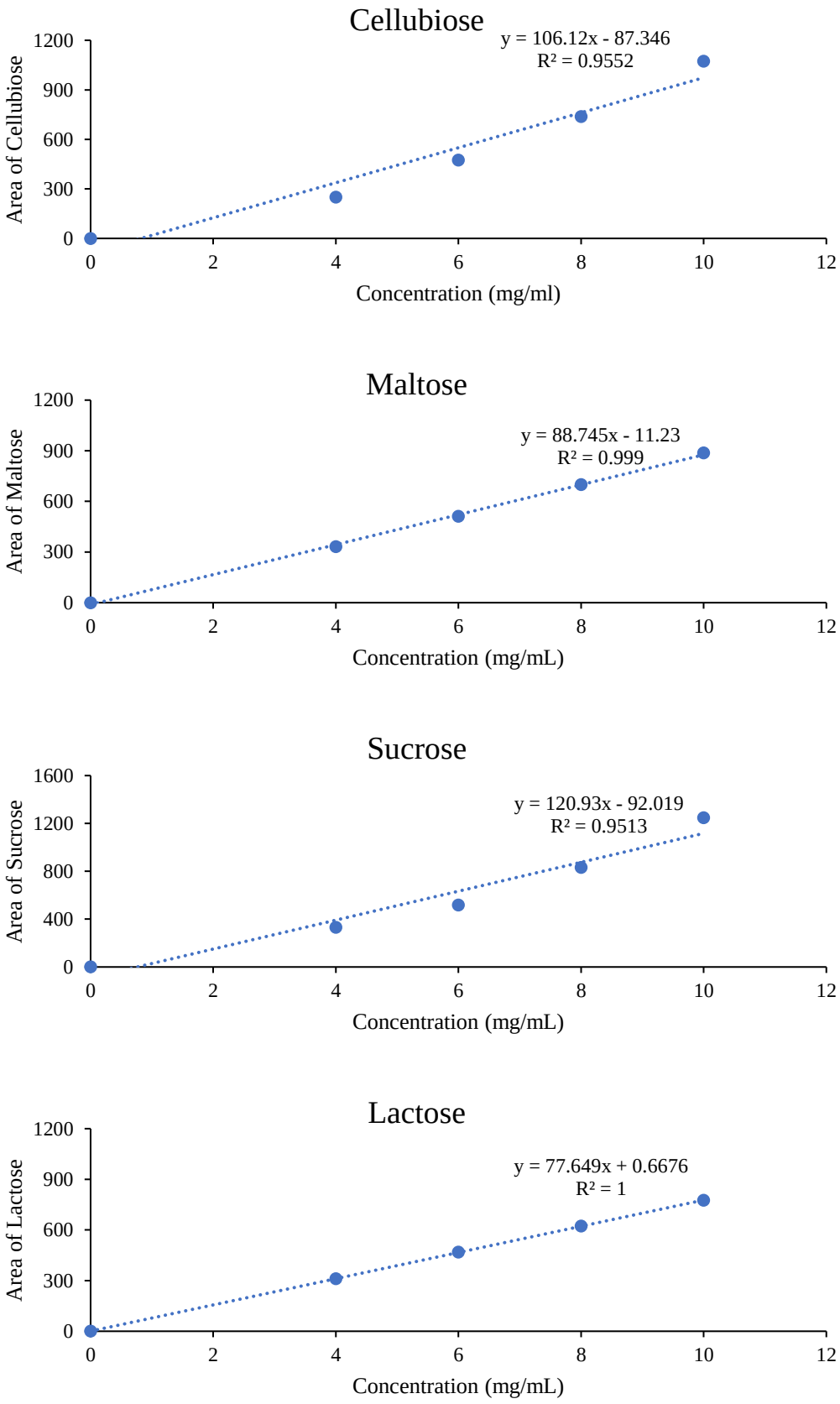


Figure ATC5.4. Standard curves of disaccharides (cellobiose, maltose, sucrose and lactose).

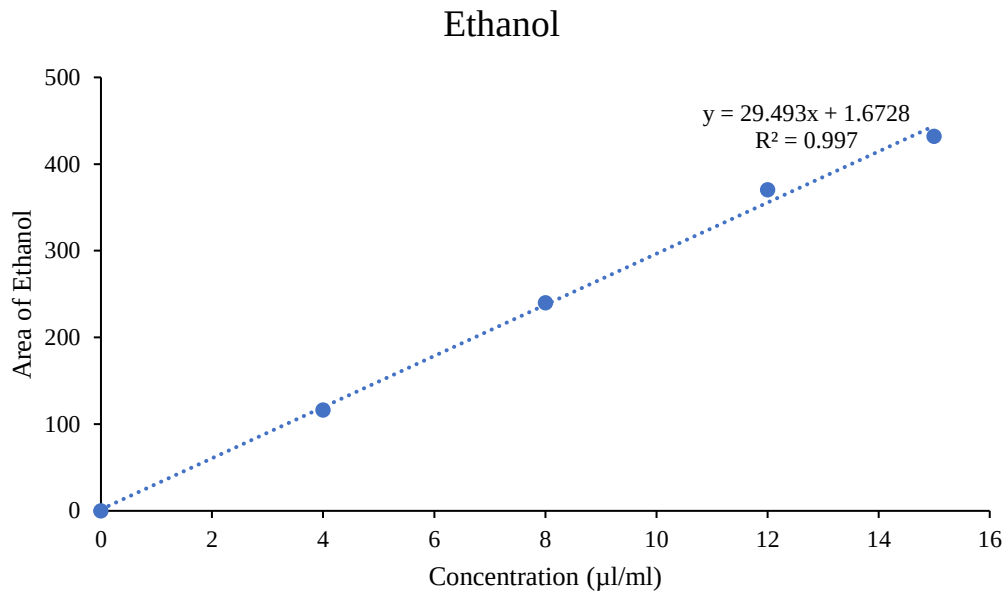


Figure ATC5.5. Standard curves of ethanol (Standard for 11 yeast fermentation on 13 sugars).

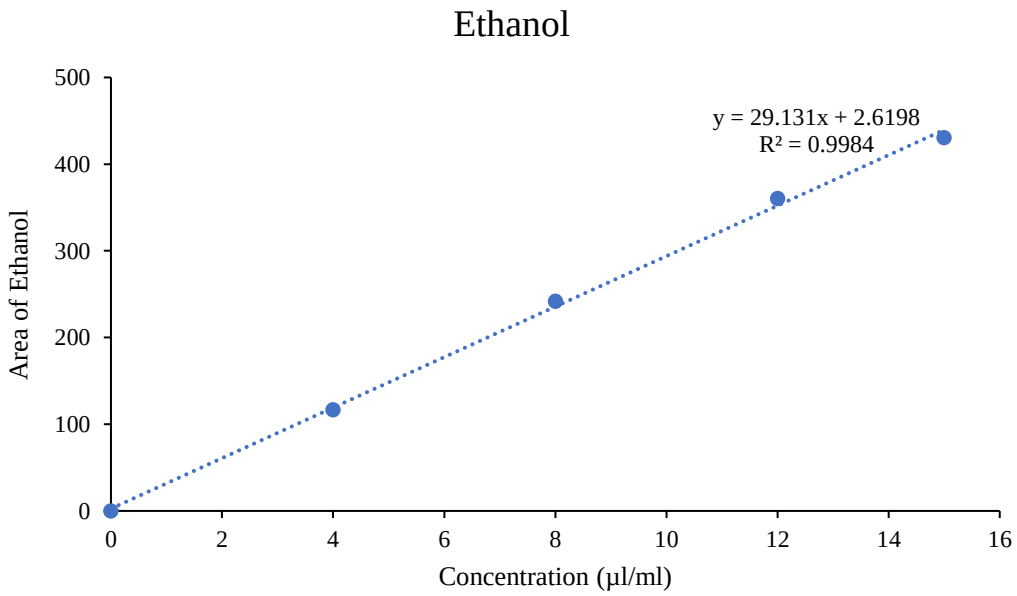


Figure ATC5.6. Standard curve of ethanol (Standard for 11 yeast fermentation on pre-treated RS).

5.4 Explanation of LP, DT and efficiency

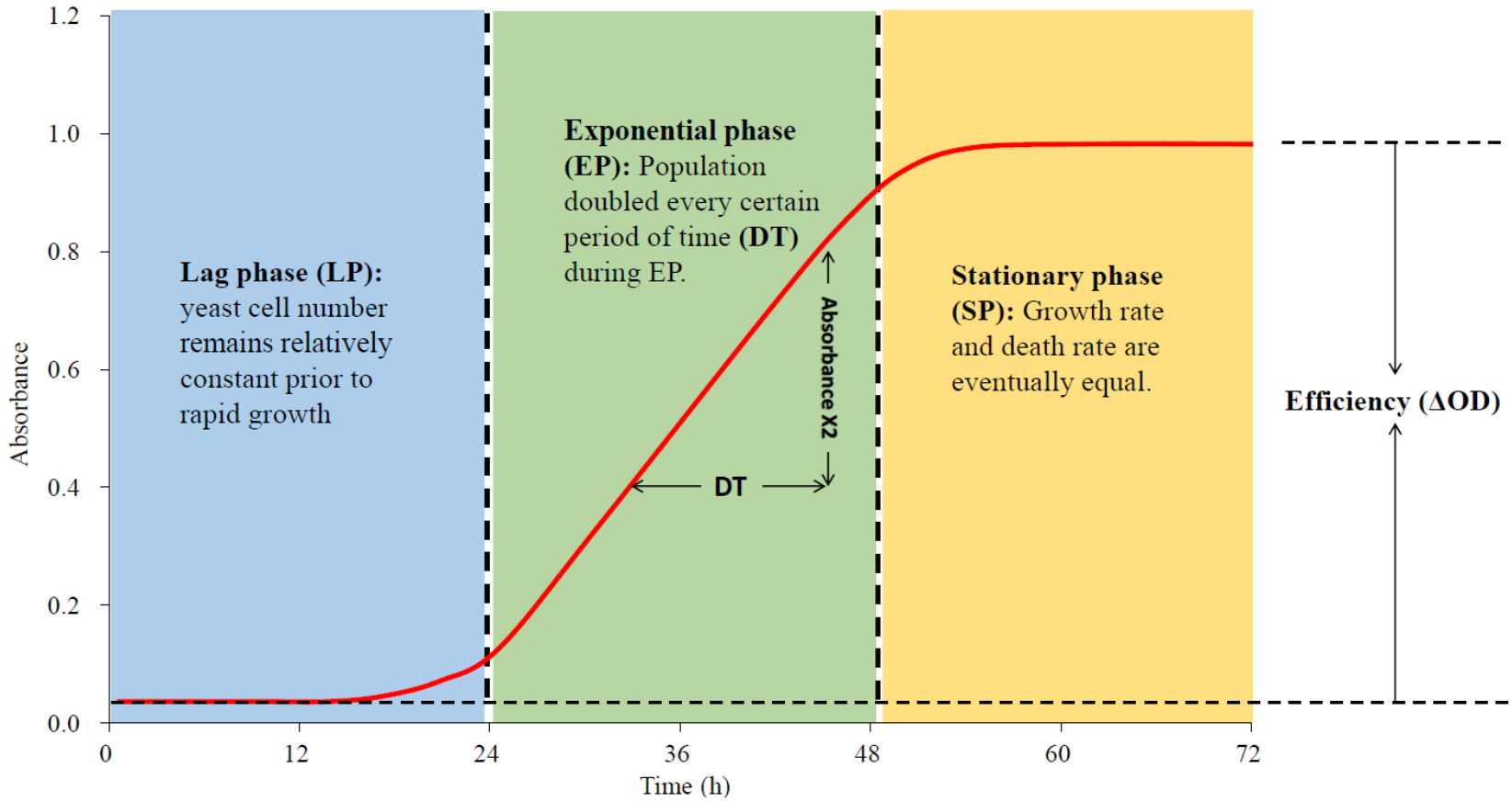


Figure ATC5.7. Illustration of lag phase (LP), doubling time (DT) and efficiency (ΔOD) (Wu et al., 2017).

5.5 Pathway of yeasts for producing ethyl acetate

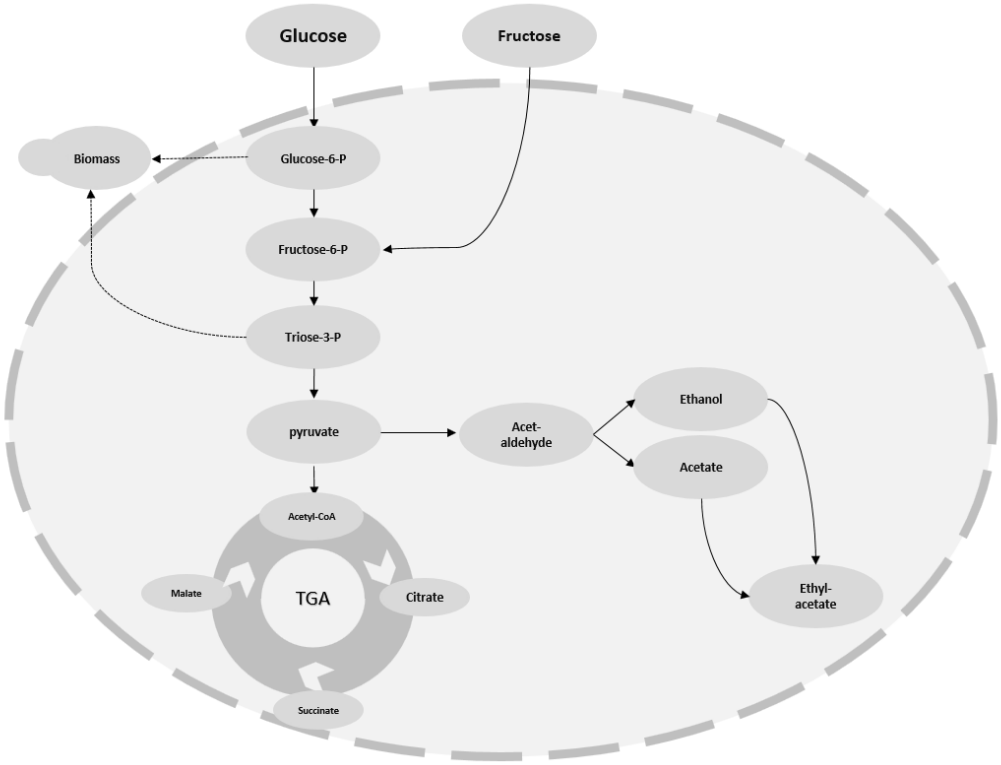


Figure ATC5.8. Brief diagram of metabolic pathway of yeasts.

5.6 Concentration of 16 chemicals (Standard deviations are not presented)

Table ATC5.3. Supplementary data for Chapter 5. These 9 tables below show the concentration of 16 chemicals produced by 11 yeast strains from 13 sugars.

	Cellubiose (m/ml)										
	NCYC 2791	NCYC 65	NCYC 31	NCYC 10	NCYC 568	NCYC 16	NCYC 2433	NCYC 4	NCYC 2577	NCYC 49	NCYC 2826
2,3-Butanediol	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Acetate	0.01	0.00	0.05	0.00	0.02	0.05	0.03	0.01	0.00	0.00	0.01
Arabinitol	0.00	0.00	0.00	0.02	0.04	0.03	0.00	0.00	0.00	0.02	0.03
Citrate acid	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Ethanol	0.02	0.03	1.10	0.01	0.05	0.02	0.10	0.02	0.06	0.07	0.16
Formic acid	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Fumaric acid	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Glycerol	0.01	0.00	0.02	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00
Lactic acid	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01
Pyruvic acid	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Succinate	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Acetaldehyde	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Ethyl acetate	0.00	0.00	0.00	0.01	0.07	0.08	0.04	0.01	0.00	0.00	0.00
Isoamyl alcohol	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Isobutanol	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Malic acid	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Glucose (m/ml)

	NCYC 2791	NCYC 65	NCYC 31	NCYC 10	NCYC 568	NCYC 16	NCYC 2433	NCYC 4	NCYC 2577	NCYC 49	NCYC 2826
2,3-Butanediol	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.03	0.04	0.01
Acetate	0.04	0.03	0.06	0.02	0.05	0.44	0.03	0.01	0.12	0.04	0.04
Arabinitol	0.00	0.01	0.00	1.62	0.11	0.03	0.02	0.03	0.51	0.00	0.00
Citrate acid	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Ethanol	1.96	0.17	3.38	0.43	3.77	0.86	3.52	3.06	1.40	1.13	3.32
Formic acid	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Fumaric acid	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00
Glycerol	0.56	0.01	0.67	0.05	0.68	0.00	0.69	0.31	0.25	0.00	0.66
Lactic acid	0.00	0.00	0.01	0.00	0.00	0.00	0.32	0.01	0.00	0.00	0.03
Pyruvic acid	0.00	0.00	0.03	0.00	0.02	0.00	0.01	0.00	0.01	0.01	0.01
Succinate	0.02	0.00	0.01	0.00	0.02	0.01	0.02	0.01	0.01	0.01	0.01
Acetaldehyde	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00
Ethyl acetate	0.00	0.00	0.00	0.01	0.13	0.64	0.06	0.01	0.01	0.00	0.00
Isoamyl alcohol	0.01	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00
Isobutanol	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.01	0.01	0.00	0.00
Malic acid	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Maltose (m/ml)

	NCYC 2791	NCYC 65	NCYC 31	NCYC 10	NCYC 568	NCYC 16	NCYC 2433	NCYC 4	NCYC 2577	NCYC 49	NCYC 2826
2,3-Butanediol	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.06	0.00	0.00	0.00
Acetate	0.03	0.00	0.04	0.01	0.04	0.70	0.04	0.06	0.05	0.00	0.01
Arabinitol	0.00	0.00	0.00	0.51	0.00	0.02	0.00	0.09	0.00	0.00	0.00
Citrate acid	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00
Ethanol	0.05	0.13	0.52	0.03	0.86	1.25	0.37	3.36	0.10	0.03	0.62
Formic acid	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Fumaric acid	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Glycerol	0.15	0.00	0.02	0.00	0.00	0.00	0.00	0.15	0.00	0.00	0.00
Lactic acid	0.01	0.00	0.00	0.00	0.01	0.00	0.02	0.01	0.00	0.00	0.00
Pyruvic acid	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.01	0.00	0.00	0.00
Succinate	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.02	0.00	0.00	0.00
Acetaldehyde	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Ethyl acetate	0.01	0.00	0.00	0.02	0.25	0.60	0.04	0.02	0.01	0.00	0.00
Isoamyl alcohol	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Isobutanol	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00
Malic acid	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Fructose (m/ml)

	NCYC 2791	NCYC 65	NCYC 31	NCYC 10	NCYC 568	NCYC 16	NCYC 2433	NCYC 4	NCYC 2577	NCYC 49	NCYC 2826
2,3-Butanediol	0.02	0.00	0.00	0.01	0.00	0.00	0.01	0.02	0.00	0.00	0.02
Acetate	0.04	0.01	0.07	0.01	0.06	0.55	0.05	0.01	0.11	0.01	0.03
Arabinitol	0.00	0.00	0.02	1.10	0.16	0.04	0.00	0.03	0.40	0.00	0.00
Citrate acid	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.01	0.00	0.00	0.00
Ethanol	1.89	0.45	3.11	0.43	3.66	0.23	3.87	3.41	3.07	0.25	2.55
Formic acid	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Fumaric acid	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00
Glycerol	0.52	0.00	0.56	0.00	0.60	0.00	0.64	0.38	0.66	0.07	0.47
Lactic acid	0.00	0.00	0.01	0.00	0.01	0.00	0.23	0.01	0.00	0.00	0.03
Pyruvic acid	0.00	0.01	0.03	0.00	0.02	0.00	0.01	0.00	0.01	0.01	0.01
Succinate	0.03	0.00	0.02	0.00	0.02	0.02	0.01	0.01	0.01	0.01	0.01
Acetaldehyde	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Ethyl acetate	0.01	0.00	0.01	0.03	0.22	0.74	0.04	0.02	0.01	0.00	0.00
Isoamyl alcohol	0.01	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00
Isobutanol	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.01	0.00	0.00	0.00
Malic acid	0.00	0.00	0.00	0.00	0.00	0.05	0.00	0.00	0.00	0.00	0.00

Xylose (m/ml)

	NCYC 2791	NCYC 65	NCYC 31	NCYC 10	NCYC 568	NCYC 16	NCYC 2433	NCYC 4	NCYC 2577	NCYC 49	NCYC 2826
2,3-Butanediol	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Acetate	0.03	0.00	0.02	0.01	0.02	0.02	0.02	0.01	0.00	0.00	0.01
Arabinitol	0.00	0.00	0.00	0.09	0.00	0.00	0.00	0.02	0.04	0.07	0.00
Citrate acid	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00
Ethanol	0.15	0.04	0.27	0.01	0.05	0.01	0.19	0.15	0.03	0.01	0.18
Formic acid	0.01	0.00	0.01	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.01
Fumaric acid	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Glycerol	0.00	0.00	0.01	0.00	0.00	0.00	0.01	0.05	0.00	0.00	0.00
Lactic acid	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.01
Pyruvic acid	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Succinate	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00
Acetaldehyde	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Ethyl acetate	0.01	0.00	0.00	0.02	0.07	0.07	0.03	0.02	0.01	0.00	0.01
Isoamyl alcohol	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Isobutanol	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Malic acid	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Mannose (m/ml)

	NCYC 2791	NCYC 65	NCYC 31	NCYC 10	NCYC 568	NCYC 16	NCYC 2433	NCYC 4	NCYC 2577	NCYC 49	NCYC 2826
2,3-Butanediol	0.00	0.01	0.00	0.00	0.01	0.01	0.00	0.03	0.02	0.03	0.01
Acetate	0.05	0.04	0.07	0.02	0.05	0.50	0.08	0.02	0.12	0.02	0.04
Arabinitol	0.01	0.00	0.01	1.06	0.15	0.03	0.00	0.05	0.28	0.15	0.00
Citrate acid	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00
Ethanol	2.63	0.19	2.08	0.99	2.20	0.65	3.37	3.22	2.40	1.10	2.38
Formic acid	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Fumaric acid	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00
Glycerol	0.58	0.00	0.60	0.00	0.68	0.00	0.69	0.39	0.71	0.00	0.60
Lactic acid	0.00	0.00	0.00	0.00	0.00	0.00	0.21	0.01	0.00	0.00	0.03
Pyruvic acid	0.00	0.00	0.03	0.00	0.02	0.00	0.01	0.00	0.00	0.01	0.00
Succinate	0.02	0.01	0.02	0.00	0.02	0.02	0.02	0.01	0.01	0.01	0.01
Acetaldehyde	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Ethyl acetate	0.02	0.01	0.02	0.03	0.12	0.23	0.11	0.07	0.03	0.01	0.01
Isoamyl alcohol	0.01	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00
Isobutanol	0.01	0.00	0.00	0.00	0.01	0.00	0.00	0.01	0.01	0.01	0.00
Malic acid	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Galactose (m/ml)

	NCYC 2791	NCYC 65	NCYC 31	NCYC 10	NCYC 568	NCYC 16	NCYC 2433	NCYC 4	NCYC 2577	NCYC 49	NCYC 2826
2,3-Butanediol	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.11	0.00	0.03	0.01
Acetate	0.12	0.00	0.01	0.01	0.01	0.01	0.03	0.09	0.13	0.04	0.02
Arabinitol	0.00	0.00	0.00	0.95	0.00	0.00	0.00	0.15	0.15	0.00	0.00
Citrate acid	0.01	0.00	0.00	0.01	0.00	0.00	0.00	0.09	0.00	0.01	0.00
Ethanol	2.15	0.06	0.03	0.09	0.04	0.06	0.34	1.64	1.02	0.77	2.96
Formic acid	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00
Fumaric acid	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00
Glycerol	0.34	0.01	0.01	0.00	0.02	0.00	0.02	0.16	0.67	0.00	0.42
Lactic acid	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.01	0.00	0.00	0.04
Pyruvic acid	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.01	0.00
Succinate	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.01	0.01
Acetaldehyde	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Ethyl acetate	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Isoamyl alcohol	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01
Isobutanol	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Malic acid	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Sucrose (m/ml)

	NCYC 2791	NCYC 65	NCYC 31	NCYC 10	NCYC 568	NCYC 16	NCYC 2433	NCYC 4	NCYC 2577	NCYC 49	NCYC 2826
2,3-Butanediol	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.01
Acetate	0.05	0.03	0.04	0.01	0.03	0.51	0.05	0.01	0.08	0.01	0.03
Arabinitol	0.01	0.00	0.00	0.49	0.00	0.07	0.00	0.03	0.19	0.00	0.00
Citrate acid	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00
Ethanol	2.05	0.18	0.36	0.14	0.52	0.70	3.64	3.98	0.39	0.05	2.68
Formic acid	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Fumaric acid	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Glycerol	0.58	0.01	0.00	0.00	0.00	0.00	0.69	0.12	0.00	0.00	0.66
Lactic acid	0.00	0.00	0.00	0.00	0.00	0.00	0.26	0.01	0.00	0.00	0.03
Pyruvic acid	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00
Succinate	0.02	0.01	0.00	0.00	0.00	0.01	0.01	0.00	0.00	0.00	0.01
Acetaldehyde	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Ethyl acetate	0.00	0.00	0.00	0.00	0.04	0.28	0.01	0.00	0.00	0.00	0.00
Isoamyl alcohol	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00
Isobutanol	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00
Malic acid	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00

Lactose (m/ml)

	NCYC 2791	NCYC 65	NCYC 31	NCYC 10	NCYC 568	NCYC 16	NCYC 2433	NCYC 4	NCYC 2577	NCYC 49	NCYC 2826
2,3-Butanediol	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Acetate	0.01	0.00	0.01	0.00	0.01	0.00	0.01	0.00	0.00	0.00	0.00
Arabinitol	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Citrate acid	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Ethanol	0.03	0.03	0.03	0.01	0.02	0.00	0.02	0.00	0.00	0.01	0.00
Formic acid	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Fumaric acid	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Glycerol	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Lactic acid	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00
Pyruvic acid	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Succinate	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Acetaldehyde	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Ethyl acetate	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00
Isoamyl alcohol	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Isobutanol	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Malic acid	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Appendix 6

Standard operating procedure

6.1 Sugar analysis by using GC

Title: SUGARS ANALYSIS by Gas Chromatography	
Reference No: SFC-KWW-002	Version No: 004

QUADRAM INSTITUTE BIOSCIENCE STANDARD OPERATING PROCEDURE	
TITLE: SUGARS ANALYSIS by Gas Chromatography	
APPLIES TO STAFF IN: E124 / E130	
REFERENCE No: SFC-KWW-002	VERSION No: 004
DATE EFFECTIVE: 24 Oct 17	REVIEW DATE: 23 Oct 19
AUTHOR: Sam Collins	APPROVED BY: Keith Waldron
QA and H&S AUTHORISATION: Rachel Walding	DATE ADDED TO QA DATABASE: 24 Oct 17

AUTHORISATION: I confirm that this SOP is an accurate description of the procedure or method in use in my area.

DECLARATION. The safety information contained in this document is to the best of my knowledge an accurate statement of the known and foreseeable hazards to health and of the safety precautions, **which are to be followed** to minimise the risks to health in the process. All users of this SOP are adequately trained to be competent in the process.

Approved by ISP/NC Leader, or Research Leader, or Senior Scientist, or Senior Administrator:

Signature: Name: Keith Waldron Date: 24 Oct 17

Title: SUGARS ANALYSIS by Gas Chromatography	
Reference No: SFC-KWW-002	Version No: 004

1 PURPOSE OF PROCEDURE/METHOD AND ITS SCOPE

To quantitatively analyse the neutral sugars in carbohydrate-containing materials by extraction with 72% (w/w) sulphuric acid followed by derivatisation to alditol acetates and determination by gas chromatography (GC) with flame ionisation detection (FID).

The derivatisation will not produce alditol acetates for uronic acids and so a different procedure must be used for the quantitative determination of these sugars.

See IFR_SOP_SFC-EP_URONIC ACID ANALYSIS by colorimetric assay.doc

2 EQUIPMENT AND REAGENTS NEEDED

Equipment:

Gas Chromatograph: Perkin-Elmer Autosystem XL (GC1) FAN 200005018

Column: Restek Rtx-225, 30m, 0.32mm I.D., 0.25µm d.f.

PC: DELL Optiplex GX270 (with Totalchrom driver software) FAN 200012584

VDU: Viewsonic G70fmb FAN 200012585

Printer: HP Officejet Pro K550 FAN 200013141

Carrier Gas Purifier: Supelco oven (serial no.1135020639442)

Balance: (5 decimal places) METTLER AE240 FAN200004932

Calibrated Micro-pipettes: Gilson P100, P200, P1000 & P5000

Glass culture tubes (e.g. Sovirel - 6 per sample/72% and 1M in triplicate)

Glass beads (acid washed)

GC sample vials (with septa)

Reagents:

- Sulfuric acid
- 2-deoxy glucose
- Ammonia
- Sodium borohydride
- Acetic acid
- 1-methylimidazole
- acetic anhydride
- Dichloromethane

- Acetone

3 STEPS IN PROCEDURE (including safety information)

Ensure relevant quality checks are performed prior to starting procedure or at least on the day of their expected use.

(e.g. check temperature of hot block, ensure pipettes are working properly and holding to within acceptable error from calibration point, ensure calibration of other equipment).

Part A: Saeman Hydrolysis

0. Make sure you have sufficient amounts of the various solutions before starting work.
1. Weigh out between 0.5 and 5 mg material direct into Soveril tube. **Record the weight.**
2. Add **200 µL** 72% H_2SO_4 (w/w) & two glass beads to aid mixing.
3. Incubate at room temperature for **3 hours**. **Mix occasionally.**
4. Add **2.2 mL** water. **Mix** (this dilution makes up 72% H_2SO_4 to 1.0M)
5. Incubate at **100°C** for **1 hour**. **Then cool on ice.**
6. Centrifuge 3500 rpm / 3 mins.
7. Take **0.5 mL** hydrolysate for uronic acid analysis, dilute and freeze (in a container that will hold at least 2.5mL – preferably made of glass and 1→5mL dilution performed).
8. Incubate at **100°C** for a further **1 ½ hours**. **Then cool on ice.**

➔ At this step, the solutions can be frozen and the procedure followed another day.

9. Add **200 µL** 2-deoxy glucose (2-DOG) as internal standard (IS)

   (stock solution: 1.0 mg/mL, therefore the amount added is 200 µg 2-deoxy glucose).

10. Centrifuge 3500 rpm / 3 mins.
11. Transfer **1.0 mL** from the solution above to a fresh tube and add **300 µL** 25% NH_3 (w/w), and **mix**. (The remaining solution must be put in the freezer).

Check the pH with pH indicator paper – should be alkaline (blue) at this point.

12. Add **100 µL** 3M NH_3 containing **150 mg/mL** $NaBH_4$. **(Reduction)**

$NaBH_4$/3M NH_3 fresh on the day

13. **Incubate for 1 hour at 30°C.**
14. Cool on ice and keep the tubes on ice while adding **200µL** glacial acetic acid. **Mix.**

➔ At this step the solutions can be frozen and the procedure followed another day.

15. Transfer **300 µL** from the solution above to a fresh tube. **Cool on ice.**

    (The remaining solution must be put in the freezer).

16. While still on ice, add **450 µL** 1-methylimidazole and **3.0 mL** acetic anhydride.

    Remove from ice, wipe ice deposits from outside of tubes and **mix contents of tube**.

(CARE: 1-methylimidazole is a potential carcinogen). **(Acetylation)**

(Note: V & S, p.1084, 1-methylimidazole does same job as pyridine).

17. Incubate at 30°C for **30 minutes**.
18. **Cool on ice**.
19. Add **3.5 mL** distilled water and **3.0 mL** dichloromethane (DCM). **Mix (vortex).**
20. Centrifuge 3000 rpm / 3 mins.
21. Transfer lower organic layer (DCM) to a second, clean labelled tube.
22. Add **2.0 mL** DCM to the first tube (containing the original aqueous layer)

    Mix (vortex) → Centrifuge (as 18.) → Aspirate the upper (aqueous) layer

(Discard this first aqueous layer to "Waste Non-Chlorinated Solvent" bottle – the waste is approximately 8:7:1 acetic acid/water/1-methylimidazole)

23. Combine the two organic (DCM) layers (containing the alditol acetates).
24. Add **3.0 mL** distilled water (to backwash the organic layer).

    Mix (vortex) → Centrifuge (as 18.) → Aspirate the upper (aqueous) layer & discard

25. Repeat step 24.

    For the last wash, carefully aspirate the aqueous layer up the inside of the tubes

26. Evaporate the DCM, under a stream of nitrogen (or argon) gas, at 40°C in a hot block/sample concentrator.

27. If residual water present, add **1.0 mL** acetone and repeat evaporation from step 25.

28. Dissolve in **1.0 mL** acetone and store at -20°C.

29. Analyse by GC.

Part B: Mild Hydrolysis (Experiment to verify the non-cellulosic glucose)

(eg. Residual starch xyloglucan, mixed linkage β-glucan)

1. Weigh out between 0.5 and 5 mg material direct into Sovirel tube. Record weight.
2. Add **2.2 mL** water and **200 µL** (72% w/w) H_2SO_4 & two glass beads to aid mixing.
3. Incubate at 100°C for **1 hour**. Then cool on ice.
4. Centrifuge 3500 rpm / 3 mins.
5. Take **0.5 mL** hydrolysate for uronic acid analysis and freeze.
6. Incubate at 100°C for more **1 ½ hour**. Then cool on ice.

➔ At this step, the solutions can be frozen and the procedure followed another day

7. Add **200 µL** 2-deoxy glucose (2-DOG) as internal standard (IS) and mix.

   (stock solution: 1 mg/mL, therefore the amount added is 200 µg 2-deoxy glucose).

8. Continue with the method for sugars analysis from step 11.

Volumes required per set of standards/sample replicates:

	5 x standards /ml	3 x sample replicates for 72% and 1 M /ml
72% H_2SO_4	1.0	1.2
Internal standard	1.0	1.2
25% NH_3	1.5	1.8
3M NH_3 + $NaBH_4$	0.5	0.6
Acetic acid	1.0	1.2
Acetic anhydride	15.0	18.0

Acetone	15.0	12.0
Dichloromethane	25.0	30.0
1-methyl imidazole	2.25	2.7

REAGENTS REQUIRED

All reagents are analytical grade. Make up the appropriate amount of solution to prevent wastage, particularly with the ammonia solutions which reduce in concentration over time.

Sulphuric acid is EXTREMELY CORROSIVE at the following concentrations, please ensure appropriate PPE is used. ALWAYS add acid to water, add it slowly and with the container on a bed of ice. The sulphuric acid produces fumes at these concentrations so it should be weighed in a sealable container, with additions made in a fume cupboard. All reagents to be prepared in the fume cupboard.

72% H_2SO_4 (w/w)

Density 96%(w/w) H_2SO_4 = 1.83 g/mL

96% H_2SO_4

72% H_2SO_4 (w/w) ≡ 72g pure H_2SO_4 in 100g total

72 x (100/96) = 75

75g (96%) H_2SO_4 in 100g total

ie.

25.00 g H_2O + 75.00 g 96%H_2SO_4
25.00 mL H_2O + 40.98 mL 96%H_2SO_4

$\sigma = m/V \rightarrow V = m/\sigma \rightarrow V = 75/1.83 = 40.9836$ mL

Density 98%(w/w) H_2SO_4 = 1.84 g/mL

98% H_2SO_4

72% H_2SO_4 (w/w) ≡ 72g pure H_2SO_4 in 100g total

72 x (100/98) = 73.469388

73.47g (98%) H_2SO_4 in 100g total

26.53 g H_2O + 73.47 g 98%H_2SO_4
26.53 mL H_2O + 39.93 mL 98%H_2SO_4

$\sigma = m/V \rightarrow V = m/\sigma \rightarrow V = 73.47/1.84 = 39.929348$ mL

1 M H_2SO_4 (ie. w/v)

Using 96% H_2SO_4 **Fw (H_2SO_4) = 98.08 g/mol** σ(96%(w/w) H_2SO_4) = 1.83 g/mL

1M H_2SO_4 ≡ 98.08 g/L pure H_2SO_4 **$\sigma = m/V$**

98.08 x (100/96) = 102.16667

102.167 g (96%) H_2SO_4 in 1000mL (or 1L) total

$\sigma = m/V \rightarrow V = m/\sigma \rightarrow V = 102.167/1.83 = 55.828962$ mL/L

V = 5.583 mL/100 mL or 55.83 mL/L

(Using 98% H_2SO_4, V = 5.439 mL/100mL or 54.39 mL/L)

Ammonia (NH_3) 25%(w/w) Ammonia 35%(w/w) specific gravity = 0.880 g/mL

25% NH_3(w/w) ≡ 25g pure NH_3 in 100g total

25 x 100/35 = 71.428571

71.429 g NH_3 35%(w/w) in 100 g total

28.571 g H_2O + 71.429 g 35% NH_3
28.571 mL H_2O + 81.17mL 35%NH_3
3.52 mL H_2O + 10.00mL 35%NH_3

$\sigma = m/V \rightarrow V = m/\sigma \rightarrow V = 71.429/0.880 = 81.169318$ mL

3 M NH_3 (ie. w/v) F_W = 17,03 g/mol Ammonia 35%(w/w) specific gravity = 0.880 g/mL

Important: check the concentration of the ammonia on the bottle!

3M NH_3 (w/v) ≡ 51,09g pure NH_3 in 1000mL or (1L / 1dm^3)

51.09 x (100/35) = 145.97143

145.97143 g in 1000mL

$\sigma = m/V \rightarrow V = m/\sigma \rightarrow V = 145.97143/0.880 = 165.87663$ mL/L

V = 165.88 mL/L (35%) NH_3 or

V = 1.659 mL/10mL (35%) NH_3

2-deoxyglucose (2-DOG) (Internal Standard - IS) (1 mg/mL)

25 mg 2-deoxyglucose and make up with triple deionised H_2O to 25 mL

Sodium Borohydride ($NaBH_4$) 1.5g→10.0mL, made up with 3M NH_3 **Sodium Borohydride: highly reactive with water – ensure glassware is dry.**

Acetic acid (glacial)

1-methylimidazole

Acetic anhydride

Acetone

Dichloromethane (DCM – CH_2Cl_2)

Preparation of sugar standards

1. Weigh out approximately **25.0 mg** of each sugar standard (rhamnose, fucose, arabinose, xylose, mannose, galactose, glucose). Record weight.
2. Make up each stock solution with distilled water to **25 mL** in a volumetric flask.
3. Take **100 µL** of each sugar stock solution and add to one Soveril tube.
4. Repeat using volumes of **200, 300** and **400 µL**.

5. Make up 0, 100, 200 and 300 µL mixtures to 2.2 mL volume using: **2.2, 1.5, 0.8 and 0.1 mL** of water respectively.
6. Add **200 µL** of 72% H_2SO_4 to these mixtures and **0.255 mL** 72% H_2SO_4 to the 400 µL mixture.
7. Add **200 µL** of the internal standard.
8. Take **1.0 mL** of each of the five standard mixtures and continue with method for sugar analysis from step 11.

Vol. stock (µL)	Vol. stock mixture (µL)	Vol. water (µL)	Vol. 72% H_2SO_4 (µL)
0	0	2,200	200
100	700	1,500	200
200	1,400	800	200
300	2,100	100	200
400	2,800	0	255

6.2 Biotage initiator

Title: age Initiator+	
Reference No: SFC-KWW-043	Version No: 002

INSTITUTE OF FOOD RESEARCH STANDARD OPERATING PROCEDURE	
TITLE: Biotage Initiator+ Special case – Biomass needing a FERA licence or subject to otherwise additional regulation Eg. Spent mushroom compost from Uganda, Sugar Cane Bagasse, Rice straw.	
APPLIES TO STAFF IN: The Biorefinery Centre – Trained staff only	
REFERENCE No: SFC-KWW-043	**VERSION No: 002**
DATE EFFECTIVE: 21 Oct 16	**REVIEW DATE: 20 Oct 18**
AUTHOR: Adam Elliston	**APPROVED BY: K. Waldron**
QA and H&S AUTHORISATION: R Walding	**DATE ADDED TO QA DATABASE: 21 Oct 16**

AUTHORISATION: I confirm that this SOP is an accurate description of the procedure or method in use in my area.

DECLARATION. The safety information contained in this document is to the best of my knowledge an accurate statement of the known and foreseeable hazards to health and of the safety precautions, **which are to be followed** to minimise the risks to health in the process. All users of this SOP are adequately trained to be competent in the process.

Approved by ISP/NC Leader, or Research Leader, or Senior Scientist, or Senior Administrator:

Signature:	Name: K. Waldron	Date: 21 Oct 16

Title: age Initiator+	
Reference No: SFC-KWW-043	Version No: 002

1 PURPOSE OF PROCEDURE/METHOD AND ITS SCOPE

Organic synthesis and pre-treatment of biomass.

Some biomass material originates in countries or areas with specific plant and other pathogen contamination. This usually will require a licence from FERA and additional consideration of human pathogen. The process itself (even at its mildest severity) is sufficiently harsh to inactivate any likely pathogen.

2 EQUIPMENT AND REAGENTS NEEDED

Equipment:

1) Biotage Initiator+
2) Biotage Vials
3) Stirrer bars
4) Caps
5) Vial collars
6) Air compressor
7) Vial capper
8) Vial un-capper
9) Fume cupboard

Reagents:

None

3 STEPS IN PROCEDURE (including safety information)

1) Place sample in vial
2) Check vial size is appropriate for volume added
3) Add appropriate magnetic flea
4) Seal the vial
5) Place collar around vial lid
6) Turn on power to the compressor
7) Turn on Biotage
8) Click “organic synthesis”

9) Enter parameters using "wizard" option

10) Start synthesis

- If a vial should break it will be contained, shutdown the machine if it has not already done so. Clean the inside of the machine thoroughly, replacing the safely sponge insert before proceeding.

13) When the synthesis has stopped, the lid will automatically open

- Do not manually open the lid while it is still under pressure, many safety prompts will appear before you can do so – please take note of them.

14) Remove the vial from the machine

15) Remove the collar

16) As biological materials can break-down into potentially harmful substances - particularly organic acids ensure that you open vials gently using the de-capping tool **inside a fume cupboard**. Rinse vials in the fume cupboard to prevent the release of volatiles to the air.

SAFETY INFORMATION

***Principal Hazards*:**

1) Liquids under pressure
2) Organic acids and unknown synthesis products. (refer to SDS's prior to use of sunstances)
3) Electrical hazards and microwave exposure will only occur if there is a fault with the machine.
4) Containment of biomass and dust prior to treatment.

***Method Steps involving Higher Risk (and precautions to minimise risk*):**

1. Follow on-screen commands to minimise risk to safety
2. Ensure glass vials are clean and adequately stirred to prevent vial rupture
3. Work in a fume cupboard opening vials.
4. Restricted biomass to be stored in triple containment in designated area.
5. Special care to be taken not to generate dust and aerosol. Any dust and spilled biomass to be cleaned with HEPA filter fitted vacuum cleaner and/or wet paper. All untreated biomass and cleaning material including vacuum cleaner bag to be autoclave prior to disposal.

Essential Precautions: Allow vials to cool before opening in the fume cupboard

Personal Protective Clothing: Lab coat, gloves, safety specs

Any restrictions on use: Trained personnel only

Exceptions or Warnings should be included at relevant step in the procedure

6.3 Klason lignin analysis

Title: Gravimetric Analysis of Klason Lignin	
Reference No: SFC-KWW-004	Version No: 005

INSTITUTE OF FOOD RESEARCH STANDARD OPERATING PROCEDURE	
TITLE: Gravimetric Analysis of Klason Lignin	
APPLIES TO STAFF IN: E124 / E130	
REFERENCE No: SFC-KWW-004	**VERSION No: 005**
DATE EFFECTIVE: 14 Jan 16	**REVIEW DATE: 13 Jan 18**
AUTHOR: G. Moates	**APPROVED BY: Keith Waldron**
QA and H&S AUTHORISATION: R Walding	**DATE ADDED TO QA DATABASE: 14 Jan 16**

AUTHORISATION: I confirm that this SOP is an accurate description of the procedure or method in use in my area.

DECLARATION. The safety information contained in this document is to the best of my knowledge an accurate statement of the known and foreseeable hazards to health and of the safety precautions, **which are to be followed** to minimise the risks to health in the process. All users of this SOP are adequately trained to be competent in the process.

Approved by ISP/NC Leader, or Research Leader, or Senior Scientist, or Senior Administrator:

Signature:	Name: Keith Waldron	Date: 14 Jan 16

Title: Gravimetric Analysis of Klason Lignin	
Reference No: SFC-KWW-004	Version No: 005

1 PURPOSE OF PROCEDURE/METHOD AND ITS SCOPE

Determination of lignin based on the hydrolysis and solution of polysaccharides with strong mineral acids, leaving a residue which, after washing and drying, is weighed as lignin. The use of sulphuric acid for determination of lignin was first applied by Klason (Arkiv Kemi, 3, No. 5, 17, 1906) and although the method has been modified extensively, the lignin isolated with this acid is usually referred to as 'Klason lignin'.

The hydrolysis of the polysaccharides is accomplished by an initial treatment with strong sulphuric acid (ca 68-78%) until the partially hydrolysed polysaccharides are no longer precipitated upon dilution. The sulphuric acid is diluted to 1 M and the polysaccharides are boiled to complete hydrolysis. The residue represents the Klason lignin (a mixture of lignin + residual protein and ash). Suitable pre-extractions are necessary to remove extraneous materials that otherwise would appear with lignin. The method is represented by the standards of TAPPI (T13m) and ASTM (D1106).

2 EQUIPMENT NEEDED

- Balance (4/5 figure – Mettler AE240)
- Pyrex glass culture tubes (e.g. Sovirel)
- Temperature controlled hot block
- Sintered glass funnels, porosity 3, diameter 50mm
- Vacuum manifold with acid resistance valve taps and adapters to fit sintered funnels
- Vacuum or suction pump with suitable trap for acid spray (e.g. soda lime tower)
- Temperature controlled oven
- pH indicator paper
- Muffle furnace
- Sulphuric acid 72%w/v, prepared from 96-98% (e.g. Fluka)

3 STEPS IN PROCEDURE (including safety information)

1. Weigh out accurately about 100mg of each sample in triplicate, into Sovirel culture tubes (Sample size can be up to 1g dry weight with appropriate addition of acid).

2. Pre-weigh sintered glass funnels with a precision of 0.1mg.

3. Disperse each sample in 1.5mL of 72% sulphuric acid and incubate at approximately 30°C for one hour with periodic agitation.

4. Add 15.0mL of water, thoroughly mix contents, and incubate at 100°C for 2.5 hours in the Sovirel culture tubes with caps lightly screwed on.

5. The insoluble lignin is allowed to settle, and the mixture is filtered into a pre-weighed sintered glass funnel, using (gentle) suction. The lignin is washed free of acid with hot water (approximately 500 ml required or more).

6. Dry the glass funnels and contents at 50°C in a temperature controlled oven overnight or until a constant weight is obtained.

7. Allow the sintered glass funnels and contents to equilibrate to room temperature, (preferably in a sealed dessicator) and immediately re-weigh them. The drying and the weighing are repeated until the weight is constant.

Additional steps for ash correction & cleaning of sinter funnels

8. Place the samples in a muffle furnace set to 200°C to char. Ensure fume hood is set to extract. Allow the samples to reach 200°C. The furnace is hot so **wear insulated gloves.**

9. Once at temperature and there is little or no smoking, raise the temperature (50°C increment) and leave to equilibrate at each temperature – little or no smoking (should not smoke above ~250°C but care required to avoid sample ignition if organic matter persists).

10. Repeat step 9 until the temperature reaches 350°C. Then close the furnace door, set the temperature to 500°C and ash about 16 hours (overnight).

11. Switch off furnace and allow samples to cool in situ until 'safe' to handle (<50°C). **[Handle wearing insulated gloves].**

12. Remove the samples from the furnace, using tongs, and allow them to cool in a closed dessicator.

13. Weigh to determine 'gross' ash content recovered (check ash is pale grey / white, i.e. ashing is complete)

Klason lignin is calculated from the difference in weight of each glass funnel.

i.e.

Klason lignin = (wt funnel + residue) – wt funnel & weight of ash (mg/g sample) sample wt

This method can be adapted for use with a smaller amount of sample (see Notes below).

6.4 GOPOD assay for glucose

Title: D-Glucose Assay (Megazyme GOPOD Method)	
Reference No: SFC-KWW-027	Version No: 004

QUADRAM INSTITUTE BIOSCIENCE STANDARD OPERATING PROCEDURE	
TITLE: D-Glucose Assay (Megazyme GOPOD Method)	
APPLIES TO STAFF IN: E124 / E130	
REFERENCE No: SFC-KWW-027	**VERSION No: 004**
DATE EFFECTIVE: 10 Nov 17	**REVIEW DATE: 31 Mar 18**
AUTHOR: Adam Elliston	**APPROVED BY: Keith Waldron**
QA and H&S AUTHORISATION: R Walding	**DATE ADDED TO QA DATABASE: 11 Nov 17**

AUTHORISATION: I confirm that this SOP is an accurate description of the procedure or method in use in my area.

DECLARATION. The safety information contained in this document is to the best of my knowledge an accurate statement of the known and foreseeable hazards to health and of the safety precautions, which are to be followed to minimise the risks to health in the process. All users of this SOP are adequately trained to be competent in the process.

Approved by ISP/NC Leader, or Research Leader, or Senior Scientist, or Senior Administrator:

Signature: Name: Keith Waldron Date: 10 Nov 17

Title: D-Glucose Assay (Megazyme GOPOD Method)	
Reference No: SFC-KWW-027	Version No: 004

1 PURPOSE OF PROCEDURE/METHOD AND ITS SCOPE

The Megazyme D-Glucose (glucose oxidase/peroxidase; GOPOD) Assay Kit employs high purity glucose oxidase and peroxidase and can be used with confidence for the specific measurement of D-glucose in extracts of plant materials or foods. The colour which forms is stable at room temperature for at least two hours after development.

2 EQUIPMENT AND REAGENTS NEEDED

Equipment:

- Thermal shake Incubator
- Pipette – Gilson P20 and P1000
- Vortex Mixer
- Eppendorf tubes
- 96 Well Reader Plate
- LT4000 Plate Reader or other plate reader

Reagents:

Dilute the contents of bottle 1 (GOPOD Reagent Buffer) to 1 L with distilled water. Use immediately.

Dissolve the contents of bottle 2 in 20 mL of solution 3 and quantitatively transfer this to the bottle containing the remainder of solution 3. Cover this bottle with aluminium foil to protect the enclosed reagent from light. This is Glucose Determination Reagent **(GOPOD Reagent)**. Stable for ~ 3 months at 2-5°C or > 12 months at -20°C. If this reagent is to be stored in the frozen state, preferably it should be divide into aliquots that should be freeze/thawed only once during use. When the reagent is freshly prepared it may be light yellow or light pink in colour. It will develop a stronger pink

Standard Curve

GOPOD method works a low glucose concentrations so standard curve should be 0 - 5mg/mL.

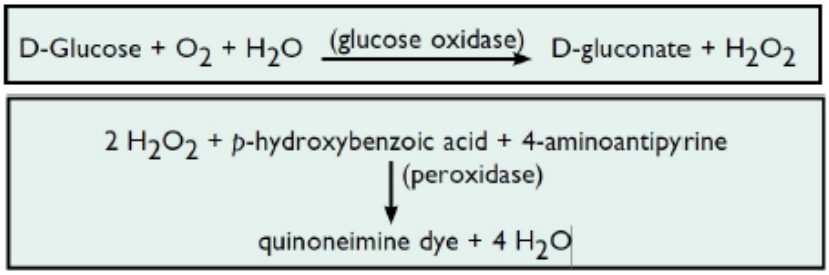
Weigh out an appropriate amount of D-Glucose and use same dilution liquid as samples are dissolved in (e.g. Acetate Buffer)

Make 10 appropriate dilutions of this standard.

3 STEPS IN PROCEDURE (including safety information)

PRINCIPLE:

The reactions involved are:



Assay Procedure

Ensure relevant quality checks are performed prior to starting procedure or at least on the day of their expected use.

(e.g. check temperature of hot block, ensure pipettes are working properly and holding to within acceptable error from calibration point, ensure calibration of other equipment).

1. Transfer 20µL sample / standard in triplicate into eppendorf tubes.
2. Add 600µL of GOPOD Reagent to each tube (including the glucose controls and reagent blanks)
3. Vortex mix all tubes thoroughly.
4. Incubate the tubes in Thermalshake Incubator at 50°C for 20 min.
5. Transfer 200µL into a 96 well reader plate (don't use outside cells), ensure there is a standard curve on each plate used.
6. Read at 510nm
7. Glucose is calculated from the standard curve.

6.5 Sample preparation for NMR screening (yeasts)

Title: NMR Screening of Multiplexed NCYC Yeast Strains	
Reference No: FH-KWW-002	Version No: 002

INSTITUTE OF FOOD RESEARCH STANDARD OPERATING PROCEDURE	
TITLE: NMR Screening of Multiplexed NCYC Yeast Strains	
APPLIES TO STAFF IN: E331 / E124	
REFERENCE No: FH-KWW-002	**VERSION No: 002**
DATE EFFECTIVE: 09 May 16	**REVIEW DATE: 08 May 18**
AUTHOR: A Elliston	**APPROVED BY: K Waldron**
QA and H&S AUTHORISATION: R Walding	**DATE ADDED TO QA DATABASE: 09 May 16**

AUTHORISATION: I confirm that this SOP is an accurate description of the procedure or method in use in my area.

DECLARATION. The safety information contained in this document is to the best of my knowledge an accurate statement of the known and foreseeable hazards to health and of the safety precautions, **which are to be followed** to minimise the risks to health in the process. All users of this SOP are adequately trained to be competent in the process.

Approved by ISP/NC Leader, or Research Leader, or Senior Scientist, or Senior Administrator:

Signature: Name: K Waldron Date: 09 May 16

Title: NMR Screening of Multiplexed NCYC Yeast Strains	
Reference No: FH-KWW-002	Version No: 002

1 PURPOSE OF PROCEDURE/METHOD AND ITS SCOPE

To inoculate multiplexed yeast samples into minimal media in order to screen for useful products. Then to prepare samples for analysis by NMR.

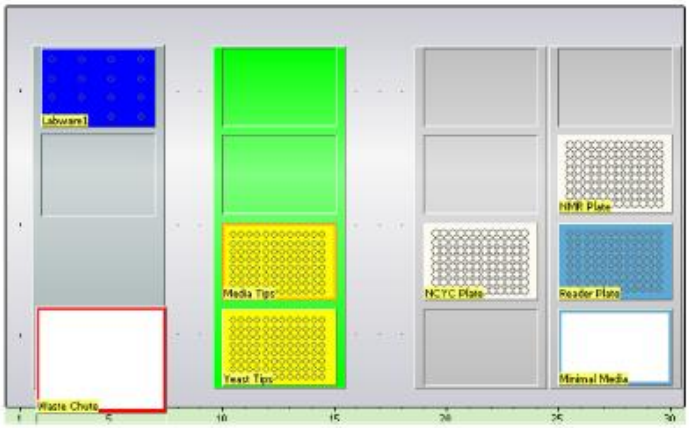
2 EQUIPMENT NEEDED

- Balance
- Magnetic stirrer
- Tecan Robot – *Training required*
- Centrifuge equipped with plate rotor – *Training required*
- 25 ℃ Incubator / Room
- Anaerobic Jar
- Micro-titre plate reader (~600 nm) – *Training required SOP Ref: SFC-KWW-021*
- Consumables
  - 2 Deep well plates (2 mL) *(Starlab, E2896-1810)* – **Sterile**
  - 1 Quickseal DMSO Plate seals *(IST, IST-126-080LS)*
  - 2 Micro-titre reader plates (Nunc)
  - 2 Robot troughs – **Sterile**
  - 4 Racks of robot pipettes *(Starlab, R1129-1810)* – **Sterile**
  - 96 NMR Tubes *(GPE, 502-7)*
  - Anaerobic Atmosphere Generation Bag *(Sigma, 68061-10SACHETS)*
  - Anaerobic indicator test *(Sigma, 59886-1PAK-F)*

3 STEPS IN PROCEDURE (including safety information)

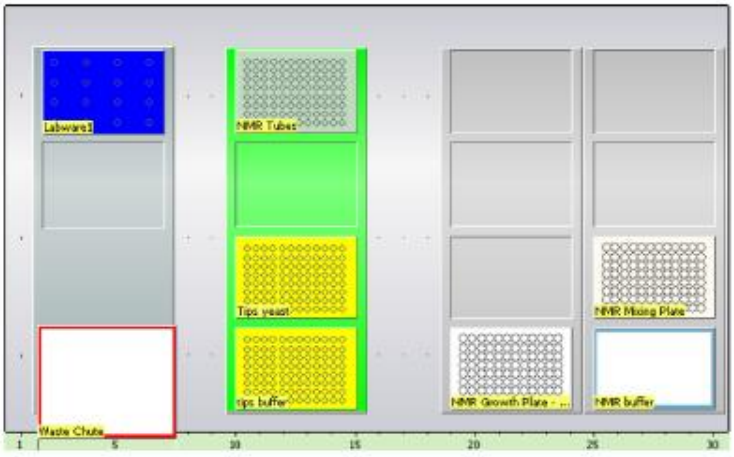
1. Autoclave deep well plates, robot troughs and Nitrogen base media (see below for recipe)
2. Load consumables onto Tecan Robot as below

   Training is required for this equipment



3. Inoculate 3 deep well plates with 1.65 mL Nitrogen base media and 55 µL of desired yeast using the Tecan robot, programme "*NMR_Plate_Preparation*".
4. 155 µL is then transferred into a micro-titre reader plate using this programme, which should then be read at 590 nm on Biotek FL-600 plate reader using the "yeast turbidy" kinetic protocol to give a growth curve over 96 hours.
   Training is also required for this equipment – SOP Ref: SFC-KWW-021
5. Deep well plates are covered with DMSO plate seals
6. Plates are placed in an anaerobic jar along with a gas pack and indicator strip.
7. Then incubated at 25℃ for 96 hours.

8. Tecan robot is loaded as below and programme "*NMR_Plate_buffer*" is run which carries out steps 10 – 16 below (except centrifugation steps)



9. When incubation is complete 155 µL is transferred to a micro-titre reader plate and a final endpoint turbidity reading is taken at 590 nm.
10. Yeast plates are then centrifuged at 3000 rpm for 15 minutes
11. 400 µL supernatant (no yeast cells) are transferred to a new deep well
12. 400 µL NMR buffer added (see reagents section below for recipe)
13. Plate is mixed by robot (10 x 100 µL)
14. Plate is centrifuged again at 3000 rpm for 15 minutes
15. 600 µL transferred to NMR tubes then assayed by NMR

Reagents - recipes

- **Minimal media**
  - Formedium nitrogen base w/o Amino Acids 6.9 g/L
  - L-Histidine 10 mg/L
  - L-Methionine 20 mg/L
  - L-Tryptophan 20 mg/L
  - Glucose 10 g/L

- **NMR Buffer (0.4 M) pH 6.4**
  - $NaH_2PO_4.H_2O$ 42 g/L
  - K_2HPO_4 16.5 g/L
  - 0.5 mM TSP 86 mg/L
  - Sodium Azide 200 mg/L
  - 100 µM EDTA 1 mL/L of 100 mM
    - 100 mM EDTA 0.372 g in 10 mL D_2O

Volumes

	Per well	Per Plate (96 wells)	Per replicate set (3 plates/replicate)	Whole Collection (36 plates in collection)
Minimal Media	1.5 mL	144.0 mL	432.0 mL	15,552.0 mL
NMR Buffer	0.4 mL	38.4 mL	115.2 mL	4,147.2 mL

6.6 Phenolics extraction from lignocellulosic biomass and analysis by HPLC

Title: Sequential Phenolic Acid Extraction from Plant Cell Wall and Analysis by HPLC	
Reference No: SFC-KWW-003	Version No: 004

QUADRAM INSTITUTE BIOSCIENCE STANDARD OPERATING PROCEDURE	
TITLE: Sequential Phenolic Acid Extraction from Plant Cell Wall and Analysis by HPLC	
APPLIES TO STAFF IN: E124 / E130	
REFERENCE No: SFC-KWW-003	VERSION No: 004
DATE EFFECTIVE: 24 Oct 17	REVIEW DATE: 23 Oct 19
AUTHOR: Sam Collins	APPROVED BY: Keith Waldron
QA and H&S AUTHORISATION: Rachel Walding	DATE ADDED TO QA DATABASE: 24 Oct 17

AUTHORISATION: I confirm that this SOP is an accurate description of the procedure or method in use in my area.

DECLARATION. The safety information contained in this document is to the best of my knowledge an accurate statement of the known and foreseeable hazards to health and of the safety precautions, which are to be followed to minimise the risks to health in the process. All users of this SOP are adequately trained to be competent in the process.

Approved by ISP/NC Leader, or Research Leader, or Senior Scientist, or Senior Administrator:

Signature: Name: Keith Waldron Date: 24 Oct 17

Title: Sequential Phenolic Acid Extraction from Plant Cell Wall and Analysis by HPLC	
Reference No: SFC-KWW-003	Version No: 004

1 PURPOSE OF PROCEDURE/METHOD AND ITS SCOPE

To quantitatively analyse the alkali-extractable phenolic acids in samples of plant cell wall material using HPLC.

2 EQUIPMENT AND REAGENTS NEEDED

Equipment:

- Perkin-Elmer series 200 LC pump
- Perkin-Elmer advanced LC Processor ISS200
- Phenomenex Column Luna 5µ C18(2), 250 x 4.0mm with pre-column
- Perkin Elmer Diode Array Detector (UV)
- PE Nelson 100 series Interface
- PE Nelson 600 series Link
- PC (Dell Pentium 4 Optiplex GX260) and peripherals
- Gilson Pipettes (P5000, P1000 & P200)
- Screw cap glass culture tubes ("sovirel" tubes)
- 5-figure balance (Mettler AE-240)
- Bench top centrifuge
- Vortex mixer and rotary tube mixer or shaking incubator (Gerhardt Thermoshake)
- Sample concentrator/hot block (Techne)

Reagents:

- Sodium Hydroxide
- Methanol
- Hydrochloric acid
- ethyl acetate
- Protocatechuic Acid
- Chlorogenic Acid
- p-OH-Benzoic Acid

- p-OH-Phenyl Acetic Acid
- Vanillic Acid
- Vanillin
- p-OH-Benzaldehyde
- t-p-Coumaric Acid
- t-Ferulic Acid
- Caffeic Acid
- Acetonitrile
- TFA

3 STEPS IN PROCEDURE (including safety information)

Alkali concentrations recommended: 0.1 M NaOH, 1 hr; 0.1 M NaOH, 24 hr; 1 M NaOH, 24 hr; 2 M NaOH, 24 hr; 4 M NaOH, 24 hr. Using all these steps will take at least a week.

1. Weigh **10-12 mg** of sample (from fruit or vegetable origin) or **5.0 mg** of sample (from cereal origin) accurately into Sovirel tubes.
2. Add sodium hydroxide (in **nitrogen-flushed, de-oxygenated** water) of the required concentration (0.1 M to 4 M), **4 mL**, over-flush with nitrogen, and mix it. Wrap in aluminium foil and leave for 1 hr or 24 hr (depending on requirements) with agitation (rotary tube mixer).
3. Centrifuge (**1000 rpm, 5 min**). Pipette maximum volume of liquid out without transferring solid to clean sovirel tube. May be achieved by double tipped Gilson if pellet not solid. Add next concentration of alkali to residue and continue as from step 2.
4. Add **50 µl** *trans*-cinnamic acid solution (**10 mg** in **50 mL** 50:50 MeOH:MilliQ water) and mix it. Add conc. Hydrochloric acid dropwise until the pH falls to 2. (Test very small aliquots with pH paper.)
5. Extract the aqueous phase with ethyl acetate (**3 mL**) three times; centrifuge (**3000 rpm, 5 min**), to separate the layers, (may need to swirl gently to break up stable foam at interface). Combine the extracts in a clean sovirel tube.
6. Evaporate the ethyl acetate dry in the sample concentrator (**40** °C, N_2).
7. Redissolve sample in **0.5** or **1 mL**, methanol/water (50:50). Filter **200 µl** through a filter disc (0.20 µm, PVDF) into a Chromacol vial and cap.

8. Analyse by HPLC using the method set up for phenolic acids.

Preparation of Phenolic Acid Standards

Protocatechuic Acid
Chlorogenic Acid
p-OH-Benzoic Acid
p-OH-Phenyl Acetic Acid (made up with 50% MeOH)
Vanillic Acid 20.0mg of each} → 100.0mL ≡ 0.2 mg/mL (of each STD)
Vanillin
p-OH-Benzaldehyde
t-p-Coumaric Acid
t-Ferulic Acid
Caffeic Acid (optional)

Take aliquots of the 0.2mg/mL STD solution and perform dilutions (using 50% MeOH) to achieve the following standard concentrations

STD conc. [mg/mL]	Aliquot of 0.2mg/mL STD [mL]
0.1	25.0 → 50.0
0.05	12.5 → 50.0
0.02	5.0 → 50.0

Run at least a single set of standards with each batch of samples. If the standards are freshly prepared it would be advisable to run a triplicate set of STDs for QA purposes.

These standards can be aliquoted into micro-centrifuge tubes and frozen for later use.

Title: Sequential Phenolic Acid Extraction from Plant Cell Wall and Analysis by HPLC	
Reference No: SFC-KWW-003	Version No: 004

HPLC method

Solvent C =	Acetonitrile	:	Methanol	:	Water
	MeCN or ACN	:	MeOH	:	H_2O
	40 %	:	40 %	:	~20 %

containing 1mM trifluoroacetic acid (TFA)

800 mL MeCN

800 mL MeOH } $\xrightarrow{H_2O(milliQ)}$ 2L

154 µL TFA

Solvent D =	Acetonitrile	:	Water
	10%	:	~90%

containing 1mM trifluoroacetic acid (TFA)

200mL MeCN } $\xrightarrow{H_2O(milliQ)}$ 2L

154 µL TFA

Gradient programme

% C	% D	Duration (minutes)	Gradient
10	90	0.5	0 (isocratic)
→75	25←	25.0	1
→100	0←	5.0	1
10←	→90	10.0	-3
10	90	2.0	0 (isocratic)