BIOCONVERSION OF RICE HUSK TO POLYHYDROXYBUTYRATE VIA PRETREATMENT AND ENZYMATIC HYDROLYSIS

By

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LIST OF SYMBOLS AND ABBREVIATIONS

%	percentage
°C	degree celcius
(v/v)	volume per volume
(w/v)	weight per volume
α	alpha
β	beta
ε	epsilon
γ	gamma
ω	omega
μL	microliter
μm	micrometer
Đ	polydispersity
Da	dalton
g	gram
Gy	gray
Hz	hertz
J	joule
kg	kilogram
kGy	kilogray
kPa	kilopascal
kV	kilovolt
kW	kilowatt
kWh	kilowatt hour
L	liter
log R_0	severity factor
M	molarity
M_n	number-average molecular weight
M_w	weight-average molecular weight
mg	milligram
MHz	megahertz
mins	minutes

MJ	megajoule
mL	milliliter
mM	millimolar
mol%	mol percent
MPa	megapascal
nm	nanometer
W	watt
wt%	weight percent
2,5-DHBA	2,5-dihydroxybenzoic acid
3,4-DHBA	3,4-dihydroxybenzoic acid
3H4MV	3-hydroxy-4-methylvalerate
3HB	3-hydroxybutyrate
3HHx	3-hydroxyhexanoate
3HV	3-hydroxyvalerate
4HA	4-hydroxyalkanoic acid
4HB	4-hydroxybutyrate
4-HBA	4-hydroxybenzoic acid
5HA	5-hydroxyalkanoic acid
ADH	alcohol dehydrogenase
AFEX	ammonia fiber explosion
AIL	acid-insoluble lignin
AIR	acid-insoluble residue
AlDH	aldehyde dehydrogenase
AOAC	Association of Analytical Chemists
ARP	ammonia recycle percolation
ASL	acid-soluble lignin
ASTM	American Society for Testing and Materials
BSA	bovine serum albumin
CBM	carbohydrate-binding module
CD	catalytic domain
CDW	cell dry weight
CE	European Conformity
CME	caprylic methyl ester
CRE	catabolite-responsive element

dH_2O	deionized distilled water
DIN	Deutsches Institut Fur Normung
DNS	dinitrosalicylic acid
DP	degree of polymerization
EBI	electron beam irradiation
EC	Enzyme Commission
FAO	Food and Agricultural Organization of the United Nations
FDA	United States Food and Drug Administration
FID	flame ionization detector
GAE	gallic acid equivalents
GC	gas chromatography
GH	glycoside hydrolase
GPC	gel permeation chromatography
HA	hydroxyalkanoic acid
HHV	higher heating value
HMF	5-hydroxymethylfurfural
HPAEC	high performance anion exchange chromatography
HPLC	high performance liquid chromatography
HV	heating value
HPS	Husk Power Systems
HSD	honest significant difference
HTP	high temperature and pressure
IRS	infrared spectroscopy
IUPAC	International Union of Pure and Applied Chemistry
LAP	Laboratory Analytical Protocol
MAS	magnetic angle spinning
MCC	microcrystalline cellulose
MI	microwave irradiation
MM	mineral salts medium
N	normality
NADH	nicotinamide adenine dinucleotide phosphate
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NMR	nuclear magnetic resonance
NR	nutrient rich

NREL	National Renewable Energy Laboratory
OD	optical density
ODW	oven dry weight
OPEFB	oil palm empty fruit bunch
P(3HB)	poly(3-hydroxybutyrate)
P(3HB-*co*-3HHx)	poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate)
P(3HB-*co*-3HV)	poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate)
P(3HB-*co*-4HB)	poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate)
PDH	pyruvate dehydrogenase
PE	polyethylene
PEP	phosphoenolpyruvate
PHA	polyhydroxyalkanoate
PLA	polylactic acid
PP	polypropylene
PPP	pentose phosphate pathway
PTFE	polytetrafluoroethylene
PTS	phosphotransferase
RH	rice husks
RHA	rice husk ash
RID	refractive index detector
ROS	reactive oxygen species
rpm	revolutions per minute
RSM	response surface methodology
RT	room temperature
SAA	soaking in aqueous ammonia
SEM	scanning electron micrograph
SHS	superheated steam
SRS	sugar recovery standards
TAPPI	Technical Association of the Pulp and Paper Industry
TCA	tricarboxylic acid
TGA	thermogravimetric analysis
UDP	uridine diphosphate
UV	ultraviolet
XRF	X-ray fluorescence spectrometry

BIOPENUKARAN SEKAM PADI KEPADA POLIHIDROKSIBUTIRAT MELALUI PRARAWATAN DAN HIDROLISIS ENZIM

ABSTRAK

Beras merupakan antara sumber makanan yang terbesar di seluruh dunia. Di Malaysia, purata penghasilan padi adalah lebih daripada 2 juta tan setahun. Daripada hasil padi yang dituai, sekitar 20% komposisinya adalah sekam padi yang biasanya akan terbuang. Sekam padi (RH) terdiri daripada lignoselulosa yang boleh ditukar kepada substrat untuk fermentasi. Kajian ini telah dijalankan untuk menilai potensi sekam padi sebagai sumber karbon untuk penghasilan polihidroksialkanoat (PHA), iaitu sejenis bioplastik yang dihasilkan oleh pelbagai jenis bakteria. Untuk mengatasi sifat ketahanan dan kekerasan biojisim ini, kaedah prarawatan fizikokimia telah dijalankan ke atas sekam padi dalam keadaan yang berbeza dan keberkesanannya telah dibanding berdasarkan penghasilan gula selepas hidrolisis enzim. Antara kaedah prarawatan yang telah diuji, didapati kalium hidroksida (KOH) dengan gabungan suhu dan tekanan tinggi, merupakan kaedah yang paling berkesan untuk meningkatkan penghadaman enzim bagi RH, iaitu 70% hasil gula atas jumlah kandungan karbohidrat. Hasil gula meningkat ke 87% apabila muatan enzim dan substrat dioptimakan untuk hidrolisis menggunakan dua enzim komersial, Celluclast 1.5L and Novozyme 188. Pencirian hidrolisat enzim menunjukkan bahawa glukosa hadir dalam kadar yang paling tinggi, iaitu sekitar 80%, diikuti oleh 15% xilosa dan 5% arabinosa. Hidrolisat ini juga mengandungi jumlah fenolik sekitar 3.7 mg persamaan asid galik (GAE)/g substrat yang dirawat. Hidrolisat ini seterusnya diberikan kepada dua jenis bakteria, *Burkholderia cepacia* USM (JCM 15050) dan

Cupriavidus necator NSDG-GG, iaitu *Cupriavidus necator* H16 yang telah diubahsuai secara genetik, untuk menilai pertumbuhan dan penghasilan PHA menggunakan sumber karbon ini. Hidrolisat ini diselaraskan kepada pH 7.0 dan 6.8 bagi kultur *B. cepacia* USM dan *C. necator* NSDG-GG masing-masing. *C. necator* NSDG-GG menunjukkan berat kering sel (CDW) dan kandungan PHA yang lebih tinggi apabila tumbuh dalam medium sintetik yang mengandungi glukosa tulen, iaitu 10.4 g/L dan 70 wt%. Walau bagaimanapun, *B. cepacia* USM dapat menggunakan hidrolisat RH secara lebih cekap, dengan maksimum 4.9 g/L CDW dan 40 wt% PHA pada skala kelalang goncang. *B. cepacia* USM juga didapati mempunyai keutamaan terhadap glukosa berbanding xilosa apabila kedua-dua gula ini hadir dalam sesuatu campuran. Apabila ditapai dalam penapai 5-L, CDW dan kandungan PHA *B. cepacia* USM masing-masing meningkat sehingga 7.8 g/L dan 50 wt%. Pengurangan jumlah fenolik dalam hidrolisat pada akhir penapaian mencadangkan bahawa *B. cepacia* USM mampu memetabolismekan sebatian fenolik. Kajian ini telah menunjukkan bahawa RH boleh ditukarkan kepada PHA melalui prarawatan alkali teroptima, hidrolisis enzim, dan biopenghasilan oleh *B. cepacia* USM.

BIOCONVERSION OF RICE HUSK TO POLYHYDROXYBUTYRATE VIA PRETREATMENT AND ENZYMATIC HYDROLYSIS

ABSTRACT

Rice is one of the largest sources of food worldwide. In Malaysia, average paddy production is more than 2 million tonnes annually. From the yield of harvested paddy, approximately 20% of it consists of the husks, which are typically disposed. Rice husks (RH) consist mainly of lignocellulose, which can be converted to substrates for fermentation. This study was conducted to evaluate the potential of RH as a carbon source for the production of polyhydroxyalkanoate (PHA), a bioplastic produced by many types of bacteria. To overcome the recalcitrant nature of this biomass, physicochemical pretreatments were performed on the rice husks under different conditions and their efficiencies were compared in terms of sugar yield upon enzymatic hydrolysis. Based on all the pretreatment methods tested, the use of potassium hydroxide (KOH) combined with high temperature and pressure, was found to be most effective in increasing the enzymatic digestibility of the material, resulting in 70% sugar yield per total carbohydrate content. The sugar yield was increased to 87% when enzyme and substrate loading were optimized for enzymatic hydrolysis using two commercial enzymes, Celluclast 1.5L and Novozyme 188. Characterization of the enzymatic hydrolysate revealed that glucose was present in the highest proportion, which was approximately 80%, followed by 15% xylose and 5% arabinose. The hydrolysate also contained total phenolics of approximately 3.7 mg gallic acid equivalent (GAE)/g of pretreated substrate. The hydrolysate was then fed to two bacterial strains, *Burkholderia cepacia* USM (JCM 15050) and

Cupriavidus necator NSDG-GG, a genetically engineered strain of *Cupriavidus necator* H16, to assess their growth and PHA production on this carbon source. The pH of the hydrolysate was adjusted to pH 7.0 and 6.8 for the culture of *B. cepacia* USM and *C. necator* NSDG-GG respectively. *C. necator* NSDG-GG exhibited higher cell dry weight (CDW) and PHA content when cultivated on synthetic medium containing pure glucose, which was 10.4 g/L and 70 wt%. However, *B. cepacia* USM was able to utilize the RH hydrolysate more efficiently, with a maximum CDW of 4.9 g/L and 40 wt% PHA at shake-flask scale. It was also found that *B. cepacia* USM had a preference for glucose compared to xylose when the sugars were present in a mixture. When cultivated in a 5-L fermentor, the CDW and PHA content of *B. cepacia* USM increased to 7.8 g/L and 50 wt%, respectively. The decrease in total phenolics in the hydrolysate at the end of fermentation suggested that *B. cepacia* USM was able to metabolize phenolic compounds. This study has proven that RH can be converted to PHA through optimized alkali pretreatment, enzymatic hydrolysis, and biosynthesis by *B. cepacia* USM.

CHAPTER 1

INTRODUCTION

1.1 Introduction

The overwhelming demand of synthetic plastics in modern society has resulted in the steady increase in plastic production over the years. These petrochemical-derived plastics, such as polyethylene (PE) and polypropylene (PP), possess a wide range of polymeric properties that enable them to be applied in almost all industries. They are lightweight and durable, as well as resistant to a wide range of chemicals and temperatures. In addition to that, these types of plastics have a high strength-to-weight ratio; when compared to non-plastic materials like glass, a lower amount of material, and subsequently lower cost, is required to produce a particular product (Andrady and Neal, 2009). Thus, it is unsurprising that global plastic production exceeded 300 million metric tonnes in 2014 (Statista, 2016). However, the disposal of plastic material has resulted in detrimental effects on the environment. Even with the ongoing efforts to manage plastic waste, plastics continue to pollute the environment across all terrain. Synthetic plastics are non-biodegradable, but can break down into smaller fragments under certain conditions, such as exposure to sunlight (Van Cauwenberghe *et al*., 2013). It has been estimated that, worldwide, up to 5 trillion fragments of plastic materials weighing over 250 thousand tonnes are present on the surface of the sea (Eriksen *et al*., 2014). Along with the impending depletion of fossil fuels, it is clear that the current production of petrochemical plastics is not sustainable. These factors have driven an interest in bioplastics, which possess similar characteristics to synthetic plastics, can be produced from renewable resources, and offer the advantage of being biodegradable.

Polyhydroxyalkanoates (PHA) are a type of bioplastics that are synthesized intracellularly by many types of microorganism when grown in conditions of unbalanced nutrition, especially when nitrogen or phosphorus are limited but carbon is present in excess (Anderson and Dawes, 1990). Biosynthesis of PHA in response to nutritional limitations has been investigated and exploited for the purpose of large-scale bioplastic production. Commercialization of PHA was initiated in the 1980s, but has faced many challenges in replacing synthetic plastics in the marketplace. Perhaps the biggest challenge is the cost of production, which is largely contributed by the cost of the feedstock. One of the strategies to overcome the high cost of feedstock is the use of waste materials that contain an abundance of carbon. These waste materials come in many forms, including waste streams from food manufacture or by-products of agricultural processing. For instance, corn steep liquor is waste material generated from the wet milling of corn. The liquor is rich in nutrients such as amino acids and minerals, but is not preferred for human consumption due to its low purity. It has gained popularity as a low-cost microbiological growth medium since its widespread use in penicillin production (Liggett and Koffler, 1948).

The use of these waste products also serves an added function of reducing the volume of waste streams generated from these industries. One such example is whey, which is a lactose-rich material from the waste streams of the dairy industries. Whey disposal in Europe has been banned due to the increased acidity in soil or water where it is disposed. Although it is occasionally used as fodder for cows, it is not encouraged as whey can disrupt the natural digestive system in the animal (Elliot, 2013). Another example is fruit pomace, which is the solid residue obtained after the fruit has been pressed during the manufacture of fruit juice. It is rich in

carbohydrates and has high moisture content, making it easily fermentable. Such characteristics make it susceptible to spoilage, which poses a serious challenge in disposal (Shalini and Gupta, 2010). However, these same properties can be exploited for production of value-added products by microbial cells. Therefore, utilization of such materials for PHA production would not only reduce the cost of feedstock, but would also reduce the environmental impact brought on by waste disposal. Agricultural biomass represents another type of by-product that has great potential as feedstock for PHA production, owing to their abundance of cellulose. However, due to their recalcitrant nature, agricultural biomass is very resistant to degradation and thus requires some degree of processing before it can be fermented.

It has been suggested that PHA manufacturing plants should be incorporated into the setup of existing production facilities that generate these by-products to enable direct procurement of the material. By doing this, the cost of intermediate storage and transportation of the material can be minimized. The choice of agricultural residue to be used also depends on seasonal or regional availability (Koller *et al*., 2010).

(a) Choice of rice husks as the biomass substrate in this study

In Malaysia, paddy is an important crop commodity produced in country, with 2.6 million tonnes as of 2014 (Food and Agricultural Organization, 2016). Approximately 20% of the harvested paddy consists of the rice husks (RH), resulting in over 500,000 tonnes of RH. The massive amounts of RH generated present serious problems in disposal. The conventional method of disposal is incineration, which is proven to be hazardous to health and the environmental. Thus, development of proper and sustainable methods of agricultural waste management is necessary to

address these issues. One solution is to convert the polysaccharides present in the RH into fermentable sugars, which can then be used in fermentation processes.

The use of an agricultural by-product for bioconversion of biotechnological products also aids in lowering the cost of feedstock. It has been reported that the cost of feedstock for PHA production accounts for up to 50% of total production costs (Choi and Lee, 1997). The issue of production costs is one of the main reasons why mass marketing of PHA is difficult. To encourage the commercial growth of PHA, cheaper feedstocks need to be used. Thus, intensive research is ongoing to develop more facile and cost-effective methods to obtain sugars from biomass.

As with other lignocellulosic material, RH are susceptible to a variety of digestion methods to break down cellulose and hemicellulose into fermentable sugars. However, processes that have been proven to be effective on certain types of biomass might not be applicable to others, due to the diverse and complex nature of lignocellulose. Furthermore, the utilization of feedstock derived from waste material could be limited due to the presence of additional, non-fermentable components. In other cases, the microbial strain does not possess the biochemical pathways necessary for metabolizing the carbon substrates in the feedstock. It has been suggested that a system, or a database, should be developed to document and organize information relating to biomass and other waste products that are used for fermentation, including composition of the material and performance in various applications (Solaiman *et al*., 2006).

In view of that, the present study was aimed at production of PHA from sugars present in RH through a systematic conversion process. Pretreatment of RH was first carried out to maximize removal of the non-carbohydrate components as well as increase the accessibility of the substrate to cellulolytic enzymes.

Pretreatment was performed under different conditions to determine the method that results in highest sugar yield upon enzymatic hydrolysis.

(b) Choice of enzymes used for hydrolysis in this study

Hydrolysis of the pretreated substrate was performed enzymatically using two commercial enzymes, namely, Celluclast 1.5L and Novozyme 188, produced by Novozyme Corporation (Denmark). Celluclast 1.5L exhibits mainly exo- and endoglucanase activities, which is needed to cleave the cellulose fibers into oligosaccharides, disaccharides, and monosaccharides. Cellobiose, a disaccharide, is able to inhibit cellulase activity. Therefore, Novozyme 188, which is a β-glucosidase, is needed as a supplementary enzyme to Celluclast l.5L to cleave cellobiose into individual glucose monomers. The use of Celluclast 1.5L and Novozyme 188 for hydrolyzing biomass substrates have been widely reported. Furthermore, these enzymes have been studied for the potential of enzyme recycling by adsportion, and have been proven to be stable at high temperatures (Kristensen *et al*., 2007; Arantes and Saddler, 2011; Chylenski *et al*., 2012; Rosales-Calderon *et al*., 2014)

(c) Choice of bacterial strains used in this study

The potential of the hydrolysate as a feedstock for biosynthesis of PHA was evaluated based on cell biomass and PHA accumulation in two selected strains, *Burkholderia cepacia* USM (JCM 15050) and *Cupriavidus necator* NSDG-GG. An important criterion in choosing the strains to be used in this study was the ability of the cell to uptake sugars that are commonly obtained from biomass, particularly glucose. Previously, *B. cepacia* USM was shown to have nutrional diversity, as it was able to metabolize a wide range of oils and sugars, including sugar alcohols (Chee et al., 2010). The *C. necator* strain NSDG-GG is a genetically modified strain of *C. necator* H16, which is a well-known PHA producer but can only utilize

fructose, thus limiting its application for biomass-derived feedstocks. To enable the strain to uptake glucose, the *nag* operon was modified (Mifune *et al*., 2008; Orita *et al*., 2012). This is the first study to be performed with the NSDG-GG strain on a biomass hydrolysate.

1.2 Objectives of the study

The specific objectives of the study are:

1. To develop a method of pretreatment for maximum digestibility of the lignocellulosic material in RH.
2. To improve sugar yield from pretreated RH by optimization of selected parameters in enzymatic hydrolysis.
3. To compare the efficiency of RH hydrolysate utilization in *Burkholderia cepacia* USM (JCM 15050) and *Cupriavidus necator* NSDG-GG and select the most suitable strain for downstream investigations.
4. To evaluate the growth and production of poly(3-hydroxybutyrate) [P(3HB)] in the selected strain using the RH hydrolysate under different nutrition conditions and in batch fermentation.

CHAPTER 2

LITERATURE REVIEW

2.1 Lignocellulosic biomass

Biomass is the general term given to organic material derived from plants (Wertz *et al*., 2010). To include a wider scope, the International Union of Pure and Applied Chemistry (IUPAC) has defined biomass collectively as the organic matter produced by living systems and the living systems themselves that have the capacity to be exploited as materials. In this context, materials refer to substances that serve to benefit human beings in their livelihood (Vert *et al*., 2012). Lignocellulosic biomass is available in large quantities from the forest and agriculture industry, and has great potential to be harnessed for energy. A lot of research has been devoted to the conversion of lignocellulosic biomass to usable energy and fuel, which is mainly focused on understanding the complexity of lignocellulose or designing and optimizing methods of bioconversion. Other approaches include the engineering of biological systems to overcome the challenges associated with using lignocellulosic biomass.

As an integral structure of plant cell walls, lignocellulose refers to the three components making up the bulk of the plant tissue, i.e. cellulose, hemicellulose, and lignin. Cellulose is the biopolymer found in highest abundance on Earth (Dixon *et al*., 1994; Leisola *et al*. 2012). Typically, the cellulose fibers are surrounded by a matrix of hemicellulose, which links the cellulose to lignin. The compositions of lignocellulose in biomass vary depending on the plant species, age of plant, soil properties, and other environmental factors (Fengel and Wegener, 1983).

2.1.1 Chemistry and structure of lignocellulose

(a) Cellulose

The fundamental synthesis of cellulose is based on the polymerization of D-glucose, linked by a β-1,4-glycosidic bond. D-glucose can refer to both acyclic and cyclic forms of glucose. In aqueous solutions, glucose exists predominantly in the cyclic form called pyranose. Pyranose (or more specifically, glucopyranose) results from an intramolecular nucleophilic addition reaction between the hydroxyl and carbonyl groups present in glucose, in which water acts as a catalyst (McMurry, 2010). The β-1,4-glycosidic bonding dictates a 180° rotation from one glucopyranose molecule to the next. Thus, the basic repeating unit of cellulose is in fact cellobiose and not glucose. The stereochemistry implied by this linkage generates a linear chain of β-1,4-D-glucan with a highly ordered structure. Many chains of the β-1,4-D-glucan homopolymer associate strongly with each other via hydrogen bonds and van der Waals forces, forming rigid and crystalline microfibrils (Nishiyama *et al*., 2002; Nishiyama *et al*., 2003; Somerville, 2006). Aggregation of these microfibrils then forms a matrix of macrofibrils (Delmer and Amor, 1995).

Cellulose synthesis occurs in the plasma membrane. Although the process is complex and not well understood, it essentially involves the polymerization of the cellulose chain, i.e. initiation and elongation of the chain. Cellulose synthases (CesA proteins) are the enzymes responsible for cellulose synthesis. In vascular plants, cellulose synthase complexes are present in the form of a hexameric rosette and are also referred to as terminal complexes (Kimura *et al*., 1999). Multiple cellulose chains are synthesized at the same time by each rosette, forming an elementary microfibril (Harris *et al*., 2010). It is postulated that each rosette synthesizes 36 glucan chains simultaneously (Somerville, 2006), thus giving rise to

the assumption that a single rosette consists of 36 active CesA proteins, six of which belongs to each of the hexamer rosette units (Doblin *et al*., 2002; Li *et al*., 2014). The substrate for synthesis is uridine diphosphate (UDP) glucose, which is catalyzed by a glucosyltransferase in the initiation step with the aid of a primer called sitosterol-β-glucoside. Elongation then proceeds by the action of the synthase (Peng *et al*., 2002).

The molecular weights of the glucan chains are often expressed in terms of degree of polymerization (DP), which refers to the number of glucose units. The DP of cellulose in its native state cannot be absolutely determined because the process of extraction may cause degradation of the chains (Somerville, 2006). Based on analysis of extracted cellulose, it was found that the DP differs greatly based on localization of cellulose. For example, cellulose found in secondary cell walls may have a DP of 15000 up to 20000. On the other hand, primary cell wall cellulose has lower molecular weight, ranging from 500 to 4000 (Brett, 2000; Habibi *et al.*, 2010).

Crystalline cellulose exists in four different polymorphs: cellulose I, II, III, and IV. The alternation of crystalline structures is due to the presence of the hydroxyl groups in cellulose, which are free to partake in intra- and intermolecular hydrogen bonding. The polymorph most commonly found in nature is cellulose I, which is further divided into cellulose I_α and I_β; subgroups of the other polymorphs are formed via different methods of preparation (O'Sullivan, 1997). Cellulose II is irreversibly formed via regeneration or mercerization, and is assumed to be more stable than cellulose I (Brett, 2000). Cellulose I and II can be treated with ammonia or other amines to form cellulose III_I and cellulose III_{II}, respectively. Cellulose III_I and III_{II} transform into cellulose IV_I and IV_{II} when treated with glycerol at high temperatures, of up to 260°C. Unlike cellulose II, cellulose III and IV are able to

revert back to their parental I or II polymorphs (Perez and Mazeau, 2005; Habibi *et al*., 2010).

Cellulose also consists of amorphous regions, often occurring on the surface of the crystalline chains. The ratios of crystalline to amorphous regions differ between plant species and location of the plant cells. It is mostly the amorphous portion as well as the surface of the crystals that participate in chemical reactions when cellulose is treated, as reagents are only able to penetrate through the noncrystalline domains (Ciolacu *et al*., 2011). Such properties of cellulose often limit its potential for modification and application. As a result, methods to increase the accessibility of cellulose have been intensively studied. Conversion of crystalline cellulose to amorphous cellulose has been proven to occur when treated in heated and compressed water (Deguchi *et al*., 2008). The use of phosphoric acid to decrystallize microcrystalline cellulose (MCC) has also been studied (Zhang *et al*., 2009). The crystallinity of MCC was proven to decrease by this treatment, and the rate of dissolution was further improved with heating. However, at temperatures above 50°C a loss of mass was observed in the material, as acid hydrolysis was simultaneously occurring.

(b) Hemicellulose

Hemicellulose is another integral component in the lignocellulosic materials. Unlike cellulose, hemicellulose is a polysaccharide of heterogeneous structure consisting of hexose (such as glucose) and pentose sugars (such as xylose, mannose, arabinose, or galactose). In the plant cell wall, hemicelluloses associate with celluloses via hydrogen bonding. Due to the branched nature of hemicellulose, they are sometimes referred to as cross-linking glucans (Wertz *et al*., 2010).

Hemicelluloses share the characteristic of having β-1,4-linked glucose, xylose, or mannose backbones. Xylans are a type of hemicellulose occurring in the highest abundance in plants. The xylan backbone is made up of xylose monomers linked via β-1,4-xylosidic bonds, similar to the β-1,4-glycosidic bonds found in cellulose. Xylans have different degrees of branching depending on the substitution of monosaccharides (Hurlbert and Preston, 2001; Dhiman *et al.*, 2008). Some of the typical substitutions found in xylan are α-1,2-linked glucuronic acid and 4-*O*-methyl-D-glucuronic acid. Xylans with such substitutions are also termed glucuronoxylans (Ebringerova *et al*., 2005). Another common type of hemicellulose is xyloglucan. Xyloglucans have a repeating unit of four glucose units with an α-1,6-xylose branching in the first three glucose molecules. The attachment of other pentoses or hexoses to the xylose units further contributes to the variability and branching of the structure (Scheller and Ulvskov, 2010).

Hemicellulose is synthesized in the Golgi apparatus in the plant cell. The process involves the transfer of a glycosyl group from a nucleotide sugar to an elongating polysaccharide chain, catalyzed by a glycosyltransferase enzyme (Horton, 2008). The initial product of this reaction is a nascent hemicellulose chain that undergoes further modifications by hydrolases, which dictate the heterogeneity and structure of the hemicellulose. These modifications play an important role in maintaining the solubility of the hemicellulose chains as they are shuttled from the Golgi apparatus and deposited in the cell wall (Scheller and Ulvskov, 2010).

Several proposed models of the plant cell wall have aided in elucidating the arrangement and function of hemicellulose. The plant cell wall model according to Talbott and Ray (1992), called the multi-coat model, suggested that noncovalent bonding was predominantly influential in the mechanical integrity of the expanding

primary cell wall. This model suggested that hemicellulose forms a sheath around cellulose microfibrils while pectin, another type of structural polysaccharide found in primary cell walls, fills up the interstitial spaces between hemicellulose-coated microfibrils. Furthermore, this model supported the absence of glycosidic bonds between hemicellulose and other cell wall polysaccharides due to the ability of hemicellulose to be completely separated via alkali extraction. Such separation would not be possible with glycosidic bonds, which are insensitive to alkalis. Another model emphasizing noncovalent interactions is the sticky network model proposed earlier by McCann and Roberts (1991). A schematic of the multi-coat model is shown in Figure 2.1A.

More recently, Park and Cosgrove (2012a) proposed that hemicellulose does not completely coat and isolate cellulose microfibrils from each other; rather, a certain degree of contact occurs between microfibrils. This model is illustrated in Figure 2.1B. Analysis of ^{13}C-labelled cell wall polysaccharides of *Arabidopsis thaliana* by magnetic angle spinning (MAS) solid-state nuclear magnetic resonance (NMR) revealed that xyloglucan and cellulose have limited interaction, suggesting that hemicellulose does not coat the cellulose microfibrils as extensively as previously believed (Dick-Pérez *et al*., 2011). The development of an *Arabidopsis* mutant with xyloglucan deficiency showed normal development and physiology, albeit growing to a smaller size compared to the wild type, providing further evidence that xyloglucans do not play an essential role in strength and mechanical support of the cell wall (Park and Cosgrove, 2012b).

The distribution of hemicelluloses across different plant species is extremely diverse. In hardwood and grasses, xylans are found most abundantly. In hardwood, methylglucuronoxylan is predominant, with some occurrence of glucomannan.

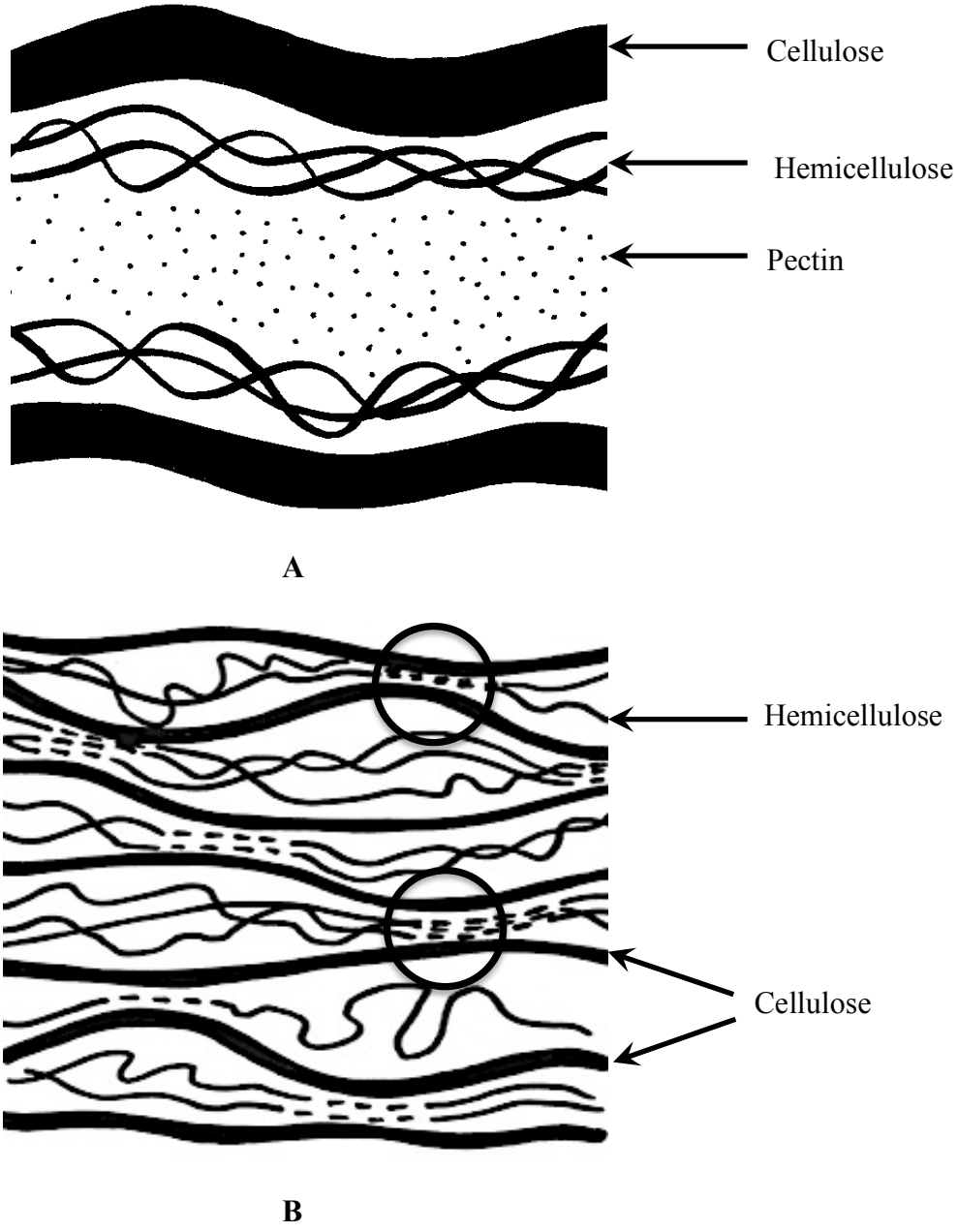

Figure 2.1: Schematic drawings of plant cells wall models. **A** the model proposed by Talbott and Ray (1992), in which the hemicellulose (thin lines) forms a sheath around the cellulose microfibrils (thick lines) and the interstitial spaces between the hemicellulose sheaths are filled by pectin (large dots); **B** the model proposed by Park and Cosgrove (2012a), in which the hemicellulose (thin lines) only interacts with the cellulose microfibrils (thick lines) at certain contact points, as indicated by the circles.

Grasses, i.e. plants belonging to the Gramineae family, contain a majority of arabinoxylans (Brigham *et al*., 1996). Hemicelluloses in softwoods, on the other hand, are mostly mannan-based, such as galactoglucomannan and arabino-4-*O*-methylglucuroxylan (Puls, 1997). Among the components in lignocellulose, hemicellulose is the most susceptible to thermochemical treatments. The side chain residues were found to be more prone to chemical degradation compared to the linear backbone chain, and proceeds in descending order from arabinose, galactose, mannose, and xylose (LeVan *et al*., 1990). However, solubility of these sugar moieties followed a different trend, whereby mannose > xylose > glucose > arabinose > galactose. Solubility of arabinose was most dependent on temperature, whereas xylose showed the least dependence (Gray *et al*., 2003).

(c) Lignin

The structural carbohydrates are linked with lignin, a polymer consisting of a complex network of aromatic alcohols. Lignin was first discovered in the 1800s by Anselme Payen, who described it as a material with high carbon content which functions to embed cellulose in wood (Sjöström, 1993). Precursors of lignin are classified under the family of monolignols, although the term hydroxycinnamyl alcohol is sometimes used interchangeably. Three types of monolignols that are most commonly found in nature are *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol (Li and Chapple, 2010). Through hydrothermal oxidation, lignin can break down into phenols and phenolic compounds such as vanillic acid and syringic acid (Villar *et al*., 2001).

Lignin is broadly classified according to softwood, hardwood, and grass lignin. The lignin found in softwoods is guaiacyl lignin, consisting of coniferyl

alcohol units. Guaiacyl-syringyl lignin, made up of coniferyl and sinapyl alcohol, is found in hardwoods. Grass lignin, on the other hand, consists mainly of *p*-coumaryl alcohol units (Higuchi *et al*., 1977).

Biosynthesis of lignin follows the phenylpropanoid pathway and is initiated by the synthesis of monolignols from phenylalanine. Before entering the phenylpropanoid pathway, phenylalanine is first converted to *trans*-cinnamic acid by an enzyme called phenylalanine ammonia-lyase found on the endoplasmic reticulum membrane. The monolignols are then synthesized via a series of reactions involving reduction and addition of hydroxyl or methyl groups (Boerjan *et al*., 2003; Kärkönen and Koutaniemi, 2010). Deposition of lignin in secondary plant cell walls serves to provide mechanical strength and rigidity. At the same time, it also protects the cells against microbial infection and desiccation (Vance *et al.*, 1980; Tronchet *et al*., 2010). However, deposition of lignin other than in genetically predetermined locations may occur in response to stress, such as physical injuries, pathogen attacks, or disruptions in the cell wall structure (Caño-Delgano *et al*., 2003; Vanholme *et al*., 2010).

As the main defence system in plants, resistance to degradation is as essential feature of lignin. Together with the crystalline nature of cellulose, the toughness afforded by lignin is one of the main challenges in utilizing lignocellulosic biomass for various bioprocesses. Thus, recent studies in lignin research have been focused on genetic and metabolic engineering to alter the composition of lignin in plants. One such method is by engineering the incorporation of new lignin monomers during the polymerization steps. Grabber *et al*. (2008) reported the incorporation of coniferyl ferulate into the lignin of maize, which provided increased ester linkages that made the resulting lignin more susceptible to alkaline extraction. It was also

reported in this study that enzymatic hydrolysis of structural polysaccharides were facilitated by the modified lignin, even without removal of the lignin itself. Another study conducted using rosmarinic acid as a partial monolignol substitute also gave similar results (Tobimatsu *et al*., 2012), thus shedding more light on the feasibility of such methods to improve digestibility of lignocellulose.

(d) Other components

Lignocellulosic materials also contain non-structural components known as extractives, which typically represent up to 5% of the biomass. The four main categories of extractives are: (i) terpenoids and steroids, (ii) fats and waxes, (iii) phenolic constituents, and (iv) inorganic compounds (Sjöström, 1993).

Another component that is present in lignocellulosic biomass is ash, which is the product of combustion and is primarily made up of inorganic elements, such as silicon, calcium, potassium, magnesium, sodium and aluminium. Heavy metals may also be present in the ash (Vassilev *et al*., 2012). The ash from woody biomass has a higher composition of calcium and potassium, compared to ash from non-woody biomass, which are found to have more silicon and potassium (Jenkins *et al*., 1996).

2.1.2 Types of lignocellulosic biomass used for bioconversion

Lignocellulosic biomass can be distinguished based on their origin, composition, and structure (Bonin and Lal, 2012). Although the classification of biomass differs depending on context, they can be broadly classified as woody materials, perennial bioenergy crops, agricultural residues, and municipal solid wastes. Table 2.1 provides a descriptions and examples of each category of biomass.

Table 2.1: Types of lignocellulosic biomass commonly used for bioconversion (adapted and modified from Sjöström, 1993; Sanchez and Cardona, 2008; White, 2010; Hadar, 2013).

Category	Description	Examples
Woody biomass	• Divided into hardwoods and softwoods • Softwoods originate from gymnosperms and conifers • Hardwoods originate from angiosperms	• Wood chips • Stumps and dead tree materials • Sawdust
Perrenial bioenergy crops	• Perrenial crops have a lifespan of more than two years • Grown specifically for use as a biomass resource	• *Miscanthus* sp. • Switchgrass • Sorghum
Agricultural residues	• Parts of the crop that cannot be used for the food or food derivatives • Often left on-site during harvesting, or collected as an output from mills	• Straw (wheat, barley, rice) • Husks (barley, rice) • Corn stover • Empty fruit bunch (oil palm) • Sugarcane bagasse
Solid municipal waste	• Biodegradable organic components from household waste • Not as ideal as other types of biomass but useful in regions where crops are scarce	• Paper and cardboard waste • Solid kitchen and garden waste

2.2 Rice husk as a material of interest

2.2.1 The paddy plant

Oryza sativa L., the paddy plant cultivated as crops, belongs to the family Poaceae (or Gramineae). Poaceae is a large family of monocotyledonous plants that is often referred to as true grasses. Ehrhartoideae is a subfamily of Poaceae. One of the tribes categorized under Ehrhartoideae is Oryzeae, which encompasses 12 genera. Members of this tribe are widespread in both temperate and tropical regions (Clayton and Renvoize, 1986). Oryza is one of the genera classified under Oryzeae. It comprises of 23 species and is morphologically characterized by bisexual inflorescence bearing undeveloped lemmas and small, straight leaves (Kellogg, 2001).

Rice is one of the largest sources of food in the world. Up to 164 million hectares of land are irrigated for paddy plantation worldwide, yielding more than 700 million tonnes of paddy annually since 2010. According to the statistics provided by the Food and Agricultural Organization of the United Nations (FAO), global paddy production has been steadily increasing since 2002. China is the largest paddy producer in the world, followed by India, Indonesia, Bangladesh, and Vietnam. In Malaysia, approximately 680 thousand hectares of land are used for the cultivation of paddy. Local production of paddy in 2010 was 2.5 million tonnes and rose to over 2.6 million tonnes in 2014 (Department of Statistics Malaysia, 2015; Food and Agricultural Organization, 2016).

One of the byproducts of the rice milling industry is rice husk, generated during the conversion process of paddy to rice. Traditionally, rice husks were first removed from the grain by winnowing, a process whereby the grains are thrown into the air. The light husks would be separated and blown away by the wind, while the

heavier rice grain would be captured in mesh pans. Throughout history, efforts were made to mechanize this process, but it was the invention of the Engelberg huller that marked significant advancement in rice milling (Wimberly, 1983; Barker *et al*., 1985). Out of the total paddy harvested, approximately 20% of it consists of the husk (Bansal *et al*., 2006; International Rice Research Institute, 2015).

2.2.2 Common uses of rice husks

Once paddy has been harvested, the residues are often burnt in the open fields. Due to high cost of transportation, it is not common for such agricultural wastes to be brought far from the mills to other places for further processing into useful materials (Miao *et al*., 2012). According to an extensive survey by Yevich and Logan (2003), the utilization of agricultural residues differs according to type. Residues from crops like wheat and barley are preferably used as fodder for livestock, which is more commonly found in Near and Middle East regions of Asia. In Southeast Asia, where paddy is one of the main cultivated crops, incineration of the residues to clear the fields appears to be the most common practice. However, in Japan and Korea where a significant amount of paddy is grown, such practices are uncommon. Instead, it is used for composting, animal feed, or as a source of fuel. Although using biomass for fuel is not common in the Southeast region, including Malaysia, Vietnam has proven to be an exception. As wood is scarce in the rural areas of North Vietnam, residues from paddy are primarily used as fuel for cooking (World Bank, 1994).

2.2.3 Rice husks as biofuel

To assess the potential of rice husks as a biofuel, the energy content in terms of higher heating value (HHV) should be considered. Heating value (HV) refers to the amount of energy released in the form of heat during combustion of a specific

amount of material. HHV is similar to HV, but takes into account the latent heat of vaporization. Based on published literature, the HHV of rice husk is within the range of 15.8 to 15.9 MJ/kg (Jenkins *et al*., 1998; Shen *et al*., 2012). In comparison, rice straw has a HHV in the range of 15.1 to 15.3 MJ/kg while other biomass such as barley straw and wheat straw have HHVs of approximately 16.1 to 18.9 MJ/kg (Liu *et al*., 2013). Fuel pellets made from biomass often have to adhere to the standard HHV that is set by individual countries. However, the most common standard used is the one established by Deutsches Institut Fur Normung (DIN), known as DIN 51731, which requires biomass fuel pellets to have a HHV of more than 17.5 MJ/kg (Liu *et al*., 2013). Despite that, rice husk still has potential to be used as biofuel, given that all other techno-economic parameters are optimized.

One of the most prominent initiatives to attest such claims is the Husk Power Systems (HPS), developed and set up by a group of engineers in Bihar, India. Through the process of biomass gasification, whereby the rice husks are heated without combustion to extreme temperatures by controlled oxygen or steam inputs, HPS has successfully provided electricity to one hundred thousand people in 125 villages using this agricultural residue. To fuel a 32 kW plant, 50 kg of rice husks per hour is required, which is bought from local rice mills for the low price of 1 Indian Rupee (or equivalent to approximately 0.06 Malaysian Ringgit). By paying for electricity generated by HPS, it is also estimated that residents of Bihar would save one-third of the money they would normally spend on kerosene (Boyle, 2010).

2.2.4 Novel uses of rice husks

Many studies have been done to explore the use of rice husks as adsorbents for the removal of pollutants in wastewater, primarily heavy metals and dyes. Such pollutants are difficult to degrade and, if ingested, will accumulate in biological

systems and adversely affect living organisms. Rice husk ash (RHA) has been extensively studied as a versatile adsorbent in wastewater treatment, particularly in decolourization of dyes, whereby the RHA served as a support material for immobilizing catalysts (Daud and Hameed, 2010). In a study by Li and Jia (2008), RH was not ashed but instead used directly as a bioadsorbent and a growth substrate for *Schizophyllum* sp. F17, which performed the decolorization function. RH also has the potential to remedy oil spills. Carbonized RH was used for the purification of biodiesel from waste frying oil and showed excellent adsorption due to its high silica content, as well the presence of porous structures (Manique *et al*., 2012). Previously, Kumagai and colleagues (2007) attributed the oil adsorption capacity of carbonized RH to the residual fluid components rather than the porosity. Without undergoing the ashing process, it was also found that alkali-treated RH was capable of adsorbing up to 20 g of oil per g of adsorbent material (Bazargan *et al*., 2014).

In the field of construction and production of building materials, RHA has showed strong potential as fillers in the production of environmentally friendly concrete. Conventional concrete uses sand as one of its basic components, which, like RHA, consists primarily of silica. The high silica content gives RHA its pozzolanic activity, which quantifies the rate of reaction of between a siliceous material and calcium hydroxide in the presence of water, resulting in compounds with cementitious properties (Malhotra and Mehta, 1996; Wansom *et al*., 2009). High amount of amorphous silica, surface area, and silanol groups have been identified as ideal characteristics of RHA for increased pozzolanic activity (Nair *et al*., 2008). The use of RHA in cement blends increases the strength and durability of the material while simultaneously decreasing the material cost

(Siddique and Khan, 2011). By bringing down the overall cost of building materials, more affordable dwellings can be built.

Besides its ability to strengthen concrete, rice husks have also been investigated for its potential as a filler material in rubber. The incorporation of RHA in both synthetic and natural rubber has been studied early in the 1970's (Haxo and Mehta, 1975) and is still of great interest today. Arayapranee *et al*. (2005) concluded that the incorporation of RHA in natural rubber did not result in significant improvements in mechanical characteristics, but in cases where such properties were not of major concern, RHA could be used as an inexpensive substitute for commercial fillers. More recently, Pongdong and colleagues (2015) found that the incorporation of RHA in epoxidized natural rubber resulted in increased curing reactions and enhanced mechanical properties. The improvements in properties were associated with increased crosslinking, which was probably due to the presence of metal oxides in RHA.

2.3 Pretreatment options for lignocellulosic biomass

Conceptually, hydrolysis of cellulosic materials is simple. In practice, however, this process is made much more complicated by the recalcitrant nature of lignocellulose. As it was intended as a main structural component in plants, it has been made extremely stable and resistant to degradation. Rice husk is no exception. Once the material has been pretreated, the cellulose and hemicellulose become more accessible to hydrolysis. In general, pretreatments can be classified into physical, chemical, or a combination of both (Hendriks and Zeeman, 2009). Table 2.2 summarizes the types of pretreatment along with their advantages and disadvantages.

Table 2.2: Advantages and disadvantages of various types of biomass pretreatments (adapted and modified from Galbe and Zacchi, 2007; Hendriks and Zeeman, 2009; Agbor *et al*., 2011; Menon and Rao, 2012; Zakaria *et al*., 2015).

Pretreatment	Advantages	Disadvantages
Physical		
(i) Comminution	• Decreases crystallinity of cellulose • Improves mass transfer of bulk materials	• Requires high energy input
(ii) Irradiation	• Disrupts lignin • Decreases crystallinity of cellulose	• Hazardous (at high radiation doses)
Chemical		
(i) Acid	• Solubilizes hemicellulose • Disrupts lignin • Increases accessibility of cellulose	• Highly corrosive and damaging to equipment • Generates inhibitory compounds
(ii) Alkali	• Removes lignin • Reduces formation of inhibitory compounds • Can be performed at lower temperatures	• Formation and deposition of salts on the substrate • Long residence time for lower temperatures
(iii) Organosolv	• Solubilizes hemicellulose and lignin • Initiates an autocatalytic reaction • Allows recovery of chemicals	• Requires thorough washing of pretreated material • High cost of organic solvents
(iv) Ozone	• Disrupts lignin and hemicellulose • Can be performed at lower temperatures • Reduces formation of inhibitory compounds	• Requires large amounts of ozone • High cost of material and equipment

Table 2.2 (*continued*).

Pretreatment	Advantages	Disadvantages
Physico-chemical		
(i) Steam explosion	• Solubilizes hemicellulose • Initiates an autocatalytic reaction	• Generates inhibitory compounds
(ii) Superheated steam	• Does not require catalyst • Environmentally friendly	• Low sugar yields from hydrolysis when used alone
(iii) Ammonia fiber expansion (AFEX)	• Solubilizes hemicellulose • Reduces loss of cellulose • Allows recovery of chemicals	• High cost of equipment • Not efficient on biomass with high lignin content