BIOCONVERSION OF OLD OIL-PALM TRUNK RESIDUES VIA ENZYMATIC HYDROLYSIS BY *Penicillium rolfsii* c3-2(1) IBRL USING REDUCING SUGARS AS AN INDICATOR

by

LEE KOK CHANG

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LIST OF ABBREVIATIONS

AAD	Aryl-alcohol dehydrogenases
AAO	Aryl alcohol oxidase
AFEX	Ammonia fiber explosion
ANF	Antinutritional factors
ANOVA	Analysis of variance
ATCC	American Type Culture Collection
BGL	*β*-glucosidase
bp	Base pair
BSA	Bovine serum albumin
CBD	Cellulose-binding domain
CBH	Cellobiohydrolase
CBM	Carbohydrate-binding module
CBP	Consolidated bioprocessing
CD	Catalytic domain
cm	Centimeter
CMC	Carboxymethylcellulose
CMCase	Carboxymethylcellulase
cP	Centipoise
CREA	Creatine sucrose agar
CYA	Czapek yeast agar
DCM	Direct microbial conversion
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DNS	Dinitrosalicylic acid
DP	Degree of polymerization
EG	Endo-*β*-1,4-glucanase
FPU/g	Filter paper unit per gram
FPU/ml	Filter paper unit per milliliter
g	Gram
g m^{-3}	Gram per cubic meter
G25N	Glycerol nitrate agar
h	Hour
HMF	5-hydroxy-2-methyl-furfural
IBRL	Industrial Biotechnology Research Laboratory
ITS	Internal transcribed spacer
JIRCAS	Japan International Research Center for Agricultural Sciences
kDa	Kilodalton
kg	Kilogram
kHz	Kilohertz
kPa m^2/g	Kilo pascal meter square per gram
LHW	Liquid hot water
LiP	Lignin peroxidases
MEA	Malt extract agar
mg	Milligram
mg/ml	Milligram per milliliter

min	Minute
µl	Microliter
mm	Millimeter
mM	Milimolar
mN m^2/g	Millinewton meter square per gram
MnP	Manganese peroxidases
MOPS	3-morpholinopropanesulfonic acid
MPa	Megapascal
MSM	Minimum salts medium
NBRC	NITE Biological Resource Center
NCBI	National Center for Biotechnology Information
Nm/g	Newton meter per gram
OsO_4	Osmium tetroxide
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
psi	Pound per square inch
rpm	Revolutions per minute
rRNA	Ribosomal ribonucleic acid
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Scanning electron microscope
SHF	Separate hydrolysis and fermentation
SSCF	Simultaneous saccharification and co-fermentation
SSF	Simultaneous saccharification and fermentation
TAPPI	Technical Association of the Pulp and Paper Industry
TEMED	Tetramethylethylenediamine
TLC	Thin layer chromatography
U/mg	Unit per milligram
U/ml	Unit per milliliter
w/v	Weight per volume
w/w	Weight per weight

LIST OF PUBLICATIONS AND CONFERENCE PAPERS

1. Lee, K. C., Arai, T., Ibrahim, D., Prawitwong, P., Deng, L., Murata, Y., Mori, Y. and Kosugi, A. (2015). Purification and characterization of a xylanase from the newly isolated *Penicillium rolfsii* c3-2(1) IBRL. BioResources 10(1):1627-1643.

2. Lee, K. C., Arai, T., Ibrahim, D., Kosugi, A., Prawitwong, P., Lan, D., Murata, Y. and Mori, Y. (2014). Purification and characterization of a thermostable laminarinase from *Penicillium rolfsii* c3-2(1) IBRL. BioResources 9(1):1072-1084.

3. Prawitwong, P., Kosugi, A., Arai, T., Deng, L., Lee, K. C., Ibrahim, D., Murata, Y., Sulaiman, O., Hashim, R., Sudesh, K., Ibrahim, W. A. B., Saito, M. and Mori, Y. (2012) Efficient ethanol production from separated parenchyma and vascular bundle of oil palm trunk. Bioresource Technology 125:37-42.

4. Lee, K. C., Arai, T., Kosugi, A., Darah, I., Prawitwong, P., Mori, Y. (2012). *Penicillium rolfsii*, The potential lignocellulolytic fungus on hydrolysis of oil-palm residues from oil palm trunk as a second generation biofuel feedstock. The 2nd Annual International Conference Unsyiah & 8th IMTGT Uninet Biosciences Conference. Universitas Syiah Kuala, 22-24 November 2012.

5. Lee, K. C., Arai, T., Kosugi, A., Darah, I., Prawitwong, P., Mori, Y. (2012). Fungal bioconversion of old oil-palm trunks by enzymatic hydrolysis on development of alternate energy source. The 2nd Annual International Conference Unsyiah & 8th IMTGT Uninet Biosciences Conference. Universitas Syiah Kuala, 22-24 November 2012.

6. Lee, K. C., Arai, T., Kosugi, A., Darah, I., Prawitwong, P., Murata, Y., Mori, Y. (2012). Degradation activity of *Penicillium rolfsii* strain isolated from the soil in Malaysia against the sap extraction residue of oil palm trunks. Annual Meeting of Japan Society for Bioscience, Biotechnology, and Agrochemistry (JSBBA) 2012. Kyoto Women's University, 23-25, March 2012.

7. Lee, K.C., Darah, I. and Arai, T. (2013). Purification and characterization of xylanase and laminarinase produced by *Penicillium rolfsii*. Proceedings of the 8th Annual PPSKH Postgraduate Colloqium, Shool of Biological Sciences, Universiti Sains Malaysia. School of Biological Sciences, USM, Penang 5-6th June 2013.

8. Lee, K.C., Darah, I. and Arai, T. (2012). Enzymatic hydrolysis of oil-palm residues from oil palm trunk as a second-generation biofuel feedstock by potential lignocellulolytic fungal isolate, *Penicillium rolfsii*. Proceedings of the 7th Annual PPSKH Postgraduate Colloqium, Shool of Biological Sciences, Universiti Sains Malaysia. School of Biological Sciences, USM, Penang,7-8th November 2012.

9. Lee, K.C., Darah, I. and Arai, T. (2010). A study on the involvement of various enzyme in the degradation of parenchyma tissue of oil palm trunk by fungal isolates. Proceedings of the 4th Annual PPSKH Postgraduate Colloqium, Shool of Biological Sciences, Universiti Sains Malaysia. School of Biological Sciences, USM, Penang,15th December 2010.

BIOPENUKARAN RESIDU BATANG KELAPA SAWIT TUA MELALUI HIDROLISIS BERENZIM OLEH *Penicillium rolfsii* c3-2(1) IBRL MENGGUNAKAN GULA PENURUN SEBAGAI PENANDA

ABSTRAK

Produktiviti minyak yang semakin menurun daripada pokok kelapa sawit tua selepas 25 tahun penanamannya telah menyebabkan pembuangan sisa batang kelapa sawit yang banyak daripada aktiviti penanaman semula. Batang kelapa sawit didapati mengandungi sap yang tinggi dengan kandungan gula yang tinggi. Residu batang kelapa sawit yang telah diperah sapnya merupakan bahan sisa yang akan dibuang dalam kuantiti yang besar. Biojisim residu batang kelapa sawit dalam bentuk buangan agro-industri merupakan sumber yang berpotensi untuk dijadikan sebagai bahan mentah bagi penukaran ke bahan api bio, dan bahan-bahan kimia tambahan yang bermutu tanpa persaingan dengan sumber makanan. Pencilan kulat berfilamen yang dilabelkan sebagai c3-2(1) telah disaring dan dipilih menunjukkan aktiviti enzim yang tinggi terhadap residu batang kelapa sawit. Dengan menggunakan kaedah pencirian morfologi, pemerhatian melalui mikroskop dan identifikasi molekul, pencilan c3-2(1) telah dikenalpasti sebagai *Penicillium rolfsii*. Pencilan tersebut dinamakan sebagai *Penicillium rolfsii* c3-2(1) IBRL. Aktitviti spesifik terhadap residu batang kelapa sawit yang lebih tinggi telah ditunjukkan dengan menggunakan ampaian yang diperoleh daripada *P. rolfsii* c3-2(1) IBRL berbanding dengan enzim komersial, di mana 2 hingga 3 kali ganda aktiviti yang lebih tinggi berbanding dengan Celluclast 1.5L (Sigma) dan 3 hingga 4 kali ganda aktiviti yang lebih tinggi berbanding dengan Accellerase 1500 (Genencor) telah ditunjukkan. Selain itu, *P. rolfsii* c3-2(1) IBRL juga menunjukkan aktiviti enzim yang lebih tinggi terhadap xilan, arabinan dan laminarin berbanding dengan enzim komersial. Hidrolisis untuk

residu batang kelapa sawit dalam keadaan optimum didapati pada pH 5.0 dan suhu 50°C, dengan kestabilan haba yang lebih tinggi bagi enzim mentahnya. Selepas 48-72 jam proses sakarifikasi biojisim, peningkatan sebanyak 1 hingga 1.5 kali ganda jumlah penukaran gula telah ditunjukkan oleh enzim daripada *P. rolfsii* c3-2(1) IBRL berbanding dengan enzim komersial. Jumlah hasil penukaran gula maksimum, iaitu kira-kira 56% telah diperolehi daripada hidrolisis berenzim oleh enzim mentah *P. rolfsii* c3-2(1) IBRL terhadap residu batang kelapa sawit berbanding dengan jumlah hasil penukaran gula sebanyak 43% oleh Celluclast 1.5L dan 40% oleh Accellerase 1500 apabila kepekatan enzim 14 FPU/g substrat ditambahkan untuk proses sakarifikasi dalam tempoh 48 jam. Residu lignin yang dipencilkan daripada residu batang kelapa sawit ini didapati memberi kesan terhadap hidrolisis biojisim dan membuktikan bahawa *P. rolfsii* c3-2(1) IBRL berupaya menghasilkan enzim 'ikatan-lignin' yang lemah, seterusnya menyumbang pada kecekapan yang lebih tinggi terhadap hidrolisis residu batang kelapa sawit. Xilanase dan laminarinase telah berjaya ditulenkan daripada *P. rolfsii* c3-2(1) IBRL. Xilanase ini didapati mempunyai sifat-sifat fizikal dan kimia seperti berikut: kespesifikan substrat terhadap xilan daripada birchwood, V_{max}= 691.6 μmol/min/mg dan K_m= 5.72 mg/ml; pH optimum, 5.0; suhu optimum, 50°C; kestabilan suhu selepas rawatan xilanase pada 50°C untuk 4 jam (residu aktiviti > 90%); berat molekul berdasarkan analisis SDS-PAGE dianggarkan 35 kDa. Laminarinase juga telah diuji terhadap laminarin yang diperolehi daripada *Laminaria digitata* sebagai substrat di mana V_{max}= 372.2 μmol/min/mg dan K_m= 0,0817 mg/ml; pH optimum, 5.0; suhu optimum, 70°C; kestabilan suhu selepas rawatan laminarinase pada suhu ≤ 55°C selama 4 jam (residu aktiviti > 90%); berat molekul berdasarkan analisis SDS-PAGE dianggarkan 75 kDa. Xilanase yang ditulenkan didapati meningkatkan kesan sinergi bersama-sama dengan

enzim komersial dalam proses sakarifikasi terhadap residu batang kelapa sawit berdasarkan jumlah penukaran gula yang dihasilkan dan ini menunjukkan ia merupakan salah satu enzim yang berperanan utama untuk hidrolisis residu batang kelapa sawit.

BIOCONVERSION OF OLD OIL-PALM TRUNK RESIDUES VIA ENZYMATIC HYDROLYSIS BY *Penicillium rolfsii* c3-2(1) IBRL USING REDUCING SUGARS AS AN INDICATOR

ABSTRACT

Due to the decreased oil productivity of old oil-palm trees after 25 years, large quantities of trunks as the waste were generated from the replanting activities. The felled old oil-palm trunk was found containing large quantity of sap with high concentration of sugar contents. The oil-palm trunk residues which are the residual substances after squeezing sap will be discharged in large quantity. These oil-palm trunk residues in the form of agro-industrial waste constitute a potentially enormous source of feedstock for bioconversion into biofuel, and other value-added chemicals without competition with food sources. A filamentous fungus namely c3-2(1) was screened and selected for its strong activities against oil-palm trunk residues. Fungal isolate c3-2(1) was identified as *Penicillium rolfsii* by morphological characterization, microscopical observations and confirmed by molecular identification. It was designated as *Penicillium rolfsii* c3-2(1) IBRL. Oil-palm trunk residues-hydrolyzing specific activity of the culture supernatant from *P. rolfsii* c3-2(1) IBRL was found superior to those of commercial enzymes Celluclast 1.5L (Sigma) and Accellerase 1500 (Genencor) which exhibited 2 to 3-fold and 3 to 4-fold higher activity, relatively. On the other hand, *P. rolfsii* c3-2(1) IBRL exhibited a greater xylan, arabinan and laminarin-hydrolyzing activities than those commercial enzymes. The optimal conditions for oil-palm residues hydrolysis was found at pH 5.0 and temperature of 50°C, with higher thermal-stability of crude enzymes. After 48–72 h of biomass saccharification, 1 to 1.5-fold higher total sugar conversion was performed by enzyme of *P. rolfsii* c3-2(1) IBRL compared to commercial enzymes.

Maximum total sugar conversion yield of approximately 56% was obtained from enzymatic hydrolysis on oil-palm trunk residues by crude enzyme of *P. rolfsii* c3-2(1) IBRL compared to total sugar conversion yield 43% from Celluclast 1.5L and 40% from Accellerase 1500 when 14 FPU/g substrate of enzyme loading added at 48 h reaction. The isolated lignin residual from oil-palm trunk residues affected the biomass hydrolysis, which revealed that *P. rolfsii* c3-2(1) IBRL is capable to produce weak 'lignin-binding' enzymes which might contribute to the higher efficiency hydrolysis on oil-palm residues. Purification of xylanase and laminarinase were successfully achieved from *P. rolfsii* c3-2(1) IBRL. The xylanase had the following physical and chemical properties: substrate specificity on xylan from birchwood, V_{max}=691.6 μmol/min/mg and K_m=5.72 mg/ml; optimum pH, 5.0; optimum temperature, 50°C; temperature stability after the treatment at 50°C for 4 hr (residual activity > 90%); molecular weight by SDS-PAGE analysis, about 35 kDa. The laminarinase was tested on laminarin from *Laminaria digitata* as substrate in which V_{max}=372.2 μmol/min/mg and K_m=0.0817 mg/ml; optimum pH, 5.0; optimum temperature, 70°C; temperature stability after the treatment at ≤ 55°C for 4 hr (residual activity > 90%); molecular weight by SDS-PAGE analysis, about 75 kDa. Considerable increasing synergism effect was observed on added purified xylanase with commercial enzymes during saccharification of oil-palm residues based on total sugar conversion, suggesting it is one of the key enzymes for the hydrolysis of oil-palm trunk residues.

CHAPTER ONE

INTRODUCTION

1.1 The potential of oil-palm trunk biomass as an alternative source for production of lignocellulolytic enzymes

Energy crisis is one of the most serious threats towards the sustainability of human kinds and civilization. Excessive global consumption of energy, such as fossil fuels, particularly in large urban areas with expansion of human population and increase of industrial prosperity, high levels of pollution and greenhouse gasses in the atmosphere have increased drastically during the last few decades (Sarkar *et al*., 2012). Furthermore, the shortage of fossil fuels parallel to the global consumption of fuels and consequences of climate change induced by greenhouse gas emissions (Singh *et al*., 2011; Sulaiman *et al*., 2012), have led the tremendous focus on using lignocellulosic biomass for the production of cellulases and other lignocellulolytic enzymes. Lignocellulosic biomass originated from agricultural and forestry residues and herbaceous is abundantly available renewable carbon source which can be converted for further usage of fuel and chemical production (Rahikainen *et al*., 2011), concomitantly to mitigate dependence on depleting fossil oil (Jørgensen *et al*., 2007).

Each year, more than 40 million tonnes of inedible plant materials are produced from agricultural residues and much of which are thrown away (Sanderson, 2011). For instance, some of the readily available lignocellulosic biomass is left at the field as a waste and is burned after harvesting through agricultural burning activity (Dawson and Boopathy, 2007), and this included wastes from oil palm plantation and industry. Each year, there are approximately more than 30 million tons of biomass in the form

of empty fruit bunches, oil-palm trunks and oil-palm fronds generated in the oil palm industry in Malaysia (Sulaiman *et al*., 2012). For instance, oil-palm trees with low productivity of palm oil after 20 to 25 years of age (Lim *et al*., 1997; Jung *et al*., 2011); these trees are chopped down and trunks are left to rot in the field (Yamada *et al*., 2010). Consequently, the felled palm trunks represent one of the most important biomass resources in Malaysia and Indonesia (Sumathi *et al*., 2008; Shuit *et al*., 2009). These oil-palm trunks which are considered as wastes could be made use for conversion of fermentable sugars and biofuels, in helping societies less dependent solely on oil (Rostrup-Nielsen, 2005). Furthermore, when these discarded, woody bits of plants are converted into value-added renewable resources, it can then be fulfilling the term of 'second-generation' biofuels which might gradually eliminate the use of 'first-generation' biofuels without competition with edible food crops such as sugar cane and corn (Graham-Rowe, 2011; Sanderson, 2011). Accumulation of lignocellulosic materials in abundance in places where agricultural residues present a disposal problems, results not only detrimental to the environment but also in loss of potentially valuable material, such as production of bioethanol from fermentable sugars that derived from lignocellulosic waste.

Oil-palm is one of the most active agricultural crops and becoming the most attractive option for Malaysia due to great amount of agricultural waste being produced every year (Goh *et al*., 2010), and it was reported that the largest portion of total agricultural waste in Malaysia comes from oil palm plantation (Misson *et al*., 2009). In the year 2007, Kelly-Yong *et al.* (2007) reported a total of 10,827 thousand tons of trunks were generated based on the total area of 4,304,914 Ha oil palm cultivation, where for every 25 years the chopped oil palm trees contribute to 2.515

tons of trunks generated from each hectare of oil palm cultivation. A report on the performance of the Malaysian oil palm industry showed that the total oil palm planted area was 4.85 million hectares in 2010 (Sulaiman *et al*., 2012). Due to the availability of a wide plantation area, a significant amount of biomass could be produced and further converted into value-added product. According to Goh *et al*. (2010) , second generation of bioethanol was applicable on lignocellulosic materials which can be used for bioethanol production to partly substitute fossil fuels in vehicle. Due to the unpredictability of palm oil price in international market, the renewable energy policy that proposed by Malaysian government is still not satisfactory even though palm oil can be used as raw material supply to biofuel industry.

Lignocellulose is consisted of more than 60% of plant biomass generated on earth which may be a potential feedstock for biofuels production, enzymes application and other biochemical products (Tengerdy and Szakacs, 2003). Cellulose, hemicelluloses and lignin are generally found intermeshed by strong chemical bondings, such as non-covalent and covalent cross-linkages (Pérez *et al.*, 2002). As lignocellulosic biomass such as oil-palm trunk residues contains high amount of sugars in the form of celluloses and hemicelluloses, it represents a promising feedstock for the bioethanol production. Kosugi *et al*. (2010) found a large quantity of sap with the high glucose content from the sap of the felled trunk. Two distinct components can be differentiated in the oil-palm trunk residue, which are parenchyma and vascular bundle (Akmar and Kennedy, 2001; Hashim *et al*., 2011). These two components are found in almost equal proportions in the fiber residues (Hashim *et al*., 2011). Therefore, ethanol fuel production from lignocellulosic biomass is advantageous as it does not lead to competition for food resources (Lynd, 1996). Due to the renewable

and ever-present nature of lignocellulosic biomass without competition with food crops, they are promising for bioethanol production and put a tremendous amount of effort into the research aspect for bioconversion.

1.2 Microorganisms and their lignocellulolytic enzymes

The main products of lignocellulose degradation are sugar components which can be used as a carbon or food source by numerous microorganisms. Fermentable sugars can be produced mainly by fungi in its natural habitat via solid state fermentation processes (Ibrahim, 2008). Many microorganisms including bacteria and fungi have been found potentially to degrade cellulose and other plant cell wall fibres. Lignocellulolytic enzymes-producing fungi are wide spread and most of them contribute significantly to the decomposition of lignocellulosic residues in nature by producing various lignocellulolytic enzymes (Dashtban *et al.*, 2009; Sánchez, 2009). Members of the *Trichoderma* genera such as *T. viride*, *T. longibrachiatum*, *T. reesei* are notable for their high enzymatic productivity. On the other hand, genera of *Penicillium*, such as *P. verruculosum* and *P. funiculosum* were reported for their superior performance of cellulase preparations over *Trichoderma* enzymes (Gusakov, 2011). Several bacteria were also reported capable of producing lignocellulolytic enzymes, such as *Pseudomonas fluorescens*, *Escherichia coli*, *Bacillus subtilis* and *Serratia marcescens* (Sethi *et al.*, 2013).

1.3 Biotechnological applications

Lignocellulose degradation is of utmost prominence for biotechnological conversion of lignocellulosic materials into value-added products, which fostering the development and application of enzymatic processes by a wide range of industries in

recent decades due to their high specificity, fast in action and often save raw materials, energy and chemicals (Jegannathan and Nielsen, 2013). Various bioproducts from the lignocellulose-degrading microorganisms and their applications are widely reported (Kuhad and Singh, 1993; Subramaniyan and Prema, 2002; Sun and Cheng, 2002), as lignocellulolytic enzymes that produced could be applied in various industries including textile, detergents, pulp and paper, fodder, bioconversion, environment, food, chemical and pharmaceutical.

1.4 Objectives of research

The objectives of the current research are as follow:

1) To isolate and to screen potential fungal isolates that can degrade the oil-palm trunk residues by submerged fermentation system.
2) To characterize the potential oil-palm trunk residues-degrading fungal isolate by morphological study and molecular level identification.
3) To characterize the crude lignocellulolytic enzymes produced by the selected potential fungal isolate.
4) To purify the key enzymes for hydrolysis of oil-palm trunk residues.

1.5 Scope of study

Potential oil-palm trunk residues-degrading fungus was isolated from soil samples which were collected from oil palm plantation areas in northern part of Peninsular Malaysia. In these studies, laboratory fungal isolates were also used for determining production of lignocellulose-degrading enzymes as well. Capability of the fungal isolates with high potential in degrading oil-palm trunk residues by crude enzyme were further investigated based on their protein production and specific activities on

oil-palm trunk residues by submerged fermentation. The fungal isolates with high capability of degrading the oil-palm trunk residues were selected for further study. Fungal isolate identification was based on the morphology of the fungal culture by comparing their physical characteristics (mechanism of spore forming, size and shape of spores) to those of reference materials, via microscopic observation. The selected fungal isolates were also identified using molecular biological protocol by DNA amplification and sequencing of the internal transcribed spacer (ITS) region. Microscopic observations of changes on the oil-palm residues' structure were investigated. These observations included the analyses of sugars released during the hydrolysis of lignocellulosic materials, which give insight information on enzyme mechanisms at an ultrastructural level. Enzymes are unstable molecules with a definite physic-chemical organization. Characterizations of the crude enzyme were conducted on the basis of pH and temperature parameters. The effect of lignin on the hydrolysis of oil-palm trunk residues was investigated and this was due to the hindrance of lignin which might affect the efficiency of enzymatic hydrolysis on lignocellulosic materials. Next, the saccharification experiments were carried out by using crude enzyme produced by the selected potential fungal isolate compared to commercial enzymes such as Celluclast 1.5L and Accellerase 1500. Since the enzymes are proteinaceous in nature, standard extraction and purification procedures for enzymes were the same as those used for proteins. It is included the purification of enzymes by ammonium sulfate precipitation, extraction of enzyme, dialysis, column chromatography and electrophoresis to obtain homogenous purified protein fraction. Synergism effect of added purified enzymes together with commercial enzymes was also investigated in the saccharification experiment.

CHAPTER TWO

LITERATURE REVIEW

2.1 Lignocellulosic biomass

Lignocellulosic biomass in the form of agricultural and forestry residues are the most abundant and boundless or renewable natural resources. Lignocellulose mostly consists of lignin, hemicelluloses and celluloses, in which can be derived from woody and non-woody plants. Due to their valuable lignocellulosic chemical properties, agricultural biomasses are the substrate of paramount importance of biotechnological value. Improvement in many processes related to lignocellulose biotechnology has gained great interest in pass few years.

2.1.1 Structure and composition of lignocellulosic biomass

The chemical components of lignocellulosic biomass varies from source to source (Sitton *et al.*, 1979; Sreenath *et al.*, 1999; Lynd *et al.*, 2005; Reddy and Yang, 2005), which the major components comprise cellulose (35–50%), followed by hemicellulose (20–35%), lignin (10–25%) as well as minimal fraction of protein, essential oils and ash. The structural components of lignocellulose are illustrated in Figure 2.1 and Figure 2.2. These complex and native structural components of biomass are generally contributing to the availability and resistance for enzymatic hydrolysis. Lignin polysaccharide matrix is usually found bounding surround cellulose fiber, whereas the structural integrity of cell walls is attributed to the formation of both covalent and non-covalent linkages from xylan. The composition of hardwoods and softwoods are significantly different. The lignin content of softwoods is generally higher than that of hardwoods, whereas hemicelluloses

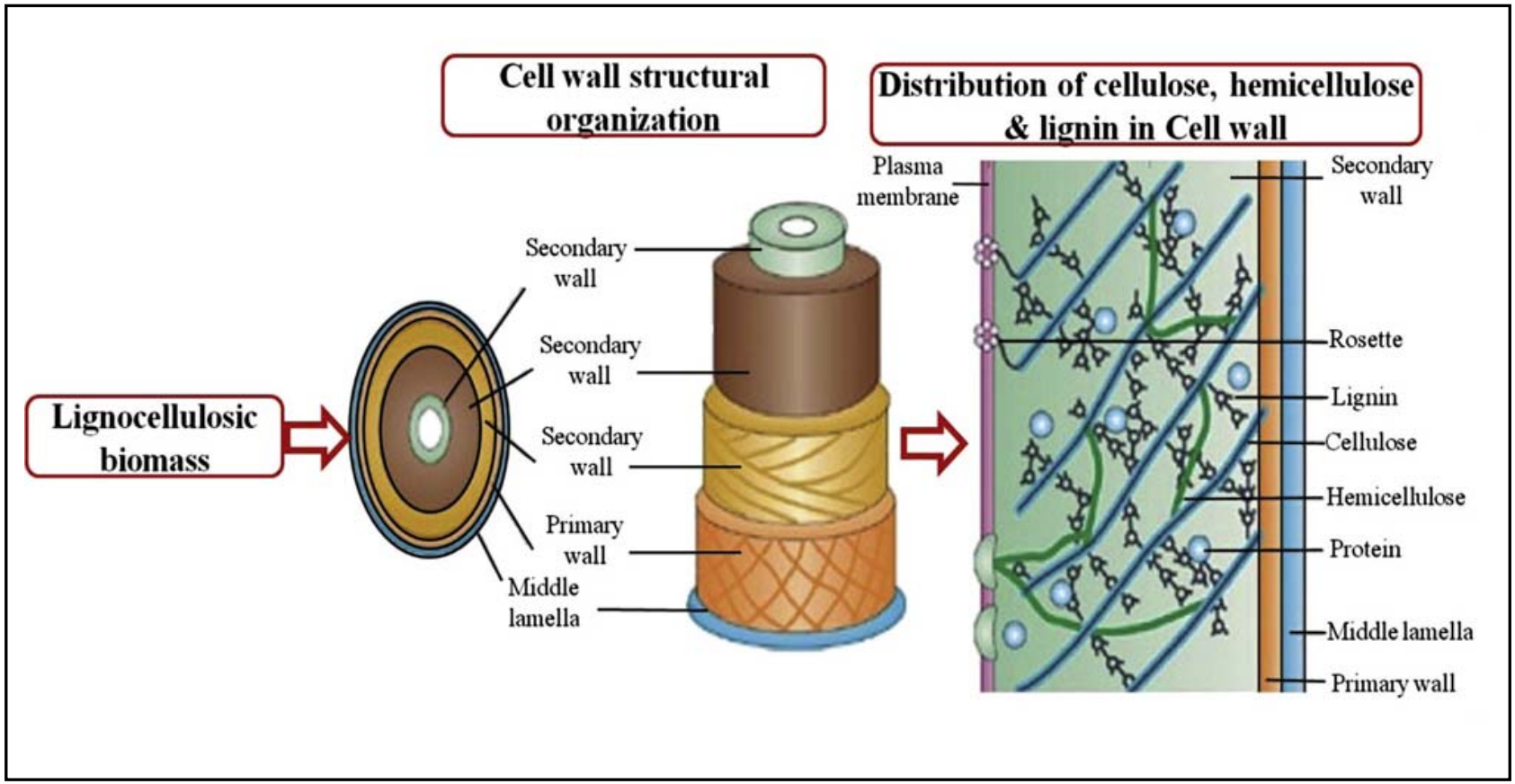

Figure 2.1: Diagrammatic illustration of the framework of lignocellulose (Menon and Rao, 2012)

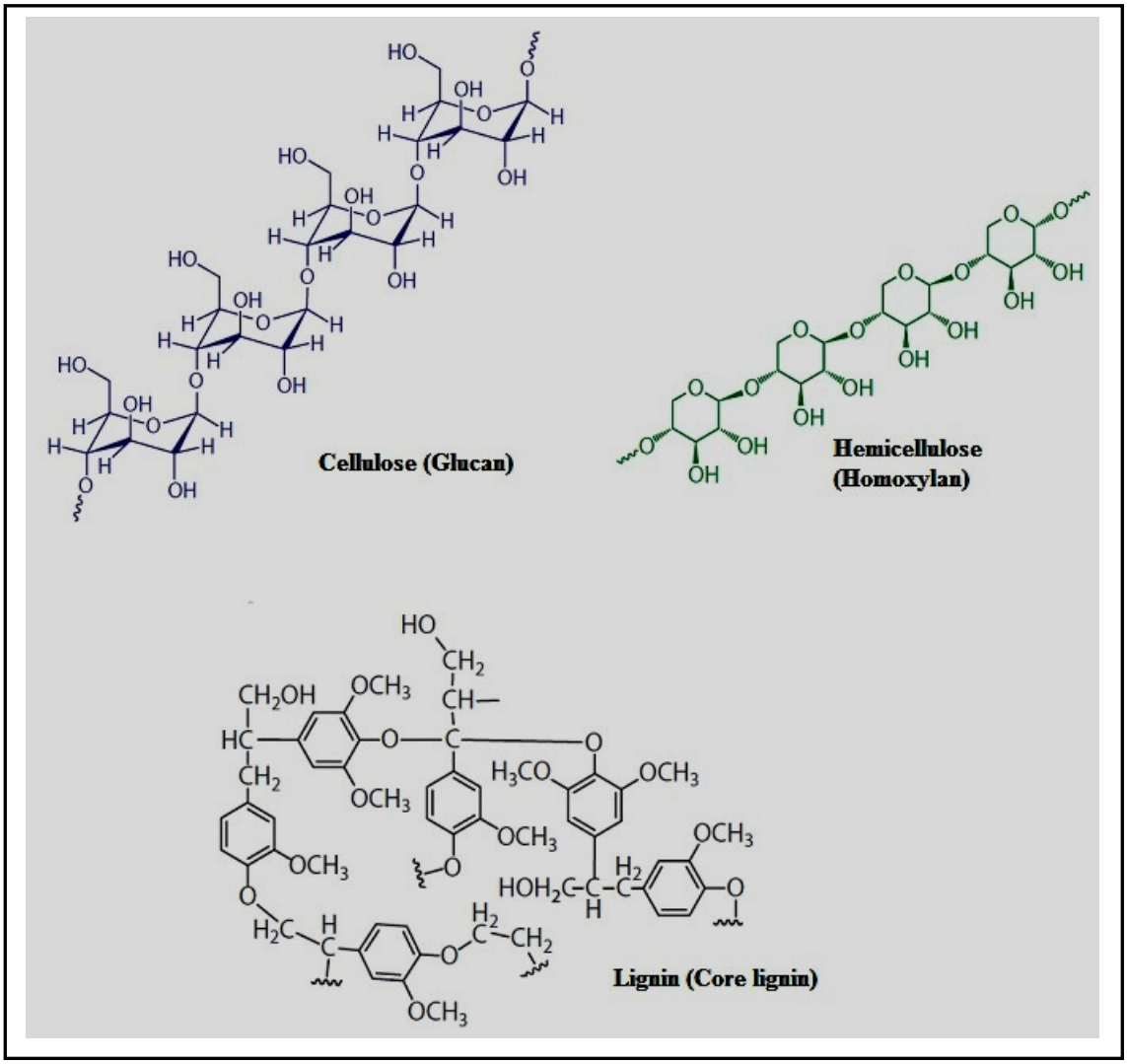

Figure 2.2: Schematic structural formula cellulose (glucan), hemicelluloses (homoxylan) and lignin (core lignin) (Chundawat *et al*., 2011).

content of hardwoods is higher than the softwoods. The compositions of various lignocellulosic materials are illustrated in Table 2.1.

Table 2.1: Composition of representative lignocellulosic materials.

Feedstocks	Carbohydrate composition (% dry wt)			Reference
	Cellulose	Hemicellulose	Lignin	
Alfalfa	21.8	12.4	9.7	Dijkerman *et al*. (1997)
	33	18	8	Sreenath *et al*. (1999)
Barley straw	38.08	22.63	22.27	Kim *et al*. (2011)
	38.0	21.9	17.3	Kim *et al*. (2014)
Bamboo	46.68	16.4	17.66	Kuttiraja *et al*. (2013)
	38.4	25.9	20.8	Littlewood *et al*. (2013)
Banana waste (pseudo stem)	54-60	8.2-16	12-21	Santa-Maria *et al*. (2013)
	44.32	22	9.66	Gabhane *et al*. (2014)
Banana waste (leaf)	27-34	11-19	25-26	Santa-Maria *et al*. (2013)
	32.56	12	21.80	Gabhane *et al*. (2014)
Banana waste (pith)	36.14	7	16.43	Gabhane *et al*. (2014)
Corn cob	35-39	38-42	4.5-6.6	Okeke and Obi (1994)
	45	35	15	Howard *et al*. (2003)
	36.4	34.9	14.8	Sahare *et al*. (2012)
Corn stover	39	19.1	15.1	Lee (1997)
	37.5	22.4	17.6	Mosier *et al*. (2005b)
	34.1	20.4	11.6	Jin *et al*. (2011)
	41.7	20.5	18	Merino and Cherry (2007)
Cotton stalk	31.1	10.7	30.1	Silverstein *et al*. (2007)
	30.58	16.85	29.99	Kaur *et al*. (2012)
Coffee pulp	24.0	8.9	19.4	Dijkerman *et al*. (1997)
Douglas fir	50	17.8	28.3	Lee (1997)
	47.3	19.5	30.3	Kumar *et al*. (2012)
Rice straw	41	21.5	9.9	Lee (1997)
	32.1	24	18	Howard *et al*. (2003)
Rice husk	34.4	17.5	23	Yáñez *et al*. (2006)
	42.2	18.47	19.4	Banerjee *et al*. (2009)
	47.3	23.0	29.7	Takahashi *et al*. (2014)
Wheat straw	33-40	20-25	15-20	McKendry (2002)
	35-39	22-30	12-16	Prasad *et al*. (2007)
	37-41	27-32	13-15	Balat (2011)

Table 2.1 Continued……

Newspaper	40-55	24-39	18-30	Howard *et al*. (2003)
	40-55	25-40	18-30	Balat (2011)
	40	23	20	Lee (1997)
Sugarcane bagasse	38.1	26.9	18.4	Lee (1997)
	25-45	28-32	15-25	Singh *et al*. (2009)
	34.1	29.6	19.4	Maeda *et al*. (2011)
Sunflower stalks	38.5	33.5	17.5	Sharma *et al*. (2002)
	32.56	20.73	13.32	Díaz *et al.* (2011)
	33.45	21.71	14.26	Ruiz *et al*. (2013)
Olive tree biomass	34.4	20.3	20.4	Ruiz *et al*. (2006)
	25.0	15.8	18.8	Cara *et al*. (2008)
	25.0	15.8	16.6	Manzanares *et al*. (2011)
	33.96	17.86	18.56	López-Linares *et al*. (2013)
Switchgrass	30-50	10-40	5-20	McKendry (2002)
	45	31.4	12	Howard *et al*. (2003)
	31	20.4	17.6	Mosier *et al*. (2005b)
	31	22	18	Merino and Cherry (2007)
Softwood	35-40	25-30	27-30	McKendry (2002)
	42	27	28	Balat (2011)
Hardwood	45-50	20-25	20-25	McKendry (2002)
	45	30	20	Balat (2011)
Oat straw	35.0	28.2	4.1	Gomez-Tovar *et al*. (2012)
Nut shells	25-30	25-30	30-40	Howard *et al*. (2003)
Sorghum straw	35.1	24.0	25.4	Vázquez *et al*. (2007)
	44.51	38.62	6.18	Poonsrisawat *et al*. (2013)

Composition of the same lignocellulosic materials might be different substantially depending on the source of the lignocelluloses and the specific species variety (Van Dyk and Pletschke, 2012). On the other hand, particular sugar analysis methods (Foyle *et al*., 2007), analysis procedures such as pretreatment methods as well as the growing location and harvesting season (Agblevor *et al*., 2003; Kaur *et al*., 2012; Van Dyk and Pletschke, 2012) for one particular crop had put the great impact on the compositional changes or differences from the same lignocellulosic biomass. For instance, hydrolysis of switchgrass was impacted substantially by the time of harvest as reported by Wyman *et al*. (2011). Therefore, compositional analysis for one particular lignocellulosic biomass should be analyzed independently for each conducted experimental unit as recommended by Van Dyk and Pletschke (2012).

2.1.1.1 Cellulose

The celluloses are complex molecules consisting of homopolymer of glucose units linked with *β*-1,4-glucosidic units. These complex celluloses composed of linear *β*-1,4-glucan chains which form aggregation of microfibrils (3 to 5 nm in diameter) via interaction of intra- and intermolecular hydrogen bonds and van der Waals forces resulting from pyranose ring stacking (Chundawat *et al.*, 2011). Glucose and cellodextrins are the products when cellulose is hydrolyzed. Depending on the different type of sources, the degree of polymerization of cellulose might range from 100 to 10,000 (O'Sullivan, 1997; Somerville *et al.*, 2004; Chundawat *et al.*, 2011) and even might reach up to 15,000 (Béguin and Aubert, 1994; Jørgensen *et al*., 2007). For instance, cellulose in nature consisted of glucopyranose chain ranging from 10,000 units in wood, whereas 15,000 glucopyranose units was found polymerized in native cotton (Agbor *et al.*, 2011). Laureano-Perez *et al.* (2005) reported that the

'straightness' of the chain was determined by the hydrogen bonding within a cellulose microfibril. On the other hand, the crystallinity and amorphous regions of cellulose were determined by interchain hydrogen bonds. The degree of crystallinity contributes to the low saccharification of cellulose due to existence of steric hindrance from the cellulose molecule itself causing resistance to microbial attack and enzymatic hydrolysis, whereas amorphous cellulose is less resistant to degradation (Jørgensen *et al.*, 2007; Menon and Rao, 2012). A dense layer of water was formed surround the hydrophobic surface of cellulose which might contribute to the obstruction for the enzymes to interact with substrate.

2.1.1.2 Hemicellulose

Hemicelluloses are matrix polysaccharides or heteropolymers, mostly constructed from both hexoses and pentoses such as arabinose, galactose, glucose, mannose and xylose (Brown, 1983; Bastawde, 1992; Rubin, 2008). The cellulose fibres are linked together with these matrix polysaccharides into microfibrils and cross-linked with lignin to create the complex linkages that provide structural strength (van Wyk, 2001). β-1,4-xylans are the second most abundant element of hemicelluloses for lignocellulosic biomass. They make up around 20-30% of the dry weight of tropical hardwood and annual plants, which are accounted for one-third of the renewable biomass available on earth (Dhiman *et al*., 2008).

Xylans can be derived from different plants and grasses, which generally have the same backbone structure of β-(1-4) linked xylose residues. The degree of branching can be attributed by the origin of the sources and the differences in the branched residues, which might compose of D-glucuronic acid, L-arabinose and 4-O-

methylesters of D-glucuronic acid (Bajpai, 2009). Bastawde (1992) reported that fewer acidic side chains were found available in softwood xylans compared to hardwood xylans. Softwood xylans comprise one acidic group of per nine to 12 D-xylose residues, whereas there are per five to six D-xylose units found in hardwood xylans. The major hemicellulose components in softwood are mannan-based, and those in hardwood are xylan-based.

The major constituents of the xylans compose of D-xylose and L-arabinose. On the other hand, the mannans are made up of D-glucose, D-galactose and D-mannose. Collectively, the principal sugar components of these hemicellulose heteropolysaccharides are: D-xylose, D-glucose, L-arabinose, D-mannose, D-galactose, D-glucoronic acid, D-galactouronic acid, 4-O-methyl- D-glucoronic acid, and a minority of L-rhamnose, L-fucose and various O-methylated sugars. They usually have degree of polymerization of 100 to 200 (Jørgensen *et al*., 2007), and side chain can be acetylated (Bastawde, 1992; Kuhad *et al*., 1997). The mannan hemicelluloses, galactoglucomannans and glucomannans, in softwoods and hardwoods, are both branced heteropolysaccharides. Their backbones are constructed by both β-D-mannopyranose and 1,4-linked β-D-glucopyranose units. The acetyl group partially replaces the C-2 and C-3 position of the mannose and glucose in the backbone.

The hemicelluloses have been described as the most thermo-chemically sensitive components of lignocellulosic biomass (Hendriks and Zeeman, 2009), and suggested to ‘coat’ cellulose-fibrils within plant cell walls. To enhance the effectiveness of cellulose digestibility, not less than 50% of hemicelluloses should be eliminated has

been suggested. Hence, comprehensive steps should be taken into account upon what type of pretreatment is used, so that the formation of degradation products from hemicelluloses such as furfurals and hydroxymethyl furfurals can be avoided. These compounds have been reported to impede the fermentation process (Palmqvist and Hahn-Hägerdal, 2000a-b).

2.1.1.3 Lignin

Lignins are highly complex network molecules consisted of phenyl-propane-based monomeric units linked together by different types of bonds, including alkyl-aryl, alkyl-alkyl, and aryl-aryl ether bonds (Jørgensen *et al*., 2007), which provides rigidity, support, and protection to the plants (Blanchette *et al*., 2004). The molecular weight of lignins may be 100 kDa or more. The relative proportions of the three cinamyl alcohol precursors mingled into lignin, i.e., coniferyl alcohol, *p*-coumaryl and sinapyl alcohol (Arora and Sharma, 2010), vary not only with plant species (Sanderson, 2011) but also with location of the lignins within the plant cell wall as well as the plant tissues. Lignin is found at the highest concentration in the middle lamella. On the other hand, it is found the most abundantly available in the secondary walls of the vascular plants.

Two types of bonding: *β*-aryl ether and α-aryl ether are suggested hydrolysable in the linkage of lignin (Adler, 1977). The predominant *β*-aryl ether type bond is more resistant to cleavage. Under mild hydrolytic conditions, the cleavage of the ether bond is exclusively restricted to the α-aryl ether type (Kirk, 1987). Lignin is usually insoluble in water due to its characteristic as the ‘glue’ that combines the different constituents of lignocellulosic biomass together. Microbial and chemical degradation

were found less efficient due to the embedded of polymer cellulose microfibrils within lignin (Malherbe and Cloete, 2002; Jørgensen *et al*., 2007) by forming a physical barrier that restricts accession of microbial enzymes and chemicals for further degradation (Avgerinos and Wang, 1983; Mooney *et al*., 1998). Efficient pretreatment methods need to be adopted for removal of lignin from different type of feedstocks, so that enhancing biomass digestibility. Chang and Holtzapple (2000) showed that lignin removal gradually able to improve the biomass digestibility successfully. Other than this lignin as a physical barrier, toxicity effect to the microorganisms from lignin derivatives, less efficient binding of cellulolytic enzymes to lignin-carbohydrates complexes specifically, and non-specific adsorption of lignocellulolytic enzymes to lignin are some of the unfavorable effects caused by lignin components (Agbor *et al*., 2011).

2.2 Pretreatment of lignocellulosic biomass

Many factors influence the reactivity and digestibility of the cellulose fractions of lignocellulose biomass. These factors include the porosity of the biomass materials, lignin and hemicellulose content, crystallinity of cellulose, cellulose degree of polymerization, substrates availability surface area, feedstock particle size, cell wall thickness and change of substrate in accessibility with its conversion (Alvira *et al*., 2010). For accomplishing the industrially satisfactory time frame, pretreatment is required for destruction of the robust structure of lignocellulosic biomass prior to utilization for the conversion to fermentable sugars by enzymatic hydrolysis, such as for the biomass-to-ethanol conversion processes.

The objective of the pretreatment is to render biomass materials more accessible to either chemical or enzymatic hydrolysis for efficient product generation. The goals of the pretreatment are: to remove and separate hemicellulose from cellulose; to disrupt and get rid of the lignin sheath; to collapse the hydrogen bonds that rendering the crystallinity of cellulose; enable maximal coverage of cellulases to cellulose surface area; and to increase the pore size of cellulose to facilitate the penetration of hydrolysis agents (Gong *et al*., 1999; Haghighi Mood *et al*., 2013). Hence, an efficient enzymatic hydrolysis with reduced energy consumption and a maximal sugar recovery can be accomplished without any obstacles (Yang and Wyman, 2008; Zhu and Pan, 2010; Limayem and Ricke, 2012). The aims of pretreatment on lignocellulosic biomass are depicted in Figure 2.3.

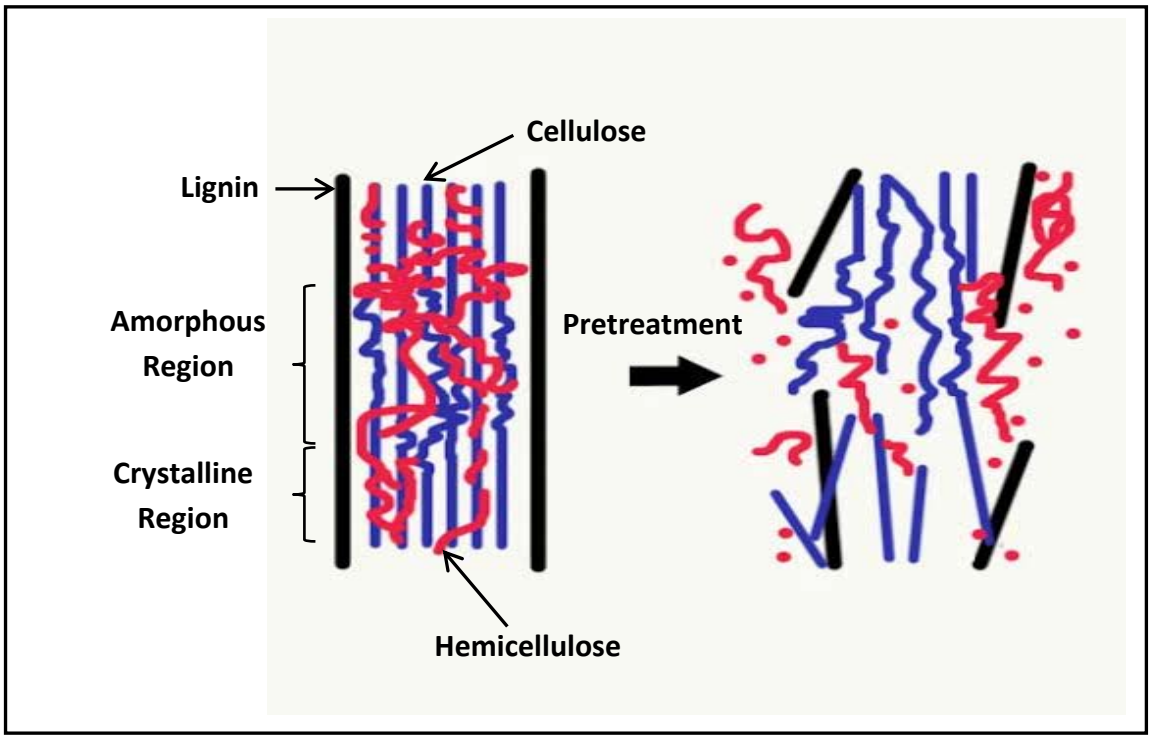

Figure 2.3: Schematic of aims of pretreatment on lignocellulosic biomass (Mosier *et al*., 2005b; Balat, 2011).

An advanced pretreatment process not only to increases accessibility of one enzymes but also to enhance the complete solubilization of the polymer to monomer sugars. However several other factors should fulfill its criteria of feasibility: preserving hemicellulose fractions, avoiding size reduction, reduced energy intensive, limiting formation of inhibitors due to degradation products, and being cost-effective (Menon and Rao, 2012). High yields for multiple crops, sites ages, and harvesting time, minimum amount of toxic compounds derived from sugar decomposition during pretreatment, fermentation compatibility, such as the choice of an organism able to ferment pentoses in hemicellulose are several key properties to take into consideration for low-cost and successful pretreatment process (Yang and Wyman, 2008; Alvira *et al*., 2010).

A multitude of diverse pretreatment technologies have been established during the last decade due to the diverse nature of different biomass feedstocks (Alvira *et al*., 2010; Menon and Rao, 2012), and its economic assessment and environmental impact (Menon and Rao, 2012). A generalized classification of pretreatment groups them into physical, chemical, biological and combinatorial pretreatments which is termed as physico-chemical method. Some of the promising pretreatment methods are summarized in Table 2.2, which describe the pros and cons of the selected particular pretreatments by a few reviewed articles.

Table 2.2: Most promising pretreatment technologies (advantages and disadvantages).

Method of pretreatment	Advantages	Limitation and disadvantages	References
Mechanical	**Reduce cellulose crystallinity, applicable for various feedstocks, success at pilot scale**	**High power consumption than inherent biomass energy, low sugar yield, high equipment cost**	**Menon and Rao (2012)**
	Increase accessible surface and pore size, decrease the crystalinity and degree of polymerization	**High energy requirement**	**Behera *et al*. (2014)**
Mineral acids	**Hydrolysis of cellulose and hemicellulose, alter lignin structure, applicable for various feedstocks, high sugar yield, allow to reuse of chemicals, success at pilot scale**	**Hazardous, toxic and corrosive, formation of inhibitors, high equipment cost**	**Menon and Rao (2012)**
	Majorly allow to hydrolyze hemicelluloses (xylan)	**Formation of inhibitory compounds, such as furfural, hydroxymethyl furfural, formic acid, levulinic acid and phenolic compounds; processing cost increased for removal of these compounds**	**Behera *et al*. (2014)**
	High solubility of hemicellulose and lignin, high glucose yield	**Process for recovery of acids used is expensive, costly corrosion-resistant equipment is required, formation of high concentration of inhibitors**	**Badiei *et al*. (2014)**
Alkali	**Removal of lignin and hemicellulose, increases accessible surface area, high sugar yield, applicable for various feedstocks, allow to reuse of chemicals, success at pilot scale**	**Long residence time, irrecoverable salts formed**	**Menon and Rao (2012)**
	Effective to remove acetyl groups from hemicelluloses, reduce steric hindrance of enzymes subsequently enhance cellulose digestibility	**Irrecoverable salts formed**	**Behera *et al*. (2014)**

Table 2.2 Continued……

	Alteration of lignin structure and effective to remove lignin, applicable to various biomasses, high sugar yield, success in pilot scale, low amount of inhibitors are formed	**Operational cost is high, catalysts used are expensive**	**Badiei *et al*. (2014)**
Liquid hot water	**Removal of hemicelluloses making enzymes accessible to cellulose, high sugar yield, formation high concentration of inhibitors, success at pilot scale**	**Long residence time, less lignin removal, not allow to reuse of chemicals**	**Menon and Rao (2012)**
	Achieved maximum solubilization of hemicelluloses by >80%, applicable for different kinds of lignocellulosic material, low or no production of inhibitors	**–**	**Haghighi Mood *et al*. (2013)**
	Removal of lignin, no any catalyst or chemical required, minimal of product degradation, no washing step or neutralization required, success at pilot scale	**Incomplete removal of lignin**	**Behera *et al*. (2014)**
Organosolv	**Hydrolyze lignin and hemicelluloses, high sugar yield, allow to reuse of chemicals, applicable for various feedstocks, success at pilot scale**	**Solvents need to be drained, evaporated, condensed and reused, formation of inhibitors, high equipment cost**	**Menon and Rao (2012)**
	Remove lignin extensively and almost complete solubilization of hemicellulose, applicable for various feedstocks	**Solvents need to be drained, evaporated, condensed and reused, high operational cost**	**Behera *et al*. (2014)**
	Removal of lignin and hydrolyze hemicelluloses, improve enzymatic degradability of cellulose, applicable to various biomasses with high potential sugar yield	**Removal of solvent is needed, costly process**	**Badiei *et al*. (2014)**
Wet oxidation	**Removal of lignin, dissolves hemicellulose and causes cellulose decrystallization**	**High equipment cost, not allow to reuse of chemicals**	**Menon and Rao (2012)**

Table 2.2 Continued……

	Efficient to fractionate lignocellulosic materials by solubilization, and hydrolysis of hemicelluloses, removal of lignin, less inhibitory compounds generation	**High capital cost, such as high cost of pressure equipment and the oxygen as well as catalyst used**	**Haghighi Mood *et al.* (2013)**
	Solubilization of hemicellulose, removal of lignin, and lignocellulosic materials are fractionated	**–**	**Badiei *et al.* (2014)**
Ozonolysis	**Reduces lignin content, no toxic residues, high sugar yield with low amount of inhibitor production**	**Large amount of ozone required, not allow to reuse of chemicals, high equipment cost**	**Menon and Rao (2012)**
	Reduces lignin content, applicable to various feedstocks, process is carried out at room temperature and normal pressure, no formation of any inhibitory compound	**–**	**Behera *et al.* (2014)**
	Reducing lignin content, applicable to various feedstocks, lack of degradation product, occurring at ambient temperature and normal pressure	**Large amount of ozone required, costly process and less applicable**	**Xu and Huang (2014)**
CO_2 explosion	**Hemicellulose removal, cellulose decrystallization, cost-effective, high sugar yield with low inhibitor formation**	**Does not modify lignin, not allow to reuse of chemical, high equipment cost**	**Menon and Rao (2012)**
	Effective to solubilize lignin, low cost process, no generation of toxic, use of low temperature and high solid capacity	**High cost of equipment**	**Behera *et al.* (2014)**
Steam explosion	**Hemicellulose removal and alteration in lignin structure, high sugar yield, applicable to various feedstocks, success at pilot scale**	**Incomplete destruction of lignin-carbohydrate matrix, high equipment cost, formation of inhibitory compounds**	**Menon and Rao (2012)**
	Applicable to various feedstocks, low energy requirement, no recycling or environment cost, cost effective	**Less effective to softwood, incomplete disruption of the lignin-carbohydrate matrix, generation of inhibitory compounds**	**Behera *et al.* (2014)**

Table 2.2 Continued……

	Increased removal of hemicelluloses from solid fraction, limited use of chemicals, low energy input	**Incomplete destruction of lignin-carbohydrate matrix, formation of fermentation inhibitors at higher temperature**	**Xu and Huang (2014)**
Ammonia Fiber Expansion Pretreatment (AFEX)	**Removal of lignin and hemicelluloses, high sugar yield with low amount of inhibitory compounds formed, allow to reuse of chemicals**	**Not efficient for biomass with high lignin content, high equipment cost**	**Menon and Rao (2012)**
	Changes of biomass structure and enhance the digestibility, applicable to various feedstocks, no formation of any toxic materials, reduction of particle size is not required, allow to reuse of chemicals	**Not efficient for biomass with high lignin content**	**Behera *et al.* (2014)**
	Induces the disruption in lignin-carbohydrate linkage, lower moisture content, lower sugar degradation products, almost complete recovery of solid material	**–**	**Xu and Huang (2014)**
Ionic liquids	**Dissolution of cellulose, increased amenability to cellulase, applicable to various feedstocks, low amount of inhibitor formed, allow to reuse of chemicals**	**–**	**Menon and Rao (2012)**
	Reduction of cellulose crystallinity, less dangerous process condition and chemicals, mild operational conditions and ionic liquid are easily recycled, lower energy consumption	**Lead to inactivation and unfolding of the protein, high temperature cause some negative side-effects, such as deteriorated stability of ionic liquids, and occurrence of side-reactions**	**Haghighi Mood *et al.* (2013)**
	Applicable to various feedstocks, allow selective extraction of unaltered lignin and cellulose, process under ambient temperature without alkaline or acidic reagents required and inhibitor compound formation	**Costly solvent are required, lower biomass conversion efficiency**	**Badiei *et al.* (2014)**

2.2.1 Physical pretreatments

2.2.1.1 Mechanical comminution

Most pretreatment approaches require the collected biomass to undergo some degree of mechanical size reduction prior to introduction into a pretreatment reactor. The goal of the mechanical pretreatment is to increase the available surface area and decrystallize the cellulose components. Hence, this helps to increase the enzymatic digestibility of the comminuted biomass and further reducing the degree of polymerization (Alvira *et al*., 2010). A combination of grinding, chipping or milling can be conducted to enhance the accessibility of enzymes to substrates, which depending on the final particle size of the material (10–30 mm after chipping and 0.2–2.0 mm after milling or grinding) (Sun and Cheng, 2002; Kumar *et al*., 2009). Due to higher energy consumption in milling stage, the use of very small particles is unfavorable, and subsequently imposes negative effect on the following pretreatment method. The amount of energy consumption that used for lignocellulosic biomass pretreatment by mechanical comminution depends on the final particle size and biomass characteristics (Cadoche and López, 1989; McMillan, 1994). As shown in Figure 2.4, the power requirements increase rapidly with decreasing particle size. The intensive mechanical energy requirements causes comminution to be cost-prohibitive for use as a individual pretreatment in a biomass to ethanol conversion process (Alvira *et al*., 2010; Talebnia *et al*., 2010; Agbor *et al*., 2011; Balat, 2011).

2.2.1.2 Pyrolysis

Pyrolysis is a pretreatment method, in which lignocellulosic materials treated at temperatures greater than 300°C and cellulose rapidly decomposes to produce residual char and gaseous products under this condition (Sun and Cheng, 2002;

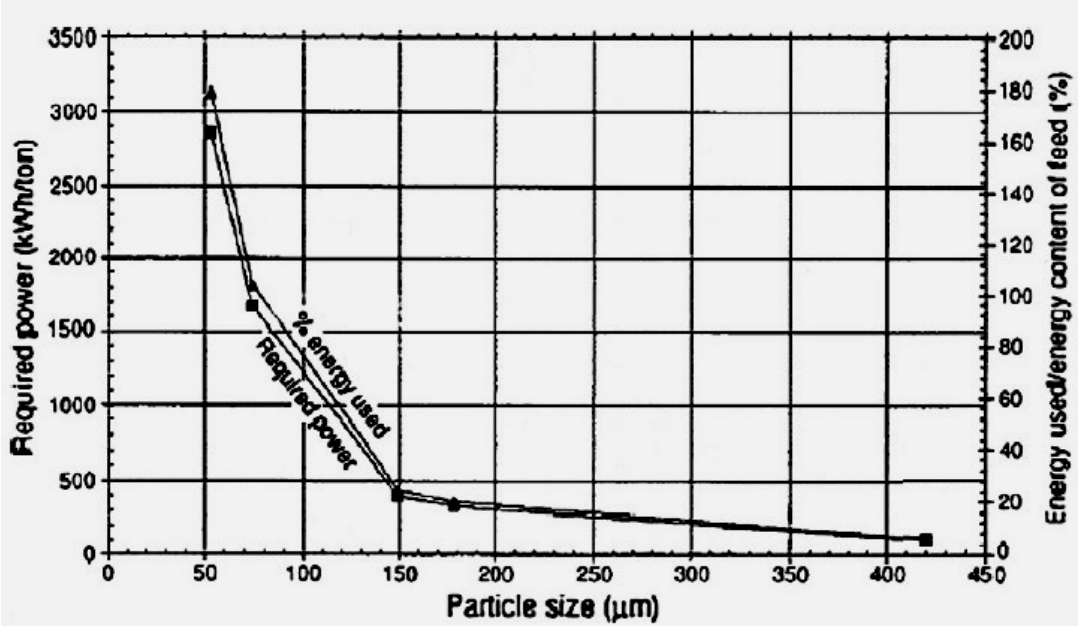

Figure 2.4: Energy requirements for ball milling municipal solid waste (McMillan, 1994).

Pasha and Rao, 2009; Balat, 2011). The decomposition is time-consuming and less volatile products are formed during the process at lower temperatures. Fan *et al*. (1987) reported that a total of 80–85% conversion of cellulose to reducing sugars with more than 50% glucose was achieved by applying mild acid hydrolysis (1 N H_2SO_4, 97°C, 2.5 h) pretreatment on biomass residues. Pyrolysis can be boosted up with the existence of oxygen (Shafizadeh and Bradbury, 1979), which further resulted in depolymerization, oxidation, and dehydration during the process. The conversion of cellulose to glucose yield through enzymatic degradation was successfully improved by pyrolysis pretreatment of ground material (Leustean, 2009).

2.2.1.3 Extrusion

In this context, extrusion is a novel and promising physical pretreatment method for biomass conversion to ethanol production. Physical and chemical modifications were