PRODUCTION OF BIOETHANOL BY USING PRETREATED COCONUT HUSK AS CARBON SOURCE

DING TECK YUAN

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PRODUCTION OF BIOETHANOL BY USING PRETREATED COCONUT HUSK AS CARBON SOURCE

By

DING TECK YUAN

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ABSTRACT

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Ding Teck Yuan

In the current study, coconut husk, a lignocellulosic biomass, was employed as the feedstock for production of bioethanol. The powderised coconut husks were subjected to thermal pretreatment, chemical pretreatment and microwave-assisted-alkaline (MAA) pretreatment prior to enzymatic and hydrolysis process. The composition profile of coconut husks was significantly altered upon the MAA pretreatment as compared to the untreated sample, with the cellulose content increasing from 18-21% to 38-39% while lignin content decreased from 46-53% to 31-33%. Enzymatic hydrolysis of MAA-pretreated coconut husk also achieved the highest yield of fermentable sugars (measured as glucose) with 0.279 g sugar/g coconut husk. Scanning electron microscopy (SEM) imaging also proved the obvious and significant disruption of coconut husks' structure. The results demonstrated that the combination of microwave radiation with alkaline solution was effective in altering the physical structures of coconut husks. Hence, MAA-

pretreated coconut husk was chosen as the substrate for subsequent hydrolysis and fermentation process.

For the optimization of simultaneous saccharification and bioethanol fermentation process, the critical variables that affected bioethanol production were identified by using Plackett-Burman design and tested using the analysis of variance (ANOVA). The factors with *p*-value less than 0.05 in this test were coconut husk loading (p = 0.0087) and pectinase loading (p = 0.0198). These two significant factors were further optimized using a Central Composite Design (CCD). The maximum response predicted from the model would yield 0.0525 g ethanol per g coconut husk daily under the optimal conditions of 3.06 g MAA-pretreated coconut husks, 0.58 mL cellulase, 0.38 mL pectinase and 1 g yeast extract in 100 mL of medium (pH 6) incubated at 30^{o}C. The experimental result gave bioethanol productivity of approximately 0.0593 g ethanol per g coconut husks daily, which was 13% higher than the estimated value (0.0525 g ethanol per g coconut husk). The results of validation experiments proved the usefulness and effectiveness of CCD as an optimization tool in enhancement of bioethanol production from indigenous renewable resources.

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Last but not least, I truly appreciate my family for their unconditional love, care and support. Words cannot express how grateful I am to my parents for all the sacrifices that they have made on my behalf. I would not have made it this far without them.

DECLARATION

I, Ding Teck Yuan, hereby declare that the dissertation is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UTAR or other institutions.

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DING TECK YUAN

FACULTY OF ENGINEERING AND SCIENCE

UNIVERSITI TUNKU ABDUL RAHMAN

Date: _____

PERMISSION SHEET

It is hereby certified that **DING TECK YUAN** (ID: **09UEM09101**) has completed this dissertation entitled "PRODUCTION OF BIOETHANOL BY USING PRETREATED COCONUT HUSK AS CARBON SOURCE" under the supervision of Associate Professor Dr. Hii Siew Ling (Supervisor) from the Department of Chemical Engineering, Faculty of Engineering Science, and Assistant Professor Dr. Lisa Ong Gaik Ai (Co-Supervisor) from the Department of Biological Science, Faculty of Science.

I hereby give permission to my supervisors to write and prepare a manuscript of these research findings for publishing in any form, if I did not prepare it within six (6) months time from this date, provided that my name is included as one of the authors for this article. The arrangement of the name depends on my supervisors.

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(DING TECK YUAN)

APPROVAL SHEET

This dissertation entitled "**PRODUCTION OF BIOETHANOL BY USING PRETREATED COCONUT HUSK AS CARBON SOURCE**" was prepared by DING TECK YUAN and submitted as partial fulfillment of the requirements for the degree of Master of Science in Biotechnology at Universiti Tunku Abdul Rahman.

Approved by:

_____

(Dr. Hii Siew Ling) Date: …….
Associate Professor / Supervisor
Department of Chemical Engineering
Faculty of Engineering and Science
Universiti Tunku Abdul Rahman

_____

(Dr. Lisa Ong Gaik Ai) Date:…….
Assistant Professor / Co-Supervisor
Department of Biological Science
Faculty of Science
Universiti Tunku Abdul Rahman

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LIST OF ABBREVIATIONS

MAA	Microwave-assisted-alkaline
DNS	3,5-dinitrosalicyclic acid
SEM	Scanning Electron Microscopy
RSM	Response Surface Methodology
CCD	Central Composite Design
GCMS	Gas Chromatography Mass Spectrometry
SSaF	Simultaneous Saccharification and Fermentation
SHF	Separate Hydrolysis and Fermentation
NADH	Nicotinamide adenine dinucleotide
YPD	Yeast extract-peptone-dextrose
NaOH	Sodium hydroxide
R^2	Coefficient of determination
HPLC	High Performance Liquid Chromatography
CFU	Colony forming unit
ca.	approximately
psi	Pound per square inch
% v/v	Volume percentage concentration
% w/v	Percentage weight over volume
% v/w	Percentage volume over weight

NDF	Neutral detergent fibre
ADF	Acid detergent fibre
ADL	Acid detergent lignin
ANOVA	Analysis of variance
Y	Ethanol yield
E	Concentration effect of tested variable
P	Ethanol productivity
N	Number of trials
V	Variance of concentration
SE	Standard error
CV	Coefficient of variance
σ	Standard deviation
μ	mean
PB	Plackett-Burman

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CHAPTER 1

INTRODUCTION

1.1 Energy Sources

In recent years, the negative impacts of fossil fuels such as global warming, greenhouse gases emissions and the fast depletion of fossil resources have resulted in an increased interest in the research of alternate power or sustainable energy such as biofuel (Palma et al., 2012). Bioethanol has been considered a better choice than conventional fuels, as it reduces the dependence on reserves of crude oil. Bioethanol also promises cleaner combustion, lower emissions of air pollutants, high octane rating and more resistant to engine knock, which may overall lead to a healthier environment because it is carbon neutral and essentially free from sulfur and aromatics (Bailey, 1996; Prasad et al., 2007; Gupta et al., 2009).

Today, bioethanol is one of the most dominant biofuel and its global production has increased sharply since year 2000. Generally, current production of bioethanol comes from sugar and starch-based materials such as sugarcane and grains (Dermirbas, 2009). However, considering the growing demand for human food,

lignocellulosic biomass has arisen as a more suitable feedstock for bioethanol production and a viable long-term option for bioethanol production as compared to the other two groups of raw material (Hamelinck et al., 2005). Lignocellulosic material is the most abundant plant biomass resources that can be used in bioethanol production industry. Examples of lignocelluloses are woody biomass, logging residues, energy crops (i.e. switchgrass and poplar), agricultural residues (i.e. wheat straw, rice straw and corn stover), agricultural by-products (i.e. rice hull, sugarcare bagasse) and municipal solid waste (Tan et al., 2008; Duku et al., 2011).

The lignocellulosic feedstock used in the current study for bioethanol production was the coconut husk. Coconuts are abundantly growing in coastal areas of all tropical countries. In Malaysia, about 115,000 ha of land were being used for coconut plantation in Year 2010 (Sulaiman et al., 2013). It was estimated that approximately 5.3 tons of coconut husk will become available per hectare of coconut. Some of the coconut husk was used as fibre source for rope and mats but most of the coconut husks are routinely disposed of after the coconut water is sold (Tan et al., 2008). This makes coconut husk a cheap and potential substrate that could be used for bioethanol production due to the presence of relatively high levels of cellulose and hemicelluloses in it (van Dam et al., 2004).

1.2 Problem Statement

The pathway of converting sugar and starch-based materials to bioethanol is a simple, effective and well-established fermentation process. However, the complex structure of lignocellulosic biomass limits the biomass utilization for bioethanol production. Lignocellulosic biomass is a heterogeneous complex of carbohydrate polymers (cellulose and hemicelluloses) and lignin. Therefore, pretreatment step is necessary to make the lignocellulosic biomass amendable to subsequent hydrolysis process so that conversion of carbohydrate polymers into fermentable sugars can be achieved more rapidly and with increased yields (Mosier et al., 2005; Champagne, 2006; Gutierrez et al., 2009).

1.3 Scope of Study

Coconut husk consisted of well-defined polymeric structures of cellulose (28%), hemicellulose (38%) and lignin (32.8%) (Pollard et al., 1992). Thus, the main challenge of hydrolysis of coconut husk is the hemicelluloses and lignin content. An effective pretreatment method which is able to increase the yield from cellulose hydrolysis is a key step in bioconversion of lignocellulosic materials to ethanol (Salvi et al., 2010). The pretreatment is needed to liberate the cellulose

from the lignin seal and at the same time to reduce the lignin content, to reduce cellulose crystallinity and to increase cellulose porosity (Hamelinck et al., 2005; Wyman et al., 2005).

Hence, the present study was initiated in determining the best pretreatment technique in altering the physical structure of coconut husk in order to increase the cellulose digestibility. The pretreatment techniques involved were thermal pretreatment, acid pretreatment, alkaline pretreatment and microwave-assisted-alkaline (MAA) pretreatment. The pretreated coconut husk was analyzed from the aspect of efficiency of enzymatic hydrolysis of the pretreated coconut husk by dinitrosalicylic (DNS) colorimetric method method and alteration in physical structure by scanning electron microscopy (SEM) analysis.

Following pretreatment, the optimization of bioethanol production of the pretreated coconut husk was conducted. Operating parameters which may affect the conversion of lignocellulosic feedstock into bioethanol such as pH of medium, incubation temperature, agitation speed and amount of coconut husk, cellulase, pectinase and yeast extract added in the medium during fermentation process were taken into consideration. Response Surface Methodology (RSM) based on Central Composite Design (CCD) was applied to determine the best combination of the affecting parameters in enhancing bioethanol production of pretreated coconut husk as sole carbon source. The crude bioethanol from fermentation broth was

concentrated by simple distillation approach and further analyzed by Gas Chromatography-Mass Spectrometry (GC-MS).

1.4 Research Objectives

The objectives of the present study are:

1. To evaluate the effects of physical, thermal, chemical and microwave-assisted-alkaline pretreatment on the physical structures of coconut husk and the efficiency of enzymatic hydrolysis from the pretreated coconut husk.

2. To study the bioconversion of pretreated coconut husk to bioethanol production by simultaneous saccharification and fermentation (SSF) process.

3. To screen and optimize various affecting parameters in enhancing bioethanol production from pretreated coconut husk by using Plackett-Burman Design and Response Surface Methodology, respectively.

CHAPTER 2

LITERATURE REVIEW

2.1 Energy Crisis

Majority of the world's electricity and energy sources are currently produced via fossil fuels. Examples of fossil fuels are coal, petroleum, natural gas, etc. These fossil fuels were generally formed by organic remains of prehistoric organisms deposited in beds of sedimentary rocks under the action of heat and pressure over millions of centuries. Fossil fuels are burnt to release energy in the form of heat which can then be used to power cars or other machines and generate electricity for our daily lifestyles (Markner-Jäger, 2008; Ayres and Ayres, 2010).

The use of fossil fuel has offered numerous advantages to our life but it also produces gaseous emissions which are harmful to both the population and the environment. For example, sulphur dioxide and sulphur trioxide gases released upon burning of fossil fuels can combine with atmospheric moisture to form sulphuric acid, leading to "acid rain", which can be very harmful to our ecological system (Menz and Seip, 2004). The use of fossil fuels also increased the concentration of carbon dioxide in the atmosphere. The transportation sector was

responsible for approximately 60% of the worldwide fuel consumption. This sector accounts for more than 70% and 19% of global carbon monoxide (CO) and carbon dioxide (CO_2) emissions, respectively (Balat, 2011). The excessive CO and CO_2 together with other greenhouse gases can absorb and trap the warmth that is generated by the sun and radiated from the earth's surface, thereby warming up the planet. According to the global climate change theory, the climate patterns and temperature could be affected by the heat trapped in our atmosphere. This is believed to be the main contributory factor to the global warming experienced by the earth today (U.S. Department of Energy (DOE), 1997; Florides and Christodoulides, 2009).

It takes hundreds of millions of years to form the non-renewable fossil source which will be depleted eventually. Hence, it is believed that in short future, the cost of finding and extracting new fossil fuels deposits will render them too expensive for daily usage.

In response to the greenhouse gas emissions and petroleum crisis, green energy from sustainable resources has gained more and more popularity. This has led to increasing interest in alternate power or sustainable energy researches such as solar energy, geothermal energy, wave power, wind power, methanol, biodiesel and bioethanol.

2.2 Bioethanol as Alternative of Fossil Fuel

The most commonly used energy alternatives are the bioethanol and biodiesel. Ethanol (C_2H_5OH), or known as ethyl alcohol, is a clear, colorless, flammable oxygenated hydrocarbon with a boiling point of 78.5^oC in the anhydrous state. Many regions of world have traditionally produced alcoholic beverages from locally available fruits and the most well-known substrate for these beverages is grape. Facing the inevitable depletion of the world's energy supply, similar alcoholic fermentation processes are now used in some countries to produce fuel grade ethanol also known as bioethanol.

Bioethanol has a number of advantages over the conventional fuels. First, it is biomass energy which comes from renewable resource, mostly crops or other agricultural sources, that is totally different from the finite fossil fuels. Unlike fossil fuels, the bioethanol produced in this way is an oxygenated fuel that contains 35% oxygen which enables a more complete combustion (Demirbas, 2005). Producing and using bioethanol as motor driving fuel or gasoline from plant crops can also help to reduce CO_2 buildup. According to U.S. Department of Energy (DOE) (1997), for every gallon of gasoline that is displaced by using bioethanol, 7.3 to 10 kg of CO_2 emissions is avoided. Hence, facing the current global warming issue, this biomass energy plays an important role in reducing the greenhouse gasses emissions (Lin and Tanaka, 2006; Sukumaran et al., 2009).

Bioethanol also contains only a trace amount of sulphur (30 mass ppm) (Archer Daniels Midland Company, n.d.). It is reported that burning bioethanol instead of gasoline is able to entirely eliminate the release of acid rain-causing sulphur dioxide (Nigam and Singh, 2011; Wei et al., 2014). The utilisation of crop biomass for production of bioethanol by local companies also reduces dependency on foreign oil and creates job opportunities from growing the necessary crops (Prasad et al., 2007).

It is convenient for bioethanol to be integrated into the existing road transport fuel system with modified internal combustion engine. This biofuel also can be used in unmodified engines by blending the ethanol with gasoline in various ratios. In United States of America, more than 95% of the gasoline contains up to 10% ethanol (E10) to boost octane and meet air quality requirements (U.S. Department of Energy (DOE), 1997). As one of the biggest biofuel producing country, all motor gasoline sold in Brazil contains 20 to 25% ethanol (E20-E25) since 1979 (Walter et al., 2008).

Biodiesel is derived from the transesterification of vegetable oils or animal fats and composed of saturated and unsaturated long-chain fatty acid alkyl esters (Fazal et al., 2011). It was reported to be one of the most promising alternative fuels due to its renewability and sustainability (Janaun and Ellis, 2010; Lozada et al., 2010). However, by comparing to the raw materials of biodiesel which

including recycled vegetable oils or fats, the production of bioethanol which uses corn, sorghum, sugarcane or agriculture wastes as raw materials may be more economical. For example, the yield of biodiesel from soybean was 0.52 tons/hectare while the yield of bioethanol from corn grains was 2.95 tons/hectare (Kim and Dale, 2005; Balat, 2011). 1The fact that with only minor modification, eventually the existing fossil fuel infrastructure can be used for bioethanol distribution and utilization also puts this biofuel in front of other renewable energy sources (Mojović et al., 2012)

2.2.1 Feedstock for Bioethanol Production

Bioethanol can be produced from different kinds of raw materials. Generally, the raw materials are classified into two categories: the first generation bioethanol was produced from the starch-rich biomass while the second generation bioethanol was made from lignocellulosic sources (Table 2.1).

Table 2.1: Comparison of first and second generation bioethanol.

Categories	First generation bioethanol	Second generation bioethanol
Feedstock	Sugarcane, wheat, sweet sorghum, corn etc.	Lignocellulosic biomass such as sugarcane bagasse, rice hulls, wheat straw etc.
Advantages	Environmentally friendly, economic.	Not competing with food and environmentally friendly.
Challenges	Unsustainable because competing with food supply.	Advance technology still under development to reduce the cost of conversion.

(Source: Naik et al., 2010)

Most of the bioethanol today are first generation bioethanol. They are synthesized from starchy material such as sugarcane, wheat and corn (Dong et al., 2008; Soccol et al., 2010; Gauder et al., 2011; Tao et al., 2011). The primary use of starch-rich materials is for animal feed and food products. Therefore bioethanol production from these materials will compete with the food supply and eventually increase the demand for food-crops supply (Mabee et al., 2011). Given the concerns on food security, first generation bioethanol development has become unsustainable. Thus, the lignocellulosic biomass has become the potential feedstock for bioethanol production.

Compared to first generation, the second generation bioethanol which uses lignocelluloses as substrate has the advantages of cheap, abundant and sustainable feedstock, no threat to food security, and greater environmental benefits (Naik et al., 2010). The lignocellulosic biomass is made up of very complex sugar polymers and is not generally used as food source. The production of bioethanol utilizing these feedstock usually begins with the separation of cellulose and hemicellulose from lignin, then a hydrolysis stage to break down the cellulose and hemicellulose into fermentable sugars, followed by fermenting ethanol from the sugars with suitable microbes, and finally the stage that recover the ethanol from fermentation broth (Sassner et al., 2008; Binod et al., 2010; Gnansounou, 2010). Table 2.2 lists the examples of various agro-industrial lignocellulosic biomass which have been used as substrate for bioethanol production. As shown in the table, the unused part of different agricultural biomass could be further converted

to the environmentally friendly bioethanol after pretreated with suitable treatment method.

Table 2.2: Bioethanol production from various lignocellulosic feedstock.

Substrate	**Ethanol yield (g ethanol/g substrate)**	**Pretreatment Techniques Employed**	**References**
Rice hulls	0.11	Acid pretreatment	Dagnino et al. (2013)
Rapeseed straw	0.14	Alkaline peroxide pretreatment	Karagoz et al. (2012)
Rice straw	0.19	Calcium capturing by carbonation followed by HCl-neutralization	Park et al. (2010)
Sorghum liquor waste	0.14	Microwave irradiation	Su et al. (2010)
Sugarcane bagasse	0.37	Diluted HCl acid	Hernandez-Salas et al. (2009)
Wheat straw	0.26	Steam-explosion pretreatment	Tomas-Pejo et al. (2009)
Bermuda grass leaves	0.12	Diluted acid pretreatment	Anderson et al., (2008)
Corn stover	0.17	SO_2-catalysed steam treatment	Sassner et al. (2008)

2.3 Overview of Coconut Palm

Cocos nucifera L. (Figure 2.1), generally referred to as coconut palm can be commonly found throughout the tropics, where it is interwoven into the lives of the local people. The coconut palm is grown in more than 93 countries. It is a native plant from the coastal region of Southeast Asia, which has then been carried eastward by ocean currents to the Pacific islands and westward to coastal India, Sri Lanka, East Africa, and other tropical islands (Chan and Elevitch, 2006). In Malaysia, coconut is the fourth important industrial crop after oil palm, rubber and paddy in terms of total planted areas (Sivapragasam, 2008).

Figure 2.1: *Cocos nucifera* L.

2.3.1 Coconut Husk

The cross-section of a coconut fruit is shown in Figure 2.2. The coconut husk envelops the hard shell of the coconut fruit with 5 to 10 cm thick fibrous. The external appearance of the husk varies from bright green when immature to dull brown when fully ripe. The husk is full of long, course fibers which running in one direction. The kernel (copra, coconut water and shell) and the husk comprise around 65% and 35% of the total weight, respectively. The dried husk of coconut fruit would be in the range of 200 to 400 g (Foale and Harries, 2011). Annual world production of approximately 54 million tonnes of coconuts yields more than 16 million tonnes of husk of which only a small part is exploited (van Dam et al., 2004).

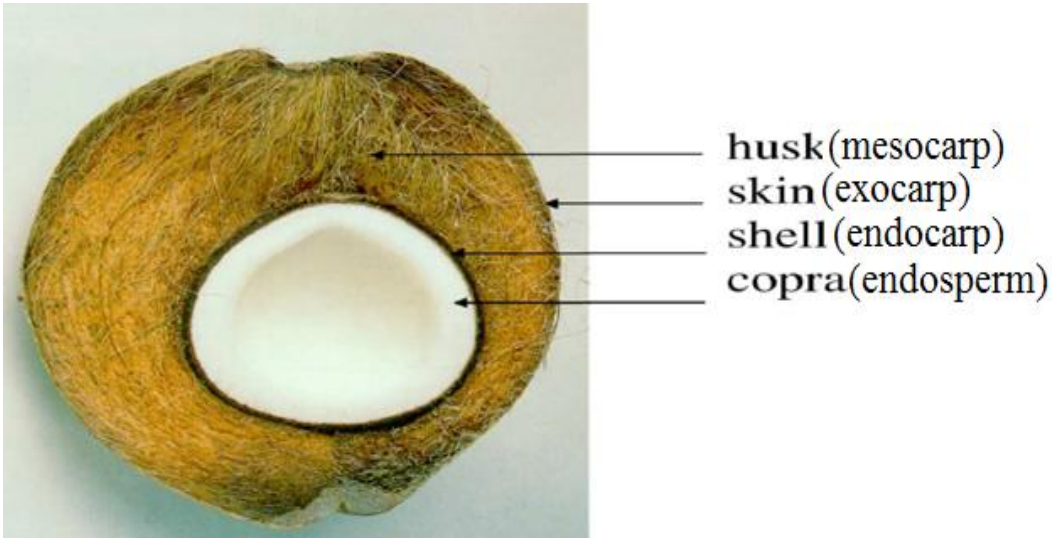


Figure 2.2: Cross-section of the fruit of *Cocos nucifera* L.

According to Pollard et al. (1992), coconut husk (Figure 2.3) consisted of well-defined polymeric structures of cellulose (28%), hemicellulose (38%) and lignin (32.8%). The husk have been used as a precursor to produce high strength-high density board materials (van Dam et al., 2004) and coconut husk-based activated carbon for impurities and dye removal from aqueous solution (Hasany and Ahmad, 2006; Hameed et al., 2008). Facing the trend of green technologies development, the available sugars locked inside coconut husk could be subsequently converted to different valuable products such as environmental friendly bioethanol.

Figure 2.3: Coconut husk.

2.4 Compositions of Lignocellulosic Materials

Lignocellulosic materials contain a complex mixture of carbohydrate polymers (cellulose, hemicellulose and lignin) from the plant cell wall, as shown in Figure 2.3. It may include wood agricultural crops, like cotton woods and kenaf, forestry wastes, i.e. chips and sawdust from lumber mills or dead trees, agricultural residues such as bagasse and stalks or husk of corn plants, and other plant substances. Table 2.3 shows the approximate compositions of various biomass feedstock. Instead of burning these lignocellulosic wastes, the best alternative solution is to utilise it for bioethanol production. The lignocellulosic biomass generally consists of more than 30% of cellulose, which could be broken down to its glucose monomer units by enzymatic hydrolysis. The ethanol fermenting microorganisms can utilise this glucose and convert it into ethanol

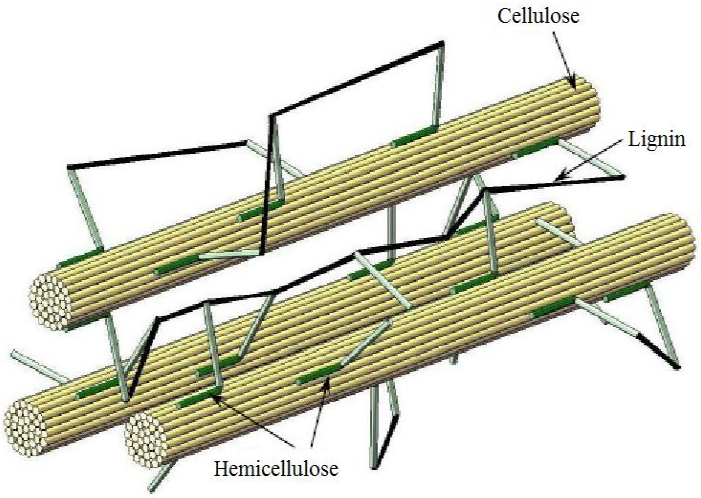


Figure 2.4: A schematic diagram of plant cell wall showing cellulose fibrils laminated with hemicellulose and lignin polymers (Source: Murphy and McCarthy, 2005).

Table 2.3: Comparison of lignocellulose in several sources on dry basis.

Lignocellulosic materials	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Hardwoods stems	40 - 55	24 - 40	18 - 25
Softwood stems	45 - 50	25 - 35	25 - 35
Coconut husk	28	38	32.8
Nut shells	25 - 30	25 - 30	30 - 40
Corn cobs	45	35	15
Oat hulls	30	34	13.2
Grasses	25 - 40	35 - 50	10 - 30
Rice hulls	30	20	21.4
Pine	50	15 - 25	15 - 30
Paper	85 - 99	0	0 - 15
Wheat straw	30	50	15
Coastal Bermuda grass	25	35.7	6.4
Switchgrass	45	31.4	12

(Source: Pollard et al., 1992; Sun and Cheng, 2002; Baltz et al., 2010)

2.4.1 Cellulose

Cellulose is the most common form of carbon in lignocellulosic materials, accounting for 15 - 55% by weight of the biomass (Waites et al., 2001). It is a linear homopolymer of β-1,4-linked glucose units (Figure 2.5). These linear chains of microfibrils are packed by hydrogen bonds and van der Waals forces to

produce crystalline structures (Taherzadeh and Karimi, 2008). These fibrils are attached to each other by hemicelluloses, amorphous polymers of different sugars as well as other polymers such as pectin, and covered by lignin. This high degree of aggregation has produced a compact fiber structure that even small molecules such as water cannot penetrate these highly ordered cellulose chains (Arantes and Saddler, 2010).

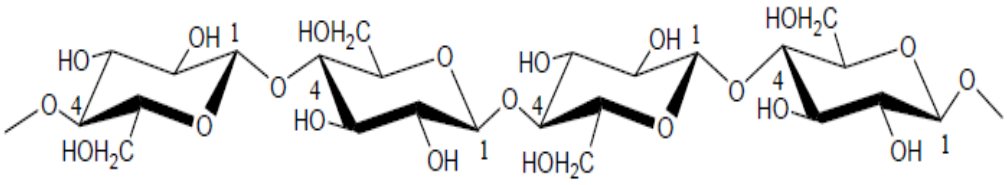


Figure 2.5: The structure of cellulose (Source: Perez and Samain, 2010).

2.4.2 Hemicellulose

Hemicellulose (Figure 2.6) is a major source of carbon in biomass, at levels of between 25 to 35% by weight (Waites et al., 2001). It is a complex polysaccharide which mostly composed of pentoses and hexoses i.e. D-xylose, L-Arabinose, D-galactose, D-mannose and D-glucose. The chains of hemicellulose usually bind with pectin to cellulose to form a network of cross-linked fibres. In contrast to cellulose, which is crystalline and strong, hemicellulose has a random, amorphous, and branced structure. Hemicelluloses are relatively easier to be hydrolyzed by

acids to their monomer components (Jacobsen and Wyman, 2000; Taherzadeh and Karimi, 2008).

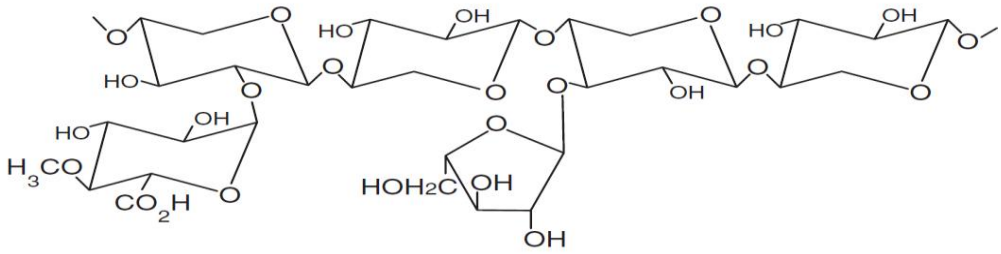


Figure 2.6: The structure of hemicellulose (Source: Chiaramonti, 2007).

2.4.3 Lignin

Lignin is a complex polymer present in the cellular wall which provides structural integrity and structural rigidity in plants. It makes up to 10-35% by weight of the biomass. The model for macromolecular structure of lignin is not completely known but is a polymer of three phenolic alcohols (Figure 2.7) differing in their degree of methoxylation (ρ-coumaryl, sinapyl and coniferyl alcohols) that encrusts the cellulose (Waites et al., 2001). Hardwood lignin is mainly composed of coniferyl and sinapyl alcohol while the softwood lignins are rich in coniferyl alcohol (90%) and the ρ-coumaryl alcohol is typical of lignin in grasses and bamboos (Boerjan et al., 2003). The close association of lignin with cellulose microfibrils makes the biomass more resistance to enzymatic degradation by

limiting the enzyme accessibility. Besides, the presence of lignin also provides higher resistance for the biomass to chemical degradation or pretreatments.

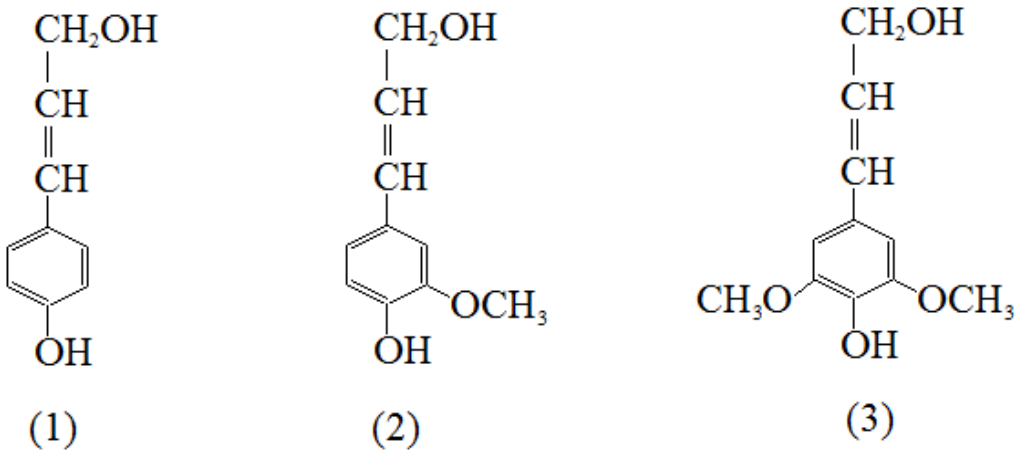


Figure 2.7: ρ-coumaryl (1), coniferyl (2) and sinapyl (3) alcohols: dominant building blocks of the three dimensional lignin (Souce: Boerjan et al., 2003).

2.5 Lignocelluloses Bioconversion Technology

The bioconversion of lignocellulosic materials to ethanol consists of three major stages i.e. pretreatment, enzymatic hydrolysis and fermentation in which the latter two can be integrated to be the simultaneous saccharification and fermentation process (SSF).

One of the technical problems arise is the inability of yeast to directly ferment cellulose of lignocellulosic materials into ethanol (Taherzadeh and Karimi, 2008).

The carbohydrate polymers (cellulose and hemicellulose) of lignocellulosic materials are tightly bound to the lignin, by hydrogen and covalent bonds. These carbohydrate fractions are the fermentable sugars that are less easily accessible. Hence, the conversion of lignocellulosic biomass, for example coconut husk, into bioethanol usually begins with a pretreatment stage to alter the physical structure of the fibres for the ease of subsequent hydrolysis step. Then, the fermentable sugars resulting from the hydrolysis of these fractions can be used as carbon source for bioethanol production by selected microorganisms (Mussatto et al., 2008).

2.5.1 Pretreatment Process

In theory, carbohydrates can be converted to simple sugars with 100% efficiency by enzymatic reactions (Isaacs, 1984). However, the yields of sugars will be highly depending on cellulose accessibility and crystalinity of the structure during application (Sun and Cheng, 2002). Surface area available for enzyme-substrate interaction is influenced by pore size and the surrounding lignin. The crystalline structure will also make the cellulose and hemicellulose less accessible for enzyme molecules (Sun and Cheng, 2002; Taherzadeh and Karimi, 2008). Hence, pretreatment step is necessary in improving the cellulose and hemicelluloses hydrolysis efficiency for bioethanol production.

Pretreatment process is the first phase of bioethanol production that involves delignification of lignocellulosic feedstock to liberate carbohydrate polymers (cellulose and hemicellulose) from lignin (Champagne, 2006). The goal of pretreatment process is to alter or remove structural and compositional impediments in lignocelluloses, in order to improve the rate of subsequent enzyme hydrolysis process and thus increase yields of fermentable sugars from cellulose and hemicelluloses (Mosier et al., 2005).

During the pretreatment, lignocellulose matrix is broken down and released its three main components that are cellulose, hemicellulose and lignin (Figure 2.8). Depending on the pretreatment method, hemicellulose is partially hydrolyzed into pentoses (Gutierrez et al., 2009). At the same time, through the pretreatment, crystallinity degree of cellulose can be decreased and porosity of lignocellulosic structure will be increased to make the lignocellulosic feedstocks more susceptible to enzymatic hydrolysis (Mooney et al., 1999).

Pretreatment methods are inclusive of physical (comminution), chemical (acid or alkaline treatment) or a combination of both methods (thermal treatment and microwave-assisted-alkaline treatment) (Mood et al., 2013) (Table 2.4).

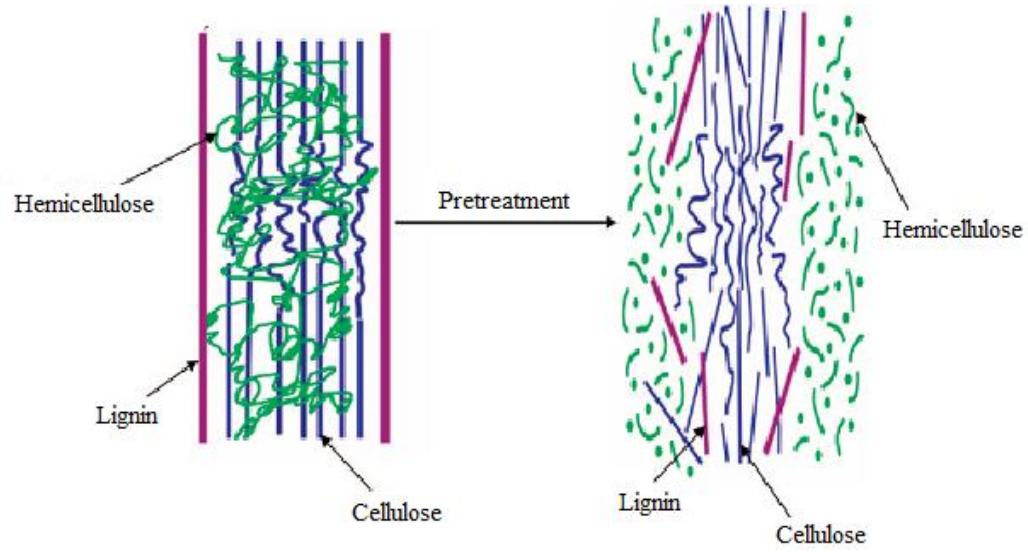


Figure 2.8: Schematic presentation of effects of pretreatment on lignocellulosic biomass (Source: Kumar et al., 2009).

Table 2.4: The common pretreatments and their possible effects.

Pretreatment	Main Objectives
Physical pretreatment (comminution)	• Reduce particle size, crystallinity of lignocellulosic biomass and degree of polymerization
	• Increase the specific surface
Acid pretreatment	• Solubilize the hemicellulose fraction of the biomass
	• Cellulose more accessible to enzyme
Alkaline pretreatment	• Cause swelling, leading to an increase in internal surface area
	• Delignification
Thermal pretreatment	• Chemically – hydrolysis of acetyl groups in hemicellulose
	• Mechanically – separation of fibers due to explosive decompression
Microwave-assisted-alkaline pretreatment	• Accelerates destruction of crystalline structure
	• Improve the effect of alkaline pretreatment (delignification)

(Source: Lloyd and Wyman, 2006; Mosier et al., 2005; Taherzadeh and Karimi, 2008; Alvira et al., 2010; Talebnia et al., 2010)

Physical Pretreatment

The most commonly applied physical pretreatment is the comminution process. The objective of comminution is to reduce the particle size of lignocellulosic materials and hence to cause a reduction of crystallinity of lignocelluloses in order to increase the specific surface area for enhancing enzyme accessibility to cellulose during hydrolysis step. This can be achieved by a combination of chipping, grinding or milling depending on the final required particle size of the material (Sun and Cheng, 2002). Theoretically, comminution causes an increased in hydrolysis rate and bioethanol yield by increasing the accessible surface area for cellulase enzymes. As no production of inhibitors like furfural is produced, comminution is a suitable pretreatment for bioethanol production. However, taking into account the high energy requirements of comminution process and the continuous rise of the energy prices, it is likely that comminution is still not economically feasible (Hendriks and Zeeman, 2009).

Chemical Pretreatment

The most common chemical pretreatment techniques used in bioethanol conversion from lignocellulosic biomass are acid and alkaline pretreatment. Sulphuric acid or hydrochloric acid and sodium hydroxide are the most commonly used acid and base in the chemical pretreatment (Mosier et al., 2005). These methods are very effective in reducing cellulose crystallinity and disrupting

the association of lignin with cellulose, as well as dissolving the hemicellulose (Sun et al., 2000; Mosier et al., 2005).

Concentrated acids have been used to treat lignocellulosic materials. However, they are too toxic and corrosive to the feedstock, hazardous and require reactors that are resistant to corrosion (Sun and Cheng, 2002). In contrast to the concentrated acid pretreatment, diluted acid pretreatment offers milder effect on lignocelluloses structures and simpler acid recovery process. The diluted acid pretreatment increases hemicellulose solubilisation rate and this enhances the digestibility of cellulose in the later hydrolysis stage (Lloyd and Wyman, 2006). Pretreatment of substrate can be performed either at lower temperature (e.g. 120^{o}C) for longer retention time (30-90 minutes) or high temperature (e.g. 180^{o}C) during a short period of time (Hendriks and Zeeman, 2009; Alvira et al., 2010). According to Saha et al. (2006), by applying acid pretreatment at high temperature, some sugar degradation compounds such as furfural and HMF and aromatic lignin degradation compounds were detected, and hence the metabolism of microorganisms in the subsequent fermentation process will be affected.

As mentioned previously, the removal of lignin is necessary for cellulose to become readily available for the enzymes, which permit the yeast to convert the glucose from cellulose into bioethanol (Liu and Wyman, 2003). Hence, the purpose of alkaline pretreatment is to increase cellulose digestibility by enhancing

lignin solubilization (Chang and Holtzapple, 2000; Sharma et al., 2002). The overall effect of alkaline pretreatment on lignocellulosic materials is to cause swelling, leading to an increase in internal surface area, a decrease in crystalinity, separation of structural linkage between lignin and carbohydrates, and disruption of the lignin structure. The alkaline pretreatment exhibits minor cellulose and hemicellulose solubilization than acid or thermal processes (Carvalheiro et al., 2008). The mechanism of alkaline treatment is believed to be saponification of intermolecular ester bonds cross-link the xylan hemicellulose and lignin. The removal of this linkage will increase the porosity of lignocellulosic materials (Sun and Cheng, 2002). Alkaline pretreatment processes can be performed at room temperature and time ranging from seconds to days and the commonly used chemicals are sodium hydroxide, potassium hydroxide and calcium hydroxide. After the pretreatment process, the alkali must be neutralized prior to hydrolysis of cellulose for subsequent fermentation process (Taherzadeh and Karimi, 2008; Hendriks and Zeeman, 2009).

Thermal Pretreatment

During steam explosion thermal pretreatment, the lignocellulosic feedstock is subjected to pressurised steam in a vessel for a period of time without addition of chemicals, and then depressurized it. At the elevated temperature, autohydrolysis of acetyl groups present in hemicellulose will promote the formation of acetic acid, which can further catalyse the degradation of lignocelluloses crystallinity

(Alvira et al., 2010). Hence, the thermal pretreatment also can be considered as a physio-chemical pretreatment. Furthermore, the fibres are separated owing to the explosive decompression when the pressure is reduced. In combination with the partial hemicellulose hydrolysis and structure decompression, the lignin is redistributed and to some extent is removed from the lignocellulosic material. Steam explosion pretreatment has been proven for ethanol production from a wide range of raw materials such as poplar (Oliva et al., 2003), olive residues (Cara et al., 2006), corn stover (Varga et al., 2004), and wheat straw (Ballesteros et al., 2006).

Thermal pretreatment offers several attractive advantages which include the potential for significantly lower capital investment, better energy efficiency and less hazard process chemicals and condition. The major drawback of thermal pretreatment is the generation of toxic compounds such as furan derivatives which can lead to extended of lag phase during fermentation process (Tomas-Pejo et al., 2008; Alvira et al., 2010).

Microwave-Assisted-Alkaline Pretreatment

Microwave-assisted-alkaline pretreatment, a combination of physic-chemical treatment technique, has gained much attention due to its efficiency in enhancing enzymatic hydrolysis of lignocelluloses materials in compared with conventional heating-chemical pretreatment process (Hu and Wen, 2008; Ma et al., 2009;

Jackowiak et al., 2011; Singh et al., 2011). Usually, the microwave treatment is done with addition of alkaline solution.

Microwave-assisted-alkaline pretreatment utilises the reaction between microwave and the polar molecules in the solution to create thermal and non-thermal effects on the materials (Fernández et al., 2011). Preliminary study reported that among different alkalines, NaOH gave the highest total reducing yields after the treatment in combination with microwave radiation (Keshwani and Cheng, 2010).

Different from the conventional heating which based on superficial heating, the microwave irradiation uses the electromagnetic field to accelerate the ions movement in chemical solution. Collisions of ions and rapid rotation of dipoles create more volumetric and rapid heat and hence improve the effect of alkaline pretreatment by increasing the yield of reducing sugars during enzymatic hydrolysis process (Hu and Wen, 2008). It is reported that the delignification effect of microwave-assisted-alkaline pretreatment is caused by the saponification of intermolecular ester bonds linkages within then biomass when alkaline solution is added to lignocellulosic biomass (Sun and Cheng, 2002). The removal of such linkages increases the porosity of biomass, leading to an increase in internal surface area for enzymatic action (Iroba et al., 2013).

2.5.2 Saccharification Process

After the macroscopic and microscopic structures of the lignocellulosic feedstock are being disrupted through pretreatment step, hydrolysis of the hemicellulose and cellulose to pentose and hexose can be achieved more rapidly and with greater yields (Mosier et al., 2005). The second phase in bioethanol production from lignocellulosic materials is the depolymerization of the carbohydrate polymers (cellulose and hemicellulose), by using either acid or cellulases enzymes in producing fermentable sugars (Champagne, 2006).

In the acid hydrolysis, the cellulosic substrate is converted to sugars by either diluted acid or concentrated acid. Generally the diluted acid process involves the usage of 1 - 9%v/v of acid and is conducted under high temperature and pressure. The major disadvantage of diluted acid hydrolysis is that the sugar conversion is only 50% and due to the high temperature and pressure during the conversion process, large portion of sugars could be possibly degraded rather than fermented to products (Yoswathana et al., 2010). The concentrated acid hydrolysis usually involves 40-70% acid. The advantage of the concentrated process is its potential for high sugar conversion efficiency while the major drawback is that at the end of the process, it requires the separation of sugars and acid from the mixtures. This process requires techniques such as ion-exchange separation which will eventually increase the cost of overall hydrolysis process (Mishra et al., 2011).

Enzymatic hydrolysis is a key step in the production of bioethanol from lignocellulosic materials. Compare to chemical conversion routes, the use of enzymes for hydrolysis is considered as the most viable strategy to offer advantages such as conversion routes of higher yields, minimal by-product formation, low energy requirements, mild operating conditions, and environmentally friendly processing (Saha, 2000; Wingren et al., 2005). Depending on the enzyme used, celluloses can be hydrolyzed into glucose and hemicelluloses can be hydrolyzed to release xylose, arabinose, galactose, glucose and mannose.

The cellulose-hydrolyzing enzymes are often applied to pretreated lignocellulosic-based materials. These enzymes consist of three major components, i.e., (i) endoglucanases which break down the non-covalent interactions within the crystalline structure of cellulose; (ii) exoglucanases which hydrolyse the cellulose microfibrils by attacking the chain ends and produce disaccharides cellobiose; (iii) β–glucosidases which hydrolyse the disaccharides of cellulose and release the glucose monomers (Ferreira et al., 2009; Yeh et al., 2010; Harun and Danquah, 2011).

2.5.3 Fermentation Process

The third phase in lignocelluloses bioconversion process is the fermentation of mixed hexose and pentose to produce bioethanol (Champagne, 2006). The microorganisms of primary interest in fermentation of ethanol include *Saccharomyces cerevisiae* (ferment mostly hexoses), *Pichia stipites* (ferment xylose), *Schwanniomyces alluvius* (hydrolyse starch), and *Kluyueromyces* yeast species (ferment lactose) (Waites et al., 2001).

Generally, yeasts are able to grow and efficiently produce ethanol at pH values of 4.0 to 6.0 and temperatures of 28 to 35^{o}C. Under anaerobic conditions, yeast metabolizes glucose to ethanol primarily by the Embden-Meyerhof pathway. The overall net reaction involves the production of 2 moles of each ethanol, carbon dioxide and ATP per mol of glucose fermented (Equation 2.1). On a weight basis, each gram of glucose can give rise to 0.51 g of alcohol and 0.49 g of CO_2 (Equation 2.1) (Kosavic and Vardar-Sukan, 2001).

$$C_6H_{12}O_6 \rightarrow 2\,C_2H_5OH + 2CO_2 + ATP \qquad \text{(Equation 2.1)}$$

Yeasts are highly susceptible to ethanol inhibition. Ethanol concentration of 1 to 2% (w/v) is sufficient to retard microbial growth and at 10% (w/v) alcohol, the growth of the organism is nearly halted (Kosavic and Vardar-Sukan, 2001;

Osunkoya and Okwudinka, 2011). The advantages of *S.cerevisiae* over other yeast strains is that it has higher efficiency in ethanol production, utilising a variety of hexoses and a higher ethanol tolerant compared to other yeast strains (Claassen et al., 1999).

The most widely used bioethanol production approaches include separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF). In SHF process, enzymatic hydrolysis of polysaccharides of the lignocellulosic feedstock is performed separately from the fermentation process. The advantage of SHF is that each step in this process can be carried out under optimal conditions. While the drawbacks of this method is the inhibition of enzymes by the substrates during hydrolysis stage, which calls for lower substrate loading and higher enzyme loading to achieve reasonable yields (Balat, 2011).

In SSF, the enzymatic hydrolysis and fermentation are carried out simultaneously in a single reactor. In this case, the fermenting microorganisms are able to consume the sugars once it is released through saccharification process. Thus, this process has an enhanced rate of hydrolysis by suppressing substrate inhibition effect. Other advantages of combining the saccharification and fermentation processes are lower enzyme loading requirement, higher bioethanol yields and reduced risks of contamination (Ferreira et al., 2010). The main disadvantage of SSF is the need to meet favorable conditions such as temperature and pH, for both

the enzymatic hydrolysis and fermentation processes (Krishna et al., 2001; Ohgren et al., 2007).

2.6 Batch Production of Bioethanol

The growth of microorganisms in liquid media can be carried out under different operating conditions, i.e. batch, fed-batch or continuous mode. The batch-mode growth involves a closed system in which cells are grown in a fixed volume of nutrient culture medium under specific environmental conditions i.e. temperature, pressure, aeration, nutrition type, pH, etc. In fed batch system, fresh medium is fed continuously or intermittently and the volume of the culture medium increases with time. In continuous culture system, fresh medium is continuously supplied to the fermentation vessel, while the products and cells inside the fermentation vessel are simultaneously withdrawn (Srivastava, 2008).

In the current study, simultaneous saccharification and fermentation (SSaF) were carried out under batch mode system. The typical growth pattern of a batch culture can be diagrammatically shown in Figure 2.9. During the batch fermentation, populations of microorganisms go through four distinct phases of growth, i.e. lag phase, exponential phase, stationary phase and death phase. Lag phase is a period of intense metabolic activity during which the cells adapt to their

new environment. There is no net increase in the cell numbers in this phase. The end of lag phase is rapidly followed by exponential phase (log phase), during which the logarithm of viable cells is a constant function of time. After some time of exponential phase, limitations to cell growth will occur by accumulation of intracellular toxins or depletion of nutrients and these causes the cells to decrease growth and enter stationary phase. Following the stationary phase, cell death begins to exceed the production of new viable cells and eventually, death phase occurs (Caldwell, 1999; Lin et al., 2000; Srivastava, 2008).

During batch fermentation, maximum ethanol production rate occurs for a brief period in this process and decline as ethanol accumulates in the fermentation broth. Millar (1982) reported that concentration of ethanol above 12%v/v can denature glycolytic enzymes of yeast cells and lead to inhibition of cellular activity. In a comprehensive study, Dombek and Ingram (1987) demonstrated that minimal inhibitory ethanol concentration for fermentative activity of *Saccharomyces cerevisiae* KD2 were 6.5% and 9% for 12h and 24h cells, respectively. According to Carlsen et al. (1991), 8-10%v/v ethanol had reduced the fermentative activity of *Saccharomyces cerevisiae* by 50%.

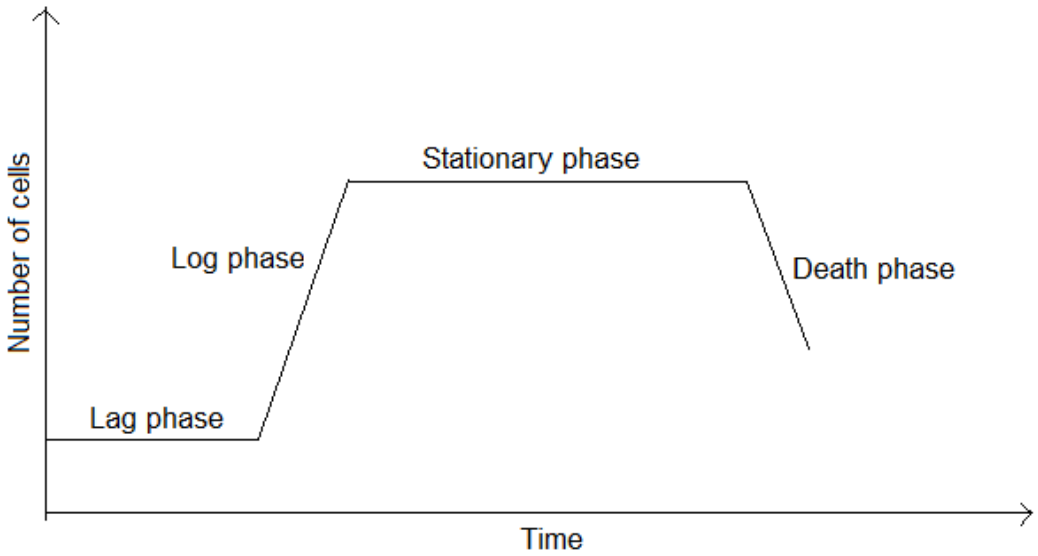


Figure 2.9: General nature of batch culture.

2.7 Factors Affecting Bioethanol Fermentation by Yeast

Yeast cells belong to the eukaryotes which are classified as Fungi. The species *Saccharomyces cerevisiae* are currently being widely used to increase the yield of bioethanol production from sugars (Liu and Shen, 2008; Lee et al., 2012; Moon et al., 2012). As living organism, yeast requires water and sugar, as well as an adequate climate to survive, and with nutritious environment as necessary additions in order for yeast to thrive. In order to survive and produce bioethanol, yeast cells have to adjust to a diversity of environmental factors. The main environmental factors that affect yeast fermentation are discussed below.

2.7.1 Temperature

Like other microorganisms, *S. cerevisiae* tend to have temperature range at which their growth is optimized because the enzyme activity is depending on the temperature of environment (Goddard, 2008). According to Black (1996), generally, the growth of microorganisms gradually increases from the minimum to the optimum temperature and decreases very sharply from the optimum at the maximum temperature. For most of the microorganisms, both extremely high and low temperatures can be very harmful; the former can cause protein denaturation while the latter can lead to intracellular ice crystal formation upon freezing. For *S. cerevisiae*, the temperature close to 35^{o}C is the optimal temperature for the working of the intracellular enzymes in order to catalyse the reactions in metabolic pathway (Narendranath and Power, 2005).

2.7.2 pH

The extent of acidity or alkalinity, referred to as the pH of a solution, also affects yeast cell growth and metabolism. *S. cerevisiae* can grow at varying pH values but it works best at slightly acidic pH (pH 4.0 – pH 6.0) and high pH levels may cause denaturation of enzymes which aid in fermentation capability of yeast cells (Mountney and Gould, 1988; Narendranath and Power, 2005).

2.7.3 Carbon Source

Yeasts are chemoorganotrophs as they consume organic compounds as source energy. The carbon source has a dual role in biosynthesis and energy generation for yeast fermentation process. For the budding yeast *S.cerevisiae*, glucose is the preferred carbon source for its metabolism and growth. Glycolysis is the general pathway for conversion of glucose to pyruvate. It is the sequence of reactions that metabolize one molecule of glucose to two molecules of pyruvate with the concomitant net production of two molecules of ATP. The conversion of glucose to final product of ethanol involves two major processes, which are pyruvate synthesis and alcoholic fermentation (Kuchel and Ralston, 1997).

First, under aerobic conditions, the catabolism of glucose (6-carbon molecule) begins with glycolysis to convert sugar into pyruvate (3-carbon molecule). After the pyruvate is being produced, alcoholic fermentation will be taken place under anaerobic condition by yeast cells. The first step in this alcoholic fermentation is the decarboxylation of pyruvate to acetaldehyde and carbon dioxide. This reaction is catalysed by pyruvate decarboxylase, which requires the coenzyme thiamine pyrophosphate. The second step is the reduction of acetaldehyde to ethanol by NADH, in a reaction catalyzed by alcohol dehydrogenase. This process regenerates one molecule of NAD^+ (Montgomery et al., 1996; Berg et al., 2001).

The conversion of glucose into ethanol in this anaerobic alcoholic fermentation process is shown in Equation 2.2:

$$\text{Glucose} + 2\text{Pi} + 2\ \text{ADP} + 2\ H^{+} \rightarrow 2\ \text{ethanol} + 2\ CO_2 + 2\ \text{ATP} + 2\ H_2O$$

(Equation 2.2)

2.7.4 Nitrogen Source

Another element that plays an important role in the adaptation of yeast to the environment and the course of fermentation is the nitrogen source. Nitrogen source generally serves anabolic roles in the biosynthesis of structural proteins, amino acids and nucleic acids. Appropriate amount of organic nitrogen source such as yeast extract and peptone can help to support rapid growth and high cell yield (Costa et al., 2002). According to Cruz et al. (2002), supplementation of nitrogen source in peptide form (peptone) was very efficient for yeast metabolism, inducing higher production of biomass and ethanol production as well as preserving yeast viability.

2.8 Concluding Remarks

In recent years, bioethanol has been considered a better choice than conventional fuels, as it reduces the dependence on crude oil reservoirs. Lignocellulosic materials, the highly abundant plant biomass resource on earth, can be an excellent substrate in bioethanol production industry (Naik et al., 2010). However, many factors such as lignin content, crystallinity of cellulose, and particle size limit the digestibility of cellulose present in the lignocellulosic materials. A suitable pretreatment technique is extremely important in increasing the exposure of cellulose and thus improving the enzymatic hydrolysis efficiency. The abundantly growing coconut husk which consists of considerable amount of cellulose (28%) has made it a cheap and potential substrate for bioethanol production (Pollard et al., 1992).

Follow the determination of the best pretreatment technique, the fermentation process which can be influenced by various of factors, i.e., temperature, carbon source, pH of medium, nitrogen source, enzyme loading size and etc), has to be optimized in order to maximize the production of bioethanol from the feedstock.

CHAPTER 3

GENERAL MATERIALS AND METHODS

3.1 Chemical Reagents

All chemical reagents used in experiments were with analytical grade. Two commercial enzymes, which were pectinase (Pectinex Ultra SP) and cellulase (Celluclast 1.5L) were purchased from Novozyme (M) Sdn. Bhd., Malaysia. Yeast extract, soy peptone and dextrose were all obtained from Prodadisa, Spain. Phenol (detached crystals) was purchased from Fisher Scientific (United Kingdom), sodium hydroxide pellets (NaOH) and sodium sulphite (Anhydrous) from R & M Chemicals (United Kingdom), 3,5-dinitrosalicylic acid from SIGMA (USA) and potassium sodium tartrate from SYSTERM® (Malaysia) and acetone from RCI Labscan (Thailand).

Chemicals used for characterisation of coconut husk were Neutral Detergent Fibre (NDF), Acid Detergent Fibre (ADF) and Acid Detergent Lignin (ADL) solutions. Chemical reagents required for NDF preparation were sodium laurel sulphate and etoxy ethanol from R & M Chemicals (United Kingdom), disodium dihydrogen ethylenediamine tetracetate from J. T. Baker Chemical (America), sodium borate

decahydrate and disodium hydrogen phosphate from SYSTERM® (Malaysia), decalin from SIGMA (USA) and sodium sulphate from Fisher Scientific (United Kingdom). Chemical reagents required for ADF preparation are cetyl trimethylammonium bromide from R & M Chemicals (United Kingdom) and sulphuric acid from Fisher Scientific (United Kingdom). Formulation for the preparation of NDF and ADF solutions are shown in Table 3.1 and Table 3.2, respectively. Sulphuric acid from Fisher Scientific (United Kingdom) was used in ADL determination.

Table 3.1: Formulation of NDF solution.

Chemical	**Amount**
Sodium laurel sulphate	30 g
Disodium dihydrogen ethylenediamine tetracetate	18.61 g
Sodium borate decahydrate	6.81 g
Etoxy ethanol	10 mL
Disodium hydrogen phosphate	4.56 g

*Dissolved all the chemicals above in 1 L distilled water and the solution was adjusted to pH7.0.

Table 3.2: Formulation of ADF solution.

Chemical	**Amount**
Cetyl trimethylammonium bromide	20 g
0.5 M Sulphuric acid	1.0 L g

3.2 Microorganisms and Maintenance

The *Saccharomyces cerevisiae* (ATCC 36858) strain was purchased from BioSynTech Sdn. Bhd., Malaysia. The strain was cultured in yeast extract-peptone-dextrose (YPD) broth at 30^{o}C for 24 h followed by storage in 30% glycerol at -70^{o}C for further use as mother culture.

3.3 Inoculums Preparation

Yeast-Peptone-Dextrose (YPD) agar containing (g/L): yeast extract, 10; peptone, 20; dextrose, 20; and agar powder, 10 was prepared. The media were autoclaved at 121^{o}C for 15 min prior to solidification process.

In the preparation of inoculums, the YPD agar was inoculated with stock culture of *Saccharomyces cerevisiae*. The inoculated agar plates were sealed and kept at room temperature for 24 hours. Following that, *Saccharomyces cerevisiae* inoculum was prepared by inoculating 2 to 3 single colonies of *Saccharomyces cerevisiae* from YPD agar into a broth medium containing (g/L): yeast extract, 10; peptone, 20; and dextrose, 20. The broth was agitated at 150 rpm for 24 h at 30^{o}C using a rotating shaker. The cell pellet was harvested by centrifugation at 3000 rpm for 5 min. Then, the cell pellet was re-suspended in sterilized distilled water

and centrifuged again to remove excess dextrose residues before transferred to fermentation medium.

3.4 Analytical Procedures

All hydrolysis and fermentation processes of the pretreated coconut husk were conducted in triplicate. In both processes, 5 mL of sample was taken at predetermined time intervals for analysis. In the stage of determining the most suitable pretreatment method, the supernatant collected from hydrolysis process was used for analysis of reducing sugar concentration.

During the fermentation processes, the supernatant was used for the analysis of reducing sugar and ethanol concentration. The cell pellet was used for the determination of dry cell weight.

3.4.1 Determination of Reducing Sugar Concentration

The soluble reducing sugar resulted from hydrolysis step was measured by using 3,5-dinitrosalicyclic acid (DNS) method (Miller, 1959). The DNS solution was prepared using (g/L): dinitrosalicyclic acid, 10; phenol, 2; sodium sulfide, 0.5;

sodium hydroxide, 10; and sodium potassium tartrate, 182. One mL of DNS reagent and 2 drops of 1 N NaOH were added to 1 mL of diluted sample in a test tube. This mixture was then placed in 100^{o}C water bath and boiled for exactly 5 min. The mixture was then immediately cooled under running tap water. Ten mL of distilled water was then added into the solution. The absorbance of the suspension was read at 540 nm after 20 min. Concentration of reducing sugar measured as glucose was determined by constructing glucose standard curve from 0.2 to 1.0 g/L (Figure 3.1), The plot shows high persistency of plotting with the satisfactory determination coefficient (R^2) values.

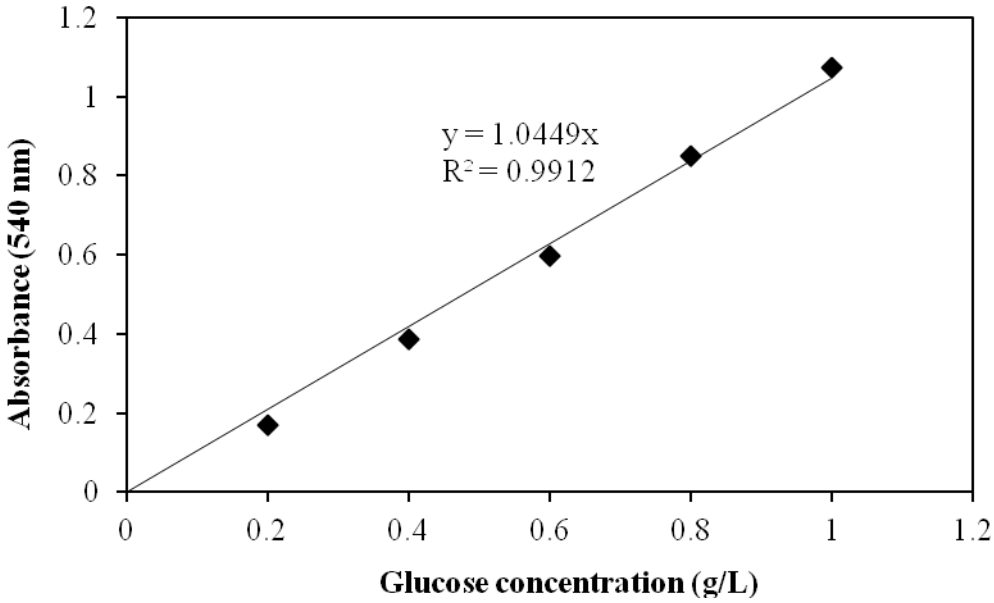


Figure 3.1: Glucose standard curve.

3.4.2 Determination of Ethanol Concentration

At predetermined time interval, 2 mL of sample solution was withdrawn and cooled at 4^{o}C ice bath in order to prevent the evaporation of ethanol. In this study, the soluble ethanol concentration was measured by High Performance Liquid Chromatography (HPLC).

The analyses were performed using a Shimadzu LC-20AD system equipped with a degasser, a pump, a column oven and a RID-10A RI detector. The column used was Rezex ROA-Organic Acid H+ (8%) column (300 x 7.8 mm) (Phenomenex, USA) and the guard column was Rezex ROA-Organic Acid H+ (8%) column (50 x 7.8 mm) (Phenomenex, USA). The aqueous mobile phase was 0.005 N sulfuric acid in water. Samples from fermentation broth were centrifuged followed by filtration using a Acrodisc® 25 mm syringe filter with 0.20 µm Supor® membrane (Pall Corporation, USA). Filtered aliquots of 10 µL were injected on HPLC operating at a flow rate of 0.4 mL/min and the HPLC column was heated to 65 °C.

Ethanol standard curve was constructed (Figure 4.2) by using HPLC. The curve shows high persistency of plotting with the satisfactory determination coefficient (R^2) values.

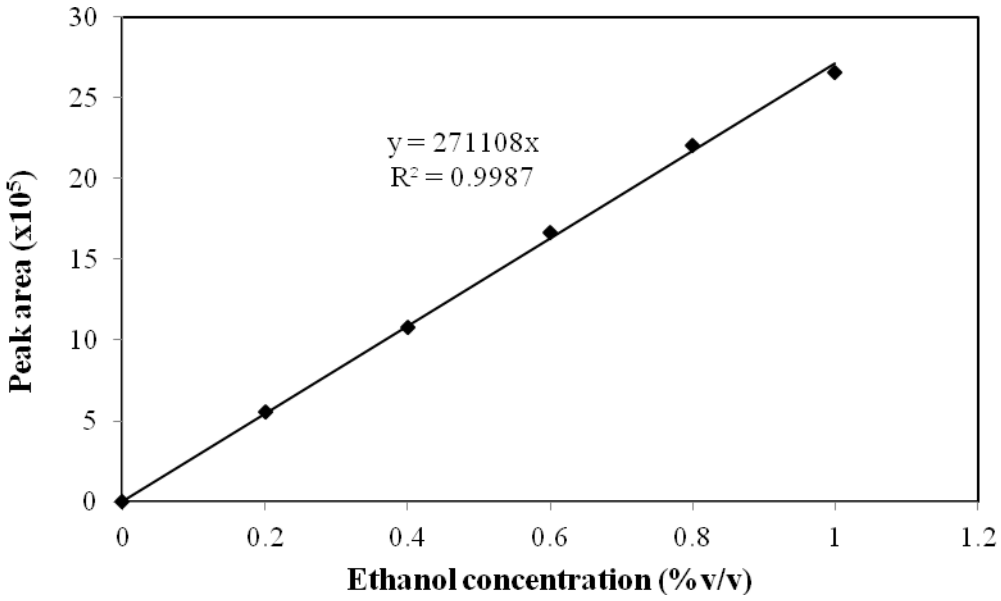


Figure 3.2: Standard curve for ethanol determination.

3.4.3 Determination of Ethanol Productivity

Ethanol yield and ethanol productivity (based on per unit gram of pretreated coconut husk) were calculated based on the Equation 3.1 and 3.2.

$$\text{Ethanol Yield, Y (g ethanol / g coconut husk)} = \frac{\text{Maximum ethanol concentration (g/L)}}{\text{Initial amount of dry pretreated coconut husk (g/L)}}$$

(Equation 3.1)

Ethanol Productivity, P = $\frac{\text{Maximum ethanol yield (g/g)}}{\text{Time taken to achieve maximum yield (day)}}$
(g ethanol / g coconut husk / day)

(Equation 3.2)

3.4.4 Viable Cell Counts

The number of actively growing cells in the sample was identified by colony forming unit (CFU). Serial dilutions were cultured in order to ensure the formation of appropriate number of colonies (30 – 300 colony forming unit (CFU)). The serial dilution of sample was cultivated on YPD agar in a petri dish that is sealed and incubated at 35^{o}C for 24 hours before enumeration. Figure 3.3 illustrates the serial dilution steps involved in CFU determination. The calculation of original number of colony forming units (CFU) is expressed in Equation 3.3.

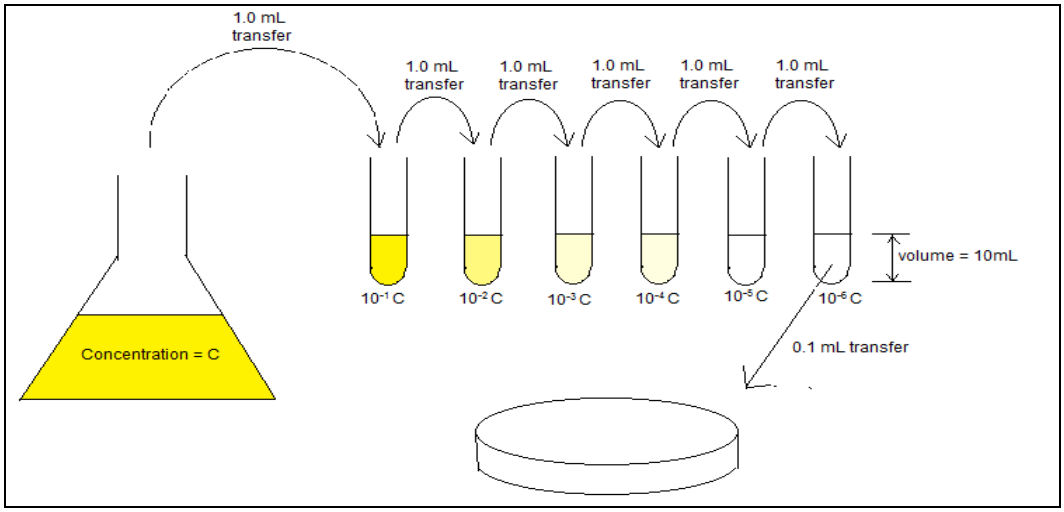


Figure 3.3: Protocol in performing serial dilution.

$$\text{Original cell density (CFU/mL)} = \frac{\text{Colonies per plate (CFU)}}{\text{Volume plated (mL)} * \text{Dilution factor}}$$

(Equation 3.3)

3.5 Experimental Designs of Project Works

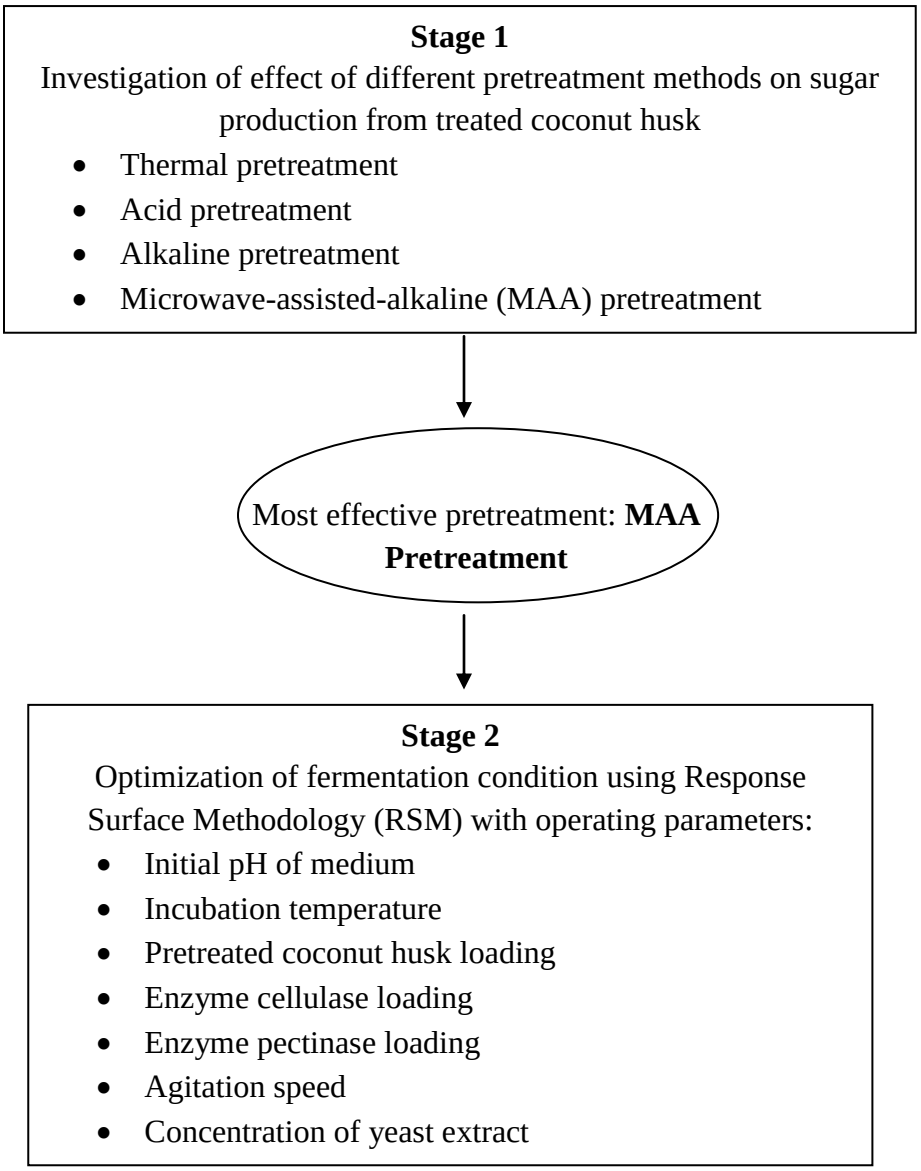


Figure 3.4: Overall process in bioethanol production by using coconut husk as lignocellulosic raw material.

CHAPTER 4

COMPARISON OF PRETREATMENT STRATEGIES ON CONVERSION OF COCONUT HUSK FIBER TO FERMENTABLE SUGARS

4.1 Introduction

Lignocellulosic biomass are promising alternative feedstock for bioethanol production. However, lignocelluloses possess a very rigid structure and very resistant to hydrolysis (Chandel et al., 2008). The recalcitrant nature of lignocellulosic biomass necessitates an efficient pretreatment step to improve the yield of fermentable sugars and maximizing the enzymatic hydrolysis efficiency. Pretreatment processes are required to increase the porosity of cellulose, delignification and minimize other factors than affecting the enzymatic hydrolysis of cellulose in order to produce more reducing sugars during the fermentation of the sugar to ethanol.

In the present study, five pretreatment methods, namely comminution, thermal pretreatment, acid pretreatment, alkaline pretreatment and microwave-assisted-alkaline (MAA) were conducted to evaluate their effects on the structures of

coconut husk. The potential of the proposed pretreatment technique in promoting the release of fermentable sugar from pretreated coconut husk were investigated.

4.2 Materials and Methods

4.2.1 Collection and Processing of Coconut Husk

A commonly available agricultural waste, coconut husk, was collected from a coconut plantation site located in Sitiawan, Perak, Malaysia. The waste was then cut into small pieces (ca. 5 cm) and washed with distilled water to remove dirt or debris.

4.2.2 Pretreatments on Coconut Husk

Physical Pretreatment

Physical pretreatment applied in this study were comminution and thermal pretreatment. The collected coconut husk was consisted of exocarp (outermost hardest layer) and mesocarp (fibrous husk). Prior to communition, the exocarp was removed (Figure 2.2). The remaining coconut husk was then dried in an oven

at 35^{o}C until constant weight. Thereafter, the dried coconut husk was grinded and sieved to two particle size, i.e., moderate size (300 to 600 µm) and large size (850 to 1500 µm). The sieved coconut husk was further washed and cleaned thoroughly to remove dust and dried at 35^{o}C until constant weight.

In thermal pretreatment, the coconut husk particles were packed with aluminum foil and then autoclaved using high pressure steam (15 psi) at 121^{o}C for about 15 min without addition of any chemicals. The heat-treated coconut husk was then dried in an oven at 35^{o}C to remove moisture created by vapor. The dried coconut husk was stored in desiccators filled with silica beads until future use.

Chemical Pretreatment

Sulphuric acid (1% v/v) and sodium hydroxide (5% w/v) were two chemical solutions applied during chemical pretreatment process. A liquid to solid ratio of 50:1 (v/w) was used to ensure that the coconut husk was fully submerged in the chemical solutions, respectively. The mixture was incubated in a incubator shaker at 150 rpm and at 40^{o}C for 24 h. The treated coconut husk was separated from the mixture by filtration and further washed with distilled water until pH was neutral. Subsequently, the cleaned coconut husks were then dried in an oven at 35^{o}C until constant weight and stored in desiccators until further use.

Microwave-Assisted-Alkaline Pretreatment

Microwave-based pretreatment was carried out with a domestic microwave oven (Sharp, R-218(S)) at 2450 MHz. During this pretreatment process, the slurry consisting of 6 g (dry basis) of coconut husk in 5% (w/v) NaOH solution, with a liquid to solid ratio of 50:1 (v/w), was exposed to microwave radiation for 20 min. The temperature of the slurry was measured to be approximately 91^{o}C. Following that, the slurry was removed from the microwave oven and the treated coconut husk particles were rinsed with distilled water to remove excess alkaline solution. The residues were then dried in an oven at 35^{o}C until constant weight and stored in desiccators for subsequent use.

4.2.3 Enzymatic Hydrolysis Process

Following pretreatment process, enzymatic hydrolysis efficiency of pretreated coconut husk was investigated. Enzymatic hydrolysis of treated coconut husks was carried out by using 1% w/v of dried pretreated coconut husk in solution containing sterile distilled water, filter-sterilised pectinase (9500 PGU/ml) and cellulase (700 EGU/g). The hydrolysis process was conducted in 250 mL Erlenmeyer flask at 35^{o}C in a incubator shaker set at 150 rpm. The broth was withdrawn at every 12 h for the analysis of reducing sugar concentration. The

determination of reducing sugar concentration was performed according to the methodology as mentioned in Section 3.4.1.

4.2.4 Characterisation of Pretreated Coconut Husk

Determination of Cellulose, Hemicellulose and Lignin

The content of cellulose, hemicellulose and lignin can be determined by the analysis of Neutral Detergent Fibre (NDF), Acid Detergent Fibre (ADF) and Acid Detergent Lignin (ADL). A NDF solution is used to dissolve the easily digested pectins and plant cell contents (lipid, starch, sugars, protein and organic acid), leaving a fibrous residue of plants (cellulose, hemicellulose and lignin) while the ADF solution is used to dissolve cell solubles, hemicellulose and soluble minerals leaving a residue of cellulose and lignin (Goering and Van Soest, 1970). The cellulose, hemicellulose and lignin content were calculated by Equation 4.1 and 4.2. The formulations for NDF, ADF and ADL solution are shown in Chapter 3.

$$\text{Hemicellulose (\%)} = \text{NDF (\%)} - \text{ADF (\%)} \qquad \text{(Equation 4.1)}$$

$$\text{Cellulose (\%)} = \text{ADF (\%)} - \text{Lignin (\%)} \qquad \text{(Equation 4.2)}$$

Determination of Neutral Detergent Fibre (NDF)

Dried coconut husk (1.0 g) was mixed with 100 mL of NDF solution (at room temperature), 2 mL of decalin and 0.5 g of sodium sulphate. The mixture was heated and left to boil for 60 min. Then, the mixture was filtered through a sintered filter funnel and rinsed with hot water followed by acetone. The residues were then placed in an oven (105^{o}C) for 24 h. The residues were then removed from the oven and weighed. The NDF was calculated by Equation 4.3, where W_1 is the original weight of coconut husk (gram) and W_2 is the weight of residue after filtration (gram).

$$\text{NDF (\%)} = \frac{W_2}{W_1} \times 100 \qquad \text{(Equation 4.3)}$$

Determination of Acid Detergent Fibre (ADF)

Coconut husk (1.0 g) was mixed with 100 mL of ADF solution and the mixture was left to boil for 60 min. Following that, the mixture was filtered and rinsed with hot water and acetone. The residues were then placed in an oven (105^{o}C) for 24 h and weighed thereafter. The ADF was calculated by Equation 4.4, where W_1 is the original weight of coconut husk (gram) and W_{2A} is the weight of residue after filtration (gram).

$$\text{ADF (\%)} = \frac{W_{2A}}{W_1} \times 100 \qquad \text{(Equation 4.4)}$$

Determination of Lignin Content

The residues after filtration from determination of ADF experiment was soaked in 72% sulphuric acid (H_2SO_4) solution for 3 h at room temperature. The residues was then rinsed with water and dried in an oven (105^oC) for 24 h, weighed and placed in a furnace (550^oC) for 3 h. The weight loss is equivalent to the lignin content. The lignin content was calculated by Equation 4.5, where W_{2A} is the weight of samples in ADF determination experiment (gram) and W_3 is the weight of residue after filtration (gram).

$$\text{Lignin (\%)} = \frac{W_3}{W_{2A}} \times 100 \qquad \text{(Equation 4.5)}$$

4.2.5 Scanning Electron Microscopy (SEM) Analysis

The effects of pretreatments upon the coconut husk surface were examined using a SEM microscope (JEOL JSM-6400 SEM) in Universiti Putra Malaysia. The specimen of SEM was cut into a number of 1 cm^2 tissues. The tissue was put into separate vials and fixed in 4% buffered glutaraldehyde for 24 h at 4^oC. The specimen was then washed with 0.1 M sodium cacodylate buffer for 3 charges of 10 min each. The specimen was post-fixed in 1% buffered osmium tetroxide for 2 h at 4^oC. Following that, the washing of specimen with 0.1 M sodium cacodylate was repeated. Then, dehydration was conducted to remove unbound water with a

series of ethanol ranging from 10% to 100%. The specimen after dehydration process was then transferred into specimen basket and was put into critical point dryer for about 45 min. Finally, the samples were gold-coated in a sputter coater and were ready for SEM viewing.

4.2.6 Data analysis

All experiments in this study were conducted out in triplicated and the statistical analysis was done using SAS ® Proprietary Software Release 6.12. One-way analysis of variance (ANOVA) and Duncan's multiple range tests were used for the comparison among pretreatment methods. Mean values in the same column not followed by the same letter are significantly different.

4.3 Results and Discussions

4.3.1 Effect of Different Pretreatment Techniques Coconut Husk on Enzymatic Hydrolysis Efficiency

In the current study, several pretreatments were applied on coconut husk prior to enzymatic hydrolysis. Efficiency of the pretreatment techniques was measured as concentration of reducing sugars produced after enzymatic hydrolysis.

Physical Pretreatment

Figure 4.1 compares the amount of reducing sugar produced by using two different particle size of pretreated coconut husk after enzymatic hydrolysis. After 2 days of incubation, reducing sugar yield by using moderate particle size (300 to 600 µm) of coconut husk (0.584 g/L) was slightly higher than the system with 850 to 1500 µm coconut husk (0.555 g/L), and both systems achieved equilibrium stage thereafter at 72-hour.

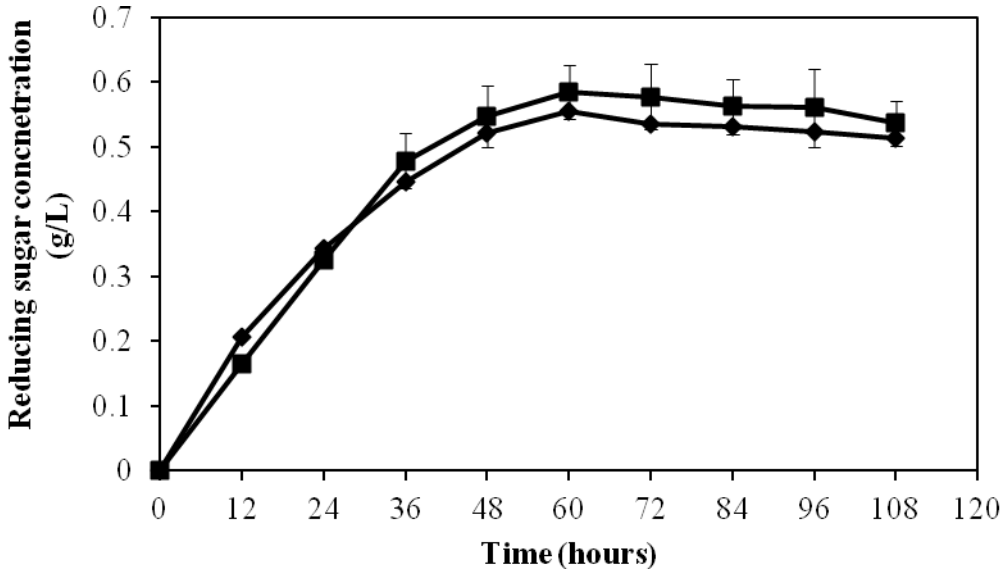


Figure 4.1: Level of reducing sugar released from coconut husk with two different particle sizes after enzymatic hydrolysis process. Symbols: (■), particle size 300 µm to 600 µm; (◆), particle size 850 µm to 1500 mm. Error bars indicate the mean ± standard deviation of three experiments. Data points without error bars indicate the errors were smaller than the size of symbols

Thermal Pretreatment

By using 850 to 1500 µm of thermally pretreated coconut husk, the concentration of reducing sugar increased exponentially from 0 g/L to 0.654 g/L after 48 h of enzymatic hydrolysis. The level of reducing sugar production remained virtually constant thereafter. In contrast, the production of reducing sugar by using smaller particle size of thermally pretreated coconut husk was significant lower (0.372 g/L) after 3 days of enzymatic hydrolysis process (Figure 4.2).

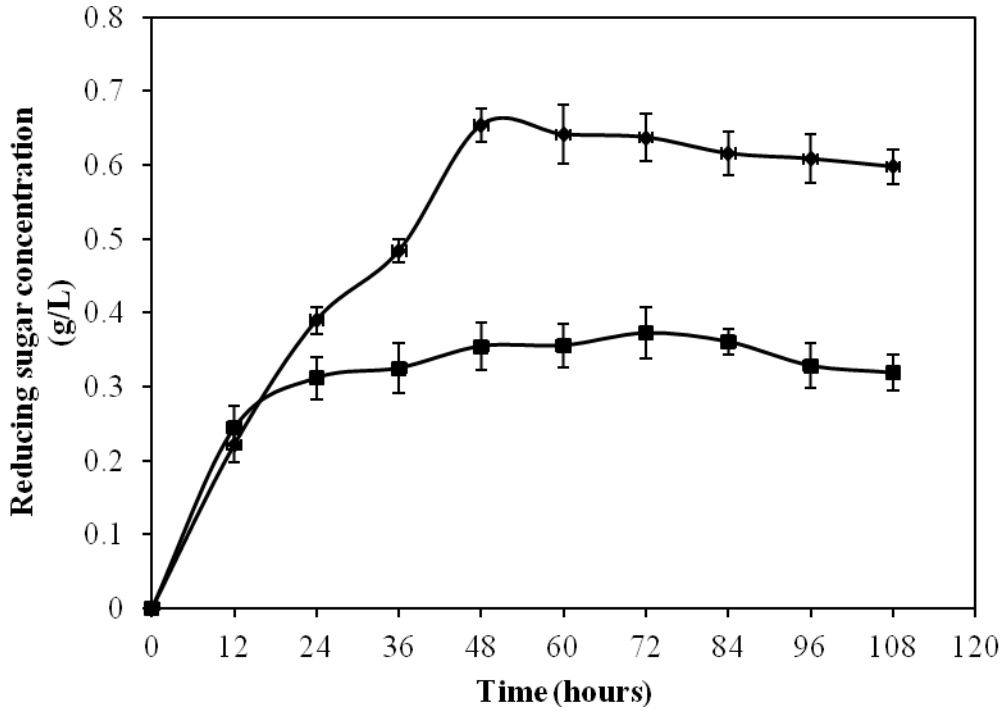


Figure 4.2: Level of reducing sugar produced through hydrolysis of thermally-treated coconut husk. Symbols: (■), particle zise 300 µm to 600 µm; (◆), particle size 850µm to 1500 mm. Error bars indicate the mean ± standard deviation of three experiments. Data points without error bars indicate the errors were smaller than the size of symbols.

Acid Pretreatment

Acid pretreatment was conducted by suspending the coconut husk in sulphuric acid solution (1% v/v) with 50:1 of liquid to solid ratio. Referring to Figure 4.3, after 3 days of enzymatic hydrolysis, significant high level of reducing sugar (0.70 g/L) was produced by using both particle size of acid-pretreated coconut husk.

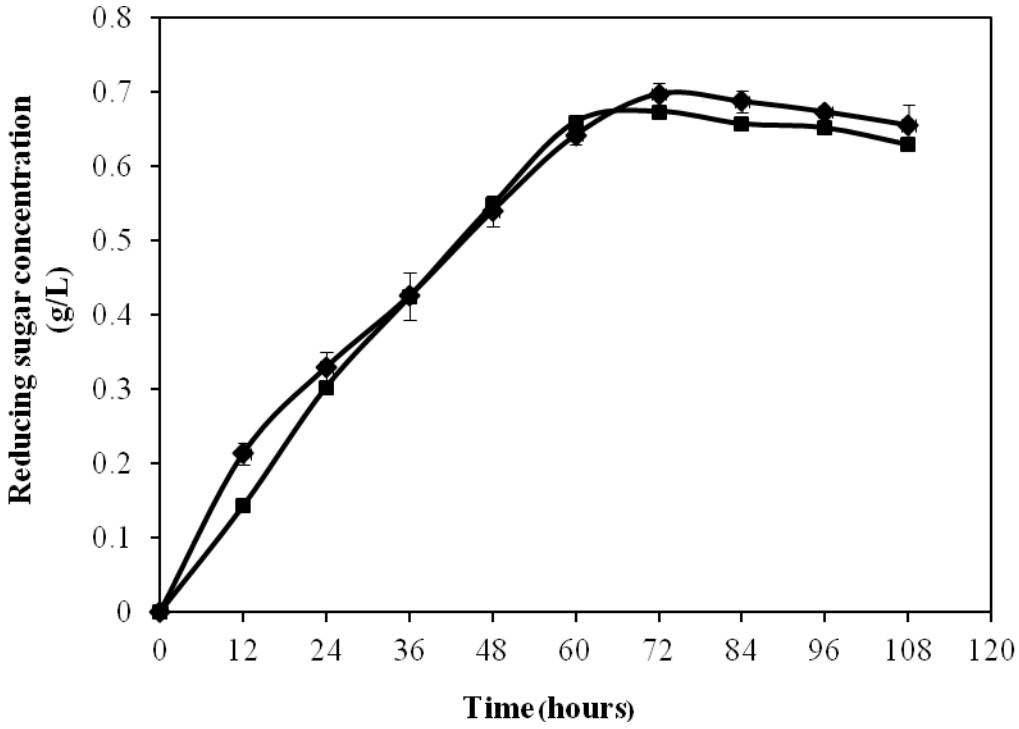


Figure 4.3: Level of reducing sugars using acid pretreated coconut husk. Symbols: (■), particle zise 300 μm to 600 μm; (◆), particle size 850μm - 1500 mm. Error bars indicate the mean ± standard deviation of three experiments. Data points without error bars indicate the errors were smaller than the size of symbols.

Alkaline Pretreatment

Figure 4.4 shows the level of reducing sugar through hydrolysis of alkaline-treated coconut husk. A drastic increment of the sugar level was recorded within 24 h of hydrolysis for both sizes of samples. In addition, system with larger particle size (850 to 1500 µm) coconut husk gave higher concentration of glucose (1.41 g/L) as compared to system with 300 to 600 µm coconut husk (1.17 g/L) after pretreatment using NaOH solution.

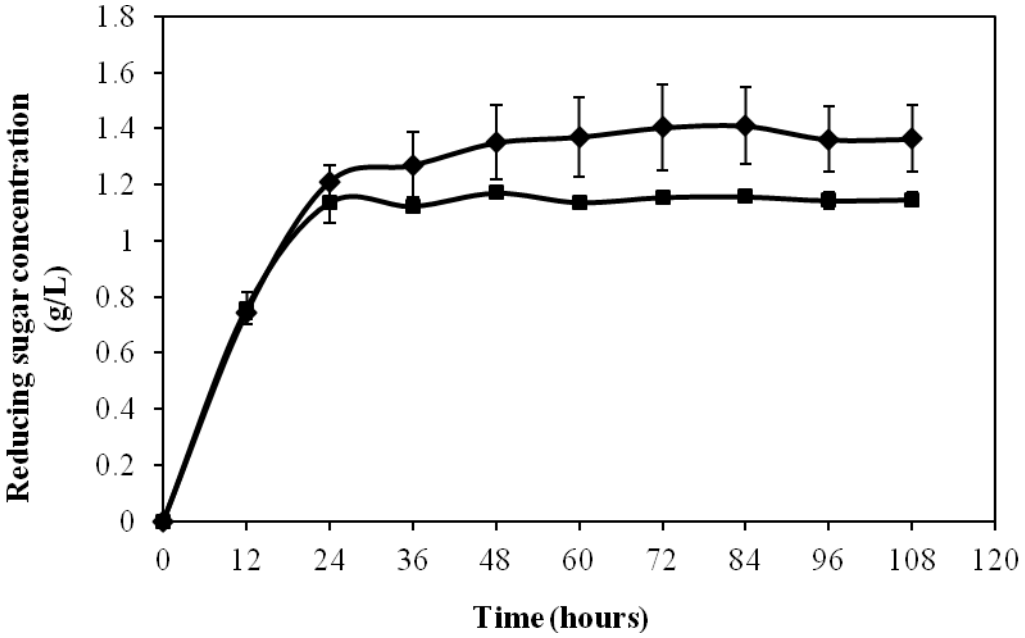


Figure 4.4: Level of reducing sugars produced through hydrolysis of alkaline-treated (5% w/v of NaOH for 24 hours) coconut husk. Symbols: (■), particle zise 300 µm to 600 µm; (◆), particle size 850µm to 1500 mm. Error bars indicate the mean ± standard deviation of three experiments. Data points without error bars indicate the errors were smaller than the size of symbols.

Microwave-Assisted-Alkaline (MAA) Pretreatment

During the microwave-assisted-alkaline pretreatment, coconut husk was pre-soaked in alkaline solution followed by microwave heating pretreatment. As observed from Figure 3.6, coconut husk with particle size of 850 to 1500 μm was found able to release higher level of reducing sugar (2.79 g/L) as compared to coconut husk with particle size of 300 to 600 μm, which produced 2.16 g/L of sugar.

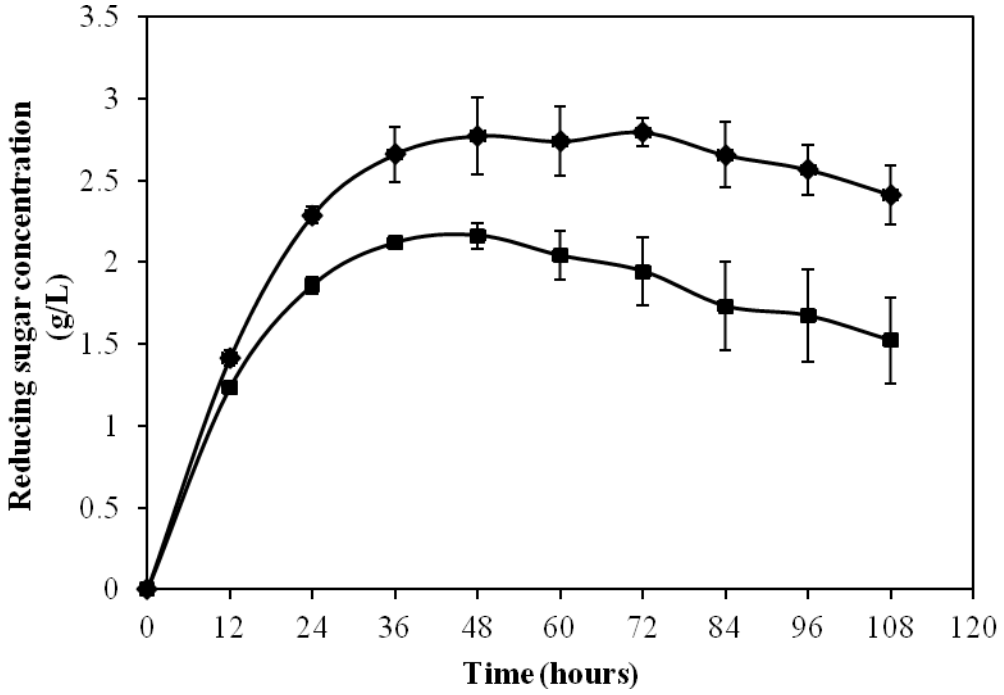


Figure 4.5: Level of reducing sugars produced through hydrolysis of microwave-assisted-alkaline-treated coconut husk. Symbols: (■), particle zise 300 μm to 600 μm; (◆), particle size 850μm to 1500 mm. Error bars indicate the mean ± standard deviation of three experiments. For data points without error bars, the errors were smaller than the size of symbols.

4.3.2 Characterization of Pretreated Coconut Husk

In the current study, NDF solution was used to estimate the total lignocellulosic materials (including cellulose, hemicellulose and lignin). When the sample in NDF was boiled in NDF solution, the cell wall of lignocellulosic material was separated from the cell content. ADF solution was used to estimate the content of lignin and cellulose. When the sample was boiled with ADF solution, materials such as protein, lipid, sugars, starch and hemicellulose were dissolved while cellulose and lignin remained (Chaves et al., 2002). Table 4.1 tabulates the cellulose, hemicellulose and lignin contents of the coconut husk pretreated with different methods. In comparison to cellulose content of the control (18 to 21%), the MAA treatment increased the cellulose content of coconut husk to approximately 39%, while reduced the lignin content significantly. Similar to MAA, alkaline pretreatment was able to raise the cellulose content of coconut husk (33 to 36%) significantly and to reduce the lignin content to 36 to 37% as compared to the control sample. In contrast, thermal or acid pretreatment alone was not effective in the alteration to cellulose and lignin content of the coconut husk after treatment.

Table 4.1: Cellulose, hemicellulose and lignin contents of the pretreated coconut husk.

Pretreatment	Particle size (μm^2)	Cellulose	Hemicellulos	Lignin
Comminution (Control)	300 - 600	21.26 ± 1.51^{a}	17.33 ± 0.74^{a}	46.36 ± 0.57^{a}
	850 -1500	18.19 ± 2.81^{b}	11.34 ± 1.34^{b}	53.08 ± 2.37^{a}
Thermal	300 - 600	19.21 ± 1.39^{c}	13.92 ± 0.37^{c}	50.90 ± 0.82^{b}
	850 - 1500	23.36 ± 1.48^{c}	14.31 ± 0.93^{c}	51.71 ± 1.39^{c}
Acid	300 - 600	16.98 ± 4.19^{d}	22.36 ± 2.99^{d}	48.65 ± 0.08^{d}
	850 - 1500	25.60 ± 1.75^{d}	13.20 ± 0.42^{e}	51.50 ± 0.68^{e}
Alkaline	300 - 600	36.87 ± 0.88^{e}	22.63 ± 0.25^{f}	36.76 ± 0.86^{f}
	850 - 1500	33.74 ± 0.77^{e}	24.23 ± 1.10^{f}	37.59 ± 0.49^{g}
Microwave-assisted-alkaline	300 – 600	38.93 ± 1.94^{f}	25.04 ± 0.93^{g}	32.98 ± 1.62^{h}
	850 – 1500	39.98 ± 1.45^{f}	25.25 ± 0.79^{g}	31.79 ± 1.07^{i}

[a-i] mean values in the same column not followed by the same letter are significantly different ($P<0.05$).

4.3.3 Comparison of Pretreatment Techniques

All pretreatments techniques (thermal, acid, alkaline and MAA) were carried out by employing two particle sizes of powderised coconut husk. The comminuted coconut husk without any additional pretreatment was used as control. As shown in Figure 4.6, thermal and acid treatment alone is not suitable as the yield of reducing sugar achieved were considerably low. However, reducing sugars yield from alkaline pretreated coconut husk almost doubled to about 1.0 to 1.4 g/L in comparison to the control. It is very obvious that reducing sugar from MAA

pretreated coconut husk is much higher than all other pretreatments methods (4 to 5 fold than that of control).

The aim of thermal pretreatment was reported to cause hemicellulose degradation and lignin transformation. The cellulose is more accessible for enzymatic hydrolysis with the aid of these effects (Taherzadeh and Karimi, 2008). During thermal pretreatment, the sample is first subjected to high-pressure steam in a vessel. After predetermined duration of heat treatment, the steam is released to reduce the pressure inside the vessel, causing the water condensed in the samples to rapidly decompress, and thereby disrupting the structure of fibers inside the lignocellulose sample (Carvalheiro et al., 2008; Kumar et al., 2009).

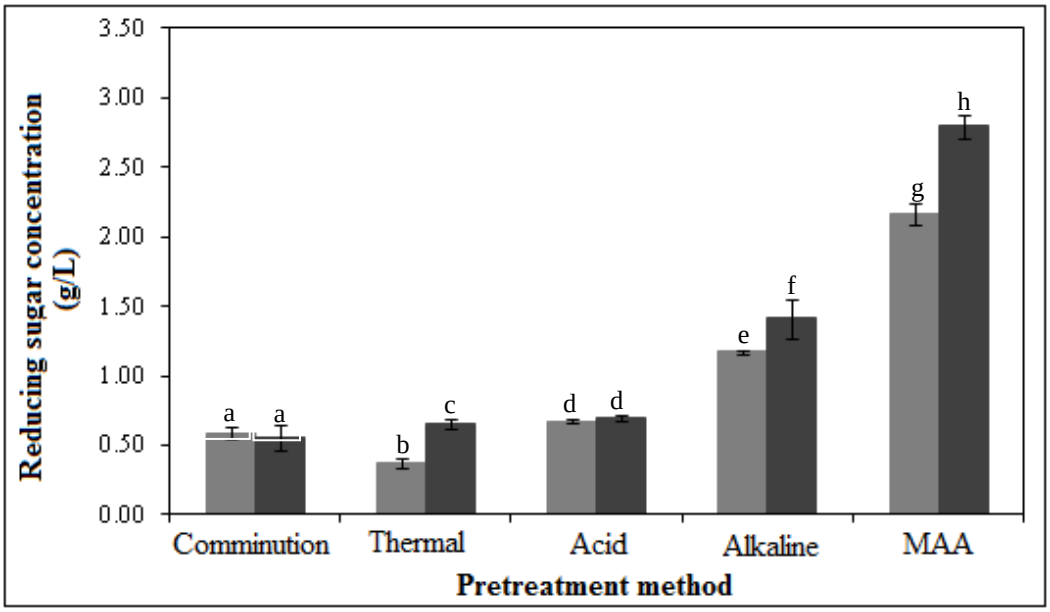


Figure 4.6: Maximum level of reducing sugars produced from the pretreated coconut husk. Symbols: ( ), 300 µm < size < 600 µm; (■),850 µm < size < 1500 µm. Error bars indicate the mean ± standard deviation of three experiments. [a-h] mean values in the graph not followed by same letter are significantly different (P<0.05).

The accessibility of cellulose by enzymes can be increased due to the alteration of lignocellulose structure (Prasad et al., 2007). However, the thermal pretreatment process did not enhance the production of reducing sugar from coconut husk as compared to the control (Figure 4.6). According to Kumar et al. (2009), one of the limitations of thermal pretreatment was an incomplete disruption of the lignin-carbohydrate matrix. As listed in Table 4.1, there was a large fraction of lignin present originally inside the coconut husk (46 to 53%). Thermal pretreatment which aims in hydrolyzing and removing hemicellulose is not able to remove the remaining high portion of lignin in coconut husk, and thus resulting in lower yield of reducing sugars (0.375 – 0.654 g/L).

Acid pretreatment is the most employed method for solubilizing hemicellulose, as it makes the cellulose better accessible by increasing the porosity of the structure (Lloyd and Wyman, 2006; Hendriks and Zeeman, 2009). The use of diluted acid (1 to 4%v/v) has been the most economical and adequate for hemicellulose hydrolysis as it is inexpensive and effective (Mussatto and Roberto, 2006; Kumar et al., 2009). According to Lloyd and Wyman (2006), acid pretreatment help to accelerate the rate of hemicellulose solubilization, therefore the remaining cellulose in residual solids are more accessible to enzyme hydrolysis. However, this pretreatment method was not effective in removing lignin fraction of lignocellulose biomass. Large fraction of lignin present originally in the coconut husk is a problem for the subsequent enzymatic hydrolysis (Table 3.1). Therefore the increment in production of reducing sugars from enzymatic hydrolysis of acid-pretreated coconut husk was not significant in the current study (Figure 4.6).

Alkaline pretreatment of lignocellulosic materials using alkaline solution such as NaOH causes swelling, leading to an increase in internal surface area, a decrease in crystallinity and removal of the major recalcitrance of lignocellulosic materials i.e., lignin fractions (Sharma et al., 2002; Alvira et al., 2010). In addition, alkaline pretreatment also helps to remove acetyl and various uronic acid substitutions on hemicellulose as these compounds lower the accessibility of enzyme to cellulose surface (Chang and Holtzapple, 2000). These effects had significantly increased cellulose digestibility in the enzymatic hydrolysis step. As shown in Table 4.1, the alkaline pretreatment had increased the cellulose percentage of coconut husk from 18-21% to 33-36% and decreased the lignin percentage from 46-53% to 36-37%. The reduction of lignin content from biomass increases the accessibility of enzymes to cellulose (Sun and Cheng, 2002). As a result, reducing sugar yield from enzymatic hydrolysis of alkaline pretreated coconut husk was improved by about 2 to 3 fold as compared with the control (Figure 4.6).

As illustrated in Table 4.1, around 20% increment of cellulose content was noticed in the MAA pretreated coconut husk (39%) in comparison to the control (18 to 21%). Microwave treatment was reported capable of producing a rapid volumetric heating throughout the material. The volumetric heating results in alteration of physio-chemical characteristics of the material which enables and accelerates the breaking down of the lignin-hemicellulose complex and increasimg the exposure of cellulose surface to cellulase (Ma et al., 2009;

Jackowiak et al., 2011). Therefore, lignin content of the control was reduced from 46 to 53% to 31 to 33% after MAA treatment.

Gabhane et al. (2011) reported that microwave treatment has better efficacy (more than 10% improvement in sugar yield) on garden biomass than autoclave and hot plate treatment techniques. In the experiment done by Zhao et al. (2008), sugar yield from switchgrass was improved by combining alkaline pretreatment with radiofrequency-based dielectric heating. The treatment uses the radiofrequency dielectric heating which creates a uniform heating profile in the targeted biomass and accelerates the disruption of lignocelluloses structure, and thus resulting in higher xylose and glucose yields compared to the pretreatment with alkaline and conventional heating. This is attributed to the fact enzymatic hydrolysis can be improved by the accelerated delignification process in microwave-assisted-alkaline pretreatment. Consequently, lignin content of the MAA pretreated coconut husk decreased significantly as compared to the control. As a result, the reducing sugar produced from enzymatic hydrolysis of MAA pretreated coconut husk increased significantly as compared to other pretreatment techniques (Figure 4.6).

4.3.4 Scanning Electron Microscope (SEM) Analysis

The morphological changes of untreated and pretreated coconut husk were examined by scanning electron microscopy (SEM) to observe the structural modification of the coconut husk (Figure 4.7). Figure 4.7 (a) shows the morphology of untreated coconut husk samples (850 to 1500 μm). The untreated coconut husk retained a compact and well-organized structure. The fibrils were found to be aligned closely without cracks between them and forming a "beehive-like" morphology.

From Figure 4.7 (b) and (c), the thermal and acid pretreated coconut husk both revealed an ordered and compact structure similar to the control sample. Thermal and acid pretreatment were reported to have significant effect in hydrolysing the hemicellulose fibres (Hendriks and Zeeman, 2009). However, results from Table 4.1 show that almost no lignin was removed from the coconut husk after thermal and acid treatment. This can be due to the fact that these two pretreatments were too mild to cause any effect in altering and disrupting the lignin-hemicellulose complex. As a result, the structure of coconut husk after acid and thermal treatments remained intact and the yield of reducing sugars were considerably lower compared to alkaline-treated and MAA-pretreated coconut husk.

Figure 4.7 (d) shows the structural changes of coconut husk after alkaline pretreatment. The alkaline treatment induced severe structural alteration and had simultaneously increased in surface area. The initially organized structure of untreated coconut husks (Figure 4.7 (a)) had been damaged to a sufficient extent that surface of alkaline treated coconut husk had become rougher and loosen. The morphology changes of the pretreated coconut husks could be contributed to the significant decrease of lignin contents after the treatment (Table 4.1). The fibrils were separated from the initial connected structure and fully exposed. This alteration facilitated the enzyme accessing into the cellulose. As a result, the yield of reducing sugar was increased by one-fold (Figure 4.6).

MAA pretreatment had successfully altered the initially organized and contiguous morphology of coconut husk into unorganized and fluffy structures (Figure 4.7 (e)). Hu and Wen (2008) noticed that the lignocellulosic material became thinner and striated under SEM analysis after the lignocellulosic materials had been presoaked in alkali and followed by microwave irradiation treatment. It is believed that this pretreatment had ruptured the physical barrier of biomass by degrading the lignin and hence increased the exposure of the cellulose and hemicellulose to hydrolysing enzymes such as cellulase (Hu and Wen, 2008). Hence, when the alkaline pretreatment was combined with the microwave irradiation, the yield of reducing sugar after enzymatic hydrolysis was markedly increased approximately 4.5-fold (2.79 g/L) compared to the untreated coconut husk (0.55 g/L).

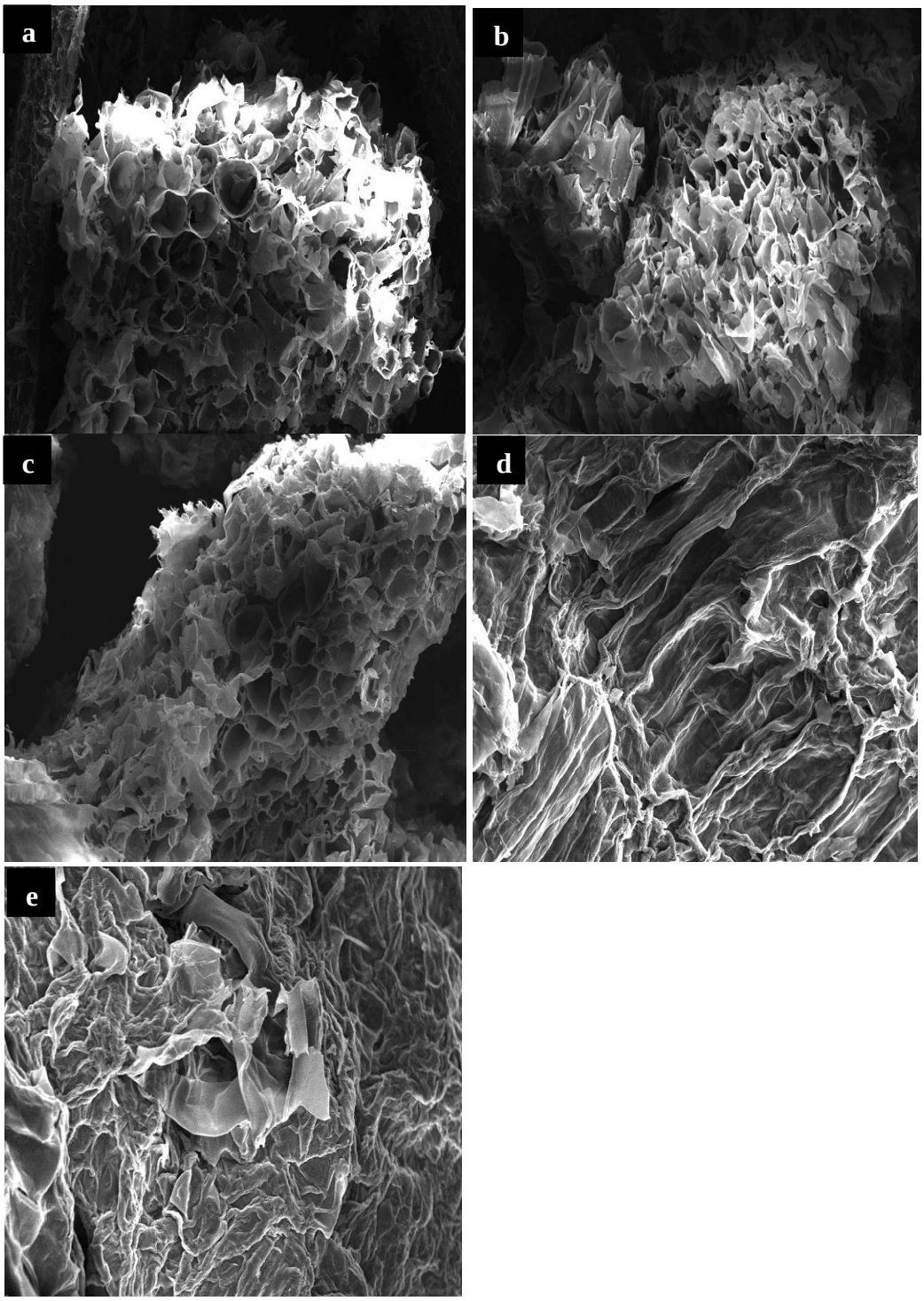


Figure 4.7: SEM images of coconut husk after several pretreatment process. Symbols: (a) Control sample, (b) Thermal treatment, (c) Acid treatment, (d) Alkaline treatment, (e) Microwave-assisted-alkaline treatment (magnification = 100 X)

4.4 Concluding Remarks

The general goal of pretreatment process is to disorganize or remove structural and compositional impediments in lignocellulosic biomass in order to improve the rate of enzymatic hydrolysis and hence increase yields of fermentable sugars from celluloses. In the present study, the most effective pretreatment method in releasing the highest concentration of reducing sugars from coconut husk after enzymatic hydrolysis was the microwave-assisted-alkaline pretreatment. In the enzymatic hydrolysis by using MAA pretreated coconut husks with sizes in between 850 μm^3 and 1.5 mm^3, the maximum concentration and productivity of reducing sugar recorded was 2.79 g/L and 0.058 g/L.h, rexpectively.

CHAPTER 5

STATISTICAL OPTIMISATION OF BIOETHANOL PRODUCTION USING MAA-PRETREATED COCONUT HUSK

5.1 Introduction

Following the pretreatment study, the fermentable sugars resulting from the hydrolysis of carbohydrate fractions can be used as a source for bioethanol production by suitable microorganisms (Mussatto et al., 2008). In ethanol fermentation process, the production of ethanol is cell dependent and the growth of cells is highly influenced by the medium composition and cultivation environment. Thus, searching of optimal growth media composition and cultural parameters are the main tasks for bioethanol fermentation process. The conventional optimisation method usually requires large numbers of experiments and time-consuming due to the fact that only one independent variable is being optimised while the others are maintained at a constant level (Ma et al., 2008). A more effective alternative optimisation method is the application of statistical designs such as Plackett-Burman design and Response Surface Methodology (RSM) (Yu et al., 2009). The screening of main parameters from a large numbers of process variables can be conducted in a more efficient manner with these

statistical experimental design tools (Ferreira et al., 2009; Chaibakhsh et al., 2012).

In the present study, the first step of optimisation process was to identify the factors (initial pH of medium, incubation temperature, loading of pretreated coconut husk, cellulase loading, pectinase loading, agitation speed and concentration of yeast extract) that have significant effects ($P<0.05$) on bioethanol fermentation with coconut husk as carbon source by using Plackett-Burman experimental design technique. Following identification of the significant parameters, the Path of Steepest Ascent (PSA) method was used to locate the region of factor levels that would possible produce high level of bioethanol. Then, Response Surface Methodology (RSM) based on Central Composite Design (CCD) was used to determine the optimum conditions for improvement of bioethanol production from the pretreated coconut husks.

5.2 Materials and Methods

5.2.1 Optimization of Simultaneous Saccharification and Fermentation Process

The main objective of the current study was the identification of optimal medium composition and culture conditions. Initial pH of medium, incubation temperature, coconut husk loading, enzyme dosage, agitation speed and concentration of yeast extract used during fermentation process were the parameters under investigation. These factors were evaluated by the application of a two-level factorial Plackett-Burman design. The optimum region of the factors was determined by the method of steepest ascent. Subsequently, the Response Surface Methodology (RSM) was implemented to search for an optimum condition that supported the maximum production of bioethanol from pretreated coconut husks by *Saccharomyces cerevisiae*.

Plackett-Burman Experimental Design

The Plackett-Burman experimental design was used in this work to screen the significant hydrolysis and fermentation parameters on bioethanol production from MAA-treated coconut husks. Design-Expert (STAT-EASE Inc., Minneapolis, USA, Version 7.1.3) was applied for the experimental design and the analysis of data obtained. Seven independent variables (Table 5.1) in twelve combinations

were organized according to the Plackett-Burman design matrix (Table 5.2). For each variable, a high (+1) and a low (-1) level was tested.

The effect of each variable was determined by following equation:

$$E_{(Xi)} = 2 \left(\sum P_i^+ + P_i^- \right) / N \qquad \text{(Equation 5.1)}$$

where, $E_{(Xi)}$ is the concentration effect of the tested variable. $P_i^+ + P_i^-$ are the ethanol productivity from the trials where the variable (X_i) measured was present at high and low concentrations, respectively; and N is the number of trials. Experimental error is estimated by calculating the variance among the dummy variables as follows,

$$V_{eff} = \sum (E_d)^2 / n \qquad \text{(Equation 5.2)}$$

where, V_{eff} is the variance of the concentration effect, E_d is the concentration effect for the dummy variable and *n* is the number of dummy variables.

All trials were performed in triplicate, with the mean values of ethanol productivity as the response of the optimisation study. The standard error (SE) of the concentration effect was the square root of the variance of an effect and the significance level (*p*-value) of each concentration effect was determined using Student's *t*-test as given by Equation 5.3:

$$t_{(Xi)} = E_{(Xi)} / SE \qquad \text{(Equation 5.3)}$$

Where $E_{(Xi)}$ is the effect of variable X_i and $t_{(Xi)}$ is the *p*-value of each concentration effect.

Table 5.1: Experimental range and levels of independent variables in the Plackett-Burman experiment.

Variable	**Level**		
	-1	**0**	**1**
A: Initial pH	5	6	7
B: Temperature (^{o}C)	30	33	37
C: Coconut husk loading (%w/v)	1.0	1.5	2.0
D: Cellulase loading (%v/w)	10	20	30
E: Pectinase loading (%v/w)	10	20	30
F: Agitation speed (rpm)	100	150	200
G: Yeast extract concentration (%w/v)	0.5	1.0	1.5

Table 5.2: Plackett-Burman design matrix representing the coded values for 7 independent variables.

Run No.	**Factors (Coded values)**						
	A	**B (^{o}C)**	**C (%w/v)**	**D (%v/w)**	**E (%v/w)**	**F (rpm)**	**G (%w/v)**
1	1	-1	1	1	-1	1	1
2	1	1	1	-1	-1	-1	1
3	-1	-1	-1	-1	-1	-1	-1
4	1	-1	-1	-1	1	-1	1
5	1	1	-1	-1	-1	1	-1
6	-1	1	1	1	-1	-1	-1
7	-1	1	-1	1	1	-1	1
8	-1	-1	1	-1	1	1	-1
9	-1	-1	-1	1	-1	1	1
10	1	-1	1	1	1	-1	-1
11	1	1	-1	1	1	1	-1
12	-1	1	1	-1	1	1	1

*****A-initial pH; B-temperature; C-coconut husk loading, D-cellulase loading; E-pectinase loading; F-agitation speed; G-yeast extract concentration.

Path of Steepest Ascent

The optimum region of the significant factors was determined by the method of steepest ascent. The path of steepest ascent was started from the center point of Plackett Burman design. The experiment was carried out along the steepest ascent path until the response value (concentration of ethanol) reached the maximum point. The experimental design of the steepest ascent method is shown in Table 5.3. From the results of Plackett-Burman experiment, two factors i.e., coconut husk loading and pectinase loading were considered as significant factors. Details on step size of coconut husk loading and pectinase loading is discussed in the Section 5.3.2.

Table 5.3: Path of steepest ascent experimental design.

Run	Coconut husk loading, %w/v	Pectinase loading, %v/w
1	1.5	20.0
2	2.0	11.9
3	2.5	3.8
4	3.0	0.0

Response Surface Methodology

Response surface methodology (RSM) based on Central Composite Design (CCD) was used to determine the optimum condition for maximum level of bioethanol production from the pretreated coconut husks. The two most significant factors (coconut husk concentration and pectinase loading) were investigated at five different levels (-1.4, -1, 0, 1, 1.4) (Table 5.4) and the experimental design used for this study is shown in Table 5.5. All trials were performed in triplicate, with the mean values of ethanol productivity considered as the response.

Table 5.4: Levels of the factors tested in central composite design.

Factors	**Levels of Factors**				
	-1.4	**-1**	**0**	**1**	**1.4**
Coconut husk loading, (%w/v)	2.00	2.15	2.5	2.85	3.00
Pectinase loading, (%v/w)	0.00	1.17	4	6.83	8.00

Table 5.5: The central composite design of RSM for optimization of bioethanol production.

Run	Coconut husk loading (%w/v)	Pectinase loading (%v/w)
1	2.15	6.83
2	2.85	1.17
3	2.50	4.00
4	2.15	1.17
5	2.85	6.83
6	2.50	4.00
7	2.50	4.00
8	3.00	4.00
9	2.50	8.00
10	2.50	4.00
11	2.00	4.00
12	2.50	0.00
13	2.50	4.00
14	2.50	4.00

5.2.2 Gas Chromatography-Mass Spectrometry (GC-MS) Analysis

In the current study, recovery of ethanol from crude fermentation broth was conducted by using simple distillation process. The distillation process works by boiling the fermentation broth. The distillation process was conducted according to the apparatus set-up shows in Figure 5.1. The condensed distillate was collected and further characterized by gas chromatography mass spectrometry (GC-MS) system on a Shimadzu (model GC-MS-2010 Plus) instrument with a Zebron-WAX plus column (0.25 mm x 30 m, 0.25µm).

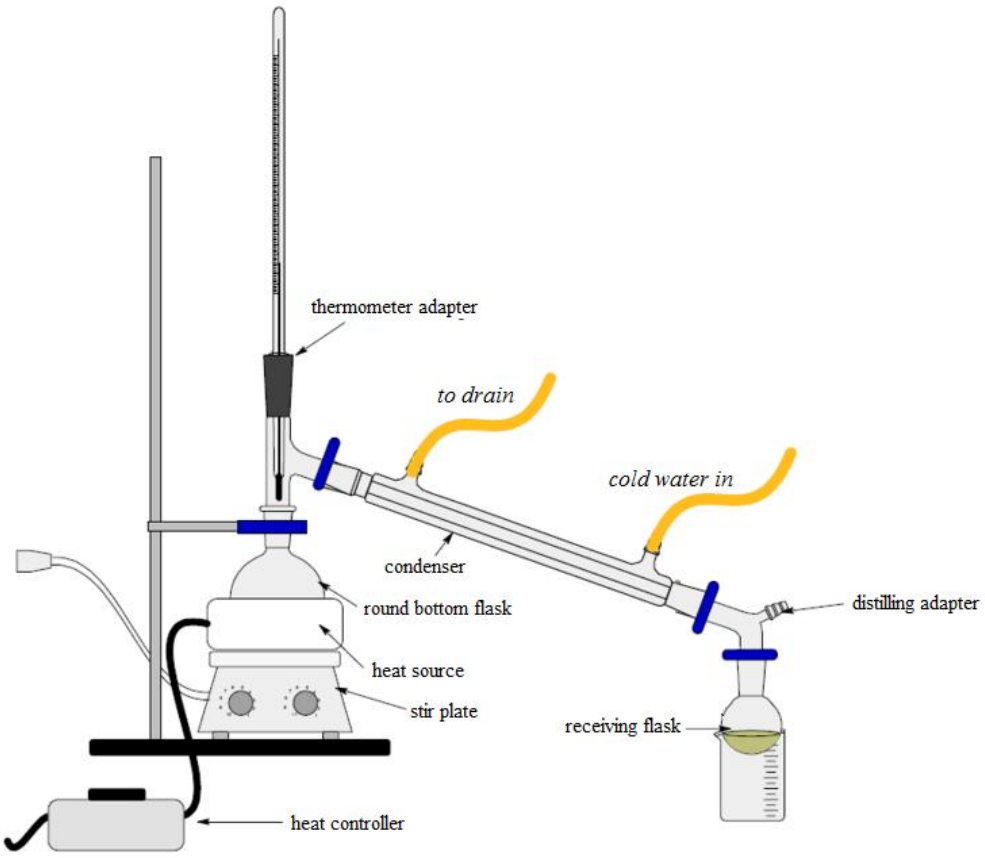


Figure 5.1: Schematic diagram of simple distillation process (Source: http://periodicalmaniacs.wikispaces.com/Lab+5).

5.2.3 Data Analysis

Design-Expert, Version 7.0 (STAT-EASE Inc., Minneapolis, USA) was used for the experimental designs and statistical analysis of the optimization experimental data.

5.3 Results and Discussion

5.3.1 Screening of Significant Factors by Plackett-Burman Design

Plackett-Burman design is a widely used statistical design for the screening of important parameters which have significant effect on the response of an experiment (Singh and Satyanarayana, 2008). In the present study, it was used as a screening method to determine which of the 7 variables (initial pH of medium, incubation temperature, coconut husk loading, pectinase loading, cellulase loaidng, agitation speed and concentration of yeast extract) significantly affect ethanol productivity by using MAA-treated coconut husk as carbon source.

The experimental data analysis of Plackett-Burnam design is shown in Table 5.6. Table 5.6 indicates that there was a wide difference in ethanol productivity ranged

from 0.0041 to 0.0486 g ethanol/g coconut husk daily in the 12 experiments. This difference in ethanol productivity reflected that optimization process was essential for enhancing ethanol productivity.

Table 5.6: Plackett-Burman design matrix representing 7 independent variables and the response.

Run No.	**Variables**							**Response**
	A	**B**	**C**	**D**	**E**	**F**	**G**	**Ethanol Productivity**
1	7	30	2.0	30	10	200	1.5	0.0486
2	7	37	2.0	10	10	100	1.5	0.0448
3	5	30	1.0	10	10	100	0.5	0.0041
4	7	30	1.0	10	30	100	1.5	0.0048
5	7	37	1.0	10	10	200	0.5	0.0131
6	5	37	2.0	30	10	100	0.5	0.0374
7	5	37	1.0	30	30	100	1.5	0.0135
8	5	30	2.0	10	30	200	0.5	0.0123
9	5	30	1.0	30	10	200	1.5	0.0160
10	7	30	2.0	30	30	100	0.5	0.0136
11	7	37	1.0	30	30	200	0.5	0.0151
12	5	37	2.0	10	30	200	1.5	0.0191

*****A-initial pH; B-temperature (^{o}C); C-coconut husk loading (%w/v), D-cellulase loading (%v/w); E-pectinase loading (%v/w); F-agitation speed (rpm); G-yeast extract concentration (%w/v).
unit of productivity = % g ethanol / g coconut husk / day

The analysis of variance (ANOVA) was applied to test the interaction effects of the variables and the results are shown in Table 5.7. The *p*-value less than 0.05

indicated that the model terms are significant. Among the selected parameters, the coconut husk loading and concentration of pectinase were the significant variables. The coefficient R^2 of the model was 0.9295, indicating that 92.95% of the variability in the response could be explained by the model. A first order model was fitted to the results obtained from the 12 experiments and the equation of the model based on coded values is as follows:

$$Y = 0.040 + 0.021\ A - 0.017\ B \qquad \text{(Equation 5.4)}$$

where, Y represents ethanol productivity while A and B are the coconut husks loading and the pectinase loading, respectively.

Table 5.7: Statistical analysis of the model (ANOVA).

Source	**Sum of squares**	**Degrees of freedom**	**Mean square**	**F-value**	**Prob > F**
Model	2.281×10^{-3}	7	3.259×10^{-4}	7.53	0.0347
pH	1.164×10^{-4}	1	1.164×10^{-4}	2.69	0.1763
Temperature	1.595×10^{-4}	1	1.595×10^{-4}	3.69	0.1273
Coconut husk loading	9.965×10^{-4}	1	9.965×10^{-4}	23.03	0.0087
Cellulase loading	1.761×10^{-4}	1	1.761×10^{-4}	4.07	0.1138
Pectinase loading	6.114×10^{-4}	1	6.114×10^{-4}	14.13	0.0198
Agitation speed	2.916×10^{-6}	1	2.916×10^{-6}	0.067	0.8080
Yeast Extract	2.187×10^{-4}	1	2.187×10^{-4}	5.05	0.0878

As show in Figure 5.2, the Pareto chart is used to graphically summarize and display the relative significance of different factors. In Pareto chart, the bars represent the estimated effect of each factor are shown as a length proportional corresponding to the absolute value. Open bars are the variables that were included in the Plackett-Burman model while closed bars are effects not included in the model. Every tested parameter is color coded in order to designate whether the parameter was positive or negative. The orange bar is considered as particular parameter that has positive effect whereas the blue bar is parameter that has a negative effect on bioethanol productivity. Coefficients with *t*-value of effect above Bonferroni limit are designated as certainly significant; coefficients with *t*-value of effect between Bonferroni limit and *t*–value limit are likely to be significant; coefficients with *t*-value of effect below the *t*-value limit are statistically insignificant and therefore should be removed from the analysis (Shah and Pathak, 2010). In this study, coconut husk loading and pectinase loading had the most significant influence on ethanol productivity.

The coconut husk loading variable is indicated as orange open bar, which implies that a higher concentration of coconut husk will be useful in increasing bioethanol production. As shown in Figure 5.2, the pectinase loading is represented as blue open bar which indicates that the factor of pectinase loading had a negative effect. This implies that a lower dosage of pectinase to be more suitable for increasing bioethanol production.

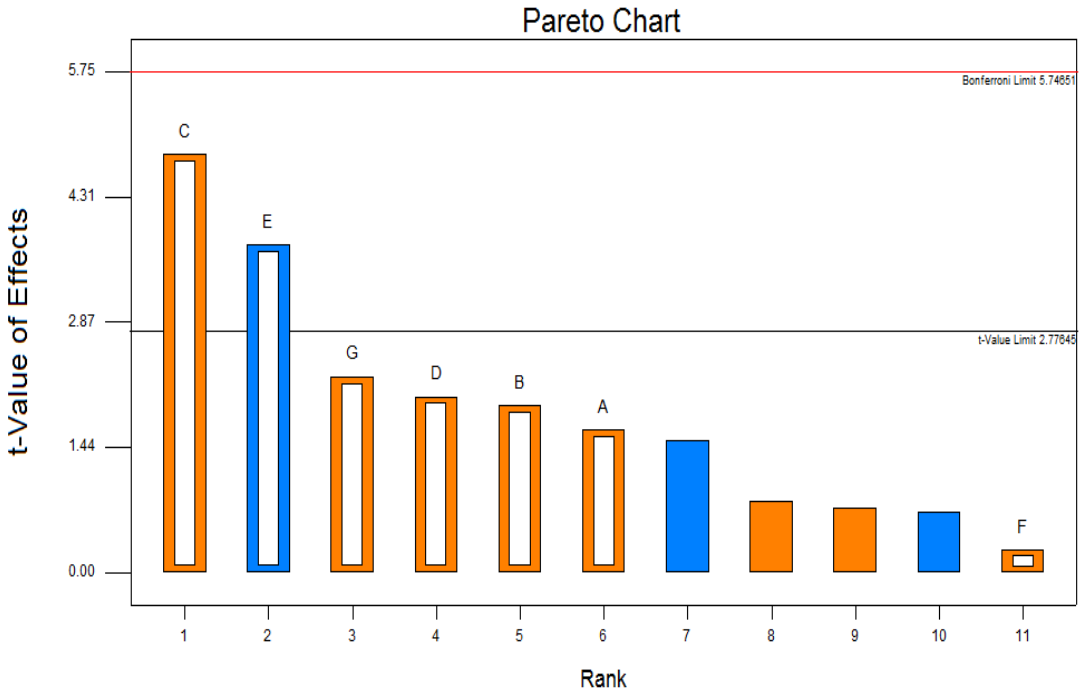


Figure 5.2: Pareto chart. A: pH; B: temperature; C: coconut husk loading; D: cellulase loading; E: pectinase loading; F: agitation speed; G: yeast extract concentration (Orange color bar: positive effect; Blue color bar: negative effect).

The analysis of main effects plot for bioethanol productivity was also employed (Figure 5.3). Figure 5.3 shows that pH, temperature, coconut husk loading, cellulase loading, agitation speed and yeast extract concentration had positive effect on ethanol productivity whereas pectinase loading had negative effect on ethanol productivity. The lines between the low and high levels of coconut husk loading and pectinase loading showed a greater slope than others, indicating that these two factors had significant effect on bioethanol productivity (Liu et al., 2011).

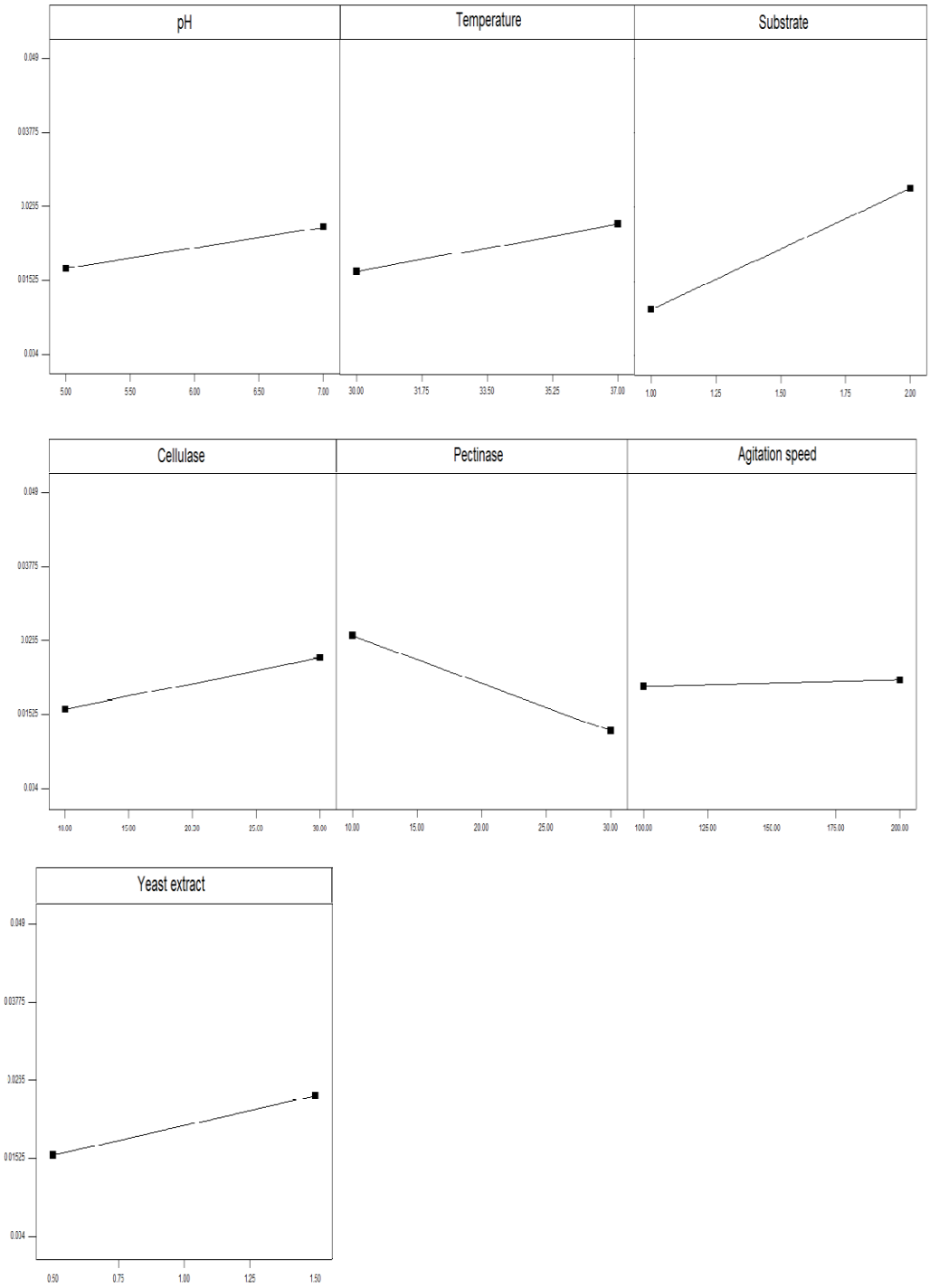


Figure 5.3: Main effect plots.

Effect of Individual Significant Parameters

The positive correlation that exists between coconut husk loading and bioethanol production implies that a higher amount of coconut husk is more effective in increasing bioethanol production in the experiment. In the current study, the pretreated coconut husk was the main carbon source for bioethanol production. The carbon source has been reported as vital limiting factor in biosynthesis and energy generation of yeast fermentation process (Kuchel and Ralston, 1997). The bioethanol productivity can be increased drastically depending upon the concentration of pretreated coconut husk in the medium due to its positive significant effect in the analysis. As shown in Table 5.6, it is very obvious that the ethanol productivity increased approximately 3.5-fold when the coconut husk loading was increased from 1.0 g (Run 5) to 2.0 g (Run 2), i.e., pectinase loading remained the same (10% v/w).

The factor of pectinase loading had a negative effect, which indicates that lower dosage of pectinase is more suitable for increasing bioethanol productivity. Pectin is a component that present in the primary cell wall and helps to cement plant cells together while pectinase enzyme is commonly used in the degradation of pectin which further lead to the increasing exposure of other cell wall components such as cellulose. As discussed in Chapter 4, MAA treatment had accelerated the breaking down of lignin-hemicellulose complex and drastically increased the exposure of cellulose content after the pretreatment process, and hence lowered

the amount of pectinase needed for pectinolytic activity. The pectinase is also reported to have degradation effect on polysaccharides in middle lamella and this activity will lead to the production of by-products such as antioxidant, phenolic compound, flavonoids and etc (Nattaporn and Pranee, 2011). The experiment conducted by Nattaporn and Pranee (2011) revealed that cantaloupe flesh with 3%v/w pectinase loading had total phenolic content of 8 times higher the control without pectinase loading. The phenolic compounds were found to have negative effect on growth and fermentation rates in some yeast strains. For example, Zhang et al. (2012) found that the phenolic compounds derived in lignocellulosic biomass might negatively influence the performance of *Candida athensensis* SB18 for xylitol production. Pastorkova et al. (2013) discovered that some phenolic compounds of grape possess antibacterial and antiyeast effects. The minimal inhibitory concentration of phenolic compound on the growth of *Saccharomyces cerevisiae* was 512µg/mg. The total phenolic content of coconut husk was determined to be 13.0 mg/g dry weights, of which 1.3-1.7% was ferulic acid (Dey et al., 2003). This might be the reason that pectinase was found to have a negative effect that signifies its effectiveness in lower concentrations in the experiments. Referring to Table 5.6, it is notable that the productivity of ethanol increased 2.5-fold when the pectinase loading was reduced from 30% v/w (Run 12) to 10% v/w (Run 1), when the other significant parameter (coconut husk loading) remained at the same level (2.0 g).

Through ANOVA analysis (Table 4.7), variables with confidence levels above 95% (*p*-value less than 0.05) were considered as the significant parameters. From the analysis results, pH, temperature, cellulase concentration, agitation speed and yeast extract concentration were concluded as insignificant parameters and were not included in the subsequent optimization experiments. Hence, the two significant factors, coconut husk loading and pectinase loading, were selected for further optimization to attain a maximum response of bioethanol productivity from MAA-pretreated coconut husk.

5.3.2 Path of Steepest Ascent

As observed from Table 5.7 and Figure 5.2, loading of coconut husk and pectinase were the major factors that influenced the productivity of ethanol from MAA-pretreated coconut husk. The method of steepest ascent is an efficient procedure developed to move the experimental region of a response in the direction toward the optimum point (Wang and Wan, 2009). In this study, the path of steepest ascent was based in the center of the Plackett-Burman design and moved along the path in which substrate concentration increased while pectinase concentration decreased. Table 4.8 shows the step size for substrate and pectinase concentration. The experimental results of Plackett-Burman design were analyzed by ANOVA and it was fitted with Equation 5.4:

$$Y = 0.040 + 0.021 A - 0.017 B \quad \text{(Equation 5.4)}$$

where, A and B are the coded factors of coconut husk loading and pectinase loading, respectively. From Equation 5.4, for every unit of substrate concentration increment,

pectinase step size = -0.017/0.021

= - 0.81

As refer to Plackett Burman design in Table 5.1, the un-coded level difference of coconut husk concentration and pectinase loading are 0.5% w/v and 10% v/w, respectively. Hence, for every 1 unit (0.5% w/v) increase in coconut husk concentration, the pectinase decreases by 0.81 unit (8.1% v/w), as illustrated in Table 5.8.

Table 5.8: Step size for substrate and pectinase loading**.**

	Coconut husk loading	**Pectinase loading**
Coded value	1	-0.81
Actual value	0.5% w/v	-8.1% v/w

Table 5.9 shows the experimental design and results of steepest ascent method. From the table, ethanol productivity reached the maximum (0.04333% g ethanol/ g coconut husk / day) in Run No. 3 and the decreasing of ethanol productivity appeared in Run No. 4, suggesting that the optimal point was between Run No. 2 and Run No. 4. Hence, Run No.3 was chosen as the center point for further optimization.

Table 5.9: Experiment design and results for the path of steepest ascent**.**

Run	Coconut husk loading (%w/v)	Pectinase loading (%v/w)	Ethanol productivity (% g ethanol/ g coconut husk / day)
1	1.5	20.0	0.03803
2	2.0	11.9	0.04224
3	2.5	3.8	0.04333
4	3.0	0.0	0.04296

5.3.3 Optimization of Ethanol Productivity by using Response Surface Methodology (RSM)

The purpose of Plackett-Burman design was to screen important variables that affect ethanol productivity as well as their significant levels, but did not consider the interaction effects among the variables as in RSM (Ferreira et al., 2009; Yan et al., 2011). After the optimal region of variables was identified, RSM was applied to determine the optimal levels of the selected variables (coconut husk

loading and pectinase loading). Since each significant variable was studied at five different levels along with other variables, the interactions among different significant variables at their corresponding levels could be studied with RSM approach (Singh and Satyanarayana, 2008). The respective low and high levels for the two variables are defined in Table 5.4. The experimental design and results of response surface optimization are shown in Table 5.10. The observed ethanol productivity variedly notably with the conditions tested (0.021 %w/w/day to 0.050 %w/w/day), which indicated that the combination of the factor levels had significantly affected the ethanol production process. The Design Expert 7.0, a statistical program software, was used for regression and graphical analysis of the data obtained.

Table 5.10: The Central Composite Design and results of RSM for optimization of bioethanol production.

Run	Coconut husk loading, %w/v	Pectinase loading, %v/w	Ethanol productivity, %w/w/day	
			Actual	Predicted
1	2.85	1.17	0.046	0.043
2	2.50	4.00	0.042	0.039
3	2.50	4.00	0.041	0.039
4	2.15	6.83	0.030	0.030
5	2.15	1.17	0.023	0.023
6	2.50	4.00	0.040	0.039
7	2.85	6.83	0.050	0.048
8	3.00	4.00	0.050	0.047
9	2.50	4.00	0.042	0.039
10	2.50	4.00	0.041	0.039
11	2.50	8.00	0.045	0.043
12	2.50	4.00	0.040	0.039
13	2.00	4.00	0.021	0.020
14	2.50	0.00	0.035	0.034

The experimental results were analyzed by ANOVA and the CCD was fitted with the second order polynomial equation (Equation 5.5):

$$Y = - 0.185 + 0.145A + 3.113*10^{-3}B – 5.750*10^{-4}AB – 0.023A^2 – 7.162*10^{-5}B^2$$

(Equation 5.5)

where, A and B are the coded factors of coconut husk loading and pectinase loading, respectively. The quadratic model in the equation contains two linear

terms, one interaction term and two quadratic terms. By ignoring the insignificant terms, the equation was modified to reduced-fitted model equation (Equation 5.6):

$$Y = - 0.185 + 0.145A + 3.113*10^{-3}B – 0.023A^2 \quad \text{(Equation 5.6)}$$

Table 5.11 tabulates the model summary and analysis of variance (ANOVA) for the quadratic model. The model F-value of 239.49 ($p < 0.0001$) implies the model is significant. There is only a 0.01% chance that a "Model F-value" this large could occur due to noise. Model terms with "Prob > F" of less than 0.05 are significant. The lack-of-fit with F-value of 2.73 implies an insignificant lack-of-fit relative to the pure error. Among the 5 model terms in this study, 3 model terms (A, B and A^2) were significant. This implies that the individual effects of the component substrate (coconut husk) and pectinase loading as well as the quadratic effect of the substrate (coconut husk) were found to be more significant than the other factors. The interactive effects of substrate with pectinase and squared term of pectinase are not significant as the *p*-value is greater than 0.05 and hence were ignored in Equation 5.6.

Table 5.11: Model summary and analysis of variance (ANOVA) for the quadratic model.

Source	**Sum of Squares**	**Degrees of freedom**	**Mean square**	***F*-value**	***p*-value (Prob>*F*)**
Model	9.98×10^{-4}	5	1.20×10^{-4}	239.49	<0.0001
A-coconut husks	8.59×10^{-4}	1	8.59×10^{-4}	1030.70	<0.0001
B-pectinase	7.78×10^{-5}	1	7.78×10^{-5}	93.38	<0.0001
AB	1.32×10^{-6}	1	1.32×10^{-6}	1.59	0.2481
A^2	5.89×10^{-5}	1	5.89×10^{-5}	70.63	<0.0001
B^2	2.42×10^{-6}	1	2.42×10^{-6}	2.91	0.1319
Residual	5.83×10^{-6}	7	8.33×10^{-7}		
Lack of fit	3.92×10^{-6}	3	1.31×10^{-6}	2.73	0.1781
Pure Error	1.91×10^{-6}	4	4.78×10^{-7}		
Cor. total	1.00×10^{-3}	13			

$R^2 = 0.9942$, Adjusted $R^2 = 0.9900$, Predicted $R^2 = 0.9582$.

The R^2 value is always between 0 to 1 and for a good statistical model, R^2 value should be close to 1.0. As tabulated in Table 5.11, the coefficient of determination (R^2) was calculated to be 0.9942. This implied that 99.42% of the experimental data of ethanol production was compatible with the data predicted by the model. The value of predicted R^2 (0.9582) is in reasonable agreement with the adjusted R^2 (0.9900). This ensured a satisfactory adjustment of the quadratic model to the experimental data (Chen and Chen, 2009). The coefficient of variance (CV) indicates the degree of precision with which the experiments are compared and it is being calculated as the ratio of standard deviation to the mean ($CV = \sigma / \mu$).

Therefore, a lower CV represents a higher reliability of the experiments. In the current study, a lower CV (2.35) demonstrates a greater reliability of the experiments performed. “Adequate precision” measures the signal to noise ratio and a ratio greater than 4 is desirable. The “adequate precision” in this test was found to be 45.671 and this indicates that the model can be used to navigate the design space (Pujari and Chandra, 2000; Gunawan et al., 2005; Huang et al., 2006; Yan et al., 2011).

Figure 5.4 shows the 3D surface graph for ethanol productivity which has curvilinear profile in accordance to the quadratic model fitted. The figure explains the effect of two independent variables; loading of coconut husk and enzyme pectinase loading on the response, i.e., the ethanol productivity. In order to determine the optimal levels of each variable for maximum ethanol productivity, the contour plot was constructed by plotting the response (ethanol productivity) on the Z-axis versus the two independent variables, while other variables were at their optimal levels. The optimum values of the test variables can be obtained from the center point of the contour plot. From the contour plot, the model predicted that maximum ethanol productivity (0.0525 g ethanol/g coconut husks/ day) can be achieved using the optimum cultivation conditions containing 3.06 g MAA-pretreated coconut husks, 0.61 mL cellulase, 0.34 mL pectinase and 1 g yeast extract in 100 mL medium with initial medium pH of 6 and cultivated at 30^{o}C.

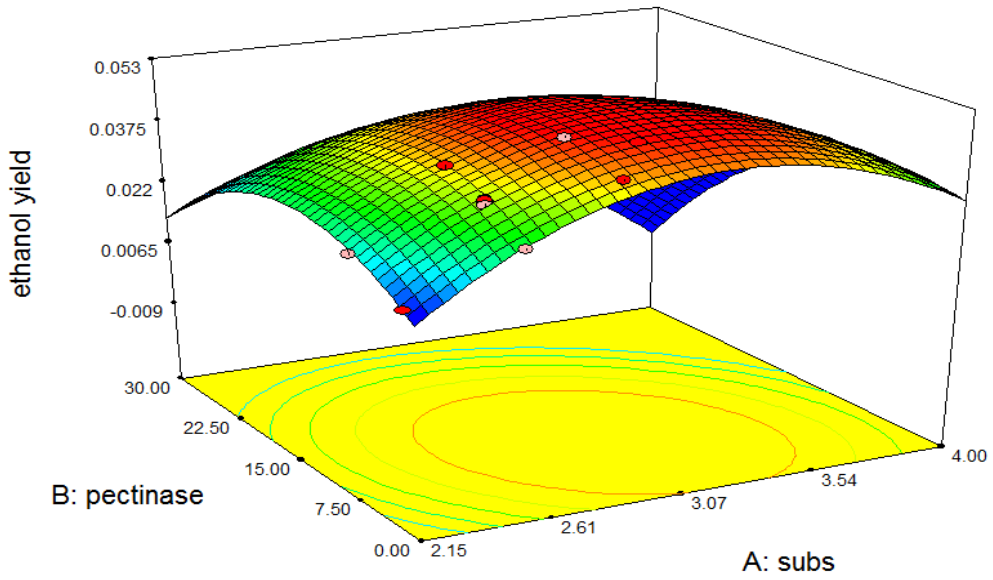


Figure 5.4: Response surface curve for bioethanol productivity showing the interaction between substrate and pectinase loading.

5.3.4 Validation of Bioethanol Fermentation using Optimized Condition

The proposed optimized condition for maximum productivity of bioethanol was verified by conducting 2 experiments by using 3.06 g MAA pretreated coconut husks, 0.61 mL cellulase, 0.34 mL pectinase and 1 g yeast extract in 100 mL medium at 30^{o}C and an initial pH of 6. Figure 5.5 shows the results of validation test. The mean value of ethanol productivity from the experiments was 0.0593 g ethanol/g coconut husks/ day which agreed well with the predicted value from the model (0.0525 g ethanol/g coconut husks/ day). The overall error was small (less

than 15%) indicating the proficiency of the model for optimizing ethanol production with MAA-pretreated coconut husk as carbon source.

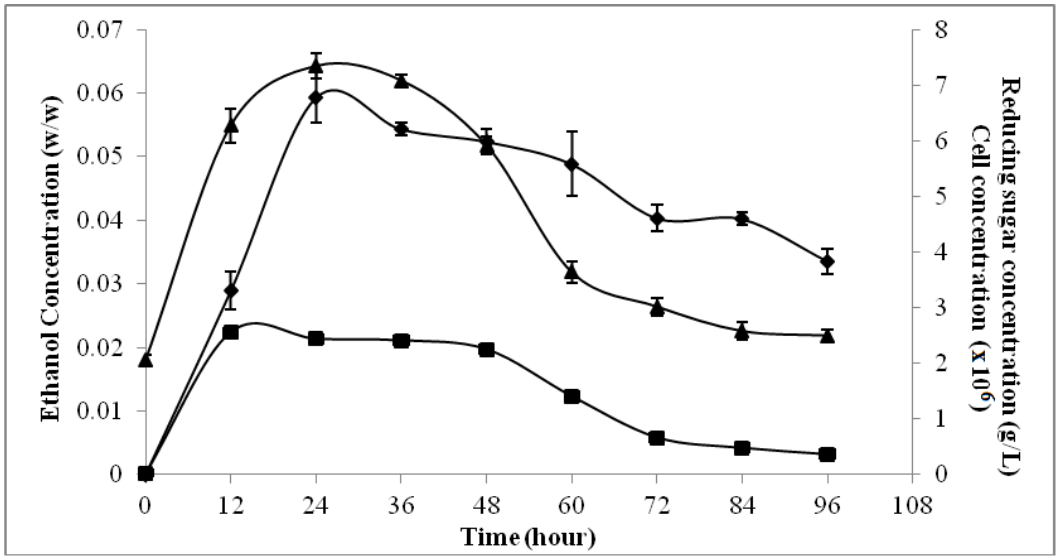
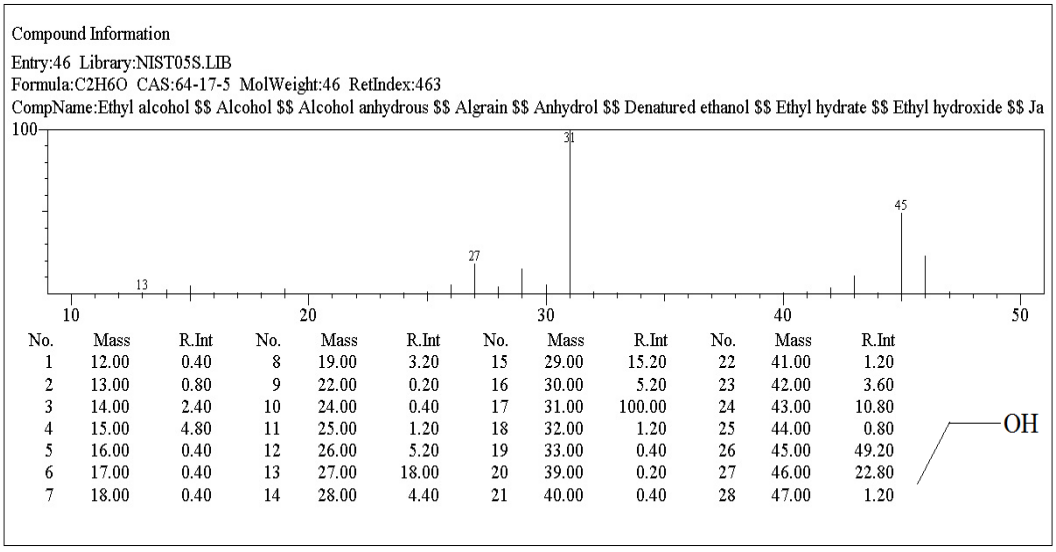


Figure 5.5: Profile of enzymatic hydrolysis and bioethanol fermentation by *Saccharomyces cerevisiae* at optimum conditions.
Symbol: (▲), cell concentration, (■), reducing sugars concentration, (◆), ethanol concentration.

5.3.5 Gas Chromatography-Mass Spectrometry Analysis of Bioethanol

Bioethanol produced from fermentation of pretreated coconut husks was characterized by gas chromatography-mass spectrometry (GC-MS). GC-MS analysis shows the presence of the ethanol (ethyl alcohol) at a retention time of 4.217 min. The mass spectrum of fermentation distillate showed a molecular ion peak at *m*/*z* 46 that correspond to molecular formula of ethanol (CH_3CH_2OH)

(Figure 5.6). The fragment ion peak at *m/z* 31 (-CH_3) is a result of losing an alkyl group attached to the carbinol carbon.



No.	Mass	R.Int	No.	Mass	R.Int	No.	Mass	R.Int	No.	Mass	R.Int
1	12.00	0.40	8	19.00	3.20	15	29.00	15.20	22	41.00	1.20
2	13.00	0.80	9	22.00	0.20	16	30.00	5.20	23	42.00	3.60
3	14.00	2.40	10	24.00	0.40	17	31.00	100.00	24	43.00	10.80
4	15.00	4.80	11	25.00	1.20	18	32.00	1.20	25	44.00	0.80
5	16.00	0.40	12	26.00	5.20	19	33.00	0.40	26	45.00	49.20
6	17.00	0.40	13	27.00	18.00	20	39.00	0.20	27	46.00	22.80
7	18.00	0.40	14	28.00	4.40	21	40.00	0.40	28	47.00	1.20

Figure 5.6: Gas Chromatography-Mass Spectrometry analysis.

5.4 Concluding Remarks

Simultaneous saccharification and fermentation of bioethanol was successfully performed. The Plackett-Burman (PB) design and response surface methodology were effectively applied to optimise the hydrolysis and fermentation parameters. Based on the results of PB experiment, pretreated coconut husk loading was the most significant parameters that positively affect or bioethanol productivity whilst pectinase loading has a significant negative effect on the response.

By applying multiple regression analysis, a second order polynomial model was established to identify the relationship between the two factors (coconut husks loading and pectinase concentration) and ethanol productivity. As suggested by the RSM, at optimum condition of 3.06 g MAA-pretreated coconut husks, 0.61 mL cellulase, 0.34 mL pectinase and 1 g yeast extract in 100 mL medium at 30^{o}C and an initial pH of 6, the maximum ethanol productivity recorded was 0.0525 g ethanol/g coconut husk/ day and this is in a good agreement with the experimental value of 0.0593g ethanol/g coconut husk/ day.

CHAPTER 6

CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE RESEARCH

6.1 Conclusions

This dissertation is focused on studying the suitability of employing coconut husk as a carbon source for bioethanol production. Two major issues were addressed in the current study. One is to increase the efficiency of enzymatic hydrolysis of coconut husk by developing a suitable pretreatment technique. The other is to optimize the fermentation conditions of the treated coconut husk in order to attain the highest level of ethanol productivity.

A number of pretreatment methods had been applied on coconut husk in order to increase the available cellulose content and thus improve the enzymatic hydrolysis efficiency. Each type of pretreatment has its own advantages and disadvantages. Some have been stated to be effective in disrupting lignin-carbohydrate complex, while others are responsible in breaking down the highly

ordered cellulose crystalline structure, which is a prerequisite for enzyme hydrolysis process (Mussatto et al., 2008). Based on the results obtained from the study carried out on the four pretreatments techniques (thermal treatment, acid treatment, alkaline treatment, microwave-assisted-alkaline (MAA) treatment), the MAA treatment is an effective pretreatment method in both altering the native structure of coconut husk as well as increasing the yield of reducing sugars. From the results, it can be concluded that among all the pretreatment options, microwave-assisted-alkaline (MAA) pretreatment facilitated the greatest sugar yield (2.79 g/L) from enzymatic hydrolysis of pretreated coconut husk. The result of characterization of MAA-pretreated coconut husk implies that coconut husk contains a substantial amount of cellulose (39%). Hence MAA-pretreated coconut husk was selected as a carbon source for subsequent bioethanol production.

Following enzymatic hydrolysis study, batch fermentation had been performed to investigate the production of bioethanol from MAA-pretreated coconut husk by *Saccharomyces cerevisiae*. A central composite design (CCD) was conducted to study the effects of seven variables (initial pH of medium, incubation temperature, agitation speed, coconut husk loading, cellulase loading, pectinase loading and yeast extract concentration) on ethanol productivity. Quadratic model was developed to correlate the variables to the response. Through the analysis of response surfaces derived from the model, coconut husk loading and pectinase loading were found to have the most significant effect on productivity of bioethanol. Process optimization was carried out and the experimental values

obtained for the ethanol productivity was found to agree satisfactorily with the value predicted by the model. Ternary diagrams i.e., 3D surface plot, was also developed and utilized to aid in visualization. According to the model, the estimated optimal media composition is 3.06 g MAA pretreated coconut husks, 0.61 mL cellulase, 0.34 mL pectinase and 1 g yeast extract in 100 mL medium at 30^{o}C and an initial pH of 6. The maximum ethanol productivity recorded was 0.0525 g ethanol/g coconut husk/ day and this is in a good agreement with the experimental value of 0.0593g ethanol/g coconut husk/ day.

As a conclusion, of all the pretreatments carried out in this study, enzymatic hydrolysis of MAA-pretreated coconut husk promoted the highest yield of reducing sugar. The delignification effect of MAA treatment had contributed in enhancing cellulose digestilibity and overall ethanol productivity in the simultaneous saccharification and fermentation process. Response surface methodology was a useful method to optimize the conditions for maximum ethanol productivity from MAA-pretreated coconut husk.

Based on the results of current study, MAA-pretreated coconut husk can be considered as potential feedstock for bioethanol production especially in Malaysia where it is available in large quantity and relatively inexpensive.

6.2 Recommendations for Future Research

In order to achieve a higher yield of reducing sugar in the hydrolysis process, the conditions of microwave-assisted-alkaline pretreatment such as duration of treatment and concentration of NaOH solution can be further developed and optimized. A factorial experiment can be designed to further study the effects of concentration of alkaline solution such as NaOH solution and pretreatment time and any potential interactions.

The strains of fermenting microorganisms are extremely important in a fermentation process. For example, genetic engineering principles can be applied to construct a more compatible recombinant strain that is able to utilize different kind of reducing sugars (hexoses and pentoses), and thus bringing the production of ethanol to maximum yield. Hence, fermentation process using genetically engineered strains of microorganisms could be explored and the fermentation process could be optimized for MAA-pretreated coconut husk.

Most of the ethanol related product (beverages and biofuels) produced today is done by the batch system due to its low investment cost and lower risk of contamination. However, studies have shown that the ethanol yield was reported to be higher by using fed-batch culture which has the advantages of reduction of

substrate and end product inhibition, higher productivity of ethanol, decreased fermentation time and higher hydrolysis rate (Tomas-Pejo et al., 2009; Chang et al., 2012). Hence, a construction of mathematical modeling using fed-batch culture is suggested for advanced investigation of the effect on bioethanol productivity by using MAA-pretreated coconut husk as substrate.

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APPENDIX

Publications:

1. Ding, T.Y., Hii, S.L. and Ong, L.G.A., 2012. Comparison of pretreatment strategies for conversion of coconut husk fiber to fermentable sugars. *Bioresources*, 7 (2), pp. 1540-1547. (ISI-Cited Publication; Impactor factor: 1.418)

2. Hii, S.L., Ding, T.Y. and Ong, L.G.A., 2010. Comparative evaluation of pretreatment strategies on enzymatic saccharification of Hylocereus polyrhizus's pericarps for bioethanol production. *Iranica Journal of Energy and Environment,* 1 (4), pp. 275-279.

Awards:

1. Top 8 finalist for oral presentation in postgraduate division.

   Ding, T.Y., Ong, L.G.A. and Hii, S.L., 2010. Assessing the potential of using coconut husk as biomass resource for bioethanol production. *Shell Inter-varsity Student Paper Presentation Contest (S-SPEC 2010 with Theme: Integrating creative mind in science and technology for a sustainable future),* 15-16 March 2010 Dewan Senat Universiti Teknologi Malaysia (UTM).

2. Awarded Best Poster Presentation

   Hii, S.L., Ding, T.Y. and Ong, L.G.A., 2009. A comparison of several pretreatment methods for enhancement of enzymatic digestibility of pericarps of Hylocereus polyrhizus, TWAS Regional Young Scientist Conference, 2-5 November 2009, Armada Hotel, Petaling Jaya, Selangor, Malaysia, pp. 45.

Conferences:

1. Ding, T.Y., Ong, L.G.A., Hii, S.L., 2011. Investigating the potential of using coconut husk as substrate for bioethanol production. 2011 *International Conference on Biotechnology and Environment Management (ICBEM2011)*, 16-18 September 2011, Paramount Hotel, Singapore, pp. 29.