Ethanol Production from Palm Pressed Fiber using Filamentous Fungi

Retno Wulandari

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of
Master of Science in Biotechnology
Prince of Songkla University
2009

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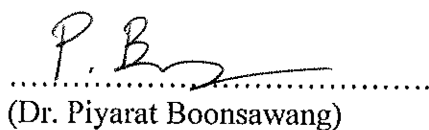

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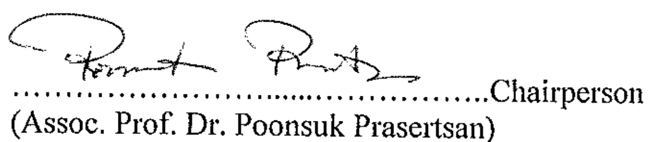

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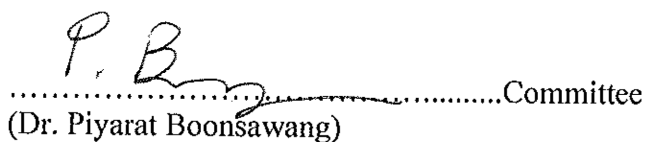

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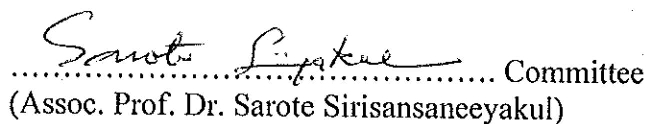

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Thesis title Ethanol Production from Palm Pressed Fiber using Filamentous Fungi

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Academic Year 2009

ABSTRACT

Palm pressed fiber (PPF); residues of palm oil mill process were pretreated using 10% NaOH by three different kinds of physical method (autoclaved 121 °C for 15 minutes, boiled using hot plate 100 °C for 1 hour and microwave 2459 MHz/1200 watt for 10 minutes). The solid residue of pretreated PPF was collected and analyzed for lignocellulose composition. The result showed that pretreatment by boiling could decrease lignification (the residue lignin content of 18.63%), which was more than using autoclave and microwave (the residue lignin content of 20.21% and 20.4%, respectively). In contrary, pretreatment using autoclave gave cellulose of 60%, which was higher than using hotplate and microwave (56% and 58%, respectively). After pretreatment, PPF was hydrolyzed using cellulase (6 U/g), xylanase (6 U/g) and mix of cellulase/xylanase (6:6 U/g) at 40°, 45° and 50° C. The higher reducing sugar (5.71 g/l) was found at 50° C in the mixture of cellulase and xylanase for 48 hours using autoclave pretreated PPF. Meanwhile, the effect of enzyme unit and addition of β-glucosidase were also been analyzed. Enzyme unit was loaded 4, 6 and 8 units, respectively. The highest reducing sugar was found at 8 units of cellulase + 8 units of xylanase + 8 units of β-glucosidase (7.25 g/l).

The fungi was able to grow well and produced high yield of ethanol (yield based on concentration of reducing sugar) when it was using PPF hydrolysate. Ethanol concentration from *Mucor indicus* STR 3237, *Mucor hiemalis* STR 3047 and *Rhizopus oryzae* STR 3099 was 4.23 ± 0.30, 4.10 ± 0.28 and 4.15 ± 0.33 g/l, when it was cultivated in PPF enzymatic hydrolysate under aerobic condition. Meanwhile, ethanol concentration from PPF enzymatic hydrolysate under anaerobic condition was found similar to aerobic condition which gave 4.33 ± 0.07, 4.27 ± 0.05, 4.28 ± 0.41 g/l of

ethanol, respectively. All strains also used to convert directly PPF to ethanol. The amount of ethanol was low at 0.03 ± 0.30 g/l in aerobic and no detection of ethanol in anaerobic condition when *Mucor hiemalis* was used in cultivation of untreated PPF. In addition, the amount of ethanol (0.04 ± 0.27 g/l) was also found only in aerobic condition when alkali/autoclave pretreated PPF was used by *Mucor hiemalis* as carbon source. Meanwhile cultivation of *Rhizopus oryzae* using untreated and alkali/autoclave pretreated PPF gave the ethanol amount 0.085 ± 0.04 and 0.11 ± 0.03 g/l in aerobic condition. Cultivation in anaerobic condition gave 0.117 ± 0.11 and 0.25 ± 0.07 g/l respectively. Furthermore, the ethanol concentration from cultivation in prehydrolysate liquid was also low possibly due to the presence of inhibitors. From all experiments, the ethanol concentration from all three strains (*Mucor indicus, Mucor hiemalis* and *Rhizopus oryzae*) was found higher in anaerobic condition, even though it was not significantly different when compared to aerobic condition. Finally, the two stage fermentation (aerobic and anaerobic condition) was not enhanced ethanol production when PPF enzymatic hydrolysate and prehydrolysate liquid were used as substrates, since the assimilation of sugar was almost consumed at 2 days in aerobic condition. Meanwhile, ethanol concentration was increased when untreated PPF was used. However, from alkali/autoclave pretreated PPF under two stage condition, the ethanol concentration was not significantly different with ethanol concentration from anaerobic condition at one stage fermentation.

Acknowledgements

First of all, I would like to express gratitude to Allah SWT for giving me blessing of the strength and persistence to complete this study.

I would like to express my deep appreciation and highly gratitude to my Advisor, Dr. Piyarat Boonsawang at Department of Industrial Biotechnology, Faculty of Agro-Industry, Prince of Songkla University for her kindness, strong efforts, patience, understanding to guide and assist me through the past year in correcting, reading, criticizing and supporting this thesis.

Special thanks to Assoc. Prof. Dr. Poonsuk Prasertsan and Assoc. Prof. Dr. Sarote Sirisansaneeyakul for their helpful suggestion and dedicating valuable time for thesis examination.

Many thanks for all Indonesian students in the same scheme of Double Degree Scholarship Program. Also, many thanks for all Thai students in Environmental Laboratory and all Industrial Biotechnology students for all their help and support during my research.

I would like to appreciate the Graduate School, Prince of Songkla University, Brawijaya University and Indonesian government under commission of Department of Education (DIKNAS) through Indonesian Embassy in Thailand, for providing the budget and scholarship for this thesis to accomplish.

Finally, I would like to express deeply gratitude to my beloved family for all their support and encouragement for me during my stayed in Thailand. Thank you for my father Arman Sukardi and my mother Ainun Saidi for their caring, loving me all the time. Thank you for my brothers and sisters (Arnita Eka Yanti, Heru Trihandanu, Herry Ageng Santoso, Hendro Ari Saputro, Hadi Imam Wahyudi, Sri Endah Handayani and Sri Kanti Handayani) and also for all my nieces and nephews for their cheering, supporting and understanding.

Retno Wulandari

CONTENTS

	Pages
Contents	vi
List of Tables	vii
List of Figures	ix
List of Abbreviation	xii
Chapter 1 Introduction	1
Literature Review	3
Objectives	39
Chapter 2 Materials and Methods	40
Materials	40
Methods	41
Chapter 3 Results and Disccusion	
1. Effect of pretreatment for palm pressed fiber (PPF)	49
2. Enzymatic hydrolysis of pretreated palm pressed fiber (PPF)	51
2.1. Effect of type of enzyme on reducing sugar production	51
2.2. Effect of addition of β-glucosidase and enzyme concentration	56
3. Ethanol production from palm pressed fiber (PPF)	58
3.1. Fermentation of PPF hydrolysate	58
3.2. Fermentation of untreated and treated PPF	65
3.3. Fermentation of prehydrolysate from pretreatment	73
4. Effect of one and two stage condition	75
Chapter 4 Conclusion	81
Reference	83
Appendix	91
Vitae	93

LIST OF TABLES

Table		Page
1.	Composition of lignocellulose materials from various biomass	5
2.	Palm oil mill processes and activities	9
3.	Advantages and disadvantages of hydrolysis using acid	21
4.	Advantages and disadvantages of hydrolysis using enzyme	22
5.	Ethanol productivities in cultivation of different zygomycetes strains on (a) glucose and (b) xylose	33
6.	Yield of ethanol production in cultivation of different zygomycetes strains in dilute-acid hydrolyzate	34
7.	Palm pressed fiber (%) composition with and without pretreatment using alkali (NaOH) solution	50
8.	Reducing sugar concentration at enzymatic hydrolysis of pretreated PPF at different temperature and enzyme type for 48 hour	53
9.	Reducing sugar concentration (g/l) at enzymatic hydrolysis of alkali/autoclave treated PPF at different enzyme concentration (cellulase:xylanase at 4,6 and 8 U/g substrate) and addition of β-glucosidase (4,6 and 8 U/g substrate) pH 5.0 for 48 hour at 50 °C.	56
10.	Ethanol concentration and ethanol yield of *Mucor indicus, Mucor hiemalis* and *Rhizopus oryzae* using PPF enzymatic hydrolysate as carbon source for 5 days cultivation at aerobic and anaerobic condition with initial sugar concentration of 8.3 g/l.	60
11.	Ethanol yield from palm pressed fiber (PPF) and others material by fungi.	64
12.	Ethanol concentration, ethanol yield, ethanol productivity of *Mucor indicus, Mucor hiemalis* and *Rhizopus oryzae* using untreated and alkali/autoclave treated PPF as carbon source for 5 days cultivation at aerobic and anaerobic condition	70

LIST OF TABLES (CONTINUED)

Table **Page**

13. The maximal CMCase activities from cultivation of *Mucor indicus, Mucor hiemalis* and *Rhizopus oryzae* using alkali/autoclave pretreated PPF of 5 g/100 ml pH 5.5 at 30 °C and 50 °C after 5 days cultivation. 72

14. Ethanol concentration and ethanol yield of *Mucor indicus, Mucor hiemalis* and *Rhizopus oryzae* using prehydrolysate liquid (initial sugar 5.6 g/l) as carbon source for 5 days cultivation at aerobic and anaerobic condition at pH 5.5. 74

15. Maximum ethanol concentration (g/l) of one stage fermentation compared with two stage fermentation of PPF enzymatic hydrolysate, alkali/autoclave pretreatead PPF, untreatead PPF and prehydrolysate liquid using *Rhizopus oryzae.* 76

16. Ethanol yield ($g_{ethanol}/g_{sugar}$) and ethanol productivity ($g_{ethanol}/g_{sugar}.day$) of two stage fermentation of palm pressed fiber (PPF) enzymatic hydrolysate, alkali/autoclave pretreatead PPF, untreatead PPF and prehydrolysate liquid using *Rhizopus oryzae.* 77

17. Ethanol yield from palm pressed fiber (PPF) and others material by *Rhizopus oryzae* one stage and two stage fermentation. 80

LIST OF FIGURES

Figure		Page
1.	Cellulose structure	6
2.	Hemicellulose structure	7
3.	Lignin structure	8
4.	Effect of temperature on the enzymatic hydrolysis of lime-pretreated (100 mg/g, 121 °C, 1 h) rice hulls (15, w/v) at pH 5.0 for 72 h using a cocktail of three commercial enzyme preparations (cellulase, β-glucosidase and hemicellulase) at each enzyme dose level 0.05 ml/g hulls.	28
5.	Cultivation of *Zygomycetes* strains on glucose and xylose as the carbon source.	32
6.	Model of glucose metabolism in the filamentous fungus *Rhizopus oryzae* Ext- stands for extracellular G-6-P for glucose-6-phosphate, F-6-P for fructose-6-phosphate and F-1,6-bP for fructose-1,6-bisphosphate.	36
7.	Cultivation of *Mucor indicus* in anaerobic (a and b) and aerobic (c and d) conditions on glucose, xylose, detoxified and non detoxified hydrolyzates.	38
8.	Experimental scheme	48
9.	Saccharification from hydrolysis of palm pressed fiber autoclave pretreated by 10% NaOH for 15 min by cellulase (6 U/g), xylanase (6 U/g) and mixture of cellulase and xylanase (6:6) U/g substrate in 0.05M citrate buffer pH 5.0) with different temperature (40 °C, 45 °C and 50 °C).	54
10.	Saccharification from hydrolysis of PPF by 10% NaOH for 60 min on hot plate (a) and 10% NaOH for 10 min in microwave (b) by cellulase (6 U/g), xylanase (6 U/g) and mixture of cellulase and xylanase (6:6) U/g substrate in 0.05M citrate buffer pH 5.0) with different temperature (40 °C, 45 °C and 50 °C).	55

LIST OF FIGURES (CONTINUED)

Figure	Page
11. Saccharification from hydrolysis of alkali/autoclave pretreated PPF using mixture of cellulase and xylanase (4:4, 6:6, 8:8 U/g) substrate and addition of β-glucosidase (4,6 and 8 U/g substrate) in 0.05M citrate buffer pH 5.0 at 50 °C.	57
12. Ethanol and reducing sugar concentration (g/l) of *Mucor indicus, Mucor hiemalis and Rhizopus oryzae* in aerobic (a) and anaerobic (b) condition using PPF enzymatic hydrolysate 8.3 (g/l) pH 5.5 for 5 days on 150 rpm.	61
13. pH profile of *Mucor indicus, Mucor hiemalis and Rhizopus oryzae* in aerobic (a) and anaerobic (b) condition using PPF enzymatic hydrolysate 8.3 (g/l) pH 5.5 for 5 days on 150 rpm.	62
14. Ethanol and reducing sugar concentration of *Mucor indicus, Mucor hiemalis and Rhizopus oryzae* using untreated (a) and alkali/autoclave pretreated (b) PPF in aerobic for 5 days fermentation pH 5.5 substrate 5 g/100 ml at 150 rpm.	66
15. Ethanol and reducing sugar concentration of *Mucor indicus, Mucor hiemalis and Rhizopus oryzae* using untreated (c) and alkali/autoclave pretreated (d) PPF in anaerobic for 5 days fermentation pH 5.5 substrate 5 g/100 ml at 150 rpm.	67
16. pH profile of *Mucor indicus, Mucor hiemalis and Rhizopus oryzae* under aerobic and anaerobic condition using 5 g/100 ml alkali/autoclave pretreated PPF pH 5.5 for 5 days on 150 rpm.	68
17. Biomass dried weight from cultivation of *Mucor indicus, Mucor hiemalis* and *Rhizopus oryzae* in prehydrolysate liquid at pH 5.5 for 5 days in aerobic and anaerobic condition.	74
18. Reducing sugar concentration (g/l) of *Rhizopus oryzae* using untreated PPF alkali/autoclave treated PPF prehydrolysate and PPF enzymatic hydrolysate in two stage fermentation pH 5.5 at 150 rpm.	78

LIST OF FIGURES (CONTINUED)

Figure **Page**

19. Ethanol concentration (g/l) of *Rhizopus oryzae* using untreated PPF, alkali/autoclave treated PPF, prehydrolysate liquid and PPF enzymatic hydrolysate in two stage fermentation pH 5.5 at 150 rpm. 79

LIST OF ABBREVIATION

ANOVA	Analysis of variance
CMC	Carboxymethyl cellulose
DNS	Dinitrosalicylic Acid
GC - FID	Gas Chromathography- Flame Ionized Detector
HPLC	High Performance Liquid Chromatography
OD	Optical density
NaOH	Sodium hydroxide
PPF	Palm Pressed Fiber
PDA	Potato dextrose agar
rpm	Revolution per minute
SPSS	Statistical package for the social sciences
TISTR	Thailand Institute of Scientific and Technological Research

CHAPTER 1

INTRODUCTION

Negatives implication of fossil fuel aligned with environment problems and the lack of fossil material supply leads many countries to search alternative energy resources. Bioenergy has the potential to make a real contribution as a renewable energy and also environmentally friendly. Bioethanol is the one example of bioenergy used as liquid fuel. It is an alcohol produced from sugar, starches or from lignocellulose (Dermibas, 2003). Lignocellulosic materials, which are relatively cheap and plentiful, are considered as the main source of feedstocks for low-cost bioethanol production. Lignocellulose such as paper, wood, agricultural residues and other fibrous plant material are in general very widespread and abundant. Oil palm upstream industry is one of the major contributors to the lignocellulosic-rich solid waste materials, generated in the field and the oil mill. The mill residues include mesocarp fiber, shell, palm kernel cake, boiler ash, empty fruit bunches, palm oil mill effluent and bunch ash. Except the palm kernel cake, boiler and bunch ash and palm oil effluent, all of the residues contain high percentage of lignocellulose and therefore useful to be used as a source of carbon for ethanol production (Zobir, 2001).

Lignocellulosic biomass consists of cellulose, hemicellulose and lignin content. Cellulose is a linear polymer of glucose unit linked by β-1,4-glucosidic bonds. It constitutes 35-50% in agricultural lignocellulosic biomass. Hemicelluloses are heterogeneous polymers of primarily pentose (C5 sugars such as xylose, arabinose), hexose (C6 sugars such as mannose, glucose, galactose) and sugar acids. Hemicellulose constitutes 20-35% in agricultural lignocellulosic biomass (Agbogbo *et al.*, 2006). However, only cellulose and hemicellulose can be hydrolyzed to reducing sugars by enzymes.

Pretreatment process is important role due to both enzymatic hydrolysis and fermentation of lignocellulose material. Acid and alkaline treatments are wellknown and often used in industry. Alkaline pretreatment through soaking material

into alkaline solution such as NaOH is often used to break the bonds between lignin and carbohydrates and disrupts the lignin structure, which makes the carbohydrates is accessible to enzyme attack (Chang *et* al., 2001 cited by Galbe and Zacchi, 2007).

It is well-known that *Saccharomyces cerevisiae* is the most successful microorganisms to produce ethanol from hexose (C6 sugar). However, it is unable to ferment pentoses (C5 sugar). In recent study for ethanol production, fungi from family of Zygomycetes such as *Mucor* sp. and *Rhizopus* sp. showed good performances in converting cellulose and hemicelulose into pentose and hexose (C5 and C6 sugars) and produced ethanol (Millati *et al.*, 2005; Panagiotou *et al.*, 2005a; Panagiotou *et al.*, 2005b; Karimi *et al.*, 2006a; Karimi *et al.*, 2008). These fungi have several advantages compared to *S.cerevisiae* such us capability to use xylose and to produce ethanol with comparable yield and productivity at 37° C while *S.cerevisiae* only have optimum temperature range from 28° until 35° C (Karimi, 2005). Therefore, ethanol production using filamentous fungi could be good alternative for fermentation of lignocellosic biomass.

This study aimed for studying the effect of alkali treatment process of palm pressed fiber (PPF). The best result from pretreatment process was used for next hydrolysis step using different enzyme doses. The effect of enzyme hydrolysis using cellulase and xylanase on reducing sugar production was investigated. Fermentation process was conducted using the best result from hydrolysis process using three fungi strains. The best strain performance was used to obtain the effect of suitable or best fermentation condition for ethanol production.

Literature Review

1. Bioethanol as fuel

Biofuel is a generic term for any liquid fuel produced from sources other than mineral reserves such as oil, coal and gas. In general biofuels can be used as a substitute for, or an additive to, gasoline and diesel fuel in most transport and non-transport applications. The most commonly used biofuels are biodiesel and bioethanol. Bioethanol can be defined as ethanol produced by fermenting sugars using microorganism, which is then recovered by distillation. In lignocellulose material, converting biomass to ethanol involves the processing of three major components in the feedstocks which are hemicellulose, (a polymer of C5 sugars), cellulose (a polymer of C6 sugars) and lignin (a complex polyaromatic material). The hemicellulose and cellulose fractions provide sugars for fermentation to ethanol. Cellulosic ethanol is particularly promising because it is derived from low cost and plentiful feedstocks which can continuously produce and achieve higher yield of ethanol and it is also environmentally friendly (Wyman, 2007). Unlike gasoline, ethanol is an oxygenated fuel that contains 35% oxygen, which produces particulate and NOx emissions from combustion. When burned, ethanol derived from fermentation produces no net increase in carbon dioxide in the atmosphere. It is an octane enhancing additive and removes free water which can plug fuel lines in cold water (Dermibas, 2003).

The global annual potential bioethanol production from the major crops, corn, barley, oat, rice, wheat, sorghum, and sugar cane has been estimated. In order to avoid conflicts between human food use and industrial use of crops, only the wasted crop, which is considered as feedstock, is used. Lignocellulosic biomass such as crop residues and sugar cane bagasse are included in feedstock for producing bioethanol. The potential bioethanol production could replace around 32% of the global gasoline consumption when bioethanol is used in E85 fuel for a midsize passenger vehicle (Kim and Dale, 2004).

1.1. Ethanol and its properties

Ethanol or ethyl alcohol is the most common of alcohols. It is the form of alcohol that is in alcoholic beverage and is easily produced from corn, sugar or fruits through fermentation of carbohydrates and has been described as one of the most exotic synthetic oxygen-containing organic chemicals because of its unique combination of properties as a solvent, a germicide, a beverage, an antifreeze, a fuel, a depressant, and especially because of its versatility as a chemical intermediate for other organic chemicals. Its chemical structure is CH_3CH_2OH. It is less toxic than methanol. Ethanol is a colorless liquid with a melting point of 144 °C and a boiling point of 78 °C. Ethanol is frequently used to form blended gasoline fuels in concentration between 10-85% and now it has been investigated as a fuel for direct ethanol fuel cells (DEFC) (Minteer, 2006).

2. Characteristic of lignocellulose

Lignocellulosic materials are composed of mainly cellulose, hemicellulose, and lignin. Lignocellulosic materials considered for ethanol production are hardwood, softwood, forestry residues, agricultural residues, and municipal solid waste (MSW). The composition of the raw material depends on the source. Both cellulose and hemicellulose, which typically constitute 36-61% and 13-39% of the total dry matter, respectively, can be used for ethanol production. Generally, hard wood contains about 50% cellulose (dry base), 23% hemicellulose, and 22% lignin. Herbaceous materials and agricultural residues contain a somewhat higher proportion of hemicellulose (30 – 33%) relative to cellulose (38 – 45%), and have lower levels of lignin (10 – 17%) (McMillan, 1994). The amount of lignin in softwoods is appreciably higher than in hardwoods, usually between 25-35%, the cellulose content is about 46% and the hemicellulose content about 21% (Lynd *et al.*, 1999). Softwood hemicellulose contains a high proportion of mannose units and more galactose units than hardwood hemicellulose whereas hardwood hemicellulose contains a high proportion of pentoses (Olsson and Birbel, 1996). Structural composition of various types of cellulosic

biomass materials is given in Table 1. To achieve maximum ethanol yield, all monosaccharides have to be fermented.

Table 1. Composition of lignocellulose materials from various biomass.

Substrate	Cellulose (%, w/w)	Hemicellulose (%, w/w)	Lignin (%, w/w)
Rice straw	32-47	19-27	5-24
Cornstalks	39-47	26-31	3-5
Palm Pressed Fiber	40	24	21
Oil palm empty fruit bunch	42.85	24.01	11.70
Grasses	25-40	25-50	10-30
Hardwoods	45±2	30±5	20±4
Softwood	42±2	27±2	28±3
Wheat straw	37-41	27-32	13-15
Cotton	80-95	20-50	-

Source : Dermibas (2003); Karimi *et al.*. (2005)

2.1. Cellulose

Cellulose is an organic compound with the formula $C_6H_{10}O_6$. It is a structural polysaccharide derived from β-glucose, which condenses through β (1→4)-glycosidic bonds (Figure 1). This linkage motif contrasts with that for α (1→4)-glycosidic bonds present in starch and other carbohydrates. Cellulose chains have more than 700 until 2000 unit of glucose which attached one to another and formed polymer chain. It consists of a three-dimensional network of chains of glucose units and some complex glucose derivatives. Cellulose fiber is divided into two parts of regions in microfibril, which are crystalline and amorphous regions (Wikipedia, 2008a).

The crystalline and amorphous regions, cellulose fiber contain various types of irregularities such as kinks or twist of microfibril or voids such as surface micropores, large pits and capillarities. The crystalline nature of cellulose implies a

structural order in which all of the atoms are fixed in dicrete positions with respect to one another. An important feature of the crystalline array is that the component molecules of individual microfibril are packed sufficiently tightly to prevent penetration not only by enzyme but even by small molecules such as water.

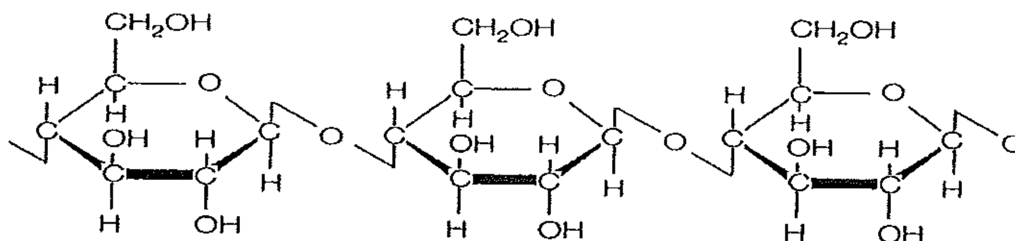

Figure 1. Cellulose structure
Source: Greenspirit (2009)

2.2. Hemicellulose

Hemicelluloses are imbedded in the cell walls of plants, sometimes in chains that form a 'ground' - they bind with pectin to cellulose to form a network of cross-linked fibres. While cellulose is crystalline, strong, and resistant to hydrolysis, hemicellulose has a random, amorphous structure with little strength. It is easily hydrolyzed by dilute acid or base as well as myriad hemicellulase enzymes. Hemicellulose contains many different sugar monomers. In contrast, cellulose contains only anhydrous glucose. For instance, besides glucose, sugar monomers in hemicellulose include xylose, mannose, galactose, rhamnose, and arabinose. Hemicelluloses contain most of the D-pentose sugars and occasionally small amounts of L-sugars as well. Xylose is always the sugar monomer present in the largest amount, but mannuronic acid and galacturonic acid also tend to be present. Unlike cellulose, hemicellulose (also a polysaccharide) consists of shorter chains around 200 sugar units as opposed to 700 - 2000 glucose molecules per polymer seen in cellulose. In addition, hemicellulose is a branched polymer, while cellulose is unbranched (Wikipedia, 2008b).

One of important derivative chemical from hemicellulose is furfural. Furfural ($C_5H_4O_2$) is essential chemical product which appears from agriculture residue and hemicellulose. Chemically, furfural participates in the same kinds of reactions as other aldehydes and other aromatic compounds. The aromatic stability of furfural is not as great as in benzene, and furfural participates in hydrogenation and other addition reactions more readily than many other aromatics (Wikipedia, 2008b).

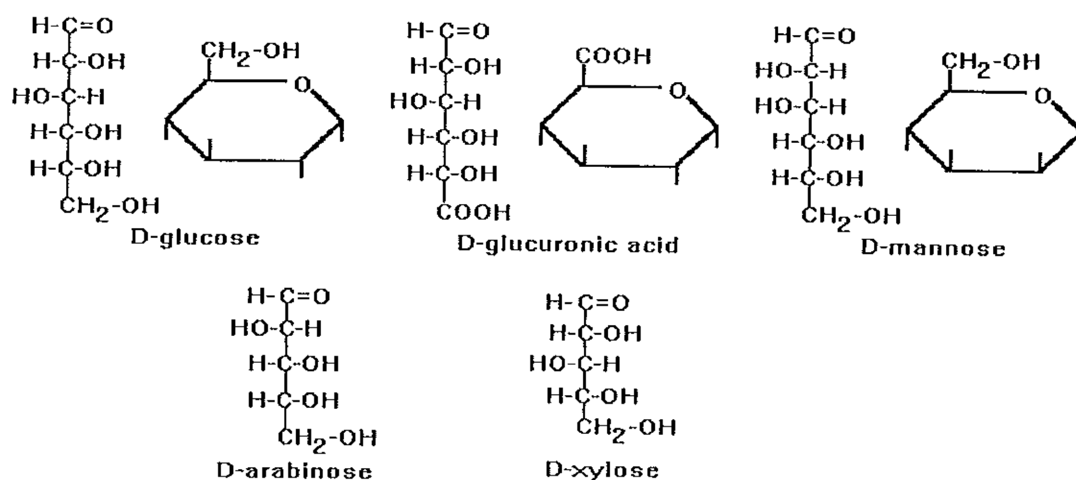

Figure 2. Hemicellulose structure

Source: Rensselaer Polytechnique Institute (2009)

2.3. Lignin

Lignin is a complex chemical compound most commonly derived from wood and an integral part of the cell walls of plants. It is the most abundant organic polymer on Earth after cellulose, employing 30% of non-fossil organic carbon and constituting from a quarter to a third of the dry mass of wood. The compound has several unusual properties as a biopolymer, not least its heterogeneity in lacking a defined primary structure. Lignin fills the spaces in the cell wall between cellulose, hemicellulose and pectin components, especially in tracheids, sclereids and xylem. It is covalently linked to hemicellulose and thereby crosslinks different plant polysaccharides, conferring mechanical strength to the cell wall and by extension the

plant as a whole. It is particularly abundant in compression wood, but curiously scarce in tension wood (Wikipedia, 2008c).

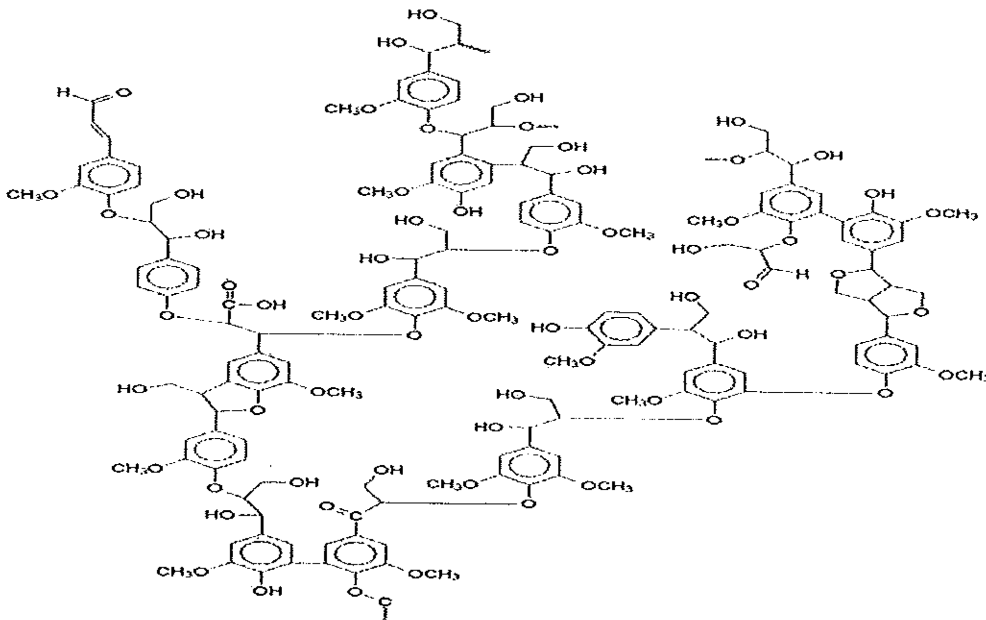

Figure 3. Lignin structure
Source: University of Waikato (2009)

3. Oil palm (*Elaeis guineensis*)

The oil palms (*Elaeis guineensis*) comprise two species of the *Arecaceae* or palm family. Mature trees are single-stemmed, and grow up to 20 meter tall. The leaves are pinnate, and reach between 3 and 5 meter long. The flowers are produced in dense clusters, each individual flower is small, with three sepals and three petals. Unlike, the coconut palm, the oil palm does not produce offshoots, propagation is by sowing the seeds. The fruit takes 5–6 months to mature from pollination to maturity; it comprises an oily, fleshy outer layer (the pericarp), with a single seed (kernel), also rich in oil (Wikipedia, 2009a). Oil palms are commonly used in commercial agriculture in the production of palm oil. The oil palm is a tropical palm tree therefore it can be cultivated easily in Malaysia. The oil palm tree in Malaysia originated from West Africa where it was growing wild and later developed into an agricultural crop.

3.1. Palm oil industry

Palm oil is an edible plant oil derived from the fruit and kernels (seeds) of the oil palm *Elaeis guineensis*. Palm oil is naturally reddish because it contains a high amount of beta-carotene (though boiling it destroys the carotenoids and renders the oil colourless). Palm oil is one of the few vegetable oils relatively high in saturated fats (like coconut oil) and thus semi-solid at room temperature. The oil is widely used as a cooking oil, as an ingredient in margarine, and is a component of many processed foods. It is also an important component of many soaps, washing powders and personal care products, is used to treat wounds, and also controversially as a feedstock for biofuel. (Wikipedia, 2009b). Palm oil industry includes several processes in palm oil extraction (Table 2). Some primary palm oil mills processes are explained by Mahlia (2001) as following:

Table 2. Palm oil mill process and activities

Process	Activity
1. Sterilization	When the fruit bunches are cut from an oil palm and stored for several days, much of fruit loosens naturally and may be shaken or knocked off bunches. If the fruits were simply pounded in a mortar and pressed cold, oil with a very high FFA content would be obtained. This would happen because of the fat splitting enzymes present in the pericarp would remain active and it hydrolyzed much of the oil when the fruit was pulped in the mortar. The oil yield obtained on pressing would be very small. It would be possible to avoid such as rise in FFA during the pulping process and obtain high oil yield from naturally stripped fruit. This fruit must be cooked before being digested and pressed. Both processes can be done using steam above atmospheric pressure. The pressure vessel used for cooking palm oil fruit with steam is known as a sterilizer and the process as sterilization.

Table 2. (continued).

Process	Activity
2. Stripping	The objective of stripping is to separate the sterilized fruits from the sterilized bunch stalks.
3. Digestion	After the bunches have been stripped, the sterilized fruit together with the accompanying calyx leaves, must be reheated and the pericarp loosened from the nuts and prepared to pressing. This is performed in steam heated vessels with stirring arms, known as digesters or kettles
4. Oil extraction	The most usual method of extracting oil from the digested palm oil fruit is by pressing. The type of press used in this palm oil is the screw type press.
5. Clarification	The crude palm oil extracted from the digested palm oil fruit by pressing contains varying amounts of water, together with impurities consisting of vegetable matter, some of which is in the form of insoluble solid and some of which is dissolved in the water. The water present in the crude palm oil can largely be removed by settling or centrifuging, since most of it is free or undissolved. A small proportion of it, however, is dissolved in the oil and this can only be removed by evaporation in the dehydrator with or without the assistance of vacuum.
6. Nutfiber separation	When digested fruit is pressed to extract the oil, a cake made up of nuts and fiber is produced. The composition of this cake varies considerably, being dependent on the type of fruit. The cake is given a preliminary breaking treatment before being fed into the nut/fiber separator that may bring about separation by mechanical means or by use of an air steam.
7. Kernel extraction and drying	When the fiber has been separated from nuts, the latter can then be prepared for cracking and cracked. Any uncracked nuts must be removed and recycled and the shell separated from the kernels.

Source: Mahlia (2001)

3.2. Palm oil industry waste

Palm oil biomass can be utilized to produce various types value added products. The palm oil biomasses and its further application were listed as:

3.2.1. Empty fruit bunch (EFB)

EFB is the major component of all solid wastes of oil palm process. EFB are rich in sugar, it makes EFB become a good resources for sugar production. Rahman *et al.* (2006) was conducting batch hydrolysis of oil palm empty fruit bunch fiber by performed acid hydrolysis at operating temperature 120 °C using various concentration of sulfuric acid (2–6%) and reaction time (0–90 min). Moreover, it was found that optimum H_2SO_4 concentration and reaction time obtained under operating temperature of 120 °C was 6% and 15 min, respectively. Optimum concentration of xylose, glucose, hydrolysate were 29.4, 2.34 (g/l), respectively. Furthermore, EFB can be used as a feedstock for second generation ethanol to produce biooil and bioethanol through pyrolysis and fermentation or hydrolysis, respectively. Flexible-fuel vehicles can run on about 85% ethanol made from these biomasses.

3.2.2. Palm pressed fiber (PPF)

PPF contains, on a dry weight basis, approximately 40% cellulose, 21% lignin, 24% pentosan and 5% ash (Kirkaldy and Sutanto, 1976 cited by Azis, 2002). Choo *et al.* (2004) investigated that palm-pressed fiber was a good candidate for phospholipids production. The result showed that they succeed to extract PPF with hexane and 95% ethanol, yielded 46,800 ppm of phospholipids. In addition, PPF also suggested as a good sources for bioethanol production. Subkaree (2008) studied about the optimum condition in simultaneous saccharification and fermentation (SSF) of PPF fermentation using *S. cereviciae* and it was found that the 100 g/l of PPF at pH 5.0, 35 ^{0}C using cellulase from *T. reesei* 6 FPU/g substrate and β-glucosidase from *A. niger* 3 IU/g substrate gave the highest ethanol concentration of 10.38 g/l and yield of 0.19 $g_{EtOH}/g_{cellulose}$ in 24 hr of SSF.

3.2.3. Palm kernel shell (PKS)

PKS is the most difficult waste to decompose. The shell size is uniform and is not as bulky as the EFB. They are usually left unused in the factory or

disposed of by the land-fill method. In terms of energy, PKS is an energy intensive substance. Local industries that require process heat (or steam) generally have furnaces (or boilers) designed for firewood or fuel oil. PKS contains 20.3% of fixed carbon and is physically similar to the coconut shell, which has been used to produce the activated carbon successfully. It is anticipated that the stringent environment control measures will increase the demand for activated carbon in the future (Prasertsan *et al.*, 1996).

3.2.4. Palm oil mill effluent (POME)

Palm oil mill effluent (POME) is the mixed effluent generated from two major sources; sterilizer and decanter or separator during the extraction of palm oil. POME usually contained very high organic matter, suspended solids and oil (1-2%) (Prasertsan *et al.*, 1990).

4. Pretreatment of lignocellulose

Galbe *et al.* (2007) reviewed about pretreatment process of lignocellulose material for efficient ethanol production. The report tells us that effectives pretreatment should have a number of features, it has to:

1. Result in high recovery of all carbohydrates.
2. Result in high digestibility of the cellulose in the subsequent enzymatic hydrolysis.
3. Produce no or limited amounts of sugar and lignin degradation products. The pretreatment liquid should be possible to ferment without detoxification.
4. Result in high solids concentration as well as high concentration of liberated sugars in the liquid fraction.
5. Have a low energy demand or be performed in a way so that the energy can be reused in other process steps as secondary heat.
6. Have a low capital and operational cost.

4.1. Pretreatment types

The digestibility of cellulose present in lignocellulosic biomass is hindered by many physicochemical, structural, and compositional factors. In the conversion of lignocellulosic biomass to fuel, the biomass needs to be treated so that the cellulose in the plant fibers is exposed. Pretreatment uses various techniques, including acid, ammonia fiber explosion, chemical treatment, biological treatment, and steam explosion, to alter the structure of cellulosic biomass to make cellulose more accessible. Then, acids or enzymes can be used to break down the cellulose into its constituent sugars. Enzyme hydrolysis is widely used to break down cellulose into its constituent sugars (Kumar *et al.*, 2009). Furthermore, Kumar *et al.* (2009) described, the goal of the pretreatment process is to remove lignin and hemicellulose, reduce the crystallinity of cellulose, and increase the porosity of the lignocellulosic materials. Pretreatment must meet the following requirements: (1) improve the formation of sugars or the ability to subsequently form sugars by hydrolysis, (2) avoid the degradation or loss of carbohydrate, (3) avoid the formation of byproducts that are inhibitory to the subsequent hydrolysis and fermentation processes, and (4) be cost-effective. The following pretreatment technologies have been used for pretreatment of lignocellulosic biomass.

4.1.1. Physical methods

Physical methods is commonly treatment used by grinding and milling raw material to reduce the size of the raw material to increase the hydrolysis rate by increasing the surface area for further reaction (Phillip *et al.*, 1979 cited by Aziz *et al.*, 2002). Comminution of lignocellulosic materials through a combination of chipping, grinding, and/or milling can be applied to reduce cellulose crystallinity. The size of the materials is usually 10-30 mm after chipping and 0.2-2 mm after milling or grinding. Vibratory ball milling was found to be more effective than ordinary ball milling in reducing cellulose crystallinity of spruce and aspen chips and in improving their digestibility. The final particle size and biomass characteristics determine the

power requirement for mechanical comminution of agricultural materials (Kumar *et al.*, 2009).

4.1.2. Steam pretreatment (autohydrolysis)

Chornet and Overend (1988) cited by Jeoh (1998) describe steam explosion as being a thermomechanochemical process. The breakdown of structural components is aided by heat in the form of steam (thermo), shear forces due to the expansion of moisture (mechano), and hydrolysis of glycosidic bonds (chemical). By the steam pretreatment, the raw material is usually treated with high-pressure saturated steam at a temperature typically between 120 °C and 240 °C (corresponding to a pressure between 5 and 34 bar), which is maintained for several seconds to a few minutes, after which the pressure is released. During pretreatment some of the raw material, predominantly hemicellulose, is solubilized and found in the liquid phase as oligomeric and monomeric sugars. The cellulose in the solid phase then becomes more accessible to the enzymes (Galbe *et al.*, 2007).

Wang *et al.* (2008) studied about the effect of NaOH pretreatment of bermudagrass by autoclaving at 121°C using 0.75%, 1%, 2% and 3% (w/v) NaOH for 15, 30, 60 and 90 minutes. After that, enzymatic hydrolysis has been conducted using 40 FPU and 70 CBU/g of dry biomass, it was used to investigate the production of reducing sugar after pretreatment. The result showed the optimal NaOH pretreatment conditions at 121°C for glucose and xylose production are 15 minutes and 0.75% and was not significantly different with pretreatment using 1% of NaOH. Furthermore, Wang *et al.* (2008) suggested that by increasing temperature, reduced the optimal pretreatment time. Pretreatment with temperature below 121°C did not perform as efficiently as 121°C pretreatment on reducing sugars production. The highest reducing sugars yield can reach up to approximate 86% of theoretical maximum for sodium hydroxide pretreatment of bermudagrass.

4.1.3. Conventional heating

Pretreatment by using conventional heating is previously likeable to use, due to the simple preparation and procedures. However, conventional heating is having several disadvantages such as slower process and introduction of

heat into the sample from the surface, resulting in less structure change. Subkaree (2008) reported the pretreatment of PPF using NaOH and $Ca(OH)_2$ from 1 to 15% (w/v) by heated on hot plate with boiling time from 15 to 90 minutes. The results show that palm pressed fiber residue after NaOH pretreatment was 36 - 66% (w/w) while palm pressed fiber residue after $Ca(OH)_2$ pretreatment was 79 - 86% (w/w). However, NaOH pretreatment can reduce lignin content more than $Ca(OH)_2$ pretreatment. The 10% NaOH/Boiling 15 min was the optimal condition for palm pressed fiber pretreatment. At this condition, the highest cellulose content of 54.13 ± 0.87 % (w/w) was obtained with lignin reduction of 44.14 ± 1.10 % (w/w). For pretreated by $Ca(OH)_2$ had cellulose content and lignin reduction after pretreated by less than 37% and 25% (w/w), respectively.

4.1.4. Microwave treatment

Recently, microwave is used to treat lignocelluloses was reported by Hu and Wen (2007). Thermal effects arise from the different characteristics of microwave dielectric heating and conventional heating. Microwave heating uses the ability of some compounds (liquids or solids) to transform electromagnetic energy into heat. Energy transmission is produced by dielectric losses, which is in contrast to conduction and convection processes observed in conventional heating. The magnitude of heating depends on the dielectric properties of the molecules, also in contrast to conventional heating. In contrast, conventional heating is slow and is introduced into the sample from the surface (De la Hoz, *et al.*, 2005). When microwave is used to treat lignocelluloses, it selectively heats the more polar (lossy) part and creates a "hot spot" with the inhomogeneous materials. It is hypothesized that this unique heating feature results in an "explosion" effect among the particles, and improves the disruption of the crystal structures (Hu and Wen, 2007).

Keshwani *et al.* (2007) reported the pretreatment of switchgrass samples immersed in water, dilute sulfuric acid and dilute sodium hydroxide solutions were exposed to microwave radiation at varying levels of radiation power and residence time. Pretreated solids were enzymatically hydrolyzed using 25 FPU/g and reducing sugars in the hydrolysate were analyzed. Microwave radiation of switchgrass

at lower power levels resulted in more efficient enzymatic hydrolysis. The application of microwave radiation for 10 minutes at 250 watts to switchgrass immersed in 3% sodium hydroxide solution (w/v) produced the highest yields of reducing sugar. Results were comparable to conventional 60 minute sodium hydroxide pretreatment of switchgrass. The findings suggest that combined microwave-alkali is a promising pretreatment method to enhance enzymatic hydrolysis of switchgrass.

4.1.5. Chemical methods

Kumar *et al.* (2009) listed following pretreatment technologies using chemical have been used for pretreatment of lignocellulosic biomass.

4.1.5.1. Ozonolysis.

Ozone treatment is one way of reducing the lignin content of lignocellulosic wastes. This result in an increase of the in vitro digestibility of the treated material, and unlike other chemical treatments, it does not produce toxic residues. Ozone can be used to degrade lignin and hemicellulose in many lignocellulosic materials such as wheat straw, bagasse, green hay, peanut, pine, cotton straw, and poplar sawdust. The degradation is mainly limited to lignin. Hemicellulose is slightly affected, but cellulose is not. The rate of enzymatic hydrolysis increased following 60% removal of the lignin from wheat straw using an ozone pretreatment. Enzymatic hydrolysis yield increased from 0% to 57% as the percentage of lignin decreased from 29% to 8% after ozonolysis pretreatment of poplar sawdust. Ozonolysis pretreatment has an advantage that the reactions are carried out at room temperature and normal pressure. Furthermore, the fact that ozone can be easily decomposed by using a catalytic bed or increasing the temperature means that processes can be designed to minimize environmental pollution. A drawback of ozonolysis is that a large amount of ozone is required, which can make the process expensive.

4.1.5.2. Acid Hydrolysis.

Concentrated acids such as H_2SO_4 and HCl have also been used to treat lignocellulosic materials. Pretreatment with acid hydrolysis can result in improvement of enzymatic hydrolysis of lignocellulosic biomasses to release

fermentable sugars. Although they are powerful agents for cellulose hydrolysis, concentrated acids are toxic, corrosive, hazardous, and thus require reactors that are resistant to corrosion, which makes the pretreatment process very expensive. In addition, the concentrated acid must be recovered after hydrolysis to make the process economically feasible (Sivers and Zacchi, 1995 cited by Sun and Cheng, 2002). Dilute-acid hydrolysis has been successfully developed for pretreatment of lignocellulosic materials. Sulfuric acid at concentrations usually below 4% has been of the most interest in such studies as it is inexpensive and effective. It has the advantages of not only solubilizing hemicellulose but also converting solubilized hemicellulose to fermentable sugars (Saha, 1999). Recently, acid pretreatment has been used on a wide range of feedstocks ranging from hardwoods to grasses and agricultural residues. Lu *et al.* (2007) carried out pretreatment of corn stover for sulfuric acid concentrations of 2%, 4%, and 6% at 80, 100, and 120 °C. The optimum conditions for corn stover pretreatment were at H_2SO_4 concentration of 2.0% and a reaction time of 43 min at 120 °C. Up to 77% xylose yield was obtained, whereas the glucose yield was only 8.4%. Moreover, solid phase from treated corn stover showed good susceptibility toward enzymatic hydrolysis, leading to solutions containing up to 42.1 g of glucose/100 g of substrate, equivalent to a conversion yield of 70% under the optimum conditions.

4.1.5.3. Alkaline Hydrolysis.

Some bases can be used for the pretreatment of lignocellulosic materials, and the effect of alkaline pretreatment depends on the lignin content of the materials. Alkali pretreatment processes utilize lower temperatures and pressures than other pretreatment technologies. Alkali pretreatment can be carried out at ambient conditions, but pretreatment times are on the order of hours or days rather than minutes or seconds. Compared with acid processes, alkaline processes cause less sugar degradation, and many of the caustic salts can be recovered and/or regenerated. Sodium, potassium, calcium, and ammonium hydroxides are suitable alkaline pretreatment agents. Of these four, sodium hydroxide has been studied the most (Elshafei *et al.*, 1991). Dilute NaOH treatment of lignocellulosic materials has been

found to cause swelling, leading to an increase in internal surface area, a decrease in the degree of polymerization, a decrease in crystallinity, separation of structural linkages between lignin and carbohydrates, and disruption of the lignin structure (Fan *et al.*, 1981). The digestibility of NaOH treated hardwood was reported to increase from 14% to 55% with a decrease of lignin content from 24-55% to 20%. Dilute NaOH pretreatment was also found to be effective for the hydrolysis of straws with relatively low lignin contents of 10-18%. Pretreatment of corn stover with 10% sodium hydroxide (NaOH) for 60 minutes under pressure at 121°C in the autoclave decreased the lignin fraction by more than 95% and increased the enzymatic conversion more than four times to 79.4% as compared to untreated stover (Silverstein, 2004)

4.1.5.4. Oxidative Delignification.

Lignin biodegradation could be catalyzed by the peroxidase enzyme with the presence of H_2O_2 (Azzam, 1986). The pretreatment of cane bagasse with hydrogen peroxide greatly enhanced its susceptibility to enzymatic hydrolysis. About 50% of the lignin and most of the hemicellulose were solubilized by 2% H2O2 at 30 °C within 8 hour, and 95% efficiency of glucose production from cellulose was achieved in the subsequent saccharification by cellulase at 45 °C for 24 hour.

4.1.5.5. Organosolv Process.

In the organosolvation process, an organic or aqueous organic solvent mixture with inorganic acid catalysts (HCl or H_2SO_4) is used to break the internal lignin and hemicellulose bonds. The solvents commonly used in the process are methanol, ethanol, acetone, ethylene glycol, triethylene glycol, and tetrahydrofurfuryl alcohol (Chum *et al.*, 1988). Organic acids such as oxalic, acetylsalicylic, and salicylic acids can also be used as catalysts in the organosolvation process. Pulps with residual lignin ranging from 6.4% to 27.4% (w/w) have been prepared from mixed softwoods using a biorefining technology called the lignol process, which is based on an aqueous ethanol organosolvation extraction (Pan *et al.*, 2005). This process uses a blend of ethanol and water in the ratio of 50:50 (w/w) at ± 200 °C and 400 psi to extract most of the lignin from wood chips or other

lignocellulosic biomass. Pan *et al.* (2005) found that all pulps were readily hydrolyzed without further delignification and more than 90% of the cellulose in low lignin pulps (<18.4% residual lignin) was hydrolyzed to glucose in 48 hour.

4.1.6. Biological methods

Biological pretreatment can be performed by applying lignin degrading microorganism (e.g white rot and soft rot fungi) attach to raw material. The method is environmentally friendly and energy saving because in this method any chemical was not used and it is performed at low temperature. But, disadvantage using this method for industrial scale is low for degrading rate and some material lost as these microorganisms to some extent consume hemicellulose and cellulose beside lignin (Galbe *et al.*, 2007).

5. Hydrolysis of lignocellulose

Hydrolysis is treatment process which is conversion and change, involves cleaving polymers such as cellulose and hemicellulose into their monomers (Taherzadeh and Karimi, 2007). Complete hydrolysis of cellulose results in glucose, whereas the hemicellulose converts to several pentose and hexose. Before having hydrolysis process, raw materials are usually milled initially to sizes of a few millimeters to make a bigger surface area. Several by-products may be formed or released in hydrolysis step. Detoxification is an effort to reduce by-product which occur from hydrolysis step. Also, the properties of substrate can affect hydrolysis. These properties are neutralizing capacity, proportion of easily hydrolysable cellulose and hemicellulose, amount and rate of hydrolysis of the difficult materials, the length of macromolecules, degree of polymerization of cellulose, configuration of the cellulose chain and association of cellulose with other protective polymeric structures within the plant cell walls such as lignin, pectin, hemicellulose, protein, mineral elements and also particle size (Taherzadeh, 1997 cited by Taherzadeh and Karimi, 2007).

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There are several hydrolysis processes, such as:

5.1. Chemical hydrolysis

The lignocellulose content can be hydrolyzed chemically or enzymatically. Dilute-sulfuric acid hydrolysis is a chemical hydrolysis for either the pretreatment before enzymatic hydrolysis or the conversion of lignocellulose to the corresponding sugars (Taherzadeh, 1999). In dilute acid hydrolysis, the hemicellulose fraction is depolymerized at lower temperature than the cellulose fraction. If higher temperature or longer retention times are applied, the formed monosaccharides will be further hydrolyzed to other compounds. It is therefore suggested that the hydrolysis process be carried out in at least two stages, the first stage at relatively milder conditions during which the hemicellulose fraction is hydrolyzed and a second stage can be carried out by enzymatic hydrolysis or dilute-acid hydrolysis at higher temperatures during which the cellulose is hydrolyzed (Sanchez *et al.,* 2004). Hydrolysis of rice straw by dilute sulfuric acid at high temperature and pressure was investigated by Karimi *et al.* (2006) in one and two stages. The hydrolysis was carried out in a 10-l reactor, where the hydrolysis retention time (3–10 min), pressure (10–35 bar) and acid concentration (0–1%) were examined. The results show the ability of first stage hydrolysis to depolymerize xylan to xylose with a maximum yield of 80.8% at hydrolysis pressure of 15 bar, 10 min retention time and 0.5% acid concentration. The best results of the second stage of the hydrolysis were achieved at the hydrolysis pressure and the retention time of 30 bar and 3 min in the second stage hydrolysis, where a total of 78.9% of xylan and 46.6% of glucan were converted to xylose and glucose, respectively in the two stages. Formation of furfural and HMF were functions of the hydrolysis pressure, acid concentration, and retention time, whereas the concentration of acetic acid was almost constant at pressure of higher than 10 bar and a total retention time of 10 min. The advantages and disadvantages of acid hydrolysis are showed in Table 3.

Table 3. Advantages and disadvantages of hydrolysis using acid.

Advantages	Disadvantages
1. Pretreatment of raw materias is not required	1. The reaction is less specific
2. The reaction occurs rapidly	2. Occurrence of sugars conversion into another product
3. Catalyst is cheap and available	3. Requirement of high temperature (with low acid condition)
4. The reaction occurs at low temperature (with high acid concentration)	4. In the case of using high acid concentration, the acid separation is required before fermentation
5. Higher product concentration with high acid concentration	5. pH adjustment of hydrolysate to neutral value is required
	6. Formation of toxic by-products (eg. Furfural)
	7. Requirements of costly equipments for corrosion problem
	8. Difficult to disposal waste from the reaction

Source: Kingsuwanrat (2002 cited by Wattanakitjanukul, 2008)

5.2. Enzymatic hydrolysis

Enzymatic hydrolysis of such cellulosic material is the most promising approach to get high product yields vital to economic success (Hinman *et al.*, 1992; Lynd *et al.*, 1996 cited by Adsul *et al.*, 2005). In fact, because of the higher ethanol yield and lower by-product formation, economic evaluation of a future full-scale plant for production of ethanol from various lignocellulosic raw materials is frequently based on enzymatic hydrolysis instead of conventional acid hydrolysis (Marques, 2007).

Hydrolysis of lignocellulosic materials to soluble products can be carried out by chemical (mild acid/alkali) or enzymatic action. Enzymatic saccharification is advantageous when compared to chemical methods, since it is by nature a more specific and cleaner process. It also allows milder operation conditions, leading to reduced formation of biological inhibitory compounds (such as sugar- and lignin-degradation products) and the catalyst is potentially reusable. The advantages and disadvantages of enzymatic hydrolysis are showed in Table 4.

Table 4. Advantages and disadvantages of hydrolysis using enzyme

Advantages	Disadvantages
1.Mild condition	1.Pretreatment of raw materials is required
2.Highly specific reaction leading to obtain high purity of product	2..Product inhibition problem
3.Product not easily convert to other compounds	3.Enzyme may be lost due to the absorption on undigested materials
4.Capable to perform both fermentation and hydrolysis in the same time	4.High risk of contamination
5.None corrosion problem	5.Low reactioin due to interfere from byproduct

Source: Kingsuwanrat (2002 cited by Wattanakitjanukul, 2008)

There are several essential enzymes have been used for hydrolysis process as following:

5.2.1. Cellulase

Cellulase refers to a class of enzymes produced chiefly by fungi, bacteria, and protozoans that catalyze the cellulolysis (or hydrolysis of cellulose). However, there are also cellulases produced by other types of organisms such as plants and animals (Wikipedia, 2009c). There are five general types of cellulases based on the type of reaction catalyzed, which are:

1. Endo-cellulase breaks internal bonds to disrupt the crystalline structure of cellulose and expose individual cellulose polysaccharide chains
2. Exo-cellulase cleaves 2-4 units from the ends of the exposed chains produced by endocellulase, resulting in the tetrasaccharides or disaccharide such as cellobiose. There are two main types of exo-cellulases (or cellobiohydrolases, abbreviate CBH) - one type working processively from the reducing end, and one type working processively from the non-reducing end of cellulose.
3. Cellobiase or beta-glucosidase hydrolyses the exo-cellulase product into individual monosaccharides.
4. Oxidative cellulases that depolymerize cellulose by radical reactions, as for instance cellobiose dehydrogenase.
5. Cellulose phosphorylases that depolymerize cellulose using phosphates instead of water.

Subkaree (2008) studied ethanol production from pretreated palm oil fiber using simultaneous saccharification and fermentation process. Cellulase from *T. reesei* and β-glucosidase from *A. niger* was used for the enzymatic hydrolysis. The 50 g/l of pretreated palm fiber and cellulase: β-glucosidase of 4FPU:4IU, 6FPU:6IU, 8FPU:8IU and 10FPU:10IU was investigated. It was found that cellulase: β-glucosidase of 10FPU:10IU was gave the highest ethanol production. However, it was no difference when cellulase: β-glucosidase of 6-10FPU:6-10IU was used. Moreover, cellulase: β-glucosidase of 6FPU:2IU, 6FPU:3IU and 6FPU:6IU was examined. The result showed that cellulase: β-glucosidase of 6FPU:3IU was the optimum values for ethanol production.

5.2.2. Xylanase

Xylanase is the given name to a class of enzymes which degrade the linear polysaccharides beta-1,4-xylan into xylose, thus breaking down hemicellulose, which is major component of the cell wall of the plants (Wikipedia, 2009d). Debranching and nondebranching1,4-ß-xylanases have been identified in

cultures of *A. niger* and *Ceratocystis paradoxa*. The complete hydrolysis of hemicellulose into monosaccharides requires the concerted action of several enzymes. These include β-xylanases, β-Dgalactanases, β-mannanases, as well as glycosidases β-xylosidase (Cullen and Kersten, 1992). These enzymes can be classified into two main groups: those acting on the on the xylose backbone, those cleaving the side chains. Degradation of the xylose backbone depends on xylanases, that cleave bonds within the polymer, and β-xylosidase that release xylose units from xylobiose and xylooligomers (Pastor *et al.*, 2007).

Limited studies have been reported of the interactions between cellulase and xylanase, in sharp contrast to the amount of attention given to the synergy among cellulase components. However, for real applications, it is vital to understand the cooperative action between these two classes of enzymes on pretreated lignocellulosic biomass. Synergism is often defined as the ratio of the rate or yield of product release by enzymes when used at the same time to the sum of rate or yield of these products when the enzymes are used separately in the same amounts as they were employed in the mixture. However, because this study was concerned with release of two products, glucose and xylose, over a range of loadings of xylanase, xylanase leverage on glucan hydrolysis was defined as the ratio of the percent increase in glucose release to the percent increase in xylose release. Kumar and Wyman (2009) reported the hydrolysis of corn stover from ammonia fiber expansion (AFEX) treatment using enzyme cocktails based on cellulase supplemented with β-glucosidase at an activity ratio of 1:2, respectively, and augmented with up to 11.0 g xylanase protein/g cellulase protein for combined cellulase and β-glucosidase mass loadings of 14.5 and 29.0 mg protein (about 7.5 and 15 FPU, respectively)/g of original potential glucose. It was found that glucose release increased nearly linearly with residual xylose removal by enzymes for all pretreatments despite substantial differences in their relative yields.

5.3. Factor affecting on enzymatic hydrolysis

There are several factors that took important role for enzymatic hydrolysis process. Gregg and Saddler (1996) summarized several factors such as;

5.3.1. Enzyme loading

Improvement in hydrolysis rates of lignocellulose materials can be obtained by increasing to a certain extent the amount of enzymes such as cellulase, β-glucosidase and xylanase used in the conversion process. Recently, many experiment was conducted to evaluate the effect of cellulase, β-glucosidase and xylanase loading on lignocellulose conversion, Zhang *et al.* (2009) observed the overloading of cellulase, β-glucosidase and xylanase concentration to eliminate the inhibitors which occur during saccharification process such as cellobiose and xylobiose of corncob treated with aqueous ammonia. Enzymatic digestibility was directly correlated with cellulase loading. The conversions of glucan and xylan from corncob were promoted by the increase in cellulase loading and reached a high level at cellulase loading of 15 mg protein/g glucan (50 FPU/g glucan), at which the yield of glucose and xylose were 93.3% and 82.1%, respectively. Further increasing the loading of cellulase beyond 15 mg protein/g glucan (50 FPU/g glucan) had no significant effect on the enzymatic digestibility. In the case of high cellulase loading (15 mg protein/g glucan), enzymatic hydrolysis of cellulose had achieved a high degree of glucose yield, so the supplement of the accessory enzymes could not gain a significant enhancement.

Moreover, Zhang *et al.* (2009) also determined effect of supplementation of β-glucosidase with Spezyme CP loading of 9.1 mg protein/g glucan (30 CBU/g glucan). Without β-glucosidase, the concentration of cellobiose in hydrolysate is 0.6 mg/ml, and 24-h yield of glucose was only 57.1%. It may be concluded that a little of cellobiose can significantly inhibit cellulase. With the increasing β-glucosidase loading, the concentration of cellobiose decreased. In fact, all cellobiose could be hydrolyzed in time by the addition of β-glucosidase with 1.45 mg protein/g glucan (30 CBU/g glucan), at which the yields of glucose and xylose increased by 19.4% and 6.2%.

The supplementary of xylanase can enhance significantly the performance of cellulase and increase the bioconversions of cellulose and hemicellulose. The initial hydrolysis of cellulose was quickened, due to that the

addition of xylanase increased the accessibility of cellulase to cellulose chains by removing the hemicellulose barrier and thus exposing more cellulose chains. When the cellulase complex was supplemented with xylanase (0.67 mg protein/g glucan) at saccharification of corncob treated by aqueous ammonia, the 24-h yields of glucose and xylose increased by 6.5% and 26.5%. Higher conversion was obtained with more xylanase loading, but the increasing trend became lower at high loading. This phenomenon may be because the competition for productive binding sites between xylanase and cellulases (Carpita and Gibeaut, 1993).

5.3.2. Substrate loading

Gregg and Saddler (1996) suggested the initial hydrolysis rate is influenced by the concentration of the substrate. There is an inverse relationship between the concentration of substrate and the enzymatic hydrolysis yield. When their compared the hydrolysis rates and yields obtained when increasing concentrations of steam exploded aspen were hydrolyzed by similar enzyme concentrations, it was apparent that, although the initial rates were similar, after 24 h the rate and yield at higher substrate concentrations decreased significantly. Glucose yields of 50-80% are generally obtained within the first 24 h and further 72 h incubation is required to obtain final yields of 80-95%. The susceptibility of cellulosic substrates to enzymatic hydrolysis is thought to depend on a number of substrate structural features including cellulose crystalline, the degree of cellulose polymeration, lignin content and the surface area accessible to cellulases. The importance of each of these factors in determining the susceptibility of the substrate has not been fully resolved. It has been frequently suggested that the surface area of the substrate available to cellulases is the most influential factor determining the hydrolysis rate, because the adsorption of the cellulases to the cellulose is an essential step in the hydrolysis reaction. A strong correlation between accessible surface area and the hydrolysis rate has been obtained in severals studies although there is some debate whether the methods of determining surface area truly reflect the area accessible to cellulases. Similarly, the initial susceptibility of the substrates could not be inferred from the hydrolysis rates because these rates were only determined after an extended

period of hydrolysis. Thus, significant changes had already occurred to the substrate, which in itself influenced the initial and final hydrolysis rates. It has been suggested that the crystallinity of cellulose may also partially determine the hydrolysis rate of a substrate. In a highly crystalline substrate, the closely packed, hydrogen-bonded cellulose molecules might be less accessible to cellulase attack than the loosely organized amorphous cellulose.

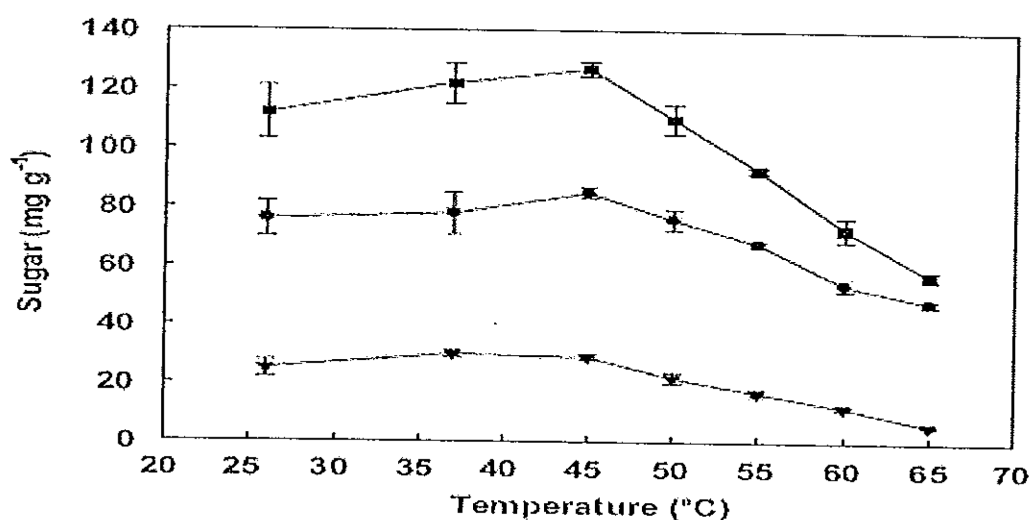
5.3.3. pH

Saha and Cotta (2008) investigated the effects of pH (3.5–6.5) and temperature (25–70 °C) on the enzymatic hydrolysis of lime-pretreated (100mg/g substrate, 121 °C, 1h) rice hulls (15.0%, w/v) using the three enzyme cocktails (cellulase, β-glucosidase and hemicellulase) at each enzyme preparation dose level of 0.05 ml/g substrate. The combined enzyme cocktail worked well over a pH range of 4.0–5.5 with an optimum pH of 5.0 for the release of all sugars. The relative sugar yields at pH 6.0 and 6.5 were 83% and 56% of the maximum level observed at pH 5.0, and the enzyme cocktail worked better at the lower pH side of optimum pH 5.0 than at the higher pH side.

5.3.4. Temperature

Nowadays, many studies have been conducted to investigate the effect of temperature on enzymatic hydrolysis in term of the stability of cellulase and hemicellulase to lower or higher temperature condition. Sharma *et al.* (2002) reported the saccharification of steam explosion pretreated sunflower stalks was carried out at temperature ranging from 40 °C to 60 °C using 5% (w/w) substrate and 25 FPU/g substrate of cellulase. The result found that maximum saccharification (56.4%) was observed at 50 °C with corresponding reducing sugars 426:2 mg/g. Decreased saccharification was observed at temperatures higher than the optimum. Reduced saccharification at higher temperature could be attributed to the thermal inactivation of endoglucanase I and cellobiohydrolase I. The temperature of 50 °C was also found optimum for enzymatic saccharification of different lignocellulosic materials. In comparison, the study about effect of temperature was also been done by Saha and Cotta (2008) using lime pretreated rice hulls. The result found that with

regard to temperature, the three enzyme combination (cellulase, β-glucosidase and hemicellulase) worked optimally at 45 °C. The yields of total sugars at 37, 50 and 60 °C were 96%, 87% and 57% of that at 45 °C, respectively.



glucose (●), xylose (▼) and total sugars (■)

Figure 4. Effect of temperature on the enzymatic saccharification of lime-pretreated (100 mg/g, 121 °C, 1h) rice hulls (15, w/v) at pH 5.0 for 72 h using a cocktail of three commercial enzyme preparations (cellulase, β-glucosidase and hemicellulase) at each enzyme dose level 0.05 ml/g hulls.

Source: Saha and Cotta (2008)

5.3.5. Surfactant

Tween 20 is known to enhance the enzymatic saccharification of cellulose. The effect of Tween 20 (0, 1.25 and 2.5 g/l) on the enzymatic action at two enzyme dose levels (0.05 and 0.15ml of each enzyme preparation of cellulase, β-glucosidase and hemicellulase/g substrate) was tested. Addition of Tween 20 did not have any effect on the release of sugars from lime-pretreated rice hulls. The reason is not clear, but the exact mechanism of enhancement of enzymatic hydrolysis of cellulose by Tween 20 is also not clear. It plays an

important role in preventing the non-specific binding of cellulases to lignin residues, allowing more enzymes to be available for the conversion of cellulose, resulting in a higher conversion rate. (Saha and Cotta, 2008).

6. Microbial conversion of lignocellulose to ethanol by filamentous fungi

Fermentation of lignocellulose hydrolyzate is more difficult than the well-established processes of ethanol production from sugarcane. Hydrolyzate contains a broader range of inhibitory compounds, whose composition and concentration depend on the type of lignocellulose materials and on the chemistry and nature of the pretreatment and hydrolysis processes. Beside of inhibition viability, the hydrolyzates of hemicellulose contain not only hexoses but also pentoses, where xylose is dominant sugar ini the hydrolyzates from hardwood hemicelluloses. Therefore, the fermenting microorganism should be able to produce ethanol from hydrolyzate with a high yield and productivity, withstand potential inhibitors and produce ethanol from pentoses as well as being safe for humans (Taherzadeh, 2007).

There are several naturally occurring microorganisms which can produce ethanol using glucose and xylose as substrates. In recent studied of ethanol production, the capability of fungi class zygomycetes was recently explored. Such as:

6.1. *Rhizopus* sp.

The cultivation conditions for *Rhizopus oryzae* grown in synthetic medium and paper pulp spent sulfite liquor (SSL) were investigated by Taherzadeh *et al.* (2003). They tried to achieve high biomass and ethanol yields using shake flasks and bioreactors. The fungus assimilated the hexoses such as glucose, mannose and galactose, and the pentoses such as xylose and arabinose, as well as acetic acid which are present in SSL. The assimilation of hexoses was faster than pentoses during cultivation in a synthetic medium. However, all sugars were assimilated concomitantly during growth in SSL supplemented with ammonium, magnesium, calcium, phosphate, sulfate and trace amounts of some other metal ions. The medium composition had an important influence on biomass yield. The highest biomass yields, 0.18 and 0.43 g biomass/g sugar were obtained, when the cells were cultivated in shake flasks with a

synthetic medium containing glucose as carbon and energy source and SSL-S, respectively.

Rhizopus oryzae also be well-known as producer of L (+) Lactic acid from glucose and starch (Dong, 1996) and capable to produce cellulolytic and xylanolytic enzymes (Shimonaka *et al.*, 2006; Bakir *et al.*, 2001).

In addition, *Rhizopus oryzae* was not quite similar to *S. cerevisiae* or *Mucor in* terms of metabolite products, since it produced lactic acid as one of the major metabolites in fermentation process. In previous study, this fungus showed the best ethanol yield from either Avicel or the rice straw. The production of ethanol was occurred in the first 2–3 days, where the ethanol production from Avicel was faster in aerobic than anaerobic conditions. On the other hand, the fungi assimilated ethanol in the presence of oxygen in aerobic condition. The effect of enzyme loading was also examined in the experiments with this fungus. Doubling the enzyme concentration from 15 to 30 FPU/g DM increased the maximum ethanol yield by 20 and 17% in aerobic and anaerobic conditions (Karimi *et al.*, 2006b).

6.2. *Mucor* sp.

Mucor indicus has recently been identified as a candidate for industrial production of ethanol from lignocellulosic hydrolyzate (Millati *et al.*, 2005). *Mucor* sp. also has been studied for its ability to produce hydrolytic enzymes that attacked native cellulose, acid swollen cellulose (amorphous cellulose), carboxymethylcellulose and cellobiose (Somkuti *et al.*, 1969).

In recent study about ethanol production from diluted acid pretreatment rice straw by simultaneous saccharification and fermentation (SSF) comparing *Mucor indicus, Rhizopus oryzae* and *Saccharomyces cerevisiae,* Karimi *et al.* (2006b) has found that both filamentous fungi have capability to assimilate xylose as well as glucose, and tolerate to apply temperature and ethanol concentration better than *Saccharomyces cerevisiae.* Moreover, in this experiment ethanol production from pretreated rice straw by SSF using *Mucor indicus* was compared with the SSF of Avicel as a reference. This filamentous fungus was quite similar to *S. cerevisiae* in terms of formation of the metabolites, in which ethanol was the major metabolite and

glycerol was the most important byproduct. The profile of ethanol was quite similar to the baker's yeast, where the major part of ethanol was produced in the first 2 days. The concentration of ethanol was practically constant within the last 4 days of SSF in anaerobic conditions, while the cells could partly take up ethanol in aerobic conditions. Increasing of the enzyme loading from 15 to 30 FPU/g DM increased the maximum ethanol yield on Avicel from 56 to 61% in anaerobic SSF, while the effect of the higher enzyme loading did not improve the ethanol yield by more than 2% in aerobic SSF. The best ethanol yield obtained from *Mucor indicus* was 67.6%, which was from the rice straw in anaerobic SSF.

6.3. Factors affecting ethanol production by filamentous fungi

6.3.1. Effect of substrate

Moreover, *Rhizopus* sp., *Mucor* sp. and *Rhizomucor* sp., have recently studied for their ability to produce ethanol from various sugars (Millati *et al.*, 2005). In this recent study, nine strains of zygomycetes, which are *Rhizopus* sp., *Mucor* sp. and *Rhizomucor* sp. were compared in terms of sugar consumption and ethanol production from glucose, xylose and dilute-acid hydrolyzate (DAH) as a carbon sources. This experiment conducted with cotton plugged shake flask. The result showed that all of the strains from *Rhizopus* and *Mucor* genera behaved similarly in the consumption of glucose and production of ethanol with ethanol yields ranging between 0.37 and 0.43 g/g of glucose. It meant that this strain can consume the high concentration of glucose from previous concentration 50 g/l of glucose in relatively short time or after 24 hour cultivation (Figure 5 and Table 5).

Furthermore, during the cultivation on xylose all the *Mucor* and *Rhizopus* strains produced ethanol from xylose albeit more slowly corresponding to the slower xylose consumption (Figure 5). The *Mucor* strains consumed xylose faster than *Rhizopus* such that xylose was depleted and ethanol peaked after 4 days, whereas the ethanol concentrations increased throughout the *Rhizopus* cultivations. The fastest ethanol producer from xylose among the tested strains was *M. indicus* with a productivity of 0.18 g/l h, while *R. oryzae* (B) resulted in the highest yield (Table 5). The biomass yields of all the strains were between 0.09 and 0.40 g/g of xylose (Table

5). The biomass yield from xylose was generally higher than the yield from glucose by the respective fungus.

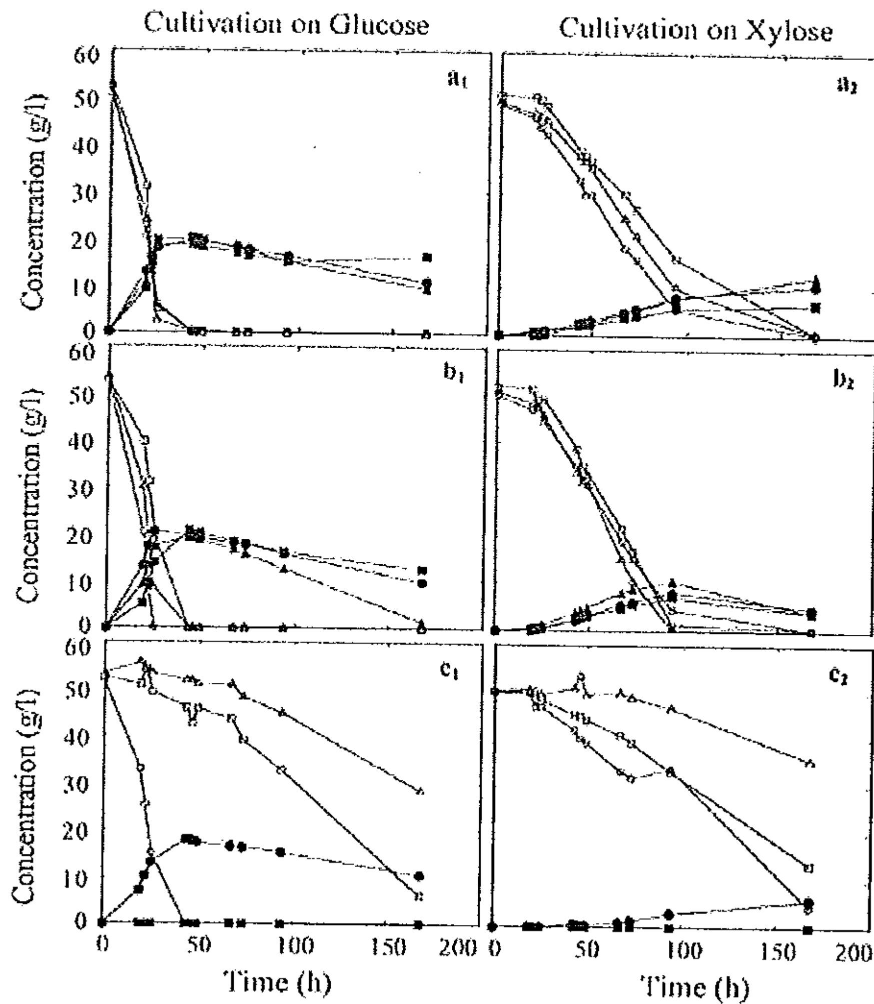


Figure 5. Cultivation of zygomycetes strains on glucose (a1, b1 and c1) and xylose (a2, b2 and c2) as the carbon source. The open symbols represent glucose or xylose profiles in the respected figures and the filled symbols represent ethanol profiles. The strains are (a) (□ / ■) *R. oryzae* A, (○ / ●) *R. oryzae* B, and (△ / ▲) *R. oryzae* C; (b) (□ / ■) *M. corticolous*, (○ / ●) *M. hiemalis*, and (△ / ▲) *Mucor indicus;* (c) (□ / ■) *R. pusillus*, (△ / ▲) *R. miehei*, and (○ / ●) the tempeh zygomycete IT.

Source: Millati *et al.* (2005)

Table 5. Ethanol productivities in cultivation of different zygomycetes strains on (a) glucose and (b) xylose.

Strain	Ethanol productivity (g/lh)	Ethanol Yield ($g_{ethanol}/g_{sugar}$)
(a) Cultivation on glucose		
R. oryzae A	1.87	0.41
R. oryzae B	1.00	0.43
R. oryzae C	1.24	0.37
M. corticolous	1.48	0.43
M. hiemalis	1.44	0.39
M. indicus	1.41	0.39
R. pusillus	0.00	0.00
Zygomycetes IT	0.95	0.38
S. cerevisiae	1.29	0.42
(b) Cultivation on xylose		
R. oryzae A	0.07	0.16
R. oryzae B	0.11	0.28
R. oryzae C	0.11	0.23
M. corticolous	0.10	0.15
M. hiemalis	0.12	0.18
M. indicus	0.18	0.22
R. pusillus	0.00	0.00
Zygomycetes IT	0.05	0.10

Source: Modified from Millati *et al.* (2005).

Nine strains of zygomycetes also have been use as microorganism for ethanol production using dilute-acid hydrolyzate (DAH) as carbon source. Most of the zygomycetes strains were able to grow in the hydrolyzate, when the initial growth had occurred in 50 ml synthetic glucose medium for 24 h, which assimilate all the glucose and then 100 ml DAH was added and incubated for another 5

days. The *Mucor* species showed the fastest ethanol production from DAH. Within less than 24 h after addition of the DAH, *Mucor indicus* and *M. hiemalis* reached maximum ethanol concentration (12 g/l) and had consumed all of glucose, mannose and galactose. Meanwhile, *M. corticolous* needed twice as long time to achieve the same results. The *Rhizopus* species consumed hexoses slower than *Mucor* species, two strains of Rhizopus can consumed the hexoses within 3 days of cultivation. The consumption of xylose was not as fast as those in hexoses. But, *Mucor indicus* showed a good performance for sugar consumption within DAH containing hexoses and pentoses, also in ethanol productivity, this strains with *Mucor hiemalis* result higher yield of ethanol concentration in 0.44 g/g respectively (Table 6).

Table 6. Yield of ethanol production in cultivation of different *Zygomycetes* strains in dilute-acid hydrolyzate

Strain	Ethanol Yield ($g_{ethanol}/g_{sugar}$)
R. oryzae A	0.41
R. oryzae B	0.34
R. oryzae C[b]	0.00
M. corticolous	0.36
M. hiemalis	0.44
M. indicus	0.44
R. pusillus	0.00
Zygomycetes IT[b]	0.00

b). *R.oryzae* C, *R. miehei,* zygomycetes IT did not consumed the sugars within the hydrolyzate.

Source: Modified from Millati *et al.* (2005)

6.3.2. Effect of nutrient addition

Sues *et al.* (2005) studied the influence of the medium composition on ethanol production from *Mucor indicus*. Hexoses, pentoses and dilute acid hydrolyzate (DAH) from lignocellulose materials were conducted as substrates.

At cultivation process, they maintained aerobic condition by continous air sparging at batch bioreactor.

From the experiment of medium composition on ethanol production and growth of *Mucor indicus.* They used $(NH_4)SO_4$, KH_2PO_4, $MgSO_4$, $ZnSO_4$, $CaCl_2$, EDTA and $FeSO_4$ as well as some yeast extract, trace element and vitamins in various concentration. The result showed that medium composition had great impact on both cultivation time course and the yield of *Mucor indicus.* The base medium for cultivation of the fungus contained $MgSO_4$ and KH_2PO_4 at standard concentration. $(NH_4)SO_4$ and glucose were chosen as nitrogen and carbon source. The very long time needed for complete consumption of glucose indicated deficiencies in the medium composition which partially supplied by zinc. Zinc is an important trace metal which participates in the activity of zinc-dependent alcohol dehydrogenase (ADH) in the fermentative pathway. Further stimulation of ethanol production was achieved by addition of trace metals, and further stimulation of both glucose consumption and ethanol production when two were combined, implying that the standard concentration of zinc in the trace metal solution was suboptimal. The mixture of vitamins were well synthesized by *Mucor indicus.* Yeast extract was the most efficient supplement, however, glucose consumption and ethanol production were still speeded up by trace metals and vitamins.

6.3.3. Effect of oxygen addition

In dimorphic species of filamentous fungi such as *Mucor,* spore germination can lead to the production of either filamentous (mycelium) or spherical (yeast) cells, depending on environmental conditions (Bartnicki-García 1963; Sypherd et al. 1978 cited by Zazueta-Sandoval and Guiterrez-Corona, 1999). The mycelial or the yeast phase of development can occur in the presence of oxygen or under microaerobiosis and depends on the carbon source and/or the presence of morphogenetic compounds. Yeast development requires hexoses and invariably correlates with a fermentative metabolism, whereas the mycelial phase has been shown to be metabolically flexible in that it can occur during either fermentation or oxidative metabolism; the latter can occur with several carbon sources. The correlation of the

yeast phase with high rates of ethanol production holds true under various growth conditions, e.g., during anaerobic or aerobic growth in media containing morphogenetic compounds such as phenethyl alcohol (PEA), dibutyryl cyclic AMP, or certain amino acids (Orlowsky 1991 cited by Zazueta-Sandoval and Guiterrez-Corona, 1999). In the presence of oxygen and glucose, *Mucor racemosus* grows oxidatively as mycelial cells with little ethanol being produced; on the other hand, mycelium or yeast cells grown anaerobically show high rates of ethanol production that correlate with higher levels of alcohol dehydrogenase (ADH) activity as compared to those of aerobic mycelial cells (Borgia *et al.*, 1985 cited by Zazueta-Sandoval and Guiterrez-Corona, 1999). Figure 6 described glucose metabolism by filamentous fungi and the products which resulted from fermentation process.

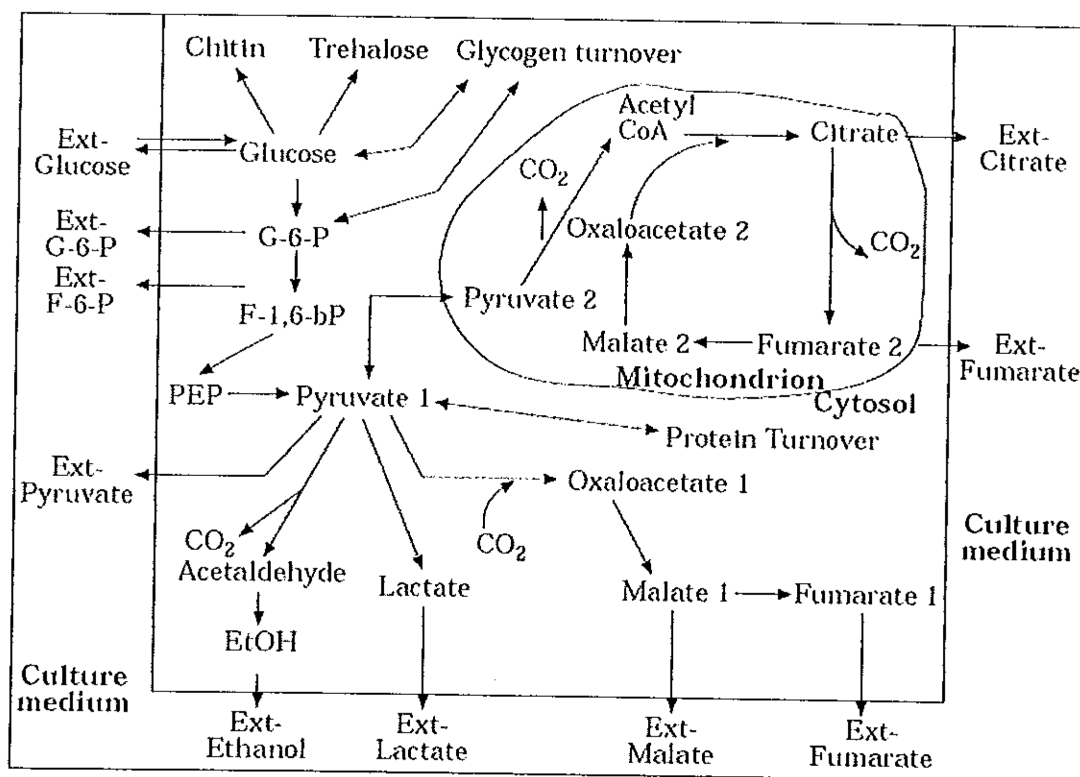


Figure 6. Model of glucose metabolism in the filamentous fungus *Rhizopus oryzae* Ext- stands for extracellular G-6-P for glucose-6-phosphate, F-6-P for fructose-6-phosphate and F-1,6-bP for fructose-1,6-bisphosphate.

Source: Wright *et al.*, (1996)

Furthermore, Abtahi (2008) reported the growth of *Mucor indicus* using high concentration of glucose in aerobic and anaerobic condition. In aerobic condition, the morphology of this fungus depends on the concentration of glucose in the medium. In low concentrations of glucose the morphology is cotton-like but in higher concentrations of glucose the morphology is heavy suspension. In the anaerobic condition the morphology of this fungus is independent on the concentration of glucose (up to 400 g/l glucose). The initial stages of spore development are independent of atmospheric composition until the spore wall is swollen; oxygen starts the mycelia life cycle and carbon dioxide in the absence of oxygen, starts the yeast life cycle. Anaerobic incubation of *Mucor indicus* under atmosphere of N_2 results filamentous growth. The rate of growth and total amount of growth in the anaerobic incubation under N_2 atmosphere were less than the aerobically growth, but morphological features were similar. Anaerobic incubation under pure CO_2 result different appearance, the growth was yeast-like cells with no trace of filamentation. Aerobic growth was more abundant than anaerobic growth. For most species of *Mucor*, in order to get yeast-like cells we most have presence of carbon dioxide and absence of oxygen.

Meanwhile, under anaerobic condition *Mucor indicus* is not able to utilize xylose but with the present of HMF, as electroreceptor, the consumption of xylose and production of ethanol increased, but it is still very low.

Karimi *et al.* (2006) has studied about ethanol production and mycelia biomass from rice straw by hydrolysis and cultivation with *Mucor indicus.* The hydrolyzate was prepared from dilute acid process. The cultivation were carried out aerobically and anaerobically on the hydrolyzate which have two treatment which are detoxified hydrolyzate by adding $Ca(OH)_2$ to raise the pH to 11 and non detoxified hydolyzate. The result showed that negligible xylose consumption and ethanol production by *Mucor indicus* in anaerobic condition, whereas sharp glucose consumption and high ethanol production on glucose. For hydrolyzate, the fungus had difficulties in taking xylose neither from detoxified and non-detoxified hydrolyzate. However, the glucose fraction was rapidly consumed in both of hydrolyzate. *Mucor*

indicus had a better performance in taking up xylose, glucose, detoxified hydrolyzate and non detoxified hydrolyzate aerobically. The Figure 7 below represents the cultivation of *Mucor indicus* in anaerobic and aerobic condition.

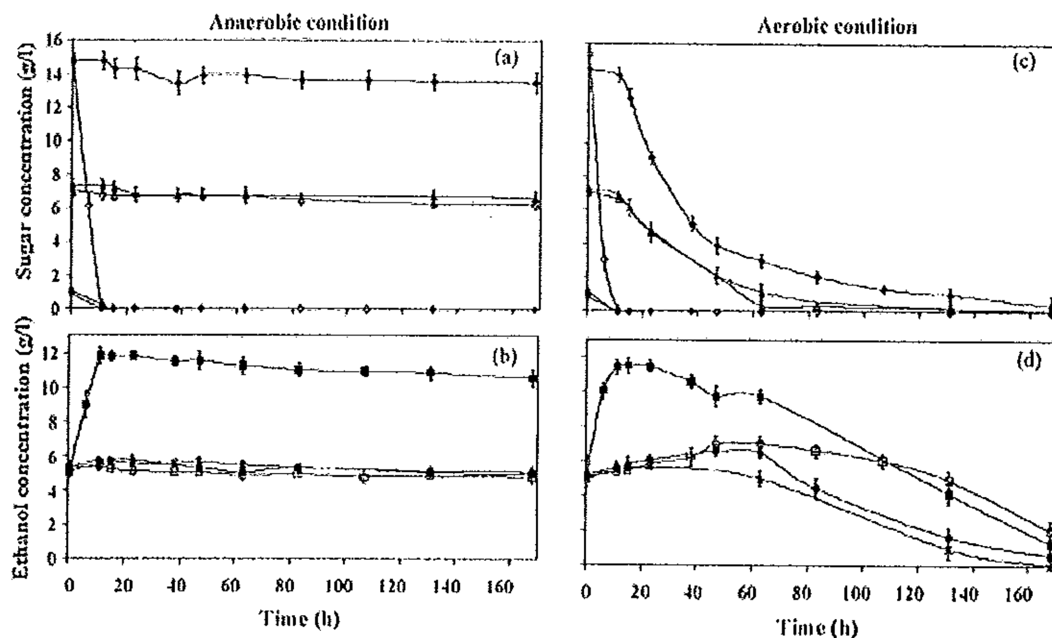


Figure 7. Cultivation of *Mucor indicus* in anaerobic (a and b) and aerobic (c and d) conditions on glucose, xylose, detoxified and non detoxified hydrolyzates. The symbols in (a) and (c) represent the profiles of glucose in synthetic media (◇); xylose in synthetic media (◆); xylose in hydrolyzate (▲), xylose in detoxified hydrolyzate (△); glucose in hydrolyzate (●); and glucose in detoxified hydrolyzate (○). The symbols in (b) and (d) represent ethanol concentration in cultivation on glucose (■); xylose (□); hydrolyzate (*) and detoxified hydrolyzate (●).

Source: Karimi *et al.*, (2006).

Objectives

1. To study the effect of alkali on pretreatment process.
2. To study the effect of enzyme hydrolysis on reducing sugar production.
3. To study the microorganism strain and optimum condition for ethanol production.
4. To study the effect of one and two-stage process.

CHAPTER 2

MATERIALS AND METHODS

Materials

1. Palm pressed fiber

Palm pressed fiber (PPF) was obtained from the Pure Palm Oil Co., Ltd., Songkhla, Thailand. It was dried in the sunlight for about two days prior to further pretreatment.

2. Microorganisms and inoculum preparation

Mucor indicus STR 3237, Mucor hiemalis STR 3047 and Rhizopus oryzae STR 3099 was obtained from Microbiological Resources Centre (MIRCEN), Thailand. Filamentous fungi was incubated on potato dextro agar (PDA) at pH 5.5 and 30°C for 5 days, where the strain grows to form a cotton-like mycelium and spores. The spore suspension was prepared by adding 2 ml of Tween 80 (10%) to slant and shaking it vigorously with tube shaker and then the suspension was returned in the 120 ml of Tween 80 solution. 20 ml of suspension were used to count the spore using haemocytometer. One ml of the suspension, containing approximately 5×10^7 spores/ml, was added to each flask (adjusted from Karimi *et al.*, 2006b).

3. Media

3.1. Fungi Cultivation

Media for fungi cultivation was prepared using commercial Potato Dextrose Agar (PDA) which contains (g/l): Potato infusion 4.0 (infusion from 200 g potatoes); D(+)glucose 20.0; agar-agar 15.0.

3.2. Media for ethanol

A medium containing (g/l): yeast extract, 5; $(NH_4)SO_4$, 7.5; K_2HPO_4, 3.5; $MgSO_4.7H_2O$, 0.75; $CaCl_2.2H_2O$, 1; in 0.05 M citrate buffer pH 5.5 was prepared for ethanol production (Karimi *et al.*, 2006b).

4. Enzymes

Commercial enzymes (cellulase, xylanase and β-glucosidase) purchased from Fluka were used in all the experiments. The enzyme was originated by *Aspergillus niger*. The maximum temperature and pH for these enzymes were known at 50 °C pH 5.0 for cellulase and β-glucosidase and 40 °C pH 5.0 for xylanase.

Analytical methods

1. Palm pressed fiber composition

Palm pressed fiber (PPF) biomass with and without pretreatment were sent to Faculty of Natural Resource for further analyzed of lignocellulose consists of cellulose, hemicellulose and lignin component using AOAC method (AOAC, 1999).

The methods were divided into acid detergent fiber (ADF), acid detergent lignin (ADL).

In acid detergent fiber solution, the chemical used were Hexadecyl-trimethylammonium bromide, 1-octanol reinst and acetone. 1 g of air-dried sample was ground to 1 mm screen and added into reflux container. 100 ml acid detergent solution was added at room temperature. After that, the sample was refluxed for 60 minutes. The container then removed, the sample was transferred, swirled, filtered and to crucible and weight (W_1). After that, the sample was washed using hot H_2O (90-100 °C) and then it was dried by vacuum. Moreover, the sample was also washed 2 times using acetone and then dried by vacuum. Then, the sample was dried 3 hours or overnight in 100 °C forced draft oven and weight (W_2). The calculation of ADF % was described in equation $\% \text{ ADF} = (W_2\text{-}W_1)/S$. Where S = g sample × g oven dried matter/g air-dried or wet matter.

Meanwhile, determination of ADL was conducted by added 1 g asbestos into sample in crucible and then cooled the crucible by using 72 % H_2SO_4 (15 °C) and stirred with the glass rod. After that, the sample was filtered with vacuum and wash with hot water until acid-free to pH paper. The sample in crucible was dried at 100 °C and then cooled at desicator and weight (W_3). Furthermore, the sample in crucible was ignited in 500 °C furnace for 2 hours, after that the crucible was cooled in

desiccator and weight (W_4). And then the loss of asses was recorded (W_5). The ADL determination was described using % ADL = (W_3-W_4-W_5)/S.

2. Fungal biomass determination

Fungal biomass was determined after cultivation. Sample was filtered by using Whatman qualitative filter paper No. 1 and solid dried at 103 °C for 24 hours. 0.25 g dried of sample was added into 5 ml conc. HCl at room temperature for 20 hours. After that, sample was filtered using Whatman filter paper number 4 to collect the supernatant. The 1 ml distilled water was added into 2 ml of filtered supernatant and boiled for 2 hours. More, the supernatant was adjusted using NaOH (30 %) until normal pH, after that adjusted the volume until 50 ml with distilled water. After adjusting the volume, 1 ml acetyl acetone reagent was added into 1 ml sample (supernatant) and boiled for 20 minutes. After boiled, 10 ml of 95% ethanol and 1 ml of Ehrlich reagent was added and waited for 30 minutes. The spectrophotometer was used at 530 nm. Glucosamine hydrochloride was used as standard curve (Van de loo, 1976).

3. Reducing sugar determination

Types of reducing sugar were determined by carbohydrate column using Agilent 1100 High Performance Liquid Chromatograph (HPLC) with refractive index detector (RID). A Zorbax carbohydrate analysis column (Agillent) was operated at 35 °C with acetonitrile and ultrapure water as a mobile phase in ratio of 75% : 25% (0.5 mL/min). The injection volume 10 µl was used.

Dinitrosalicylic acid (DNS) was used for determination of reducing sugar and enzyme unit. One ml of sample was placed in test tube. The 3 ml of DNS reagent was added and boiled for 15 minutes. After that, the sample was transferred to cold water and then 5 ml of distilled water was added and then was measured OD at 540 nm by using spectrophotometer (Miller, 1959).

4. Enzyme activity

Carboxymethylcelullase (CMCase) and xylanase activities were assayed in reaction containing 1% (w/v) of carboxymethyl cellulose (0.5 ml) and 1% (w/v) oat spelt xylan (0.5 ml) in citrate buffer pH 4.8 and appropriately dilutes enzyme

dilutions. After 30 minutes incubation at 50°C, reducing sugar was measured by dinitrosalicylic acid (DNS). One unit (U) of each enzyme activity is defined as the amount of enzyme, which produce 1 μmol reducing sugar as glucose (xylose in the case of xylanase) in the reaction mixture per minute under above specific condition (modified from Saha *et al.*, 2005)

β-glucosidase activity was assayed in the reaction contain 4 mM *p*-nitrophenyl-β-glucosidase in 50 mM acetate buffer, pH 5.0 and appropriately diluted enzyme solutions. After incubation at 50°C for 30 minutes, the reaction was stopped by adding 1 mL of ice-cold 0.5 M Na_2CO_3, and the color that developed as a result of p-nitrophenol liberation was measured at 405 nm by using spectrophotometer (Saha, 2005). One unit of each enzyme acitivity is defined as the amount of enzyme that release 1 μmol of *p*-nitrophenol per minute in the reaction mixture under these assay conditions. The percent saccharification of palm pressed fiber was calculated as follow:

$$\%\text{Saccharification} = \frac{\text{Reducing sugar (g)} \times 100}{\text{Palm pressed fiber (g)}}$$

5. Ethanol

Standard of ethanol and samples were mixed with acetone. Ethanol concentration was determined by gas chromatography with a flame ionization detector (Hewlette Packard 6850). A capillary coloum model number 807095 Stabilwax-DA was used. The coloum was operated at a temperature of 250 °C, with the helium as carrier gas. 1 μl of sample volume was injected to inlet (modified from Jennings, 2001).

The yield of ethanol were calculated as follow

$$Y_{ethanol} = \frac{\text{Ethanol (g)}}{\text{Substrate (g)}}$$

6. Statistical analysis

The data were calculated with mean values standard deviation (mean±SD) were determined from duplicate determinations. Statistical significance of the results were evaluated by one way ANOVA (analytical of varience) and Duncan's multiple range tests (*P*< *0.05*) using SPSS 10 software.

Methods

1. Effect of pretreatment for palm pressed fiber (PPF)

The raw palm pressed fiber (PPF) collected from Pure Palm Oil Co., Ltd., Songkhla, Thailand was analyzed for its composition. Following collection, the sample was dried under sunlight for 2 days. Palm pressed fiber (PPF) (10 g) was pretreated with 10 % (w/v) NaOH (100 ml). Then the solution was treated using three kinds of physical treatment, which were: autoclaved at 121 °C for 15 minutes (modified from Umikalsom *et al.*, 1998), boiled on hot plate 100 °C for 60 minutes (Zhu *et al.*, 2005) and heated in microwave (LG Intellowave 2450 Mhz, 1200 watt) for 10 minutes (modified from Zhu *et al.* 2007). The residues was collected by cheese cloth and washed extensively with tap water until neutral pH, dried at 65 °C for two days (modified from Zhu *et al*, 2005). The experiment was conducted in duplicate. After that it was ground to 20 mesh with hammer mill. After pretreatment process, there were two components; the solid and liquid phase of palm oil fiber. Cellulose, hemicellulose and lignin content of palm oil fiber were determined before and after pretreatment. Also, the reducing sugar in the liquid part or "prehydrolyzate liquid" was analyzed. The entire pretreated palm pressed fiber with different physical treatment was used as substrate for next experiment.

2. Effect of enzyme hydrolysis on reducing sugar production

2.1 Effect of type of enzyme on reducing sugar production

The 2.5 g pretreated palm pressed fiber from section 1 was used as subtrate in 45 ml of 0.05 M citrate buffer (pH 4.8). The substrate in buffer solution was autoclaved at 121 °C for 15 minutes. After that 5 ml of enzyme cellülase from Fluka (6 U/g substrate) (modified from Subkaree, 2008) at 50 °C pH 5.0 was added for

hydrolysis. In comparison, enzyme xylanase from Fluka (6 U/g substrate) at 40 °C pH 5.0 and the mixture of cellulase and xylanase (6 U/g substrate: 6 U/g substrate) at 40, 45 and 50 °C pH 5.0 were studied. Enzymatic hydrolysis was performed on a rotary shaker at 150 rpm for 48 hour (modified from Saha *et al.*, 2005). Sample was taken every 6 hour. The enzyme reaction was stopped in water bath at 100 °C for 3 minutes. Then the sample was centrifuged 6000 rpm for 20 minutes. The supernatant was determined for reducing sugar concentration by DNS method. The hydrolysis condition that performs the best result on higher yield of reducing sugar was used and selected for further experiment. Experiments were conducted in duplicate.

2.2 Effect of enzyme concentration and addition of β-glucosidase on reducing sugar production

Enzyme that gives the highest reducing from 2.1 was used. The 2.5 g pretreated palm fiber from section 1 was used as substrate in 45 ml of 0.05 M citrate buffer (pH 4.8). The substrate in buffer solution was autoclaved at 121 °C for 15 minutes. After that 5 ml of enzyme (4, 6 and 8 U/g substrate) was added for hydrolysis. β-glucosidase addition was also investigated by comparing reducing sugar amount between additions of β-glucosidase (4 cellulase: 4 xylanase: 4 β-glucosidase U/g substrate; 6 cellulase: 6 xylanase: 6 β-glucosidase U/g substrate; 8 cellulase: 8 xylanase: 8 β-glucosidase U/g substrate) and without the addition of β-glucosidase. Enzymatic hydrolysis was performed at 50 °C on a rotary shaker at 150 rpm for 48 hour (modified from Saha *et al.*, 2005). Sample was taken every 12 hour. The enzyme reaction was stopped in water bath at 100 °C for 3 minutes. Then the sample was centrifuged 6000 rpm for 20 minutes. The supernatant was determined for reducing sugar concentration by DNS method. The percentage of saccharification was calculated. The hydrolysis condition that performs the best result on higher yield of reducing sugar was used and selected for further experiment. All of the experiment was conducted in duplicate. The palm fiber hydrolyzate was obtained by centrifugation to remove the solid residues.

3. Ethanol production from palm pressed fiber (PPF)

Fermentation was performed under aerobic and anaerobic condition using three different filamentous fungi strains which were *Mucor hiemalis.*, *Mucor indicus* and *Rhizopus oryzae*. The spore suspension was prepared by adding 2 ml of Tween 80 (10%) to agar slant and shaking it vigorously with tube shaker and then the suspension was returned in the 120 ml of Tween 80 solution. 1 ml of suspension were used to count the spore using haemocytometer. One ml of the suspension containing approximately 5×10^7 spores/ml was added to each flask (adjusted from Karimi *et al.*, 2006b).

The substrates used for fermentation were; (1) PPF enzymatic hydrolysate from section 2 (2) Untreated PPF from section 1 and alkali/autoclave pretreated PPF from section 1 (3) Prehydrolyzate liquid from section 1 after pH adjustment to 5.5 using HCl 37%.

The liquid phase fermentation; prehydrolyzate liquid and PPF enzymatic hydrolysate (liquid) was carried out in serum bottle for anaerobic condition and orbital flask for aerobic condition in room temperature (± 28 – 30 °C). A medium containing (g/l): yeast extract, 5; $(NH_4)SO_4$, 7.5; K_2HPO_4, 3.5; $MgSO_4.7H_2O$, 0.75; $CaCl_2.2H_2O$, 1; and 90 ml liquid hydrolyzate was used. Total medium solution was 90 ml for fermentation process. For solid fermentation, 5 g of untreated PPF and alkali/autoclave pretreated PPF in 90 ml of 0.05 M citrate buffer pH 4.8 with media the same component as prehydrolyzate liquid was carried out in 100 ml serum bottle, the pH was adjusted to 5.5 ± 0.1. It was autoclaved and 10 ml of inoculum (10%) was added to each flask aseptically. Fermentation process was maintained for 5 days by shaking in the orbital shaker with 150 rpm of agitation speed for aerobic and anaerobic condition by sparging nitrogen gas into the serum bottle. All the experiments were performed in duplicate (modified from Karimi, 2006b).

The sample was taken everyday to determine reducing sugar and ethanol concentration, yield of ethanol on basis of the same amount of reducing sugar content was calculated. Total fungal biomass was examined at the end of fermentation

process. The best strain that gave the highest ethanol production for both phases was selected for the next experiment.

4. Effect of one and two stages condition on ethanol production

Using the best strain from section 3, the effect of one and two stage condition for solid and liquid fermentation was studied. In one stage, the effect of either aerobic or anaerobic condition on ethanol production was investigated. Aerobic condition was carried out on rotary shaker at 150 rpm, while pure nitrogen gas was sparged into the media without shaking in anaerobic condition. Fermentation process was maintained for 5 days. All the experiment was conducted in duplicate.

Moreover, the two step condition was studied. The first step is aerobic condition maintained for 2 days and subsequent to anaerobic condition maintained for 3 day in room temperature (± 28 – 30 °C). The sample was taken everyday to determine ethanol and reducing sugar concentration. Moreover, fungal biomass was examined at the end of the fermentation.

The experiments scheme are presented in Figure 8.

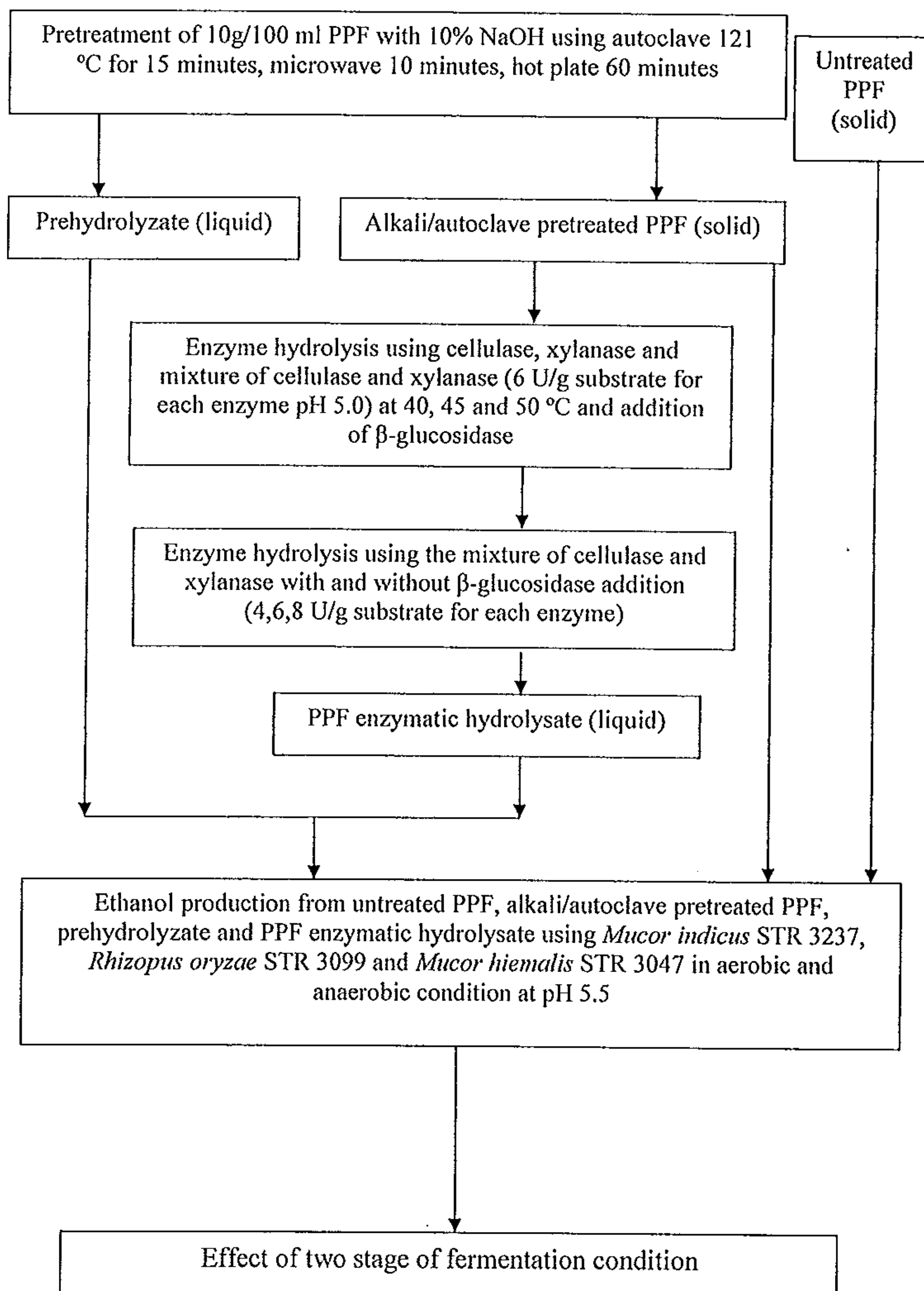


Figure 8. Experimental scheme

CHAPTER 3

RESULT AND DISCUSSION

1. Effect of pretreatment for palm pressed fiber (PPF)

Pretreatment process was conducted by soaking PPF into 10% of alkali (NaOH) liquid and treated by using autoclave, boiling and microwave.

Table 7 presents the palm pressed fiber composition with and without pretreatment using alkali solution. It was found that the composition of PPF without treatment was 37.8%, 23.3% and 23.5% of cellulose, hemicellulose and lignin content, respectively. The pretreatment of PPF with autoclaving for 15 minutes at 121°C was yielded 60.4%, 11.0% and 20.2% of cellulose, hemicellulose and lignin, respectively. Whereas, the PPF heated in microwave for 10 minutes gained 58.2%, 11.7% and 20.4 of cellulose, hemicellulose and lignin, respectively. Moreover, when the PPF was treated by boiling on hotplate for 1 hour the percentage of lignocellulose component were 56.5%, 12.2% and 18.6%, of cellulose, hemicellulose and lignin, respectively.

The pretreatment of palm pressed fiber using 10% NaOH resulted in the decreasing of lignin and hemicellulose content and increasing of cellulose content. This might be from the intracrystalline swelling of cellulosic materials and the changes in cellulose structure that involved the pore structure, particle size, lignin and hemicellulose association, crystallinity, and degree of polymerization (Fan, 1980). Alkaline pretreatments also remove acetyl and different kinds of uronic acid substitutions on hemicellulose, which lowers the extent of enzymatic hydrolysis of cellulose and hemicellulose (Chang and Holtzapple, 2000 cited by Wang *et al.*, 2008). The digestibility of NaOH-treated hardwood increased with the decrease of lignin content (Millet et al, 1976 cited by Wang *et al.*, 2008).

Table 7. Palm pressed fiber (%) composition with and without pretreatment using alkali (NaOH) solution.

Treatment	**% Dry matter**		
	Cellulose	**Hemicellulose**	**Lignin**
Without treatment	37.6	23.3	23.5
Alkali, autoclave	60.4	11.0	20.2
Alkali, boiling	56.5	12.2	18.6
Alkali, microwave	58.2	11.7	20.4

From Table 7, it showed that pretreatment of PPF by boiling for 1 hour reduced the lignin content higher than using microwave and autoclave because of the long retention time that have been conducted to treat in the process. Reaction time, together with temperature, has been reported to influence the severity, and consequently the effectiveness of biomass pretreatment and hydrolysis (Saha, *et al.*, 2005). However, the highest increasing cellulose percentage amount occurs as a result of treatment combination between NaOH (10%) and autoclave (15 minutes, 121 °C). It can be suggested that by treated PPF using autoclave, the pressure and high temperature together with NaOH performed the reaction synergism leading to the decreasing of complex material from PPF. Playne (1984) reported investigation of alkali treatment and steam explosion of bagasse in order to develop economical and effective methods of increasing the digestibility of bagasse. The alkalis examined were NaOH, NH_3 (aqueous), NaOH + NH_3, $Ca(OH)_2$, and $Ca(OH)_2$. + Na_2CO_3, at ambient temperature and in combination with steam explosion at 200 °C, 6.9 MPa, and 5 min cooking times. The result showed the highest digestibilities of up to 733 g organic matter (OM)/kg bagasse dry matter (DM) were obtained for bagasse treated with

NaOH and $Ca(OH)_2 + Na_2C0_3$. Steam explosion increased the digestibility of bagasse up to 740 g OM in the presence of alkali but only to 610 g OM in the absence of alkali.

As mentioned before, the microwave however may have new function effective for acceleration of reactivity of cellulosic materials. Ooshima, *et al.* (1984) was previously described that in conventional steam treatment such as autoclave, the cellulosic material containing water have been heated by an external heat source, such as the electrical coils surrounding the autoclave, or high pressure steam has been supplied to the cellulosic materials externally. On the other hand, in microwave treatment the cellulosic materials are heated internally. The water absorbed to cellulose, hemicellulose and other low molecules in cellulosic materials in the microwave as the kinetics energies when the polar molecules and their neighboring clusters are forced to orient to specific direction.

In fact, there was slightly different of data compositions between three methods of treatment. Moreover, further investigation was needed to optimize the ideal condition for pretreatment of lignocelullosic material using autoclave and microwave. Therefore, the three methods were used for enzymatic hydrolysis to obtain enymatic hydrolysate for ethanol production. Moreover, the prehydrolysate liquid from alkali pretreatment was used as substrate for ethanol production after adjusted the pH 5.5 by using 37% HCl.

2. Enzymatic hydrolysis of pretreated palm pressed fiber (PPF)

2.1. Effect of type of enzyme on reducing sugar production

Enzymatic hydrolysis of PPF was conducted using 2.5 g of pretreated PPF in 45 ml citrate buffer pH 5 0.05 M with 5 ml cellulase (6 U/g substrate), xylanase (6 U/g substrate) and mixture of cellulase and xylanase (6:6 U/g substrate) and it was shaking on rotary shaker 150 rpm in different temperature (40 °C, 45 °C and 50 °C).

Table 8 and Figures 9 and 10 presents the amount of reducing sugar and percentage of saccharification with various pretreatment processes at different condition and enzyme type. By using the statistical program (SPSS), the results showed that the hydrolysis with mixture of cellulase and xylanase (6:6) from

autoclaved treatment PPF at 50 °C gave the highest reducing sugar of 5.71 ± 0.21 g/l and it was significantly different comparing to treatment by boiling but not significantly different comparing to heat in microwave with the same condition. PPF with hot plate and microwave treatment gave the reducing sugar of 4.19 ± 0.60 g/l and 4.96 ± 0.36 g/l, respectively. It shows that by autoclaving the PPF before hydrolysis was conducted makes enzyme easy to attack to the fiber. Krishna *et al.* (1998) studied enzymatic hydrolysis of sugarcane leaves pretreated by autoclaving, alkali and alkali peroxide and unpretreated sugarcane leaves. The results showed that the sugarcane leaves pretreated by autoclaving, alkali and alkali peroxide had saccharification more than sugarcane leaves without pretreatment. Moreover, Abedinifar *et al.* (2009) suggested that pretreatments improved the yields of the subsequent enzymatic hydrolyses. While using 20 g/l rice straw for enzymatic hydrolysis, the pretreatment with steam and dilute-acid resulted in higher sugar yield by 30% and 56% compared to the hydrolysis of untreated straw, respectively.

Table 8, the highest amount of reducing sugar was found at hydrolysis using mixture of cellulase and xylanase at 50 °C by using PPF from autoclave, hot plate and microwave with percentage of saccharification reached 10.28 ± 0.38 %, 7.54 ± 1.07 % and 8.32 ± 0.64 %, respectively for 48 hours of hydrolysis. Meanwhile, the percentage of saccharification of PPF by using only cellulase or xylanase were 6.15 ± 0.65 %, 5.5 ± 0.75 %, 4.71 ± 0.2 % and 4.9 ± 0.26 %, 5.7 ± 0.36 %, 5.28 ± 0.33 %, respectively. This could be happened due to addition of enzyme unit at certain level increased the production of reducing sugar concentration.

Meanwhile, effect of temperature on the enzymatic hydrolysis of treated PPF was also investigated and the results are presented in Table 9. The temperature examined were 40, 45 and 50 °C at pH 5.0. pH 5.0 was selected based on report by Saha and Cotta (2008) which investigated the combination of enzyme cocktail (cellulase, xylanase and β-glucosidase) worked well over a pH range of 4.0–5.5 with an optimum pH of 5.0 for the release of all sugars. The profile of reducing sugar conversion was examined over 48 hour period. In all experiments, the maximum glucose concentration was found at 50 °C with mixture of cellulase and xylanase (6:6).

The % of saccharification at 45 °C using mixture cellulase and xylanase was 10.17 ± 1.07 %, 6.71 ± 0.06 % and 8.94 ± 0.32 % (autoclave, boiling and microwave) respectively (Figure 9 and 10). Amount of reducing sugar at 40 °C was lower than the others temperature. It might be due to the higher amount of cellulose in pretreated PPF about 4.6-5.5 times than hemicellulose. Consequently, cellulase was the key enzyme that influence on reducing sugar production. Therefore, the increasing temperature to 50 °C, which was the optimum temperature for enzyme cellulase activity resulted in the increasing of sugar concentration (Sharma *et al.* 2002).

Table 8. Reducing sugar concentration at enzymatic hydrolysis of pretreated PPF at different temperature and enzyme type at pH 5.0 for 48 hour.

Enzyme types (U/g)	Temp. (°C)	Reducing sugar (g/l)		
		Autoclave	Boiling	Microwave
6 unit of xylanase	40	$2.72^{Aa} \pm 0.14$	$3.18^{Aa} \pm 0.2$	$2.93^{Aab} \pm 0.18$
6 unit of cellulase : 6 unit of xylanase	40	$3.49^{Ab} \pm 0.14$	$3.67^{Aab} \pm 0.29$	$3.58^{Abc} \pm 0.26$
6 unit of cellulase : 6 unit of xylanase	45	$4.65^{Cc} \pm 0.10$	$2.72^{Aa} \pm 0.02$	$3.97^{Bbc} \pm 0.27$
6 unit of cellulase	50	$3.21^{Aa} \pm 0.36$	$3.05^{Aa} \pm 0.48$	$2.62^{Aa} \pm 0.11$
6 unit of cellulase : 6 unit of xylanase	50	$5.71^{Bd} \pm 0.21$	$4.19^{Ab} \pm 0.60$	$4.96^{ABd} \pm 0.36$

Different letters in the same row (A, B, C) and same column (a, b, c, d) indicated the significant differences ($p<0.05$).

The result was low compared to the previous study by Wattanakitjanukul (2008) who used alkali treated palm empty fruit bunch as substrate using 6 unit of cellulase and 3 unit of β-glucosidase at 37 °C with the reducing sugar up to 9.83 ± 0.03 g/l. This occasion might be happened due to the presents of cellobiose which is thought to be a stronger inhibitor of the cellulases than glucose. Additionally, another experiment was conducted using autoclaved pretreated PPF as substrate with addition of β-glucosidase to enhance the saccharification by using mixture of cellulase and xylanase at 50 °C on rotary shaker 150 rpm for 48 hours.

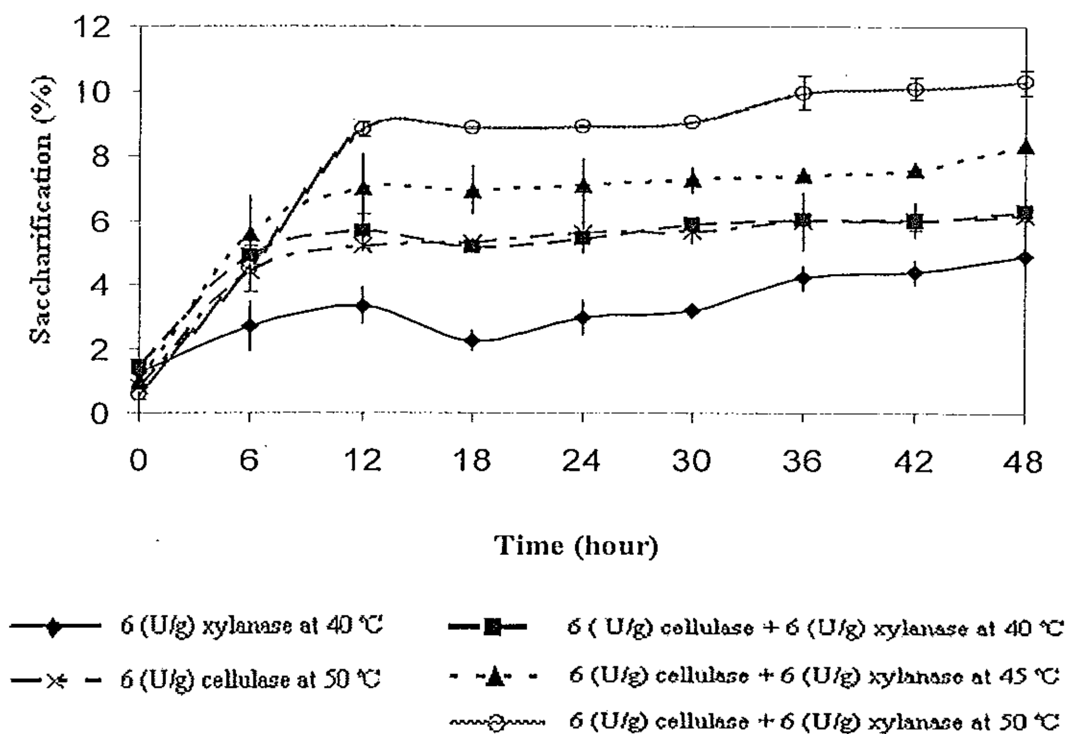


Figure 9. Saccharification from hydrolysis of palm pressed fiber autoclave pretreated by 10% NaOH for 15 min by cellulase (6 U/g), xylanase (6 U/g) and mixture of cellulase and xylanase (6:6) U/g substrate in 0.05M citrate buffer pH 5.0) with different temperature (40 °C, 45 °C and 50 °C).

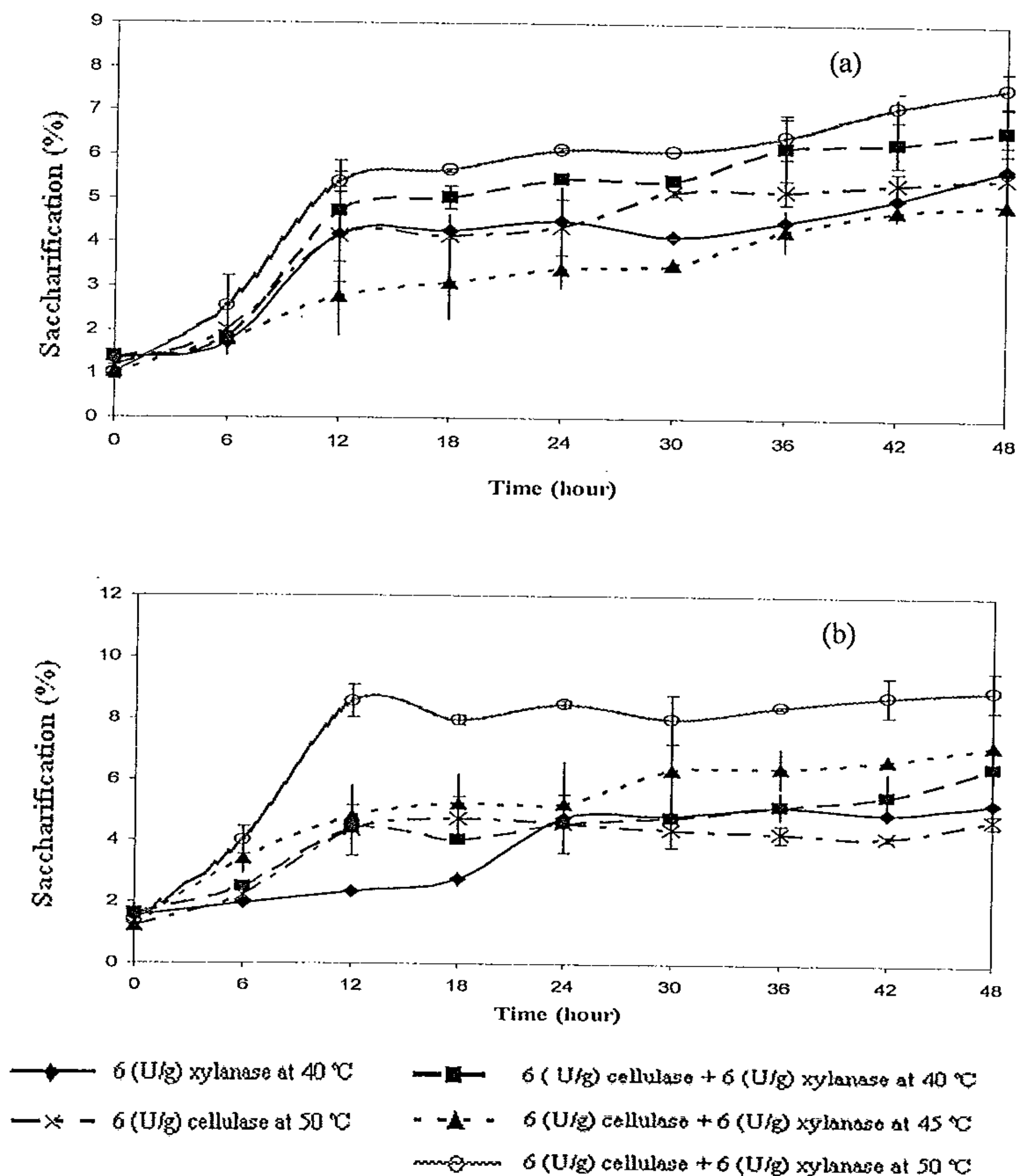


Figure 10. Saccharification from hydrolysis of PPF by 10% NaOH for 60 min on hot plate (a) and 10% NaOH for 10 min in microwave (b) by cellulase (6 U/g), xylanase (6 U/g) and mixture of cellulase and xylanase (6:6) U/g substrate in 0.05M citrate buffer pH 5.0) with different temperature (40 °C, 45 °C and 50 °C).

2.2. Effect of addition of β-glucosidase and enzyme concentration.

The different enzyme concentration of the mixture of xylanase and cellulase was conducted at 50 °C for reducing sugar production from alkali/autoclave pretreated PPF. The unit of each enzyme (cellulase and xylanase) used was 4, 6 and 8 U/g substrate. The results showed that the highest reducing sugar was found at 8 unit mixture of cellulase (8 U/g substrate) and xylanase (8 U/g substrate) enzymes which gave reducing sugar 6.4 g/l (Table 9). Increasing the dosage of cellulases in the process, to a certain extent, can enhance the yield and rate of the hydrolysis, but would significantly increase the cost of the process.

Table 9. Reducing sugar concentration (g/l) at enzymatic hydrolysis of alkali/autoclave treated PPF at different enzyme concentration (cellulase:xylanase at 4:4, 6:6 and 8:8 U/g substrate) and addition of β-glucosidase (4,6 and 8 U/g substrate) pH 5.0 for 48 hour at 50 °C.

Treatment	Reducing sugar concentration (g/l) at each enzyme unit (cellulase, xylanase, with/without β-glucosidase)		
	4 U	6 U	8 U
Without addition of β-glucosidase	$4.87^{a} \pm 0.03$	$5.62^{b} \pm 0.01$	$6.4^{c} \pm 0.01$
With addition of β-glucosidase	$5.05^{a} \pm 0.04$	$6.01^{b} \pm 0.03$	$7.25^{c} \pm 0.03$

Different letters in the same row indicated the significant differences ($p<0.05$).

Zheng *et al.* (2009) implied that cellulose conversion increased with the addition of cellulase up to a certain level. Zheng *et al.*, (2009) investigated enzymatic hydrolysis of creeping wild rygrass (CWR) in various cellulase concentrations, including 5, 15, 30, 60, 100, and 150 FPU/g cellulose were tested. The β-glucosidase concentration of 45 CBU/g cellulose remained constant under all different cellulase

concentrations. The solid loading was kept at a constant of 8%. The total reaction time was 168 h. The report showed that enzymatic digestibility increased from 60 to 93% after 168 h of enzymatic hydrolysis with the increase of cellulase concentration from 5 to 30 FPU/g-cellulose. Only 6% increase of cellulose conversion was found when cellulase concentration increased further to 60 FPU/g.

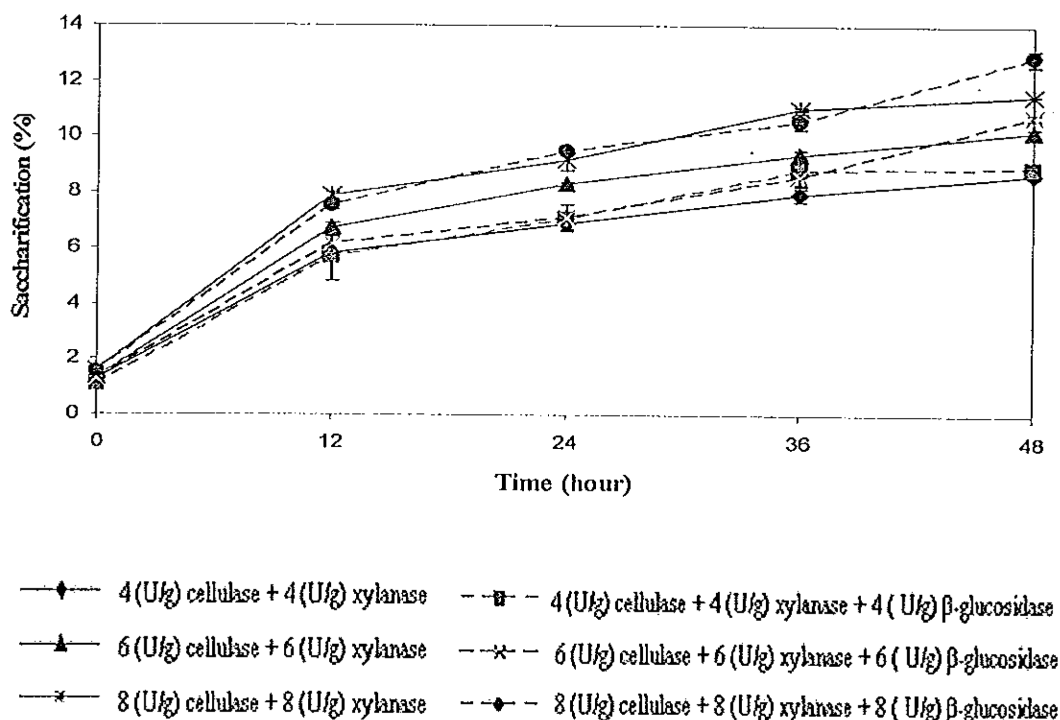


Figure 11. Saccharification from hydrolysis of alkali/autoclave pretreated PPF using mixture of cellulase and xylanase (4:4, 6:6, 8:8 U/g) substrate and addition of β-glucosidase (4,6 and 8 U/g substrate) in 0.05M citrate buffer pH 5.0 at 50 °C.

Moreover, supplementation of β-glucosidase (4, 6 and 8 U/g substrate) was also investigated. The result showed in Table 10 and Figure 11. Without supplementation of β-glucosidase, mixture of cellulase and xylanase from 4, 6 and 8 U/g biomass enhanced %saccharification 8.69 ± 0.11, 10.21 ± 0.14 and 11.52 ± 0.01

%. But when β-glucosidase was added to system, %saccharification was increased to 8.89 ± 0.28 %, 10.76 ± 0.10 and 12.87 ± 0.28 % (Figure 11). It corresponds to the reports by Zhang *et al.* (2009) determined effect of supplementation of β-glucosidase of ryegrass with Spezyme CP loading of 9.1 mg protein/g glucan (30 CBU/g glucan). Without β-glucosidase, the concentration of cellobiose in hydrolysate is 0.6 mg/ml, and 24-h yield of glucose was only 57.1%. It may be concluded that a little of cellobiose can significantly inhibit cellulase. With the increasing β-glucosidase loading, the concentration of cellobiose decreased. However, by using SPSS two paired of t sample, the effect of β-glucosidase addition was analyzed. It was showed that there was no significant different between addition of β-glucosidase and without addition of β-glucosidase although the amount of reducing sugar was increased. Zheng *et al.* (2009) suggested that low β-glucosidase concentration led to high cellobiose accumulation, which strongly inhibited cellulase activity resulting in low enzymatic digestibility and glucose concentration. Despite the synergism of xylanase and cellulase could enhanced saccharification, the present of xylanase also can inhibit the activity of cellulase because of the competition for productive binding sites between xylanase and cellulase (Carpita and Gibeaut, 1993). Moreover, Carpita and Gibeaut (1993) suggested that xylobiose which occur during hemicellulose conversion together with cellobiose accumulation inhibits the activity of β-glucosidase. The higher lignin amount also became a barrier for enzymes to attack the active side.

3. Ethanol production from palm pressed fiber (PPF)

3.1. Fermentation of PPF enzymatic hydrolysate

The performance of three strains of *Zygomycetes* with respect to ethanol production was compared in anaerobic and aerobic condition with hydrolysate from palm pressed fiber (PPF) hydrolysis using 8 U/g substrate for each enzyme (cellulase, xylanase and β-glucosidase) for 48 hours on rotary shaker 150 rpm as carbon and energy source with initial reducing sugar 8.3 g/l. The results of cultivation of the filamentous fungi *Mucor indicus, Mucor hiemalis* and *Rhizopus oryzae* on the PPF enzymatic hydrolysate are summarized in Table 10. All three *Zygomycetes* strains

were able to grow on the hydrolyzates, and produced ethanol. The sugar was almost completely assimilated within 1 day by *Mucor indicus* in aerobic condition (Figure 12). This fact was similar with Karimi *et al.* (2006b) that *Mucor indicus* could assimilate hexose and pentose faster more than *Saccharomyces cerevisiae* and *Rhizopus oryzae*. Ethanol concentration from *Mucor indicus, Mucor hiemalis* and *Rhizopus oryzae* was 4.23 ± 0.30, 4.10 ± 0.28 and 4.15 ± 0.33 g/l, when it was cultivated in PPF enzymatic hydrolysate under aerobic condition. Meanwhile, ethanol concentration under anaerobic condition was found similar to aerobic condition with the ethanol concentration of 4.33 ± 0.07, 4.27 ± 0.05, 4.28 ± 0.41 g/l, respectively. Furthermore, Table 10 described the yield and productivity of ethanol by *Mucor indicus, Mucor hiemalis* and *Rhizopus oryzae* under aerobic and anaerobic condition. It was showed that *Rhizopus oryzae* gave the highest ethanol yield by 0.51 g/g reducing sugar under aerobic condition. In contrast, *Mucor indicus* gave the highest ethanol yield by 0.52 g/g reducing sugar under anaerobic condition. The highest ethanol productivity of PPF enzymatic hydrolysate from *Mucor indicus, Mucor hiemalis* and *Rhizopus oryzae*, was found under aerobic condition by *Mucor indicus* which gave 0.51 g/g reducing sugar on 1 day of cultivation.

Changes in the pH of the fermentation medium were observed in cultures everyday until 5 days of fermentation (Figure 13). Under aerobic and anaerobic condition, the pH decreased from 5.5 to 5.2 in aerobic condition and from 5.5 to 5.45 in anaerobic condition after 1 day of cultivation. It means that *Mucor indicus, Mucor hiemalis* and *Rhizopus oryzae* produced some acids which affected on pH of the medium. This case was similar to Millati *et al.* (2005) result, whereas the *Zygomycetes* strains not only produced high concentration of ethanol but also lactic acid, acetic acid and succinic acid which affected the initial pH in the medium. In addition, Oda *et al.* (2003) conducted experiment using two groups of 15 *Rhizopus oryzae* strains were grown in a liquid medium using glucose as substrate to analyse metabolic products such as lactic, fumaric, malic, other organic acids and ethanol. The final pH levels of the media after cultivation were 6.0–6.4 and 3.0–3.2 with and without addition of calcium carbonate which stimulated the production of lactic acid

higher and reduced that of ethanol, indicating that enzymes converting these metabolites were influenced by pH of media.

Table 10. Ethanol concentration and ethanol yield of *Mucor indicus, Mucor hiemalis* and *Rhizopus oryzae* using PPF enzymatic hydrolysate as carbon source for 5 days cultivation under aerobic and anaerobic condition with initial sugar concentration of 8.3 g/l.

Strain	Ethanol concentration (g/l)*	Ethanol yield * ($g_{ethanol}/g_{sugar}$)	Ethanol productivity* ($g_{ethanol}/g_{substrate.day}$)	Ethanol Yield* ($g_{ethanol}/g_{cel+hem}$)	Biomass yield** ($g_{biomass}/g_{sugar}$)
Aerobic					
Mucor indicus	$4.23^a \pm 0.30$	0.50 ± 0.29	0.51 ± 0.29	0.118 ± 0.008	0.10 ± 0.005
Mucor hiemalis	$4.16^a \pm 0.28$	0.50 ± 0.03	0.51 ± 0.03	0.116 ± 0.008	0.06 ± 0.01
Rhizopus oryzae	$4.28^a \pm 0.33$	0.51 ± 0.82	0.48 ± 0.82	0.117 ± 0.019	0.07 ± 0.006
Anaerobic					
Mucor indicus	$4.33^a \pm 0.07$	0.52 ± 0.01	0.47 ± 0.01	0.121 ± 0.003	0.04 ± 0.008
Mucor hiemalis	$4.27^a \pm 0.05$	0.48 ± 0.08	0.26 ± 0.08	0.117 ± 0.004	0.02 ± 0.015
Rhizopus oryzae	$4.15^a \pm 0.41$	0.50 ± 0.10	0.25 ± 0.10	0.120 ± 0.020	0.01 ± 0.008

* Calculation based on the maximum value of cultivation.

** Calculation was measured at the end of fermentation (5 days).

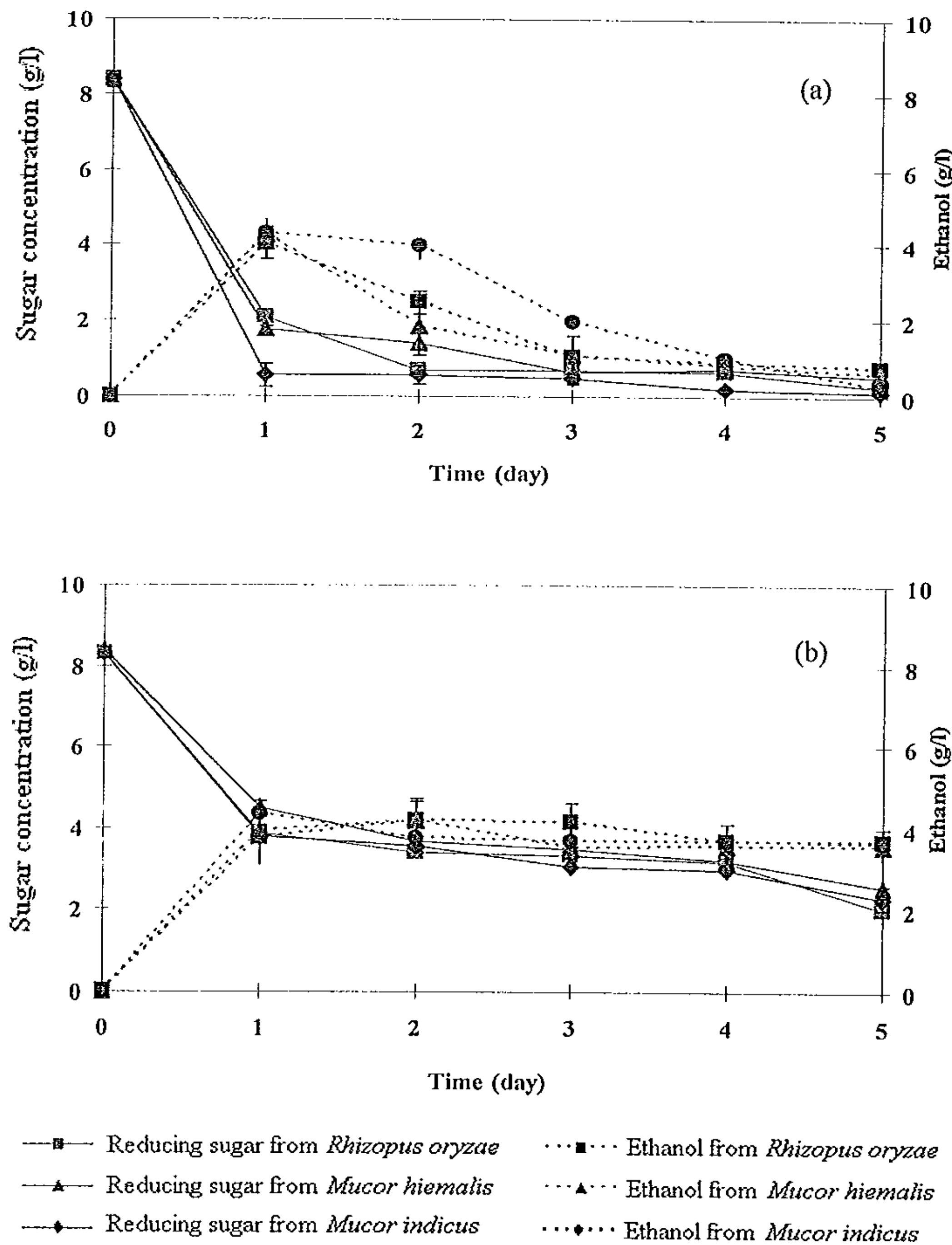


Figure 12. Ethanol and reducing sugar concentration (g/l) of *Mucor indicus, Mucor hiemalis and Rhizopus oryzae* in aerobic (a) and anaerobic (b) condition using PPF enzymatic hydrolysate 8.3 (g/l) pH 5.5 for 5 days on 150 rpm.

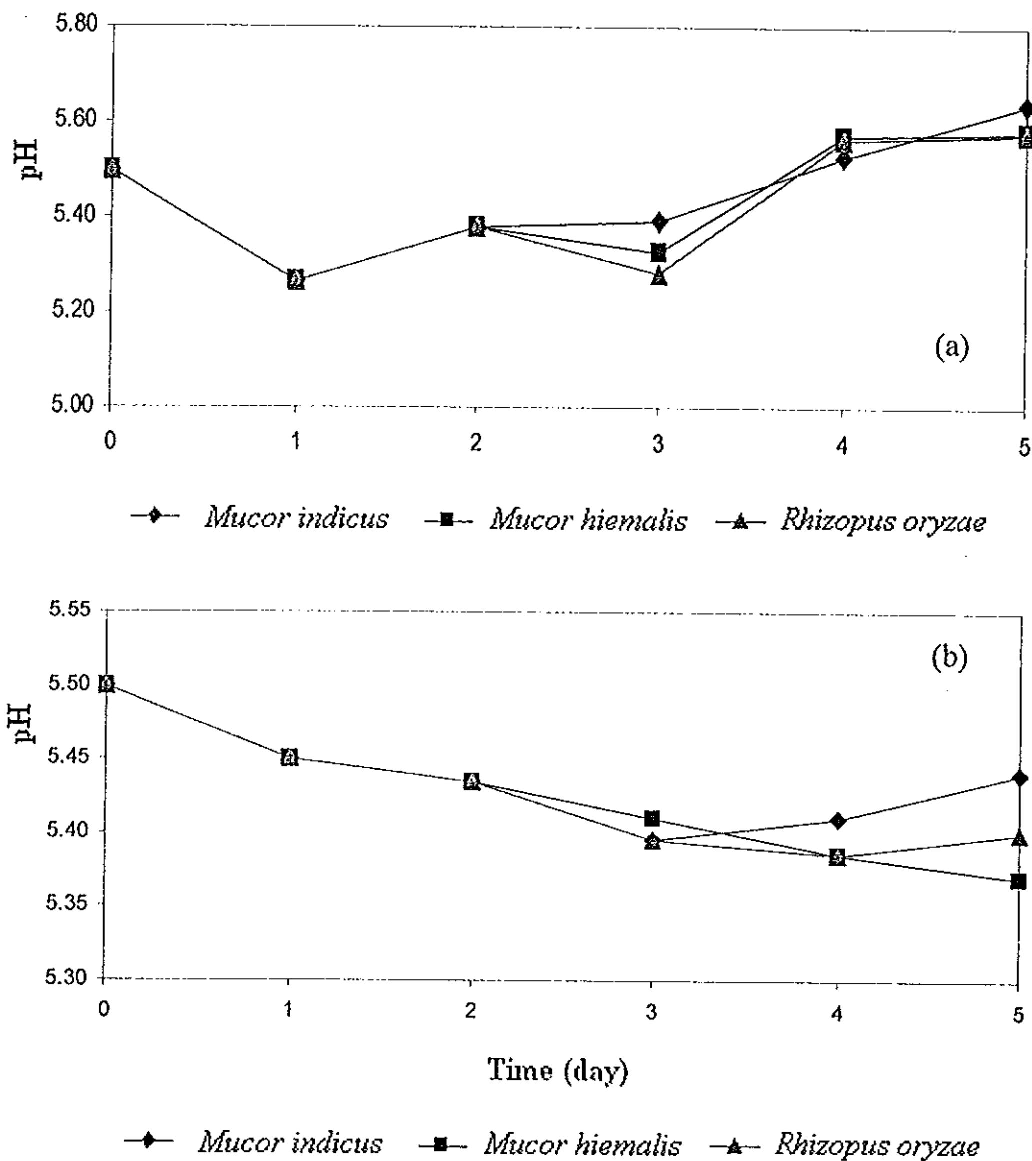


Figure 13. pH profile of *Mucor indicus, Mucor hiemalis and Rhizopus oryzae* in aerobic (a) and anaerobic (b) condition using PPF enzymatic hydrolysate 8.3 (g/l) pH 5.5 for 5 days on 150 rpm.

Biomass was collected at the end of fermentation and the results showed that *Mucor indicus* gave the highest yield of fungal biomass compare to the others two (Table 10). All strains (*Mucor indicus, Mucor hiemalis* and *Rhizopus oryzae)* produced the high biomass when they were cultivated in aerobic compared to

anaerobic condition. It can be suggested that in aerobic condition oxygen uptake by fungal made them able to grow well in PPF enzymatic hydrolysate.

In dimorphic species of filamentous fungi such as *Mucor*, spore germination can lead to the production of either filamentous (mycelium) or spherical (yeast) cells, depending on environmental conditions (Bartnicki-García 1963; Sypherd *et al.*, 1978 cited by Zazueta-Sandoval and Guiterrez-Corona, 1999). The mycelial or the yeast phase of development can occur in the presence of oxygen or under microaerobiosis and depends on the carbon source and/or the presence of morphogenetic compounds. Yeast development requires hexoses and invariably correlates with a fermentative metabolism, whereas the mycelial phase has been shown to be metabolically flexible in that it can occur during either fermentation or oxidative metabolism; the latter can occur with several carbon sources.

The correlation of the yeast phase with high rates of ethanol production holds true under various growth conditions, e.g., during anaerobic or aerobic growth in media containing morphogenetic compounds such as phenethyl alcohol (PEA), dibutyryl cyclic AMP, or certain amino acids (Orlowsky 1991 cited by Zazueta-Sandoval and Guiterrez-Corona, 1999). In the presence of oxygen and glucose, *Mucor racemosus* grows oxidatively as mycelial cells with little ethanol being produced; on the other hand, mycelium or yeast cells grown anaerobically show high rates of ethanol production that correlate with higher levels of alcohol dehydrogenase (ADH) activity as compared to those of aerobic mycelial cells (Borgia *et al.*, 1985 cited by Zazueta-Sandoval and Guiterrez-Corona, 1999). Figure 6 described glucose metabolism by filamentous fungi and the products which resulted from fermentation process.

The result from this experiment was compared to others (Subkaree, 2008; Wattanakitjanukul, 2008; Millati *et al.*, 2005) in term of ethanol yield. Table 11 was summarized ethanol from palm pressed fiber and another materials. From the summary, this study yielded the low ethanol compared to others, in the term of ethanol based on cellulose and hemicellulose as substrate.

Table 11. Ethanol yield from palm pressed fiber (PPF) and others material by fungi.

Raw materials	Pretreatment	Fermentation condition	Reducing sugar (g/l)	Strain	Ethanol concentration (g/l)	Yield ($g_{ethanol}$/ $g_{cel+hem.}$)	References
Palm pressed fiber 10%	10% NaOH 15 minutes	SSF (aerobic)	-	*S. cerevisiae* TISTR 5596	10.38	0.19	Subkaree, 2008
Palm empty fruit bunches 5%	10% NaOH 100 °C 30 minutes	SSF (aerobic)	-	*S. cerevisiae* TISTR 5055	9.38	0.29	Wattanakitjanukul, 2008
Spruce wood	0.05% H_2SO_4 autoclave 15 bar 10 minutes	SHF (aerobic)	50	*Rhizopus oryzae,*	-	0.41	Millati *et al.*, 2005
				Mucor indicus,		0.44	
				Mucor hiemalis		0.44	
Rice straw 20 g/l	0.5% H_2SO_4 autoclave 15 bar 10 minutes	SHF (aerobic)	7.5	*Rhizopus oryzae,*	-	0.36	Abedinifar *et al.*, 2009
				Mucor indicus,		0.39	
				S. cerevisiae		0.41	
Palm pressed fiber 5%	10% NaOH autoclave 15 minutes 121 °C	SHF (aerobic)	8.3	*Rhizopus oryzae,*	4.28 ± 0.33	0.117	This study
				Mucor indicus,	4.16 ± 0.28	0.118	
				Mucor hiemalis	4.23 ± 0.30	0.116	

SSF : Simultaneous saccharification and fermentation

SHF : Separate hydrolysis and fermentation

3.2. Fermentation of untreated and alkali/autoclave pretreated PPF

Three strains of *Zygomycetes* also have been used as microorganism for ethanol production. The fermentation was conducted using 5 g/100 ml untreated and treated PPF in aerobic and anaerobic condition. Most of the *Zygomycetes* strains were not able to grow well in the solid form of PPF. All of reducing sugar, ethanol concentration and pH profile from alkali/autoclave pretreated PPF by *Mucor indicus, Mucor hiemalis* and *Rhizopus oryzae* were summarized in Table 12, Figure 14, 15, 16. Moreover, the result showed that there was no ethanol detected from fermentation of untreatead and treated PPF by *Mucor indicus*. However, the trace amount of ethanol was found from fermentation by *Mucor hiemalis* and *Rhizopus oryzae*. The amount of ethanol was found at 0.03 ± 0.30 in aerobic and no detection of ethanol in anaerobic condition when *Mucor hiemalis* was used in cultivation of untreated PPF. In addition, the amount of ethanol was also found only in aerobic condition (0.04 ± 0.27) when treated PPF was used by *Mucor hiemalis* as carbon source. It indicated that either *Mucor indicus* or *Mucor hiemalis* can not directly converted cellulose to ethanol in anaerobic condition. Meanwhile cultivation of *Rhizopus oryzae* using untreated and treated PPF gave the ethanol amount 0.085 ± 0.04 and 0.11 ± 0.03 in aerobic condition, meanwhile cultivation in anaerobic condition gave 0.117 ± 0.11 and 0.25 ± 0.07 respectively. It seems that *Rhizopus oryzae* able to produce ethanol directly from cellulose because *Rhizopus oryzae* is a cellobiose utilizing organism which can facilitate saccharification of cellulose (Karimi *et al.*, 2006b).

Previously, there have no report which studied about direct fermentation of cellulose into ethanol by *Mucor indicus, Mucor hiemalis* and *Rhizopus oryzae*. But, *Fusarium oxysporum* has been reported to acquire the ability of fermenting cellulose directly to ethanol (Christakapoulos and Kekos, 1989). The cellulase of *Fusarium oxysporum* fermented glucose, xylose, cellobiose, and cellulose directly to ethanol. The result showed that *Fusarium* sp. produced maximum ethanol concentrations at 9.6 and 14.5 g/l in a medium containing 20 and 50 g/l cellulose, respectively. Based on the result above, it seems that *Mucor indicus, Mucor hiemalis* and *Rhizopus oryzae* was able to produce ethanol even only in trace concentration due to presence of reducing sugar (Table 12, Figure 14 and 15). It showed in Figures 14

and 15 that reducing sugar was increasing, which might be due to enzyme activity produced from fungi.

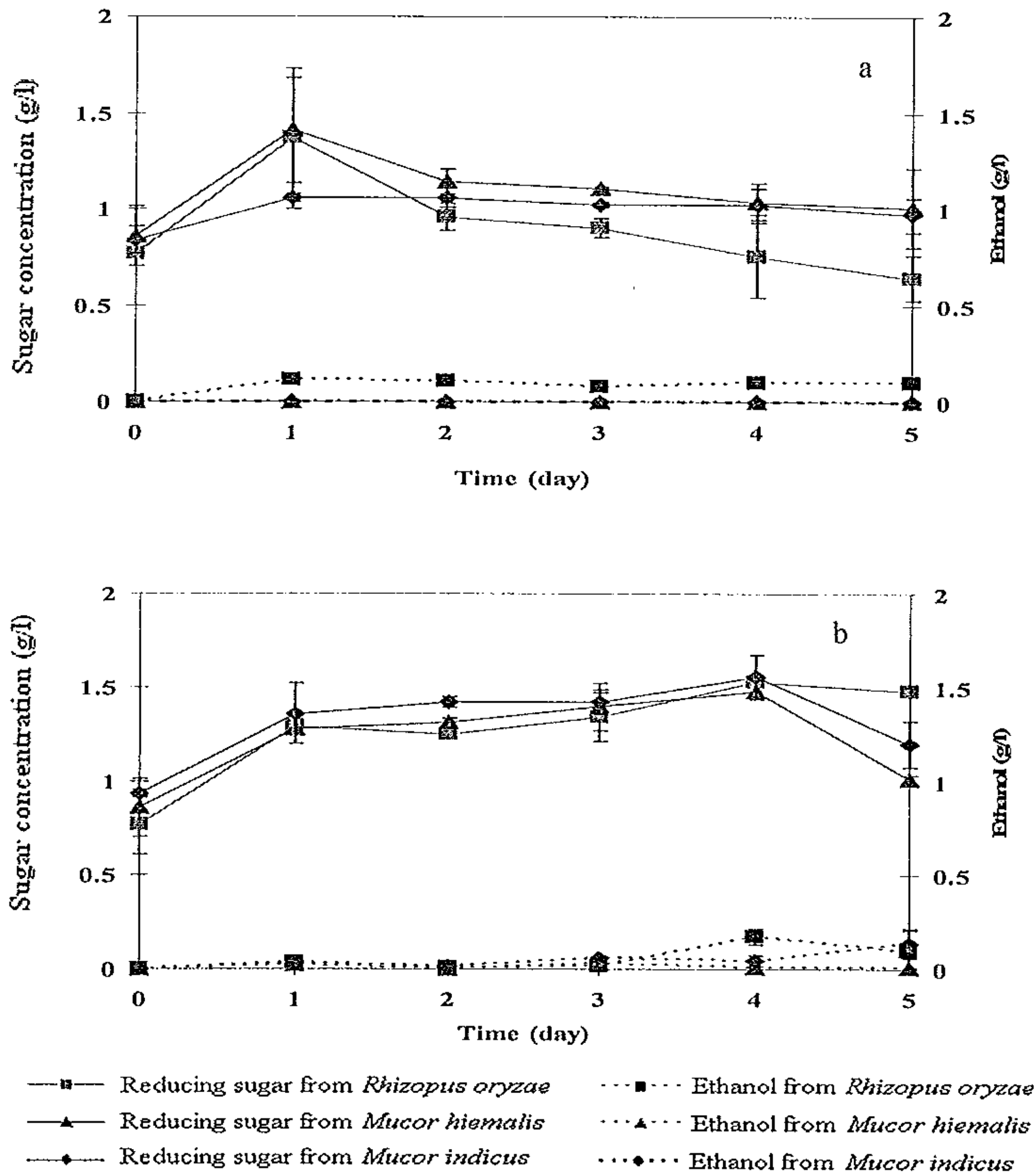


Figure 14. Ethanol and reducing sugar concentration of *Mucor indicus, Mucor hiemalis and Rhizopus oryzae* using untreated (a) and alkali/autoclave pretreated (b) PPF in aerobic condition for 5 days fermentation pH 5.5 substrate 5 g/100 ml at 150 rpm.

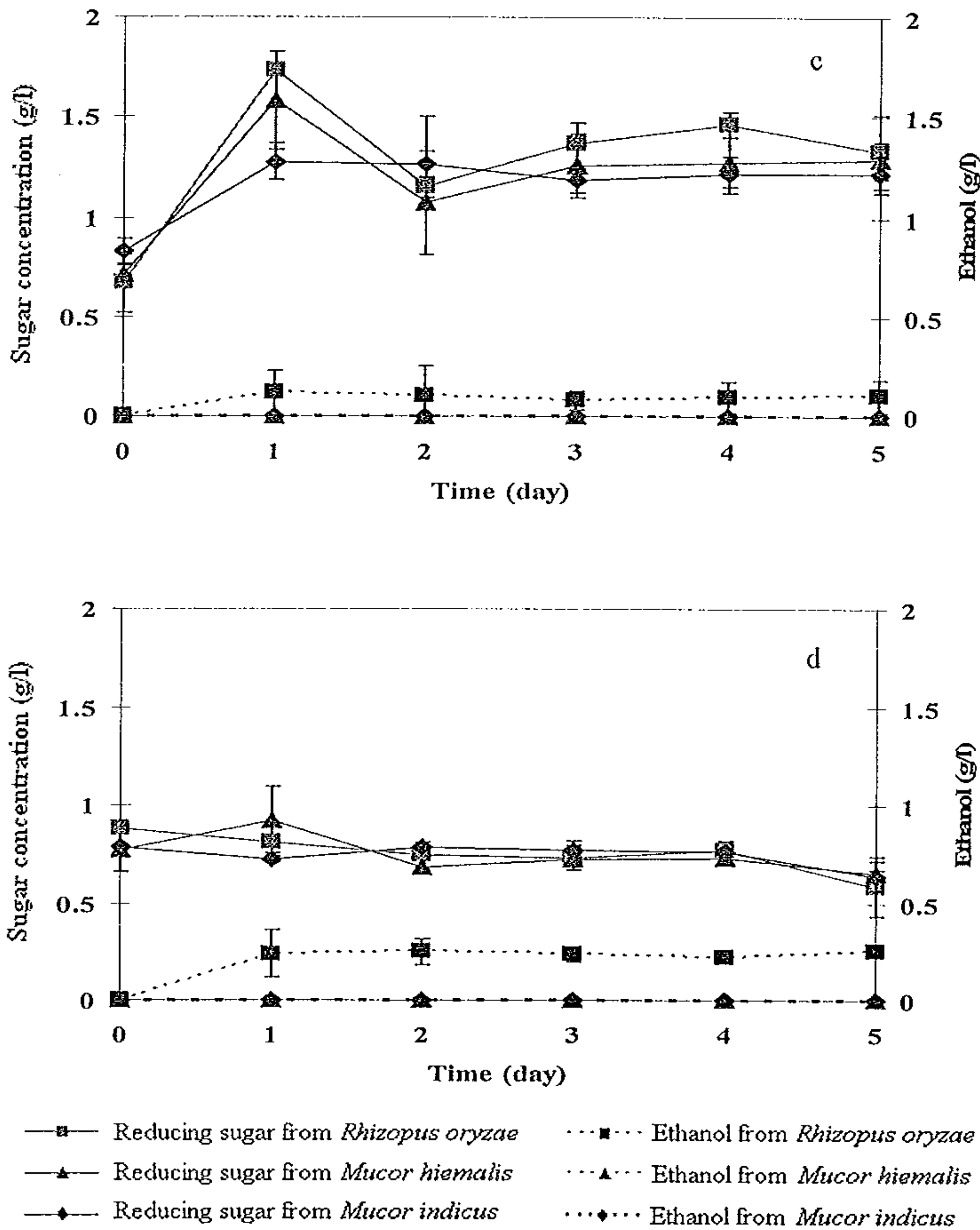


Figure 15. Ethanol and reducing sugar concentration of *Mucor indicus, Mucor hiemalis and Rhizopus oryzae* using untreated (c) and alkali/autoclave pretreated (d) PPF in anaerobic condition for 5 days fermentation pH 5.5 substrate 5 g/100 ml at 150 rpm.

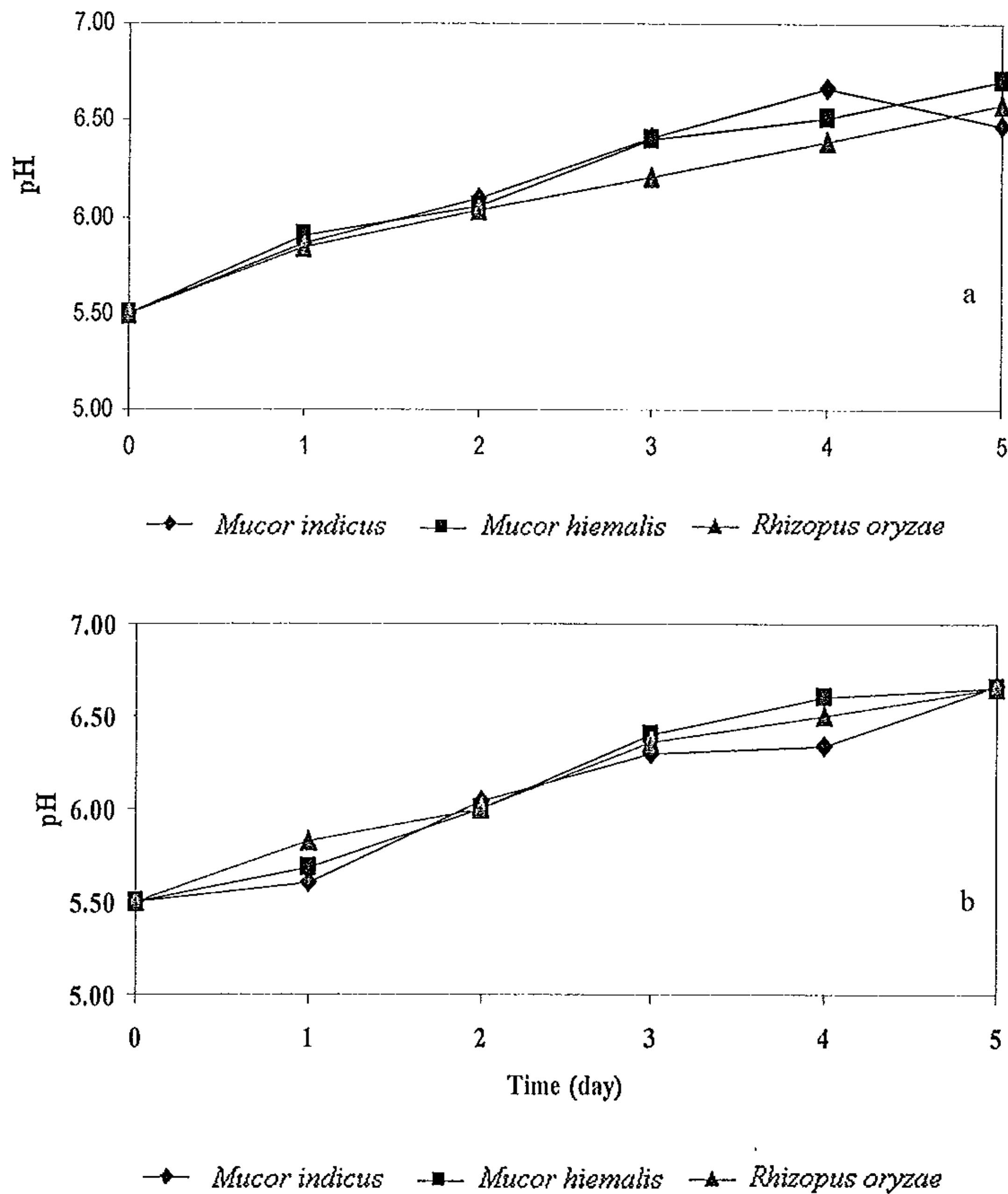


Figure 16. pH profile of *Mucor indicus, Mucor hiemalis and Rhizopus oryzae* in aerobic (a) and anaerobic (b) condition using alkali/autoclave pretreated PPF pH 5.5 for 5 days on 150 rpm.

In addition, tolerance for low levels of oxygen has been observed in some aquatic fungi like Allomyces (Deacon, 1984 cited by Durrant, 1996). A number

of yeasts and several filamentous fungi, such as *Fusarium oxysporum, Mucor hiemalis* and *Aspergillus jiumigatus* can grow by fermenting sugars, without respiration (Dube, 1990; Singh *et al.,* 1992 cited by Durrant, 1996).

Table 12 described the yield and productivity of ethanol by *Mucor indicus, Mucor hiemalis* and *Rhizopus oryzae* under aerobic and anaerobic condition using alkali/autoclave pretreated PPF as substrate. It was showed that *Rhizopus oryzae* gave the highest ethanol yield both under aerobic and anaerobic condition by 0.12 g/g reducing sugar under aerobic condition and 0.25 g/g reducing sugar under anaerobic condition. The highest ethanol productivity of alkali/autoclave pretreated PPF from *Mucor indicus, Mucor hiemalis* and *Rhizopus oryzae,* was found under anaerobic condition by *Rhizopus oryzae* which gave 0.20 g/g reducing sugar on 1 day of cultivation.

Changes in the pH of the fermentation medium were also observed in cultures everyday until 5 days of fermentation (Figure 16). Under aerobic and anaerobic condition, the pH increase from 5.5 to 6.7 in aerobic condition and from 5.5 to 6.65 in anaerobic condition after 5 days of cultivation. From the figure above, it showed that there were substances secreted or produced from *Mucor indicus, Mucor hiemalis* and *Rhizopus oryzae* that made pH in the medium became higher. This phenomenon could be happened because of *Mucor indicus, Mucor hiemalis* and *Rhizopus oryzae* produced or secreted enzymes into fermentation medium which converted cellulose or hemicellulose into reducing sugar for them to use as carbon source. Amadioha (1993) conducted experiment using *Rhizopus oryzae* to produce cellulase from infected potatoes tuber. The result showed that there was a general increase in growth and stability of the cellulase activity between pH 3 and 6 with the peak at pH 6. The growth of the fungus and its cellulase activity declined thereafter. Rhizopus oryzae produced cellulases in culture and infected potato tubers suggesting that the rot causing organism is a cellulolytic fungus.

Table 12. Ethanol concentration, ethanol yield, ethanol productivity of *Mucor indicus, Mucor hiemalis* and *Rhizopus oryzae* using untreated and alkali/autoclave treated PPF as carbon source for 5 days cultivation at aerobic and anaerobic condition.

Strain	Reducing sugar (g/l)	Ethanol concentration (g/l)*	Ethanol yield * ($g_{ethanol}/g_{sugar}$)	Ethanol productivity* ($g_{ethanol}/g_{substrate.day}$)	Ethanol Yield* ($g_{ethanol}/g_{cel+hem}$)
Untreated PPF					
Aerobic					
Mucor indicus	1.28 ± 0.09	nd	nd	nd	nd
Mucor hiemalis	1.73 ± 0.24	0.03 ± 0.30	0.02	0.02	8.4×10^{-4}
Rhizopus oryzae	1.58 ± 0.03	0.085 ± 0.04	0.05	0.05	2.38×10^{-3}
Anaerobic					
Mucor indicus	1.06 ± 0.09	nd	nd	nd	nd
Mucor hiemalis	1.41 ± 0.36	nd	nd	nd	nd
Rhizopus oryzae	1.37 ± 0.27	0.117 ± 0.11	0.085	0.085	3.3×10^{-3}
Alkali/autoclave pretreatead PPF					
Aerobic					
Mucor indicus	0.73 ± 0.01	nd	nd	nd	nd
Mucor hiemalis	0.81 ± 0.03	0.04 ± 0.27	0.05	0.012	1.12×10^{-4}

Table 12. (continued).

Strain	Reducing sugar (g/l)	Ethanol concentration (g/l)*	Ethanol yield * ($g_{ethanol}/g_{sugar}$)	Ethanol productivity* ($g_{ethanol}/g_{substrate.day}$)	Ethanol Yield* ($g_{ethanol}/g_{cel+hem}$)
Rhizopus oryzae	0.92 ± 0.01	0.11 ± 0.03	0.12	0.12	3.1×10^{-3}
Anaerobic					
Mucor indicus	1.42 ± 0.01	nd	nd	nd	nd
Mucor hiemalis	1.31 ± 0.03	nd	nd	nd	nd
Rhizopus oryzae	1.25 ± 0.02	0.25 ± 0.07	0.20	0.20	7×10^{-3}

nd Not detected

* Calculation based on the maximum value of cultivation.

** Calculation was measured in the end of fermentation (5 days).

Furthermore, the ability of *Zygomycetes* to produce cellulolytic enzyme was determined. Cellulase unit was tested using method by IUPAC using CMC as substrate. The temperature was set up to 30 °C and 50 °C. The variety of temperature was based on the fermentation condition which used room temperature with average of 30 °C. Meanwhile, 50 °C was used due to the optimum condition of cellulase. Table 13 was summarized the result from CMCase activities of three fungi.

Table 13. The maximal CMCase activities from cultivation of *Mucor indicus, Mucor hiemalis* and *Rhizopus oryzae* using alkali/autoclave pretreated PPF of 5 g/100 ml pH 5.5 at 30 °C and 50 °C after 5 days cultivation.

Strain	**Condition**	**Temperature (°C)**	**CMCase (U/ml)**
Mucor indicus	Aerobic	30	0.213 ± 0.06
		50	0.019 ± 0.007
	Anaerobic	30	0.029 ± 0.003
		50	0.028 ± 0.19
Mucor hiemalis	Aerobic	30	0.251 ± 0.11
		50	0.177 ± 0.05
	Anaerobic	30	0.320 ± 0.29
		50	0.198 ± 0.04
Rhizopus oryzae	Aerobic	30	0.319 ± 0.12
		50	0.243 ± 0.018
	Anaerobic	30	0.421 ± 0.39
		50	0.296 ± 0.26

The results showed that there was less activity detected from *Mucor indicus*. In the meantime, cellulase activity was found in supernatant of *Mucor hiemalis* and *Rhizopus oryzae*. Table 13 was interpreted the unit of cellulase which found in supernatant of *Mucor hiemalis* and *Rhizopus oryzae* when it cultivated from alkali/autoclave pretreatead PPF in aerobic and anaerobic condition. *Mucor indicus*

and *Mucor hiemalis* was able to produced reducing sugar and cellulase enzyme (Figure 14, 15 and Table 13), but, the two strains can not utilized sugar directly to ethanol. It might be because of *Mucor indicus* and *Mucor hiemalis* used sugar for their growth only.

Meanwhile, *Rhizopus oryzae* showed activity of cellulase and it can directly converted PPF to ethanol either in aerobic and anaerobic condition either in treatead PPF and untreated PPF. This result showed higher activity than previous experiment. Amadioha (1993) reported that *Rhizopus oryzae* produced endo-β-1,4-glucanase and exo-β-1,4-glucanase in culture of *Rhizopus* infected tissues of *S. tuberosum* in potatoes. The cellulase unit was found at 0.26 ± 0.4 U/ml with the optimum temperature and pH of the enzymes were 30 °C and pH 6.

1.3. Fermentation of prehydrolysate liquid from alkali pretreatment

Three strains of *Zygomycetes* also have been use as microorganism for ethanol production. The fermentation was conducted using prehydrolysate liquid from alkali pretreatment. The liquid have to be added by HCl 37% to decrease the pH from 12 to 5.5. The report showed that the *Zygomycetes* strains were able to grow in the prehydrolysate liquid but they failed to produce ethanol (Figure 17 and Table 14). The initial reducing sugar in prehydrolysate liquid was around 5.6 g/l but it can only produced ethanol around 0.09-0.13 g/l. If the outcome was compared with the ethanol production from PPF enzymatic hydrolysate, where these fungi utilized the reducing sugar (initial reducing sugar 8.3 g/l) and produced ethanol around 4 g/l. The ethanol amount of prehydrolysate liquid was lower. It was indicating that there were inhibitors action which prevented the fungi to produce ethanol. Palmqvist and Hagerdal (1999) described that during the hydrolysis of lignocellulose materials a wide range of compounds which are inhibitory to microorganism are formed or release. Based on their origin the inhibitors are usually divided in three groups: weak acids, furan derivatives and phenolic compounds. These compounds limit efficient utilization of the hydrolysate for ethanol production by fermentation. Moreover, the present of

inhibitors such as lignin in prehydrolysate barrier the capability of *Zygomycetes* strains to produce ethanol. However, the fungus still grew in prehydrolysate.

Table 14. Ethanol concentration and ethanol yield of *Mucor indicus, Mucor hiemalis* and *Rhizopus oryzae* using prehydrolysate liquid (initial sugar of 5.6 g/l) as carbon source for 5 days cultivation at aerobic and anaerobic condition at pH 5.5.

Strain	Ethanol concentration (g/l)		Ethanol Yield ($g_{ethanol}/g_{sugar}$)	
	Aerobic	Anaerobic	Aerobic	Anaerobic
Mucor indicus	0.12 ± 0.10	0.12 ± 0.23	0.02	0.02
Mucor hiemalis	0.038 ± 0.09	0.014 ± 0.09	6×10^{-3}	2.5×10^{-3}
Rhizopus oryzae	0.130 ± 0.018	0.098 ± 0.23	0.02	0.018

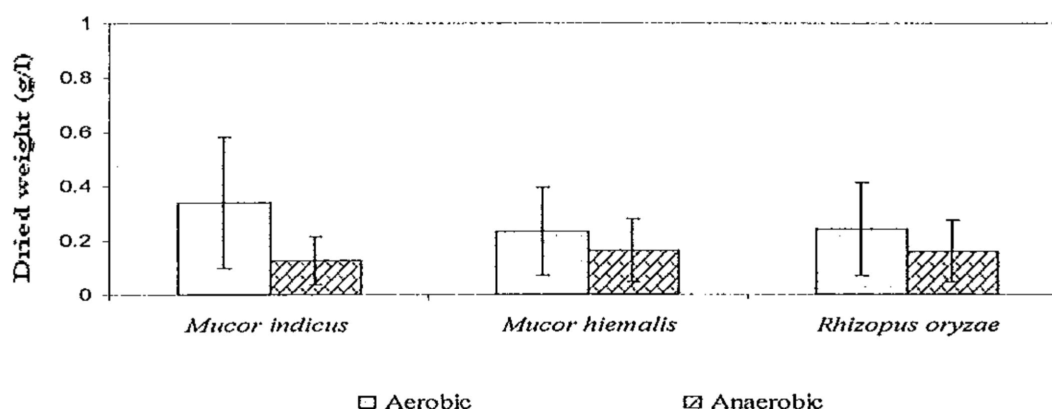


Figure 17. Biomass dried weight from cultivation of *Mucor indicus, Mucor hiemalis* and *Rhizopus oryzae* in prehydrolysate liquid PPF at pH 5.5 for 5 days in aerobic and anaerobic condition.

From the result, *Rhizopus oryzae* was a good choice to produce ethanol, because it can produced ethanol from PPF enzymatic hydrolysate, untreated PPF, alkali/autoclave pretreated PPF and prehydrolysate liquid. But, the fungi still produced the low amount of ethanol. The fungi was able to grow well and produced high yield

of ethanol (yield based on reducing sugar amount) when it was using PPF enzymatic hydrolysate. But when the strain was used to convert directly PPF to ethanol using untreated and alkali/autoclave pretreatead PPF as substrates, the result was not high even though this strain can produce cellulase (0.319 ± 0.12 in aerobic and 0.421 ± 0.39 in anaerobic). Cultivation of *Rhizopus oryzae* in prehydrolysate liquid was resulted low amount of ethanol, it might be due to the present of inhibitors such as lignin that limiting the utilization of hydrolysate by fungi. From all the experiment, ethanol amount from all three strains (*Mucor indicus, Mucor hiemalis* and *Rhizopus oryzae*) was found higher in anaerobic condition, even though it was not significantly different when compared to aerobic condition. This fact can happen due to higher acitivity of alcohol dehydrogenase when *Rhizopus oryzae* was cultivated in anaerobic condition (Zazueta-Sandoval and Guiterrez-Corona, 1999). The next experiment was tried to enhance the ethanol production by combining aerobic and anaerobic condition.

4. Effect of one and two stages condition

The one and two stages condition using strains of *Zygomycetes* were studied regarding to ethanol production. The fermentation was conducted using PPF hydrolysate, 5 g/100 ml untreated and treated PPF and prehydrolysate liquid in aerobic and anaerobic condition for one stage. For two stages conditions, the condition was maintained to aerobic for 2 days and continued to anaerobic condition for 3 days, this experiment was based on previous experiment which conducted using PPF enzymatic hydrolysate where *Rhizopus oryzae* can produce higher amount of biomass in 2 days of cultivation. The experiment was set up for 2 days in aerobic condition to produced higher concentration of biomass and then continued with 3 days of anaerobic condition to produce higher ethanol concentration. The results were showed in Table 15, Figure 18 and Figure 19. The concentration of ethanol from one stage was higher than two stages when PPF enzymatic hydrolysate was used as substrate. It was happened due to complete sugar assimilation by *Rhizopus oryzae* was in the 2 days of aerobic fermentation. Consequently, there was lack of sugar (almost finished) for *Rhizopus oryzae* to use as substrate in anaerobic condition. The same condition was found when

prehydrolysate liquid was used as substrate. Moreover, the cultivation of *Rhizopus oryzae* using alkali/autoclave pretreated and untreated PPF with two stages was enhanced ethanol concentration. It might be because of when aerobic condition was performed, this fungus produced an enzyme and biomass that provided reducing sugar and after that, 3 days fermentation in anaerobic condition was increasing the ethanol concentration due to the higher activity of alcohol dehydrogenase (Zazueta-Sandoval and Guiterrez-Corona, 1999).

Table 15. Maximum ethanol concentration (g/l) of one stage fermentation compared with two stage fermentation of PPF enzymatic hydrolysate, alkali/autoclave pretreatead PPF, untreatead PPF and prehydrolysate liquid using *Rhizopus oryzae*.

	Reducing Sugar (g/l)	Ethanol concentration (g/l)*			
		PPF enzymatic hydrolysate	Alkali/ autoclave pretreated PPF	Untreated PPF	Prehydrolysate liquid
One stage					
Aerobic/ 5 days	8.3	$4.28^{a} \pm 0.33$ (1st day)	$0.11^{a} \pm 0.03$ (4th day)	$0.085^{a} \pm 0.04$ (1st day)	$0.130^{a} \pm 0.018$ (1st day)
Anaerobic/ 5 days	8.3	$4.15^{a} \pm 0.41$ (2nd day)	$0.25^{ab} \pm 0.07$ (5th day)	$0.117^{a} \pm 0.11$ (1st day)	$0.098^{a} \pm 0.23$ (1st day)
Two stage					
Aerobic/2 days and anaerobic/ 3 days	7.24	$3.7^{a} \pm 0.1$ (1st day)	$0.286^{b} \pm 0.2$ (3rd day)	$0.205^{a} \pm 0.3$ (5th day)	$0.077^{a} \pm 0.09$ (2nd day)

* Calculation based on the maximum value of cultivation.

Different letters in the same column indicated the significant differences ($p<0.05$).

Table 16. Ethanol yield ($g_{ethanol}/g_{sugar}$) and ethanol productivity ($g_{ethanol}/g_{sugar}.day$) of two stages fermentation of palm pressed fiber (PPF) enzymatic hydrolysate, alkali/autoclave pretreatead PPF, untreatead PPF and prehydrolysate liquid using *Rhizopus oryzae*.

Substrate	Condition	Ethanol yield * ($g_{ethanol}/g_{sugar}$)		Ethanol productivity* ($g_{ethanol}/g_{sugar}.day$)	
		One stage	Two stages	One stage	Two stages
PPF enzymatic hydrolysate	AE	0.51		0.51	
	AN	0.50		0.25	
	AE/AN		0.51		0.26
Untreated PPF	AE	0.05		0.05	
	AN	0.085		0.085	
	AE/AN		0.14		0.14
Alkali/autoclave pretreated PPF	AE	0.05		0.12	
	AN	0.20		0.20	
	AE/AN		0.19		0.19
Prehydrolysate liquid	AE	0.02		0.02	
	AN	0.018		0.018	
	AE/AN		0.014		0.014

* Calculation based on the maximum value of cultivation.

AE Fermentation under aerobic condition.

AN Fermentation under anaerobic condition.

AE/AN Two stages fermentation (2 days under aerobic continued with 3 days under anaerobic condition).

Table 16 described the yield and productivity of ethanol by *Rhizopus oryzae* in one stage and two stage fermentation. Ethanol yield from two stages fermentation condition was as same as one stage when PPF enzymatic hydrolysis was used as substrate. The productivity from two stage fermentation was low compared to one stage aerobic fermentation condition. Moreover, overall result from alkali/autoclave pretreated PPF and prehydrolysate liquid was low compared to one

stage in term of ethanol yield and ethanol productivity. Meanwhile, ethanol yield and productivity from two stages using untreated PPF was higher compared to one stage fermentation. In conclusion, the two stage was not enhanced the ethanol production when PPF enzymatic hydrolysate and prehydrolysate liquid were used as substrates. It seems that cultivation of *Rhizopus oryzae* for 2 days in aerobic condition enhanced the biomass production but not ethanol production. Meanwhile, ethanol concentration was increased when untreated PPF was used. However, from alkali/autoclave pretreated PPF under two stage condition, the ethanol concentration was not significantly different with ethanol concentration from anaerobic condition at one stage fermentation.

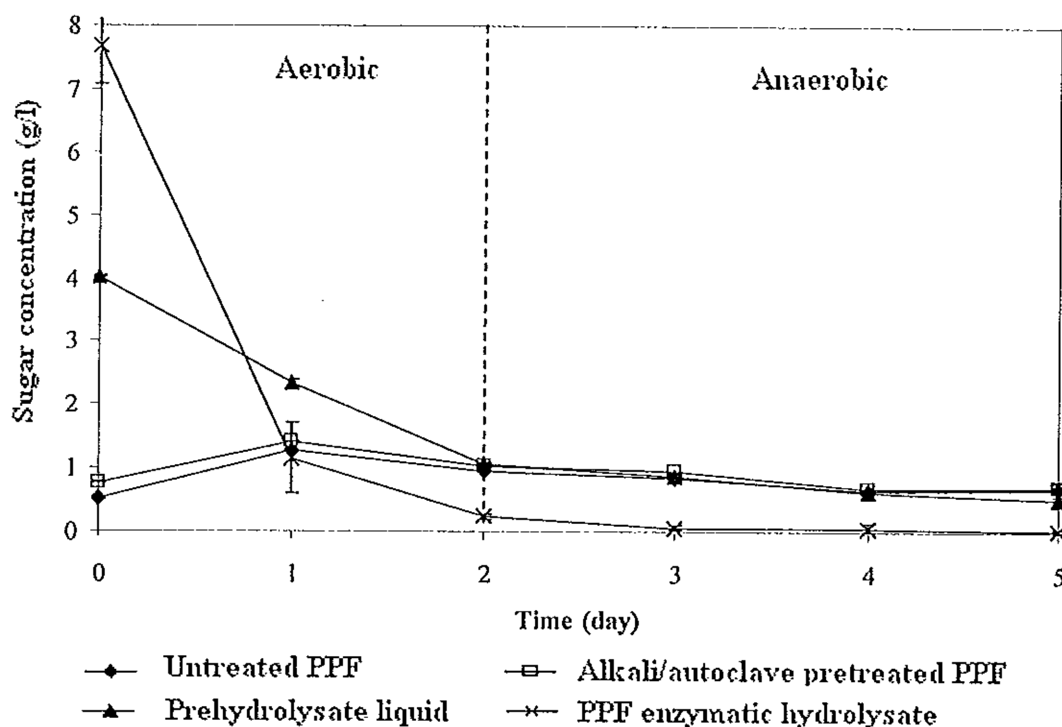


Figure 18. Reducing sugar concentration (g/l) of *Rhizopus oryzae* using untreated PPF alkali/autoclave treated PPF prehydrolysate and PPF enzymatic hydrolysate in two stage fermentation pH 5.5 at 150 rpm.

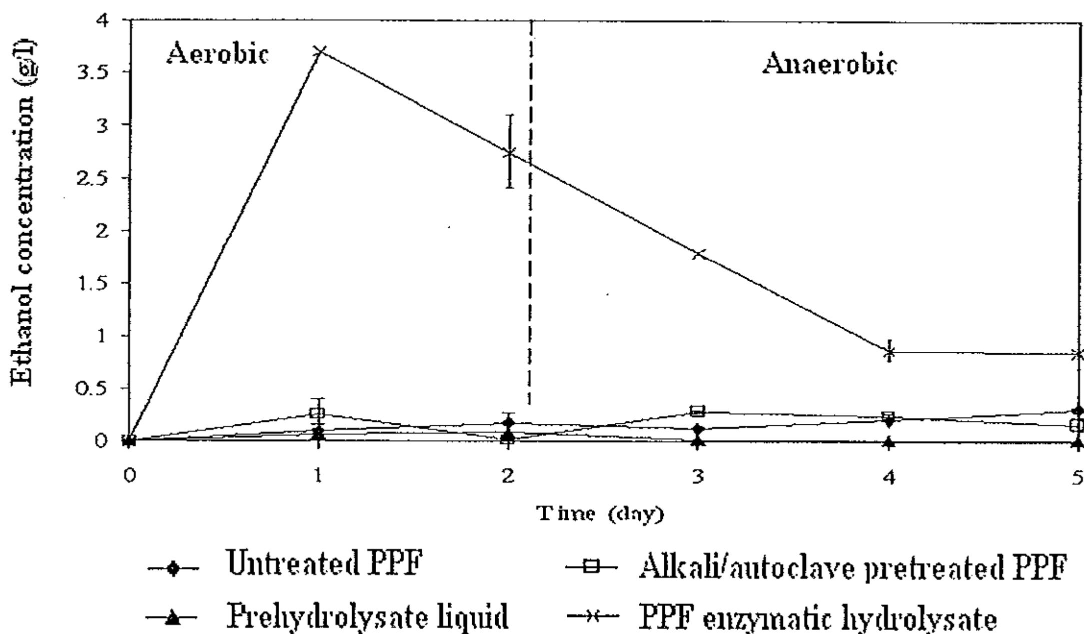


Figure 19. Ethanol concentration (g/l) of *Rhizopus oryzae* using untreated PPF, alkali/autoclave treated PPF, prehydrolysate and PPF enzymatic hydrolysate in two stage fermentation pH 5.5 at 150 rpm.

Ethanol concentration was depleted after 2 days of cultivation in aerobic condition when PPF enzymatic hydrolysate was used as substrate. This could be happened because filamentous fungi (*Rhizopus oryzae*) consumed all sugars within 2 days and then subsequently assimilate ethanol. Furthermore, Karimi *et al.* (2006a) reported that filamentous fungus such as *Mucor indicus* was able to produce ethanol in aerobic condition. However, the fungus can assimilate ethanol, while no hexoses were present in the medium. It can be suggested that in the former period catabolite repression by glucose occurred during aerobic condition in lack of glucose present. The result from this experiment was compared to others in term of ethanol yield. Table 17 was summarized ethanol from palm pressed fiber and another materials by *Rhizopus oryzae*. From the summary, this study yielded the high ethanol compared to others, in the term of ethanol based on sugar as substrate.

Table 17. Ethanol yield from palm pressed fiber (PPF) and others material by *Rhizopus oryzae* one stage and two stages fermentation.

Raw materials	Pretreatment	Fermentation condition	reducing sugar (g/l)	Strain	Yield ($g_{ethanol}/g_{sugar}$)	References
Spent sulfite liquor	Calsium sulfite paper pulp	SHF (aerobic)	133	*Rhizopus oryzae*	0.37	Taherzadeh *et al.* (2003)
Glucose	-	SHF (aerobic)	100	*Rhizopus oryzae*	0.37	Buyukkileci *et al.* (2006)
Spruce wood	0.05% H_2SO_4 autoclave 15 bar 10 minutes	SHF (aerobic)	50	*Rhizopus oryzae*	0.41	Millati *et al.* (2006)
Rice straw 20 g/l	0.5% H_2SO_4 autoclave 15 bar 10 minutes	SHF (aerobic)	7.5	*Rhizopus oryzae*	0.36	Abedinifar *et al.* (2009)
Palm pressed fiber 5%	10% NaOH autoclave 15 minutes 121 °C	SHF two stage (aerobic 2 days continued with 3 days in anaerobic condition)	7.24	*Rhizopus oryzae*	0.51	This study

SHF : Separate hydrolysis and fermentation

CHAPTER 4

CONCLUSIONS

1. The physical and chemical pretreatments should be involved in the preparation of suitable PPF as a substrate for enzymatic saccharification. From the result of this study, it can be suggested that by autoclaving at 121 °C for 15 minutes in alkali solution (10% NaOH) was found to be the best combination of pretreatments for preparation of suitable PPF for enzymatic saccharification.
2. The highest reducing sugar of PPF was found at the hydrolysis with mixture of cellulase and xylanase (6:6) from alkali/autoclave pretreatment at 50 °C of 5.71 ± 0.21 g/l. Moreover, the highest reducing sugar was found at 8 unit cellulase, 8 unit xylanase and 8 unit β-glucosidase (7.25 ± 0.03 g/l), when it was compared with 4 and 6 unit of the same enzyme mixture. However, the addition of β-glucosidase was not significantly enhanced hydrolysis rate.
3. The fungi was able to grow well and produced high yield of ethanol (yield based on reducing sugar amount) using PPF enzymatic hydrolysate. However, the solid fermentation using fungi to convert directly PPF to ethanol, the result was low even though this strain produced cellulase. Meanwhile, cultivation in prehydrolysate also resulted low ethanol concentration which might be from the present of inhibitor.
4. The two stage fermentation was not enhanced ethanol production when PPF enzymatic hydrolysate and prehydrolysate liquid were used as substrates, since the assimilation of sugar was almost consumed at 2 days in aerobic condition. Meanwhile, ethanol concentration was increased when untreated PPF was used. However, from alkali/autoclave pretreated PPF under two stage condition, the ethanol concentration was not significantly different with ethanol concentration from anaerobic condition at one stage fermentation.
5. From all experiments, the ethanol concentration from all three strains (*Mucor indicus, Mucor hiemalis* and *Rhizopus oryzae*) was found higher in anaerobic

condition, even though it was not significantly different when compared to aerobic condition.

Suggestions

1. Further investigation was needed to optimize the ideal condition for lignocelullosic material pretreatment using autoclave and microwave.
2. Further investigation was needed to optimize the ideal condition for saccharafication of lignocellulosic material using enzyme such as time of saccharafication, unit and pH. Cellulase from *Rhizopus oryzae* can be used as enzyme for saccharafication.
3. Further investigation was needed to optimize the ideal condition for two stage fermentation to enhance ethanol production also to cultivate biomass from *Mucor indicus, Mucor hiemalis and Rhizopus oryzae*.

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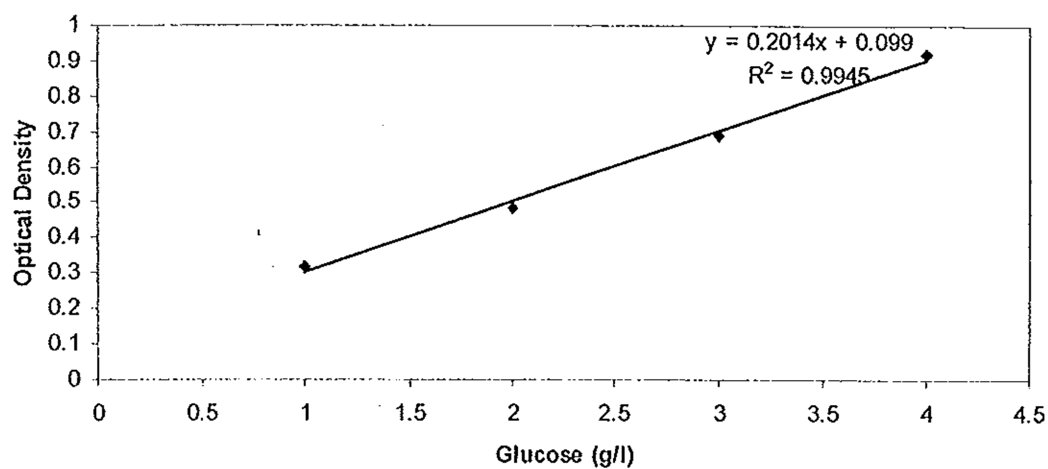
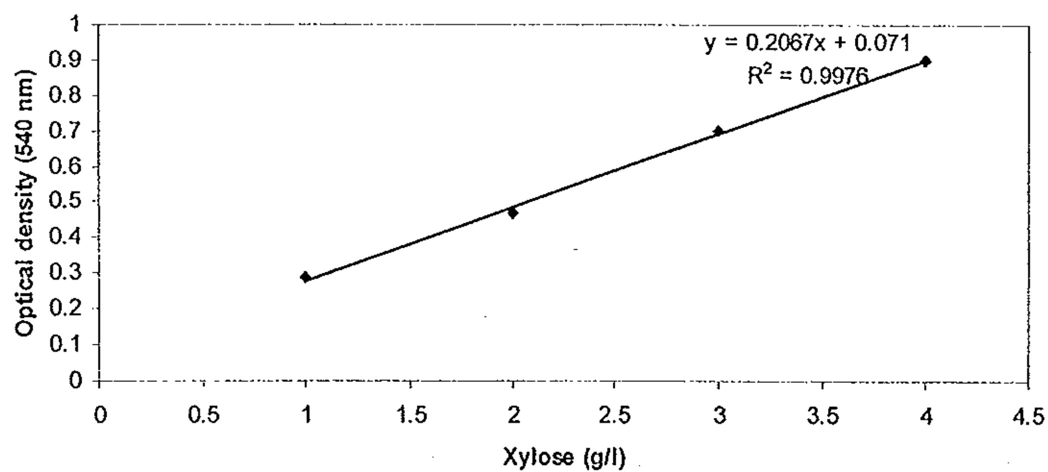
APPENDIX

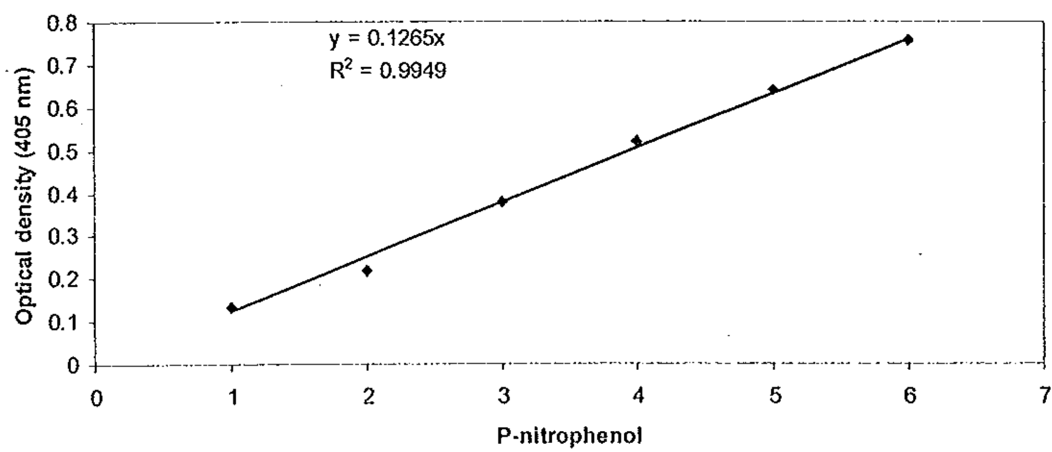
1. Standard Curve of glucose (DNS)



2. Standard Curve of Xylose (DNS)



3. Standard of P-nitrophenol



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