PRETREATMENT OF SUGARCANE LEAVES TO ENHANCE CELLULASE HYDROLYSIS FOR ETHANOL FERMENTATION BY *Saccharomyces cerevisiae*

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การปรับสภาพใบอ้อยเพื่อเพิ่มการสลายด้วยน้ำของเซลลูเลสสำหรับการหมักเอทานอลโดย *Saccharomyces cerevisiae*

นางสาว รัมภา จุฑะกนก

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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Field of Study	Industrial Microbiology
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รัมภา จุฑะกนก : การปรับสภาพใบอ้อยเพื่อเพิ่มการสลายด้วยน้ำของเซลลูเลสสำหรับการหมักเอทานอลโดย *Saccharomyces cerevisiae* (PRETREATMENT OF SUGARCANE LEAVE TO ENHANCE CELLULASE HYDROLYSIS FOR ETHANOL FERMENTATION BY *Saccharomyces cerevisiae*) อาจารย์ที่ปรึกษาวิทยานิพนธ์หลัก: รองศาสตราจารย์ ดร. อัญชริดา อัครจรัลญา, อาจารย์ที่ปรึกษาวิทยานิพนธ์ร่วม: ดร. ธีรภัทร ศรีนรคุตร, 72 หน้า

ใบอ้อยปรับสภาพด้วยกรดซัลฟูริกเจือจางย่อยสลายด้วยเซลลูเลสได้ดีกว่าใบอ้อยซึ่งปรับสภาพด้วยด่าง สภาวะที่เหมาะสมสำหรับการปรับสภาพใบอ้อยด้วยกรดซัลฟูริกเจือจางคือปริมาณวัตถุดิบ (อนุภาค 20-40 เมช) 6% น้ำหนักต่อปริมาตร กรดซัลฟูริก 1.5% น้ำหนักต่อปริมาตรที่121^{o}ซ ความดัน 15 ปอนด์ต่อตารางนิ้ว 30 นาที ใบอ้อยที่ผ่านการปรับสภาพด้วยกรดซัลฟูริกเจือจางที่สภาวะนี้ เมื่อย่อยด้วยเซลลูเลส 10 FPU ต่อกรัมน้ำหนักแห้ง (β-glucosidase 3.55 salicin Unit ต่อมิลลิลิตร) 6 ชั่วโมง จะได้น้ำตาลกลูโคส 0.057 กรัมต่อกรัมน้ำหนักแห้งใบอ้อย

ย่อยใบอ้อยที่ผ่านการปรับสภาพด้วยกรดซัลฟูริกเจือจางด้วย Acellulase^{TM}1000 (160 FPUต่อกรัมน้ำหนักแห้ง (β-glucosidase 400 pNPGUต่อกรัม) เป็นเวลา 6 ชั่วโมง ได้น้ำตาลกลูโคส 9.8 กรัมต่อลิตร แล้วนำไปหมักเป็นเอทานอลโดย *Saccharomyces cerevisiae* TISTR 5596 เป็นเวลา 24 ชั่วโมง โดยวิธีนี้ได้เอทานอล 4.71 กรัมต่อลิตร (0.48 กรัมต่อกรัมน้ำตาลกลูโคส) คิดเป็น 0.20 กรัมต่อกรัมเซลลูโลส หรือ 0.08 กรัมต่อกรัมน้ำหนักแห้งใบอ้อย

ผลการปรับสภาพใบอ้อยด้วยกรดซัลฟูริกเจือจาง มีน้ำตาลไซโลสละลายออกมา 0.11 กรัมต่อกรัมน้ำหนักแห้ง ซึ่งเท่ากับปริมาณน้ำตาลกลูโคสที่ได้หลังการย่อยสลายด้วยเซลลูเลส (0.12 กรัมต่อกรัมน้ำหนักแห้ง) จึงหมักเอทานอลจากทั้งน้ำตาลไซโลสและน้ำตาลกลูโคสด้วย *Pichia stipitis* และ *S.cerevisiae* TISTR 5596 ตามลำดับ ผลการหมักในระดับขวดเขย่าได้เอทานอลจากน้ำตาลไซโลส 3.12 กรัมต่อลิตร (0.35 กรัมต่อกรัมไซโลส) ที่ 96 ชั่วโมง และได้เอทานอลจากน้ำตาลกลูโคส 2.9 กรัมต่อลิตร (0.46 กรัมต่อกรัมกลูโคส) ที่ 12 ชั่วโมง คิดเป็นเอทานอลทั้งหมด 6.02 กรัม (0.10 กรัมต่อกรัมน้ำหนักแห้งใบอ้อย) ผลการหมักในระดับขยายส่วน 5 ลิตร ได้เอทานอลจากน้ำตาลไซโลส 4.08 กรัมต่อลิตร (0.47 กรัมต่อกรัมไซโลส) ที่ 96 ชั่วโมง และได้เอทานอลจากน้ำตาลกลูโคส 3.05 กรัมต่อลิตร (0.49 กรัม/กรัมกลูโคส) ที่ 12 ชั่วโมง คิดเป็นเอทานอลที่ได้ทั้งหมด 7.13 กรัม (0.12 กรัมต่อกรัมน้ำหนักแห้งใบอ้อย)

ภาควิชา	จุลชีววิทยา	ลายมือชื่อนิสิต…….....
สาขาวิชา	จุลชีววิทยาทางอุตสาหกรรม	ลายมือชื่ออาจารย์ที่ปรึกษาวิทยานิพนธ์หลัก.....
ปีการศึกษา	2552	ลายมือชื่ออาจารย์ที่ปรึกษาวิทยานิพนธ์ร่วม……..……....

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RUMPA JUTAKANOKE: PRETREATMENT OF SUGARCANE LEAVES TO ENHANCE CELLULASE HYDROLYSIS FOR ETHANOL FERMENTATION BY *Saccharomyces cerevisiae*. THESIS PRINCIPAL ADVISOR: ASSOC. PROF. ANCHARIDA AKARACHARANYA, D. Eng, THESIS CO-ADVISOR: TEERAPATR SRINORAKUTARA, Ph.D., 72 pp.

Sugarcane leaves pretreated by dilute sulfuric acid was more susceptible so cellulase than those pretreated by lime. Optimal condition for the dilute sulfuric acid pretreatment was 6% (w/v) substrate (20-40 mesh particle size) loading, 1.5% (w/v) H_2SO_4 at 121^{o}C, 15 lb/in^{2}, 30 min. Hydrolysis of the dilute sulfuric acid pretreated sugarcane leaves slurry by cellulase (10 FPU/g, dry weigh basis (DS): β-glucosidase 3.55 salicin Unit/ml) for 6h yield 0.057 glucose g/g DS.

Hydrolysis of the dilute sulfuric acid pretreated sugarcane leaves by Acellulase™1000 at 160 FPU/g (DS) (β-glucosidase 400 pNPGU/g) for 6 h resulted in glucose 9.8 g/l. The glucose was further fermented to ethanol by *Saccharomyces cerevisiae* TISTR 5596 for 24 h. Ethanol yield was 4.71 g/l (0.48 g/g glucose) or 0.20 g/g cellulose (0.08 g ethanol/g (DS) sugarcane leaves).

Pretreatment of sugarcane leaves by dilute sulfuric acid released xylose 0.11 g/g (DS) which was the same as glucose released after cellulase hydrolysis (0.12 g/g (DS)). So, both of xylose and glucose were fermented to ethanol by *Pichia stipitis* and *S.cerevisiae* TISTR 5596, respectively. Flask scale fermentation gave ethanol from xylose (3.12 g/l or 0.35 g/g xylose) at 96h, and from glucose (2.9 g/l or 0.46 g/g glucose) at 12h. Total ethanol yield in flask scale was 6.02 g or 0.10 g ethanol/g (DS) sugarcane leaves. Three liters scale fermentation in 5L fermenter gave ethanol from xylose (4.08 g/l or 0.47 g/g xylose) at 96h, and from glucose (3.05 g/l or 0.49 g/g glucose) at 12h. Total ethanol yield in 3L fermenter scale was 7.13 g or 0.12 g ethanol/ g (DS) sugarcane leaves.

Department : Microbiology Student's Signature……...…….
Field of Study : Industrial Microbiology Advisor's Signature……...……
Academic Year : 2009 Co-advisor's Signature……...

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LIST OF ABBREVIATIONS

w/v	=	weight/volume
w/w	=	weight/weight
v/v	=	volumn/volumn
g/l	=	gram/liter
g/g/h	=	gram/gram/hour
M	=	molar
N	=	Normality
nm	=	nanometer
min	=	minute
h	=	hour
U	=	unit
FPU	=	filter paper unit
DS	=	dry solid sugarcane leaves
lb/in^2	=	pounds/square inch
°C	=	degree celsius
rpm	=	revolution per minutes
$A_{520\ nm}$	=	$Absorbance_{520\ nm}$
$A_{660\ nm}$	=	$Absorbance_{660\ nm}$

CHAPTER I

INTRODUCTION

The world high energy demand energy with fossil fuel resources limitation entails in an increase of gasoline price. Therefore, searching for an alternative energy is important and necessary. Ethanol which produced from agricultural products by microbial activity is a potential alternative energy of Thailand. Presently, Thailand produces ethanol fuel from cassava and sugarcane molasses. The cost of the two raw materials is relatively expensive, so price of the final product, ethanol, is high compared to price of gasoline. In order to reduce an ethanol production cost, utilization of lignocellulose as substrate has always been considered because the lignocellulose is abundant and cheap when included an agricultural and agro-industrial wastes.

Sugarcane (*Saccharum officinarum*) is an economically important plant of Thailand. It is cultivated in about 10.7 thousand million square-metre (6.7 million rais) (Office of the National Economics and Social Development Board, 2006) which generates a huge amount of sugarcane leaves. Sugarcane leaves is sharp at margin. This character makes sugarcane stem difficult to be harvested. Agriculturist prefers to remove sugarcane leaves by burning before harvesting of sugarcane stem. This method causes air pollution, disrupts high electric-line and finally breakouts an electrical power. Moreover, the sugarcane stem product harvested after leaves burning out, is low in price compared to those harvested without leaves burning. Value addition of sugarcane leaves will terminate the leaves burning activity. Since, sugarcane leaves composes of three major components; cellulose, hemicellulose and lignin. The cellulose and hemicellulose can be hydrolysed to fermentable sugars, glucose and xylose, respectively. Therefore, sugarcane leaves is a potentially low cost lignocellusic substrate for ethanol production. Lignocellulosic ethanol production is divided in 3 major steps; (i) pretreatment, the step to increase of cellulose susceptibility through hemicellulose and lignin removal (ii) enzymatic hydrolysis, the step to hydrolyse cellulose to glucose and (iii) fermentation, the step to ferment the resultant glucose to ethanol. (Prasad et al., 2007)

By this work, an efficacy of dilute acid and lime pretreatment to improve cellulose susceptibility of sugarcane leaves was compared. The pretreated sugarcane leaves was then

hydrolysed to glucose by cellulase and the liberated glucose was fermented to ethanol by *Saccharomyces cerevisiae*, a separate hydrolysis and ethanol fermentation method. Ethanol was also produced by separate xylose- and glucose- fermentation to evaluate a maximal ethanol yield produced from substrate, sugarcane leaves. Xyose liberated in pretreatment step was fermented to ethanol by *Pichia stipitis* and glucose liberated from cellulase hydrolysis step was fermented to ethanol by *Saccharmyces cerevisiae*.

CHAPTER II

LITERATURE REVIEW

2.1 Sugarcane

Sugarcane (*Saccharum officinarum*) (Fig. 2.1A) is a tall grass, two to six meters tall, which looks rather like a bamboo cane belonging to family Poaceae. The cultivation of sugarcane requires a tropical or subtropical climate. It is propagated from cuttings, more than from seed. Each cutting must contain at least one bud. After that, the stems multiply at the base, which produces a cluster of 2 or 3 stems. The stems develop in full sunshine, and the sugar content increases when they matured. Cut sugarcane re-grows, so the replantation does not require for the plantation of it. The stems of sugarcane (Fig 2.1B) are usually cut at the age about 11-14 months before sending to sugar industries. Then they are bundled to be taken to a sugar mill. Canes are shredded and crushed with heavy rollers to retrieve the juice which normally contains 10-20% sucrose. This juice is sieved to filter out of the impurities and is sent to the next process for sugar production. The cost of sugarcane products depend on the CCS value (commercial cane sugar) that can measure from the percentage of sucrose in the sugarcane juice. Currently, about 70% of the world's supply of sugar is derived from sugarcane. Table 2.1 shows list of countries that are in the top producers of sugarcane, which are Brazil, India, China, and Thailand, respectively (Food And Agricultural Organization of United Nations: Economic And Social Department: The Statistical Division, 2008). Sugarcane is on the first rank in the major commercial crop of Thailand. It is cultivated about 6.4×10^7 million tons per year (Table 2.2) (Food And Agricultural Organization of United Nations: Economic And Social Department: The Statistical Division, 2007).

Fig. 2.1 Sugarcane (A) and Sugarcane stem (B).

(http://www.sucrose.com, http://th.wikipedia.org)

Table 2.1 Top ten sugarcane producing countries

Country	Production (tons)
Brazil	514,079,729
India	355,520,000
China	106,316,000
Thailand	64,365,682
Pakistan	54,752,000
Mexico	50,680,000
Colombia	40,000,000
Australia	36,000,000
United States	27,750,600
Philippines	25,300,000
World	**1,557,664,978**

Revised on 11 June 2008

Table 2.2 Top ten commercial crops and their production of Thailand in 2007

Rank	Commodity	Production (MT)	Production (Int $1000)
1	Sugar cane	64365482	1336871
2	Rice, paddy	32099401	6357229
3	Cassava	26915541	1939534
4	Maize	3661323	103292
5	Natural rubber	3024207	1622124
6	Pineapples	2815275	544446
7	Bananas	2000000	285020
8	Mangoes, mangos teens, guavas	1800000	438282
9	Coconuts	1721640	155705
10	Vegetables fresh	1015000	190464

(http://faostate.fao.org)

Sugarcane is a raw material in many industries both of food and non-food products. Brazil, which is the country that has the highest sugarcane production in the world, has widely used sugarcane to produce ethanol for transportation more than sugar production (Roza and Vieira, 2005). While, in Thailand, the sugarcane is mainly used to produce sugar. From this production processes the sugarcane by-products, molasses, are used as substrate or additive in food industries and some are used as raw material to produce ethanol. Huge amount of agricultural area in Thailand. In 2006, about 10.7 thousand million sqaure-metre (6.7 million rais) of thailand were used for sugarcane cultivation (Office of the National Economics and social Development Board of Thailand). From this result, it generates an immense amount of sugarcane leaves. Sharp margin leaves of the sugarcane makes it difficult to cut sugarcane stem. Therefore, leaves burning prior to harvest are popular. This method causes air pollution, disrupts high electric-line resulted in power breakout. Moreover, the sugarcane products harvested by burning leaves prior have lower CCS value than those harvested without leave burning. Value addition to the sugarcane leaves will solve the problem of leaves burning activity.

2.2 Sugarcane leaves

Sugarcane leaves are lignocellulosic compounds which composed of three major components: cellulose, hemicellulose, and lignin.

2.2.1 Cellulose

Cellulose is an organic compound with the formula $(C_6H_{10}O_5)_n$. It is a polymer of D-glucose subunits linked by β-1,4 glycosidic bonds (Fengel and Wegener, 1984). There are two types of cellulose structures, crystalline and amorphous, which its cellulose polymer is well and not well-organized, respectively. The amorphous structure is more susceptible to cellulase than the crystalline structure. (Hendriks and Zeeman, 2009)

2.2.2 Hemicelluloses

Hemicelluloses are complex carbohydrate structure (Fig. 2.2) that composes of heterogeneous sugars such as pentose (xylose and arabinose) and hexose (mannose, glucose, galactose). Hemicelluloses are not chemical homogeneous that make them different from cellulose. They act as connection between the cellulose fibers and the lignin and give the network of cellulose-hemicellulose-lignin more rigidity (Hendriks and Zeeman, 2009). The major composition of hemicelluloses is xylan, which has different composition from different source, such as grasses, softwood, and hardwood. However, the largest amount of sugar monomer presented in xylan is mostly xylose (Fengel and Wegener, 1984; Saha et al., 2003).

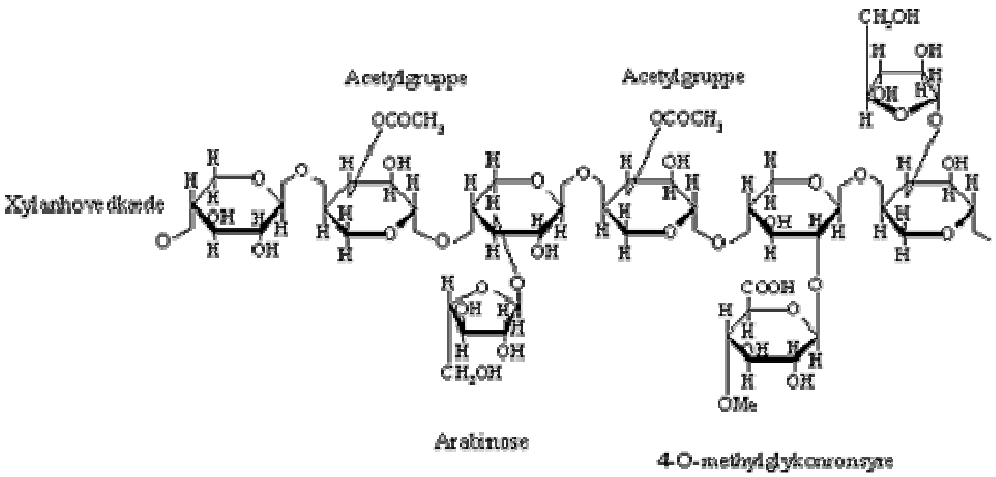


Fig. 2.2 Hemicellulose structure compose of different sugars. (http://www.life.ku.dk)

2.2.3 Lignin

Lignin is a phenolic polymer that has a complex structure (Fig 2.3). Its major functional groups are methoxyl, phenolic, and benzyl alcohol (Fig. 2.4). There are three derivatives, which are divided from the amount of methoxyl group: (i) p-coumaryl, is a minor component of grass (10-25%), (ii) coniferyl, is the predominant lignin monomer found in softwoods (90-95%), and (iii) sinapyl alcohol found in hardwood (50-70%) (Fig. 2.5). (Helm, 2000; Klinke et al., 2004) Lignin is presented in plant cells to support the rigidity of plant structure. Degradation of lignin is difficult because the amorphous heteropolymer is non-water soluble and optically inactive. (Hendriks and Zeeman, 2009)

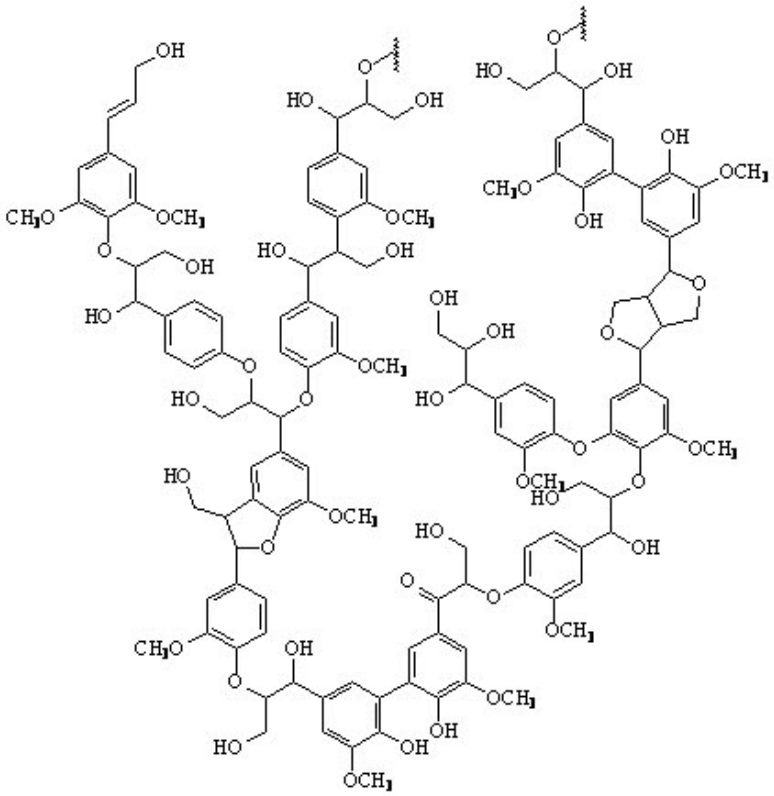


Fig. 2.3 Complex structure of lignin

(http://www.research.uky.edu)

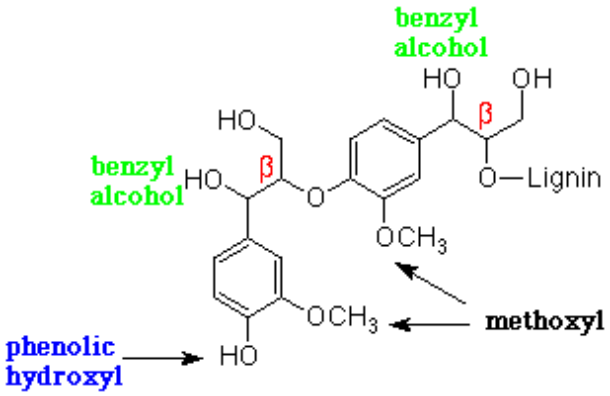


Fig. 2.4 Functional group of lignin

(http://dwb4.unl.edu)

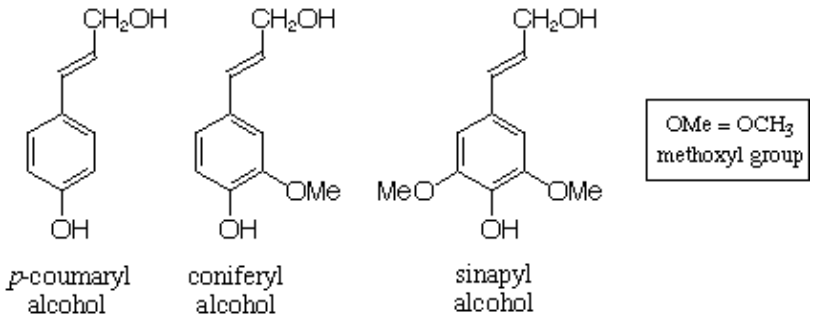


Fig. 2.5 Lignin monomer (derivatives of lignin)

(http://dwb4.unl.edu)

2.3 Ethanol, a promising alternative energy of Thailand

Due to the high demand of energy consumption in the world and a limitation of fossil fuel resources results in gasoline price increase, so searching for promising alternative energy is important and necessary. Ethanol which produced from agricultural products by microbial activity is one of the highly potential alternative energy of Thailand. There are three major substrate types for ethanol production, sugar, starch and lignocellulose. Normally the ethanol production from sugar or starch has two major steps: (i) hydrolysis of raw materials to fermentable sugars and (ii) fermentation of the resultant fermentable sugars to ethanol by microorganisms. The usage of lignocellulose as raw materials for ethanol production, there are some problems of fermentable sugars; glucose and xylose, which are dissolved from cellulose and hemicellulose, respectively. Because the cellulose is shielded by hemicellulose and lignin. An accessibility of cellulase to

cellulose is difficult. Therefore, removal of hemicellulose and lignin from lignocellulosic raw material is necessary for improvement of cellulose hydrolysis. This removal process is known as pretreatment (Prasad et al., 2007).

2.3.1 Pretreatment of lignocellulosic materials

Lignocellulose can be pretreated by physical methods (Grinding, cutting, milling, irradiation, and heat and pressure), and chemical methods (acid, alkaline, ammonia, SO_2, CO_2 treatment) (Palonen et al., 2004). The physical pretreatment reduced crystalline cellulose structure which increases in surface area.

Acid pretreatment improves the accessible of enzyme to cellulose through the solubilization of hemicellulose and precipitation of solubilizied lignin (Fengel and Wegener, 1984). Both strong and dilute acid can be used in the acid pretreatment. Disadvantage of the acid pretreatment is a risk of inhibitors for ethanol fermentation and microbial growth formation. These inhibitors are furfural, hydroxymethylfurfural (HFM), organic acids and other (volatile) products. Higher concentration of inhibitors is formed by strong acid than by dilute acid pretreatment. (Hendriks and Zeeman, 2009)

Alkaline pretreatment causes lignocellulose structure swelling, leading to an increase in internal surface area, decrease in crystallinity cellulose, disruption of structural linkage between lignin and carbohydrates, and disruption of lignin structure (Prasad et al., 2007). The removal of acetyl groups in lignin structure by alkaline chemicals has been known to improve cellulose digestibility for a long time (Yang and Wyman, 2008). Recently, lime pretreatment becomes more attractive then sodium hydroxide, because lime is recyclable and generates lower amount of inhibiting compounds (Ranatunga et al., 2000; Hodge et al., 2009; Gupta et al., 2009).

2.3.2 Enzymatic hydrolysis

Enzymatic hydrolysis by cellulase is a process required after pretreatment process to hydrolyze the pretreated lignocellulose to fermentable sugar, glucose. Cellulase is a mixture of enzymes that used to hydrolyze cellulose. Complete hydrolysis of cellulose to glucose requires at least three major groups of cellulase: (i) endoglucanase (endo-cellulase) (1,4-β-D-glucan-4-glucanohydrolase, EC 3.2.1.4) which breaks regions of low crystalline structure in cellulose fiber

and expose free cellulose polysaccharide chain-ends, (ii) exoglucanase or cellobiohydrolase (exo-cellulase) (1,4-β-D-glucan cellobiohydrolase, EC 3.2.1.91) cuts 2-4 units from the ends of the exposed chains produced by endocellulase, resulting in the tetrasaccharides or disaccharide such as cellobiose, and (iii) β-glucosidase or Cellobiase (EC 3.2.1.21) which hydrolyses cellobiose into individual monosaccharide, glucose (Fig 2.6) (Sun and Cheng, 2002)

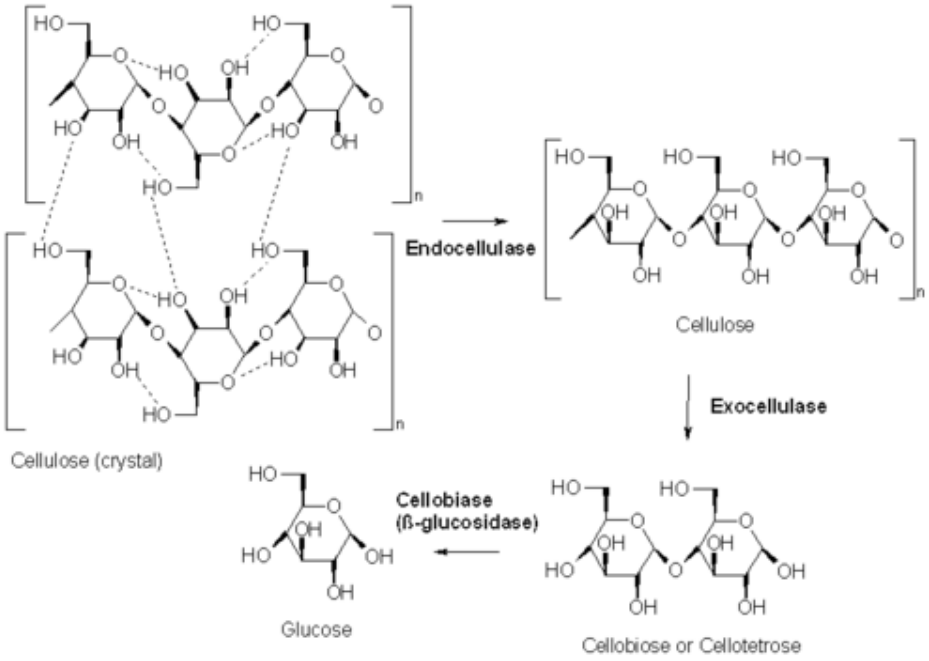


Fig. 2.6 Enzymatic hydrolysis of cellulose (http://www.search.com/reference/Cellulase)

2.3.3 Ethanol fermentation by microorganisms

Baker's yeast, *Saccharomyces cerevisiae*, is the most common organism used for fermentation of hexose sugars to ethanol. It is tolerant toward high ethanol concentration, and the inhibitors generated in pretreatment process. (Palmqvit and Hahn-Hagardal, 2000; Sues et al., 2005) It uses hexose sugar through glycolytic pathway. After that, end product of the pathway, pyruvate, is decarboxylated to acetaldehyde by enzyme pyruvate decarboxylase, which is then reduced to ethanol by enzyme alcohol dehydrogenase with NADH as the electron donor (Fig. 2.7). *S. cerevisiae* is unable to utilize the pentose sugars. (Abedinifar et al., 2009) There are several species of microorganisms, which can ferment pentose sugars, such as bacteria

(*Clostidium*, *Bacillus*, etc.), yeast (*Candida*, *Pichia*, *Schizosaccharomyces*, etc.), and filamentous fungi (*Fusarium*, *Mucor*, etc.). Bacteria use an isomerase pathway to channel the pentose sugars to the central metabolism, whereas yeast and filamentous fungi mostly use a reductase or dehydrogenase pathway (Margeot et al., 2009 and Warner and Mosier, 2007) (Fig. 2.8). Olsson and Hahn-Hagerdal (1996) reported that among ethanolic xylose fermenting microorganisms, *Pichia stipitis* has the highest efficiency. Its ethanol yield is more than 0.45g ethanol/g xylose. The theoretical ethanol production yield from xylose was 0.51 g of ethanol per g of xylose (Magaritis and Bajpai, 1982; Alexander, 1986; Ohta, et al., 1991).

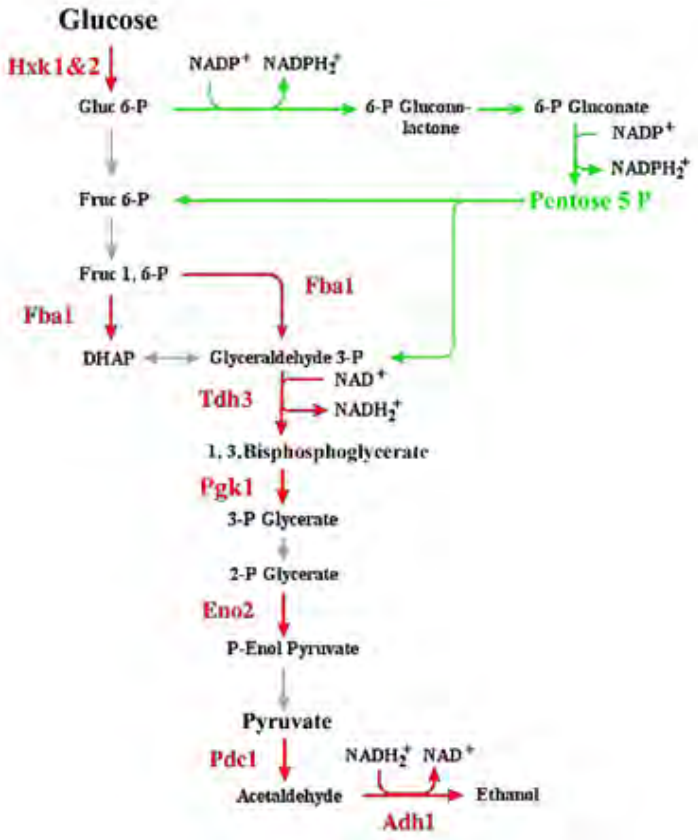


Fig. 2.7 Glycolytic and pentose phosphate pathway in *S. cerevisiae* (Houghton, 2002)

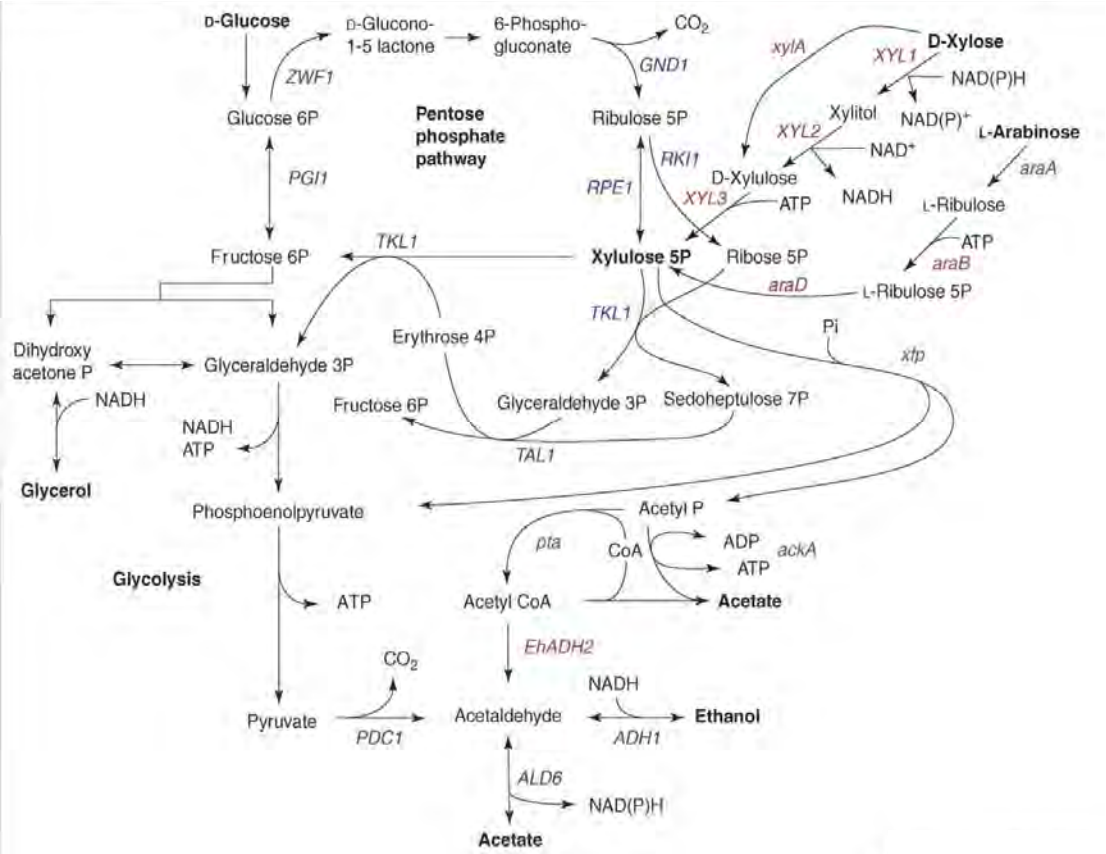


Fig. 2.8 Xylose utilizing pathway by yeast. Abbreviation of enzyme in the pathway: glucose 6-phosphate dehydrogenase (ZWF1), 6-phosphogluconate dehydrogenase (GND1), D-ribulose-5-phosphate 3-epimerase (RPE1), ribose-5-phosphate ketol-isomerase (RKI1), transketolase (TKL1), transaldolase (TAL1). Phosphoglycerate isomerase (PGI1), D-xylose reductase (XYL1), xylitol dehydrogenase (XYL2), D-xylulokinase (XYL3), xylose isomerase (xylA), L-arabinose isomerase (araA), L-ribulokinase (araB), L-ribulose-5-phosphate 4-epimerase (araD, L-arabinose. Phosphoketolase (xfp), acetate kinase (ackA), phosphotransacetylase (pta), acetaldehyde dehydrogenase (adhE), alcohol dehydrogenase (ADH1), decarboxylase (PDC1), and aldehyde dehydrogenase (ALD6). (Jeffries, 2006)

Previous reports about ethanol production from sugarcane leaves were: Krishna et al. (2001) fermented ethanol from sugarcane leaves by pretreatment of the sugarcane leaves with alkaline peroxide using 10% (w/v) substrate loading at 121^{o}C, 15 lb/in^{2} for 15 min. Then the alkaline peroxide pretreated sugarcane leaves residue was hydrolyzed and fermented by

Trichoderma reesei cellulase and *Saccharomyces cerevisiae*, respectively through simultaneous saccharification and fermentation method (SSF). It was found that ethanol (4 g/l or 0.04g/g DS) was produced within 24h. Maximum ethanol produced was at 20 g/l or 0.20 g/g DS within 96h. Whereas, the experiment that supplement cellulase with β-glucosidase generated 1.5 g/l (0.15 g/g DS) and 22 g/l (0.22 g/g DS) of ethanol within 24 and 96h, respectively. Dawson et al. (2006) fermented ethanol dilute sulfuric acid pretreated sugarcane leaves residue by *Saccharomyces cerevisiae* ATCC 765 which produced ethanol directly from cellulosic substrate. It was found that maximum ethanol (0.34 g/l) or 0.01 g/g DS) was produced in 12 days.

By this work, an efficacy of dilute sulfuric acid and lime pretreatment on cellulose susceptibility of sugarcane leaves were compared. Fermentable sugars liberated in pretreatment hydrolysate and by cellulase hydrolysis were fermented to ethanol by 2 methods. 1) Sugarcane leaves pretreatment slurry was further hydrolyzed by cellulase then hydrolysate obtained was fermented to ethanol by *Saccharomyces cerevisiae* TISTR 5596 2) Fermentable sugars in pretreatment hydrolysate and in hydrolysate obtained after cellulase hydrolysis were separately fermented to ethanol by *Pichia stipitis* and *Saccharomyces cerevisiae* TISTR 5596, respectively. Ethanol yield obtained from the 2 fermentation methods were compared.

CHAPTER III

MATERIALS AND METHODS

Materials

3.1 Sugarcane leaves

Leaves of sugarcanes *(Saccharum officinarum)*; collected from Nakhonratchasima province, Thailand; were cut, dried at 60^{O}C then sent to grind to 20-40 meshes by hammer milled.

Fig 3.1 Sugarcane leaves

3.2 Equipments

3.2.1 Analytical balance : Mettler Toledo, model AG 285, Switzerland.

3.2.2 Autoclaves : Tomy, model SS- 325 and Hirayama, model HV-28, Japan.

3.2.3 Biochemistry Analyzer : YSI 2700 SELECT, USA.

3.2.4 Fermenter : B.E. Marubishi, model 10L, Japan.

3.2.5 Gas chromatography : Shimadzu, model 7AG, Japan.

3.2.6 Hot plate : E.G.O., model RK18715, Poland.

3.2.7 High Performance Liquid Chromatography (HPLC) : Agilent Technology Ltd., model 1100 series, USA.

3.2.8 Laminar flow : Lab service Ltd., Clean model V6, Thailand.

3.2.9 Incubator shaker : New Brunswick Scientific, model Innova 2300, USA.

3.2.10 pH meter : Mettler Toledo, model SevenEasy, China.

3.2.11 Precision balance : Mettler Toledo, model PB 3002, Switzerland.

3.2.12 Refrigerated centrifugation : Sorvall, model Biofuge stratos, Germany. (Rotor #3334, Heraeus, USA).

3.2.13 Test seive : 20 mesh, Retesch, Germany

3.2.14 Test seive : 40 mesh, Retesch, Germany

3.2.15 Spectrophotometer : Spectronic Instruments, model Spectronic 20, USA.

3.2.16 Water bath : Tolabo, model TW20, Germany.

3.2.17 Water bath shaker : Amerex Instrument Inc., model Gyromax 939XL, USA. and GFL, model 1086, Germany.

3.3 Chemicals

Chemicals (Analytical grade)

Agar	Becton
Ammonium heptamolybdate tetrahydrate ($(NH_4)_6Mo_7O_{24}.4H_2O$)	Merck
Ammonium sulphate ($(NH_4)_2SO_4$)	Merck
Bacto-peptone	Becton
Calcium hydroxide ($Ca(OH)_2$)	Merck
Copper (II) sulfate ($CuSO_4.5H_2O$)	Merck
Disodium hydrogen arsenate ($Na_2HAsO_4.7H_2O$)	Merck
di-Sodium hydrogen phosphate dodecahydrate ($Na_2HPO_4.12H_2O$)	Merck
Glucose ($C_6H_{12}O_6$)	Sigma
Hydrochloric acid (HCl)	Sigma
Magnesium sulfate heptahydrate ($MgSO_4.7H_2O$)	Merck
Malt extract	Lab-Scan
Potassium dihydrogen phosphate (KH_2PO_4)	Merck
Potassium Sodium Tartate ($C_4H_4KNaO_6.4H_2O$)	Merck
Sodium hydroxide (NaOH)	Merck
Sulfuric acid (H_2SO_4)	Merck
Xylose	Sigma
Yeast extract	Difco

3.4 Microorganisms

3.4.1 *Pichia stipitis* was a gift from Dr. Teerapatr Srinorakutara, Thailand Institute of Scientific Technological Research.

3.4.2 *Saccharomyces cerevisiae* TISTR 5596 was obtained from Thailand Institute of Scientific Technological Research.

Methods

3.5 Experiments

Flow diagram of all experiments is shown in Fig. 3.2

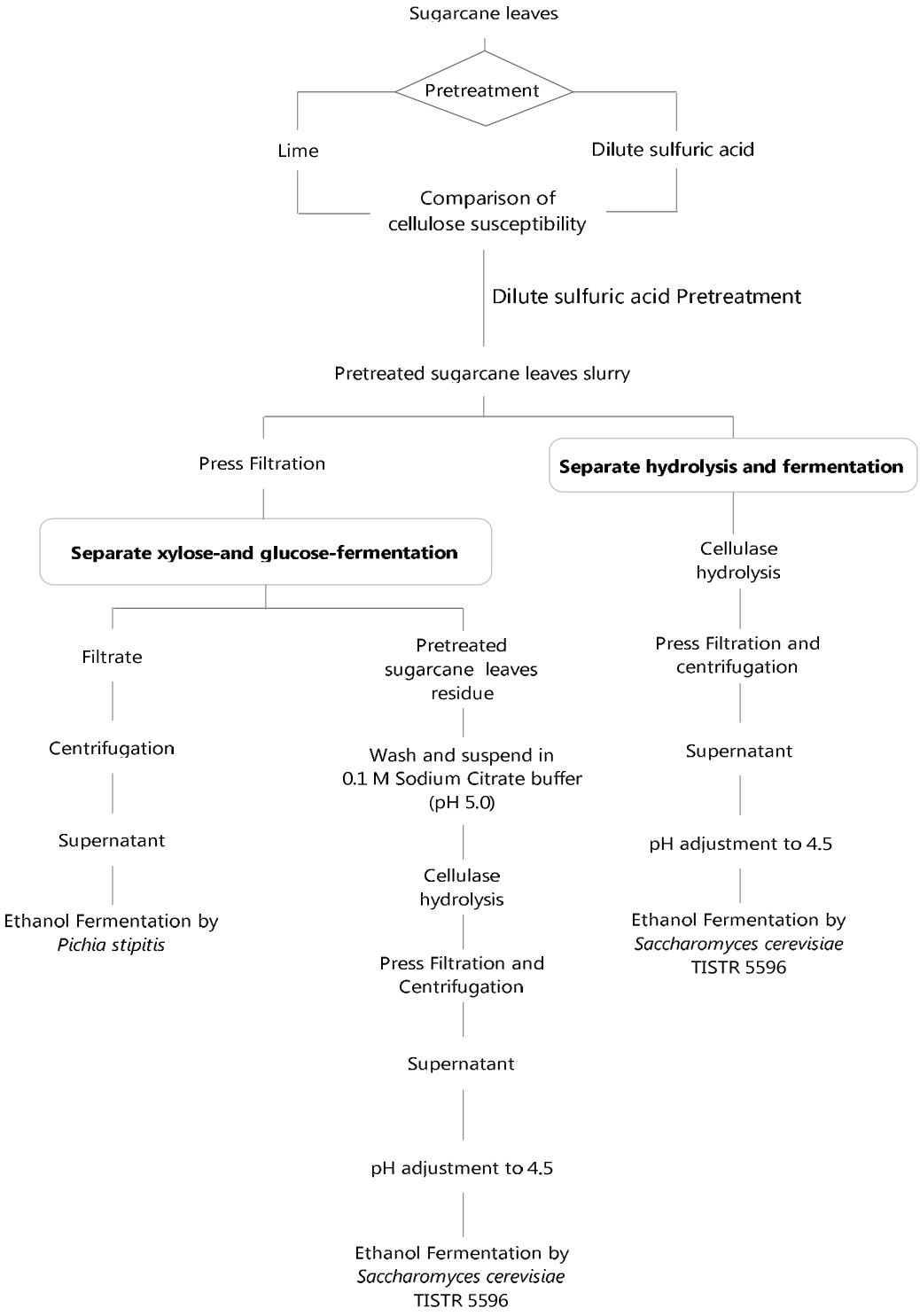


Fig. 3.2 Flow diagram of all experiments

3.6 Cultivation of *Saccharomyces cerevisiae* TISTR 5596 and *Pichia stipitis* microorganisms

3.6.1 Maintainance of microorganisms

Saccharomyces cerevisiae TISTR 5596 and *Pichia stipitis* were long-term maintained in yeast peptone dextrose (YPD) broth containing 30% (w/v) glycerol at -80°C. Before use, the cultures were activated by transferring to YPD broth and incubated at 30°C, 200 rpm for 24h. The activated *Saccharomyces cerevisiae* TISTR 5596 was further cultivated on YPD agar and incubated at 30°C for 24h, while the activated *Pichia stipitis* was further cultivated on yeast malt extract (YM) agar and incubated at 30°C for 48h. They were subcultured to new fresh medium every week and used for inoculum preparations. Each lot of activated culture was subcultured not more than 4 times.

3.6.2 Preparation of inoculum

3.6.2.1 *Saccharomyces cerevisiae* TISTR 5596

A single colony of *Saccharomyces cerevisiae* TISTR 5596 grown on YPD agar at 30°C for 24 h was inoculated into YPD broth and incubated at 30°C (200 rpm) for 24 h. The culture transferred at 1% (v/v) to the same medium and incubated at the same above condition was used as inoculum.

3.6.2.2 *Pichia stipitis*

A single colony of *Pichia stipitis* grown on YM agar at 30°C for 48 h, was inoculated into medium containing; xylose 50 g/l, yeast extract 4 g/l, malt extract 3 g/l, peptone 5 g/l; pH 5.0 and incubated at 30°C (200 rpm) for 24 h. The culture transferred at 1% (v/v) to the same medium and incubated at the same above condition was used as an inoculum.

3.7 Pretreatment of sugarcane leaves

An efficiency of dilute sulfuric acid and lime pretreatment on increasing of cellulose susceptibility of sugarcane leaves was studied. The sugarcane leaves particles were pretreated by suspending at 3% (w/v, dry weight basis (DS)) in various concentration of sulfuric acid or lime solution, then autoclaved at 121°C, 15 lb/in^2 for 30 min. The pretreated sugarcane leaves slurry was press filtered through 0.88 mm stainless sieve and the resultant filtrate was centrifuged at

4°C, 11857gx for 20 min. Reducing sugar, glucose, xylose, pretreatment by-products (furfural, hydroxymethylfurfural (HMF), 4-hydroxybenzaldehyde, syringaldehyde, vanillin) liberated in pretreatment hydrolysate were analyzed after pH adjustment of the resultant supernatant to 7.0. Reducing sugar was analyzed by method of Somogyi (1952); glucose, xylose and pretreatment by-products were analyzed by High Performance Liquid Chromatography (HPLC). Cellulase hydrolysis of the pretreated sugarcane leaves residue was performed by press filtration of the sugarcane leaves pretreatment slurry through 0.88 mm stainless sieve, filtrate obtained was further centrifuged at 4°C, 11857gx for 20 min to remove residual lime powder. The pretreated sugarcane leave residue was resuspended into the self supernatant, pH adjusted to 6.0. Cellulase GC220 (cellulase 71.3 FPU/µl, β-glucosidase 28.28 Salicin Unit/ml, endo glucanase 987.4 CMCU/ml; Genecor International, Inc., USA) was added at 10 FPU/g (DS) (β-glucosidase 3.55 Salicin Unit/ml, endo glucanase 138.5 CMCU/ml) and the cellulase hydrolytic reaction was incubated at 40°C with shaking (125 rpm). After 72 h, the reaction mixture was centrifuged at 4°C, 11857gx (20 min), reducing sugar and glucose released after cellulase hydrolysis in the supernatant was analyzed.

3.7.1 Effect of sulfuric acid and lime concentration

Various concentration of sulfuric acid (1.0, 1.5, and 2.0% w/v) or of lime (0.1, 0.25, 0.5, 1.0, 1.5, 2.0 % w/v) was used to suspend the sugarcane leaves in the pretreatment process. Reducing sugar released from pretreatment step and after cellulase hydrolysis were analysed.

3.7.2 Effect of autoclaving time

The sugarcane leaves was pretreated at 121°C, 15 lb/in^2 for 5, 10, 15, 30, 45, 60 min using the optimal concentration of sulfuric acid or lime (result of 3.7.1)

3.7.3 Effect of substrate loading

The sugarcane leaves was pretreated at various substrate loading (3, 6, 8% w/v) using the optimal concentration of sulfuric acid or lime, and autoclaving time (result of 3.7.2).

3.8 Optimization of cellulase hydrolysis

Sugarcane leaves pretreated by dilute sulfuric acid at its maximal cellulose susceptibility condition was saccharified by cellulase, Accellulase™ 1000 (265 FPU/ml: endoglucanase 2500

CMCU/g, β-glucosidase 400 pNPGU/g, Genecor International, Inc., USA) The saccharification of sugarcane leaves was performed by 2 methods: 1) pH adjustment of the pretreated sugarcane leaves slurry to 5.0 before an addition of celluase, 2) press-filtration of the pretreated sugarcane leaves slurry, suspending washed pretreated sugarcane leaves residue in 0.1 M sodium citrate buffer (pH 5.0) at its original volume before an addition of celluase. The enzymatic reaction was incubated at 50^{o}C with shaking (120 rpm) for 72h. Reducing sugar and glucose released by cellulase hydrolysis were analysed by Somogyi (1952) method and glucose analyzer, respectively.

3.8.1 Effect of enzyme dose

The sugarcane leaves was pretreated by dilute sulfuric acid at optimal condition (result of 3.8.3). The resultant pretreated sugarcane leaves was hydrolyzed by cellulase as described above by the method No.1, The cellulase was used at a dose of 0.2, 0.4, 0.6, 0.8 ml/g (DS) or 50, 106, 160, 212 FPU/g (DS). Reducing sugar and glucose released by cellulase were analyzed.

3.8.2 Effect of cellulase hydrolyzing time

The sugarcane leaves was pretreated by dilute sulfuric acid at optimal condition (result of 3.8.3). The resultant pretreated sugarcane leaves was hydrolyzed by cellulase as described above by the method No.1, using optimal dose of cellulase (result of 3.8.1) for 72 h, every 12 h reducing sugar and glucose released by cellulase were analyzed.

3.9 Separate hydrolysis and ethanol fermentation (SHF)

Sugarcane leaves was pretreated by dilute sulfuric acid at optimized condition then the resultant slurry was pH adjusted, saccharified by cellulase at optimized condition. After centrifugation, obtained supernatant containing glucose 9.1 g/l was fermented to ethanol by *Saccharomyces cerevisiae*. Fermentation condition was as followed: 10% (v/v) inoculum, 30^{o}C, pH 4.5, oxygen limit condition for 72h.

3.9.1 Effect of inoculum age on ethanol production

Saccharomyces cerevisiae TISTR 5596 grown in YPD broth for 6h (mid log phase cell, $OD_{660\ nm}$ was 0.6) or 24h (stationary growth phase cells, OD_{660nm} was 0.6) was used as inoculum at 10% (v/v). Ethanol fermentation was performed as described above.

3.9.2 Effect of $(NH_4)_2SO_4$ concentration supplemented on ethanol production

Ethanol fermentation was performed as described above. Inoculum age of *S.cerevisiae* used was a result of 3.9.1, but various $(NH_4)_2SO_4$ concentrations (0, 0.02, 0.04, 0.06 g/l) was supplemented in fermentation medium.

3.9.3 Effect of nutrients supplemented on ethanol production

Ethanol fermentation was performed as described above. Concentration of $(NH_4)_2SO_4$ supplemented was a result of 3.9.2. This experiment, other nutrients, KH_2PO_4 (0.5g/l) and yeast extract (3.0 g/l), were supplemented in fermentation medium.

3.9.4 Effect of fermentation time on ethanol production

Fermentation was performed as described in above. Nutrient supplemented was a result of 3.9.3. Every 12h interval ethanol produced in culture supernatant was analyzed by Gas chromatography (GC).

3.9.5 Effect of residual glucose in inoculum medium on ethanol production

Ethanol fermentation was performed at maximal ethanol produced condition (result of 3.9.4). But the *S.cerevisiae* inoculum used was centrifuged. Cell precipitate was resuspended at original volume in YPD broth without glucose, and used as inoculum at 10% (v/v). Ethanol production in culture surpernatant was analyzed by Gas chromatography (GC).

3.10 Separate xylose- and glucose- ethanol fermentation

3.10.1 Ethanol production from xylose released in pretreatment hydrolysate

Sugarcane leaves was pretreated by dilute sulfuric acid at the optimal condition (result of 3.7). Pretreatment hydrolysate obtained after press filtration and centrifugation of the pretreated sugarcane leaves slurry was fermented to ethanol by *Pichia stipitis* at 25^{o}C, pH 5.0, oxygen limit condition for 72h.

3.10.1.1 Effect of nutrient supplementation on ethanol production

The pretreatment hydrolysate supplemented with 0.02 g/l $(NH_3)_2SO_4$; 0.02 g/l $(NH_4)_2SO_4$+ 0.2 g/l KH_2PO_4+3.0 g/l yeast extract or without supplementation was used as substrate. Ethanol fermentation was performed as described above. Ethanol produced in culture supernatant was analyzed by Gas chromatography (GC).

3.10.1.2 Effect of pH on ethanol production

Ethanol fermentation of the pretreatment hydrolysate using *P.stipitis* was performed as described above. Fermentation medium used was a result of 3.10.1.1. But pH was varied (4.5, 5.0, 5.5, 6.0, 6.5, and 7.0). Ethanol produced in culture supernatant was analyzed by Gas chromatography (GC).

3.10.1.3 Effect of temperature on ethanol fermentation

Ethanol fermentation of the pretreatment hydrolysate using *P.stipitis* was performed as described above, pH used was a result of 3.10.1.2. But incubation temperature was varied (25, 30, 37, 40^{o}C). Ethanol produced in culture supernatant was analyzed by Gas chromatography (GC).

3.10.1.4 Effect of fermentation period time on ethanol production

Ethanol fermentation of the pretreatment hydrolysate using *P.stipitis* was performed as described in 3.10.1.3 for 108h, temperature used was a result of 3.10.1.3. Every 12h, ethanol produced in culture supernatant was analyzed by Gas chromatography (GC).

3.10.1.5 Effect of residual xylose in inoculum medium on ethanol production

Ethanol fermentation was performed at maximal ethanol produced condition (result of 3.10.1.4). But the *P.stipitis* inoculum used was centrifuged. Cell precipitate was resuspended at original volume of medium containing yeast extract 4 g/l, malt extract 3 g/l, and peptone 5 g/l (without xylose) was used as inoculum at 10% (v/v). Ethanol produced in culture supernatant was analyzed by Gas chromatography (GC).

3.10.2 Ethanol production from glucose released by cellulase hydrolysis

Pretreated sugarcane leaves residue separated from pretreatment hydrolysate was suspended in citrate buffer, saccharified by cellulase. Supernatant obtained after cellulase hydrolysis was used as substrate for ethanol fermentation by *S.cerevisiae* TISTR 5596 at 10% (v/v) inoculum. Fermentation conditions were as followed: pH 4.5, 30^{o}C, oxygen limit condition, 72h.

3.10.2.1 Effect of inoculum age on ethanol production

Saccharomyces cerevisiae TISTR 5596 inoculum (OD_{660nm} was 0.6 or 2.24) was used as inoculum. Ethanol fermentation was performed as above, and ethanol produced was analyzed by Gas chromatography (GC).

3.10.2.2 Effect of $(NH_4)_2SO_4$ concentration on ethanol production

Ethanol fermentation was performed as described in 3.10.2.1, inoculum age used was a result of 3.10.2.1. But concentration of $(NH_4)_2SO_4$ supplemented was varied (0, 0.02, 0.04, and 0.06 g/l). Ethanol produced in culture supernatant was analyzed by Gas chromatography (GC).

3.10.2.3 Effect of nutrient supplementation on ethanol production

Ethanol fermentation was performed as described in 3.10.2.2, $(NH_4)_2SO_4$ supplementation was a result of 3.11.2.2. The fermentation medium used was further supplemented without or with yeast extract (3.0 g/l); KH_2PO_4 (0.2 g/l) + yeast extract (3.0 g/l); yeast extract (3.0 g/l) + KH_2PO_4 (0.2 g/l) + $(NH_4)_2SO_4$ (0.02 g/l). Ethanol produced in culture supernatant was analyzed by Gas chromatography (GC).

3.10.2.4 Effect of fermentation time on ethanol production

Ethanol fermentation was performed as described in 3.10.2.3 for 72h, nutrient supplementation used was a result of 3.10.2.3. Ethanol produced in culture supernatant at 0, 3, 6, 9, 12, 24, 48 and 72h after incubation was analyzed by Gas chromatography (GC).

3.10.2.5 Effect of residual glucose in inoculum medium on ethanol production

Ethanol fermentation was performed at maximal ethanol production condition (result of 3.10.2.4). But the *S.cerevisiae* inoculum used was centrifuged. Cell precipitated was resuspended at original volume of YPD broth without glucose and used as inoculum at 10% (v/v). Ethanol produced in culture supernatant was analyzed by Gas chromatography (GC).

3.11 Separate xylose- and glucose-ethanol fermentation in fermenter

3.11.1 Ethanol production from xylose released in pretreatment hydrolysate

Dilute sulfuric acid pretreatment hydrolysate of sugarcane leaves was used as substrate. Ethanol fermentation by *P.stipitis* was performed in 5L fermentor (Fig. 3.3) using an optimized condition obtained as a result of (3.10.1). Fermentation condition was as followed: agitation rate (50 rpm) and without aeration. Ethanol produced in culture supernatant was

analyzed by gas chromatography (GC) and xylose in culture supernatant was analyzed by High Performance Liquid Chromatography (HPLC).

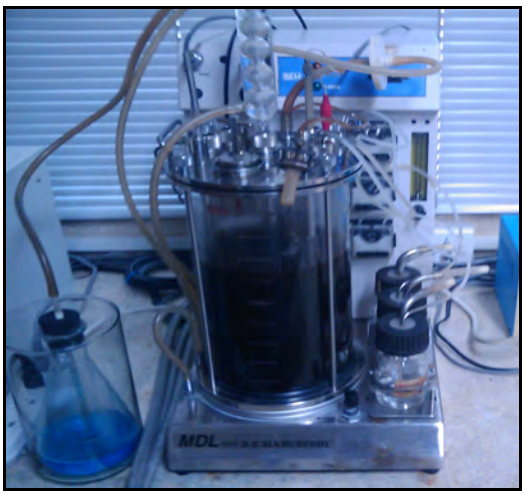


Fig. 3.3 Ethanol production in 5 liter fermenter

3.11.2 Ethanol production from glucose released by cellulase hydrolysis

Dilute sulfuric acid pretreated sugarcane leaves residue separated from its hydrolysate was suspended in 0.1M sodium citrate buffer and saccharified by celluase. After centrifugation, the resultant supernatant was used as substrate for ethanol fermentation by *Saccharomyces cerevisiae* TISTR 5596 using an optimal condition obtained as a result of (3.10.2) Fermentation condition was as followed: agitation rate (50 rpm) and without aeration. Ethanol produced and glucose in culture supernatant was analyzed by Gas chromatography (GC) and glucose analyzer, respectively.

3.12 Analytical procedures

Cellulose, hemicellulose and lignin contents of sugarcane leaves were determined by The Technical Association of Pulp and Paper Industry method (TAPPI, 1988). Reducing sugar was analyzed by Somogyi-Nelson method (Somogyi, 1952). Glucose, xylose and pretreatment by-products (furfural, hydroxymethylfurfural, 4-hydroxybenzaldehyde, syringaldehyde, vanillin) were analysed by HPLC (Agilent 1100 Series equipped with quaternary pump, on-line degasser, autoinjector, colume thermostat, refractive index detector, and a ChemStation softwares, Agilent Technology Co. Ltd., USA). Sugars were identified and quantified by Aminex column HPX-87P

(300 x 7.8) with a Carbo-P micro-guard cartridge (Bio-Rad, USA). The Column was maintained at 80°C and 20 µl of each samples was injected at a time and eluted with Milli-Q filtered water at a flow rate of 0.6 ml/min. The pretreatment by-products were identified and quantified by Aminex column HPX-87H (300 x 7.8) with a Cation H micro-guard cartridge (Bio-Rad, USA). The column was maintained at 55°C and 50 µl of each sample was injected at a time and eluted with 0.01 N H_2SO_4 at a flow rate of 0.6 ml/min. Glucose in celllulase hydrolysate was analysed by glucose analyzer.

Ethanol was quantified by GC (Hewlett-Packard, HP 5890 Series) with a Porapak QS (Cabowax 20 M) column (2 m x 0.32m) at oven temperature of 175°C and flame ionization detector (FID) at 150°C. Helium with a flow rate of 35 ml/min was used as carrier gas.

CHAPTER IV

RESULTS

4.1 Sugarcane leaves

Chemical composition of sugarcane leaves analyzed at Kasetsart Agricultural and Agro-Industrial Product Improvement Institute using method described by Technical Association of Pulp and Paper Industry (TAPPI, 1998) is shown in Table 3. Its major component on a dry weight basis (DS) was 38.5% (w/w) cellulose, 23% (w/w) hemicellulose and 15.6% (w/w) lignin.

Table 4.1 Chemical composition of sugarcane leaves

Components	**% (w/w)**
Cellulose	38.5
Hemicellulose	23.0
Lignin	15.6
Ash	22.9

4.2 Pretreatment of sugarcane leaves

4.2.1 Dilute acid pretreatment

4.2.1.1 Effect of sulfuric acid concentration

Sugarcane leaves (20-40 mesh particle size) at 3% (w/v, DS) pretreated by 1.5% (w/v) H_2SO_4 at 121°C, 15 lb/in^2 for 30 min liberated the highest reducing sugar (0.25 g/g, DS) after pretreatment and the pretreated sugarcane leaves was the most susceptible to cellulase. The reducing sugar released after cellulase hydrolysis was 0.34 g/gDS (Fig. 4.1A, 4.1B).

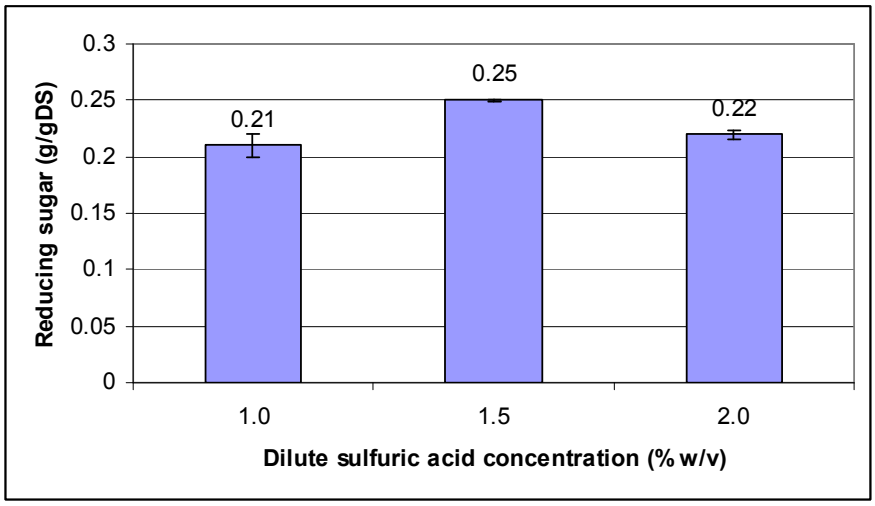


Fig. 4.1A Effect of H_2SO_4 concentration on reducing sugar liberation after dilute sulfuric acid pretreatment. Sugarcane leaves at 3% (w/v, DS) was pretreated by sulfuric acid at 121^{o}C, 15 lb/in^{2} for 30 min. The data are displayed as the mean ±1SD, and are derived from triplicate experiments.

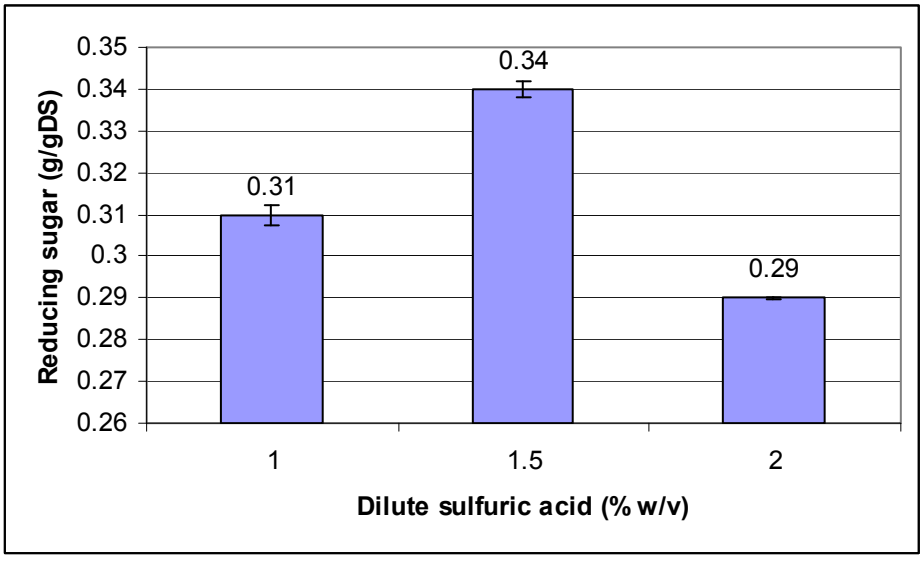


Fig. 4.1B Effect of H_2SO_4 concentration on cellulose susceptibility of dilute sulfuric acid pretreated sugarcane leaves. The pretreated sugarcane leaves was pretreated by dilute sulfuric acid and further saccharified by cellulase at 40^{o}C, 72h. The data are displayed as the mean ±1SD, and are derived from triplicate experiments.

4.2.1.2 Effect of autoclaving time

Sugarcane leaves particles was suspended in 1.5% (w/v) H_2SO_4 at 3% (w/v, DS) and steam heat at 121°C, 15 lb/in^2 for 15, 30, 45, and 60 min. The pretreated sugarcane leaves was then saccharified by cellulase. Reducing sugar liberated was stable after 30 min as shown in Fig. 4.2A. Pretreated sugarcane leaves was most susceptible to cellulase after autoclaving for 30 min as shown as maximal reducing sugar (0.34 g/g (DS) or 11.51 g/l) and maximal glucose (0.057 g/g (Ds) or 1.92 g/l) released (Fig. 4.2B, 4.2C)

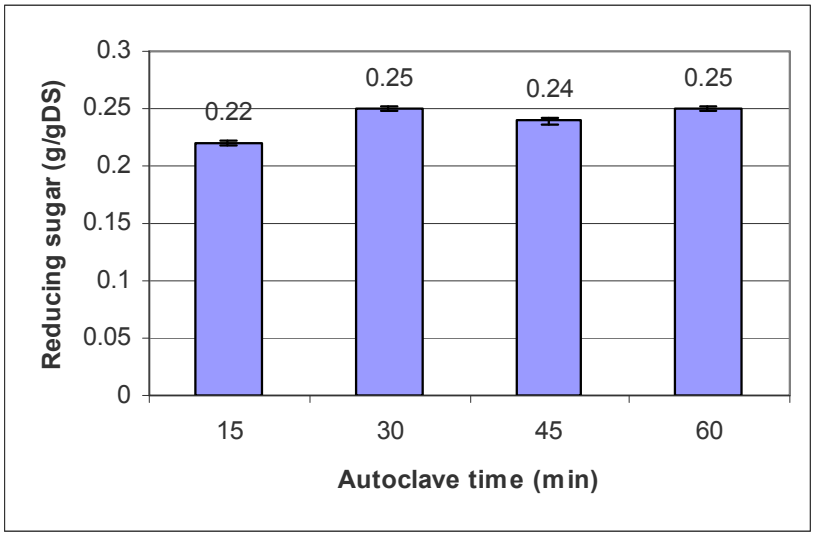


Fig. 4.2A Effect of autoclaving time on reducing sugar liberation after dilute acid pretreatment. Sugarcane leaves (3% w/v, DS) were pretreated by 1.5% (w/v) H_2SO_4 at 121°C, 15 lb/in^2. The data are displayed as the mean ±1SD, and are derived from triplicate experiments.

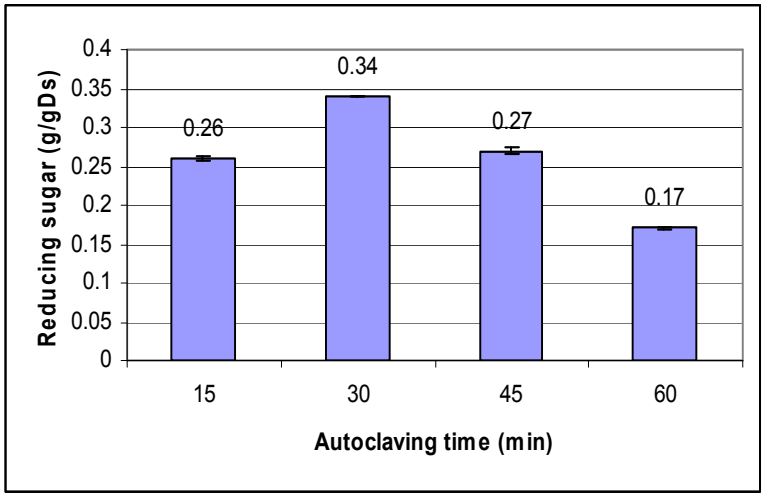


Fig. 4.2B Effect of autoclaving time on cellulose susceptibility, as described by reducing sugar released, of dilute acid sulfuric pretreated sugarcane leaves. The pretreated sugarcane leaves was pretreated by 1.5% (w/v) H_2SO_4 and further saccharified by cellulase at 40^{o}C, 72h. The data are displayed as the mean ±1SD, and are derived from triplicate experiments.

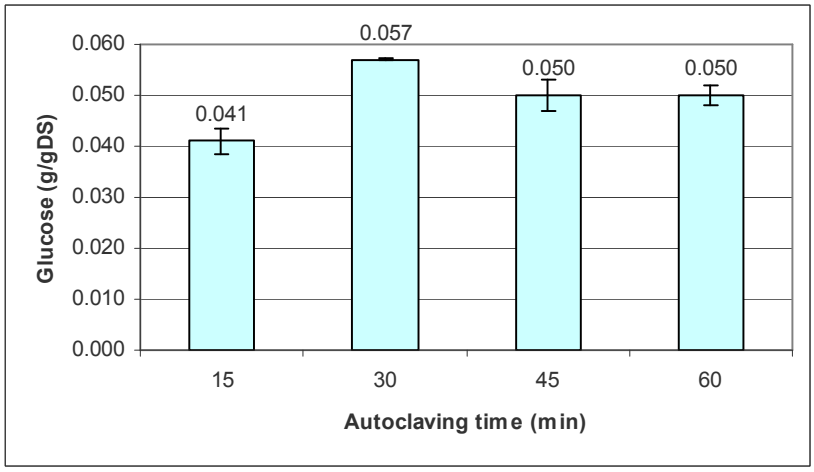


Fig. 4.2C Effect of autoclaving time on cellulose susceptibility, as described by glucose released, of dilute acid pretreated sugarcane leaves. The pretreated sugarcane leaves was pretreated by 1.5% (w/v) H_2SO_4 and further saccharified by cellulase at 40^{o}C, 72h. The data are displayed as the mean ±1SD, and are derived from triplicate experiments.

4.2.1.3 Effect of substrate loading

Various loads of sugarcane leaves (3, 6, 8 % w/v, DS) was suspended in 1.5% (w/v) H_2SO_4, autoclaved at 121°C 15 lb/in^2 (30 min), and hydrolysed by cellulase. Reducing sugar released after pretreatment and cellulose susceptibility of the pretreated sugarcane leaves decreased at 8% (w/v) sugarcane leaves loading as shown in Fig 4.3A, 4.3B. This was due to a problem of mixing, mass and heat transfer of higher substrate loading. The pretreated sugarcane leaves 6% w/v (DS) loading released reducing sugar 0.34 g/g (DS) or 23.2 g/l (Fig. 4.3B)

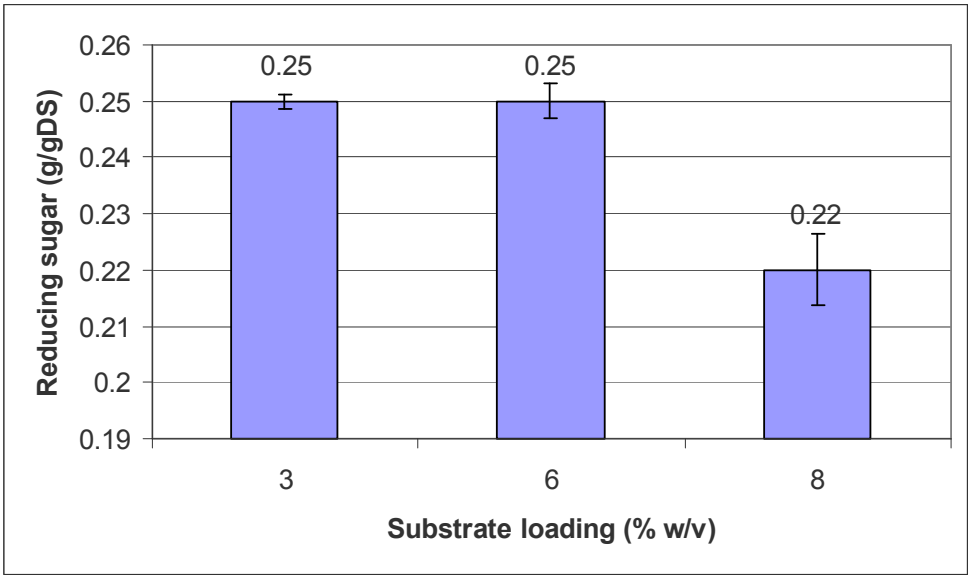


Fig. 4.3A Effect of substrate loading on reducing sugar liberation after dilute sulfuric acid pretreatment. The data are displayed as the mean ±1SD, and are derived from triplicate experiments.

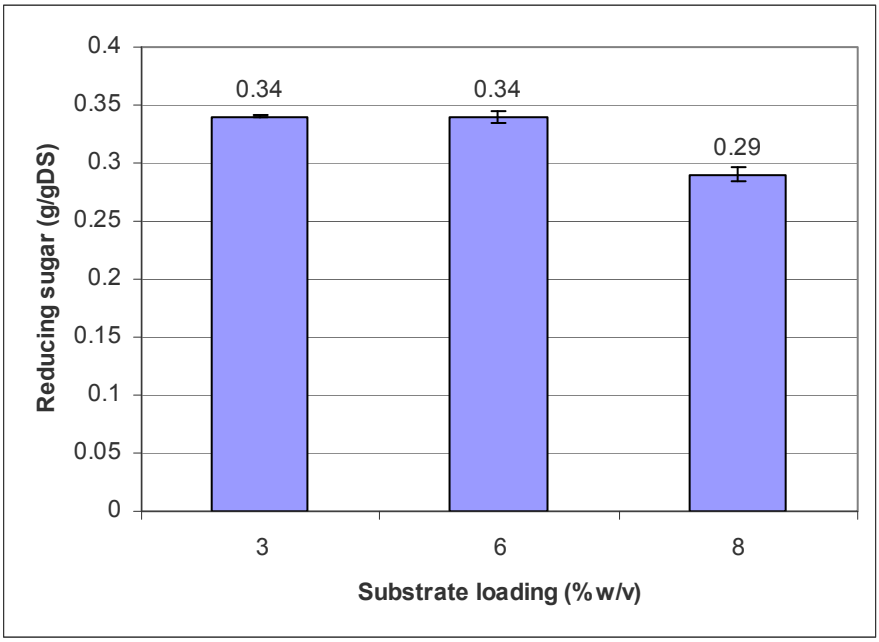


Fig. 4.3B Effect of substrate loading on cellulose susceptibility of pretreated sugarcane leaves. The data are displayed as the mean ±1SD, and are derived from triplicate experiments.

4.2.2 Lime pretreatment

4.2.2.1 Effect of lime concentration

Sugarcane leaves particle (20-40 mesh) at 3% (w/v), DS was pretreated by various concentration of $Ca(OH)_2$ (0.1, 0.25, 0.5, 1.0, 1.5, 2.0 % (w/v)) at 121°C, 15 lb/in^2 for 30 min and further saccharified by cellulase at 40°C, 72 h. Sugarcane leaves pretreated by 1.0 % (w/v) $Ca(OH)_2$ was most susceptible to cellulase. Maximum reducing sugar (0.097 g/g (DS)) was released after cellulase hydrolysis (Fig. 4.4A). The higher $Ca(OH)_2$ concentration used, the lower reducing sugar was released after lime pretreatment (Fig. 4.4B). Reducing sugar released after lime pretreatment was not coincided with the cellulose susceptibility of lime pretreated sugarcane leaves (Fig. 4.4A, 4.4B).

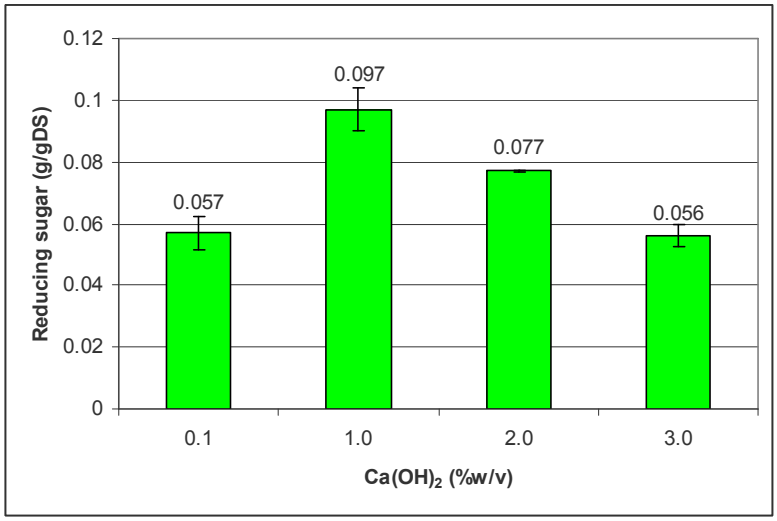


Fig. 4.4A Effect of $Ca(OH)_2$ concentration on cellulose susceptibility of lime pretreated sugarcane leaves. Sugarcane leaves was pretreated by $Ca(OH)_2$ and further saccharified by cellulase at 40°C, 72 h. The data are displayed as the mean ±1SD, and are derived from triplicate experiments.

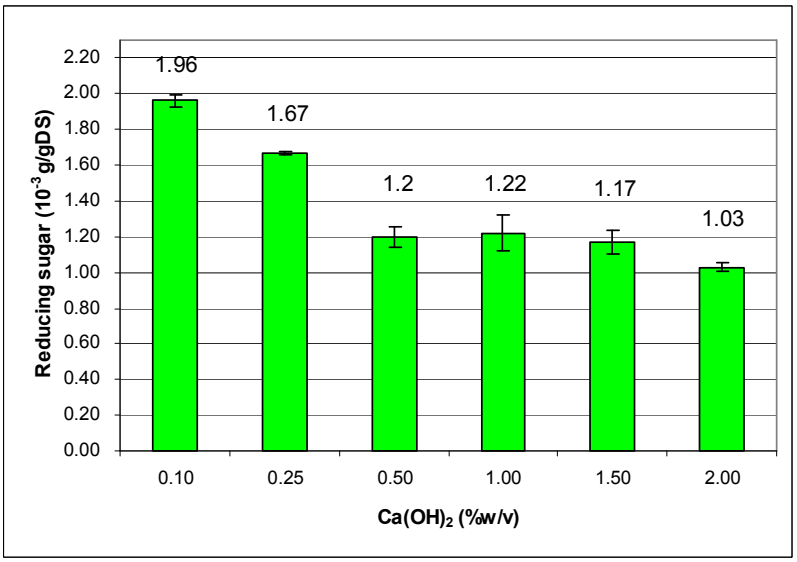


Fig. 4.4B Effect of $Ca(OH)_2$ concentration on reducing sugar liberation after lime pretreatment. The data are displayed as the mean ±1SD, and are derived from triplicate experiments.

4.2.2.2 Effect of autoclaving time on cellulose susceptibility

Sugarcane leaves at 3% (w/v), DS was most susceptible to cellulase after pretreated by 1% (w/v) $Ca(OH)_2$. Or sugarcane leaves 1 g (DS) was pretreated with 0.33 g $Ca(OH)_2$. In this experiment, sugarcane leaves was pretreated with $Ca(OH)_2$ at 0.33 g $Ca(OH)_2$ / g (DS) sugarcane leaves at 121°C, 15 lb/in^2 for various time. As shown in Fig. 4.5, sugarcane leaves was most susceptible to cellulase after autoclaving for 15 min. Maximum reducing sugar (0.107 g/g DS) was released at this condition.

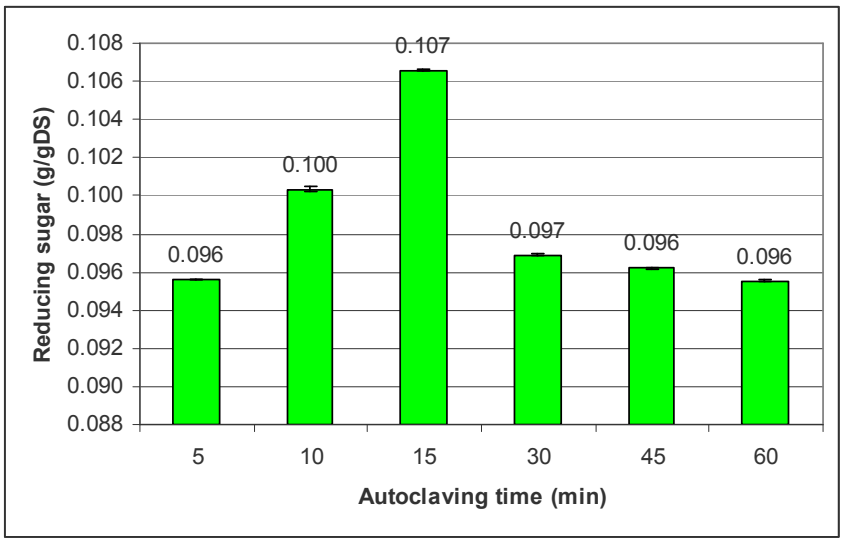


Fig. 4.5 Effect of autoclaving time on cellulose susceptibility of lime pretreated sugarcane leaves. Sugarcane leaves was pretreated with $Ca(OH)_2$ at 0.33g $Ca(OH)_2$ /g DS sugarcane leaves and further saccharified by cellulase at 40°C, 72 h. The data are displayed as the mean ±1SD, and are derived from triplicate experiments

4.2.2.3 Effect of substrate loading on cellulase susceptibility

Sugarcane leaves was pretreated by $Ca(OH)_2$ at 0.33 g $Ca(OH)_2$/g (DS) sugarcane leaves at 121°C, 15 lb/in^2 for 15 min. In this experiment, sugarcane leaves was loaded at 3, 6, or 8 % (w/v) in the pretreatment process. After pretreatment, the lime pretreated sugarcane leaves was saccharified by cellulase at 40°C, 72h. As shown in Fig. 4.6A, cellulose susceptibility of the lime pretreated sugarcane leaves decreased when sugarcane leaves loaded was increased. But maximal reducing sugar (6.69 g/l) was released after cellulase hydrolysis when sugarcane leaves was lime pretreated at 6 % (w/v). Optimal substrate loading for lime pretreatment was 6 % (w/v), Fig 4.6B.

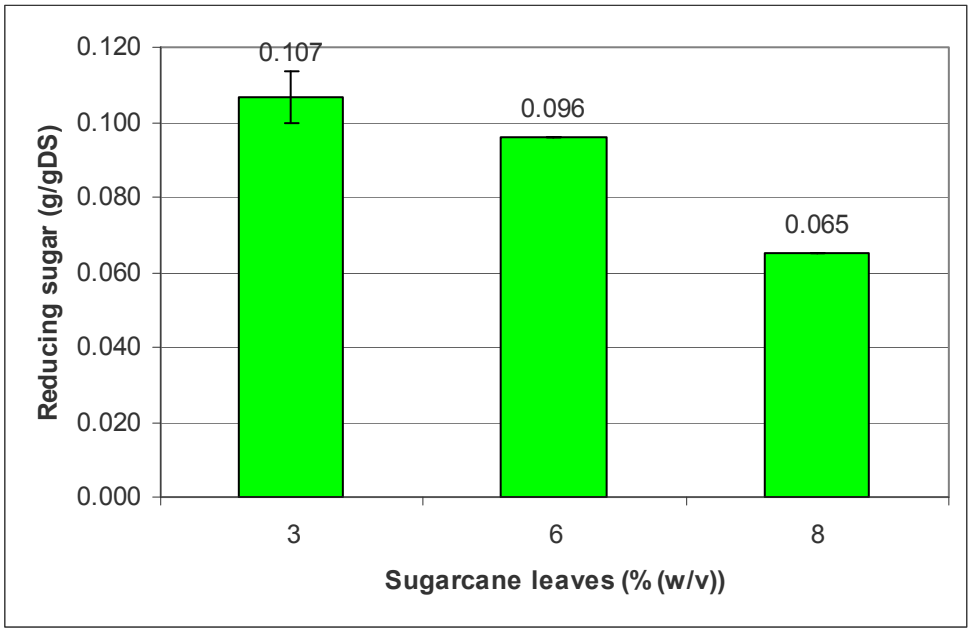


Fig. 4.6A Effect substrate loading on cellulose susceptibility of lime pretreated sugarcane leaves. Sugarcane leaves was pretreated with $Ca(OH)_2$ at 0.33 g $Ca(OH)_2$ / g (DS) sugarcane leaves for 15 min and further saccharified by cellulase at 40°C, 72 h. The data are displayed as the mean ±1SD, and are derived from triplicate experiments

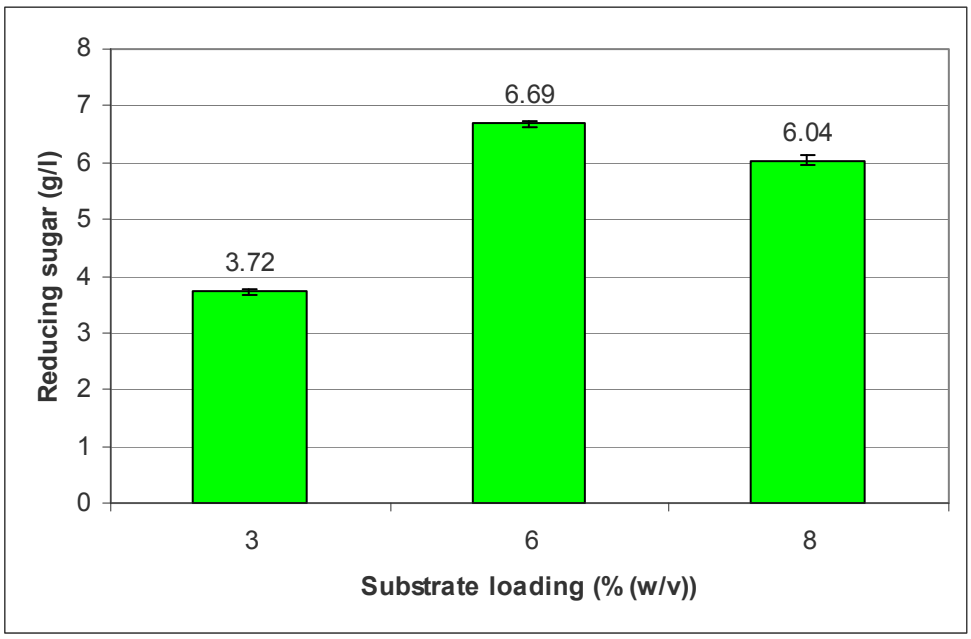


Fig. 4.6B Effect substrate loading on reducing sugar released from lime pretreated sugarcane leaves after cellulase hydrolysis. Sugarcane leaves was pretreated by $Ca(OH)_2$ and further saccharified by cellulase at $40^{o}C$, 72 h. The data are displayed as the mean ±1SD, and are derived from triplicate experiments

4.2.3 Analysis of sugars and pretreatment by-products

Glucose, xylose, pretreatment by-products (furfural, hydroxymethylfurfural (HMF), 4-hydroxylbenzaldehyde, syringaldehyde, and vanillin) detected in the dilute sulfuric acid and lime pretreatment hydrolysate are shown in Table 4.2A and 4.2B, respectively.

Table 4.2A Sugars and pretreatment by-products in dilute acid pretreatment hydrolysate

Sugars	**g/l**
Glucose	0.62
Xylose	9.0
Inhibitors	**g/l**
Hydroxymethylfurfural	0.000396
Furfural	0.006047
4-Hydrobenzaldehyde	0.000228
Vanilin	0.000198
Syringaldehyde	0.006557

Table 4.2B Sugars and pretreatment by-products in lime pretreatment hydrolysate

Sugars	**g/l**
Glucose	-
Xylose	-
Inhibitors	**g/l**
Hydroxymethylfurfural	-
Furfural	-
4-Hydrobenzaldehyde	0.0171
Vanilin	0.0076
Syringaldehyde	0.0080

(-): Not detectable

4.3 Cellulase hydrolysis

Optimal condition for dilute acid sulfuric acid pretreatment was 1.5 % (w/v) H_2SO_4; 121^{o}C, 15 lb/in^2 (30 min) and 6% (w/v) substrate loading (result of 4.2.1). Optimal condition for lime pretreatment was 0.33 g $Ca(OH)_2$ /g (DS) sugarcane leaves; 121^{o}C, 15 lb/in^2 (15 min) and 6% (w/v) substrate loading (result of 4.2.2). Reducing sugar released when sugarcane leaves pretreated by dilute sulfuric acid and by lime at the optimal condition were 0.34 and 0.096 g/g (DS) sugarcane leaves, respectively. Moreover dilute sulfuric acid pretreated sugarcane leaves slurry contained 9.8 g/l of xylose, whereas xylose was not detected in lime pretreated sugarcane leaves hydrolysate.

This experiment, the dilute sulfuric acid pretreated sugarcane leaves slurry was saccharified by cellulase, Accellulase™ 1000 (265 FPU/ml: endoglucanase 2500 CMCU/g, β-glucosidase 400 pNPGU/g, Genecor International, Inc., USA) at 50^{o}C, pH 5.0 as recommended by manufacturer.

Dose of Acellulase ™ 1000 used for saccharification was varied 0.2, 0.4, 0.6, 0.8 ml/g (DS) or 50, 106, 160, 212 FPU/g (DS) or 75, 160, 240, 320 pNPGU/g (DS). Pretreated sugarcane leaves was saccharified for 6, 12, 18, 24 h. Saccharification for 6 h; the higher cellulase dose, the higher glucose was released. Minimum cellulase dose which could liberate the maximum glucose was 0.6 ml/g (DS) (Fig. 4.7).

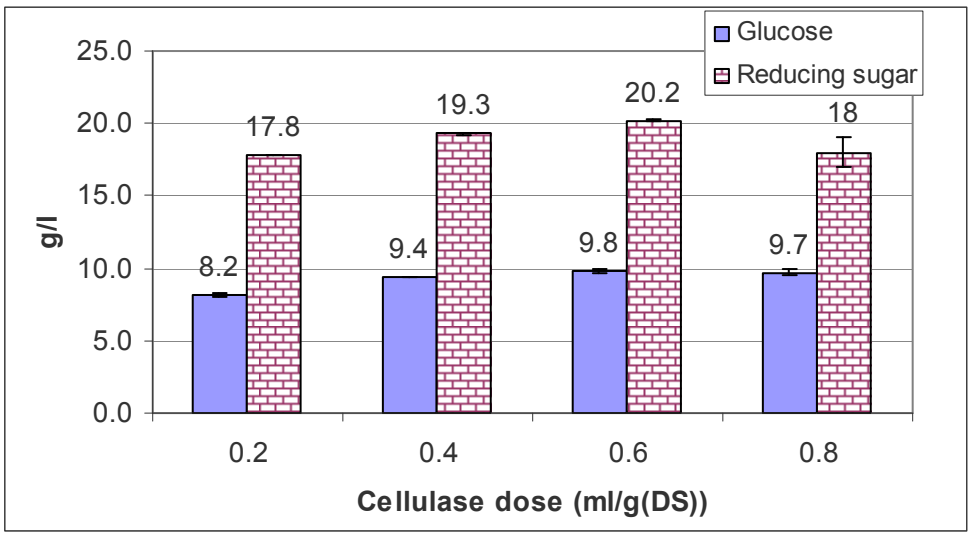


Fig. 4.7 Effect of cellulase dose on reducing sugar and glucose released by cellulase hydrolysis. Sugarcane leaves was pretreated by dilute sulfuric acid at optimal condition and further saccharifired by cellulase at 50°C, pH 5.0 for 6 h. Glucose (□), reducing sugar (▤). The data are displayed as the mean ±1SD, and are derived from triplicate experiments.

Accellulase™ 1000 at dose of 0.6 ml/g (DS) was used to saccharify dilute sulfuric acid pretreated sugarcane leaves. Increase of saccharification time resulted in increase of glucose released. Glucose released was stable after 18 h. Maximum glucose (11.1 g/l) was released at 18 h. (Fig. 4.8).

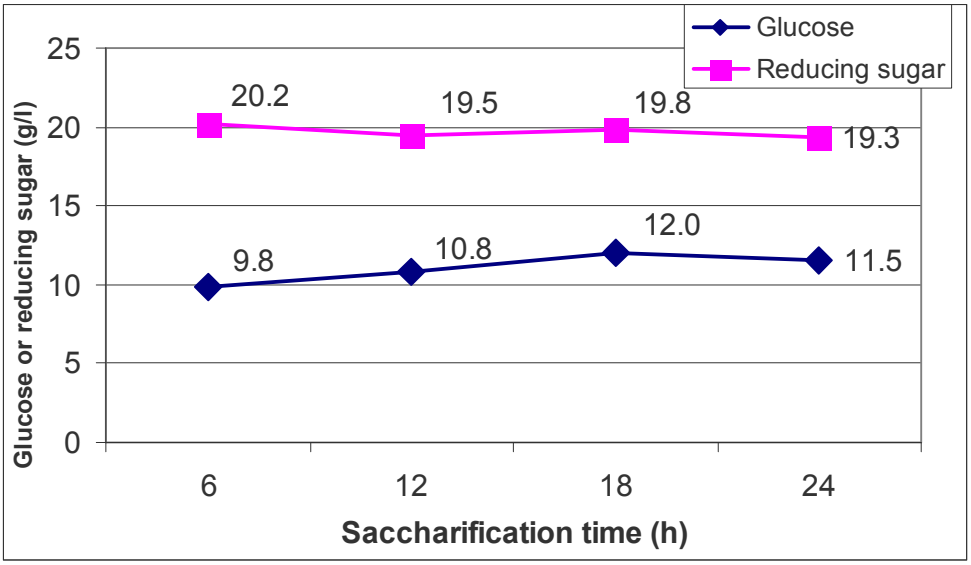


Fig. 4.8 Effect of saccharification time on reducing sugar and glucose released by cellulase hydrolysis. Sugarcane leaves was pretreated by dilute sulfuric acid at optimal condition and further saccharified by Accellulase™ 1000 at a dose of 0.6 ml/g (DS). Glucose (◆), reducing sugar (■). The data are displayed as the mean ±1SD, and are derived from triplicate experiments.

The longer saccharification time, the higher risk of glucose lost due to contamination. Accellulase™ 1000 at a dose of 0.6 ml/g (DS), glucose productivity at the first 6 h was 1.6 g/l/h, but glucose productivity decreased to 0.18 g/l/h during 7th to 18th of saccharification time. Glucose released was stable after 18 h (Fig. 4.8). Dilute sulfuric acid pretreated sugarcane leaves was saccharified by Accellulase™ 1000 at a dose of 0.6 ml/g (DS) for 6 h. The cellulase reaction was centrifuged and the resultant supernatant was used as substrate for ethanol fermentation in further experiments.

4.4 Separate hydrolysis and ethanol fermentation (SHF)

Sugarcane leaves was pretreated by dilute sulfuric acid at optimal condition then the resultant slurry was pH adjusted, saccharified by cellulase at optimal condition. After centrifugation, obtained supernatant containing glucose 9.8 g/l was used as substrate for ethanol fermentation by *Saccharomyces cerevisiae*. Fermentation condition was as followed: 10% (v/v) inoculum, 30^{o}C, pH 4.5, oxygen limit condition for 72h.

4.4.1 Effect of inoculum age on ethanol production

In this experiment, *S. cerevisiae* grown in YPD broth for 6h (mid log phase cells, OD_{660nm} was 0.6) or for 24h (stationary phase cells, OD_{660nm} was 2.24) was used as inoculum at 10% (v/v). As shown in Fig. 4.9 A, mid log phase cells of *S. cerevisiae* gave ethanol (5.02 g/l) which was higher than those of stationary phase *S. cerevisiae* cells (4.48 g/l). A 6h culture of *S. cerevisiae* (mid log phase cells) was used as inoculum in futher experiments.

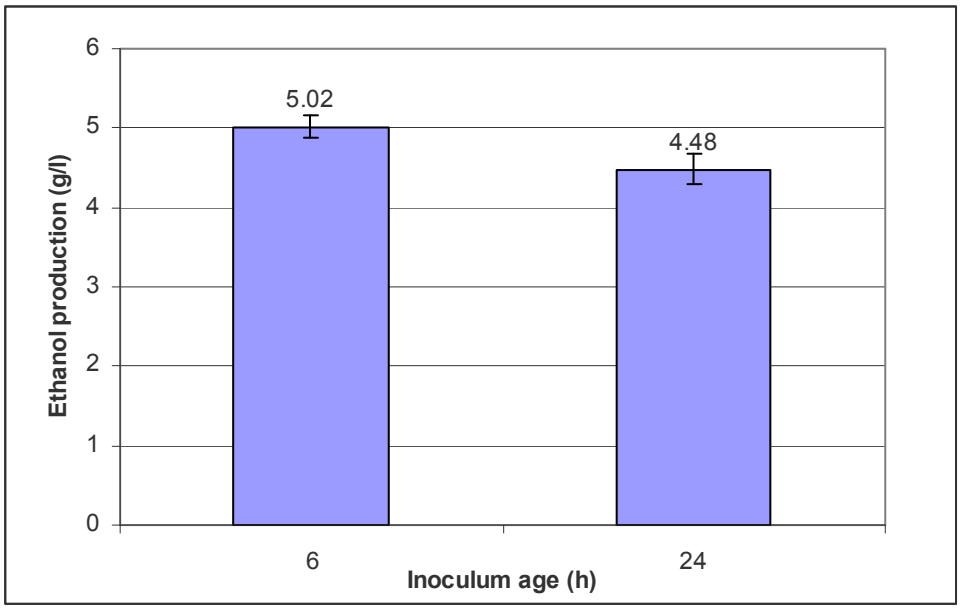


Fig. 4.9A Effect of *S. cerevisiae* inoculum age on ethanol production. The data are displayed as the mean ±1SD, and are derived from triplicate experiments.

4.4.2 Effect of $(NH_4)_2SO_4$ supplementation on ethanol production

In this experiment, ethanol fermentation was performed as described above, and *S. cerevisiae* inoculum age used was 6h, but concentration of $(NH_4)_2SO_4$ supplemented was varied. As shown in Fig 4.9B, $(NH_4)_2SO_4$ supplementation did not increase ethanol production. Maximum ethanol produced without $(NH_4)_2SO_4$ supplementation after 72h was 5.02 g/l.

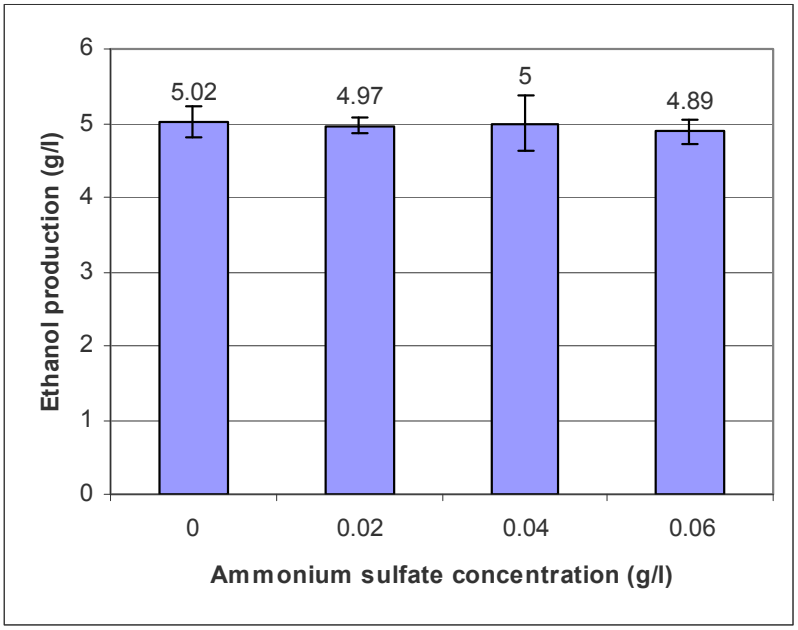


Fig. 4.9B Effect of $(NH_4)_2SO_4$ supplementation on ethanol production. The data are displayed as the mean ±1SD, and are derived from triplicate experiments.

4.4.3 Effect of nutrients supplementation on ethanol production

There was no effect of $(NH_4)_2SO_4$ supplementation on ethanol production (result of 4.4.2). In this experiment, an effect of other nutrients (KH_2PO_4 (0.5 g/l), yeast extract (3.0 g/l), $(NH_4)_2SO_4$ (0.02 g/l) and/or their combination on ethanol production was determined. It was found that the nutrients tested had no effect on ethanol production (Fig 4.9C).

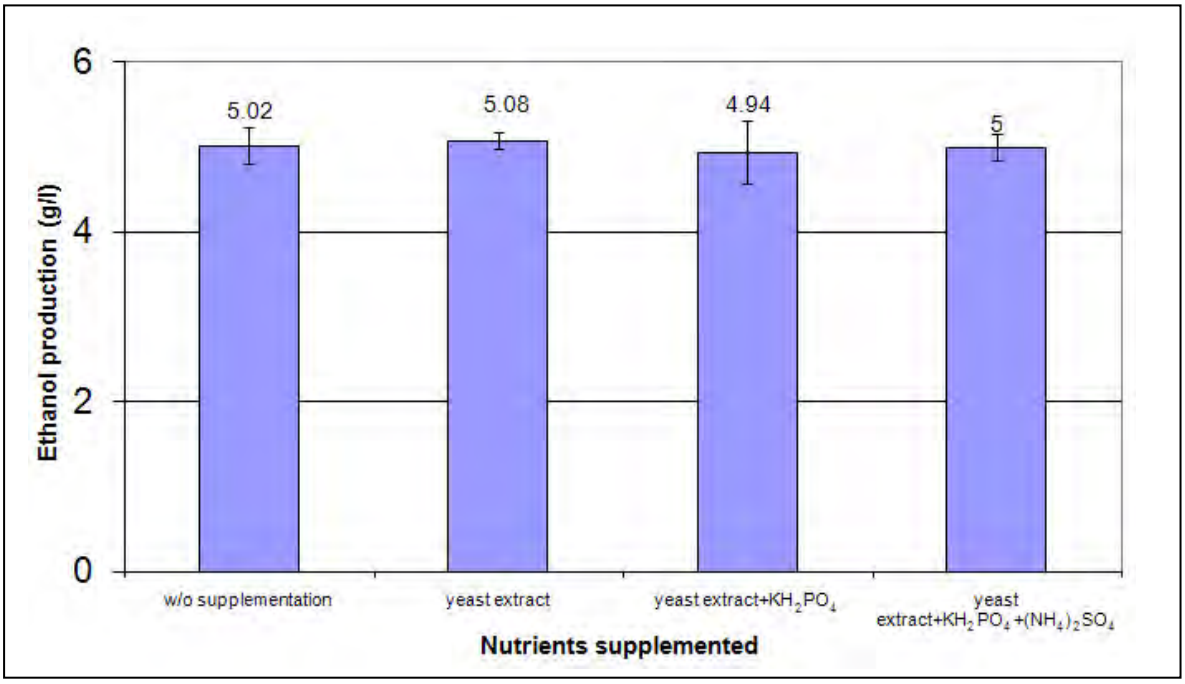


Fig. 4.9C Effect of yeast extract, KH_2PO_4, $(NH_4)_2SO_4$, or their combination on ethanol production. The concentration supplemented were: yeast extract (3.0 g/l), KH_2PO_4 (0.5 g/l), and $(NH_4)_2SO_4$ (0.02 g/l). The data are displayed as the mean ±1SD, and are derived from triplicate experiments.

4.4.4 Effect of fermentation time on ethanol production

In this experiment, ethanol fermentation was performed as described above, and using 6h *S.cerevisiae* inoculum age without any supplementation. Every 12h, ethanol produced in culture supernatant was analyzed. As shown in Fig. 4.9D, maximum ethanol (5.46 g/l) was produced after 24h of incubation.

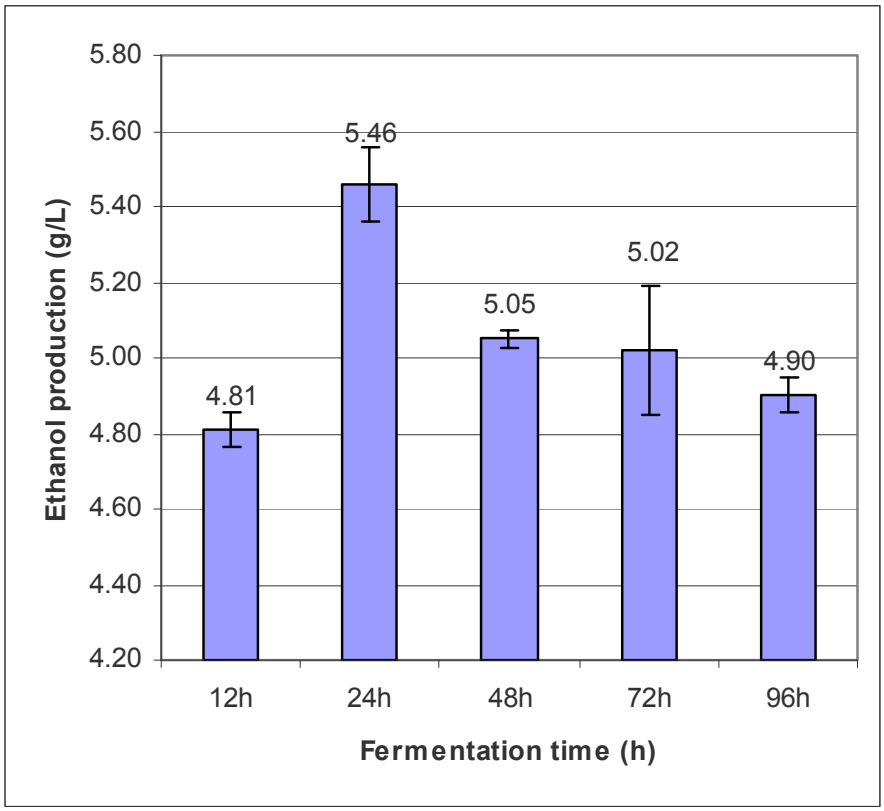


Fig. 4.9D Effect of fermentation time on ethanol production by *S.cerevisiae*. The data are displayed as the mean ±1SD, and are derived from triplicate experiments.

4.4.5 Effect of residual glucose in inoculum medium on ethanol production

The 6h *S.cerevisiae* inoculum was centrifuged. The cell precipitate was resuspended at original volume in YPD broth without glucose and used as inoculum at 10% (v/v). Ethanol fermentation of the cellulase hydrolysate which pH adjusted to 4.5 was performed at 30°C. Maximum ethanol (4.71 g/l) was produced when inoculum cells were suspended in YPD broth without glucose after 24h of incubation as shown in Fig. 4.9E. It was 0.75 g/l lower than those obtained in the experiment using *S.cerevisiae* inoculum which carried residual glucose in inoculum medium.

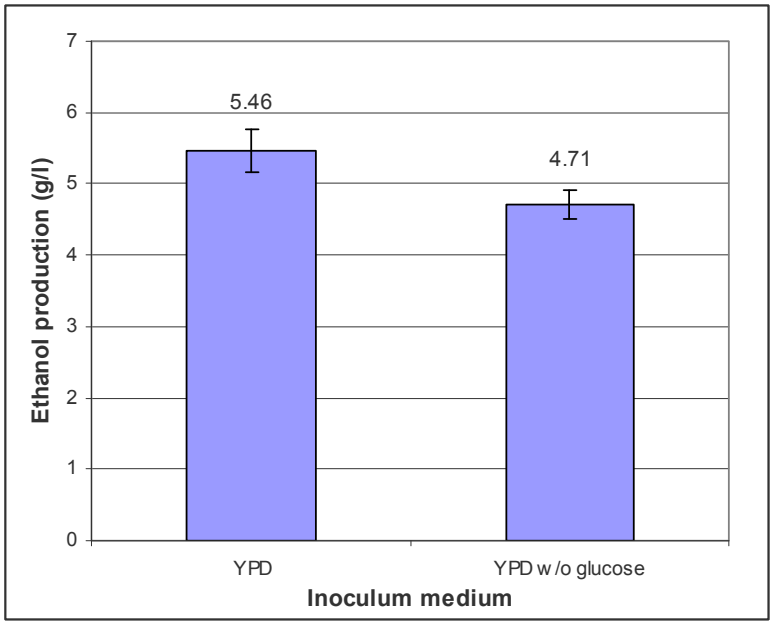


Fig. 4.9E Effect of residual glucose in inoculum medium on ethanol production by *S.cerevisiae*. The data are displayed as the mean ±1SD, and are derived from triplicate experiments.

4.5 Separate xylose- and glucose- ethanol fermentation

4.5.1 Ethanol production from xylose in pretreatment hydrolysate

Sugarcane leaves was pretreated by dilute sulfuric acid at the optimal condition (result of 3.8). Pretreatment hydrolysate obtained after press filtration and centrifugation of the pretreated sugarcane leaves slurry was fermented to ethanol by *Pichia stipitis* at 25°C, pH 5.0, oxygen limit condition for 72h.

4.5.1.1 Effect of nutrient supplementation on ethanol production

The pretreatment hydrolysate supplemented without or with $(NH_4)_2SO_4$ (0.02 g/l); or $(NH_4)_2SO_4$ (0.02 g/l) + KH_2PO_4 (0.2 g/l) + yeast extract (3.0 g/l) was used as substrate for ethanol fermentation by 24h *P.stipitis*. $(NH_4)_2SO_4$ supplementation had no effect on ethanol production, but further supplemented with KH_2PO_4 and yeast extract reduced ethanol production as shown in Fig. 4.10A.

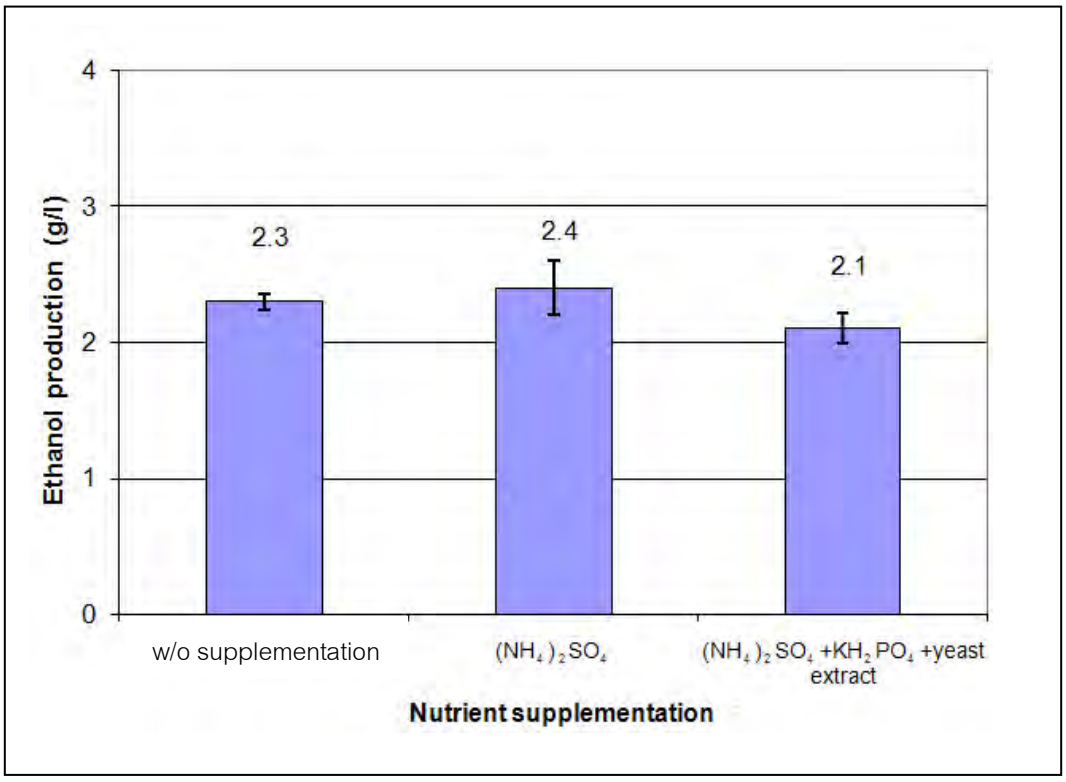


Fig. 4.10A Effect of nutrient supplementation in the dilute sulfuric acid pretreatment hydrolysate on ethanol production by *P.stipitis*. Concentration used as followed: $(NH_4)_2SO_4$ (0.02 g/l), KH_2PO_4 (0.2 g/l), and yeast extract (3.0 g/l). The data are displayed as the mean ±1SD, and are derived from triplicate experiments.

4.5.1.2 Effect of pH on ethanol production

The dilute sulfuric acid pretreatment hydrolysate without any supplementation was used as substrate for ethanol fermentation by *P.stipitis*. Ethanol fermentation was performed at various pH (4.5-7.0). Ethanol production was maximum (3.3 g/l) at pH 5.5 as shown in Fig 4.10B.

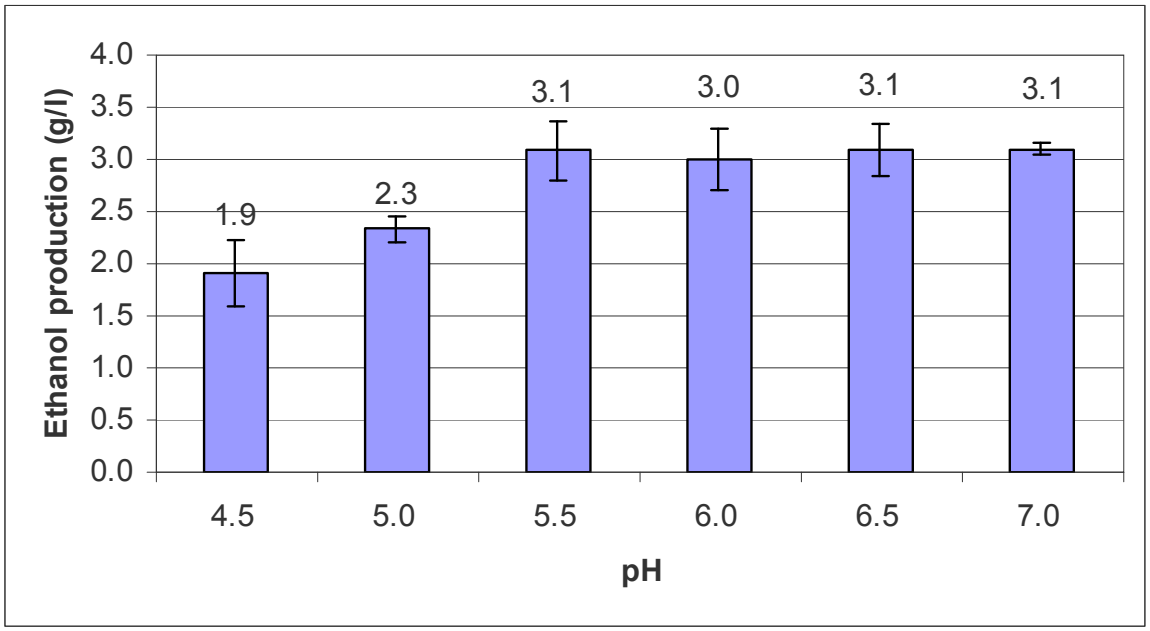


Fig. 4.10B Effect of pH on ethanol production from dilute sulfuric acid pretreatment hydrolysate by *P.stipitis*. The data are displayed as the mean ±1SD, and are derived from triplicate experiments.

4.5.1.3 Effect of temperature on ethanol production

The dilute sulfuric acid pretreatment hydrolysate was used as fermentation medium. The 24h culture of *P.stipitis* was used as inoculum at 10% (v/v). Ethanol fermentation was performed at various temperatures. It was found that maximum ethanol (3.3 g/l) was produced at 25°C as shown in Fig. 4.10C. Increase of fermenting temperature (25, 30, 37, and 40°C) resulted in a decrease of ethanol production. Optimal temperature for ethanol fermentation of *P.stipitis* was 25-30°C (Gupta et al., 2009). Effect of temperature lower than 25°C on ethanol production was not studied due to non-applicable in commercial.

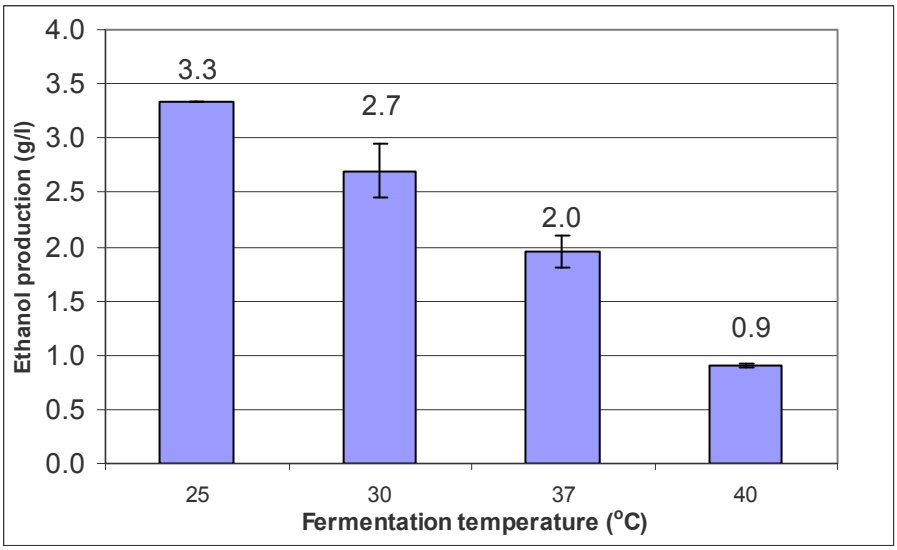


Fig. 4.10C Effect of fermentation temperature on ethanol production from dilute sulfuric acid pretreatment hydrolysate by *P.stipitis*. The data are displayed as the mean ±1SD, and are derived from triplicate experiments.

4.5.1.4 Effect of fermentation time on ethanol production

Dilute sulfuric acid pretreatment hydrolysate without any supplementation which pH was adjusted to 5.5 was used as substrate for ethanol fermentation. The fermentation was performed at 25°C using 24h *P.stipitis* inoculum at 10% (v/v). Maximum ethanol (3.82 g/l) was produced after 96h as shown in Fig. 4.10D.

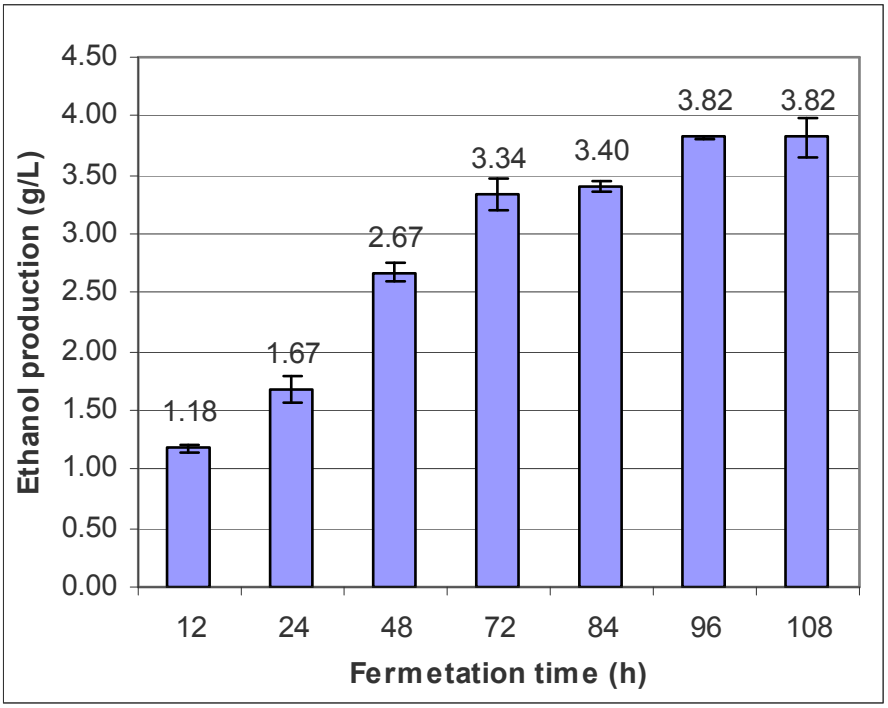


Fig. 4.10D Effect of fermentation time on ethanol production from dilute sulfuric acid pretreatment hydrolysate by *P.stipitis*. The data are displayed as the mean ±1SD, and are derived from triplicate experiments.

4.5.1.5 Effect of xylose residue in inoculum medium on ethanol production

The 24h *P.stipitis* inoculum was centrifuged. The cell precipitate was resuspended at original volume in medium containing; yeast extract 4 g/l, malt extract 3 g/l, peptone 5 g/l (without xylose) and used as inoculum at 10% (v/v). Dilute sulfuric acid pretreatment hydrolysate which pH adjusted to 5.5 was used as substrate for ethanol fermentation. The fermentation was performed at 25°C. Maximum ethanol (3.12 g/l) was produced when *P.stipitis* inoculum cells were suspended in medium without xylose after 96h as shown in Fig. 4.10E, which was 0.7 g/l lower than those obtained in the experiment using *P.stipitis* inoculum which carried residual xylose in inoculum medium.

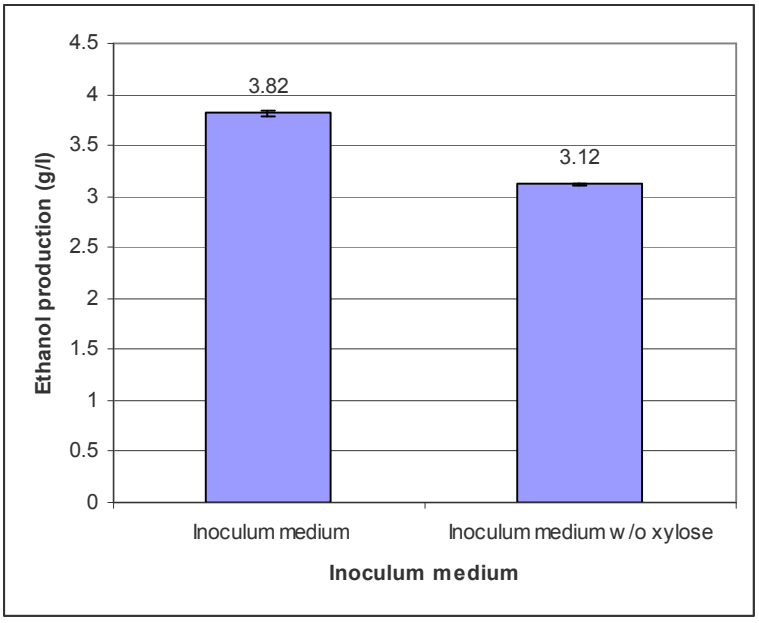


Fig. 4.10E Effect of residual xylose in inoculum medium on ethanol production by *P.stipitis*. The data are displayed as the mean ±1SD, and are derived from triplicate experiments.

4.5.2 Ethanol production from glucose in cellulase hydrolysate

Pretreated sugarcane leaves separated from pretreatment hydrolysate was saccharified by cellulase. Supernatant obtained after cellulase hydrolysis was used as substrate for ethanol fermentation by *S.cerevisiae* TISTR 5596 at 10% (v/v). Fermentation conditions were as followed: pH 4.5, 30^{o}C, oxygen limit condition, 72h.

4.5.2.1 Effect of inoculum age

S.cerevisiae inoculum grown in YPD broth for 6h (mid log phase cells, OD_{660nm} was 0.6) or 24h (stationary phase cells, OD_{660nm} of 2.24) was used as inoculum at 10% (v/v). Ethanol produced by the 6h and 24h inoculum culture were 3.13 and 2.76 g/l, respectively as shown in Fig. 4.11A

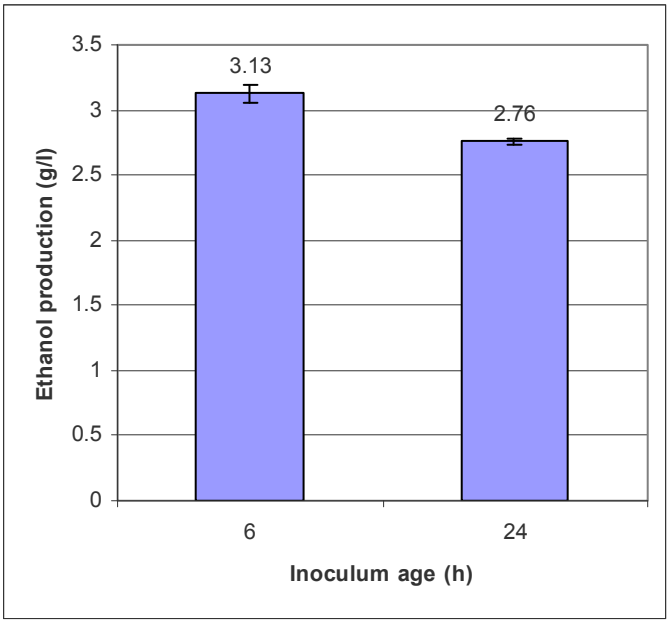


Fig. 4.11A Effect of *S.cerevisiae* inoculum age on ethanol production from cellulase hydrolysate. The data are displayed as the mean ±1SD, and are derived from triplicate experiments.

4.5.2.2 Effect $(NH_4)_2SO_4$ supplementation on ethanol production

In this experiment, ethanol fermentation was performed as described above. *S.cerevisiae* inoculum age used was 6h, but concentration of $(NH_4)_2SO_4$ supplementation was varied. As shown in Fig 4.11B, $(NH_4)_2SO_4$ supplementation had no effect on ethanol production.

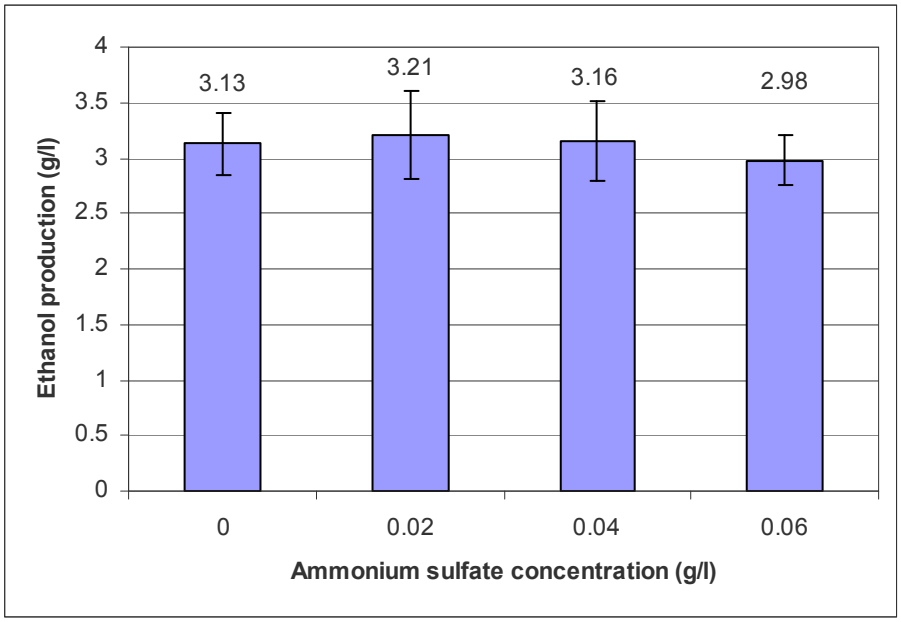


Fig. 4.11B Effect of $(NH_4)_2SO_4$ supplementation in cellulase hydrolysate on ethanol production by *S. cerevisiae*. The data are displayed as the mean ±1SD, and are derived from triplicate experiments.

4.5.2.3 Effect of nutrient supplementation

$(NH_4)_2SO_4$ supplementation had no effect on ethanol production (result of 4.5.2.2). Effect of other nutrients (KH_2PO_4 (0.2 g/l), and/or yeast extract (3.0 g/l) on ethanol production was determined. It was found that there was no nutrients supplementation tested increased ethanol production (Fig 4.11C). The cellulase hydrolysate without any supplementation was used as substrate in further experiments.

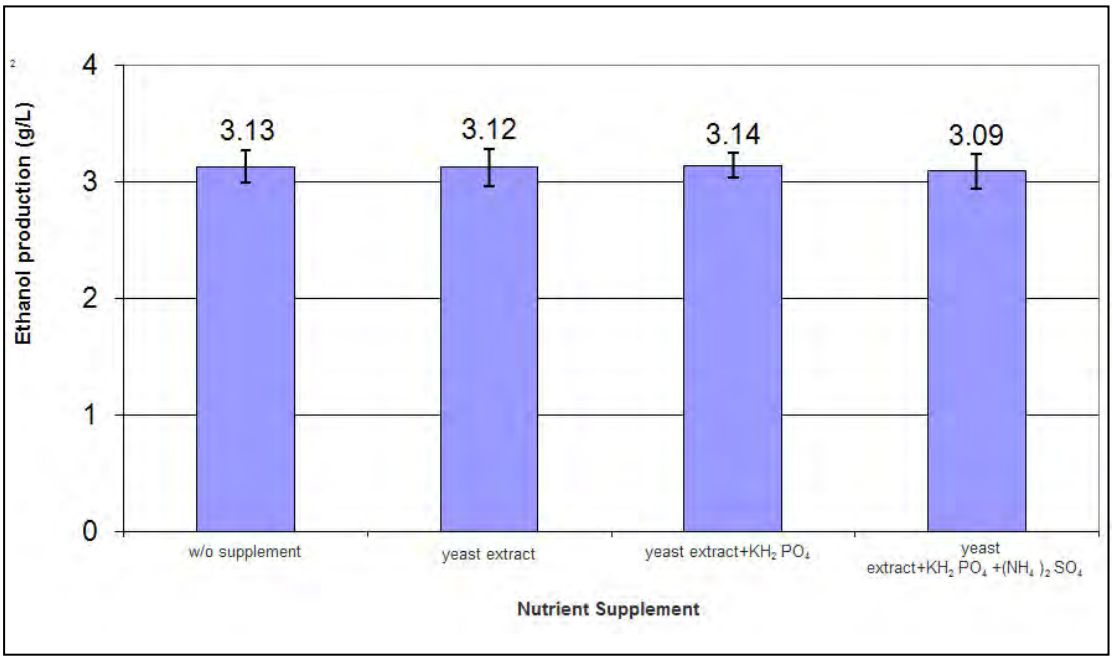


Fig. 4.11C Effect of nutrient supplementation in the cellulase hydrolysate on ethanol production by *S.cerevisiae*. Concentration used as followed: $(NH_4)_2SO_4$ (0.02 g/l), KH_2PO_4 (0.2 g/l), and yeast extract (3.0 g/l). The data are displayed as the mean ±1SD, and are derived from triplicate experiments.

4.5.2.4 Effect of fermentation time on ethanol production

In this experiment, ethanol fermentation was performed as described above using 6h *S.cerevisiae* inoculum. The hydrolysate without any supplementation was used as substrate. Every 12h interval, ethanol produced in culture supernatant was analyzed. As shown in Fig. 4.11D, maximum ethanol (3.29 g/l) was produced after 12h of incubation.

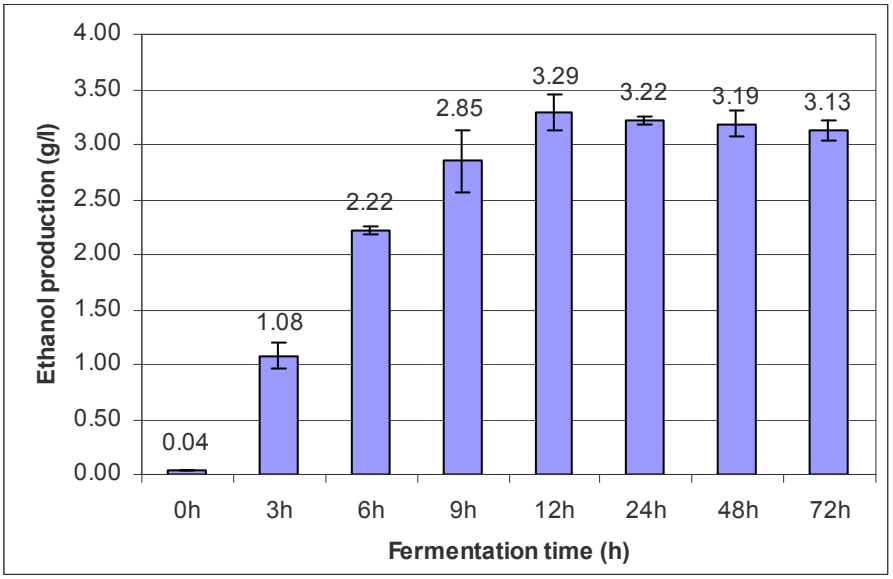


Fig. 4.11D Effect of fermentation time on ethanol production from cellulase hydrolysate by *S.cerevisiae*. The data are displayed as the mean ±1SD, and are derived from triplicate experiments.

4.5.2.5 Effect of residual glucose in inoculum medium on ethanol production

The 6h *S.cerevisiae* inoculum was centrifuged. The cell precipitate then was resuspended at original volume in YPD broth without glucose and used as inoculum at 10% (v/v). The cellulase hydrolysate which pH was adjusted to 4.5 was used as substrate. Ethanol fermentation was performed at 30°C. Maximum ethanol (2.9 g/l) was produced when *S.cerevisiae* inoculum cells were suspended in YPD broth without glucose after 12h of incubation as shown in Fig. 4.11E, which was 0.39 g/l lower than those obtained in the experiment using *S.cerevisiae* inoculum which carried residual glucose in inoculum medium.

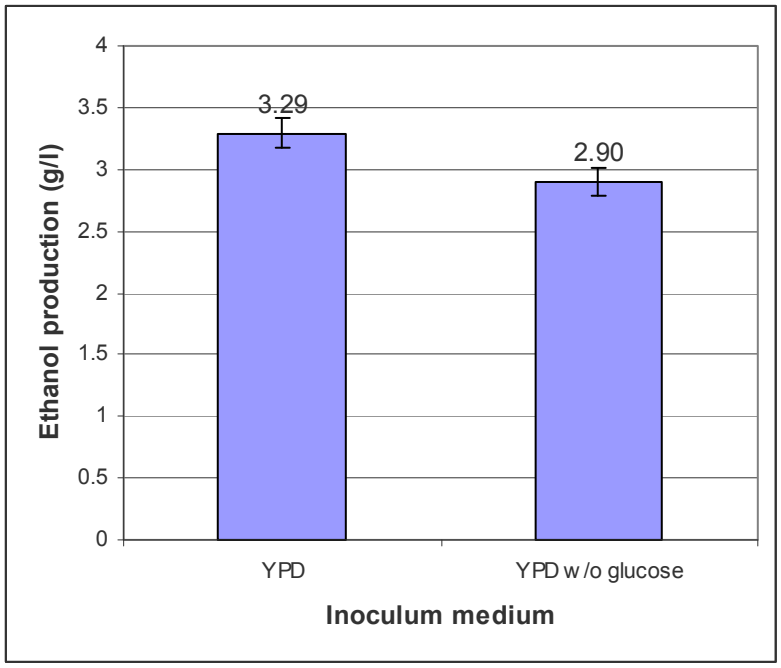


Fig. 4.11E Effect of residual glucose in inoculum medium on ethanol production from cellulase hydrolysate. The data are displayed as the mean ±1SD, and are derived from triplicate experiments.

4.6 Separate xylose- and glucose-ethanol fermentation in fermenter

4.6.1 Ethanol production from xylose in pretreatment hydrolysate

Dilute sulfuric acid pretreatment hydrolysate was fermented to ethanol by *Pichia stipitis* in 5L fermenter. During 144h of fermentation, ethanol production and growth increased with time, while xylose decreased from 8.67 to 3.27 with time. After 144h, ethanol 4.54 g/l was produced (Fig 4.12A).

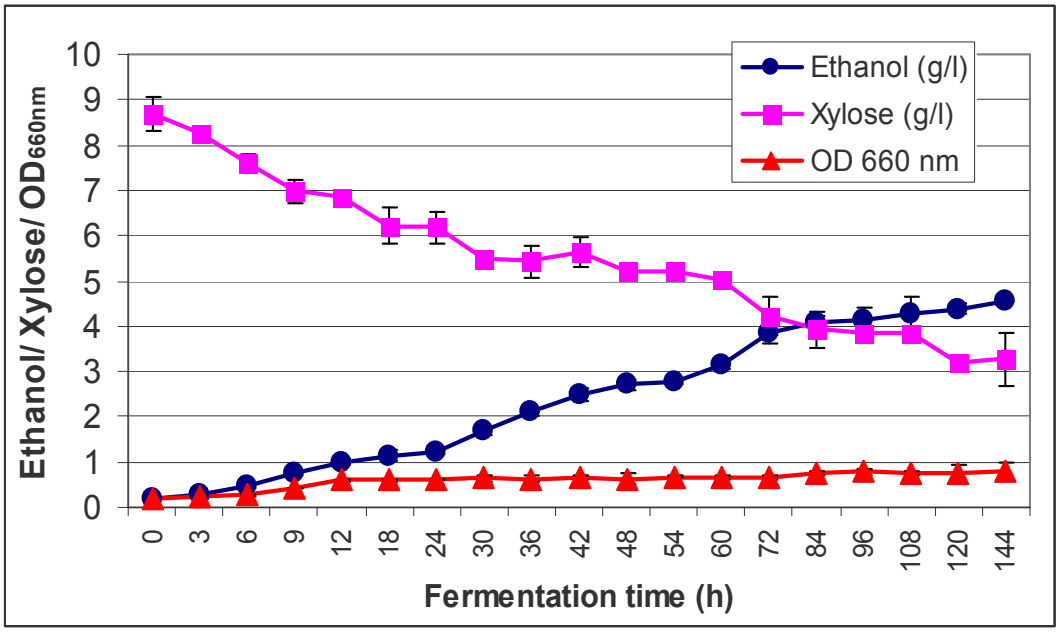


Fig. 4.12A Time course of ethanol fermentation from dilute sulfuric acid pretreatment hydrolysate by *P.stipitis* in 5L fermenter. The amount of ethanol production (●), residual xylose (■), and OD_{660nm}(▲) were determined. The data are displayed as the mean ±1SD, and are derived from triplicate experiments.

4.6.2 Ethanol production from glucose in cellulase hydrolysate

Dilute sulfuric acid pretreated sugarcane leaves residue separated from its hydrolysate was suspended in 0.5M sodium citrate buffer and saccharified by celluase. After centrifugation, The resultant supernatant was used as substrate for ethanol fermentation by *Saccharomyces cerevisiae* TISTR 5596 at its optimal condition. Maximum ethanol (3.05 g/l) was produced at 12h. At this time, *S.cerevisiae* reached stationary growth phase and glucose was completely consumed (Fig. 4.12B).

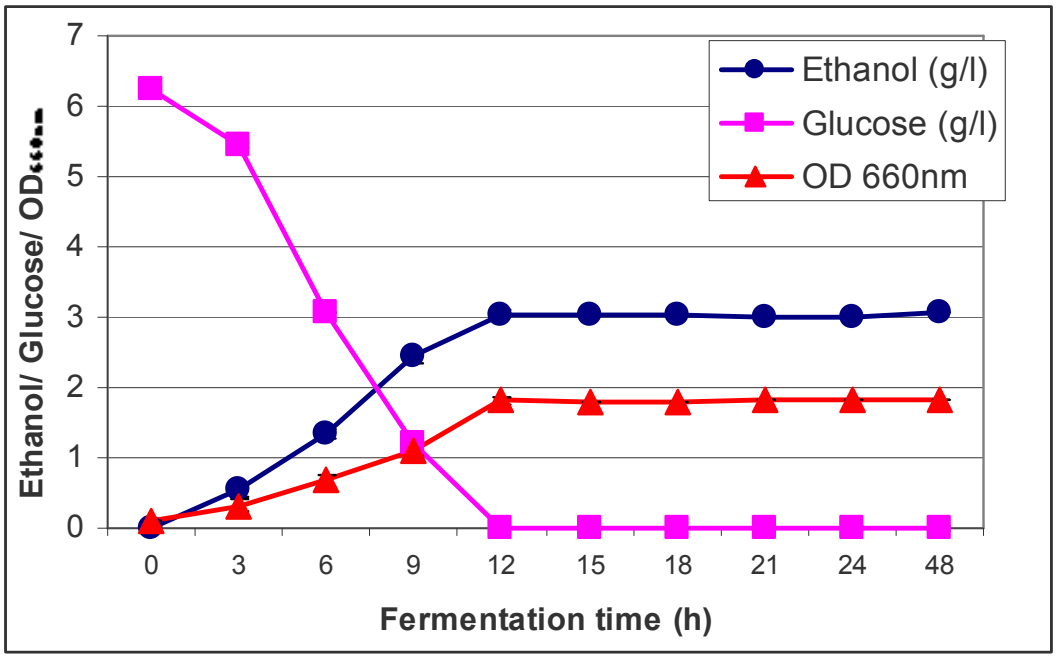


Fig. 4.12B Time course of ethanol fermentation from cellulase hydrolysate by *S.cerevisiae* in 5L fermenter. The amount of ethanol production (●), residual glucose (■), and OD_{660nm}(▲) were determined. The data are displayed as the mean ±1SD, and are derived from triplicate experiments.

Table 4.3 Summary of products and their yield in each experimental steps

Processes	Methods	Products	Yield
Pretreatment	Dilute sulfuric acid	Glucose	0.62 g/l
		Xylose	9.0 g/l
	Lime	Glucose	-
		Xylose	-
Cellulase hydrolysis	Dilute sulfuric acid	Glucose	3.42 g/l 0.057 g/g (DS)
	Lime	Glucose	2.34 g/l 0.039 g/g (DS)
Fermentation (Flask scale)	Separate hydrolysis and fermentation (SHF)	Ethanol (from glucose 9.8 g/l)	4.71 g/l 0.48 g/g glucose 0.20 g/g cellulose 0.08 g/g (DS)
	Separate xylose- and glucose- ethanol fermentation	Ethanol (from xylose 9.0 g/l)	3.12 g/l 0.35 g/g xylose 0.23 g/g hemicellulose 0.05 g/g (DS)
		Ethanol (from glucose 6.24 g/l)	2.9 g/l 0.46 g/g glucose 0.13 g/g cellulose 0.05 g/g (DS)
		Total ethanol (from xylose and glucose)	6.02 g 0.16 g/g substrate 0.10 g/g (DS)

Processes	Methods	Products	Yield
Fermentation (5L fermenter)	Separate xylose- and glucose- ethanol fermentation	Ethanol (from xylose 8.67 g/l)	4.08 g/l 0.47 g/g xylose 0.30 g/g hemicellulose 0.07 g/g (DS)
		Ethanol (from glucose 6.24 g/l)	3.05 g/l 0.49 g/g glucose 0.13 g/g cellulose 0.051 g/g (DS)
		Total ethanol (from glucose and xylose)	7.13 g 0.2 g/g substrate 0.12 g/g (DS)

CHAPTER V

DISCUSSIONS AND CONCLUSIONS

Sugarcane leaves which composed of 38.5% (w/w) cellulose, 23% (w/w) hemicellulose and 15.6% (w/w) lignin was used as substrate for ethanol production. Efficiency of dilute sulfuric acid and lime pretreatment method on improving of cellulose susceptibility of the sugarcane leaves was determined. Optimal condition for dilute sulfuric acid pretreatment of sugarcane leaves was 1.5% (w/v) H_2SO_4, 6% (w/v) substrate loading at 121°C, 15 lb/in^2 for 30 min. There was a direct relationship of reducing sugar released after dilute sulfuric acid pretreatment and cellulose susceptibility of sugarcane leaves. [Grohman and Bothast (1996) determined optimal condition of dilute sulfuric acid pretreatment of corn fibre by monitoring reducing sugar released after dilute sulfuric acid pretreatment] and [Gupta et al. (2009) determined optimal condition of dilute sulfuric acid pretreatment of *Propopis juliflora* by monitoring reducing sugar released after dilute sulfuric acid pretreatment]. Optimal condition of lime pretreatment of sugarcane leaves was 0.33 $Ca(OH)_2$/g, dry weight basis of sugarcane leaves, 6% (w/v) substrate loading at 121°C, 15 lb/in^2 for 15 min. There was no relationship between reducing sugar released after lime pretreatment and cellulose susceptibility of the lime pretreated sugarcane leaves. Increase of substrate loading in pretreatment process resulted in an increase of reducing sugar released (g/l), but a decrease of reducing sugar released efficiency of sugarcane leaves (g /g, (DS) sugarcane leaves). High substrate loading blocked heat transfer in the pretreatment reaction (Kuhad et al., 1999; Zheng et al., 2009). Whereas increase of substrate loading in cellulase hydrolysis reaction resulted in an increase of reducing sugar released (g/l), but a decrease of reducing sugar released efficiency of sugarcane leaves (g/g (DS) sugarcane leaves). This was due to a problem of reaction mixing and reduction of enzyme diffusion in the reaction (Kuhad et al., 1999; Zheng et al., 2009). Sugarcane leaves pretreated by dilute sulfuric acid and lime at optimal condition released glucose after hydrolysis by cellulase GC220 (10 U/g (DS): β-glucosidase 3.55 salicin Unit/ml, for 72h) at 0.057 g/g (DS) (0.17 g/g cellulose), 0.039 g/g (DS) (0.11 g/g cellulose), respectively. Moreover xylose 9.0 g/l was detected in the dilute acid pretreated sugarcane leaves slurry while in the lime pretreated sugarcane leaves slurry was not detected. From these results indicated that sugarcane

leaves pretreated by dilute sulfuric acid was more susceptible to cellulase than those pretreated by lime and the xylose released after dilute sulfuric acid pretreatment process could be fermented to ethanol.

Analysis of pretreatment by-products (furfural, hydroxymethylfurfural (HFM), 4-hydroxylbenzaldehyde, syringaldehyde, and vanillin) released after dilute sulfuric acid and lime pretreatment at their optimal condition, concentration of all by-products was vary low and lower than their toxic level for growth and ethanol fermentation of *Saccharomyces cerevisiae* and *Pichia stipitis*. 4-hydroxykbenzadehyde (1.0 mg/m) was a toxic level for growth and ethanol fermentation of *Saccharomyces cerevisiae*. Furfural (1 mg/ml), HFM (3 mg/ml), and syringaldehyde (0.22 mg/ml) was a toxic level for growth and ethanol fermentation of *Pichia stipitis* (Olsson and Hahn-Hagerdal, 1996). Therefore, detoxification of sugarcane leaves pretreatment hydrolysate was not done.

Dilute sulfuric acid pretreated sugarcane leaves was saccharified by Acellulase™ 1000 (265 FPU/ml: endoglucanase 2500 CMCU/g, β-glucosidase 400 pNPGU/g) by varying an enzyme dose (0.2, 0.4, 0.6, 0.8 ml/g (DS)) and reaction time (6, 12, 24, 48 h). Saccharification for 6h, increase of the enzyme dose resulted in an increase of glucose released. The glucose released was stable at 9.8 g/l when enzyme dose (0.6 ml/g (DS)) or more was used due to substrate limitation. Saccharification by the enzyme, 0.6 ml/g (DS), increased of reaction time resulted in an increase of glucose released. Glucose released was stable at 12.0 g/l when reaction time was 18h and longer. The glucose released in the first 6h was 1.6 g/l/h, while the glucose released during 6^{th} to 18^{th} was 0.18 g/l/h. To lower a risk of glucose lost due to contamination, the pretreated sugarcane leaves was hydrolyzed by Acellulase™ 1000 (0.6 ml/g (DS)) for 6h was used in further experiments. At this condition the cellulase hydrolysate contained glucose 9.8 g/l.

Sugarcane leaves was fermented to ethanol by separate hydrolysis and fermentation (SHF) method. Sugarcane leaves slurry obtained after pretreatment by dilute sulfuric acid was further saccharified by cellulase. The resultant hydrolysate containing 9.8 g/l glucose was fermented to ethanol by *Saccharomyces cerevisiae* TISTR 5596 (inoculum age 6h). After 24h, maximum ethanol 4.71 g/l (0.48 g/g glucose) or 0.20 g/g cellulose (0.08 g/g (DS)) was produced by the separate hydrolysis and ethanol fermentation without any nutrients supplementation.

Glucose released in hydrolysate obtained after dilute sulfuric acid pretreatment and cellulase hydrolysis of sugarcane leaves were 0.67 and 9.2 g/l, respectively (total glucose 9.8 g/l). Xylose released in hydrolysate obtained after dilute sulfuric acid pretreatment was 9.0 g/l. Xylose released from the pretreatment process was almost the same as glucose released from the cellulase hydrolysis. For lignocellulosic ethanol production yield maximization, both of xylose and glucose should be fermented to ethanol (Gupta et al., 2009). Therefore, the dilute sulfuric acid pretreatment hydrolysate containing glucose 0.67 g/l and xylose 9.0 g/l was fermented to ethanol by *Pichia stipits*. Ethanol (3.12 g/l or 0.35 g/g xylose) was produced after 96h without any nutrients supplementation.

Pretreated sugarcane leaves residue separated from the pretreatment hydrolysate was washed and resuspended into 0.1M sodium citrate buffer pH5.0 at its original volume then saccharified by cellulase. The resultant hydrolysate was used as substrate for ethanol fermentation by *Saccharomyces cerevisiae* TISTR 5596 (inoculum age 6h). Ethanol (2.9 g/l or 0.46 g/g glucose) was produced after 12h of incubation. Total ethanol 6.02 g or 0.16 g/g substrate (cellulose and hemicellulose) or 0.10 g/g (DS) was produced by separated xylose- and glucose-fermentation after 96 and 12h, respectively in flask scale without any nutrients supplementation.

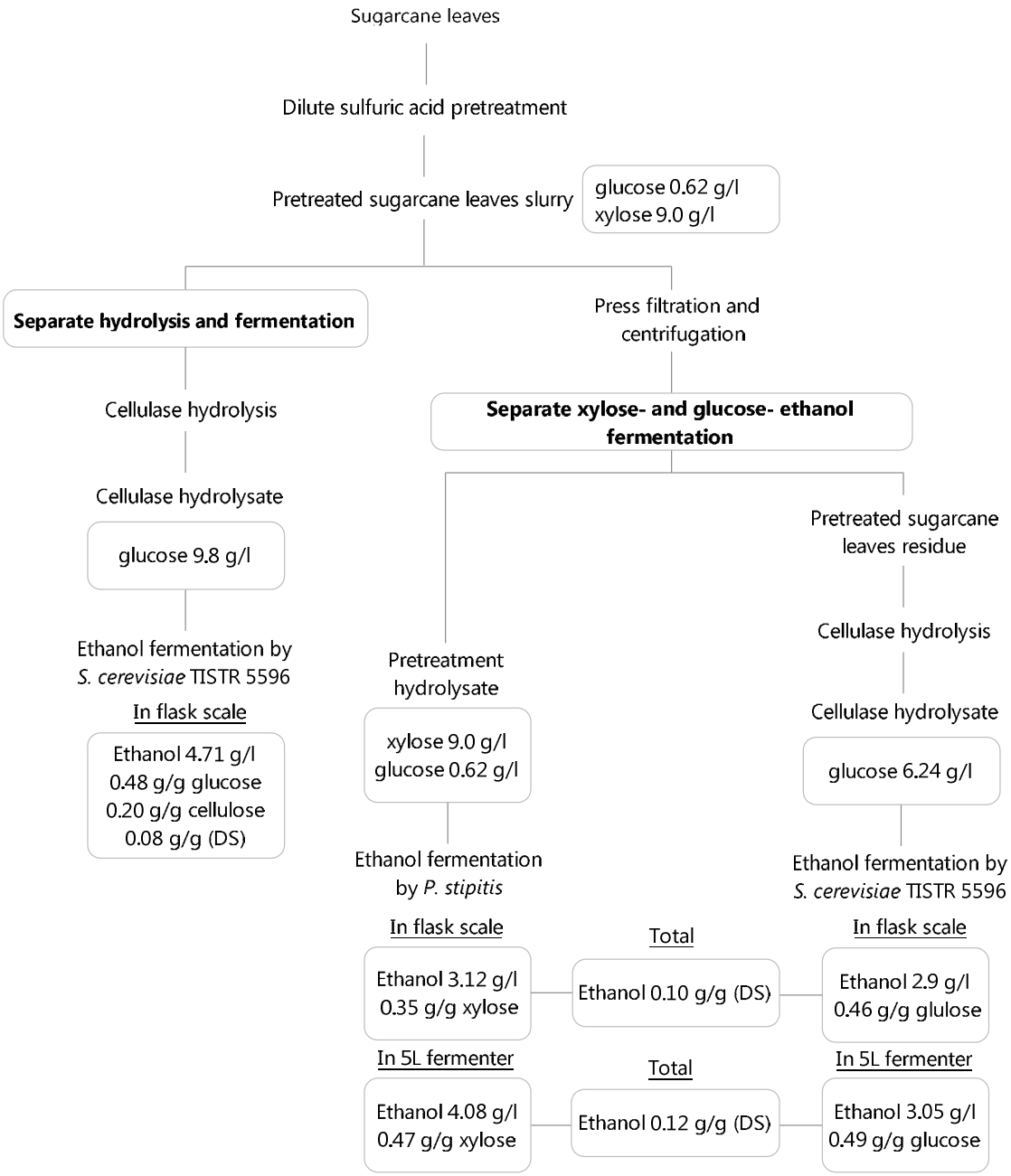
Nutrient supplementation was not required in all above experiments. This might due to nutrients left in an inoculum medium inoculated into the fermentation hydrolysates.

Scaling up of the separate xylose- and glucose- ethanol fermentation to 3L in 5L fermenter, ethanol (4.08 g/l or 0.47 g/g xylose) was produced from xylose fermentation after 96h, when xylose decreased from 8.67 to 3.86 g/l. Optimization of an oxygen level during growth phase and fermentation phase should be performed to improve xylose consumption. Whereas, ethanol production from glucose fermentation gave 3.05 g/l or 0.49 g/g glucose after 12h. Therefore separate xylose- and glucose- ethanol fermentation yield gave total ethanol 7.13 g. This was 0.12 g ethanol/g (DS) sugarcane leaves. Previous reports of ethanol production yield (g ethanol/g xylose) by *Pichia stipits* were 0.39 g/g xylose from D-xylose within 48h (du Preez et al., 1986), 0.37 g/g xylose from 3% SO_2 pretreated aspen wood residue within 32h (Delgenes et al., 1996), 0.36 g/g xylose from 13.7% Na_2SO_3 pretreated *Prosopis juiliflora* residue, hard wood, within 32h (Kapoor et al., 2008), and 0.39 g/g xylose from 3% sulfuric acid pretreated *Prosopis juiliflora* residue within 24h (Gupta et al., 2009). Previous reports of ethanol yield from glucose

fermentation by *Saccharomyces cerevisiae* was 0.49 g/g glucose from 3% sulfuric acid pretreated *Prosopis juiliflora* within 16h (Gupta et al., 2009).

Krishna et al. (2000) fermented sugarcane leaves to ethanol by simultaneous saccharification and fermentation (SSF) method. They got 22 g/l or 0.22 g ethanol/g (DS) sugarcane leaves after 96h. Dawson and Boopathy (2006) fermented sugarcane leaves to ethanol by separate hydrolysis and ethanol fermentation (SHF) method for 12 days. Ethanol yield (0.34 g/l or 0.01 g ethanol/g (DS) sugarcane leaves was reported. In this study, we reported ethanol yield of 4.7 g/l (0.48 g/g glucose or 0.20 g/g cellulose or 0.08 g/g (DS) sugarcane leaves) by the SHF method after 24h and 7.13 g ethanol (0.20 g/g substrate (cellulose and hemicellulose) or 0.12 g/g (DS) sugarcane leaves) by fermenting of both xylose and glucose to ethanol.

These results indicated that sugarcane leaves pretreated by dilute sulfuric acid was more susceptible to cellulase than those pretreated by lime. Both of xylose released after dilute sulfuric acid pretreatment and glucose that released after cellulase hydrolysis should be fermented to ethanol through the separate xylose- and glucose- ethanol fermentation process. Sugarcane leaves was a promising abundant and low cost substrate for ethanol production.


Sugarcane leaves
Dilute sulfuric acid pretreatment
Pretreated sugarcane leaves slurry
glucose 0.62 g/l
xylose 9.0 g/l
Separate hydrolysis and fermentation
Press filtration and centrifugation
Cellulase hydrolysis
Separate xylose- and glucose- ethanol fermentation
Cellulase hydrolysate
glucose 9.8 g/l
Ethanol fermentation by S. cerevisiae TISTR 5596
In flask scale
Ethanol 4.71 g/l
0.48 g/g glucose
0.20 g/g cellulose
0.08 g/g (DS)
Pretreatment hydrolysate
xylose 9.0 g/l
glucose 0.62 g/l
Pretreated sugarcane leaves residue
Cellulase hydrolysis
Cellulase hydrolysate
glucose 6.24 g/l
Ethanol fermentation by P. stipitis
Ethanol fermentation by S. cerevisiae TISTR 5596
In flask scale
Ethanol 3.12 g/l
0.35 g/g xylose
Total
Ethanol 0.10 g/g (DS)
In flask scale
Ethanol 2.9 g/l
0.46 g/g glulose
In 5L fermenter
Ethanol 4.08 g/l
0.47 g/g xylose
Total
Ethanol 0.12 g/g (DS)
In 5L fermenter
Ethanol 3.05 g/l
0.49 g/g glucose

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APPENDICES

APPENDIX A

Culture media

1. Yeast Peptone Dextrose (YPD) Agar

Yeast extract	10	g
Bacto peptone	20	g
Glucose	20	g
Agar	18	g
Distilled water	1000	ml

Adjusted pH 4.5

Sterile by autoclaving at 121ºc, 15 lb/in ^{2} for 15 min.

2. Yeast and Malt extract (YM) Agar

Yeast extract	3	g
Bacto peptone	5	g
Glucose	10	g
Malt extract	3	g
Agar	22	g
Distilled water	1000	ml

Adjusted pH 5.0

Sterile by autoclaving at 121ºc, 15 lb/in ^{2} for 15 min.

APPENDIX B

Reagents and Buffers

1. Determination of reducing sugar

The reducing sugar was measured by the method of Somogyi (1952) using glucose as authentic sugar.

1.1 Somogyi-Nelson Reagent

A. Alkaline Copper Reagent:

- Potassium sodium tartate (Rochelle salts) 40 g in 300 ml distilled water
- Disodium hydrogen phosphate dodecahydrate 71 g in 300 ml distilled water
- 10% Copper (II) sulfate 80 ml

  (8 g Copper (II) sulfate in 80 ml distilled water)
- 1N Sodium hydroxide 100 ml

  (4 g Sodium hydroxide in 100 ml distilled water)
- Sodium sulfate 180 g

Dissolve the solutions above and make up volume to 1000 ml.

B. Nelson Reagent

- Ammonium molybdate 53.2 g in 500 ml distilled water
- Sulfuric acid (conc.) 21 ml

  Sulfuric acid (conc.) is added into the ammonium molybdate.
- Sodium arsenate 6 g in 50 ml distilled water

Dissolve the solutions above and make up volume to 1000 ml.

1.2 Procedure

- Put proper dilution of sample (1 ml) in a test tube
- Add Alkaline Copper solution (1 ml) and place in boiling water for 15 minutes. Immediately cool in ice water.
- After addition of 1 ml Nelson solution, incubate at room temperature for 30 minutes and dilute by adding 5 ml of distilled water.

- Absorbance of samples was measured at 520 nm. Concentrations of the samples were compared to the standard curve for determination of values. Distilled water was used instead of sample as a blank.

1.3 Preparation of standard curve of glucose

Glucose standard solutions (1 mg/ml) are prepared in distilled water. Standards of 0, 20, 40, 60, 80, 100, 120, 150, 180 and 200 µg/ml were prepared from glucose solution. The reactions were carried out with the same procedure as described previously. Absorbances were plotted against concentrations of standards.

1.4 Calculation of reducing sugar

Formula Reducing sugar (g/l) = $\frac{A_{520} \text{ x dilution}}{\text{Slope}}$

2. 0.1 M Sodium Citrate buffer pH 5.0

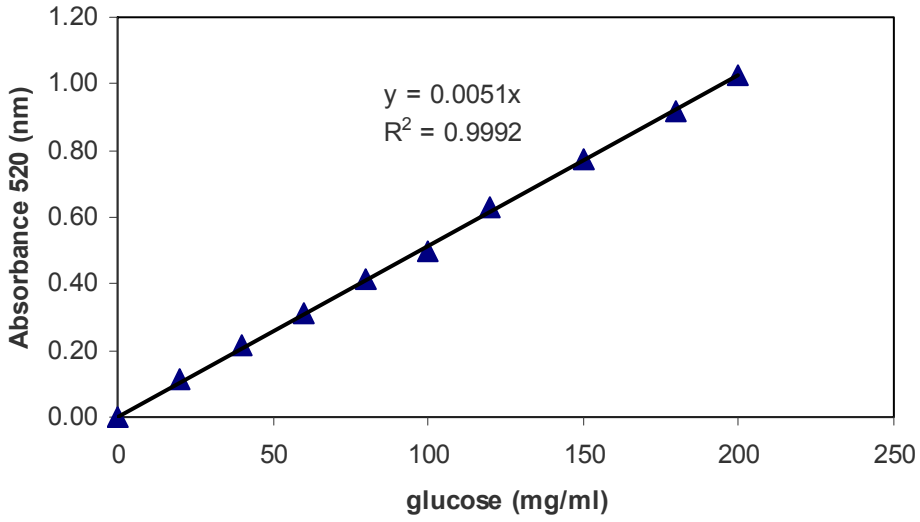
Citric acid monohydrate (0.1 M)	21.01	g/l
Trisodium citrate dehydrate (0.1 M)	29.41	g/l

Mix 0.1 M citric acid (35 ml) with 0.1 M trisodium citrate (65 ml). Adjust pH to 5.0.

APPENDIX C

Standard curve of glucose

1. Standard curve of glucose



BIOGRAPHY

Miss Rumpa Jutakanoke was born in January 30, 1985 in Bangkok, Thailand. She graduated from Department of Microbiology, Faculty of Science, Chulalongkorn University, Thailand with Bachelor Degree of Science since 2007.

Academic presentation :

1. Rumpa Jutakanoke, Teerapat Srinarakutara, Vasana Tolieng, Vichien Kitprechavanich Natchanun Leepipatpiboon, and Ancharida Akaracharanya. 2007. Comparison of dilute acid and lime pretreatments on susceptibility of Sugarcane leaves to cellulase [Proceeding]. FerVAAP 2009: The 3rd International Conference on fermentation Technology for Value Added Agricultural Products. August 26-28, 2009 at Kosa Hotel, Khon Kaen.