การหาภาวะที่เหมาะสมของการสลายทางเคมีของชานข้าวฟ่างหวานและชานอ้อยสำหรับการหมักเอทานอล

นายอภิสิทธิ์ พูนศรีสวัสดิ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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OPTIMIZATION OF CHEMICAL SACCHARIFICATION OF SWEET SORGHUM STRAW AND BAGASSE FOR ETHANOL FERMENTATION

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A Thesis Submitted in Partial Fulfillment of the Requirements
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Thesis Title	OPTIMIZATION OF CHEMICAL SACCHARIFICATION OF SWEET SORGHUM STRAW AND BAGASSE FOR ETHANOL FERMENTATION
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Field of Study	Biotechnology
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อภิสิทธิ์ พูนศรีสวัสดิ์ : การหาภาวะที่เหมาะสมของการสลายทางเคมีของชานข้าวฟ่างหวานและชานอ้อยสำหรับการหมักเอทานอล (OPTIMIZATION OF CHEMICAL SACCHARIFICATION OF SWEET SORGHUM STRAW AND BAGASSE FOR ETHANOL FERMENTATION) อ.ที่ปรึกษาวิทยานิพนธ์หลัก : อ.ดร.ศิริลักษณ์ ธีระดากร, 83 หน้า.

ชานข้าวฟ่างหวานและชานอ้อยเป็นวัสดุประเภทลิกโนเซลลูโลสที่ถูกนำมาใช้เป็นวัตถุดิบทางเลือกในการผลิตเอทานอล เนื่องจากสามารถหาได้ง่ายและมีราคาถูก โดยมีเซลลูโลสและเฮมิเซลลูโลสเป็นองค์ประกอบ ซึ่งสามารถถูกสลายให้เป็นน้ำตาลเพื่อนำไปใช้ในการหมักได้ โดยต้องมีการปรับสภาพเพื่อปรับเปลี่ยนขนาดและโครงสร้างของวัตถุดิบ งานวิจัยนี้ได้ทำการหาภาวะที่เหมาะสมในการสลายทางเคมีของชานข้าวฟ่างหวานและชานอ้อย โดยทำการผสมวัตถุดิบกับสารละลายกรดซัลฟูริกเจือจาง (0-3%โดยปริมาตร) หรือสารละลายโซเดียมไฮดรอกไซด์เจือจาง (0-4%โดยน้ำหนักต่อปริมาตร) ในอัตราส่วน 10%โดยน้ำหนักต่อปริมาตร แล้วนำไปปรับสภาพที่อุณหภูมิสูง (120 -190 องศาเซลเซียส) โดยใช้ระยะเวลาปรับสภาพนาน 10-30 นาที จากการทดลองพบว่า การปรับสภาพชานข้าวฟ่าวหวานที่อุณหภูมิ 120 องศาเซลเซียส สารละลายกรดซัลฟูริกที่ความเข้มข้น 3%โดยปริมาตร ใช้เวลาปรับสภาพนาน 10 นาที จะให้ปริมาณน้ำตาลกลูโคสและไซโลสสูงสุดเท่ากับ 0.234 กรัมกลูโคสต่อกรัมสับสเตรท และ 0.208 กรัมไซโลสต่อกรัมสับสเตรท ตามลำดับ ซึ่งคิดเป็นเปอร์เซ็นต์การเปลี่ยนเป็นน้ำตาลกลูโคสและไซโลสเท่ากับ 50.04% กรัมกลูโคสต่อกรัมกลูแคน และ 76.41% กรัมไซโลสต่อกรัมไซแลน ตามลำดับ ในกรณีของการปรับสภาพชานอ้อย พบว่า การปรับสภาพที่อุณหภูมิ 170 องศาเซลเซียส ด้วยสารละลายกรดซัลฟูริกที่ความเข้มข้น 3%โดยปริมาตร ใช้เวลาปรับสภาพนาน 20 นาที จะให้ปริมาณน้ำตาลกลูโคสสูงสุดเท่ากับ 0.367 กรัมกลูโคสต่อกรัมสับสเตรท ซึ่งคิดเป็นเปอร์เซ็นต์การเปลี่ยนเป็นน้ำตาลกลูโคสเท่ากับ 78.52% กรัมกลูโคสต่อกรัมกลูแคน และการปรับสภาพที่อุณหภูมิ 120 องศาเซลเซียส ด้วยสารละลายกรดซัลฟูริกที่ความเข้มข้น 3%โดยปริมาตร ใช้เวลาปรับสภาพนาน 20 นาที จะให้ปริมาณน้ำตาลไซโลสสูงสุดเท่ากับ 0.226 กรัมไซโลสต่อกรัมสับสเตรท ซึ่งคิดเป็นเปอร์เซ็นต์การเปลี่ยนเป็นน้ำตาลไซโลสเท่ากับ 83.05% กรัมไซโลสต่อกรัมไซแลน

หลังจากการปรับสภาพทางเคมีแล้ว นำสารละลายน้ำตาลที่อยู่ในสภาพเป็นกรดมาลดความเป็นพิษด้วยวิธีการ overliming และระเหยให้เข้มข้น ตามลำดับ โดยสารละลายน้ำตาลที่มีปริมาณของน้ำตาลทั้งหมดประมาณ 20 และ 50 กรัมต่อลิตร จะถูกนำไปหมักด้วยกระบวนการหมักแบบแยกส่วน (SHF) ด้วยเชื้อยีสต์ *Saccharomyces cerevisiae* และ *Pichia stipitis* ที่อุณหภูมิ 30 องศาเซลเซียส ค่าความเป็นกรด-ด่าง 5.5 และสภาวะการเขย่า 150 รอบต่อนาที พบว่า เมื่อทำการหมักสารละลายจากชานข้าวฟ่างหวานที่มีน้ำตาลทั้งหมดอยู่ 20 กรัมต่อลิตร ด้วยเชื้อยีสต์ *P. stipitis* เป็นเวลา 46 ชั่วโมง จะได้ปริมาณเอทานอล 10.17 กรัมต่อลิตร ขณะที่เชื้อ *S. cerevisiae* ให้ปริมาณเอทานอล 6.38 กรัมต่อลิตร ที่เวลา 12 ชั่วโมง และเมื่อหมักสารละลายน้ำตาลจากชานอ้อยด้วยเชื้อยีสต์ *P. stipitis* และ *S. cerevisiae* จะได้ปริมาณเอทานอลค่อนข้างต่ำ คือ 3.73 g/l ที่ 32 ชั่วโมง และ 1.78 g/l ที่ 9 ชั่วโมง ตามลำดับ

สาขาวิชา…….เทคโนโลยีชีวภาพ…..... ลายมือชื่อนิสิต.....

ปีการศึกษา.....2553..... ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

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APHISIT POONSRISAWAT : OPTIMIZATION OF CHEMICAL SACCHARIFICATION OF SWEET SORGHUM STRAW AND BAGASSE FOR ETHANOL FERMENTATION. THESIS ADVISOR: SIRILUK TEERADAKORN, Ph.D., 83 pp.

Sweet sorghum straw and sugarcane bagasse are lignocellulosic materials that are promoted as an alternative feedstock for ethanol production because it is available and inexpensive. Due to its composition of cellulose and hemicellulose, that could be hydrolyzed into fermentable sugars. Conversion of these potential feedstocks requires a pretreatment step to alter the microscopic size and structure of the lignocellulose. This research was studied in order to find the optimum conditions on hydrolysis of sweet sorghum straw and sugarcane bagasse using dilute-acid or dilute-alkaline pretreatment. The biomass was mixed with dilute sulfuric acid (0-3%v/v) or dilute sodium hydroxide (0-4%w/v) with solid loading of 10% w/v and then pretreatment at hight temperatures (120-190°C) for 10-30 min of pretreated times. The maximum yield of glucose and xylose from sorghum straw was 0.234 g glucose/g dry substrate and 0.208 g xylose/g dry substrate, respectively, at the pretreatment condition : 120ºC, 3%H_2SO_4 for 10 min. In this case, a total of 50.04% of glucan and 76.41% of xylan were converted to glucose and xylose, respectively. In the case of bagasse, pretreatment at 170°C, 3%H_2SO_4 for 20 min gave the maximum yield of glucose of 0.367 g glucose/g dry substrate and a total of 78.52% of glucan was converted to glucose. Pretreatment at 120°C, 3%H_2SO_4 for 20 min, gave the maximum yield of xylose of 0.226 g xylose/g dry substrate and a total of 83.05% of xylan was converted to xylose.

After chemical pretreatment, the hydrolyzates of sorghum straw and bagasse were detoxified and concentrated by overliming and evaporation method, respectively. The hydrolyzates containing 20 g/l and 50 g/l of reducing sugars were fermented with separate hydrolysis and fermentation (SHF) process using *Saccharomyces cerevisiae* and *Pichia stipitis* at 30ºC, pH 5.5 and agitation rate of 150 rpm. The fermentation of sorghum straw hydrolyzate, containing 20 g/l of reducing sugars by *P. stipitis* gave the highest ethanol concentration of 10.17 g/l at 46 hr. Whereas *S. cerevisiae* gave the ethanol concentration of 6.38 g/l at 12 hr. In case of fermentation bagasse hydrolyzate by *P. stipitis* and *S. cerevisiae* gave ethanol concentration of 3.73 g/l at 32 hr and 1.78 g/l at 9 hr, respectively.

Field of Study : ……Biotechnology……Student's Signature……..……...
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CONTENTS

Page

Page

LIST OF TABLES

Table Page

LIST OF FIGURES

Figure Page

LIST OF ABBREVIATIONS

µm	Micrometre(s)
µl	Microlitre(s)
%	Percent
°C	Degree Celsius
GC	Gas chromatography
HPLC	High-performance liquid chromatography
l	Litre(s)
m	Metre(s)
g	Gram(s)
mg	Milligram(s)
min	Minute(s)
ml	Millilitre(s)
mm	Millimetre(s)
sp	Species
v/v	Volume by volume
w/v	Weight by volume
g/l	Gram per Litre
g/l/hr	Gram per Litre per Hours
mg/ml	Milligram per milliliter
SPSS	Statistical package for the social science
OD	Optical Density
rpm	Round per minute
hr	Hours
EtOH	Ethanol

CHAPTER I

INTRODUCTION

During the past decades, energy consumption has increased steadily because the world population has grown and more countries have become industrialized. These problems have led to an interest in development of alternative energy. Bioethanol or ethanol, a renewable fuel is becoming increasingly important as a consequence of major concern for depleting oil reserves, rising crude oil prices and greenhouse effect (Sun and Cheng, 2002 and Silverstein et al., 2007). Ethanol is a cleaner energy than fossil fuel that can be produce from several different lignocellulosic materials including wood, agricultural, or forest residues. The ethanol can ease both natural resource limitations and environmental pollution because burning fuel do not release green house gas to the atmosphere (Kumar et al., 2009). Lignocellulose is the most abundant global source of renewable biomass, because of its does not compete with food crops, availability in large quantities and lower cost than agricultural feedstocks. (Sanchez and Cardona, 2008 ; and Alvira et al.,2010). For the conversion of lignocellulose to ethanol, the cellulose and hemicellulose that contain in materials must be broken down into fermentable sugars and subsequently fermented by microorganism e.g., *Saccharomyces cerevisiae, Pichia stipitis* to produce ethanol (Palmqvist and Hahn-Hägerdal, 2000 ; and Kumar et al., 2009).

Sweet sorghum is a sub-group of sorghum [*Sorghum bicolor* (L.) Moench] and is a tropical grass grown primarily in semiarid and drier parts of the world. It is a high photosynthetic efficiency, high biomass yield crop compared with other species and high dry matter accumulation rates (Billa et al., 1997). The harvest season for sweet sorghum is annually limited from September to December. Sweet sorghum is a potential feedstocks for production of bioethanol because of the straw or bagasse of sweet sorghum is an abundant and low-cost lignocellulosic material that can be synthetically used as a raw material for ethanol production and some byproduct with high additional value (Shen and Liu, 2009 ; and Vazquez et al., 2007). Sweet sorghum has a high biomass yield and is rich for carbohydrates.

Sugarcane (*Saccharum officinarum* L.) is a tropical grass that can be harvest from June to September. Sugarcane bagasse is a byproduct that obtained after crushing of the sugarcane. The fibrous residue of sugarcane is useful to provide the main source of fermentable sugars witch can be produce ethanol by fermentation (Harnandez-Salas et al., 2009 ; and Laopaiboon et al., 2010).

Success of using renewable biomass for ethanol production depends upon the physical and chemical properties of the biomass structure, pretreatment method, efficient microorganisms and optimization of the processing conditions. The purpose of the pretreatment is to break the lignin seal, cleave the linkages of hemicellulose and cellulose chains and disrupt the crystalline structure of the cellulose. Pretreatment method such as steam explosion, dilute-acid pretreatment, concentrated-acid pretreatment, alkaline pretreatment, ammonia fiber explosion and organic solvent pretreatment have been studied (Sun and Cheng, 2002 ; and Mosier et al., 2005). Among these methods, treating lignocellulosic materials with dilute-acid pretreatment has been used in a wide range of feedstocks ranging from hardwoods to grasses and agricultural residues. This method has been successfully developed for pretreatment of lignocellulosic materials (Kumar et al., 2009). The reaction conditions of dilute-acid pretreatment is carried out using mineral acids such as H_2SO_4 or HCl, at temperatures between 120°C to 200°C and reaction time between 10 minute to 30 minute (Torget and Hsu, 1994 ; Nguyen et al., 1998 ; and Sanchez et al., 2004). In the dilute-acid pretreatment process, the several toxic compounds (such as furfural, HMF and phenolic compound) are generated. These compounds are known to affect ethanol fermentation performance (C.A.Cardona et al., 2010). Therefore, before fermentation of hydrolyzates by separate hydrolysis and fermentation (SHF) should be removed or reduce these toxic by detoxification for improving the efficiency of the fermentation processes.

The goal of this research was to study the potential and performance of biomass products (sweet sorghum straw and sugarcane bagasse) as feedstocks for ethanol production. Pretreatment technology using dilute acid and alkaline to increase fermentable sugars recovery from sorghum and bagasse were investigated.

Results from this research will improve the utilization of sorghum as feedstock for biofuel production.

The objectives of this research

To investigate the optimum conditions for converting lignocellulosic materials (sweet sorghum straw and sugarcane bagasse) into fermentable sugars by using chemical (dilute-acid and dilute-alkaline) pretreatment.

CHAPTER II

THEORETICAL AND LITERATURE REVIEW

2.1 Ethanol

Ethanol (ethyl alcohol or EtOH) is a clear, colorless liquid with a characteristic, agreeable odor and its molecular formula is C_2H_5OH . Ethanol has been produced both as a petrochemical, through the hydration of ethylene (shown in equation 2.1), and biologically, by fermenting sugars with yeast (e.g., *Saccharomyces cerevisiae*) (shown in equation 2.2). Ethanol has widespread use as a solvent of substances intended for human contact or consumption, including scents, flavorings, colorings, and medicines. In chemistry, it is both an essential solvent and a feedstock for the synthesis of other products. It has a long history as a fuel for heat and light, and more recently as a fuel for internal combustion engines. Which process is more economical is dependent upon the prevailing prices of petroleum and of grain feed stocks. Ethanol is used as an automotive fuel by itself and can be mixed with gasoline to form gasohol, therefore it can reduce the world's dependence on crude oil resources (Gray et al., 2006).

$$C_2H_{4(g)} + H_2O_{(g)} \xrightarrow{H_3PO_4,\ T = 300°C} CH_3CH_2OH_{(l)} \quad \text{..... (2.1)}$$

$$C_6H_{12}O_6 \xrightarrow{\text{Yeast}} 2\ CH_3CH_2OH + 2\ CO_2 \quad \text{..... (2.2)}$$

Ethanol can be produced from lignocellulosic materials, that is the most promising feedstock (Balat et al., 2008). In Figure 2.1 describes the general process for converting the carbohydrates in lignocellulose into ethanol (Keshwani et al., 2009). Pretreatment is required to improve accessibility of enzymes to cellulose and hemicellulose fractions. Following pretreatment, cellulose and hemicellulose fractions can be hydrolyzed into fermentable sugars while lignin can be recovered and used as a fuel to meet some of the energy requirements in a bioethanol production system (Wyman et al., 1994). After hydrolysis, the fermentable sugars are fermented into

ethanol, which is then distilled for fuel purposes. Currently, there are technological and economic limitations to ethanol production from lignocelluloses in each step in the conversion process.

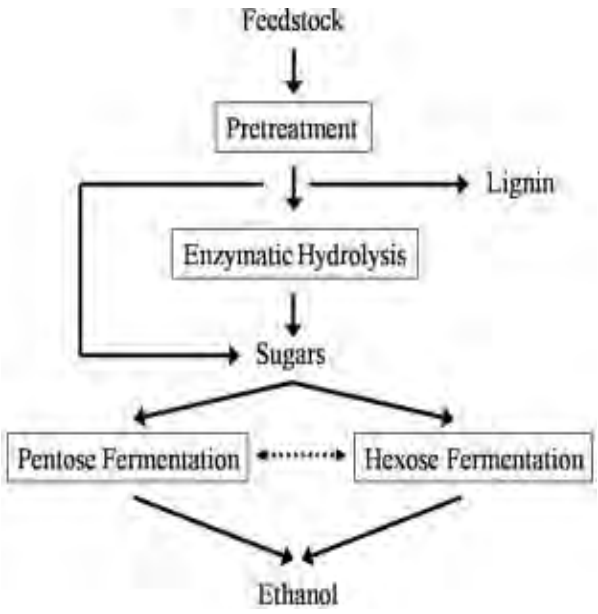

Figure 2.1 The general process to produce ethanol from lignocellulose.
(Source : Keshwani et al., 2009)

2.2 Feedstocks for bioethanol production

Bioethanol or ethanol originated from plant oils, sugar beets, cereals, organic waste and lignocellulosic biomass. The biological feedstocks are contained appreciable amounts of sugars or materials that can be converted into sugar (such as starch or cellulose and hemicelluloses) and subsequently fermented to produce bioethanol. Bioethanol feedstocks can be classified into 3 types : (i) sucrose-containing feedstocks (e.g. sugar beet, sweet sorghum and sugar cane), (ii) starchy materials (e.g. wheat, corn, cassava and barley) and (iii) lignocellulosic biomass (e.g. wood, straw, bagasse and grasses). Different feedstocks that can be utilized for bioethanol production and their comparative production potential are given in Table 2.1 (Linoj et al., 2006).

Table 2.1 Different feedstocks for bioethanol production and their comparative production potential

Feedstocks	Bioethanol production potential (l/ton)
Sugar cane	70
Sugar beet	110
Sweet potato	125
Potato	110
Cassava	180
Maize	360
Rice	430
Barley	250
Wheat	340
Sweet sorghum	60
Bagasse and other cellulose biomass	280

(Source : Linoj et al., 2006)

2.3 The composition of lignocellulosic materials

Lignocellulosic materials include wood, grass, forestry waste, agricultural residues(e.g., wheat straw, corn stover, sweet sorghum straw and sugarcane bagasse) and municipal solid waste are composed of three major different types of polymers (Figure 2.2) namely cellulose, hemicellulose and lignin. These components along with smaller amounts of pectin, protein, extractives (e.g., chlorophyll and waxes) and ash. Cellulose and hemicellulose are carbohydrates constructed from different sugars while lignin is an aromatic polymer synthesized from phenylpropanoid precusors (Sánchez, 2009 ; and Palmqvist and Hahn-Hägerdal, 2000). The component of these materials can be vary from one species to another species (Table 2.2) (Kumar et al., 2009 ; and Sánchez, 2009).

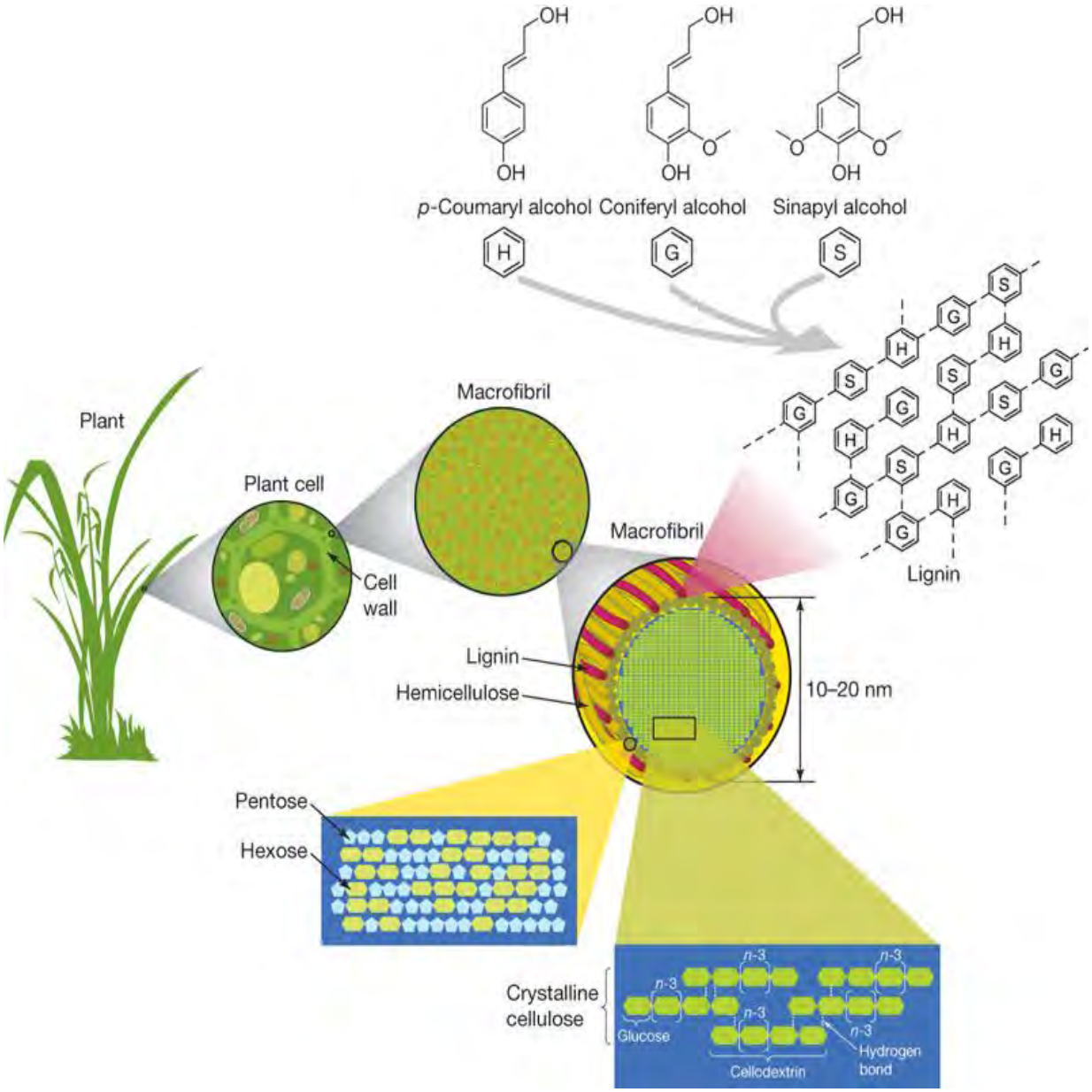

Figure 2.2 Composition of lignocellulosic materials.

(Source : http://www.nature.com/nature/journal/v454/n7206/fig_tab/nature07190_F2.html)

Table 2.2 The composition of lignocellulosic materials

Lignocellulosic material	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Hardwood stems	40-55	24-40	18-25
Softwood stems	45-50	25-35	25-35
Nut shells	25-30	25-30	30-40
Corn cobs	45	35	15
Grasses	25-40	35-50	10-30
Wheat straw	29-35	26-32	16-21
Sorted refuse	60	20	20
Leaves	15-20	80-85	0
Cotton seed hairs	80-95	5-20	0
Coastal bermudagrass	25	35.7	6.4
Switchgrass	45	31.4	12
Rice straw	32.1	24	18
Sugar cane bagasse	32–44	27–32	19–24
Barley straw	31–34	24–29	14–15
Oat straw	31–37	27–38	16–19
Rye straw	33–35	27–30	16–19
Bamboo	26–43	15–26	21–31

(Source : Kumar et al., 2009 ; and Sánchez, 2009)

2.3.1 Cellulose

Cellulose is the most abundant component not only of cell walls but also of the plant as a whole. The structure of cellulose was shown in Figure 2.3. It is a linear polymer that compose of D-glucose subunits linked by β-1,4 glycosidic bonds forming the dimmer cellobiose. These form long chains (or elemental fibrils) linked together by hydrogen bonds and van der Waals forces (Sánchez, 2009). This is cause the cellulose to be formed crystalline structures and make them particularly difficult to digest. Starch and cellulose are both long chains of glucose but starch (linked by α-1,4 and α-1,6 bonds) is easily digested by monogastrics, like humans, while the linkages between glucose molecules in cellulose are most commonly broken by enzymes produced by microbial inhabiting the guts of ruminants, such as cattle, sheep and termites Cellulose in biomass is present in both crystalline and amorphous forms (Kumar et al., 2009).

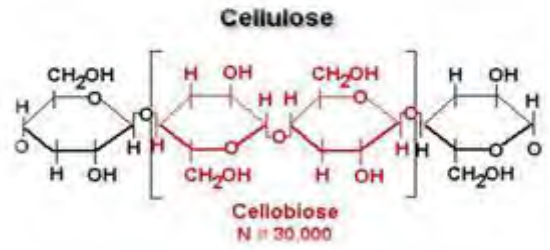

Figure 2.3 Illustration of a cellulose unit
(Source : http://forestproducts.orst.edu/research.php)

2.3.2 Hemicellulose

Hemicellulose is a polysaccharide with a lower molecular weight than cellulose (Sánchez, 2009). The structure of hemicelluloses was shown in Figure 2.4. It is highly branched because of the bonds that form among the sugars that make them up, and they form a network that coats the much larger cellulose microfibrils (structure and function of plants). It consists of different sugars such as pentoses (xylose and arabinose), hexoses (glucose, galactose and mannose) and sugar acids (D-glucuronic and D-galacturonic acids) (Hendriks and Zeeman, 2009). The backbone of hemicellulose is linked together by β-1,4 glycosidic bonds and sometimes linked by β-1,3 glycosidic bonds (Sánchez, 2009). Hemicellulose is randomly acetylated, with reduces its enzymatic reactivity. The polymer present in hemicelluloses are easily hydrolysable (Kumar et al., 2009).

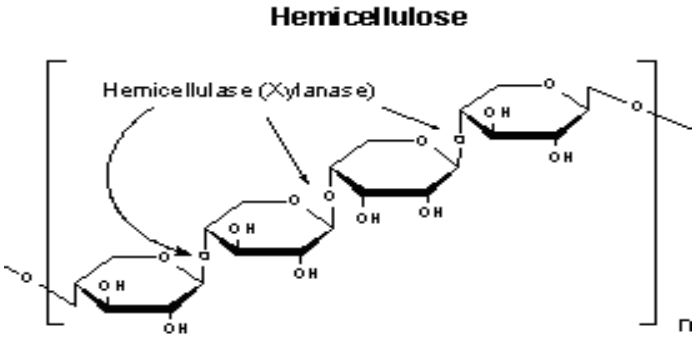

Figure 2.4 The structure of hemicellulose
(Source : http://blogs.princeton.edu/chm333/f2006...try.html)

2.3.3 Lignin

Lignin is a complex, large molecular structure containing cross-linked polymers of phenolic monomers (Kumar et al., 2009). The structure of lignin was shown in Figure 2.5A. It is linked to both hemicellulose and cellulose, forming a physical seal that is an impenetrable barrier in the plant cell wall (Sánchez, 2009). The main function of lignin is to give the plant structural support, impermeability and resistance against microbial attack and oxidative stress (Hendriks and Zeeman, 2009). Lignin is an amorphous heteropolymer, non-water soluble and optically inactive. This polymer is synthesized by the generation of free radicals, which are released in the peroxidase-mediated dehydrogenation of three phenyl propionic alcohols: coniferyl alcohol (guaiacyl propanol), coumaryl alcohol (p-hydroxyphenyl propanol), and sinapyl alcohol (syringyl propanol) (Figure 2.5B) (Sánchez, 2009). These phenolic monomers is linked by alkyl-aryl, alkyl-alkyl, and aryl-aryl ether bonds (Kumar et al., 2009).

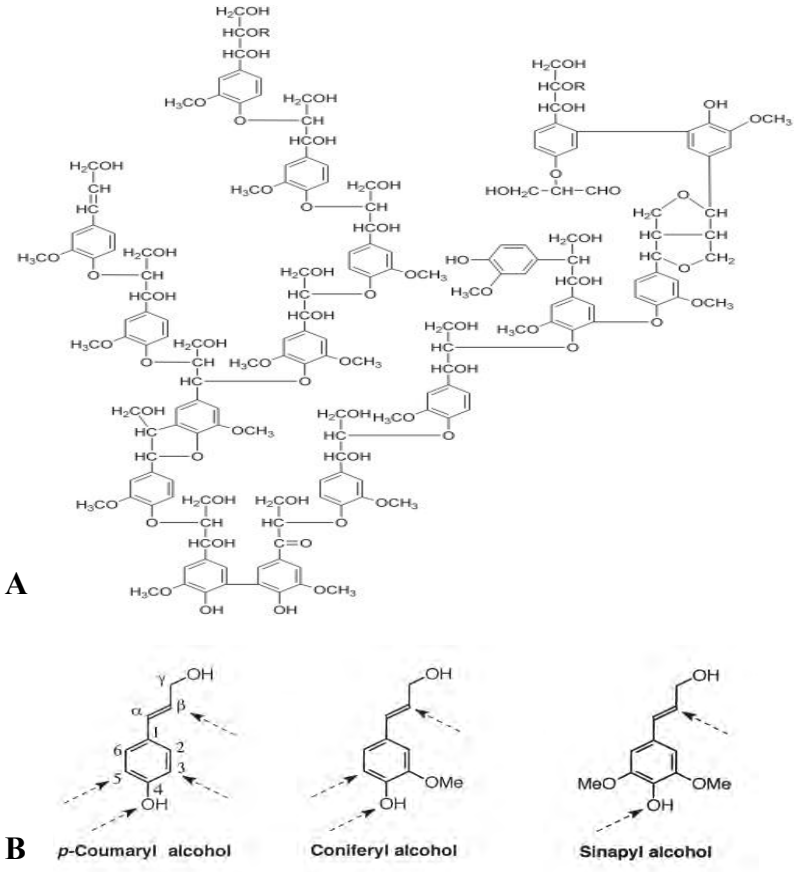

Figure 2.5 The structure of lignin ; (A) Phenolic polymer of lignin, (B) The structure of three phenyl propionic alcohols (Source : www.ibwf.de/env%26enz_index.htm)

2.4 Factors limiting the hydrolysis

The hydrolysis of lignocellulose to monomeric sugars is limited by several factors (Hendriks and Zeeman, 2009). The choice of pretreatment technology for a particular raw material depends on several factors, some of them directly related to the enzymatic hydrolysis step such as sugar-release patterns and enzymes employed. Thus, the combination of the composition of the substrate, type of pretreatment, and dosage and efficiency of the enzymes used for the hydrolysis have a great influence on biomass digestibility (Alvira et al., 2010). These factors are described separately although their effect is normally interrelate.

2.4.1 Lignin content

Lignin is the main components in lignocellulose. It limits the rate of enzymatic hydrolysis because of its close association with cellulose microfibrils and prevents enzyme access to the carbohydrate fraction of materials. To enhance digestibility, materials must undergo pretreatment to remove or alter the lignin (Chang and Holtzapple, 2000).

2.4.2 Hemicellulose content

Hemicellulose and lignin are linked by covalent bonds. Acid hydrolysis of hemicellulose can open materials structure as well. Removal of hemicellulose is required to increases pore size of materials and therefore increases cellulose digestibility (Mosier et al., 2005).

2.4.3 Acetyl content

Degree of acetylation in the hemicellulose is another important factor because lignin and acetyl groups are attached to the hemicellulose matrix and may hinder polysaccharide breakdown (Chang and Holtzapple, 2000).

2.4.4 Cellulose crystallinity

Cellulose crystallinity has been considered as important factors in determining the hydrolysis rates. Several studies have shown that crystallinity prevents the rapid access of enzymes. The lignocellulose was mechanically pretreated, thus any decrease in crystallinity was accompanied by an alteration of other substrate characteristics such as particle size reduction or increase in available surface area (Alvira et al., 2010).

2.4.5 Degree of polymerization

Degree of polymerization is essentially related to other substrate characteristics, such as cristallinity. Depolymerization depends on the nature of cellulosic substrate. In the enzymatic hydrolysis, endoglucanases cut at internal sites of the cellulose chains, preferentially less ordered, being primarily responsible for decreasing degree of polymerization of cellulosic substrates (Alvira et al., 2010).

2.4.6 Surface area and porosity (pore size)

Surface area and porosity of the materials are an important factors influencing hydrolysis process. Therefore, the main objectives of the pretreatment is to increase the available surface area and porosity for improve the hydrolysis (Alvira et al., 2010).

2.5 Pretreatment of lignocellulosic materials

2.5.1 Goals of pretreatment

Lignocellulosic materials do not contain monosaccharides readily available for bioconversion. Instead of polysaccharides, they contain cellulose and hemicelluloses, which have to be hydrolyzed, by means of acids or enzymes, to fermentable sugars. Cellulose in lignocellulosic materials are closely associated with hemicelluloses and lignin. The lignin is partly covalently associated with hemicelluloses, thus preventing the access of hydrolytic agents to cellulose. In addition, the crystalline structure of cellulose itself represents an extra obstacle to hydrolysis (Cardona et al., 2010). An effective pretreatment must preserve the utility of the hemicelluloses and avoid the formation of inhibitors (Laser et al., 2002). An economical for pretreatment should use inexpensive chemicals and require simple process and equipment (Martín et al., 2007).

The goal of pretreatment process is to alter the physical features and chemical composition of the lignocellulose to improve it more digestible (Mosier et al., 2005 ; and Sun and Cheng, 2002). Specifically, pretreatment improves enzyme access and effectiveness (Figure 2.6) by : 1) Removing or altering lignin, 2) Hydrolyzing hemicelluloses, 3) Decrystallizing cellulose, 4) Removing acetyl groups from hemicelluloses, 5) Reducing the degree of polymerization in cellulose and 6) Expanding the structure to increase pore volume and internal surface area

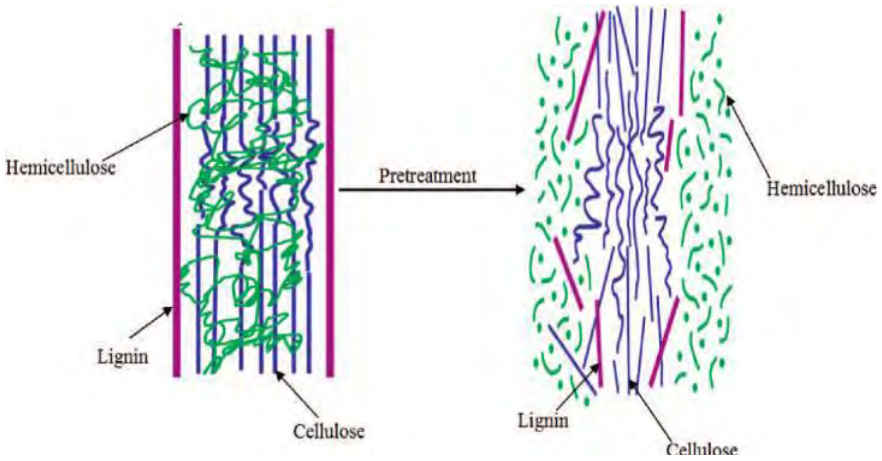

Figure 2.6 Schematic of goals of pretreatment on lignocellulosic material (Source : Mosier et al., 2005)

2.5.2 Pretreatment categories

Pretreatment methods can be classified into 4 method as shown in Figure 2.7 : (Sun and Cheng, 2002 ; and Talebnia et al., 2010)

I. Physical pretreatment :
   - Mechanical comminution (chipping, grinding and milling)
   - Pyrolysis

II. Physico-chemical pretreatment :
   - Steam explosion
   - Ammonia fiber explosion (AFEX)
   - CO_2 explosion
   - Liquid hot water

III. Chemical pretreatment :
   - Acid pretreatment (Acid hydrolysis)
   - Alkaline pretreatment (Alkaline hydrolysis)
   - Ozonolysis
   - Oxidative delignification

IV. Biological pretreatment :
   - Enzyme from microorganisms (fungi)

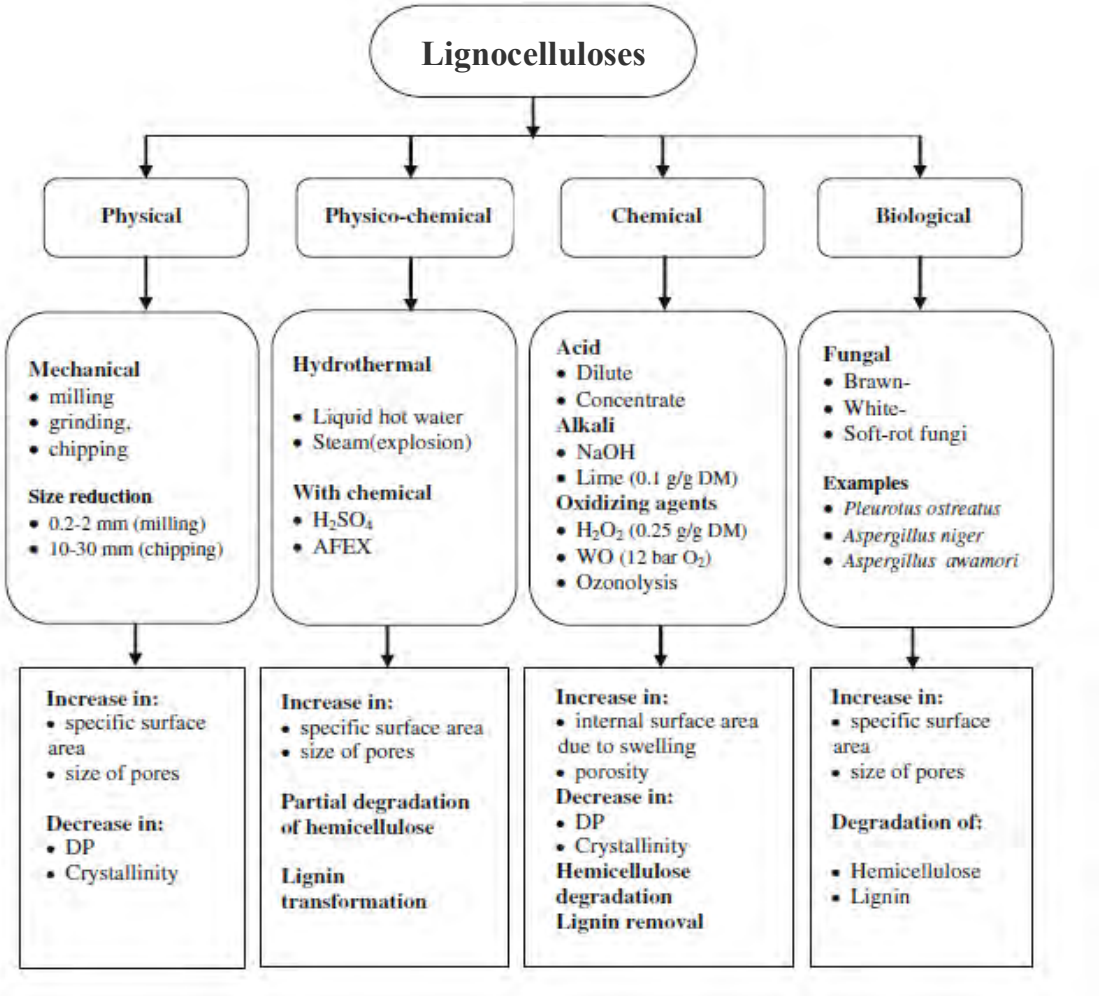

Figure 2.7 The most common pretreatment methods used on lignocelluloses and their possible effects (DP, degree of polymerization; WO, wet oxidation) (Source : Talebnia et al., 2010)

Among all these methods, the applied methods usually use combination of different principles, such as mechanical pretreatment together with chemical pretreatment effects in order to achieve high sugar release efficiencies, low toxicants production, and low energy consumption (Talebnia et al., 2010). Lignocelluloses have been studied for bioethanol production as summarized in Table 2.3. The advantages and disadvantages of various pretreatment methods were also summarized in Table 2.4 (Alvira et al., 2010).

Table 2.3 Pretreatment methods of lignocellulosic biomass for bioethanol production

Pretreatment method	Procedure/agents	Remarks	Examples of pretreated materials	References
Physical methods:				
Mechanical comminution	Chipping, grinding, milling	Milling: vibratory ball mill (final size: 0.2–2 mm), knife or hammer mill (final size: 3–6 mm)	Wood and forestry wastes (hardwood,straw) Corn stover, cane bagasse Timothy, alfalfa	Alvo and Belkacemi (1997); Papatheofanous et al. (1998); Sun and Cheng (2002)
Physical–chemical methods:				
Steam explosion	Saturated steam at 160–290 ºC, p = 0,69–4,85 MPa for several sec or min, then decompression until atm. pressure	-It can handle high solids loads -Size reduction with lower energy input compared to comminution 80–100% hemicellulose hydrolysis, destruction of a portion of xylan fraction, 45–65% xylose recovery	Poplar, aspen, eucalyptus Softwood (Douglas fir) Bagasse, corn stalk, wheat straw, rice straw, barley straw, sweet sorghum straw, *Brassica carinata* residue, olive stones	Ballesteros et al. (2001, 2002b, 2004); Belkacemi et al. (1997, 2002); De Bari et al. (2002); Hamelinck et al. (2005); Lynd et al. (2002); Nakamura et al. (2001); Negro et al. (2003); Shevchenko et al. (1999); So¨derstro¨m et al. (2003);Sun and Cheng (2002)
Ammonia fiber explosion (AFEX)	1–2 kg ammonia/kg dry biomass, 90 ºC, 30 min, p = 1.12–1.36 MPa	-Ammonia recovery is required 0–60% hemicellulose hydrolysis in dependence on moisture, >90% olygomers -No inhibitors formation -Cellulose depolymerization occurs at certain degree -Further cellulose conversion can be >90%, for high-lignin biomass (<50%) ∼10-20% lignin solubilization	Aspen wood chips Bagasse, wheat straw, barley straw, rice hulls, corn stover Switchgrass, coastal Bermudagrass, alfalfa Newsprint	Dale et al. (1996); Lynd et al. (2002); Sun and Cheng (2002)
Chemical methods:				
Dilute-acid hydrolysis	0.75–5% H_2SO_4, HCl, or HNO_3, p ∼1MPa; continuous process for low solids loads (5–10 wt% dry substrate/mixture): T = 160–200 ºC; batch process for high solids loads (10–40 wt% dry substrate/mixture): 1. T = 120–160 ºC	-pH neutralization is required that generates gypsum as a residue -80–100% hemicellulose hydrolysis, 75-90% xylose recovery -Cellulose depolymerization occurs at certain degree -High temperature favors further cellulose hydrolysis -Lignin is not solubilized, but it is redistributed	Poplar wood Bagasse, corn stover, wheat straw, rye straw, rice hulls Switchgrass, Bermudagrass	Hamelinck et al. (2005); Lynd et al. (2002); Martinez et al. (2000); Rodrı´guez-Chong et al. (2004); Saha et al. (2005a,b); Schell et al. (2003); Sun and Cheng (2002); Wooley et al. (1999b)

Table 2.3 (continued)

Pretreatment method	Procedure/agents	Remarks	Examples of pretreated materials	References
Concentrated-acid hydrolysis	10–30% H2SO4, 170–190 ºC, 1:1,6 solid–liquid ratio 21–60% peracetic acid, silo-type system	-Acid recovery is required -Residence time greater compared to dilute-acid hydrolysis -Peracetic acid provokes lignin oxidation	Poplar sawdust Bagasse	Cuzens and Miller (1997); Teixeira et al. (1999a,b)
Alkaline hydrolysis	Dilute NaOH, 24 h, 60 ºC; $Ca(OH)_2$, 4 h, 120 ºC; it can be complemented by adding H_2O_2 (0.5–2.15 vol.%) at lower temperature (35 ºC)	-Reactor costs are lower compared to acid Pretreatment ->50% hemicellulose hydrolysis, 60–75% xylose recovery -Low inhibitors formation -Cellulose swelling -Further cellulose conversion can be >65% 24–55% lignin removal for hardwood, lower for softwood	Hardwood Bagasse, corn stover, straws with low lignin content (10–18%), cane leaves	Hamelinck et al. (2005); Hari Krishna et al. (1998); Kaar and Holtzapple (2000); Lynd et al. (2002); Saha and Cotta (2006); Sun and Cheng (2002); Teixeira et al. (1999a)
Biological methods: Fungal pretreatment	Brown-, white- and soft-rot fungi	-Fungi produces cellulases, hemicellulases, and lignin-degrading enzymes: ligninases, lignin.peroxidases, polyphenoloxidases, laccase and quinone-reducing enzymes	Corn stover, wheat straw	Sun and Cheng (2002); Tengerdy and Szakacs (2003)

(Modified from Sánchez and Cardona, 2008)

Table 2.4 The advantages and disadvantages of various pretreatment methods for lignocellulosic biomass

Pretreatment method	**Advantages**	**Disadvantages**
Milling	– Reduces cellulose crystallinity	– High power and energy consumption
Steam explosion	– Causes lignin transformation and hemicellulose solubilization – Cost-effective – Higher yield of glucose and hemicellulose in the two-step method	– Generation of toxic compounds – Partial hemicellulose degradatio
AFEX	– Increases accessible surface area – Low formation of inhibitors	– Not efficient for raw materials with high lignin content – High cost of large amount of ammonia
CO_2 explosion	– Increases accessible surface area – Cost-effective – Do not imply generation of toxic compounds	– Does not affect lignin and hemicelluloses – Very high pressure requirements
Wet oxidation	– Efficient removal of lignin – Low formation of inhibitors – Minimizes the energy demand (exothermic)	– High cost of oxygen and alkaline catalyst
Ozonolysis	– Reduces lignin content – Does not imply generation of toxic compounds	– High cost of large amount of ozone needed
Organosolv	– Causes lignin and hemicellulose hydrolysis	– High cost – Solvents need to be drained and recycled
Concentrated acid	– High glucose yield – Ambient temperatures	– High cost of acid and need to be recovered – Reactor corrosion problems – Formation of inhibitors
Diluted acid	– Less corrosion problems than concentrated acid – Less formation of inhibitors	– Generation of degradation products – Low sugar concentration in exit stream
Biological	– Degrades lignin and hemicellulose – Low energy consumption	– Low rate of hydrolysis

(Source : Alvira et al., 2010)

2.5.2.1 Physical pretreatment

The objective of physical pretreatment is a reduction of particle size, crystallinity and degrees of polymerization of cellulose, and increase surface area of materials (Binod et al., 2010).

In general, mechanical comminution is the initial steps for pretreatment of any lignocellulose which reduces the particle size, through a combination of chipping, grinding and milling (Binod et al., 2010). The increase in specific surface area, reduction of degrees of polymerization are all factors that increase the total hydrolysis of lignocelluloses (Hendriks and Zeeman, 2009). The energy requirements of mechanical comminution of lignocellulosic materials depend on the final particle size and biomass characteristics (Sánchez and Cardona, 2008). The size of the materials is usually 10-30 mm after chipping and 0.2-2 mm after milling (Sun and cheng, 2002).

2.5.2.2 Physico-chemical pretreatment

Physico–chemical pretreatment methods (such as steam explosion, ammonia fiber explosion, CO_2 explosion and liquid hot water) are considerably more effective than physical. The steam explosion is the most studied method of this type. During this process, the use of saturated steam at high pressure causes autohydrolysis reactions in which part of the hemicellulose and lignin are converted into soluble oligomers. The factors affecting steam explosion pretreatment are residence time, temperature, chip size and moisture content. To consider the combined action of both temperature and time over the performance of steam explosion pretreatment, the so-called severity index has been defined including a correction term when this process is carried out under acidic conditions (Shahbazi et al., 2005; and Söderström et al., 2003).

2.5.2.3 Chemical pretreatment

Chemical pretreatment for lignocellulose employ different chemicals agents such as acids, alkaline, ozone, peroxide and organic solvents. Among these method, dilute acid pretreatment using sulfuric acid is the widely used method. The effect of structural and components of materials are depend on the type of chemical used for pretreatment. Alkaline pretreatment, ozonolysis, peroxide and wet oxidation

pretreatment are more efficient in lignin removal while dilute acid pretreatment is more efficient in hemicellulose solubilization. (Galbe and Zacchi, 2002; and Sánchez and Cardona, 2008)

2.5.2.3.1 Alkaline hydrolysis

Alkaline process is based on utilization of dilute bases in pretreatment of lignocellulosic feedstocks. Sodium, potassium, calcium and ammonium hydroxides are suitable alkaline agents for pretreatments, among which sodium hydroxide has been studied the most (Kumar et al., 2009). It causes swelling, increasing the internal surface of cellulose and decreasing the degree of polymerization and cristallinity, which provokes lignin structure disruption (Taherzadeh and Karimi, 2007). Alkaline pretreatment processes utilize lower temperatures and pressures than other pretreatment technologies (Mosier et al., 2005). The effective of this method depending on the lignin content of biomass. (Sánchez and Cardona, 2008; Sun and Cheng, 2002). Alkaline pretreatment can largely improve the cellulose digestibility and sugars degradation is less than acid treatment.

2.5.2.3.2 Acid hydrolysis

The pretreatment of lignocellulose with inorganic acids (sulfuric, hydrochloric or acetic acid) is usually called acid hydrolysis or prehydrolysis (Cardona et al., 2010). The main objective of the acid pretreatment is to solubilize the hemicellulose fraction of the materials and make the cellulose more accessible to enzymes hydrolysis (Alvira et al., 2010). Therefore, the cellulose and lignin fractions remain almost unaltered in the solid phase (Cardona et al., 2010). Based on the dose of acid used in the process it could be classified in 2 group ; concentrated acid and dilute acid

In the first group, the materials is treated with high concentration of acid at ambient temperatures, which results in high yield of sugars. Concentrated acid pretreatment offers advantage of not using any enzymes for saccharification, however, this method has less attractive for ethanol production because of inhibitor compounds are formed. Furthermore, acid recovery and equipment corrosion are important problems when using concentrated acid pretreatment, which makes the pretreatment process very expensive (Alvira et al., 2010 ; Talebnia et al., 2010 ; and Kumar et al., 2009).

In the second group, dilute acid appear as more favourable method for industrial applications and have been studied for pretreating wide range of lignocellulosic materials (Alvira et al., 2010). It can be performed at high temperature (e.g. 180ºC) during a short period of time; or at lower temperature (e.g. 120ºC) for longer retention time (30–90 min). It presents the advantage of solubilizing hemicellulose, mainly xylan, but also converting solubilized hemicellulose to fermentable sugars. Nevertheless, depending on the process temperature, some sugar degradation compounds such as furfural and HMF and aromatic lignin degradation compounds are detected, and affect the microorganism metabolism in the fermentation step (Saha et al., 2005). Anyhow, this pretreatment generates lower degradation products than concentrated acid pretreatments (Alvira et al., 2010).

Sanchez et al., 2004 investigated the optimum condition for hydrolysis of the cellulose and hemicelluloses in Bolivian straw, a sturdy grass. The hydrolysis was carried out in two stages. The results shown that xylose was the main product in first stage hydrolysis. The highest yield of xylose (0.209 g/g dry substrate) was obtained at 0.5%w/w sulfuric acid, temperature of 190°C and residence time of 10 min, whereas the second stage hydrolysis used higher temperature than first stage for cellulose hydrolysis to glucose. The highest glucose yield (0.118 g/g dry substrate) was obtained at 0.5%w/w sulfuric acid, temperature of 230°C and reaction time of 10 min. After that, the hydrolyzates were fermented by *Pichia stipitis, Candida shehatae* and *Pachysolen tannophilus.* The results shown that fermented hydrolyzates produced at temperature over 200°C gave low fermentability of hydrolyzates.

Karimi et al., 2006 investigated the optimum condition for dilute sulfuric acid hydrolysis of rice straw. The hydrolysis was carried out in two stages hydrolysis. The results of first stage shown that maximum yield of 80.8% of xylose was converted to xylose at hydrolysis pressure of 15 bar, 0.5% H_2SO_4 reaction time for 10 min. In the second stage, the highest yield of glucose (46.6%) was obtained when the second stage pressure was 30 bar, 0.5% H_2SO_4 residence time for 3 min. The highest yield of xylose (78.9%) was achieved at pressure of 25 bar, 0.5% H_2SO_4 residence time for 3 min.

Kootstra et al., 2009 studied the efficiency of sulfuric acid (mineral acid) was compared with fumaric and maleic acids (organic acid). Pretreatment was performed at temperature of 130°C, 150°C and 170°C for 30 min. The results shown that highest yield of xylose (80%) was obtained with H_2SO_4 pretreatment at 150°C, however, the

yield of xylose was decreased at 170°C due to more extensive degradation of xylose to furfural. The maximum xylose yield at 170°C was obtained with treatment using maleic acid. The yield of glucose after enzymatic hydrolysis reached to 98% and 96% for sulfuric acid and maleic acid, respectively. Fumaric acid was less effective than maleic acid. From this experiment can be concluded that dilute fumaric or maleic acid pretreatment of wheat straw is almost as effective as dilute sulfuric acid.

Laopaiboon et al., 2010 investigated the optimum condition for acid hydrolysis of sugarcane bagasse. The hydrolysis condition was performed at temperature of 90°C-120°C, 0.5%-5% v/v H_2SO_4 or HCl and incubation time of 1-5 hr. The results shown that xylose was the main product and temperature in the range 90°C-110°C were observed that the longer of residence time gave the higher xylose concentration. However, the maximum xylose concentration was obtained at 120°C, reaction time of 4 hr. The highest xylose concentration (15.16 g/l) was obtained under 0.5% v/v HCl, at 120°C for 4 hr. In this condition, glucose 2.85 g/l ; arabinose 1.35 g/l ; acetic acid 0.04 g/l and furfural 0.66 g/l were obtained. In the case of H_2SO_4 hydrolysis, the highest xylose concentration (12.64 g/l) was obtained under 0.5% v/v H_2SO_4, at 110°C for 4 hr. In this condition, glucose 2.28 g/l ; arabinose 1.33 g/l ; and acetic acid 0.06 g/l were obtained.

2.5.2.4 Biological pretreatment

Biological pretreatment offers some conceptually important advantages such as low chemical and energy used in pretreatment process. However, most of these processes are too slow limiting its application at industrial level (Sánchez et al., 2008). Chemical pretreatments have serious disadvantages in terms of the requirement for specialized corrosion resistant equipment, extensive washing, and proper disposal of chemical wastes. Biological pretreatment is a safe and environmentally-friendly method for lignin removal from lignocellulose (Taniguchi et al., 2005 and Binod et al., 2010).

In biological pretreatment processes, microorganisms such as brown-, white-, and soft-rot fungi that belong to class Basidiomycetes are used to degrade lignin and hemicellulose in waste materials. Brown rots mainly attack cellulose, whereas white and soft rots attack both cellulose and lignin. Lignin degradation by white-rot fungi occurs through the action of lignin degrading enzymes such as peroxidases and

laccase. These enzymes are regulated by carbon and nitrogen sources. White-rot fungi seems to be the most effective microorganism for biological pretreatment of lignocellulosic materials (Kumar et al., 2009). The important microbial enzymes for lignocellulose hydrolysis was shown in Table 2.5 (Alper and Stephanopoulos, 2007).

Table 2.5 Important enzymes for hydrolysis lignocellulose

Enzyme type	Function	Typical sources
Cellobiohydrolase	Solubilizes crystalline cellulose	Fungal systems (especially *Trichoderma* and *Aspergillus* spp.)
Endoglucanase	Hydrolyses the β-(1,4) glycosidic bonds in cellulose	Fungal systems (especially *Trichoderma* and *Aspergillus* spp.)
β -glucosidase	Hydrolyses β-linked disaccharides into monosaccharides	Fungal systems (especially *Trichoderma* and *Aspergillus* spp.)
Xylanase	Hydrolyses β-1,4-xylan into xylose	Fungal systems (especially *Trichoderma* and *Aspergillus* spp.)
Lignin peroxidase	Oxidizes lignin molecules through an H_2O_2 donor	White rot and brown rot fungi
Laccase	Oxidizes phenol groups	White rot fungi

(Source : Alper and Stephanopoulos, 2007)

2.6 Detoxification

During pretreatment and hydrolysis of lignocellulosic biomass, a great amount of compounds that can seriously inhibit the subsequent fermentation are formed in addition to fermentable sugars. Inhibitory substances are generated as a result of the hydrolysis of the extractive components, organic and sugar acids esterified to hemicellulose (acetic, formic, glucuronic, galacturonic), and solubilized phenolic derivatives. In the same way, inhibitors are produced from the degradation products of soluble sugars (furfural, HMF) and lignin (cinnamaldehyde, p-hydroxybenzaldehyde, syringaldehyde), and as a consequence of corrosion (metal ions) (Palmqvist and Hahn-Hägerdal, 2000). The existence of these substances is more probably when using acid and/or high-temperatures in process (Cardona et al., 2010). The main degradation pathways were presented in Figure 2.8. The toxic compounds of sugar and lignin degradation can stress fermentative organisms to a point beyond which the efficient utilization of sugars is reduced and product formation decreases. Because some of them are inhibit their metabolism. Several detoxification methods (including physical, chemical and biological) have been proposed to transform inhibitors into inactive compounds or to reduce their concentration (Mussatto and Roberto, 2004). The detoxification methods employed for ethanol production are observed in Table 2.6 (Sánchez et al., 2008).

Nigam et al., 2001 evaluated the ethanol production from wheat straw hydrolyzate. They also found that a furfural concentration of 0.25 g/l in fermentation medium was not significant effect to reduce the ethanol yield and productivity, but a concentration of furfural increase to 1.5 g/l was interfered in respiration and microorganism growth. The ethanol yield and productivity were decreased by 90.4% and 85.1% respectively.

Gupta et al., 2009 reported that the release in sugar increased with increase in acid concentration and it declined thereafter. They explained that any further increase in acid concentration caused the increase in release of some toxic compounds or inhibitors, resulting in a decrease of sugar concentration. Under some acid hydrolysis conditions in the present study, the increase in acid concentration caused a decrease in xylose concentration without any increase in the inhibitors (furfural and acetic acid).

Table 2.6 Detoxification methods of pretreated lignocellulosic biomass for bioethanol production

Detoxification method	Procedure/agents	Examples	Microorganism	Remarks	References
Physical methods:					
Evaporation	Evaporation, separation of volatile and nonvolatile fractions and dilution of non-volatile fraction	Willow hz. Aspen hz.	*S. cerevisiae* *P. stipitis*	-Reduction of acetic acid and phenolic compounds in non-volatile fraction; roto-evaporation -93% yield of ref. fermn.; removal: 54% acetic acid, 100% furfural, 29% vanillin; roto-evaporation;	Palmqvist and Hahn-Ha¨gerdal (2000a) Palmqvist and Hahn-Ha¨gerdal (2000a)
Chemical methods:					Palmqvist and Hahn-Ha¨gerdal (2000a)
Alkaline detoxification (overliming)	$Ca(OH)_2$, pH = 9–10.5, then pH adjustment to 5.5–6.5 with H2SO4 or HCl	Dilute-acid hz. of spruce Steam-exploded bagasse Acid hz. of cotton waste pyrolysate Rice hulls hz. Wheat straw hz.	Recombinant *S. cerevisiae* *S. cerevisiae*, *Pichia sp.* Recombinant *E. coli* Recombinant *E. coli* Recombinant *E.coli*	-Yield comparable to ref. fermn.; 20% removal of furfural and HMF -Removal of acid acetic, furfural and part of phenolic compounds -7.5% lower yield for Pichia sp. -39% reduction in fermentation time -Reduction in fermn. time: SSF -18%, SHF - 67% -Removal: 51% furfural, 51% HMF, 41% phenolic compounds, 0% acetic acid; overliming at 60 ºC or 25 ºC, at high temperature, the required amounts of lime and acid are reduced	Martı´n et al. (2002)) Yu and Zhang (2003) Saha et al. (2005a) Saha et al. (2005b) Martinez et al. (2000, 2001)
Ionic exchange	Weak base resins Amberlyst A20, regenerated with ammonia	Dilute-acid bagasse hz. Dilute-acid poplar Dilute-acid hz. of spruce	Recombinant *Z. mobilis* *S. cerevisiae*	-Removal: 88% acetic acid, 100% H2SO4; 100% sugars recovery -Removal: >80% phenolic compounds, ~100% levulinic, acetic and formic acids, 70% furfural; considerable lost of fermentable sugars	Wooley et al. (1999b) Palmqvist and Hahn-Ha¨gerdal (2000a)
Biological methods:					
Enzymatic detoxification	Laccase (phenol oxidase) and lignin peroxidase from Trametes versicolor: 30 ºC, 12 h	Willow hz. Steam-exploded bagasse	*S. cerevisiae* Recombinant *S. cerevisiae*	-2–3-fold increase of EtOH productivity compared to undetox. hz.; laccase selectively removes phenolic low molecular weight compounds and phenolic acids -80% removal of phenolic compounds	Palmqvist and Hahn-Ha¨gerdal (2000a); Jo¨nsson et al. (1998) Martı´n et al. (2002)
Microbial detoxification	Trichoderma reesei	Steam-exploded willow	*S. cerevisiae*	-3-fold increase of EtOH productivity compared to undetox. hz.; 4-fold increase of yield; removal of acetic acid, furfural and benzoic acid derivatives -Aerobic bacteria oxidize aromatic compounds	Palmqvist and Hahn-Ha¨gerdal (2000a)

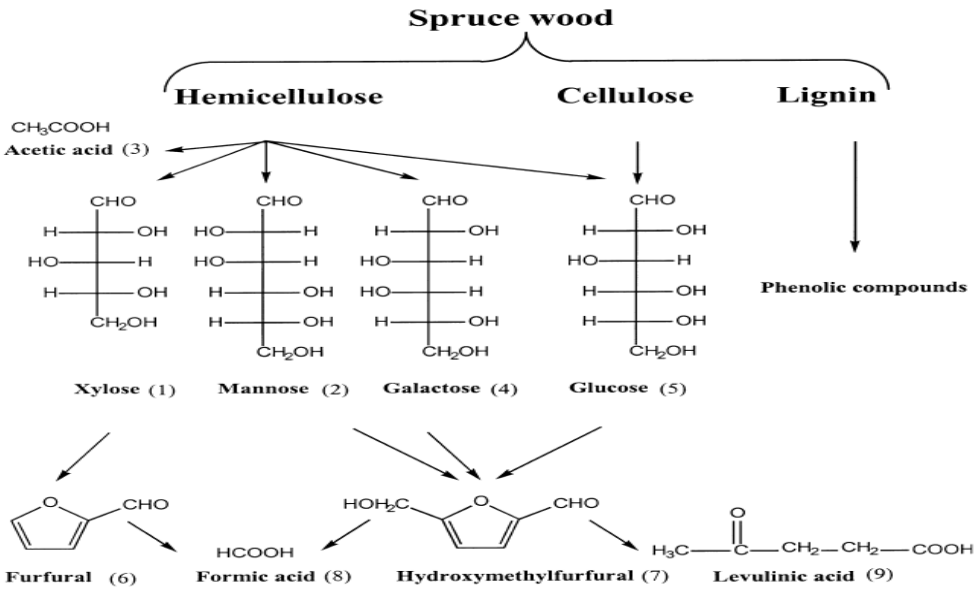

Figure 2.8 Reactions occurring during hydrolysis of lignocellulosic materials. (Source : Palmqvist and Hahn-Hägerdal, 2000)

2.6.1 Physical methods

Physical detoxification methods are based on the principle that inhibitors can be removed, either through phase equilibria-based separations based on solubility or volatility using for example liquid–liquid extraction or evaporation (Palmqvist and Hahn-Hägerdal, 2000). Hydrolyzate concentration by vacuum evaporation is a detoxification method for reducing the contents of volatile compounds such as acetic acid, furfural and vanillin, present in the hydrolyzate. However, this method also moderately increases the concentration of non-volatile toxic compounds (extractives and lignin derivatives) and consequently the degree of fermentation inhibition (Mussatto and Roberto, 2004).

2.6.2 Chemical methods

Chemical detoxifications such as overliming has been effective on chemical modifications of the inhibitors, such as furfural and 5-hydroxymethylfurfural to a less toxic or non-toxic product (Hodge et al., 2009). In the operation of overliming, it is usual to add chemicals that neutralize the acids from the initial hydrolysates, forming salts. These salts have low solubility and are normally removed by filtration. The concentration of hydrolysates by evaporation is usual to increase the sugar

concentration. In this operation, besides water, small amounts of growth inhibitors such as acetic acid, furfural and HMF are removed (Cardona et al., 2010).

Chandel et al., 2007 have evaluated the efficiency of various detoxification methods (such as ion exchange treatment, activated charcoal, laccase, overliming and neutralization) for the removal of inhibitors from dilute acid sugarcane bagasse hydrolysate and eventually for improving the fermentation of hydrolysate to ethanol using *Candida shehatae* strain NCIM 3501. The results shown that ion exchange treated hydrolysate gave the maximum ethanol concentration (8.67 g/L), followed by activated charcoal (7.43 g/L), laccase treatment (6.50 g/L), overliming (5.19 g/L), and neutralized hydrolysate (3.46 g/L).

2.6.3 Biological methods

Biological methods of treatment involve the use of specific enzymes or microorganisms that act on the toxic compounds present in the hydrolyzates and change their composition. Wood hydrolyzates detoxified with laccase and peroxidase enzymes of the white-rot fungus *Trametes versicolor* promoted an increase in glucose consumption and ethanol productivity, due to the action of these enzymes on acid and phenolic compounds (Jönsson et al., 1998). The detoxification mechanism of these enzymes probably involves oxidative polymerization of low-molecular-weight phenolic compounds (Mussatto and Roberto, 2004).

2.7 Ethanol fermentation process

Lignocellulose is often hydrolyzed by dilute-acid treatment; the hydrolyzate obtained is used for bioethanol fermentation by microorganism such as yeast. Lignocellulose hydrolyzate contains not only glucose, but also various monosaccharides, such as xylose, mannose, galactose, arabinose, and oligosaccharides. Required microorganisms should be efficiently utilized these sugars for the successful production of bioethanol (Balat et al., 2008).

The classic configuration employed for fermenting biomass hydrolyzates involves a sequential process where the hydrolysis of cellulose and the fermentation are carried out in different units. This configuration is known as separate hydrolysis and fermentation (SHF). In the alternative variant, the simultaneous saccharification and fermentation (SSF), the hydrolysis and fermentation are performed in a single

unit. The most employed microorganism for fermenting lignocellulosic hydrolyzates is *S. cerevisiae*, which ferments the hexoses contained in the hydrolyzate but not the pentoses (Sánchez et al., 2008).

2.7.1 Separate hydrolysis and fermentation (SHF)

Lignocellulosic hydrolyzate can be converted to ethanol by separate hydrolysis and fermentation (SHF) process. In this process, the hydrolysis of lignocellulose and fermentation are carried out in different units (Sánchez et al., 2008). The SHF with separate pentose and hexose sugars and combined sugar fermentation are shown in Figure 2.9. Compared to SHF the final bioethanol yield is higher, less energy is required and production costs are minimized. The primary advantage of SHF process is that each step can be performed at its optimal operating conditions; the disadvantage is that cellulolytic enzymes are endproduct inhibited so that the rate of hydrolysis is progressively reduced when glucose and cellobiose accumulate (Hahn-Hagerdal et al., 2006). The most important factors to be taken into account for saccharification step are reaction time, temperature, pH and substrate load (Sánchez et al., 2008).

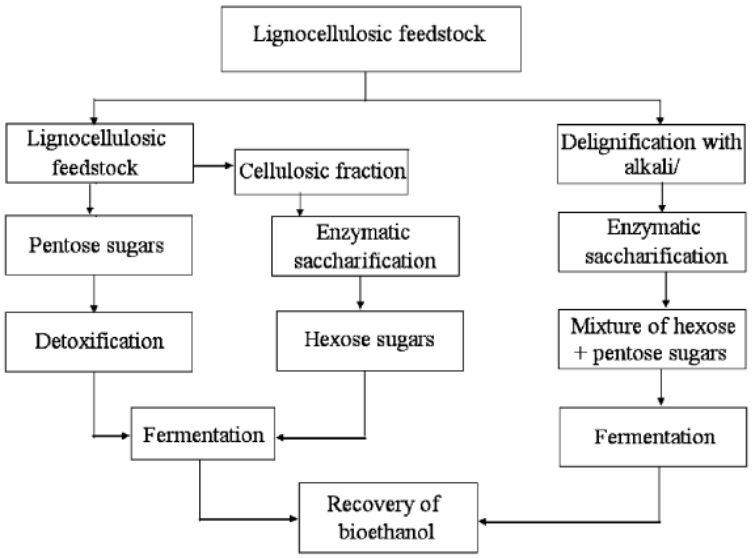

Figure 2.9 Separate hydrolysis and fermentation (SHF) with separate pentose and hexose sugars and combined sugar fermentation (Source : Balat et al., 2008)

Saha et al., 2005 evaluated the performance of both simultaneous saccharification and fermentation (SSF) and separate hydrolysis and fermentation (SHF) using dilute acid pretreated wheat straw by the recombinant *Escherichia coli* strain FBR5. SHF approach worked better than SSF and higher ethanol yield at shorter fermentation time. Detoxification pretreated hydrolyzate with overliming method dramatically reduced fermentation time of SHF from 118 to 39 hr and enhanced the yield of ethanol from 13 g/l to 17 g/l in the case of SSF.

Buaban et al., 2010 investigated ethanol production from sugarcane bagasse of both separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF). The pretreated bagasse was attained from combine pretreatment process by ball milling and enzymatic hydrolysis. The results of fermented shown that the maximum ethanol concentration of 8.4 g/l was obtained by *Pichia stipitis* BCC15191, at pH 5.5, incubation temperature of 30°C for 24 hr in separate hydrolysis and fermentation process. In simultaneous saccharification and fermentation process was obtained ethanol concentration of 8.0 g/l when fermentated at the same conditions for 72 hr.

2.7.2 Simultaneous saccharification and fermentation (SSF)

The sugars from the pre-treatment and enzymatic hydrolysis steps are fermented by bacteria, yeast or filamentous fungi, although the enzymatic hydrolysis and fermentation can also be performed in a combined step, that called simultaneous saccharification and fermentation (SSF) (Hahn-Hagerdal et al., 2006). It is often effective when combined with dilute-acid or high-temperature hot-water pre-treatment. In SSF, cellulases and xylanases convert the carbohydrate polymers into fermentable sugars. These enzymes are notoriously susceptible to feedback inhibition by the products (glucose, xylose, cellobiose, and other oligosaccharides). The SSF with combined sugars (pentoses and hexoses) fermentation shown in Figure 2.10 (Balat et al., 2008).

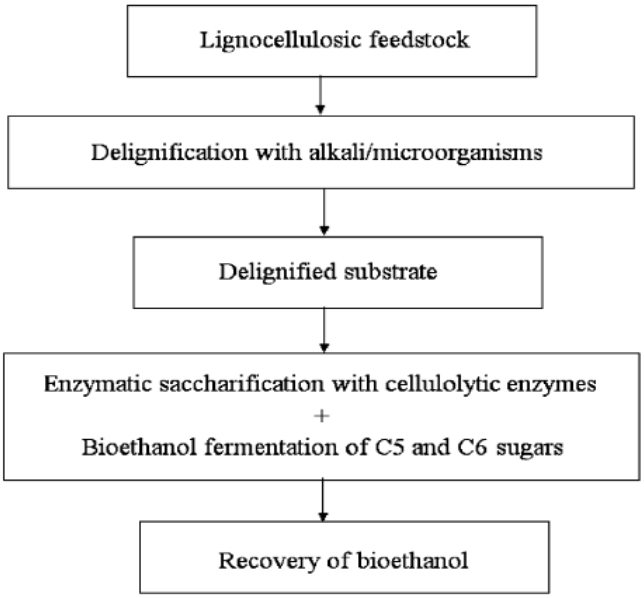

Figure 2.10 Simultaneous saccharification and fermentation (SSF) with combined sugars fermentation (Source : Balat et al., 2008).

2.8 Microorganism in bioethanol fermentation process

The supernatant from acid hydrolysis of lignocelluloses contain both hexoses and pentoses (if both cellulose and hemicellulose are hydrolyzed).These sugars are released during pretreatment and hydrolysis. Depending on the lignocellulose source, the hydrolyzate typically consists of glucose, xylose, arabinose, galactose, mannose, fucose and rhamnose (Saha, 2003). Glucose and xylose are two dominant sugars in the lignocellulosic hydrolyzates. The best known microorganisms for ethanol production from hexoses are the yeast *Sacchamyces cerevisiae* and the bacterium *Zymomonas mobilis* (Claassen et al., 1999). One of the main problems in bioethanol production from lignocellulosics hydrolyzate is the native strains of *S. cerevisiae* and *Z. mobilis* inability to utilize xylose, the main C5 sugar obtained from hemicelluloses hydrolysis (Rogers et al., 2007). Other approach to this problem is the use of pentose fermenting microorganisms like some species of yeasts and enteric bacteria. In this case, configurations involving the separate fermentation of pentoses and hexoses have been proposed. Yeasts as *Pichia stipitis*, *Candida shehatae* and *Pachysolen tannophilus* can assimilate pentoses but their ethanol production rate from glucose is at least five times less than that observed for S. cerevisiae. Moreover, their culture requires oxygen and ethanol tolerance is 2-4 times lower (Claassen et al., 1999 ;

Chandel et al., 2007 ; and Lin and Tanaka, 2006). Among the xylose fermenting yeasts, *P. stipitis* has shown the most promise for industrial application, because it ferments xylose with a high ethanol yield. Furthermore, *P. stipitis* has no absolute vitamin requirements for xylose fermentation and is able to ferment a wide range of sugars, including cellobiose (Agbogbo et al., 2006).

Pentoses and hexoses are commonly found in lignocellulosic material include xylose (Xyl), arabinose (Ara), glucose (Glc), mannose (Man) and galactose (Gal). These sugars are converted to the phosphorylated forms xylose-5-phosphate (X5P), glucose-6-phosphate (G6P), fructose-6-phosphate (F6P) and glucose-1-phosphate (G1P). These molecules are eventually converted into glyceraldehyde-3-phosphate (G3P), pyruvate (Pyr) and formate (Alper and Stephanopoulos, 2009). A number of possible biofuels can then be produced as shown in Figure 2.11.

Xylose can be incorporated into the pentose phosphate pathway through either the three-enzyme pathway containing a xylitol intermediate or a two-step process that uses a yeast or bacterial xylose isomerase gene. The two-step process bypasses the need for the reducing power that is incorporated in NAD- and NADP-reducing partners and has been shown to improve ethanol production. Xylulose-5-phosphate is formed by both pathways and can enter into central carbon metabolism through the transketolase and transaldolase reactions as shown in Figure 2.12.

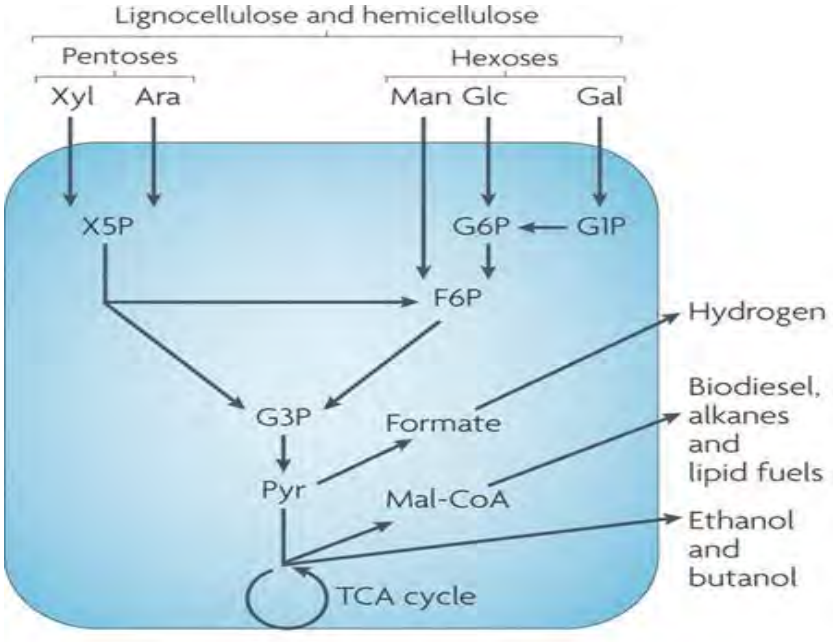

Figure 2.11 Metabolic pathway of pentoses and hexoses
(Source : Alper and Stephanopoulos, 2009)

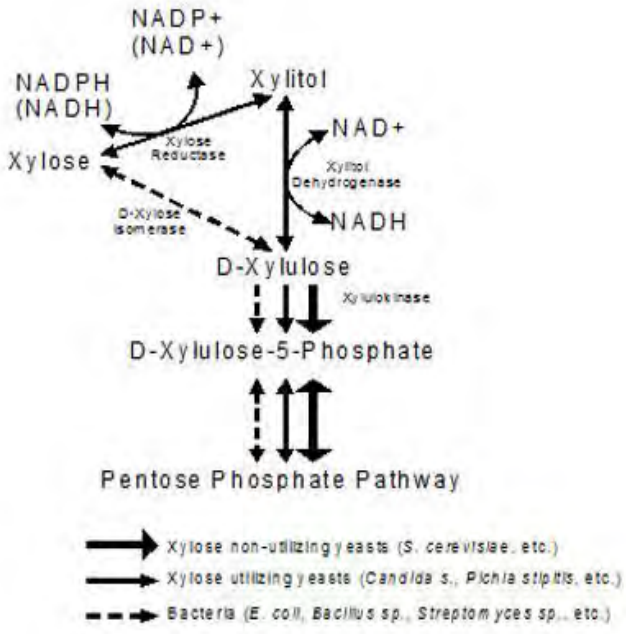

Figure 2.12 Xylose utilization pathway by bacteria and yeast
(Source : http://bioweb.sungrant.org/Technical/Biofuels/Technologies/Ethanol)

From the Figure 2.13 (Bai et al., 2008), the main metabolic pathway involved in the ethanol fermentation is glycolysis (Embden–Meyerhof–Parnas or EMP pathway), through which one molecule of glucose is metabolized, and two molecules of pyruvate are produced. the pyruvate is further reduced to ethanol with the release of CO_2. Theoretically, the yield is 0.511 for ethanol and 0.489 for CO_2 on a mass basis of glucose metabolized. Two ATPs produced in the glycolysis are used to drive the biosynthesis of yeast cells which involves a variety of energy-requiring bioreactions. Therefore, ethanol production is tightly coupled with yeast cell growth, which means yeast must be produced as a co-product. Without the continuous consumption of ATPs by the growth of yeast cells, the glycolytic metabolism of glucose will be interrupted immediately, because of the intracellular accumulation of ATP, which inhibits phosphofructokinase (PFK), one of the most important regulation enzymes in the glycolysis. This very basic principle contradicts the ethanol fermentation with the yeast cells immobilized by supporting materials, particularly by gel entrapments, which physically restrict the yeast cells and significantly retard their growth.

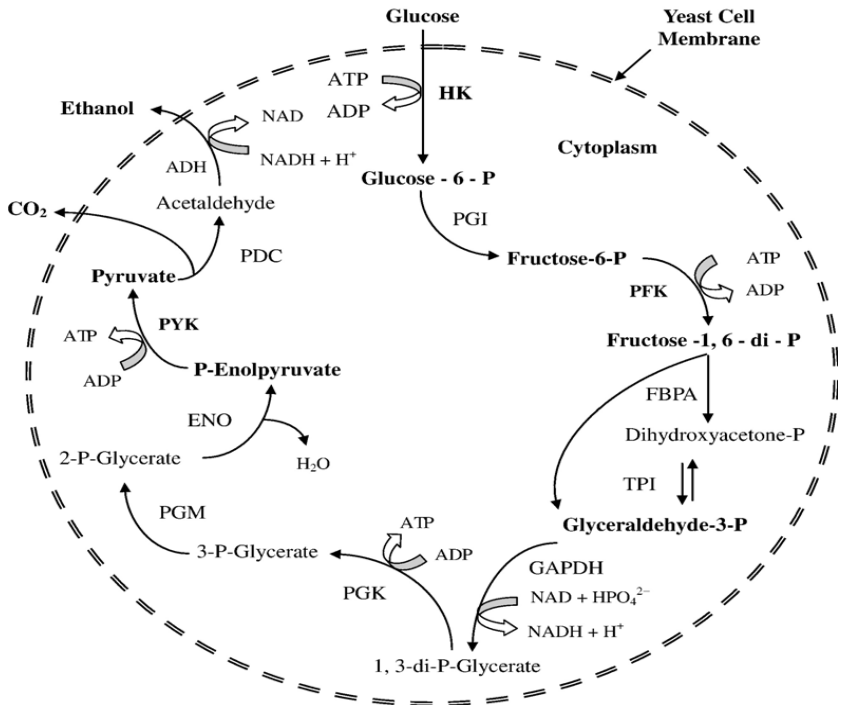

Figure 2.13 Metabolic pathway of ethanol fermentation in *S. cerevisiae*. Abbreviations: HK: hexokinase, PGI: phosphoglucoisomerase, PFK: phosphofructokinase, FBPA: fructose bisphosphate aldolase, TPI: triose phosphate isomerase, GAPDH: glyceraldehydes-3-phosphate dehydrogenase, PGK: phosphoglycerate kinase, PGM: phosphoglyceromutase, ENO: enolase, PYK: pyruvate kinase, PDC: pyruvate decarboxylase, ADH: alcohol dehydrogenase (Source : Bai et al., 2008)

CHAPTER III

EXPERIMENTAL

3.1 Materials

Sweet sorghum straw and sugarcane bagasse used in the experiments were obtained from the Suphanburi Field Crop Research Center, Suphanburi province, Thailand. The fresh straw and bagasse were chopped into a small-size about 10-15 cm and dried in a hot-air oven at 80°C for 24 h. Then, the substrate was milled in a hammer mill to pass through an 8 mm screen. The milled sweet sorghum straw and sugarcane bagasse were stored in sealed plastic bags at 4°C for pretreatment study.

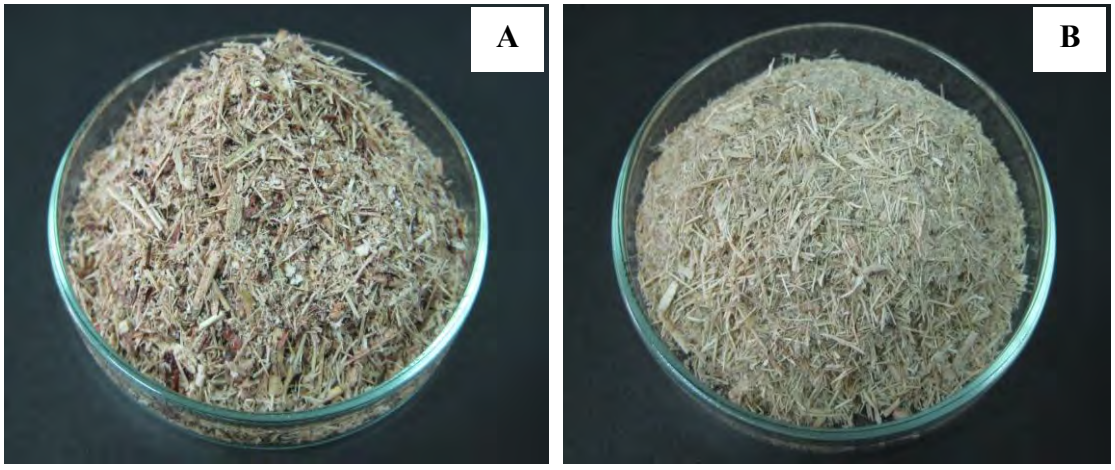

Figure 3.1 Lignocellulosic materials used as substrate in this experiment: (A) milled sweet sorghum straw ; (B) milled sugarcane bagasse

3.2 Chemicals and Reagents

Chemicals	Company	Country
Agar (Pearl Mermaid Brand)	Patanasin Enterprise	Thailand
Arabinose ($C_5H_{10}O_5$)	Sigma	Germany

Calcium hydroxide ($Ca(OH)_2$)	Sigma	USA
Cellobiose($C_{12}H_{22}O_{11}$)	Sigma	Germany
3,5 –Dinitrosalicylic acid ($C_7H_4N_2O_7$)	Sigma	USA
Ethanol absolute 99.5%	Merck	Germany
Galactose ($C_6H_{12}O_6$)	Sigma	Germany
Glucose ($C_6H_{12}O_6$)	Riedel-de Haen	France
Hydrochloric acid (HCl)	Merck	Germany
Magnesium sulfate ($MgSO_4 \cdot 7H_2O$)	Fluka	Switzerland
Mannose ($C_6H_{12}O_6$)	Sigma	Germany
Milli Q water	Mahidol U.	Thailand
Peptone	Difco	USA
Phenol (C_6H_6O)	Merck	Germany
Potassium dihydrogen phosphate (KH_2PO_4)	Merck	Germany
Potassium sodium tartrate ($KNaC_4H_4O_6 .4H_2O$)	Carlo Erba	Italy
Propanol (C_3H_8O)	Merck	Germany
Sodium hydroxide (NaOH)	Merck	Germany
Sulfuric acid (H_2SO_4)	Merck	Germany
Urea $(NH_2)_2CO$	Merck	Germany
Xylose ($C_5H_{10}O_5$)	Sigma	Germany
Yeast extract	Difco	USA

3.3 Apparatus and Instruments

Instruments	Model, Company, Country
Autoclave	Hirayama Manufacturing Corporation, Japan
Cellulose membrane acetate filter (pore size 0.45 μm, 13 mmØ)	Sartorius Stedim Biotech GmbH, Germany
Cellulose membrane acetate filter (pore size 0.45 μm, 47 mmØ)	Sartorius Stedim Biotech GmbH, Germany
Centrifuge	MTX-150, Tomy Seiko Co., Ltd. Japan
Electronic balance (Two decimal)	ARC 120, Ohaus Corporation, USA
Electronic balance (Four decimal)	AR 2140, Ohaus Corporation, USA
Filter papers (Whatman No.1, 110 mmØ)	Whatman International Ltd. England
Filter papers (Whatman No.4, 110 mmØ)	Whatman International Ltd. England
Gas chromatography (Porapak Q column)	163, Hitachi Ltd. Tokyo, Japan
Hammer mill	(Motor) Mitsubishi Electric Automation Co., Ltd. Thailand
High Performance Liquid Chromatography (HPLC)	LC-6A, Shimadzu Corporation, Japan
HPLC column (300mm x 7.8 mm)	Aminex HPX-87P, Bio-Rad Laboratories.Inc., USA
Hot air oven	Contherm Digital Series Oven, New Zealand

Hot plate	PC-101, Corning, N.Y, USA
In-house saccharification reactor	Institute of Biotechnology and Genetic Engineering (IBGE), Chulalongkorn University, Thailand
Laminar flow hood	NK System HV-50, Clean Bench, Japan
Microcentrifuge	MC-15A, Tomy Seiko Co., Ltd. Japan
Micropipette	Pipetteman, Gilson, France
Microwave	NE-767C, Matsushita Electric Industrial Co., Ltd. Japan
pH meter	AB15, Fisher Scientific Pte Ltd. Singapore
pH paper	Merck, Germany
Pump	MPN125, Thakita Electric Works., Ltd. Japan
Refractometer	ATAGO N1 Brix 0-32%, Japan
Refrigerated incubator shaker	INNOVA4330, Scientific Promotion Co., Ltd. Thailand
Rotary Evaporator	RE-52, Yamato Scientific Co., Ltd. Japan
Spectrophotometer	UV160, Shimadzu Corporation, Japan
Ultrasonic disruption	UD-201, Tomy Seiko Co., Ltd. Japan
Vortex mixer	G-560E, Scientific Industries, USA

Saccharification reactor

The in-house saccharification reactor (Figure 3.2) consisted of 3 main parts: 1. saccharification unit (reactor); 2.heat generator unit; and 3. temperature controller unit. The pressure of the reactor could be set by a temperature controller. The saccharification reactor was made from iron and equipped with heat generator unit for heating the reactor. Temperature was measured with temperature probe inside the reactor. On the top of the reactor equipped with pressure gage and globe valve for monitoring and controlling the pressure, respectively.

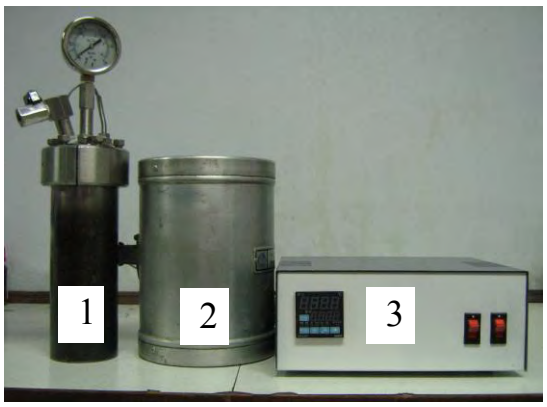

Figure 3.2 In-house saccharification reactor

3.4 Procedures

3.4.1 Diluted-alkaline pretreatment (Akaline hydrolysis)

Milled sweet sorghum straw and bagasse were mixed with dilute sodium hydroxide solution (final concentrations: 0%, 2% and 4% w/v) with solid loading of 10% w/v (30 g of substrate/300 ml of reaction mixture). The mixture was then hydrolysis in the autoclave. The pretreatment temperature of 121°C and reaction time of 30 and 60 min were used during saccharification. After pretreatment, the pretreated material was separated into solid and liquid (hydrolyzate) fractions. The hydrolyzates were analyzed for total reducing sugars.

3.4.2 Diluted-acid pretreatment (Acid hydrolysis)

3.4.2.1 Diluted-acid pretreatment of sweet sorghum straw

Milled sweet sorghum straw was mixed with dilute sulfuric acid solution (final concentrations: 0%, 1% and 3% v/v) with solid loading of 10% w/v (30 g of substrate/300 ml of reaction mixture). The mixture was then hydrolysis into the in-house saccharification reactor. The pretreatment was carried out in two types of the diluted-acid pretreatment process: high temperature (T at 150°C, 170°C and 190°C) and low temperature (T at 120°C). Different residence time (10, 20 and 30 min) was used during pretreatment. After pretreatment, the pretreated material was separated into solid and liquid (hydrolyzate) fractions. The hydrolyzates were analyzed for total reducing sugars and monomeric sugar (glucose, xylose, galactose, arabinose, and mannose). The solid fraction was thoroughly washed with distilled water until the filtrate pH about 6-7 and stored at -10°C prior to analysis the composition of pretreated straw.

3.4.2.2 Diluted-acid pretreatment of sugarcane bagasse

Diluted-acid pretreatment of sugarcane bagasse was performed same as 3.4.2.1, except the pretreatment temperature was carried out at 120°C, 150°C and 170°C and residence time of 20 min was used for pretreatment.

3.4.3 Hydrolyzate detoxification

Detoxification of acid hydrolyzate was operated by overliming and evaporation. The hydrolyzate of sweet sorghum straw and sugarcane bagasse that gave the maximum glucose and/or xylose was selected. This hydrolyzate was overliming by adding 40%w/v $Ca(OH)_2$ solution until the pH of hydrolyzate was about 5-6. During neutralization, salt and toxic compounds were precipitated and removed by centrifuge at 8,000 rpm for 20 min. The liquid fraction was concentrated by vacuum evaporation until solid content in hydrolyzate increased to about 20ºBrix (estimated by using refractometer) and then analyze for total sugars concentration by HPLC. This fraction was used for ethanol fermentation.

3.4.4 Ethanol fermentation

3.4.4.1 Yeast strains

Saccharomyces cerevisiae and *Pichia stipitis* were obtained from the Institute of Biotechnology and Genetic Engineering (IBGE), Chulalongkorn university, Thailand. Yeast strain was maintained on agar slants containing : 10 g/l yeast extract, 10 g/l peptone, 20 g/l agar and 20 g/l glucose as a carbon source (Sanchez et al., 2004).

3.4.4.2 Inoculum preparation

The inoculum was grown in 50 ml of culture medium that contained : 10 g/l yeast extract, 10 g/l peptone, and 20 g/l glucose as a carbon source. Then, incubated in rotary shaker at 30°C, agitation rate of 150 rpm for 18-22 hr. At the end of incubation, these cells were used for fermentation process.

3.4.4.3. Ethanol fermentation

Ethanol fermentation was performed under aerobic condition in 250 ml Erlenmeyer flasks with a total reaction volume of 50 ml. The fermentation medium contained : 2 g/l KH_2PO_4, 1 g/l $MgSO_4 \cdot 7H_2O$, 6.4 g/l urea, 10 g/l yeast extract, 20 or 50 g/l hydrolyzate and adjusted to pH 5.5. Subsequently, adding 10%v/v inoculum to start fermentation process and incubated at the agitation rate of 150 rpm at 30°C. Samples were withdrawn at time intervals and concentrations of ethanol were determined by gas chromatography.

3.4.5 Analytical methods

3.4.5.1 Reducing sugars

The reducing sugar concentration of hydrolyzate was determined by the 3,5-dinitrosalicylic acid (DNSA) method applied from Miller (1959), with D-glucose as the standard. In a typical reaction, 100 µl of sample and the reagent are mixed and heated in a boiling water bath for 10 min, then cooled immediately on ice bath and

added 1 ml of distilled water. At the end of the reaction, the absorbance was measured by spectrophotometer at 540 nm.

3.4.5.2 Monomeric sugars in hydrolyzate

All the samples of hydrolyzate were analyzed for monomeric sugar (glucose, xylose, galactose, arabinose, and mannose) by high-performance liquid chromatography (HPLC). Before injection into a column, all samples were neutralized with 40% NaOH, centrifuged at 12,000 rpm for 15 min and then filtered through a cellulose membrane acetate filter (pore size 0.45 μm). Subsequently, one milliliters of each sample was dilute 5 times The condition for analysis process was shown below.

Column	Bio-Rad Aminex HPX-87P (300mm x 7.8 mm)
Guard column	Carbo-P micro-guard cartridge
Eluent	H_2O (Milli Q water)
Temperature	85°C
Flow rate	0.6 ml/min
Injection volume	20 μl
Detector	RI detector (Shimadzu Model RID-6A)
Retention times	30 min

Peaks area of samples were indentified and quantified by comparison with retention times (RT) of analytical standards (glucose, xylose, galactose, arabinose and mannose). (Shown in appendix B)

3.4.5.3 Chemical composition of substrate

The composition of the untreated substrates (sweet sorghum straw and sugarcane bagasse) and the solid fraction remaining after pretreatment were determined by the Nakhonratchasima Animal Nutrition Research and Development Center. The percentages of cellulose, hemicellulose and lignin in substrate were determined by the procedures of Goering and Van Soest (Goering and Van Soest, 1971).

3.4.5.4 Calculation methods

The amount of glucose and xylose released from sweet sorghum straw and sugarcane bagasse were used to calculate the percentage conversion of glucose and xylose, which were calculated based on the original substrate (sweet sorghum straw and sugarcane bagasse) composition of 42% glucan and 24% xylan. The percentages of glucose and xylose conversion were calculated by the following equation (1 and 2):

$$\% \text{ Conversion of glucose} = \frac{\text{g produced glucose}}{\text{g glucan in materials}} \times 100 \quad \ldots\ldots\ldots (1)$$

$$\% \text{ Conversion of xylose} = \frac{\text{g produced xylose}}{\text{g xylan in materials}} \times 100 \quad \ldots\ldots\ldots (2)$$

Yield of ethanol from fermentation broth of sweet sorghum straw and sugarcane bagasse hydrolyzate were calculated using the following equation (3) : (Hernández-Salas et al., 2009)

$$\% \text{ Yield ethanol} = \frac{\text{Ethanol concentration at each time intervals (g/l)}}{\text{Initial sugar concentration (g/l)}} \times 100 \quad \ldots (3)$$

3.4.5.5 Ethanol concentration

Ethanol produced during the fermentation process was analyzed by Gas chromatography (GC). Ethanol was determined using a Hitachi 163 gas chromatography equipped with Porapak Q column and a flame ionization detector (FID) system. The injector and column temperatures were set at 220ºC and 190ºC, respectively. Nitrogen and helium were used as carrier gas. The flow rate of the carrier gas was 1.0 ml/min. The sample, mixed with 3 mg/ml propanol (ratio of 1 : 1) about 1 µl was injected manually into the gas chromatography column. The ethanol in fermentation broth was identified and calculated by compare with the peak area ratio of ethanol and propanol relative to various concentrations of ethanol standard.

3.5 Statistical analysis

This experiment was designed using a factorial design ($p \leq 0.05$) and effects of 3 parameters (temperature pretreatment, acid concentration and residence time) were analyzed. The experimental data were carried out in triplicate and analyzed using the SPSS for Windows program version 15.0.

CHAPTER IV

RESULTS AND DISCUSSION

Generally, glucan and xylan were the major component of sweet sorghum straw and sugarcane bagasse followed by acid-insoluble lignin. Arabinan, galactan and mannan accounted for only a small amount of the biomass composition.

Chemicals pretreatment process is the old technology for converting lignocellulosic biomass to ethanol. Acid catalyzes the breakdown of long chains hemicellulose to form shorter chain oligomers and then to sugar monomers. After pretreatment at different temperature, sulfuric acid concentrations and residence time the liquid fractions (hydrolyzate) and solid fractions were collected. The amount of monomeric sugars contain in hydrolyzate, ethanol concentration and composition of materials were analyzed. The results were shown and discussed in each part of this chapter.

4.1 Comparison between acid and alkaline hydrolysis of sweet sorghum straw and sugarcane bagasse

Dilute acid and dilute alkaline hydrolysis of sweet sorghum straw and sugarcane bagasse were performed using H_2SO_4 and NaOH respectively. The amount of total reducing sugars concentration released from sweet sorghum straw and sugarcane bagasse were measured by DNSA method (Miller, 1959).

Table 4.1 Total reducing sugars liberated from pretreated sweet sorghum straw and sugarcane bagasse at 120°C with various sulfuric acid concentration and residence time

Substrate	H_2SO_4 (%)	Residence time (min)	Total reducing sugars (g/l)
Sweet sorghum straw	0	30	17.133
	1		35.109
	3		30.870
Sugarcane bagasse	0	20	0.508
	1		72.612
	3		53.570

Table 4.2 Total reducing sugars liberated from pretreated sweet sorghum straw and sugarcane bagasse at 121°C, with various sodium hydroxide concentration and residence time

Substrate	NaOH (%)	Residence time (min)	Total reducing sugars (g/l)
Sweet sorghum straw	0	30	1.671
		60	2.034
	2	30	2.528
		60	2.609
	4	30	1.133
		60	1.398
Sugarcane bagasse	0	30	1.384
		60	1.563
	2	30	1.016
		60	1.052
	4	30	0.690
		60	0.732

Total reducing sugars liberated from treated sweet sorghum straw and sugarcane bagasse by dilute sulfuric acid and dilute sodium hydroxide hydrolysis were shown in Table 4.1 and Table 4.2, respectively. From these results, total reducing sugars released from dilute alkaline hydrolysis was very low when compare with dilute acid hydrolysis. It was demonstrated that dilute alkaline hydrolysis had a lower efficiency for hydrolysis than dilute acid hydrolysis. Consistent with the fact that alkaline was capable to remove the lignin barrier in the lignocelluloses and facilitate for the process of enzyme hydrolysis to sugars (Balat et al., 2008). Alkaline hydrolysis could be carried out at lower temperatures and pressures than other chemical pretreatment technologies, but pretreatment times are on the order of hours or days rather than minutes or seconds. Compared with acid hydrolysis processes, alkaline hydrolysis processes cause less sugar degradation, and many of the caustic salts can be recovered and/or regenerated (Kumar et al., 2009 ; and Mosier et al., 2005). Zhao et al. (2009) pretreated sugarcane bagasse by NaOH under mild conditions (pretreated by 7%NaOH with 3:1 liquid-to-solid ratio at 90°C for 1.5 hr) to increase the enzymatic digestibility.

Therefore, dilute sulfuric acid hydrolysis of sweet sorghum straw and sugarcane bagasse was selected for the further experiments.

4.2 Effects of dilute sulfuric acid hydrolysis on substrates composition

Untreated and treated of sweet sorghum straw and sugarcane bagasse by dilute acid pretreatment were analyzed for chemical compositions : cellulose, hemicellulose and lignin content.

Table 4.3 The composition of sweet sorghum straw and sugarcane bagasse

Condition	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Sweet sorghum straw			
Untreated (Raw materials)	44.51	38.62	6.18
treated at 120°C/ 0% H_2SO_4/ 10 min	46.36	36.51	9.18
treated at 120°C/ 3% H_2SO_4/ 10 min	69.50	0.44	19.53
Sugarcane bagasse			
Untreated (Raw materials)	48.04	30.70	9.57
treated at 120°C/ 0% H_2SO_4/ 20 min	57.19	29.53	13.17
treated at 120°C/ 3% H_2SO_4/ 20 min	68.26	0.57	23.44

As shown in Table 4.3, the composition of these substrates (untreated) were found to be within the range as other reports. Neureiter et al. (2002) reported that sugarcane bagasse composes of 40-45% cellulose, 30-35% hemicelluloses and 15-20% lignin. The treated sweet sorghum straw at 120°C, 3%H_2SO_4 for 10 min consists of 69.50% cellulose, 0.44% hemicellulose and 19.53% lignin. Treated sugarcane bagasse at 120°C, 3%H_2SO_4 for 20 min contains 68.26% cellulose, 0.57% hemicellulose and 23.44% lignin. Compared with the chemical components in the untreated materials, it was noted that dilute sulfuric acid pretreatment in sweet sorghum straw and sugarcane bagasse shown the increament of cellulose composition 56.14% and 42.09%, respectively. While the hemicellulose composition decreased by 96.86% and 98.14% in sweet sorghum straw and sugarcane bagasse, respectively.

Cardona et al. (2010) reported that dilute acid attacks the polysaccharides, especially hemicelluloses that are easier to be hydrolyzed than cellulose. Therefore, cellulose and lignin fractions remain almost unaltered in the solid phase. Salvi et al. (2010) reported that about 35% of hemicelluloses was removed from sorghum straw pretreated by dilute ammonia. Zhang and Cai (2008) reported 61% decrease of hemicelluloses from rice straw pretreated by dilute alkaline. From our results could be demonstrated that dilute acid pretreatment effectively solubilize most of the hemicelluloses as dissolved sugars and recovere cellulose composition for enzyme hydrolysis when compared to dilute alkaline pretreatment.

The composition percentages of untreated and treated biomass were shown in Figure 4.1. The appearances of treated sweet sorghum straw and sugarcane bagasse at 120°C were shown in Figure 4.2 and 4.3, respectively.

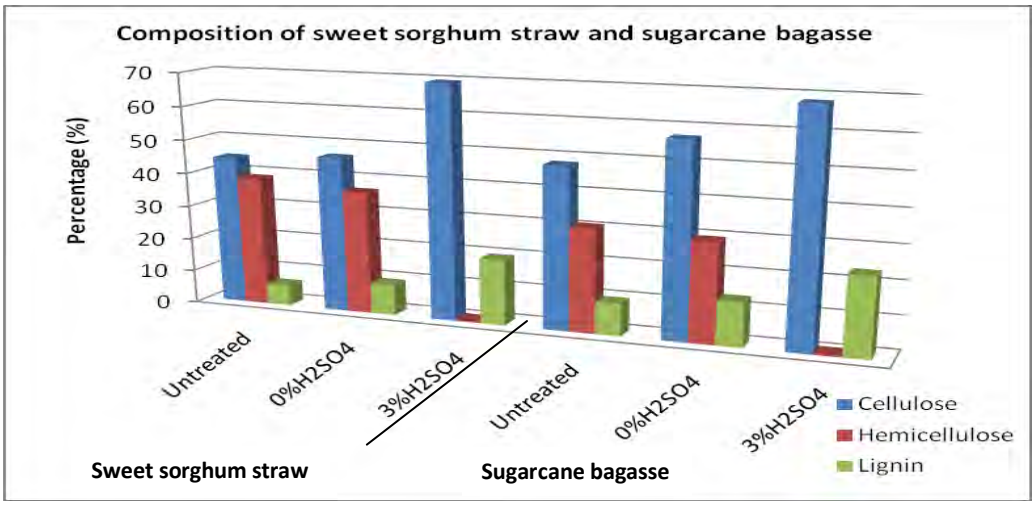

Figure 4.1 Percentage of cellulose, hemicelluloses and lignin from untreated and pretreated of sweet sorghum straw and sugarcane bagasse

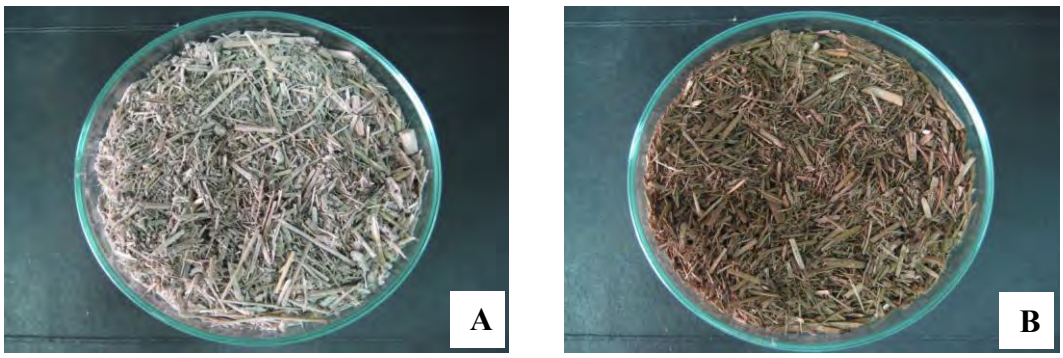

Figure 4.2 The pretreated of sweet sorghum straw at 120°C
(A) treated with 0% H_2SO_4 for 10 min ; (B) treated with 3% H_2SO_4 for 10 min

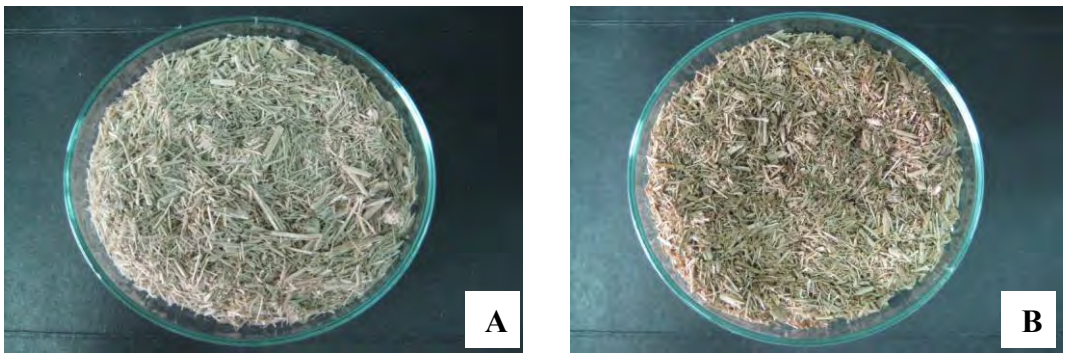

Figure 4.3 The pretreated of sugarcane bagasse at 120°C
(A) treated with 0% H_2SO_4 for 20 min ; (B) treated with 3% H_2SO_4 for 20 min

4.3 Distilled water only hydrolysis sweet sorghum straw

Sweet sorghum straw was pretreated by using distilled water as control. Yield of monosugars (glucose, xylose, galactose, arabinose and mannose) contained in hydrolyzates at various temperature and residence time was shown in Appendix C (Table C1). The yield of glucose and xylose were shown in Figure 4.4. From these results, the yield of glucose and xylose that obtained from pretreated sweet sorghum straw by using distilled water (0%H_2SO_4) was not much different in each pretreatment condition.

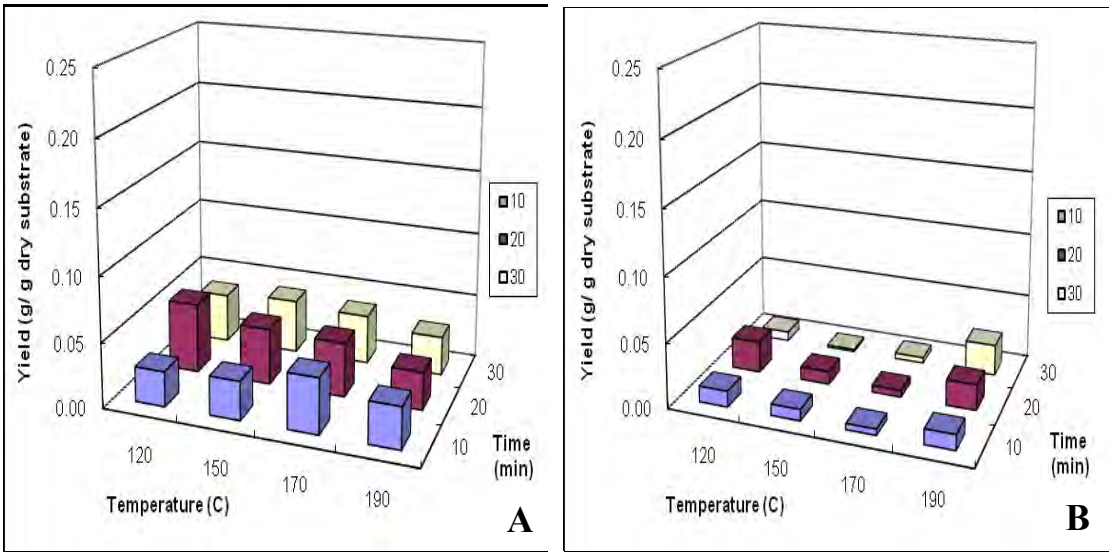

Figure 4.4 Yield of monosugar liberated from sweet sorghum straw when pretreated by distilled-water for 10-30 min (A) Yield of glucose ; (B) Yield of xylose

4.4 Dilute-acid pretreatment of sweet sorghum straw

Results for yield of monosugars contained in sweet sorghum straw hydrolyzates, which pretreated by 1%H_2SO_4 at various temperature and residence time was shown in Appendix C (Table C2). The yield of glucose and xylose were shown in Figure 4.5. The maximum yield of glucose was 0.221 g glucose/g dry substrate at 170ºC for 20 min and the maximum yield of xylose was 0.161 g xylose/g dry substrate at 150ºC for 20 min. In this case, a total of 47.41% of glucan and 59.08% of xylan were converted to glucose and xylose, respectively. Yields of glucose and xylose increased when the pretreatment temperature increase from 120ºC to 170ºC in the range 10-20 min. In severe conditions, such as high temperature (T at 190 ºC and long redidence time (> 20 min), yields of glucose and xylose dramatically decreased.

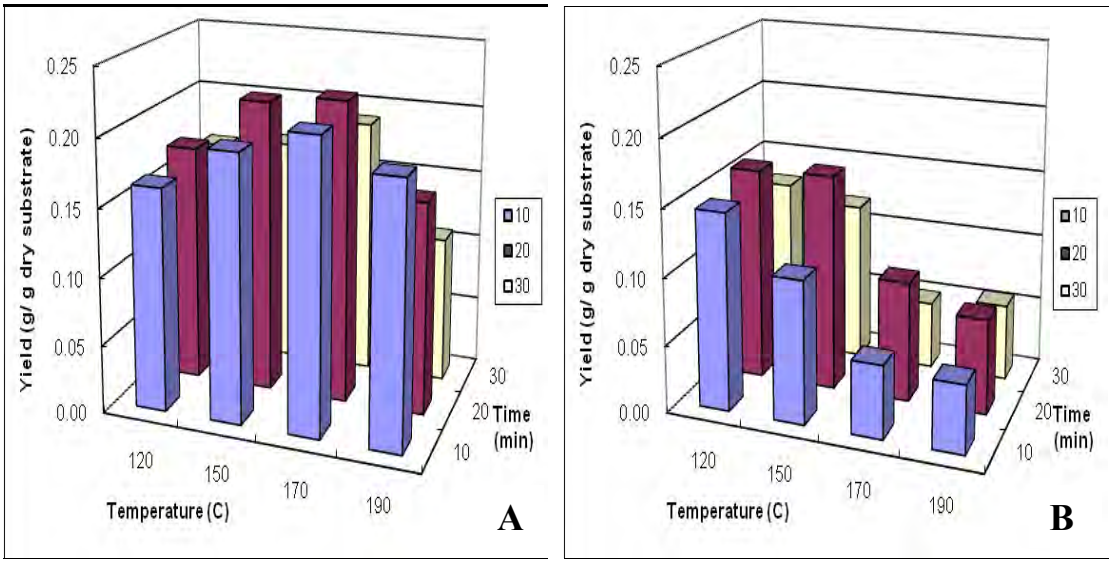

Figure 4.5 Yield of monosugars liberated from sweet sorghum straw when pretreated with 1% sulfuric acid for 10-30 min (A) Yield of glucose ; (B) Yield of xylose

Results for yield of monosugars contained in sweet sorghum straw hydrolyzates, which pretreated by 3%H_2SO_4 at various temperature and residence time was shown in Appendix C (Table C3). The yield of glucose and xylose were shown in Figure 4.6. The maximum yield of glucose was 0.234 g glucose/g dry substrate at 120ºC for 10 min and the maximum yield of xylose was 0.208 g xylose/g dry substrate at the same conditions. In this case, a total of 50.05% of glucan and 76.41% of xylan were converted to glucose and xylose, respectively. The experimental data indicate that glucose yield decreased at pretreated temperature above 120ºC with increasing residence time. The xylose yield in the hydrolyzate gave similar results with increasing of pretreatment severity.

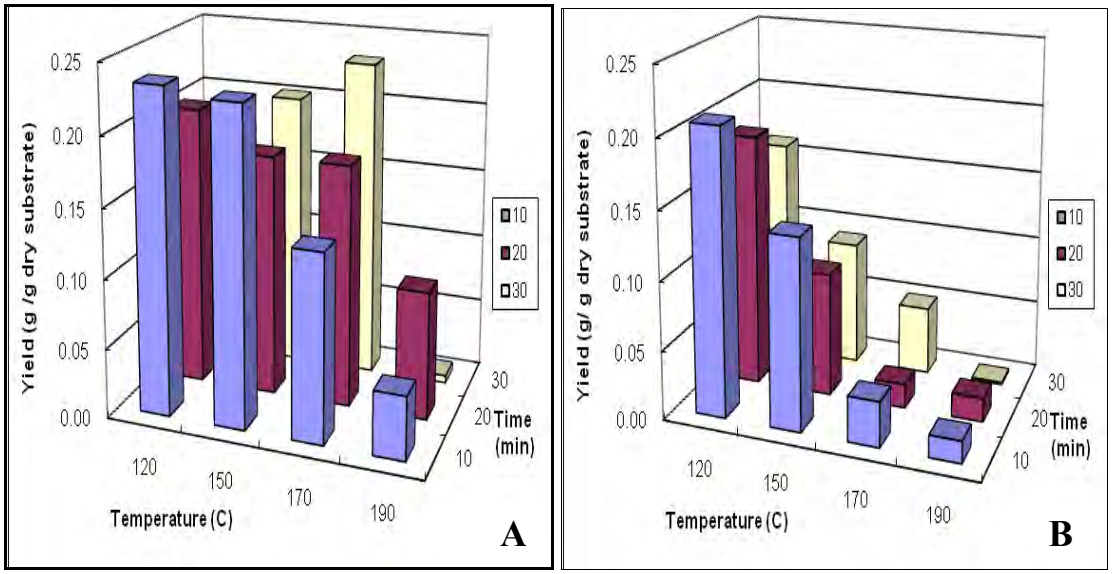

Figure 4.6 Yield of monosugars liberated from sweet sorghum straw when pretreated with 3% sulfuric acid for 10-30 min (A) Yield of glucose ; (B) Yield of xylose

From this study, pretreatment condition at 120°C with increasing sulfuric acid concentration from 0%-3% and residence time from 10-30 min, resulting in increasing yields of glucose and xylose. In contrast, pretreatment temperature in the range 150-190°C, and residence time from 10-30 min, yields of glucose and xylose were decreased when using sulfuric acid concentration above 1%.

All experimental data of sweet sorghum straw were analyzed by factorial design ($p \leq 0.05$).The statistical analysis shown that pretreatment temperature and dilute sulfuric acid concentration had a significant effect on yield of glucose and xylose. By contrast, the residence time for pretreatment did not have a significant effect on the yield of glucose and xylose released from sweet sorghum straw (p = 0.559 and 0.387 respectively). The data was shown in appendix D. From these results, the effects of two parameters (pretreatment temperature and sulfuric acid concentration) were applied for dilute-acid pretreatment of the sugarcane bagasse.

4.5 Dilute-acid pretreatment of sugarcane bagasse

The results for yield of monosugars contained in sugarcane bagasse hydrolyzates, which pretreated by 0 - 3%H_2SO_4 for 20 min was shown in Appendix C (Table C4). The yield of glucose and xylose were shown in Figure 4.7. The maximum yield of glucose was 0.367 g glucose/g dry substrate at 170°C, 3%H_2SO_4 for 20 min and the maximum yield of xylose was 0.226 g xylose/g dry substrate at 120°C, 3%H_2SO_4 for 20 min. In this case, a total of 78.52% of glucan and 83.05% of xylan were converted to glucose and xylose, respectively. From these results, at the same pretreatment temperature, glucose and xylose yields increased when the acid concentration increases from 0%H_2SO_4 to 3%H_2SO_4.

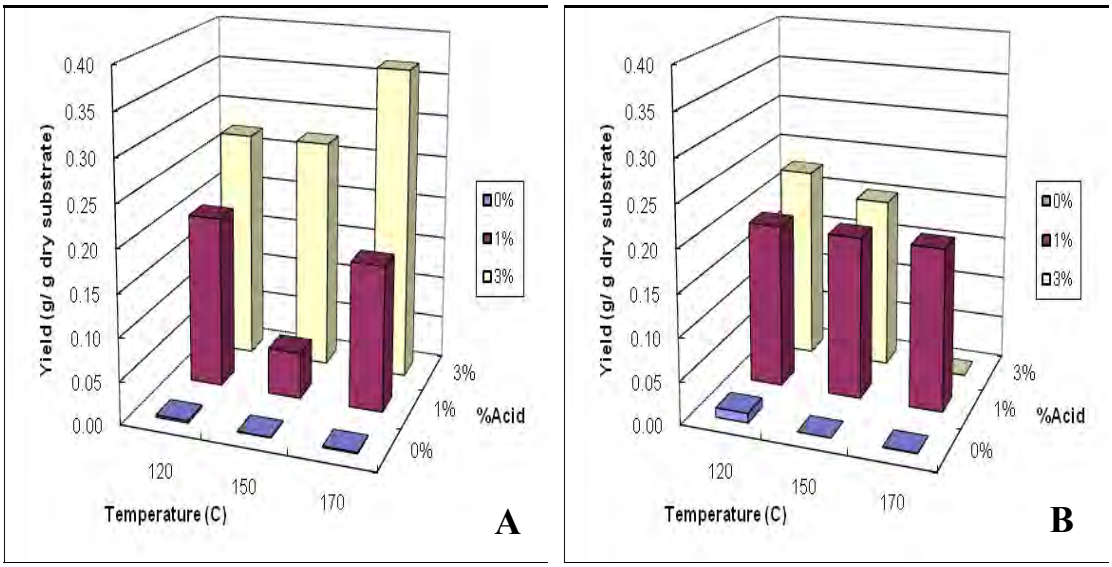

Figure 4.7 Yield of monosugars liberated from sugarcane bagasse when pretreated with 0% (distilled-water), 1% and 3% sulfuric acid concentration for 20 min
(A) Yield of glucose ; (B) Yield of xylose

From the statistical analysis of two parameters (pretreatment temperature and sulfuric acid concentration) showed that dilute sulfuric concentration had a significant effect on yield of glucose and xylose. By contrast, the pretreatment temperature did not have a significant effect on the yield of glucose and xylose released from sweet sugarcane bagasse (p = 0.554 and 0.276 respectively). The data was shown in appendix D.

Table 4.4 Summary of maximum yield and percentage conversion obtained from sweet sorghum straw and sugarcane bagasse at optimal pretreatment condition.

Substrate	**Conditions**	**$Yield_{avg}$ (g monosugar/ g dry substrate)**		**%Conversion**	
		Glucose	Xylose	Glucose[a]	Xylose[b]
Sweet sorghum straw	120°C, 3%H_2SO_4, 10 min	0.234	0.208	50.05	76.41
Sugarcane bagasse	120°C, 3%H_2SO_4, 20 min	0.272	0.226	58.15	83.05
	170°C, 3%H_2SO_4, 20 min	0.367	ND	78.52	ND

[a] %conversion (g glucose/ g glucan) , [b] %conversion (g xylose/ g xylan)

ND : Not detectable

The maximum yield of glucose and xylose obtained from sweet sorghum straw was slightly lower than those obtained from sugarcane bagasse (Table 4.4). From these results, the pretreatment with dilute acid at moderate temperature effectively hydrolyze hemicelluloses to fermentable sugars. Since the structure of cellulose is more complex than the hemicellulose fraction in the plant materials, therefore, saccharification of cellulose requires much severe conditions for their degradation to monosugars.

Based on the experimental results, the pretreatment at severe conditions (high temperature and high sulfuric acid concentration) were not suitable for the hydrolysis because of at these conditions glucose and xylose can be degraded into furfural and hydroxymethylfurfural (HMF), respectively. When furfural and HMF are degraded, formic acid is formed. Levulinic acid is formed by HMF degradation, and phenolic compounds are generated from the partial breakdown of lignin. These compounds are toxic to fermentative microorganisms and inhibit their metabolism (Palmqvist and Hahn-Hägerdal, 2000).

Karimi et al. (2006) reported that when using rice straw as substrate, pretreatment at pretreatment temperature of 201°C, 10 min retention time and 0.5% sulfuric acid, the yield of xylose was about 80% whereas the yield of glucose was about 25% which were lower than yields of sugars obtained from this study. Lavarack et al. (2002) reported that the maximum xylose from sugarcane bagasse was 0.274 g/g solid after hydrolysis at 120 ºC with 4% H_2SO_4 for 50 min which is slightly higher than results from this present study. Saha et al. (2005) reported that about 92% of hemicelluloses was converted to sugars (0.255 g/g dry substrate) and only 47% of cellulose (0.230 g/g dry substrate) was converted to glucose when pretreated wheat straw by 0.75%v/v H_2SO_4 at pretreatment temperature of 121°C for 1 hr and using cellulase (Celluclast) and β-glucosidase (Novozyme 188) for enzymatic saccharification at 45°C, pH 5.0 for 72 hr.

4.6 Ethanol fermentation

Hydrolyzate from pretreated sweet sorghum straw and sugarcane bagasse were used as carbon source for ethanol fermentation process. The fermentation was performed under aerobic condition by *Saccharomyces cerevisiae* and *Pichia stipitis*

4.6.1 Fermentation of sweet sorghum straw hydrolyzates by *Saccharomyces cerevisiae* and *Pichia stipitis*

Results of fermentation of hydrolyzates from acid pretreated sweet sorghum straw by *Saccharomyces cerevisiae* were shown in Table 4.5 and Figure 4.8, and fermentation by *Pichia stipitis* were shown in Table 4.6 and Figure 4.9. The fermentation of 20 g/l of total sugars concentration in hydrolyzate for 46 hr by *Pichia stipitis* was obtained the highest ethanol concentration about 10.17 g/l and productivity of 0.22 g/l/hr. At the same condition, ethanol fermentation by *Saccharomyces cerevisiae* gave the highest ethanol concentration about 6.38 g/l and productivity of 0.53 g/l/hr at 12 hr of cultivation time. When using 20 g/l of total sugars from the hydrolyzate of acid pretreated sweet sorghum straw, ethanol yields from *Pichia stipitis* and *Saccharomyces cerevisiae* were about 50.86 and 31.87, respectively. In contrast, fermentation of hydrolyzate of acid pretreated sweet sorghum straw at high total sugars concentration (50 g/l) resulting in dramatically low both ethanol concentration and yield, as shown in Figure 4.8 and Figure 4.9.

Table 4.5 Ethanol concentration and ethanol yield from fermentation hydrolyzates of acid pretreated sweet sorghum straw by *Saccharomyces cerevisiae*

Hydrolyzate conc. (expressed as TS)	Cultivation time (hr)	Ethanol conc. (g/l)	Yield (%w/w initial total sugars)
20 g/l	0	2.11	10.528
	12	6.38	31.873
	24	5.30	26.479
	36	3.99	19.948
	46	2.63	13.164
	58	0.94	4.718
	74	0.00	0.000
50 g/l	0	0.98	1.950
	12	1.24	2.485
	24	1.40	2.796
	36	1.15	2.301
	46	0.84	1.680
	58	0.31	0.619
	74	0.39	0.773

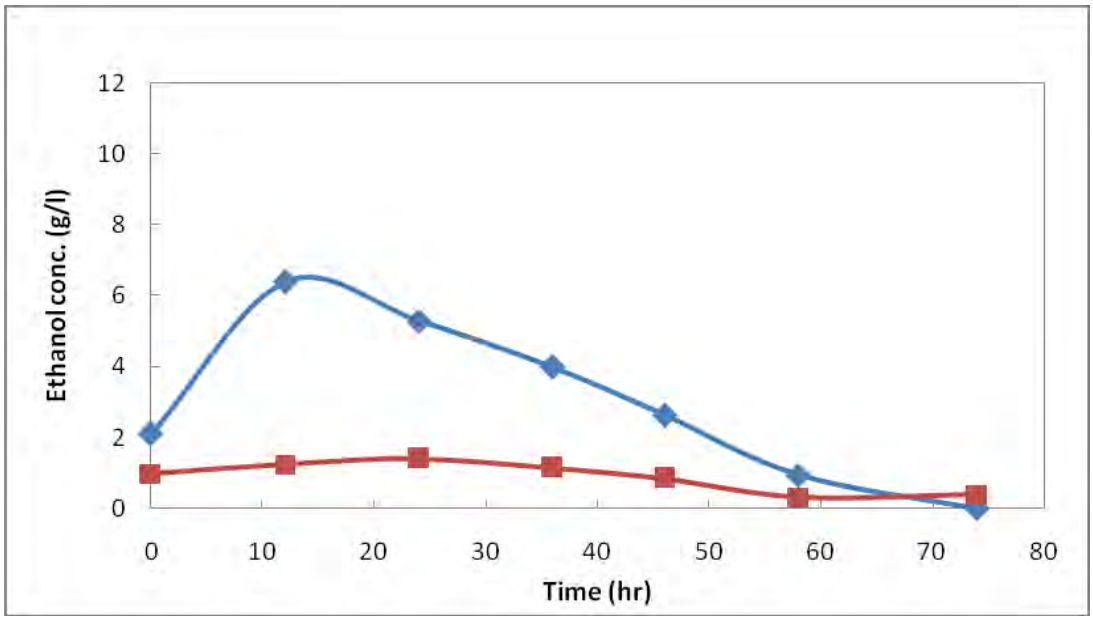

Figure 4.8 Time courses of ethanol fermentation by *Saccharomyces cerevisiae* from acid hydrolyzate of sweet sorghum straw (◆: 20 g/l Total sugars ; ■: 50 g/l Total sugars)

Table 4.6 Ethanol concentration and ethanol yield from fermentation hydrolyzates of acid pretreated sweet sorghum straw by *Pichia stipitis*

Hydrolyzate conc. (expressed as TS)	Cultivation time (hr)	Ethanol conc. (g/l)	Yield (%w/w initial total sugars)
20 g/l	0	0.78	3.883
	12	1.42	7.106
	24	7.39	36.934
	36	9.47	47.350
	46	10.17	50.868
	58	6.17	30.823
	74	2.48	12.405
50 g/l	0	1.07	2.134
	12	1.17	2.332
	24	0.79	1.577
	36	0.70	1.401
	46	0.65	1.294
	58	0.68	1.360
	74	0.18	0.363

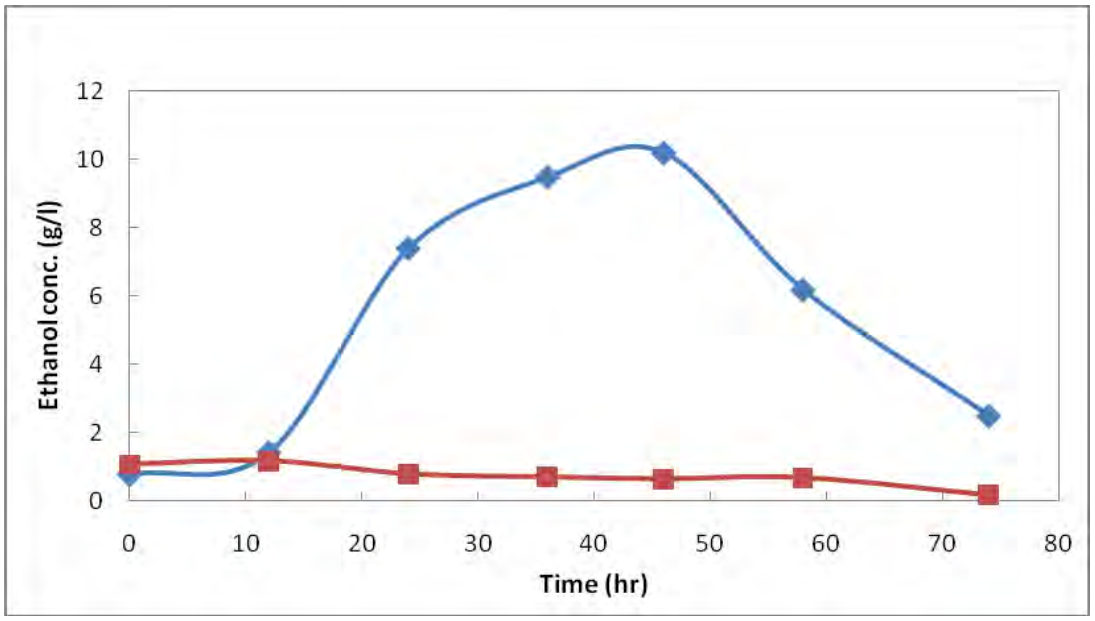

Figure 4.9 Time courses of ethanol fermentation by *Pichia stipitis* from acid hydrolyzate of sweet sorghum straw (◆ : 20 g/l Total sugars ; ■: 50 g/l Total sugars)

4.6.2 Fermentation of sugarcane bagasse hydrolyzates by *Saccharomyces cerevisiae* and *Pichia stipitis*

Results of fermentation of hydrolyzates from acid pretreated sugarcane bagasse by *Saccharomyces cerevisiae* and *Pichia stipitis* were shown in Table 4.7 - 4.8 and Figure 4.10 - 4.11, respectively. The maximum ethanol concentration was 3.73 g/l and productivity of 0.11 g/l/hr when fermentation of 20 g/l of total sugars for 32 hr by *Pichia stipitis*. At the same condition, ethanol concentration of 1.78 g/l and productivity of 0.19 g/l/hr was obtained by *Saccharomyces cerevisiae* at 9 hr of cultivation. Ethanol yields from *Pichia stipitis* and *Saccharomyces cerevisiae* were about 18.66 and 8.92, respectively. In contrast, fermentation of hydrolyzate of acid pretreated sweet sorghum straw at high total sugars concentration (50 g/l) resulting in dramatically low both ethanol concentration and yield, as shown in Figure 4.10 and Figure 4.11.

Table 4.7 Ethanol concentration and ethanol yield from fermentation hydrolyzates of acid pretreated sugarcane bagasse by *Saccharomyces cerevisiae*

Hydrolyzate conc. (expressed as TS)	Cultivation time (hr)	Ethanol conc. (g/l)	Yield (%w/w initial total sugars)
20 g/l	0	1.46	7.304
	9	1.78	8.917
	21	0.69	3.448
	32	0.00	0.000
	44	0.00	0.000
	56	0.00	0.000
	69	0.00	0.000
	77	0.00	0.000
50 g/l	0	1.30	2.600
	9	1.47	2.938
	21	1.39	2.789
	32	1.39	2.778
	44	1.41	2.827
	56	1.18	2.360
	69	0.69	1.386
	77	0.40	0.807

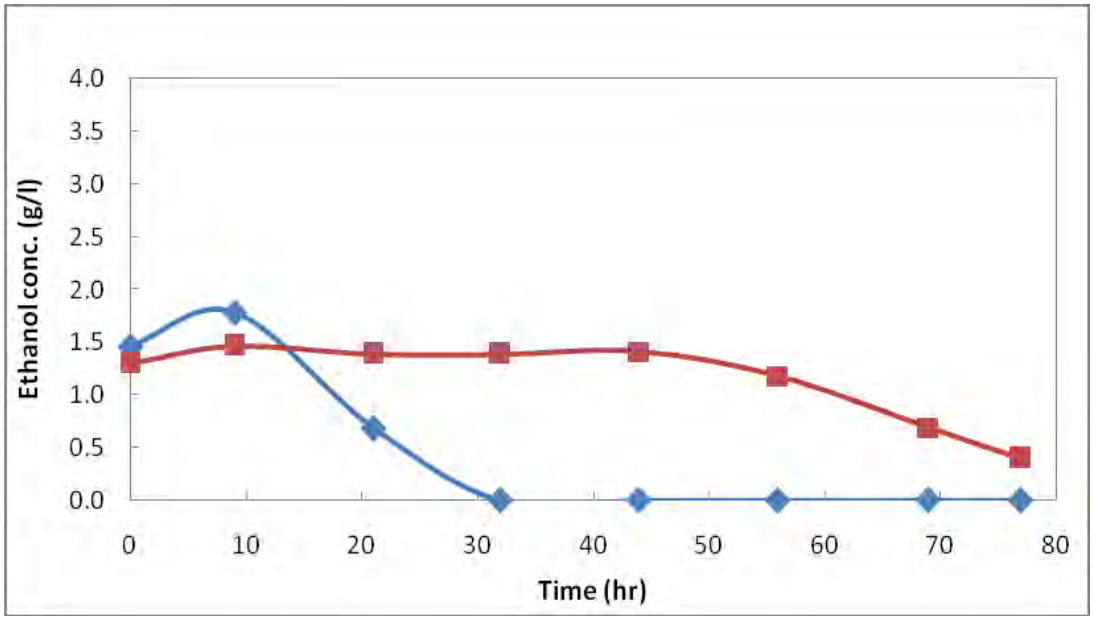

Figure 4.10 Time courses of ethanol fermentation by *Saccharomyces cerevisiae* from acid hydrolyzate of sugarcane bagasse (◆ : 20 g/l Total sugars ; ■ : 50 g/l Total sugars)

Table 4.8 Ethanol concentration and ethanol yield from fermentation hydrolyzates of acid pretreated sugarcane bagasse by *Pichia stipitis*

Hydrolyzate conc. (expressed as TS)	Cultivation time (hr)	Ethanol conc. (g/l)	Yield (%w/w initial total sugars)
20 g/l	0	1.06	5.276
	9	1.80	8.986
	21	2.17	10.856
	32	3.73	18.660
	44	3.43	17.155
	56	0.55	2.731
	69	0.01	0.031
	77	0.00	0.000
50 g/l	0	1.13	2.267
	9	1.03	2.053
	21	1.29	2.570
	32	1.13	2.257
	44	0.95	1.904
	56	0.38	0.758
	69	0.34	0.671
	77	0.04	0.073

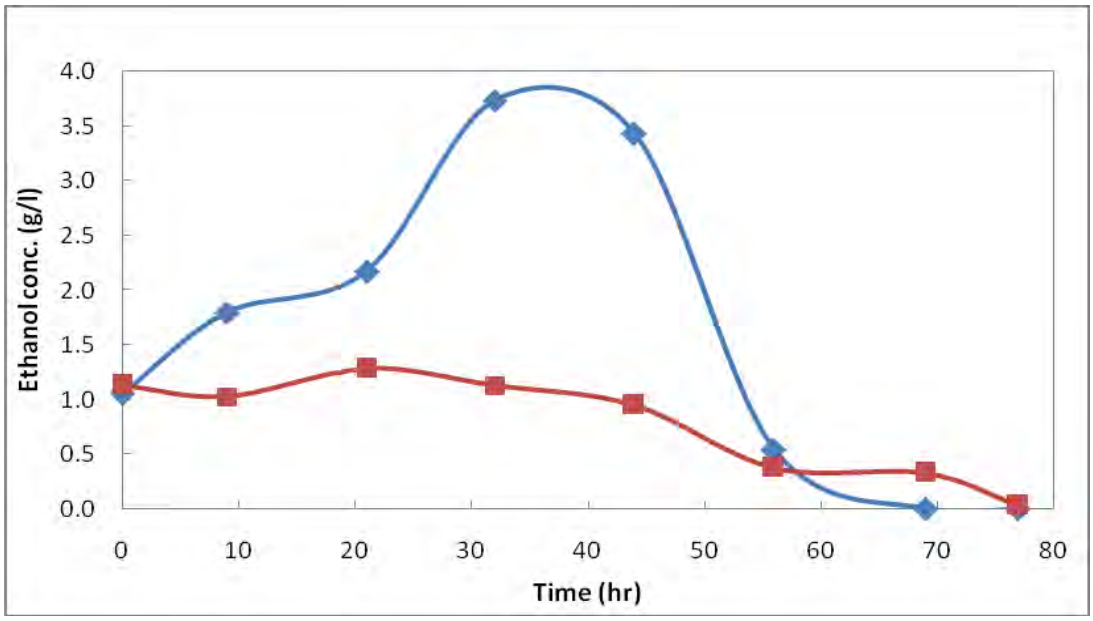

Figure 4.11 Time courses of ethanol fermentation by *Pichia stipitis* from acid hydrolyzate of sugarcane bagasse (◆ : 20 g/l Total sugars ; ■: 50 g/l Total sugars)

Table 4.9 Summary of maximum ethanol concentration and ethanol yield obtained from *Saccharomyces cerevisiae* and *Pichia stipitis* using hydrolyzates of sweet sorghum straw and sugarcane bagasse.

Substrate	*S. cerevisiae*			*P. stipitis*		
	EtOH conc. (g/l)	$Yield_{max}$	Productivity (g/l/hr)	EtOH conc. (g/l)	$Yield_{max}$	Productivity (g/l/hr)
SSS	6.38 (12 hr)	31.87	0.53	10.17 (46 hr)	50.87	0.22
SCB	1.78 (9 hr)	8.92	0.19	3.73 (32 hr)	18.66	0.11

Glucose and xylose are two main components available in the hydrolyzates of acid pretreated lignocellulosic materials. In this study, the fermenting yeasts *Saccharomyces cerevisiae* and *Pichia stipitis* were used for utilization of the fermentative sugars. These yeasts have been using in fermentation of several hydrolyzates of pretreated cellulose materials. Fermentation of 20 g/l of total sugars concentration in sweet sorghum straw hydrolyzate by *Pichia stipitis* at 46 hr gave the maximum ethanol concentration was 10.17 g/l and productivity of 0.22 g/l/hr. On the contrary, at the same condition, fermentation of 20 g/l of total sugars concentration by *Saccharomyces cerevisiae* at 12 hr obtained lesser ethanol concentration of 6.38 g/l and productivity of 0.53 g/l/hr (Table 4.9).

Buaban et al. (2010) reported the ethanol concentration of 8.4 g/l and productivity of 0.35 g/l/hr were attained by separate hydrolysis and fermentation (SHF) of sugarcane bagasse using *Pichia stipitis* BCC15191, at pH 5.5, 30 ºC for 24 hr. Dawson et al. (2007) reported that ethanol concentration of 0.335 g/l was obtained from fermented sugarcane hydrolyzate by *Saccharomyces cerevisiae sp*. for 12 days. Hernández-Salas et al. (2009) reported that fermentation of sugarcane hydrolyzate by *Saccharomyces cerevisiae* for 48 hr produced 5.0 g/l ethanol concentration and productivity of 0.10 g/l/hr. Obviously the maximum attainable ethanol concentration for *Saccharomyces cerevisiae* was low compared with the yeast *Pichia stipitis* (Sanchez et al., 2004).

A critical problem in the fermentation of hydrolyzate from acid-pretreated lignocellulosic materials has been the inability of the fermentative microorganism (such as furfural, HMF, acetic acid and phenolic compounds). These compounds were toxic to microorganisms during fermentation steps. A detoxification step is used to partially or completely remove these inhibitors, consequently improve the fermentation processes (Palmqvist et al., 2000 and Saha et al., 2005). The previous study used chemical and physical detoxification method for reduction the toxic, volatile compounds and some inhibitors present in the hydrolyzate. Physical detoxification method by vacuum evaporation lead to decrease volatile compounds (such as acetic acid, furfural and vanillin) and increases hydrolyzate concentration for fermentation. However, this method also moderately increases the concentration of non-volatile toxic compounds (Mussatto and Roberto, 2004). Consistency with this results indicate that fermentation of 20 g/l of total sugars concentration in hydrolyzate is possible to obtain ethanol concentration higher than fermentation of 50 g/l of total sugars concentration in hydrolyzate, because increasing of total sugars concentration in hydrolyzate lead to increases the non-volatile toxic compounds in hydrolyzate.

CHAPTER V

CONCLUSION

Sweet sorghum straw and sugarcane bagasse have the potential feedstock for ethanol production. Dilute sulfuric acid pretreatment was effective in solubilizing cellulose and hemicellulose in the biomass to fermentable sugars. In case of sweet sorghum straw, the maximum yield of glucose and xylose were 0.234 g glucose/g dry substrate and 0.208 g xylose/g dry substrate, respectively, at the pretreatment condition : 120ºC, 3%H_2SO_4 for 10 min. In this case, a total of 50.04% of glucan and 76.41% of xylan were converted to glucose and xylose, respectively.

In case of sugarcane bagasse, the maximum yield of glucose was 0.367 g glucose/g dry substrate and a total of 78.52% of glucan was converted to glucose at the pretreatment condition : 170ºC, 3%H_2SO_4 for 20 min. The maximum yield of xylose was 0.226 g xylose/g dry substrate and a total of 83.05% of xylan was converted to xylose at the pretreatment condition : 120ºC, 3%H_2SO_4 for 20 min.

Fermentation of 20 g/l of total sugars concentration in sweet sorghum straw hydrolyzate by *P. stipitis* gave the highest ethanol concentration of 10.17 g/l and productivity of 0.22 g/l/hr, at 46 hr of cultivation. In the case of *S. cerevisiae*, ethanol concentration of 6.38 g/l and productivity of 0.53 g/l/hr were obtained at 12 hr of cultivation.

Suggestion for future work

Dilute acid hydrolysis of lignocellulosic materials release oligomers and monosaccharides followed by the breakdown of the glucose released to form inhibitors such as furan derivatives (furfural and 5-hydroxymethylfufural), phenolic chemicals and aliphatic acids. These products are generally considered inhibitors for fermentative microorganisms. Further study is needed to investigate improving the yield of monosugars by avoiding further degradation of those sugars, which subsequently increase fermentable sugars for ethanol production.

In this current research, optimization of the chemical pretreatment has been extensively studied in an attempt to provide useful information concerning the pretreatment conditions applicable directly to other lignocellulosic materials.

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APPENDICES

APPENDIX A

CULTURE MEDIA

1. Yeast Peptone Dextrose (YPD) agar

Yeast extracts	10	g
Peptone	10	g
Glucose	20	g
Agar	20	g
Distilled water	1000	ml

Add the yeast extracts, peptone, glucose and agar in distilled water and then dissolve by streaming. The media were sterilized by autoclave at 121ºC for 15 minutes.

2. Yeast Peptone Dextrose (YPD) borth

Yeast extracts	10	g
Peptone	10	g
Glucose	20	g
Distilled water	1000	ml

Add the yeast extracts, peptone and glucose in distilled water and then dissolve by streaming. The media were sterilized by autoclave at 121ºC for 15 minutes.

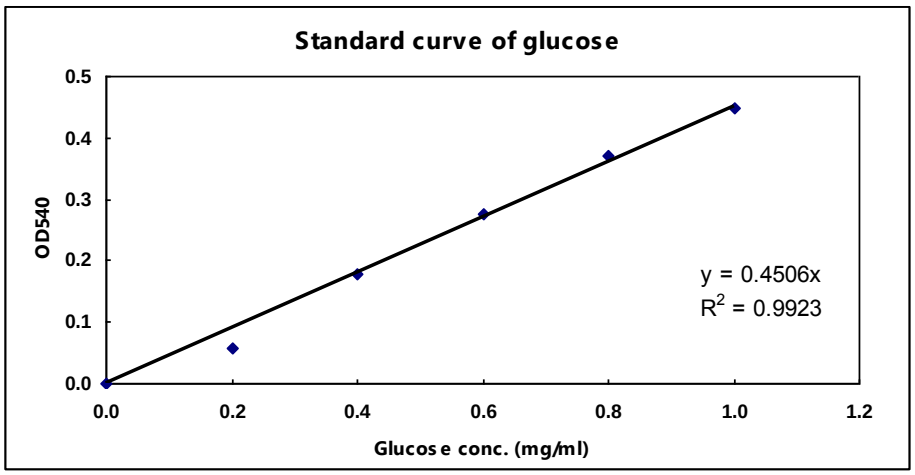
APPENDIX B

B1. Calibration curve for various concentration of glucose by DNSA method

DNSA reagent (Miller, 1959) (per liter)

3,5 –Dinitrosalicylic acid	5.3	g
Sodium hydroxide	9.9	g
Sodium potassium tartratate	153.0	g
Sodium metabisulfile	4.1	g
Phenol (melt at 50ºC)	3.8	ml
Distilled water	708	ml

Dissolve 3,5 –Dinitrosalicylic acid and sodium hydroxide with distilled water, then add sodium potassium tartratate, sodium metabisulfile and phenol in the mixer. Stir this reagent until homogeneously and store in amber bottle.



Slope = 0.4506

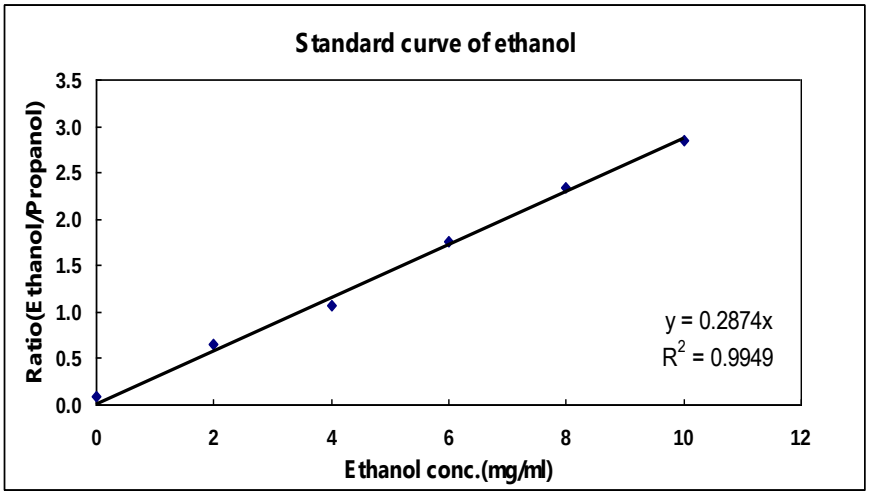
Figure B1 Standard curve of glucose concentration

$$\text{Glucose concentration (mg/ml)} = \frac{1}{\text{Slope}} \times \text{OD 540}$$

B2. Calibration curve for various concentration of ethanol by gas chromatography

Method

1. Prepare standard ethanol at vary concentrations in the range 0–10 mg/ml.
2. Mix sample of standard ethanol 1 ml with 1ml of 3 mg/ml propanol (propanol use as internal standard).
3. Inject 1 µl of mixture solution in gas chromatography to make standard curve.



Slope = 0.2874

Figure B2 Standard curve of ethanol concentration

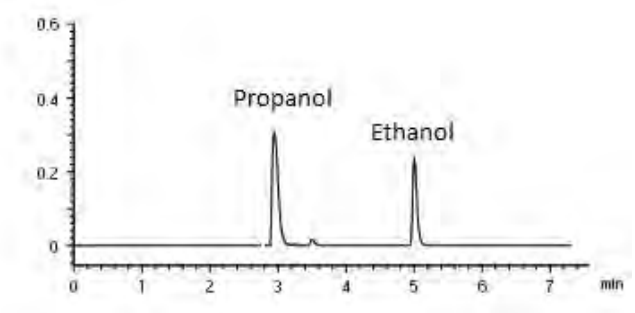
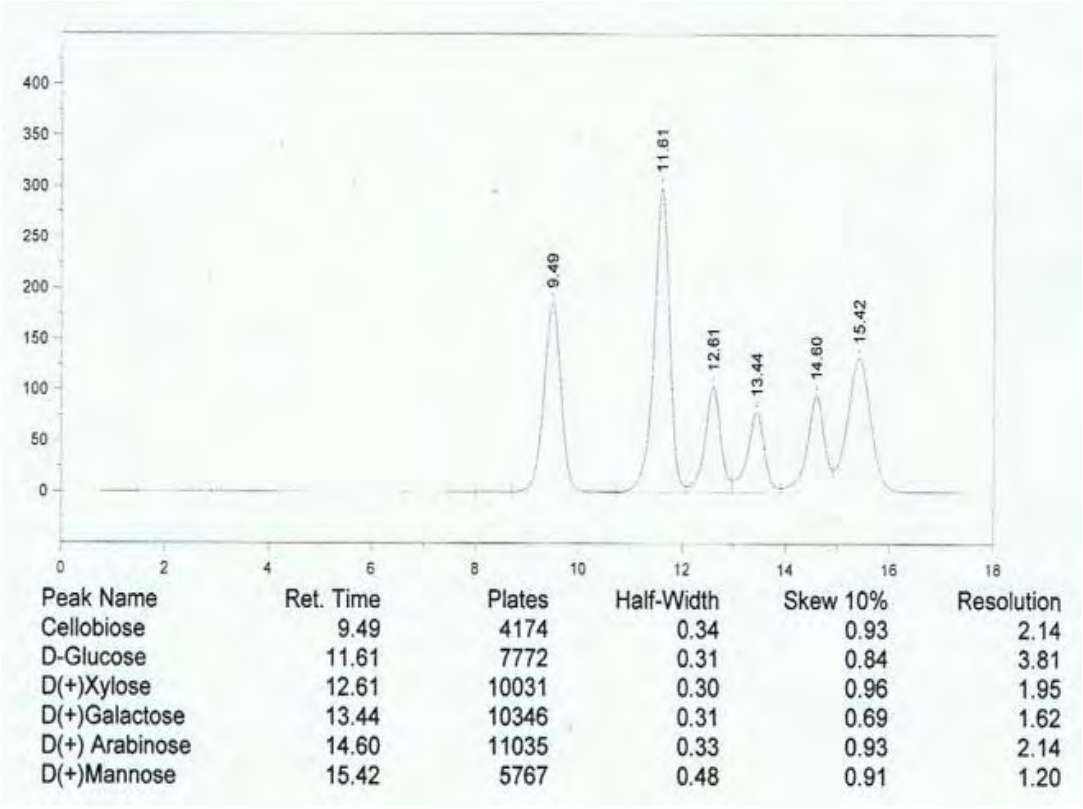


Figure B3 Standard peaks of propanol and ethanol on Porapak Q column

B3. Standard peaks of monosugars determine by HPLC (Aminex HPX-87P Column)



Peak Name	Ret. Time	Plates	Half-Width	Skew 10%	Resolution
Cellobiose	9.49	4174	0.34	0.93	2.14
D-Glucose	11.61	7772	0.31	0.84	3.81
D(+)Xylose	12.61	10031	0.30	0.96	1.95
D(+)Galactose	13.44	10346	0.31	0.69	1.62
D(+) Arabinose	14.60	11035	0.33	0.93	2.14
D(+)Mannose	15.42	5767	0.48	0.91	1.20

Figure B4 Standard peaks of cellobiose and monosugars on the Aminex HPX-87P Column

APPENDIX C

Experimental Data

Table C1 Summary of average yields of monosugars liberated from sweet sorghum straw using distilled-water hydrolysis

Temp (°C)	Time (min)	Yield $_{avg}$ (g monosugar /g dry substrate)			
		Glu	Xyl	Gal, Man, Ara	Total Sugar
120	10	0.027 ± 0.009	0.012 ± 0.002	0.005 ± 0.006	0.044 ± 0.012
	20	0.054 ± 0.010	0.024 ± 0.011	0.025 ± 0.037	0.115 ± 0.078
	30	0.038 ± 0.010	0.007 ± 0.003	0.006 ± 0.007	0.050 ± 0.008
150	10	0.030 ± 0.013	0.009 ± 0.005	0.003 ± 0.002	0.041 ± 0.014
	20	0.043 ± 0.002	0.010 ± 0.008	0.002 ± 0.003	0.054 ± 0.008
	30	0.041 ± 0.008	0.002 ± 0.001	0.005 ± 0.003	0.048 ± 0.008
170	10	0.042 ± 0.005	0.005 ± 0.002	0.009 ± 0.002	0.056 ± 0.008
	20	0.041 ± 0.005	0.005 ± 0.004	0.007 ± 0.002	0.053 ± 0.007
	30	0.038 ± 0.005	0.004 ± 0.002	0.013 ± 0.005	0.055 ± 0.010
190	10	0.032 ± 0.008	0.012 ± 0.006	0.007 ± 0.004	0.051 ± 0.011
	20	0.029 ± 0.018	0.020 ± 0.011	0.009 ± 0.002	0.057 ± 0.031
	30	0.030 ± 0.017	0.024 ± 0.007	0.009 ± 0.003	0.063 ± 0.025

Data are mean values ± S.D. of three replicates.

Table C2 Summary of average yields of monosugars liberated from sweet sorghum straw when pretreated with 1% H_2SO_4

Temp (°C)	Time (min)	Yield $_{avg}$ (g monosugar /g dry substrate)			
		Glu	Xyl	Gal, Man, Ara	Total Sugar
120	10	0.164 ± 0.070	0.146 ± 0.094	0.014 ± 0.004	0.324 ± 0.168
	20	0.175 ± 0.031	0.159 ± 0.037	0.059 ± 0.043	0.393 ± 0.055
	30	0.162 ± 0.053	0.130 ± 0.041	0.086 ± 0.085	0.377 ± 0.103
150	10	0.195 ± 0.086	0.106 ± 0.076	0.086 ± 0.039	0.387 ± 0.132
	20	0.215 ± 0.039	0.161 ± 0.029	0.079 ± 0.051	0.454 ± 0.078
	30	0.168 ± 0.038	0.119 ± 0.078	0.060 ± 0.070	0.347 ± 0.148
170	10	0.213 ± 0.154	0.054 ± 0.044	0.030 ± 0.031	0.297 ± 0.184
	20	0.221 ± 0.071	0.089 ± 0.037	0.063 ± 0.093	0.373 ± 0.127
	30	0.189 ± 0.069	0.050 ± 0.055	0.070 ± 0.073	0.309 ± 0.032
190	10	0.191 ± 0.086	0.050 ± 0.020	0.081 ± 0.063	0.322 ± 0.076
	20	0.155 ± 0.078	0.070 ± 0.019	0.081 ± 0.103	0.307 ± 0.070
	30	0.108 ± 0.098	0.057 ± 0.092	0.074 ± 0.111	0.238 ± 0.025

Data are mean values ± S.D. of three replicates.

Table C3 Summary of average yields of monosugars liberated from sweet sorghum straw when pretreated with 3% H_2SO_4

Temp (°C)	Time (min)	Yield $_{avg}$ (g monosugar /g dry substrate)			
		Glu	Xyl	Gal, Man, Ara	Total Sugar
120	10	0.234 ± 0.079	0.208 ± 0.073	0.235 ± 0.164	0.676 ± 0.230
	20	0.202 ± 0.047	0.184 ± 0.017	0.288 ± 0.093	0.674 ± 0.068
	30	0.162 ± 0.079	0.161 ± 0.078	0.279 ± 0.280	0.602 ± 0.288
150	10	0.227 ± 0.107	0.138 ± 0.047	0.240 ± 0.039	0.605 ± 0.184
	20	0.174 ± 0.072	0.090 ± 0.028	0.201 ± 0.269	0.464 ± 0.356
	30	0.201 ± 0.088	0.091 ± 0.055	0.100 ± 0.173	0.391 ± 0.302
170	10	0.134 ± 0.091	0.033 ± 0.021	0.161 ± 0.278	0.327 ± 0.356
	20	0.136 ± 0.088	0.017 ± 0.009	0.235 ± 0.329	0.389 ± 0.405
	30	0.231 ± 0.012	0.049 ± 0.050	0.269 ± 0.379	0.549 ± 0.349
190	10	0.045 ± 0.015	0.016 ± 0.015	0.229 ± 0.396	0.290 ± 0.384
	20	0.092 ± 0.067	0.017 ± 0.019	0.315 ± 0.350	0.423 ± 0.400
	30	0.005 ± 0.001	0.002 ± 0.000	0.549 ± 0.483	0.556 ± 0.484

Data are mean values ± S.D. of three replicates.

Table C4 Summary of average yields of monosugars liberated from sugarcane bagasse, using pretreatment time for 20 min

Temp (°C)	**%H_2SO_4**	**Yield $_{avg}$ (g monosugar /g dry substrate)**			
		Glu	Xyl	Gal, Man, Ara	Total Sugar
120	0	0.002 ± 0.002	0.011 ± 0.015	0.037 ± 0.049	0.050 ± 0.066
150		0.001 ± 0.000	0.000 ± 0.000	0.003 ± 0.002	0.003 ± 0.002
170		0.001 ± 0.001	0.000 ± 0.001	0.010 ± 0.004	0.012 ± 0.003
120	1	0.201 ± 0.156	0.192 ± 0.072	0.050 ± 0.041	0.443 ± 0.043
150		0.054 ± 0.057	0.190 ± 0.054	0.157 ± 0.091	0.401 ± 0.020
170		0.170 ± 0.054	0.190 ± 0.186	0.118 ± 0.023	0.477 ± 0.262
120	3	0.272 ± 0.028	0.226 ± 0.319	0.009 ± 0.008	0.507 ± 0.356
150		0.271 ± 0.190	0.201 ± 0.073	0.052 ± 0.062	0.524 ± 0.325
170		0.367 ± 0.082	0.000 ± 0.000	0.226 ± 0.311	0.592 ± 0.393

Data are mean values ± S.D. of three replicates.

APPENDIX D

Statistical Analysis of Yields of Glucose and Xylose from Sweet sorghum straw and Sugarcane bagasse

Table D1 Statistical analysis yields of glucose from Sweet sorghum straw

Tests of Between-Subjects Effects

Dependent Variable: Yield of Glucose

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	6348.326[a]	35	181.381	4.769	.000
Intercept	16460.305	1	16460.305	432.791	.000
Temp	811.587	3	270.529	7.113	.000
Acid	4159.407	2	2079.704	54.682	.000
Time	44.624	2	22.312	.587	.559
Temp * Acid	788.817	6	131.469	3.457	.005
Temp * Time	132.556	6	22.093	.581	.744
Acid * Time	64.242	4	16.061	.422	.792
Temp * Acid * Time	347.093	12	28.924	.761	.688
Error	2738.371	72	38.033		
Total	25547.002	108			
Corrected Total	9086.698	107			

a. R Squared = .699 (Adjusted R Squared = .552)

Table D2 Statistical analysis yields of xylose from Sweet sorghum straw

Tests of Between-Subjects Effects

Dependent Variable: Yield of Xylose

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	3956.264[a]	35	113.036	6.491	.000
Intercept	4558.477	1	4558.477	261.773	.000
Temp	1362.479	3	454.160	26.080	.000
Acid	1553.171	2	776.585	44.596	.000
Time	33.511	2	16.756	.962	.387
Temp * Acid	826.612	6	137.769	7.911	.000
Temp * Time	25.966	6	4.328	.249	.958
Acid * Time	88.134	4	22.034	1.265	.292
Temp * Acid * Time	66.390	12	5.532	.318	.984
Error	1253.796	72	17.414		
Total	9768.537	108			
Corrected Total	5210.060	107			

a. R Squared = .759 (Adjusted R Squared = .642)

Table D3 Statistical analysis yields of glucose from Sugarcane bagasse

Tests of Between-Subjects Effects

Dependent Variable: Glucose

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.169[a]	8	.021	15.978	.000
Intercept	.284	1	.284	214.876	.000
Temp	.002	2	.001	.631	.554
Acid	.163	2	.081	61.714	.000
Temp * Acid	.004	4	.001	.784	.563
Error	.012	9	.001		
Total	.464	18			
Corrected Total	.181	17			

a. R Squared = .934 (Adjusted R Squared = .876)

Table D4 Statistical analysis yields of xylose from Sugarcane bagasse

Tests of Between-Subjects Effects

Dependent Variable: Xylose

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.123[a]	8	.015	4.005	.027
Intercept	.208	1	.208	54.084	.000
Temp	.011	2	.006	1.489	.276
Acid	.100	2	.050	13.006	.002
Temp * Acid	.012	4	.003	.763	.575
Error	.035	9	.004		
Total	.366	18			
Corrected Total	.158	17			

a. R Squared = .781 (Adjusted R Squared = .586)

BIOGRAPHY

Mr. Aphisit Poonsrisawat was born on August 10, 1984 in Angthong, Thailand. He received Bachelor Degree of Science in Biotechnology from Department of Biotechnology, Faculty of Science and Technology, Thammasat University, Thailand in 2007. He completed his Master Degree of Science in Biotechnology, Faculty of Science, Chulalongkorn University, Thailand in 2010.

Academic presentations

Poster presentation and proceedings

Poonsrisawat, A., and Teeradakorn, S. Pretreatment of sweet sorghum straw using dilute sulfuric acid saccharification. The 21st Annual Meeting and International Conference of the Thai Society for Biotechnology –*TSB 2009 : Biotechnology: A Solution to the Global Economic Crisis?*". 24–25 September 2009, Queen Sirikit National Convention Center, Bangkok, Thailand.

Oral presentation and proceedings

Poonsrisawat, A., and Teeradakorn, S. Conversion of sweet sorghum straw to sugars by dilute-acid saccharification. The 3rd Technology and Innovation for Sustainable Development International Conference (TISD2010). 4-6 March 2010, Royal Mekong Nongkhai Hotel, Nongkhai, Thailand.