EFFICIENCY OF MULBERRY STALK PRETREATMENT WITH IONIC LIQUID AND ITS APPLICATION FOR SUCCINIC ACID FERMENTATION

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ไม้ชีวมวลเป็นของเหลือทิ้งเส้นใยพืชที่มีมาก และได้รับการคาดไว้ว่าจะเป็นแหล่งชีวมวลพลังงานทดแทนในรุ่นถัดไป กิ่งหม่อนในการวิจัยนี้เป็นของเหลือทิ้งจากบริษัทไหมไทย การปรับสภาพด้วยคอลิเนียมอะมิโนแอซิดของเหลวไอออนิก [Ch][AA] เพิ่มผลผลิตกลูโคสและไซโลส เป็น 74.72 และ 33.05 % จากการใช้ [Ch][Gly] และการปรับสภาพด้วย [Ch][Ala] ได้ผลผลิตน้ำตาลทั้งสองชนิดเป็น 66.15 และ 24.35 % ตามลำดับ ขณะที่ผลผลิตกลูโคส และไซโลสจากการไฮโดรไลซิสกิ่งหม่อนที่ไม่ปรับสภาพมีเพียง 14.93 และ 1.88% การเพิ่มของผลผลิตน้ำตาลสัมพันธ์กับการลดลงของลิกนินในกิ่งหม่อนที่ปรับสภาพ ด้วยค่าการสกัดลิกนินโดย [Ch][Ala] และ [Ch][Gly] คือ 65.15 และ 65.44% การเปลี่ยนแปลงทางกายภาพและเคมีโดยของเหลวอิออนิกมีการแสดงให้เห็นด้วยภาพจากกล้องจุลทรรศน์อิเลคตอนแบบส่องกราด (SEM) สเปกตร้าของ FT-IR และ XRD ซึ่งภาพจาก SEM แสดงให้เห็นโครงสร้างกิ่งหม่อนที่ถูกปรับสภาพถูกเปิดออก และกลายเป็นโครงสร้างที่อยู่กันอย่างหลวมๆ สเปกตร้าของ FT-IR แสดงว่า C=O หายไปอย่างชัดเจนเมื่อเทียบกับกิ่งหม่อนตามธรรมชาติ ดัชนีความเป็นผลึกของกิ่งหม่อนที่ถูกปรับสภาพมีสูงกว่ากิ่งหม่อนตามธรรมชาติ ซึ่งสัมพันธ์กับลิกนินที่ออกจากไม้ชีวมวลนั่นเอง อุณหภูมิและเวลาการบ่มมีผลต่อผลผลิตน้ำตาลและการสกัดแยกลิกนิน โดยผลของน้ำตาลทั้งสองต่ำสุดที่ 60 °ซ การปรับสภาพโดย [Ch][Gly] สามารถเกิดขึ้นได้ที่ 80 °ซ นาน 4 ชั่วโมง โดยสภาวะความดันสิ่งแวดล้อม ซึ่งผลผลิตน้ำตาลและการแยกลิกนินออกไม่น้อยกว่าที่ 90 °ซ นาน 6 ชั่วโมง สารละลาย [Ch][Gly] มีคุณสมบัติที่ดีสำหรับการนำมาใช้ใหม่ โดยไม่เปลี่ยนแปลงในการให้ผลผลิตน้ำตาลและความสามารถในการแยกลิกนิน ผลการทดสอบบ่งชี้ว่า [Ch][Gly] มีคุณสมบัติที่ดีของการเลือกแยกลิกนินได้สูง การแยกส่วนหลังการปรับสภาพถูกเก็บแยกเป็น 2 ส่วนหลัก ได้แก่ วัสดุที่มีคาร์โบไฮเดรตมาก (Carbohydrate rich material; CRM) และวัสดุที่มีลิกนินมาก (Lignin rich material; LRM) วัสดุ LRM ถูกนำกลับได้ >70% และ >90% ของลิกนินเป็นส่วนประกอบใน LRM โครงสร้างทางเคมีของ LRM ถูกทดสอบให้เห็นผ่านสเปกตร้า FT-IR และ ^{1}H-NMR ซึ่งพบว่า หมู่ฟังก์ชั่นและพันธะหลักไม่ต่างจากลิกนินในธรรมชาติหรือลิกนินทางการค้า (ลิกนินด่างของบริษัท Sigma) ในการย่อยไฮโดรไลซิสแบบกะพบว่า การเพิ่มของแข็งช่วยการเพิ่มความเข้มข้นน้ำตาล ผลผลิตน้ำตาลไม่เพิ่มขึ้น ถึงแม้ว่ามีการเพิ่มเอนไซม์จาก 22 เอฟพียู/กรัม เซลลูโลส เป็น 40 เอฟพียู/กรัม เซลลูโลส การย่อยแบบกึ่งกะถูกนำมาช่วยแก้อิทธิพลของปริมาณของแข็งที่สูงในการไฮโดรไลซิสของเอนไซม์ ความเข้มข้นกลูโคสของการใช้ของแข็งรวม 11.2% มวลแห้ง (5.6% + 5.6%) และ 14% มวลแห้ง (5.6%+ 8.4%) ได้ถึง 68.88 และ 77.16 g/l ซึ่งดีขึ้นมามากกว่าการย่อยแบบกะ สำหรับวัสดุ CRM ถูกนำมาใช้ในการผลิตกรดซัคซินิกโดย *Actinobacillus succinogenes* ATCC55618 เพื่อแสดงให้เห็นประสิทธิภาพ CRM จากการปรับสภาพด้วย [Ch][Gly] ที่ของแข็ง 8.4% มีการผลิตกรด

ซัคซินิกจาก SHF ได้ต่ำกว่าระบบแบบ SSF เล็กน้อยด้วยผลผลิตกรดซัคซินิกสูงสุด 0.82 กรัม/กรัม ซับสเตรต และกำลังผลิต 1.18 กรัม/ลิตร/ชั่วโมง จากการหมักแบบ SSF ในขวดไร้อากาศ 50 มิลลิลิตร ในถังหมักชีวภาพ 2 ลิตร การหมักแบบ SSF ด้วยของแข็งรวม 11.2% จากการย่อยแบบกึ่งกะ พบว่าได้กรดซัคซินิกความเข้มข้น 46.93 กรัม/ลิตร และผลผลิต 0.42 กรัม/กรัม ซับสเตรต จากผลทั้งหมดได้แสดงให้เห็นอย่างชัดเจนว่า [Ch][Gly] เป็นตัวทำละลายที่ดีในการแยกลิกนินออกและให้มีการกักเก็บส่วนของโครงสร้างคาร์โบไฮเดรตไว้ ยิ่งไปกว่านั้นคือวัสดุ LRM และวัสดุ CRM สามารถนำกลับมาได้ โดยที่โครงสร้างของ วัสดุ LRM มีคุณสมบัติทางเคมีไม่ต่างจากลิกนินที่มีตามธรรมชาติ และวัสดุ CRM สามารถนำมาใช้ในการหมักกรดซัคซินิกได้เป็นอย่างดี จากการให้ทั้งความเข้มข้น ผลผลิต และกำลังการผลิตของกรดซัคซินิก ผลการวิจัยนี้เป็นการแสดงอย่างชัดเจนมากถึงความมีประสิทธิภาพสูงของการปรับสภาพด้วย [Ch][Gly] และการเดินหน้าไปสู่ผลิตภัณฑ์การผลิตผลิตภัณฑ์ชีวเคมีจากชีวมวลต่อไปได้

สาขาวิชาเทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา_____

ปีการศึกษา 2561 ลายมือชื่ออาจารย์ที่ปรึกษา_____

WORANUD PAKDEEDACHAKIAT : EFFICIENCY OF MULBERRY STALK PRETREATMENT WITH IONIC LIQUID AND ITS APPLICATION FOR SUCCINIC ACID FERMENTATION. THESIS ADVISOR : ASSOC. PROF. APICHAT BOONTAWAN, Ph.D. 155 PP.

CHOLINIUM BASED IONIC LIQUID/WOOD BIOMASS/ PRETREATMENT/ SUCCINIC ACID

Wood biomass is an abandoned lignocellulosic waste and expected to be a renewable energy source in the next generation. Mulberry stalk (MS), for this research is the agricultural waste from a Thai silk company. Pretreatment of MS with cholinium glycinate [Ch][Gly] improved glucose and xylose yields to be 74.72 and 33.05%, respectively, and glucose and xylose yields from cholinium alanate [Ch][Ala] pretreatment was 66.15 and 24.35%, respectively. Whereas, glucose and xylose yield from hydrolysis of the untreated MS was only 14.93 and 1.88%, respectively. The increase in sugar yields relative to lignin removed by pretreatment, lignin extractability by [Ch][Ala] and [Ch][Gly] was 65.15 and 65.44%, respectively. The physical and chemical change by ionic liquid was illustrated using SEM, FT-IR, and XRD spectra. The SEM revealed that pretreated MS feature was an opened and loose structure after pretreatment step. FT-IR spectra showed C=O was significantly removed as compared with native MS. Crystalline index of pretreated MS was slightly higher than the native biomass, which corresponded to lignin removed from the wood biomass. [Ch][Gly] was selected and applied for the further studies. The temperature and incubation time affected the sugar yields and lignin extractability, in which were the lowest the result of both were at 60 °C. Pretreatment by [Ch][Gly] can occur at 80°C for 4 h with an ambient pressure, in which sugar yields and lignin extractability was not lower than the sugar yields and lignin extractability at 90°C for 6 h. The solvent of [Ch][Gly]

could be reused and not affect the sugar yield and lignin extractability. The results indicated that [Ch][Gly] has a good ability to remove lignin with highly selective lignin. The fraction after pretreatment was collected as 2 main fractions which were carbohydrate rich materials (CRM) and lignin rich materials (LRM). LRM was recovered with >70% recovery and >90% lignin component in LRM. Chemical structure of LRM was investigated via FT-IR and ^{1}H NMR spectra which found that the main functional group and bonding is not different from the native lignin. In batch hydrolysis, an increase of solid loading enhanced the sugar concentrations. The sugar yields were not increased even a raise of enzyme dosage from 22 FPU/g cellulose to be 40 FPU/g cellulose. Fed-batch hydrolysis was applied to overcome the effect of high solid loading in enzymatic hydrolysis. At 11.2% (5.6% + 5.6%) and 14% (5.6% + 8.4%) dry matter total solid loading, glucose concentrations were 68.88 and 77.16 g/l which were higher than batch-hydrolysis. The CRMs were applied for succinic acid production by *Actinobacillus succinogenes* ATCC55618 to investigate efficiency of CRM from [Ch][Gly] pretreatment. At 8.4% solid loading, succinic acid production from SHF was lower than SSF process with the highest yield 0.82 g/g substrate and productivity 1.18 g/l/h from SSF in 50 ml anaerobic bottle. In 2 L stirred bioreactor, the SSF process with fed batch hydrolysis of 11.2% total solid, 46.93 g/l succinic acid concentration and 0.42 g/g substrate succinic acid yield were obtained. In all the results investigated, the [Ch][Gly] showed a good ability to selectively remove lignin while retaining the carbohydrate structure. Furthermore, fractionation of LRM could be recovered after processing and chemical structure was not different from native lignin. Fraction of CRM could be applied for succinic acid fermentation with high productivity. These results were an impact confirmation of the high efficiency of [Ch][Gly] pretreatment and a possibility for bio-chemical products in the next generation of bio-energy.

School of Biotechnology Student's Signature _____

Academic Year 2018 Advisor's Signature _____

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CHAPTER I

INTRODUCTION

1.1 Rationale and background

Lignocellulose is an abundant material in agricultural countries including Thailand. These materials contain of structural carbohydrate and cross linking phenolic polymer comprising the construction in plant cell wall. Since, lignocellulosic materials based on agricultural wastes and forest residual and waste, they can be available without competitive growing land with food crops. It was expected as the third generation of renewable resource of energy in world. However, the complicated structure in cell wall made it is not easy for biomass conversion and application for biochemical production. The structural polysaccharide contain link aromatic unit with hydrogen bond to be a protector complexity functional of plant cell wall. Structural polysaccharide divides to cellulose, hemicellulose and pectin. Cellulose is a linear chain of monomer of glucose linkage by β-1-4 as polymer which contains crystallinity more than amorphous structure. Hemicellulose is the branch chain of heteropolysaccharide structure, xylan as a major component that consists of a homopolymer backbone of *β*-1,4- linked D-xylose units with random side chains such as arabinose, glucuronic acid, ferulic acid, and acetic acid. Xylan softwood is glucomannan as the principal component which consists of arabinofuranose unit linked α-1, 3 to xylose polymer backbone (Thakur, 2006). Lignin is a highly cross-linked aromatic polymer of phenylpropane units; *p*-coumarylalcohol, coniferyl alcohol, and sinapyl alcohol. These phenylpropane compounds are called monolignols as they are fundamental units in lignin (Keshwani, 2010). Most lignin in softwood biomass is in a group of G lignin which is copolymerized by guaiacyl and normally, bond with sugar residual in hemicellulose.

Polymers of aromatic carbon of lignin link with physical or chemical bond with carbohydrate polymer and mainly link with hemicellulose constituents, which make a barrier on enzyme accessibility on cellulose fibril or hemicellulose. Removing of lignin is an important process to enhance enzyme accessibility on cellulose/hemicellulose structure. Conversely, removing recalcitrant structure is a challenge redundacy toward the next generation renewable carbon sources of lignocellulosic materials. Various pretreatment methods including physical (Zhang *et al*., 2015), chemical (Zhang *et al*., 2013), physicochemical (Kumara *et al*., 2009) and (Huran *et al*., 2011) and biological/combination physical or chemical methods and biological pretreatment (Yu *et al*., 2009) have been applied to enhance releasing of monomer of sugar yield, but limitation of each method was considered such as inhibitors owing to chemical reaction, low selective delignification, and high energy consumption (Brodeur *et al*., 2011). Ionic liquids (ILs) contain wholly ion and keep a liquid state at ambient temperature (Financie *et al*., 2016). Recently, several types of ILs have been synthesized with various physical, chemical, and biological properties that are specific for its application (Freemantle, 2010). ILs shows ability to disrupt interaction of non-covalent bond linking between polymers in the network and to breakdown hydrogen bond of cellulose polymer (Lopes *et al*., 2013). Some type of IL for an example; cholinium alkanoate showed high efficiency dissolution of cellulose which involved with hydrogen formation between anion or cation of IL and hydroxyl of cellulose network (Zhang *et al*., 2014) However, these substances are general synthesized by using of petrochemical derivative substance, and always show ability dissolution of cellulose. Recently, biocompatible or natural resources of substances are used to be the substrate for synthesized of ILs such as cholium, diethylethanolamine, amino acid and glycerol (Fang *et al*., 2014). Choline-amino acid based room temperature ILs ([Ch][AA]) are combined under the consideration of nontoxicity material, biodegradability, and biocompatibility. [Ch][AA] ILs can be applied with pretreatment of lignocellulosic biomass before

enzymatic hydrolysis. Cholinium based ILs showed high lignin dissolving capabilities (Hou *et al*., 2015). Efficiency of [Ch][AA] based ILs pretreatment on wood biomass such mulberry stem, and its functional application have not been well appearance. In this paper, the small anion amino acid combined cholinium based ionic liquids were investigated impacts on mulberry stalk wood biomass pretreatment and increasing of released glucose yield even under mild condition, as well as lignin removal ability by reused [Ch][Gly]. Moreover, efficiency of fractional material of [Ch][Gly] pretreatment was also investigated. LRM was a material for lignin purification and CRM was applied in succinic acid fermentation by *Actinobacillus succinogens* ATCC55618.

1.2 Research objectives

1.2.1 To investigate pretreatment efficiency of small molecule of cholinium based amino acid ionic liquid on wood mulberry stalk (MS).

1.2.2 To enhance glucose and xylose yield from mulberry stem under ambient pressure.

1.2.3 To recover and reuse cholinium based amino acid ionic liquid after each batch of pretreatment step.

1.2.4 To recover lignin from mixture of lignin/IL after pretreatment and demonstrate extracted lignin properties.

1.2.5 To apply pretreated wood mulberry stalk for succinic acid fermentation in separation hydrolysate and fermentation (SHF) and semi-simultaneous saccharification and fermentation (semi-SSF).

1.3 Research hypothesis

Lignocellulosic wastes including of wood biomass are abundant materials from agricultural and forest. Mulberry stalk is in a group of lignocellulosic material which represent for wood forestry or agro-industry wastes. These materials contain structural carbohydrate and aromatic polymer. It can be converted into valuable products via biorefinery and fermentation process. However, its complicate structure made it is not easy to separate polymer of sugar and aromatic structure. Pretreatment step is necessary to improve enzyme accessible on polysaccharide structure. Various pretreatment steps have been used for conversion the materials, nevertheless pretreatment may be caused of inhibition of enzyme activity and microbial in the following step. The solvent of cholinium based ionic liquid was reported as high selective lignin removal and non-toxicity (Ninomiya *et al.*, 2013). This research, [Ch][AA] was synthesized and applied for enhance sugar yield. Furthermore, the fraction from pretreatment was applied for lignin recovery and organic acid fermentation such as succinic acid.

1.4 Scope of thesis

1.4.1 Mulberry stalk was supported from Thai silk company at Phetchabun Province. MS was dried in hot air oven, mill and crash with the sieves size less than 0.3 mm and keep in dry place prevent from moisture and insect until use.

1.4.2 Two types of cholinium amino ionic liquid ([Ch][AA]) of this study were cholinium glycinate ([Ch][Gly]) and cholinium alanate ([Ch][Ala]). Both of [Ch][AA] were synthesized according to Ren *et al*., (2016), Tou *et al*., (2013) and Liu *et al*., (2012). The chemical character of [Ch][Gly] and [Ch][Ala] were demonstrated with FT-IR and H^1NMR.The both of [Ch][AA] were applied in pretreatment step to investigate efficiency of [Ch][AA] pretreatment by determination of glucose, xylose yield compare with native

biomass and lignin extractabilities. Physical and chemical changes were determined with scanning electron micro scope (SEM), FT-IR spectra and crystalline index.

1.4.3 The solvent of [Ch][AA] was recovered and reused after pretreatment step to investigate efficiency of [Ch][AA] reuse and study the effect of incubation temperature on efficiency of [Ch][AA] solvent. Pretreatment MS conditions were at various temperature and incubation time with an ambient pressure.

1.4.4 Fractionation of materials occurred after pretreatment by recovery 2 materials such as recovered lignin (Lignin rich material; LRM) and material containing of polysaccharide as a main component (Carbohydrate rich material; CRM). FT-IR, ^{1}H NRM spectra and TGA was the methods for demonstration of lignin rich material properties.

1.4.5 Two types of enzymatic hydrolysis were applied to enhance sugar concentration and yield of hydrolysis of pretreated MS such as batch and fed batch enzymatic hydrolysis.

1.4.6 The CRMs were applied for succinic acid fermentation by *Actinobacillus succinogenes* ATCC55618. The fermentations were processed by 2 methods such as separate hydrolysis and fermentation (SHF) and semi-simultaneous saccharification and fermentation (semi-SSF). Fermentation profiles were monitored through succinic acid, lactic acid, acetic acid, formic acid and sugar concentration.

1.5 Expected results

Solvent of [Ch][AA] improved sugar yield >70% glucose yield as compare with native biomass (mulberry stalk without pretreatment or initial mulberry stalk) and lignin can be removed from wood biomass. IL solvent is recovered and reused which does not decrease in sugar yield and lignin extractability. IL has high performance even at mild condition without high pressure. Lignin is recovered from the mixture of extracted lignin/IL. Furthermore, pretreated biomass is non-toxicity on enzymatic hydrolysis and

bacterial strain in the following step of fermentation. Bacterial strain (*Actinobacillus succinogenes*) utilized sugar from hydrolysis of the solid residue after pretreatment. These results are important for the conversions of wood biomass or forest lignocellulosic wastes to the valuable products.

CHAPTER II

LITERATURE REVIEWS

2.1 Lignocellulosic materials

Biomass comes from a variety of sources as following Figure 2.1. European committee for standardization published two standards for classification and specification (EN 14961) and quality assurance (EN 15234) of biomass (Busa, 2018).

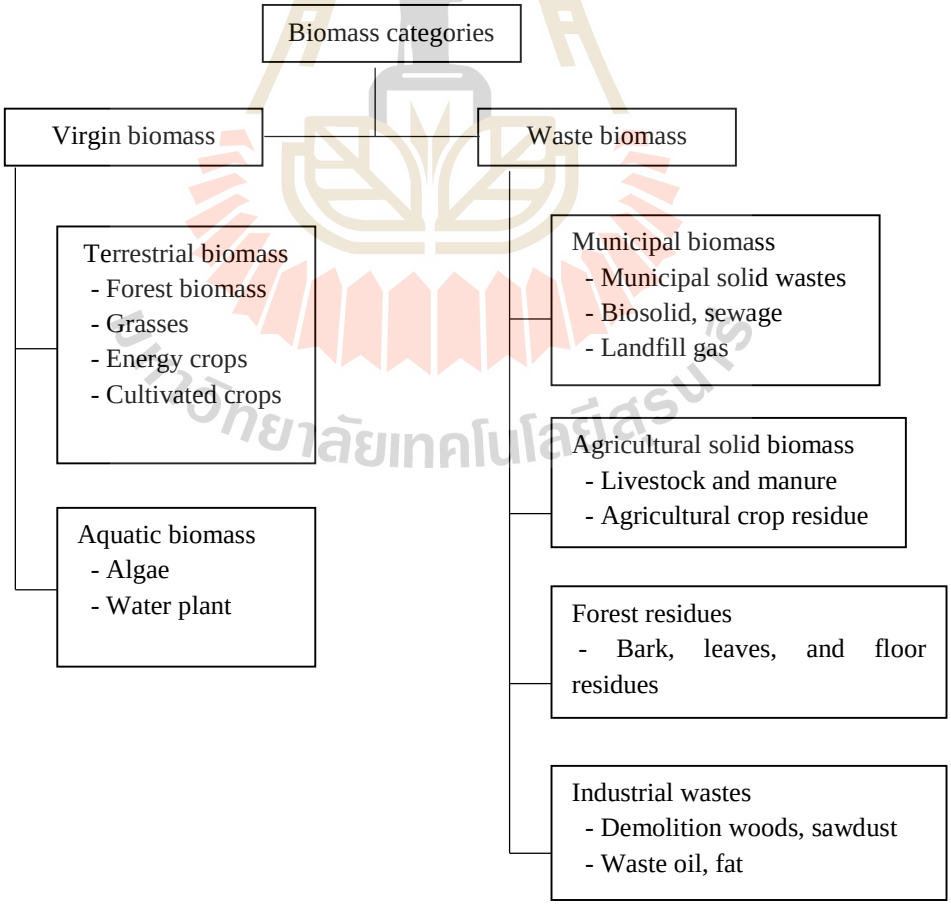


Figure 2.1 Two major groups of biomass and their subclassification (Modified from Busa, 2018).

Based on their origin, biomasses are classified under four broad categories: 1) Woody biomass 2) Herbaceous biomass 3) Fruit biomass and 4) Blend and mixtures (Busa, 2018). Biomass may also divide into two categories:

1) Virgin biomass includes wood, plant, leaves (lignocellulose), crops and vegetables.
2) Wastes biomass includes solid and liquid wastes [municipal solid waste (MSW)]; sewage, animal, and human wastes; gases derived from landfilling (mainly methane); and agricultural wastes.

Lignocellulosic waste can be classified in a group of virgin biomass or waste biomass depend on source of them. Lignocellulosic biomass is nonstarch, and most of fibrous part of plant materials. Lignocellulose refers to plant dry material (or biomass) for example: sugarcane bagasse, rye straw, corn husk, and empty pod. Lignocellulose structure composed of cellulose, hemicellulose, pectin, and lignin.

2.1.1 Main chemical component of lignocellulose

2.1.1.1 Cellulose is the primary structure in plant cell walls. Cellulose is the major component in paper, cardboard, and textiles made from plant fibers. The purest form of natural cellulose is cotton fiber, which is about 98% of pure cellulose. Woody biomass composed of about 40-50% cellulose, 30-45% cellulose in agricultural residues, and 25-50% cellulose in grasses. Cellulose is a linear polysaccharide consisting of β-D-glucose units that are linked in glycosidic bonds (Figure 2.2). Each cellulose chain can contain up to 15,000 D-glucose units. The repeat unit is cellobiose , which is two D-glucose units linked by β-1,4-glycosidic bond (Keshwani, 2010). Cellulose is a long chain polymer with high degree of polymerization (<10,000) and large molecular weight >500,000. Cellulose is a crystalline structure which made up of polymer of glucose (Busa, 2018).

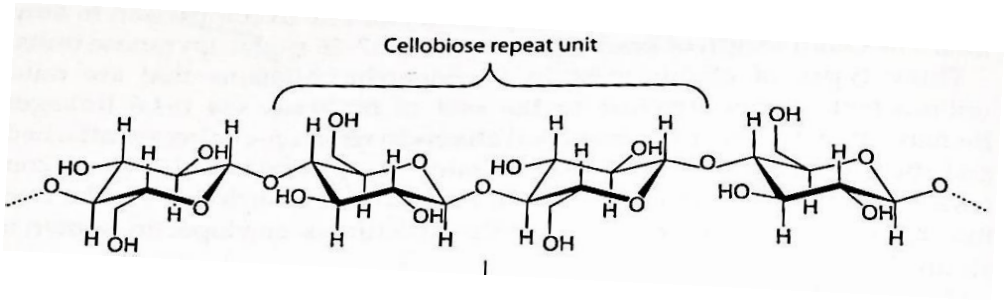


Figure 2.2 Structure of cellulose with *β*-1, 4-linked D-glucopyranose (Keshwani, 2010 p. 24).

The β configuration at the anomeric carbons gives rise to a stretched chain conformation, with hydrogen bonds linking these chains into flat sheets. This is in contrast to starch, which has a helical shape due to the α configuration at the anomeric carbon (Figure 2.3 a and b). The linear conformation enables the packing of numerous cellulose strands into crystalline fibrils (O'sullivan, 1997). In biosynthesis, native cellulose made up of three hydrogen bonds per glucosyl unit occurs: two intramolecular hydrogen bonds and one intermolecular hydrogen bond to neighboring cellulose molecule in the same sheet (Qian *et. al.*, 2005). The sheets interact mostly through van der Waals interactions which contribute significantly to the stabilization of cellulose (Nishiyama *et. al.*, 2002). The short oligomers and glucose monomer are water-soluble but cellulose polymer is not. The solubility is usually inversely relative to polymer length and low flexibility of cellulose polymer chains the intermolecular hydrogen-bonding and hydrophobic flat tops and bottom surface enabling van der Waals interactions between sheets allow intimate and ordered packing of cellulose strands and contribute to the polymer's insolubility in water and most solvents (Brandt *et. al.*, 2013).

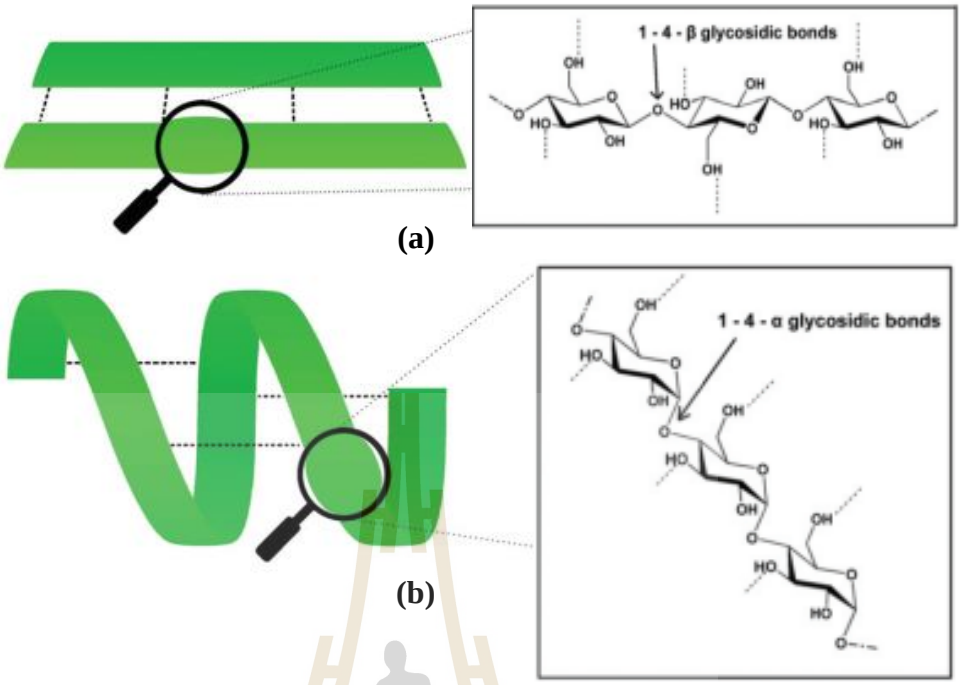
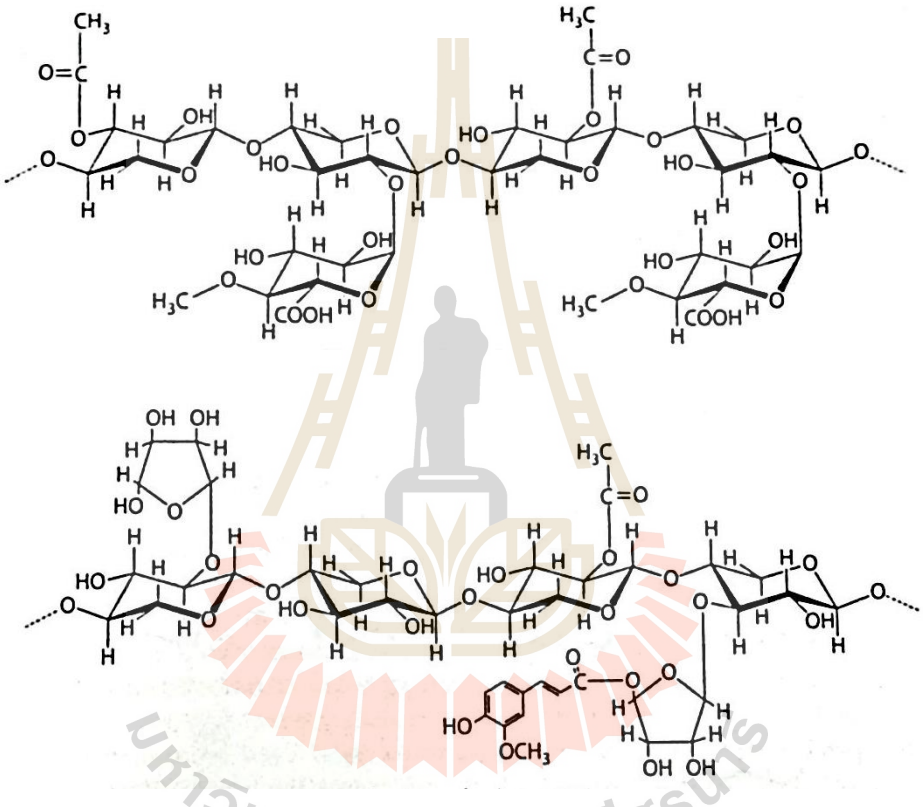


Figure 2.3 Impact of the geometry anomeric carbon on the polymer conformation. Beta configuration flat sheet of stretched chain of cellulose which consist in hydrogen bond link between strands **(a)** and helical conformation of starch with α-configuration **(b)** (Brandt *et. al.*, 2013).

2.1.1.2 Hemicelluloses; the amount of hemicellulose is about 25-30% in softwoods and hardwoods, 20-25% in agricultural residues, and 15-30% in grasses. Hemicelluloses are branched heteropolymer with monosaccharide units that include pentose (arabinose, and xylose), hexose (glucose, galactose, mannose, rhamnose, and fucose) and uronic acid (galacturonic, glucuronic, and methylglucoronic). Nearly, all hemicelluloses are either xylans or glucomannans. Xylans are heteropolysaccharides that consist of a homopolymer backbone of *β*-1, 4- linked D-xylose units with random side chains such as that arabinose, glucuronic acid, ferulic acid, and acetic acid. Glucomannans are heteropolysaccharides that consist of a polymer backbone of *β*-1,4-D-glucose and D-mannose units and may contain random side chains of galactose residues (Keshwani,

2010). In hardwoods and grasses, the major hemicelluloses are xylans (Figure 2.4 a, b) and the minor hemicelluloses are glucomannans (Figure 2.5a). In softwoods, the major hemicelluloses are galactoglucomannans (Figure 2.5b) and the minor hemicelluloses are arabinoglucuronoxylans (Figure 2.5c).



(a)

(b)

Figure 2.4 General structures of xylans (major hemicellulose) glucuronoxylan in hardwoods **(a)** and arabinoxylan in grasses **(b)** (Keshwani, 2010 p. 27).

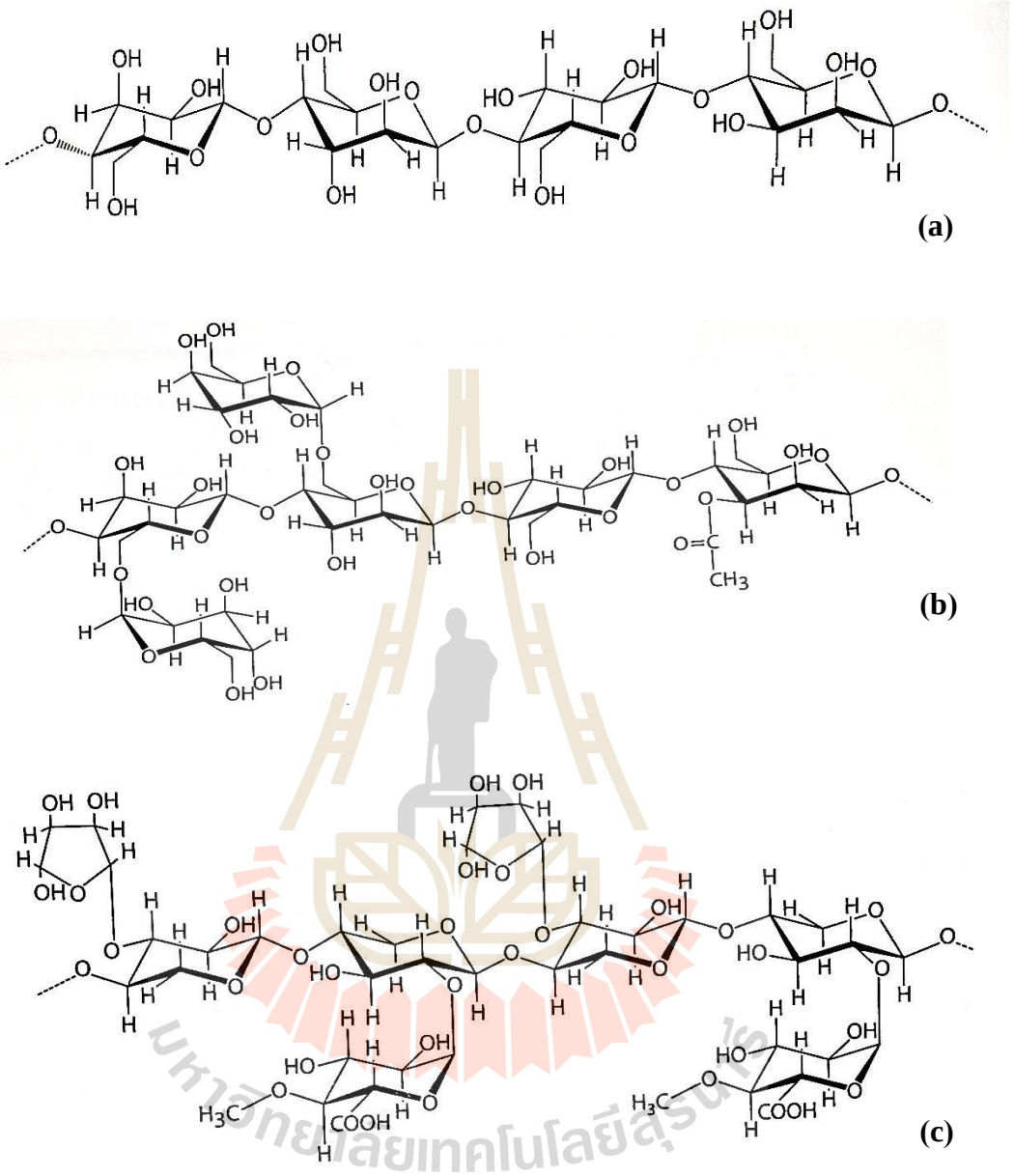


Figure 2.5 General structures of glucomannan (minor hemicellulose) in hardwoods and grasses **(a)**, galactoglucomannan in softwoods **(b)**, and arabinoglucuronoxylans in softwoods **(c)** (Keshwani, 2010 p. 28).

2.1.1.3 Lignin is defined as a highly cross-linked aromatic polymer of phenylpropane units (Fig 2.6a) with a molecular weight in excess of 10,000 units. The fundamental building block of lignin structure starts with biosynthesis of phenylpropane. Conversion of glucose into aromatic amino acids by the shikimic acid pathway and amino acid (; phenylalanine or tyrosine) is converted into three types of phynylpropane units; coniferyl alcohol, sinapyl alcohol, and *p*-coumaryl alcohol. These phenylpropane compounds are called monolignols as they are fundamental units in lignin (Figure 2.6b). There are formed link between two monolignols with many pattern of the bonding (Figure 2.7). Incorporated into the lignin polymer, the subunits are identified by their aromatic ring structure and therefore called guaiacyl, syringyl and *p*-hydroxyphenyl subunit, respectively (Brandt *et. al.*, 2013). Guaiacyl units are more likely to C-C cross-link at the C-5 position of the ring; these cross-links can form during lignification and during delignification (Boerjan *et. al.*, 2003). The C5 position is substituted in the syringyl unit therefore cannot participate in substitution reactions. The C-C cross-links cannot be hydrolyzed by acid or base, making delignification of softwood (high content of S unit) more difficult than hardwood (high content of G unit) and grasses (Brandt *et. al.*, 2013). The lignin contains a variety of linkages. The most common linkage is the β-O-4 ether bond with about 50% of all inter-subunit linkages are of this type (EI *et. al.*, 2009). The β-O-4 ether bonds lead to a linear elongation of the polymer. Other C-O and C-C linkages are presented in lower abundance, and branching occurs with lignification (Brandt *et. al.*, 2013).

Lignin property is very less hydrophilic than cellulose and hemicellulose. Thus, it is the main part structure to support vascular tissue in plant and some algae. It prevents the absorption of water by these polysaccharides in plant cell walls and allows the efficient transport of water in the vascular tissues. Lignin also forms an effective barrier against attack by insects and fungi. (Lignoworks, 2015). The amount of lignin ranges from 15-25% for most grasses and hardwoods and up to 40% in softwoods. Lignin is separated

from wood or raw material in the processes of paper production and it has economic significance in several industrial. Large amount of lignin is separated from wood in the paper industry. Moreover, separated lignin is modified and there are used in several purposes such as; dispersing agent in oil-well and cement application, adhesion agents for peletization of animal feed, tanning agents in the leather industry, and raw material for vanillin production (Keshwani; in Cheng, 2010).

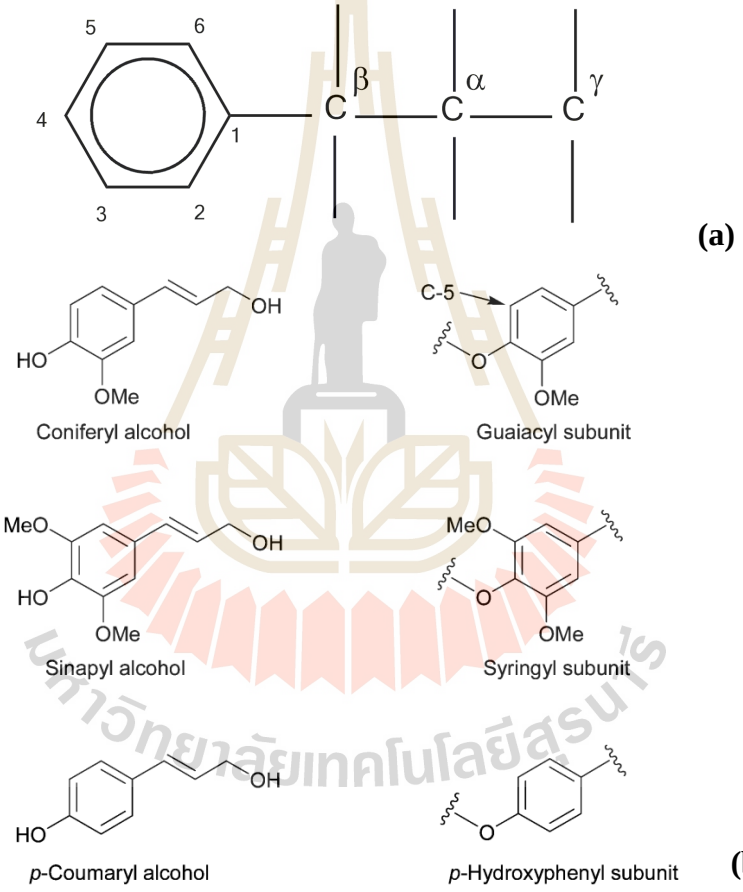


Figure 2.6 Phenyl propane unit (a) (Bykov, 2008) and the three monolignols and

monomers vary in the substitution at the C-3 and C-5 ring positions (b) (Brandt *et. al.*, 2013).

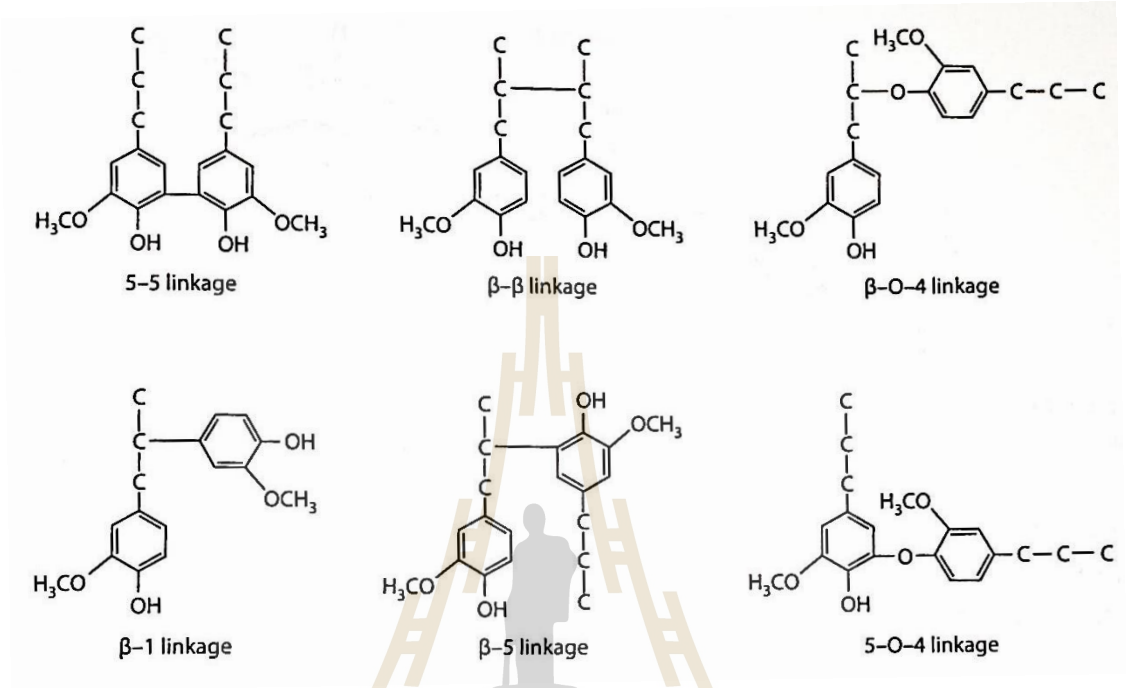


Figure 2.7 Example of carbon-carbon and ether linkages typically found in lignin (Keshwani, 2010 p. 31).

2.1.1.4 Pectin, Lignocellulose consisting mainly of cellulose, hemicellulose and lignin, it also contains smaller amounts of pectins, inorganic compounds, proteins and extractives such as waxes and lipids. Pectins are a family of heteropolymer and homopolymer polysaccharides. These polysaccharides are dominated by galacturonic acid units, which account for approximately 70% of all monomeric units in pectin. The dominant polysaccharide in the pectin family is homogalacturonan, which accounts for approximately 65% of al pectins. Homogalacturonan is a homopolymer of approximately 100 α-1,4-linked galacturonic acid units (Figure 2.8) (Keshwani, 2010).

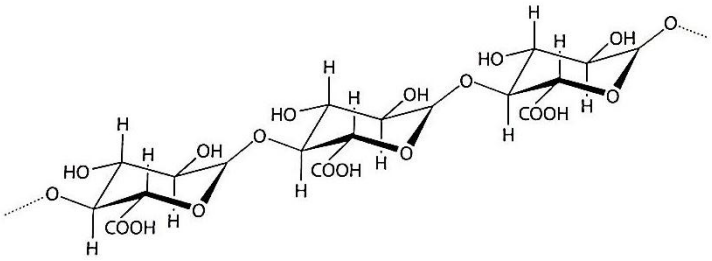


Figure 2.8 General structures of homogalacturonan (major pectin polysaccharide) (Keshwani, 2010 p. 32)

2.1.2 Interaction between polysaccharide and polysaccharide with lignin

The polysaccharide and non-polysaccharide structure is linked together between cellulose and hemicellulose and polysaccharide with lignin (Figure 2.9). These interpolymer linkage is considered for improvement the efficiency of pretreatment process (Brandt *et al.*, 2013). The linkage of each polymer structure have been identified. There are hydrogen bond between lignin and polysaccharide and also found in the linkage of cellulose and hemicellulose. However, the conclusion of connecting linkage between each polymer is still not clear (Harmsen *et. al.*, 2010).

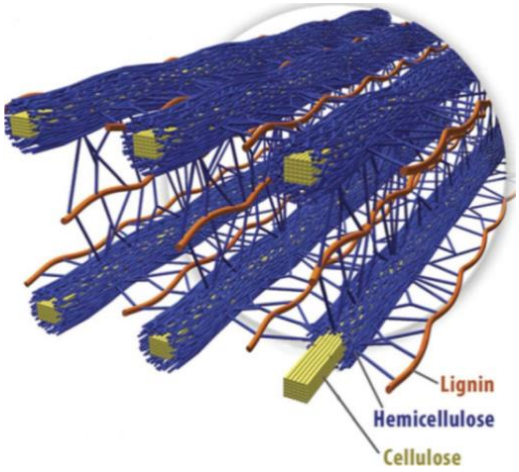


Figure 2.9 Illustration of arrangement of cellulose, hemicellulose and lignin in cell wall of lignocellulosic biomass (Brandt *et al.*, 2013).

Plant surrounding of lignocellulose made up of biopolymer to strengthen the plants' structure or ultrastructure. There are three major types of lignocellulosic biomass such as softwood, hardwood and grasses or herbaceous plant and non-herbaceous plant. In this study, mulberry stem was used as raw materials for ionic liquid pretreatment and applied for lignin separation and succinic acid fermentation. Mulberry tree is a woody plant that produces wood as its structural tissue. Differences between woody plants and herbaceous plant; 1) woody plants have wood as structural tissue, have hard and woody stem whereas herbaceous plant normally have soft and green stem, 2) Woody plants are typically perennial plant whereas herbaceous plants can be annual plant, biennial plant and perennial plant. 3) Woody plants include of shrub and tree whereas herbaceous plants include of annual plant, biennial plant and perennial plant.

The cells in woody tissues are elongated and interconnected via tiny holds in the walls (pits and perforation plates), making wood an anisotropic material. These long perforated cells create channels that enable transport of nutrients and water between root and leaves. Between adjacent cell walls is a lumen called the middle lamella, whose content holds the cell walls together (Figure 2.10). It is devoid of cellulose fibrils and therefore rich in hemicellulose. In mature wood tissue, the middle lamella becomes heavily encrust with lignin (Brandt *et. al.*, 2013). Wood cells that carry fluids are also known as fibers or tracheids. They are hollow and contain extractives and air. These cells vary in shape but are generally short and pointed. The tracheids or cells typically form an outer primary and an inner secondary wall. The middle lamella is predominantly made of lignin. The secondary wall (inside the primary layer) is made up of three layers: S1, S2, and S3 (Figure 2.11). The thickest layer, S2, is made of macrofbrils, which consist of long cellulose molecules with embedded hemicellulose. The S2 layer has the highest concentration of cellulose. The highest concentration of hemicellulose is in layer S3.

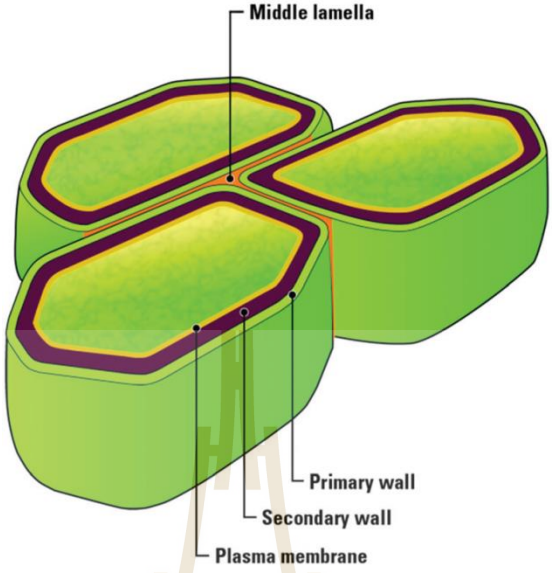


Figure 2.10 Porous structure of lignocellulosic tissue (Brandt *et al.*, 2013).

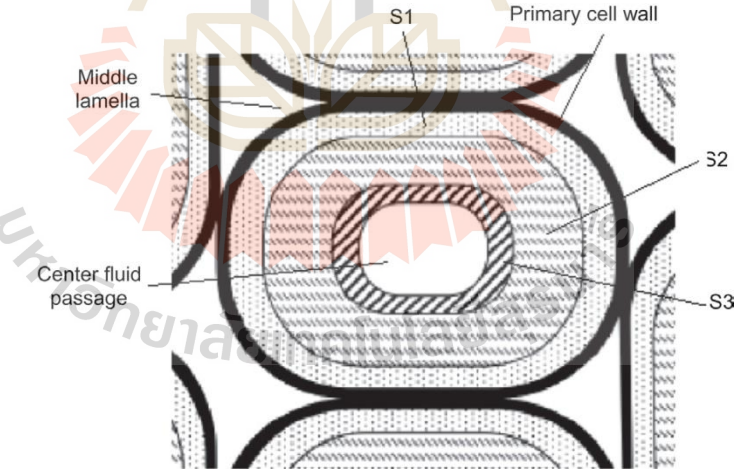


Figure 2.11 Layer of wood cell (Busa, 2018).

Biomass lignocellulosic materials composed of different amount of chemical component (Table 1). Amount of chemical component depends on type of lignocellulose, lived (age of plant), and plant organ.

Table 2.1 Chemical component of some lignocelluloses.

Material	Component (%)				References
	Cellulose	Hemicellulose	Lignin	Ash	
Corn husk	20.47	46.13	3.93	2.08	Pakdeedachakiat, 2008
Rice straw	48.28	27.80	5.63	8.74	Pakdeedachakiat, 2008
Cassava bagasse	36.6	21.3	17.3	7.0	Amenaghawon, 2014
Eucalyptus chips	40	16.1	23.1	2.0	Canettieri, 2007
Sugar cane bagasse	39.5	22.1	17.1	2.3	Martin, 2006

Normally, conversion of lignocellulosic material to biochemical products derived into 2 main steps such as:

1. Pretreatment and hydrolysis of lignocellulosic material to fermentable sugar or reducing sugar.

2. Fermentation of fermentable sugars to products.

2.2 Pretreatments of lignocellulosic materials

The conversion of lignocellulose to another product has two mainly ways: 1) chemical hydrolysis and 2) enzymatic hydrolysis. Many research reports investigated that enzymatic degradation of lignocellulosic material without pretreatment step is not effective due to inaccessible of cellulase or hemicellulase to polysaccharide structure (Elemike *et. al.*, 2015 and Ko *et. al.*, 2015). Pretreatment techniques have been used together and application with another technique to more efficiency. Nowadays, pretreatment processes have developed by application another process to improve degradation and provide structure of lignocellulose for enzymatic hydrolysis. Moreover, hydrolysate should be low contents of inhibitor that effect on microbial cell.

2.2.1 Type of pretreatment

Pretreatment method derived in to 4 types:

2.2.1.1 Physical pretreatment: this technique put the force on structure of lignocellulose and including the step for decreasing size of material for example: grinding, milling, and pyrolysis.

2.2.1.2 Chemical pretreatment: this technique uses chemical substance at optimum temperature to degradation or separation of lignocellulose structure such as acid hydrolysis, ozonolysis, alkaline hydrolysis, oxidation delignification, and organosolv process

2.2.1.3 Physical-chemical pretreatment: this technique combines between physical factors and chemical substance for example; steam explosion (autohydrolysis), ammonia fiber explosion, liquid hot water, wet oxidation, and CO_2 explosion.

2.2.1.4 Biological pretreatment: this technique use biocatalyst (enzyme) or some type of microorganisms that can produce enzyme system to complete degradation of lignocellulose. The advantage and disadvantage of each type of pretreatment (Table 2).

Table 2.2 Advantage and disadvantage of pretreatment of lignocellulose (Modified from สุขใจ, 2554 and Cheng, 2002)

Method	Advantage	Disadvantage and Limitation
Mechanical	- Decreasing of crystallize	- More energy use than energy of biomass
Acid hydrolysis	- Hydrolyzed cellulose to monosaccharide, and changing in lignin structure	- Sugar decomposition - Corrosive equipment, and toxic
Alkaline hydrolysis	- Degradation crosslinking xylan hemicelluloses and lignin - Disruption lignin structure - Less of inhibitor generation	- Less efficiency when high content of lignin
Steam explosion	- Hydrolyzed lignocellulose, lignin deconstructed	- Incompletely degradation of carbohydrate-lignin - Generation of inhibitor
CO_2 explosion	- Increasing of area hydrolysis, low cost, non-inhibitor	- Non-change in lignin and hemicellulose
Biological	- Non inhibitor - Able to ethanol fermentation during hydrolysis reaction	- Rate of reaction is very low

2.2.2 Alkaline pretreatment

Alkaline pretreatment is a type of pretreatment that applied base solution such as; sodium, potassium, calcium, and ammonium hydroxide. This technique is not need high energy in the reaction which is different from another method such as, acid pretreatment. Alkaline pretreatment has the effect on ester bond that cross-linked between cellulose and hemicellulose or lignin. The affecting of alkaline or alkaline peroxide on lignocellulosic materials is degradation of ester and glycocidic linkage. Alkaline hydrolysis

reacts on the ester bond between polysaccharide and lignin and this reaction is saponification (Cheng, 2010).

2.2.3 Ionic liquid

Ionic liquids (ILs) are compound of ion with combination of anions and cations in liquid. Melting point is less than 100 °C and it can dissolve combination of organic and inorganic compounds. ILs have been used to solvent in various chemistry process and chemistry industrial such as; lithium sulfur batteries, heat transfer media for solar thermal system. ILs are applied to be a solvent in pretreatment of lignocellulosic biomass. Choline (or other names choline cation or 2-hydroxyethyl (trimethyl) azanium) is in a group of vitamin B complex which is water-soluble nutrient. Choline contain cation of nitrogen ion.

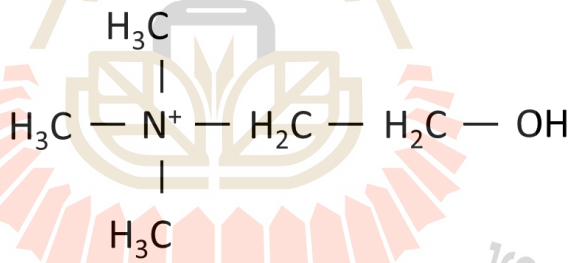


Figure 2.12 Choline structure.

Generally, ILs is synthesized from imidazolium and pyridinium but it was demonstrated drawback of biodegradability, biocompatibility, and component from non-renewable sources. Biomaterials can be used to component for ILs synthesis such as cholinium-amino acid based ILs. Choline-amino acid based room temperature ILs are combined under the consideration of nontoxicity material, biodegradability, and biocompatibility. Physico-chemical characterization is in the term of viscosity and density. Cholinium-amino acid based ILs can be applied with pretreatment of lignocellulosic biomass before enzymatic hydrolysis. Choline-Lysine (;[Cho][Lys]) and Choline-Aspartate

([Cho][Asp]) were applied with corn stover and subsequently with commercial enzyme CTec and HTec, the result showed the process need water to improve before hydrolysis less than water requirement of acid or alkaline pretreatment, moreover bioethanol can be processed in one pot. Aspartate (or aspartic acid; Asp) is negatively charge, polar amino acid. Lysine (Lys) is essential amino acid that has positively charge, polar amino acid (Figure 2.13).

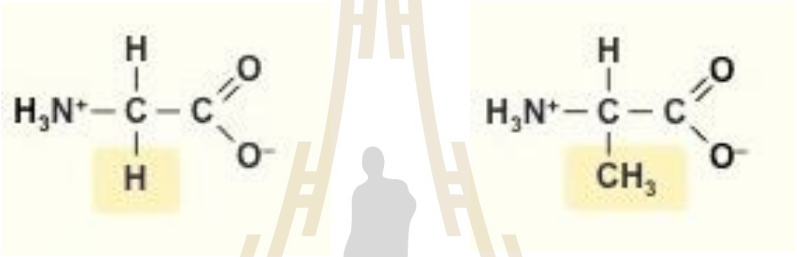


Figure 2.13 Amino acid structure of glycine (A) and alanine (B).

2.2.4 Limitation of conversion of lignocellulose on fermentation process

1. Amount of monosaccharide sugar or fermentable sugar from pretreatment on enzymatic hydrolysis. The affecting on enzymatic hydrolysis:

1.1 Porosity; the porosity between polysaccharide structure and polysaccharide/lignin structure to increasing of enzyme accessible on cellulose structure.

1.2 Characterization of cellulose structure; the crystalline part of cellulose is more resistance to hydrolysis than amorphous structure.

2. Inhibitors from pretreatment effect on organisms during the fermentation process. By-product from pretreatment process may act as inhibitors of microorganism. The variety and concentration of inhibitory compounds depend on pretreatment conditions for example: treatment materials, temperature, pH, pressure, and time duration. Inhibitors have generally been as weak acids, furan, and phenolic. However, inhibitor is removed by

physical, chemical, and biological methods (Liu and Blaschek, 2010 In Vertes, 2010) (Table 3).

Table 2.3 Pretreatment of lignocellulose and treatment to remove inhibitors.

Substrate	Hydrolysis method	Treatment to remove inhibitor	References
Bagasse	Alkali pretreated + enzyme hydrolyzed	Ammonium sulfate + activated carbon	Soni *et. al.*,1982
Rice straw	Above	Above	Soni *et. al.*,1982
Corn stove	Steam explosion	$Ca(OH)_2$	Marchal *et. al.*, 1986
Dried distillers' grains and soluble	Ammonia fiber explosion	None	Ezaji and Blaschek, 2008
Above	Hot water	None	Ezaji and Blaschek, 2008
Above	Dilute sulfuric acid	Over liming	Ezaji and Blaschek, 2008

After pretreatment; pretreated biomass is followed by enzymatic hydrolysis. Microorganisms will use initial sugar from pretreatment for initiation growth.

2.2.5 Lignocellulolytic microorganisms

Lignocellulose structure has complicate structure to prevent plant cell from the plant pathogens infection or offense from plant pest sometime called these structure as recalcitrance. Degree of recalcitrance of plant cell is concerned about accessible of enzyme to polysaccharide structure. Early pioneer noted that although many microorganisms grow rapidly on cellulose, only a few produce extracellular cellulases capable of converting crystalline cellulose to glucose (Sternberg, 1976).

2.3 Degradation of lignocellulosic materials by enzymatic system

Conversion of plant fiber can occur in natural environment which can be degraded by decomposer in ecosystem. Matrix structure in plant fiber is complicate to complete degradation which it need enzymatic system to convert polysaccharide into monosaccharide or fermentable sugar. The mainly type of enzymes involve degradation of bond on lignocellulosic materials are composed of cellulolytic enzyme, xylanolytic enzyme and ligninase.

2.3.1 Cellulolytic enzyme

This group of enzyme has an important function on degradation of cellulose and cellulose derivative. Cellulolytic enzyme composed of three mainly types of enzyme such as; cellobiohydrolase, endogucanase, and β-glucosidase (Martti, 1983 and Kubicek, 1992). The general properties of cellulase as shown below (Pranee, 2000):

1. Average molecular weight 63,000 Da
2. Optimum pH 5.5-6.0
3. Inhibited by heavy metal, sulfidyl group, oxidizing-reducing reagent, and high concentration of product which is glucose
4. Activity measurement from reducing sugar product by using of high soluble substrate such as carboxymethyl cellulose

Each type of enzyme of cellulolytic enzyme have different properties and specific with bond on cellulose structure (Lee *et. al.*, 2002):

2.3.1.1 Cellobiohydrolase; synonym is cellobiase or exoglucanase or 1, 4-β-D-glucan-cellobiohydralase. This enzyme catalyst hydrolysis reaction on crystalline cellulose hydrolysis or cellulose swollen, 4-methylumbelliferyl-cellodextrin including cellotetraose to weakening of hydrogen bond and produce final product as cellobiose. Product of this enzyme is subsequent degraded by endoglucanase.

2.3.1.2 Endoglucanase or 1, 4- β -D-glucan-4-glucanohydrolases (EC 3.2.1.4) has a function to degradation of cellulose or derivative of water soluble cellulose, but it cannot degrade complicated structure of cellulose. The enzyme has random activity on internal amorphous sites in cellulose structure. This enzyme composed of 2 groups such as 1) endo-β-1,4-glucanase degrade by random on internal polymer structure and generate product as oligomer and glucose, and 2) exo- β-1,4-glucanase degrade from non-reducing end of polymer and produced β to α configuration and generate product as cellobiose and glucose.

2.3.1.3 β-glucosidase or β-D-glucoside glucohydrolase (EC 3.2.1.21) This enzyme similar with exo- β-1,4-glucanase but rate of reaction of β-glucosidase decrease on long chain polymer. This enzyme hydrolyzes with common substrate or normal cellulose and cellobiose to cellohexose (or cellodextrins) and generate product as D-glucose.

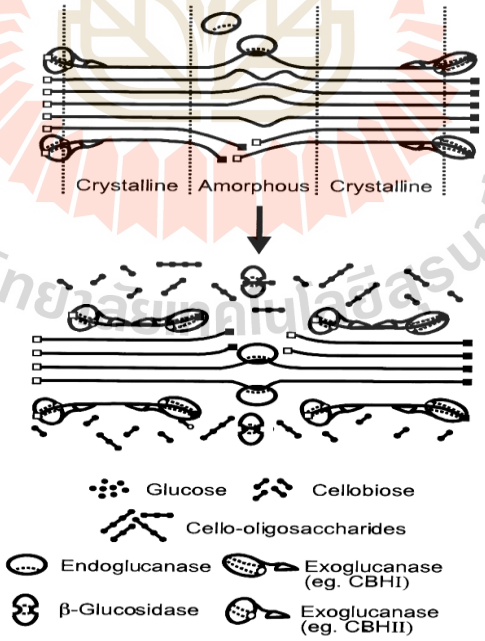


Figure 2.14 Mechanism of cellulolytic enzyme on crystalline and amorphous structure in cellulose (Lee *et. al.,* 2002).

2.3.2 Xylanolytic enzyme

This enzyme has function on xylan structure which is complicate structure. The complete degradation of xylan structure need enzyme system to degrade xylan chain and side chain (Figure 2.15). Xylanolytic enzyme composed of endoxylanase, *β*-xylosidase, and exoxylanase. The enzyme degrade side chain such as α-arabinofuranosidase, α-glucuronidase, acetylesterase, and phenolic acid esterases.

Branch side of xylan is the barrier of activity of xylanase on xylan backbone. The complexity of xylanase is necessary for complete hydrolysis of xylan structure. Xylanolytic enzyme breakdown side chain structures which include off reduce steric effect of substrate and enhance accessible of enzyme on xylan backbone. Xylanolytic enzyme composed of 7 types according to reaction of enzyme on site of substrate [Figure 2.15] (Sunna and Autranikian, 1997).

2.3.2.1 *β*-xylosidase or ***β* -D-xyloside xylohydrolase** (E.C.3.2.1.37) or exoglycosidase is found in an extracellular or cell bound depend on microbial types and growth duration. There was a report that *β*-xylosidase was the most of xylanolytic enzyme from xylanolytic enzyme microorganisms which can be found from fungi. Molecular weight on SDS-PAGE is 60-360kDa and isoelectric point is in range 3.3-7.3. This enzyme degrade on xylooligosaccharide and xylobiose by hydrolyze on terminal non-reducing glycosidic linkage and release xylose as a product. Long chain of xylooligosaccharide and high concentration of xylose have an effect on decrease enzyme activity. The enzyme activity can be tested with artificial substrate such as *p*-nitrophenyl- *β*-D-xyloside and *p*-nitrophenol is a product.

2.3.2.2 Exoxylanase or 1,4 -exoxylanase (EC3.2.1.37) are a class of glycosyl hydrolases and play an important role in hydrolysis of xylan to xylose. Exo-xylanases are active on natural xylan substances, hydrolyzing long-chain xylo-oligomers from the reducing end to produce short-chain xylo-oligomers (xylo-biose and xylo-

tetraose) and xylose. The combined use of exo- and endo-xylanases can increase the xylose yield compared to using either of them alone. The enzyme activity is decrease when long chain of xylooligosaccharide and inhibit with product. It can hydrolyze another kind of products such as α-arabinoglucuronoxylan and methyl-*β*-D-xylopyranoside.

2.3.2.3 Endoxylanase or 1,4 –β-D-xylan xylohydrolase (EC3.2.1.8)**;** molecular weight is in range 11,000-85,000 Da and pI is 3.6-10.3. Isoelectric point relative to molecular weight of enzyme, endoxylanase in basic pI is always small MW and enzyme in acidic pI is large MW. It hydrolyze internal *β*-1, 4-glycosidic linkage producing short chains of oligomer of xylose. The short chains of xylooligosaccharide are converted to xylose by *β*-xylosidase.

2.3.2.4 Acetyl esterase or acetic-ester actylhydrplase (EC.3.1.1.6) that can deacetylate ester bond of acetic (*O*-acetyl residues) from backbone xylan.It can cleave acetic acid from short acetylated xylan oligomers and is inactive against acetylated long chain xylan polymer. It has also been shown to be restricted to deacetylate to xylobiose on C3 of acetylxylan. This enzyme acts on short acetylated end-products of xylan degradation, removing any remaining acetyl groups and allowing *β*-xylosidase access to xylose oligomer. The artificial substrate can be used for analysis of enzyme activity such as *p*-nitrophenyl acetate, 4-methylumbelliferyl acetate or *α*-naphthy acetate.

2.3.2.5 Arabinofuranosidase or α-L arabino furanoside or arabinofurano hydrolase (EC 3.2.1.55) can be found from plants bacteria and fungi which are mostly in form of monomers but can be in form of dimer, tetramer and octamer. Molecular weight is higher than 495 kDa and pI 2.5-9.3. It acts on 1, 3-α-L-arabinofuranosylan and 1, 5-α-arabinosyl of arabinoxylan and produces xylose and xylan polymer as products. The artificial substrate can be used for enzyme activity such as *p*-nitrophenyl, *α*-L-arabinofuranoside (pNPA), arabinan, arabinogalactan type I and II polymeric arabinoxylan or arabinose-substituented xylo-oligosacharides. The enzyme

cleaves *α*-1,2- *α*-1,3- or *α*-1,5 which link with arabinose substitute and generate arabinose as product.

2.3.2.6 Glucuronidase or α-D-glucosiduronate glucuronohydrolase (EC 3.2.1.139); molecular weight 366 kDa and pI 2.5-3. It acts on α-1,2 linkage between glucuronic acid and xylose (4-methy-α-D-glucopyranosyluronic acid) or larchwood xylan and produce 4-*o*-methyglucuronic acid and xylose as products. These activities could be detected by thin layer chromatography (TLC) or HPLC or GC.

2.3.2.7 Phenolic acid esterase or 4-hydroxy-3-methoxycinnamoyl-sugar hydrolase (EC 3.1.1.73) break down ester bond that link between arabinose side chain and ferulic acid on xylan structure and produce ferulic acid as a product.

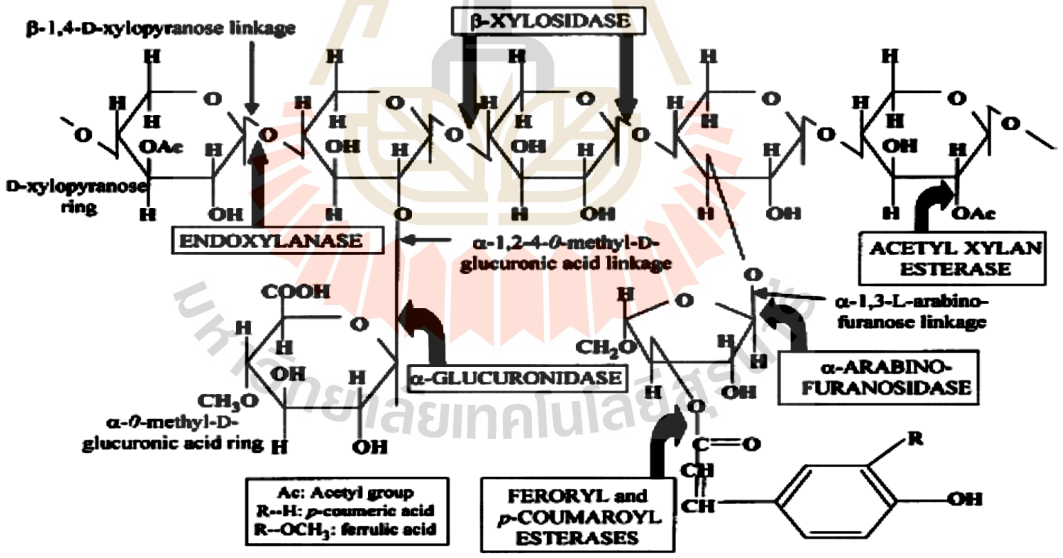


Figure 2.15 Synergy of xylanolytic enzyme (Beg *et. al.*, 2001).

In addition, biocatalyst of polysaccharide monooxygenases (PMOs or LPMOs which stands for lytic PMOs) have been greatly studied for improvement of biomass conversion and biorefinery products (Eijsink *et. al.*, 2019). PMOs are mononuclear copper (Cu) enzyme with the oxidative reaction on polysaccharide structure with co-substrates are from

O_2 or H_2O_2 (Tandrup *et. al.,* 2018). PMOs act on glycosidic linkage involving with the oxidoreductases of biomass conversion (Vaaje-Kolstad *et. al.*, 2010). The reactions of PMOs on polysaccharide structure occur by glycosidic bond hydroxylation on C1 or C4 of beta- linkage. PMOs have high oxidative power owing to copper on the enzyme structure. The copper is bound with histidine-brace which relative to reduction of Cu. The reaction of PMOs on polysaccharide occur by reduction of Cu (II) to Cu (I) and from Cu (I), 3 pathway can be occur depend on the condition of reduction. PMOs have the primarily reaction on α-1, 4 and β-1, 4 linkage of glucose backbone. In condition of O_2, Cu(I) is converted to Cu(II) and substrate can be oxidized at either C1 and C4. In the presence of H_2O_2, this is fast co-substrate react to hydroxylate on substrate while retain the state of Cu(I) (Fig 4.16) (Tandrup *et. al.*, 2018).

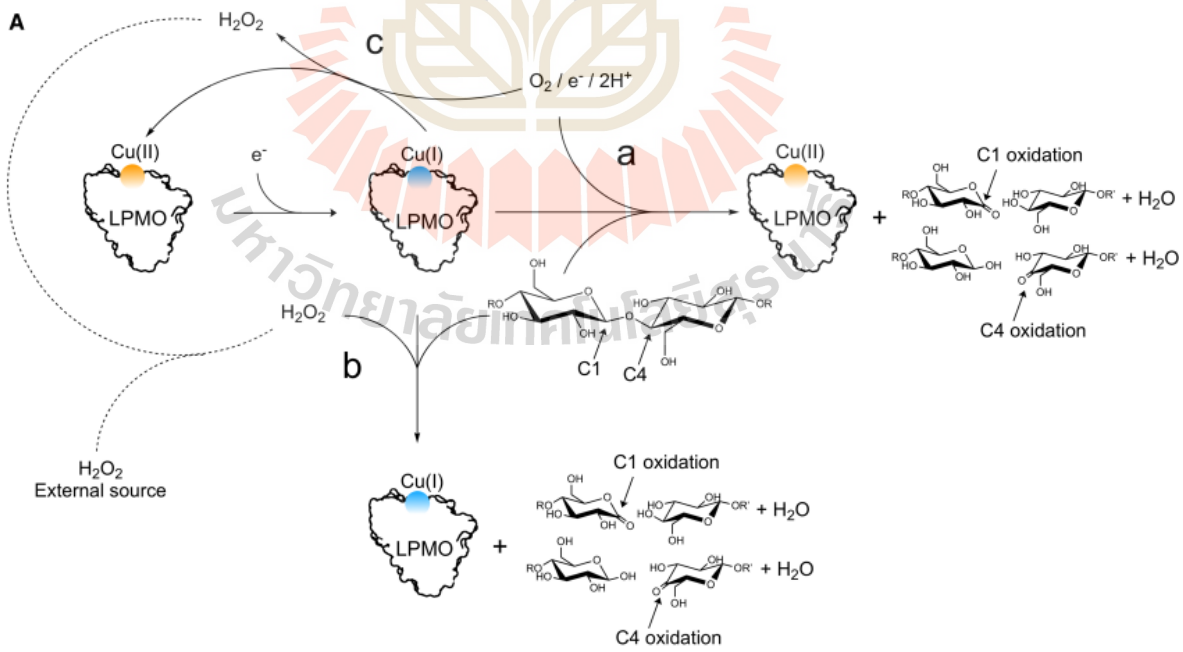


Figure 2.16 PMOs reaction (Modified from Tandrup *et. al.* (2018)).

2.4 Succinic acid fermentation

2.4.1 Succinic acid

Succinic acid (or butanedioic acid) is a diprotic acid (dicarboxylic acid) with chemical formula $C_4H_6O_4$ and structure formula HOOC-CH_2-CH_2-COOH. It is often found as an intermediate in metabolisms. It is white, odorless water soluble, and slightly dissolves in ethanol (as shown in Table4). Succinic acid has been used as an intermediate in various industrial such as 1, 4-butanediol, tetrahydrofuran, *N*-methyl pyrrolidinone, 2-pyrrolidinone, gamma-butyrolactone. It is also used in synthesis of biodegradation polymer such as PBS (polybutyrate succinate). Succinic acid can be produced from oxidation reaction of n – butane to be maleic anhydride and finally, convert to succinic acid. Succinic acid can be produced from fermentation from renewable carbon sources or agricultural products. The process for conversion of maleic anhydride to succinic acid have high cost and this is limiting factor to wildly application of succinic acid. While as, >75,000 ton/ year product from fermentation can be saved cost than chemical process (Willke and Vorlop, 2004).

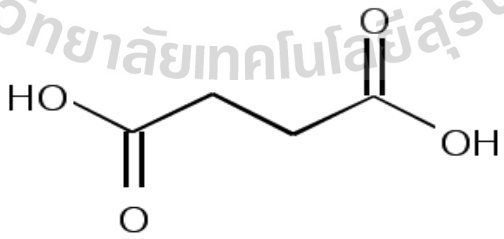


Figure 2.17 Structural of succinic acid.

Table 2.4 Chemical and physical properties of succinic acid.

Characteristic	Properties
Physical characteristic (purities at room temperature)	White crystal, colorless, odorless
Molecular weight	118.09 g/mol
Density	1.56 g/cm^3
Melting point	185-187 °C
Boiling point	235 °C
Water soluble	58 g/l (20 °C)
Acid ionization constant (pK_a)	pK_{a1} = 4.2
	pK_{a2} = 5.6

2.4.2 Succinic acid microorganisms

Succinic acid is often found as an intermediate during TCA cycle in aerobic respiration and one of the end product in anaerobic fermentation. Some organisms generate succinic acid as by-product from aerobic/ or anaerobic conditions for example: *Penicillium viniferum*, *Byssochlamys nivea*, and *Saccharomyces cerevisiae*. Recently, some bacterial strains have been studies for fermentation and enhance succinic acid production such as; *Anaerobiospirillum succiniciproducens*, *Actinobacillus succinogenes.* Moreover, researchers have modified bacterial strain through metabolic engineering of bacterial strain for instance, *Escherichia coli* for production of succinic acid (Song and Lee, 2006).

Bacteria producing succinic acid including of *Actinobacillus succinogenes, Mannheimia succiniciproducens, Anaerobiospirillum succiniciproducens, Corynebacterium glutamicum* and recombinant *E. coli* (Nghiem, 2017). Gram positive bacteria (for example; *C. glutamicum* and *Enterococcus faecalis*) have been studied to modify or block or replace genes. The modified bacterial strains have been applied for succinic acid fermentation for

example 7 fold of succinic acid production rate increased under without oxygen (Inui *et. al*., 2004).

There are 3 the most wild type of bacterial strains such as; *Actinobacillus succinogenes*, *Basfia succiniciproducens* and *Mannheimia succiniciproducens* that they are able to use numerous carbon sources, high efficiency anaerobic bacteria to produce succinic acid, and are classified as biosafety level 1 microorganisms by DSMZ and ATCC (Pateraki *et. al.*, 2016).

The various routes to succinic acid are presented in Figure 2.17. Succinic acid is a metabolite of the tricarboxylic acid (TCA cycle). The use of the reductive direction of the TCA cycle for succinic acid production is appealing, and can theoretically produce 1.71 molecules of succinic acid from each molecule of a 6-carbon sugar with incorporation of 2 CO_2 molecules in the conversion of phosphoenolpyruvate (PEP) to oxaloacetate (OAA), offering the potential for an excellent fermentation yield. Succinic acid is more reduced molecule than its sugar fermentation feedstocks. The theoretical yield of succinic acid from 6-carbon sugars such as glucose ($C_6H_{12}O_6$) and CO_2 via the reductive TCA route is 1.71 mole/mole sugar, or 1.12 g/g sugar, which is summarized in the following reaction (McKinlay *et. al.*, 2007):

$$7\ C_6H_{12}O_6 + 6\ CO_2 \rightarrow 12\ C_4H_6O_4 + 6\ H_2O$$

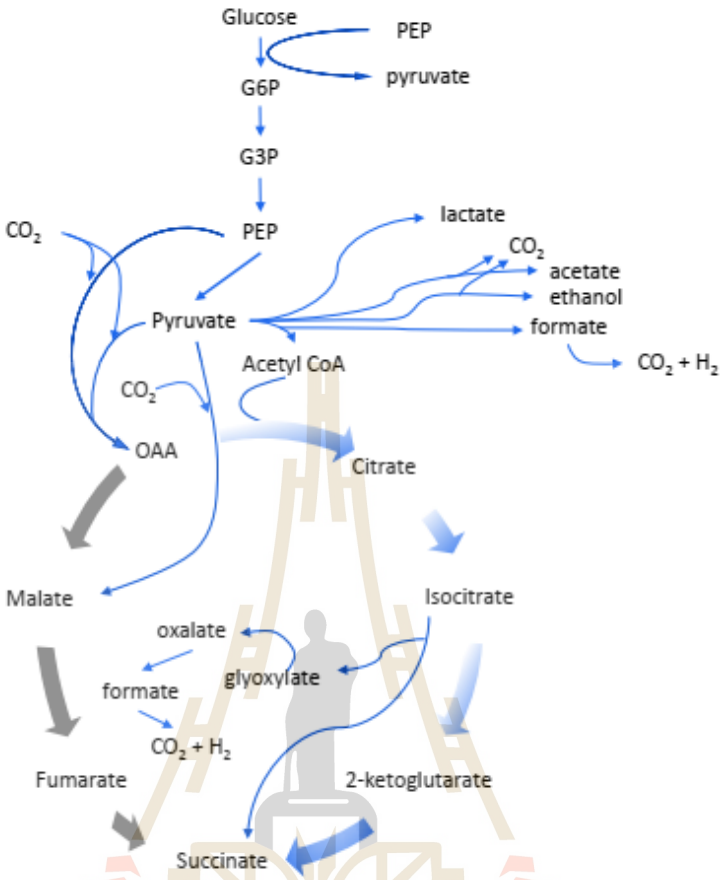


Figure 2.18 Metabolic pathways to succinic acid. Thick grey arrows showing the reductive (anaerobic condition) and thick blue arrows indicating the oxidative (aerobic) tricabocylic acid (TCA cycle). Thin blue arrows indicate contributing reaction and alternative pathways to succinic acid (Nghiem, 2017).

Relative enzymes in the succinic acid production have 5 key enzymes that are PEP (phosphoenolpyruvate) carboxykinase, malate dehydrogenase, malic enzyme, fumarase, and fumarate reductase. PEP carboxylation is the important step for succinic acid fermentation in rumen bacteria and this step is strongly regulated by CO_2 level. 1 mol of CO_2 is used for the formation of 1 mol succinic acid in theoretical. The higher CO_2 level have the effect on an increased succinic acid and relative to the expense of ethanol and formic acid, which result from

increasing of carboxylation of PEP to oxaloacetate rather than PEP conversion to pyruvate (Song and Lee. 2006; Park and Zeikus, 1999; and Van der Werf *et. al.*, 1997).

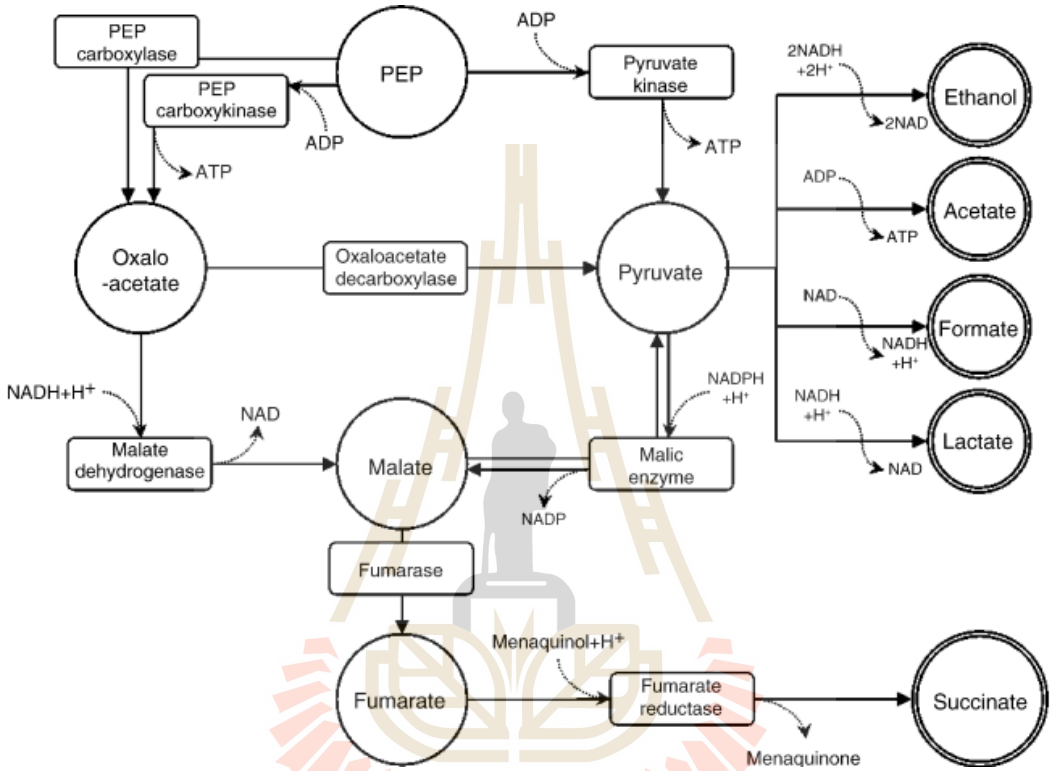


Figure 2.19 Major metabolic pathway for succinic acid fermentation and byproducts in *M. succiniciproducens* (Song and Lee, 2006).

Natural succinic acid producers are organisms which produce succinic acid as the main fermentation product, and many of them are capable of fumerate respiration. The most of succinic acid bacterial strains were isolated from rumen. These strains are anaerobic bacteria that favor to thrive at a near neutral pH, are capanophilic. They are naturally use the reductive TCA cycle for succinic acid fermentation. All natural succinic acid microorganisms from rumen require pH control for a fast and efficient fermentation (Nghiem, 2017).

Anaerobiospirillum succiniciproducens (ANS) was one of the first the isolated bacterium that produce succinic acid. This strain can produce 50 g/l succinic acid under strict anaerobic conditions. However, it was instability and deterioration such as, sensitive to high glucose concentrations and air, which made it unsuitable for industrial application (Nghiem, 2017).

Actinobacillus succinogenes was also the same isolation scheme used for ANS and a more robust organism, and a facultative anaerobe. This strain did not grow at pH value below pH 6.0, require near neutral pH and the best performances were reported when using Mg^{2+} as a counterion. Succinic acid fermentation using lignocellulosic biomass could be occurred by this strain (Zheng *et. al*., 2010). *A. succinogenes* is wild type of succinic acid producing bacteria that have been studied and applied for the succinic acid production. Moreover, this strain can utilize various types of carbon sources such as, glucose, xylose, arabinose, galactose, arabitol, sorbitol, and glycerol which produce a large amount of succinic acid under anaerobic condition (Guettler *et. al.*, 1996). *A. succinogenes* show the advantages over *E. coli* or *A. succiniciproducens*, *A. succinogenes* is a moderate osmophile and has good tolerance to a high concentration of glucose, which is beneficial for fermentation.

Mannheinmia succiniciproducens was the first isolated from rumen of a rumen Korean cow and produced mix acid. The strain produces succinic acid via the same reductive TCA pathway with *A. succinogenes.* In CO_2 poor condition, this strain produced lactate. This strain produce 14 g/l succinic acid concentration, 1.87 g l^{-1} h^{-1} productivity and 0.70 g g^{-1} yield succinic acid (Lee *et. al*, 2003).

There has been much effort in metabolic engineering recombinant *Escherichia coli* which enhance succinic acid production under aerobic and anaerobic condition (Millard *et. al.*, 1996). The developed technology was licensed to Applied Carbochemicals, Inc. (ACC), in the United States. In contrast to natural isolated succinic

acid bacteria, *E. coli* was answerable to the separation of growth and succinic acid production, by fast aerobic growth, followed by a switch to anaerobic conditions for succinic acid production (Vemuri *et. al.*, 2002). The use of *E. coli* fermented glucose in a batch and dual phase aeration fermentation system results in succinic acid concentration of 101 gl^{-1} (Hodge *et. al.*, 2009).

Aspergillus niger is fungi that produce citric acid and gluconic acid with production of citric acid more than 78 g/l and 65 % yield (w/w) per sucrose sugar. Through metabolic engineering, consideration of stoichiometric succinic acid yield is 1.5 mol from glucose 1 mol under microaerobic condition (David *et. al.*, 2003). However, application of fungi have been limited in industrial food and beverage due to complicate for management during fermentation, separation and purification which result in low productivity after downstream process (Song and Lee, 2006).

Lignocellulosic waste can be used as carbon source for succinic acid fermentation. High carbohydrate wastes or glycerol and by-product from industrial biodiesel have been applied for succinic acid production also (Lin *et. al.*, 2005). For this study, hydrolysate from degradation of lignocellulosic will be applied as carbon source for *A. succinogenes* in the succinic acid fermentation because of natural succinic acid producer and good ability utilization of sugar containing in lignocellulosic materials.

2.4.3 Applications of succinic acid

Succinic acid was produced first time from microbial fermentation in 1546. It is formed by plants, animals and microorganisms, its maximum production by anaerobic microbes. Traditional applications of succinic acid include food additives, detergents, cosmetics, pigment, toners, cement additives, soldering fluxes, and pharmaceutical intermediates. Succinic acid has a significant role in the industrial and there are involve four existing markets. The largest markets of succinic acid, succinic acid is mostly used as a surfactant, additive, foaming agent and detergent. The second application is ion chelator

to prevent corrosion and pitting in the metal industry. Third, it is also used in the food market such as; acidulate, pH regulator, antimicrobial and flavoring agent. The fourth market is the pharmaceutical industry for instance; production of vitamins, antibiotics and amino acids. The industrial potential of succinic acid is also a precursor to various industrial as shown in Figure 2.19 (Akhtar *et. al.*, 2014).

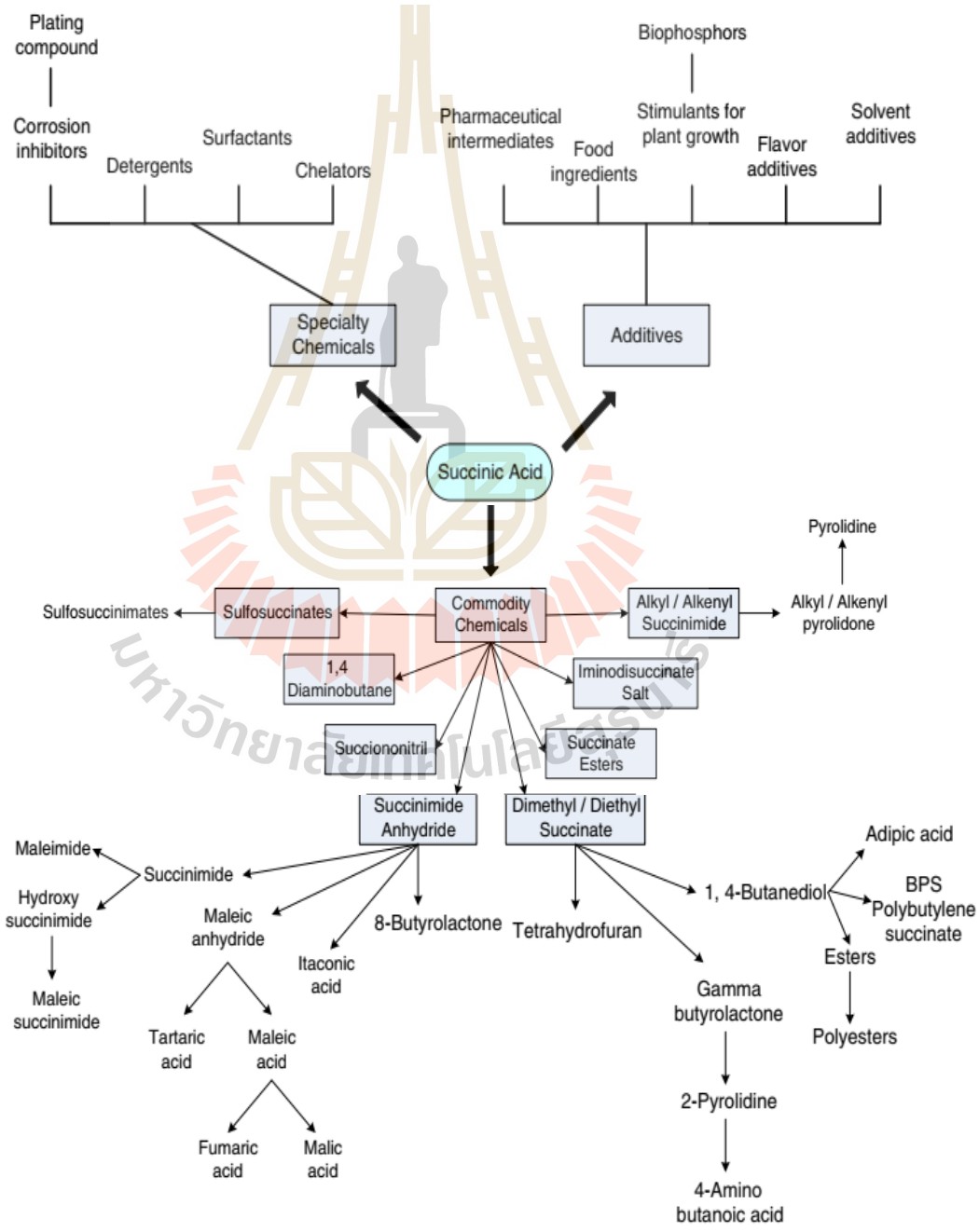


Figure 2.20 Various products from succinic acid as a precursor (Akhtar *et. al.*, 2014).

CHAPTER III

MATERIALS AND METHODS

3.1 Raw material preparation and reagents

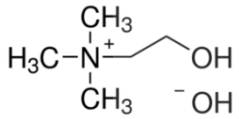
Small chopped pieces of mulberry stalk (3.5-5 cm.) were the residual waste from Thai silk company, Phetchabun province on the northern of Thailand. These small pieces was dry at 60 °C for 3 days, and then grinder was applied to mill, crush and sieve size was 200 μm. Dry powder was kept in vacuum jar until use. Commercial cellulase (Cellic CTec2 Novozyme®) was purchased from Novozyme® and hemicellulase (HTec2 Novozyme®) was donate from Brenntag Company. The activity of Cellic CTec2 was 186 FPUml^{-1} which was measured by NREL method. Avicel, sodium acetate, acetic acid, sulfuric acid, and other chemicals were of analytical grade.

Figure 3.1 Mulberry stalk powder.

3.2 Cholinium based amino acid ionic liquid synthesis

The [Ch][AA] ILs such as cholinium alanate ([Ch][Ala]) and cholinium glycinate ([Ch][Gly]) were synthesized according to Ren *et al*., 2016, Tou *et al*., 2013 and Liu *et al*., 2012. Briefly, an equal mole of choline hydroxide mixed with droplet of solution or suspension of amino acid at cool temperature. Water was removed and dry in vacuum oven at 70°C for 2 days (or Ren *et. al.*, 2016 reported water content <2%). Characterization of each [Ch][AA] was analyzed by FT-IR (Figure A1) and ^{1}H NMR spectra (Appendix).

Cation



Ch, Choline hydroxide ([Ch])

Anions

Glycine ([Gly]) Alanine ([Ala])

Figure 3.2 Cation and anion of ionic liquid amino acid ([Ch][AA]); small molecular weight (MW) amino acid for based ILs are in a group of non-polar aliphatic amino acid such as glycine and alanine.

3.3 Pretreatment of mulberry stalk with [Ch][AA]

Mulberry stalk was incubated with [Ch][AA] ILs by a ratio of 1:10 (w/w) under continuous magnetic stirrer 200 rpm at 90°C for 6 h. The mixture was washed with

deionized water until colorless by centrifugation at 12000 rpm, 4±5 °C for 8 min to separate solid residue and liquid part. Solid residue was further hydrolyzed with commercial cellulase CTec2 and hemicellulase HTec2. Homogenization was consequent applied after IL pretreatment to develop enzymatic efficiency on structural polysaccharide. Slurry of solid part was blended with homogenizer (Nessei homogenizer ACE® AMseries) at 14000 rpm, 4±10 °C for 10 mins. Liquid phase was collected for IL recycling and separation of crude lignin by pH adjustment to pH 2 with H_2SO_4.

3.4 Enzymatic hydrolysis of pretreated and untreated mulberry stalk

Enzymatic hydrolysis was performed at 50 °C, continuously magnetic stirring at 200 rpm in a 50 ml vial containing 500 mg pretreated mulberry stalk or 500 mg of untreated mulberry stalk, 2 $FPUml^{-1}$ of CTec2 and 10% of HTec2 in 10 ml of sodium citrate buffer (50 $mmol^{-1}$, pH 5.5). Aliquots (250 µl) were periodically taken and centrifuged at 4 °C; 12 *g* for 10 min. Hydrolysate solutions were analyzed for glucose and xylose concentration by HPLC. The yield of glucose and xylose was calculated as follows (Hou *et. al*., 2012):

$$\text{Sugar yield (\%)} = \frac{\text{Released sugar amount}}{\text{Theoretic sugar amount in native biomass}} \times 100$$

3.5 Compositional analysis of mulberry stalk

Structural polysaccharide and lignin contents of mulberry stalk were determined according to NREL analytical procedure (Sluiter *et. al.*, 2012). Briefly, Dry sample were hydrolyzed with 72% sulfuric acid at 30 °C for 1 h, and then the mixture was added with water to dilute content of 4% and was further incubated at 121 °C for 1 h. The resulting hydrolysate was neutralized with calcium carbonate and glucose and xylose were determined by HPLC. The HPLC (Agilent 1260) was equipped with an Aminex HPX-

87H column (Bio-Rad) and a refractive index detector. An aqueous sulfuric acid concentration of 5 mM (as a mobile phase) pumped at flow rate of 0.4 $mlmin^{-1}$ and eluted through column at 60 °C. The acid-insoluble lignin content was determined gravimetrically by using filtering crucible, while the acid-soluble lignin was determined by UV/vis at 240 nm using the extinction coefficient of 12 $Lg^{-1}cm^{-1}$. The analysis method of HPLC, structural carbohydrate and lignin content are presented on the Appendix.

$$\text{Lignin extractability (\%)} = 1 - \left[\frac{\text{Lignin amount in the recovered residues}}{\text{Lignin amount in the untreated raw material}}\right] \times 100$$

3.6 Characterization

3.6.1 Fourier transforms infrared analysis

FTIR (Bruker Tensor27) analysis was used in transmission mode. The spectra was recorded with an average scans of 60 scans between 400 and 4000 cm^{-1}. The spectra resolution was 0.3 cm^{-1}. Structural polysaccharide and lignin contents of mulberry stalk were determined

3.6.2 Scanning electron microscope

Morphology of native mulberry stalk and treated biomass were taken on a JEOL (JSM-6010LV) scanning electron microscope (USA). The samples were mounted on double-side carbon tape on a cylinder SEM specimen stub and coated with gold (JEOL, JFC-1100E, USA) prior to acquiring images at 10 kV.

3.6.3 X-ray diffraction analysis (XRD)

The samples were scanned on a D2 PHASER (Bruker, German) from 2Θ = 5-60° with a scan speed of 0.07° min^{-1} and a step size of 0.04° at 40 kV, 40 mA. The cellulose crystalline index (CrI) was calculated by the peak height method (Segal et. al., 1959) :

CrI=[(I_{002}-I_{am})/I_{002}]×100, which I_{002} is the peak intensity of the crystalline portion of biomass at 2Θ≈22.5°, and I_{am} is the peak intensity of the amorphous portion at 2Θ≈18.2°.

3.6.4 Thin layer chromatography of sugar

Aliquots and 10 mM of standard sugar solution was spotted on silica gel plates at intervals The plates were developed with water:acetonitrile (18:82) as an eluting solvent by slightly modified of Robyt (2000). After the plate were developed and dried they were stained with 10% of sulfuric acid in ethanol. For visualization, the plates were heated for 5 mins at 120 °C for color development. For extraction of aspect band, 5µl of hydrolysate was drop on plates and without staining plates was striped. The silica powder was dissolved in ultrapure water to remove solubilize sugar over night at 37°C, remove water by speed vacuum at 50 °C for 8h. Silica was precipitated with 95% ethanol. Supernatant was transferred to new vials, evaporation of ethanol with speed vacuum at 45 °C for 5 h. Carbohydrates normally have at least two NMR-active nuclei, ^{1}H and ^{13}C, can be used for studies of natural or synthetic oligosaccharides (Duus *et. al.*, 2000). Carbohydrates studied by NMR spectroscopy in aqueous solution were dissolved in deuterated water (D_2O).

3.7 Lignin fractionation

Precipitation and purification of crude lignin were carried out as the method of Sun *et al*., (2009) and An *et al*., (2015) with minor modifications. Briefly, the black liquor of [Ch][AA] after pretreatment of MS was separated from carbohydrate rich material (CRM) and CRM was washed with deionized water until colorless, lyophilized and keep in -80°C until used. The liquid fraction or black liquor was collected as lignin rich material (LRM). The LRM was concentrated and acidified with 4M HCl to pH 2.0, precipitation proceed at 4 °C for 16-18 h. The precipitated lignin was washed with acid water until less of brown color (partial hemicellulose), then it was lyophilized at -80 °C for 18-24h. The lignin yield was calculated with the following equation:

$$\text{Lignin yield (\%)} = \frac{\text{Amount of lignin product}}{\text{Amount of lignin in black liquor}} \times 100$$

3.8 Solid loading with batch and fed-batch hydrolysis

CRM was incubated with Cellic CTec2 and HTec2 10% of CTec2 by various 2 operation mode such as batch and fed-batch hydrolysis. Batch enzymatic hydrolysis, various solid loading (5.6, 8.4, 11.2, and 14% dry wt) was incubated with different cellulase dosage such as 15, 22 and 40 FPU/g cellulose and hemicellulase 10% of each cellulase dosage. The reaction mixture was processed at 55°C in 50 $mmol^{-1}$ sodium acetate buffer pH of 5.5. Aliquots (250 µl) were periodically taken and centrifuged at 4 °C; 12 *g* for 10 min. Hydrolysate solutions were analyzed for glucose and xylose concentration by HPLC. The yield of glucose and xylose was calculated as present in materials and methods section 3.4 and 3.5. Fed batch hydrolysis was carried out by various amount of solid loading which present on Table 3.1. Solid was added after initiation of 4 h. The condition for enzyme reaction was operated in the same condition with batch hydrolysis.

Table 3.1 Solid loading in fed batch operation

Operating No. / Time of solid loading	Solid loading (%)[A]		
	1	**2**	**3**
1	5.6	5.6	-
2	8.4	5.6	-
3	8.4	8.4	-
4	8.4	5.6	5.6

[A] enzyme dosage was choose from the batch operation for each time of solid adding

3.9 Seed inoculum preparation and medium

CRMs of MS were applied for succinic acid fermentation by *Actinobacillus succinogenes* ATCC55618. Lyophilized strain of *A. succinogenes* ATCC 55618 was purchased from American Type Culture Collection (ATCC), and it was maintained in at −70 ˚C. Preculture medium and seed medium (SA medium) contained per liter: 17 g tryptone, 3 g peptone, 2.5 g glucose, 5 g NaCl, 1 g K_2HPO_4, medium was autoclaved at 120 °C for 15 min. For preculture, lyophilized strain from ampule was mixed with SA medium and incubated in an anaerobic jar with CO_2 generator at 37 °C for 18h. The bacterial strain was transferred in to SA medium and incubated for 18 h, and then 500 µl of mixture was combined with 500 µl of sterilized glycerol and kept at -80 °C as a stock until use. For seed preparation, the bacteria from glycerol freeze stock was transferred into 50 ml SA medium and incubated at 37 °C for 18h with CO_2 generator.

3.10 Succinic acid fermentation in SHF and SSF process

The medium for fermentation followed as per liter: 85 g glucose, 25 g yeast extract, 3 g KH_2PO_4, 1.5 g K_2HPO_4, 1 g NaCl, 0.3 g $MgCl_2$, 0.3 g $CaCl_2$, 0.07 g $MnCl_2$, 50 g $MgCO_3$. Fermentation medium, concentrated NaOH was added to adjust the pH to 6.5. *A. succinogenes* ATCC55618 was incubated in sealed 250 ml bottles containing 50 ml of the seed medium with CO_2 gas generator in anaerobic jar at 37 °C for 12 h. Batch fermentation was applied to investigate the succinic acid fermentation from hydrolysate of ionic liquid pretreatment. The seed culture was inoculated (10% v/v) into fermentation medium. Batch fermentation was carried out at 37 °C in anaerobic bottles under CO_2 gas atmosphere, and agitation speed of 200 rpm (Zhu *et al*., 2012).

CHAPTER IV

RESULTS AND DISCUSSIONS

4.1 Effect of cholinium based ionic liquid on mulberry stalk pretreatment

Mulberry stalk in this study was industrial agricultural waste from silk production. Materials composed of small amount of protein and fat which was 4.62 and 1.03% dry weight (DW), respectively. Whereas, fiber structure was the main component which was 31.71%DW cellulose 24.29 %DW hemicellulose and polymer of lignin 15.33 %DW (Table 4.1).

Table 4.1 Chemical component of native mulberry stalk.

Chemical component	Quantities (%Dry weight; DW)
Moisture	8.17
Ash	4.26
Protein (crude protein; CP)	4.62
Fat (Crude fat or ether extract; EE)	1.03
Crude fiber	41.55
Carbohydrate (Nitrogen free extract; NFE)	40.37
Cellulose	31.71
Hemicellulose	24.29
Lignin	15.33

Generally, wood biomass consist of lignin more than 20% DW, for examples of lignin of wood, hardwood River red gum (*Eucalyptus*) was 31.3% DW (Reina and Resquin, 2011), softwood white spruce was 26.8% DW and common juniper was 32.1% DW. Whereas, the sample of mulberry stalk of this experiment composed of lignin less than 20% DW, component variation in each sample involved with many parameters including of age, part of sample from plant or organs, harvesting season and water and climate (Han and Rowell, 1997). Mulberry stalk contained of cellulose >30% DW, hemicellulose <25%DW and lignin <20%DW. This material was suitable for the representative of soft wood biomass for monosaccharide separation and used as fermentable sugar for organic acid fermentation. However, the recalcitrant of complicated structure interfered with accessibilities of cellulase and hemicellulase on polysaccharide structure. Pretreatment of raw materials was necessary for enhance enzyme accessible on polysaccharide structure. In this study, cholinium based ionic liquid was used as a solvent for pretreatment of mulberry stalk.

Mulberry stalk was treated with nonpolar aliphatic of amino acid based cholinium ionic liquid, 2 cholinium amino acid ILs including of cholinium glycinate ([Ch][Gly]) and cholinium alanate ([Ch][Ala]) (Table 4.2). Both of ILs were observed the abilities to remove >50% of lignin from mulberry stalk; as shown in Table 4.1. Lignin extractabilities by [Ch][Gly] and Ch][Ala] was 65.44 and 65.15%, respectively, which was not different between both of ionic liquid (ILs). Cellulose content of treated mulberry stalk increased after pretreatment by both of ILs. Cellulose content increased due to delignification and depletion of xylan component. Removing of lignin from biomass was a significant effect on rising of cellulose content of pretreated lignocellulosic materials. These ionic liquids were significantly effect on increasing of glucose and xylose yield as compared with untreated mulberry stalk. Pretreatment with [Ch][Gly] and [Ch][Ala] amended more than 5 times of glucose yield of untreated MS. Cholinium glycinate IL improved glucose and xylose yield from 14.93% and 1.88% of untreated MS to be 75 % and 33 % respectively.

Whereas, glucose and xylose yield by [Ch][Ala] pretreatment was 66.15% and 24.35%. Pretreatment lead to partial lignin removal, disrupt crystalline cellulose, and increase porosity of biomass (ShuBehera *et. al.*, 2014), however many methods of pretreatment generated inhibitor and required step to eliminate enzyme/or organisms inhibitor (Jönsson and Carlos, 2016; Klinke *et. al.*, 2004; Palmqvist and Hahn-Hägerdal, 2000; Kumar and Sharma, 2017). Cholinium amino acid ionic liquid behaved as a high selective lignin remover from lignocellulosic materials, enhance enzyme accessible on carbohydrate structure, form low toxic or inhibitor on enzyme or organisms. The toxicity of choinium based ionic liquid have been demonstrated. The toxicity of [Ch] cation with linear carboxylate anion ionic liquid had less effected on cell yeast than imidazolium cation and longer chain of anion increased toxicity of [Ch][CA] ILs (Ninomiya *et al.*, 2013). Cholinium based amino acid ILs were investigated low toxicity to tested bacterial strains including of *Escherichea coli*, *Staphylococcus aureus*, *Salmonella. enteritidis*, and *Listeria monocytogenes* with most of [Ch][AA] had lower inhibitory concentrations and minimum biocidal concentration than 1-butyl-3methylimidazolium tetrafluoroborate ([Bmim][BF4]), moreover, these ILs were also high biodegradability (Hou *et. al.*, 2013). Varies pretreatment methods required large amount of water afterward; instance liquid hot water, lime pretreatment and Sulfite pretreatment to overcome recalcitrance of lignocellulose (SPORL pretreatment) (Klinke *et. al.*, 2004), on the other hand ILs pretreatments were not required high amount of water to remove inhibitor substances after pretreatment step (Shi *et. al.*, 2013). Moreover, [Ch] cation based ILs performed as efficient solvent to selective lignin removal, report of Hou *et. al.* (2015) presented most of tested 28 cholinium ILs was good solvents for lignin dissolving, on contrary xylan and cellulose scantly soluble in these ILs (<10 $gmol^{-1}$ and < 0.7 $gmol^{-1}$, respectively) (Hou *et. al.*, 2015). Alanine and glycine is an aliphatic amino acid anion, these ILs with in a group of nonpolar character or hydrophobic while [Ch][Arg], [Ch][Lys], [Ch][His] are [Ch][AA] based on hydrophilic

character (del Olmo *et. al.*, 2016). As mention miscibility of solvent cannot represent lignin extractabilities. The pKa value of anion amino acid based ILs are not exactly involve with lignin dissolve abilities except in the case of basic amino acid, the pKa value; [Ch][Arg] 12.50 > [Ch][Lys] 10.54 > [Ch][His] 6.04 > [Ch][Gly] 2.35 > [Ch][Ala] 2.34 with lignin extractabilities; 72.1 ,43.3, 36.8, 65.9, and 66.8% (Hou *et. al.*, 2015). The lignin extractabilities of aliphatic amino acid based ILs may involve with molecular weight of nonpolar amino acid anion as report about lignin extractabilities of Hou *et. al.*, 2012; [Ch][Gly] 59.9%, [Ch][Ala] 58.2% and [Ch][Met] 55.2% (Hou *et. al.*, 2012). Likewise, the [Ch] cation with small aliphatic amino acid ILs of this study acted as high ability solvent to dissolve lignin from biomass, resulting in increased cellulose. Consequent, sugar yield increased by enhance accessible surface of soft wood MS for enzymatic hydrolysis. However, there was less supporting data of [Ch][Gly] and [Ch][Ala] pretreatment on forest waste and softwood biomass.

Depletion of lignin and xylan content corresponded with weight loss of solid residuals after pretreatment which was 64% and 56% by [Ch][Ala] and [Ch][Gly], respectively. Total solid removal by [Ch][Ala] and [Ch][Gly] was 36 and 46%. Sugar yield of [Ch][Gly] pretreatment was more over than that of [Ch][Ala], it may be due to more cellulose remain and lignin remove from [Ch][Gly] than pretreatment by [Ch][Ala].

Table 4.2 Effect of cholinium amino acid IL on pretreatment of mulberry stalk.

ILs	Chemical componennt (% dry weight)[a]			Residue[b] recovery (%)	Lignin extractability[b] (%)	Sugar yield (%)[c]	
	Cellulose	Hemicellulose	Lignin			Glucose	Xylose
Untreat	31.71	24.29	15.33	100	0	14.93	1.88
[Ch][Ala]	41.84	18.76	6.64	64	65.15	66.15	24.35
[Ch][Gly]	51.09	13.77	5.68	56	65.44	74.72	33.05

[a]Determined via the NREL procedure. [b]500 mg of Mulberry stalk samples were incubated in 5 g of [Ch][AA] with continuosly magnetic sterring at 90°C for 6 h. [c] Recovered residues or 0.5 g of untreated raw materials were reacted with CTec2 and HTec2 in sodium citrate buffer, pH 5.5 at 55 °C for 24 h.

Deduction of lignin was strongly considerable with improvement enzyme accessible on carbohydrate structure. These ionic liquid revealed increasing of glucose and xylose yield compared with untreated mulberry stalk. Pretreatment had a significant effect on improving of enzymatic hydrolysis. Many methods improved yield of glucose and xylose, however some methods reacted on monosaccharide and generated inhibitor of cellulose or microorganisms in fermentation.

Cholinium amino acid ionic liquid behaved as a solvent lignin from agricultural materials, and good ability enhance enzyme accessible on carbohydrate structure. However, there was no result of [Ch][AA] to forest waste or softwood biomass. In this studies, the morphological and chemical structure changed by [Ch][AA] on softwood biomass was illustrated via SEM and FT-IR spectra.

4.2 Morphological and chemical change of mulberry stalk after cholinium based amino acid ionic liquid pretreatment

The mulberry stalk softwood structure significantly altered after pretreatment with [Ch][Gly] as compared with native mulberry stalk. Alteration of mulberry stalk structure and delignification by [Ch][Gly] was investigated by SEM and FTIR spectra (Figure 4.1 and Figure 4.2). SEM images compared the morphological feature of native, mulberry stalk after enzymatic hydrolysis and residual solid from pretreatment. The external characteristic of MS from enzymatic hydrolysis was slightly different from untreated MS. Whereas, the surface of pretreated MS with of both ILs had open and became a loose structure, which significantly alter as compared with and native MS. The loose structure relative to increase glucose and xylose yield when compared with untreated mulberry stalk.

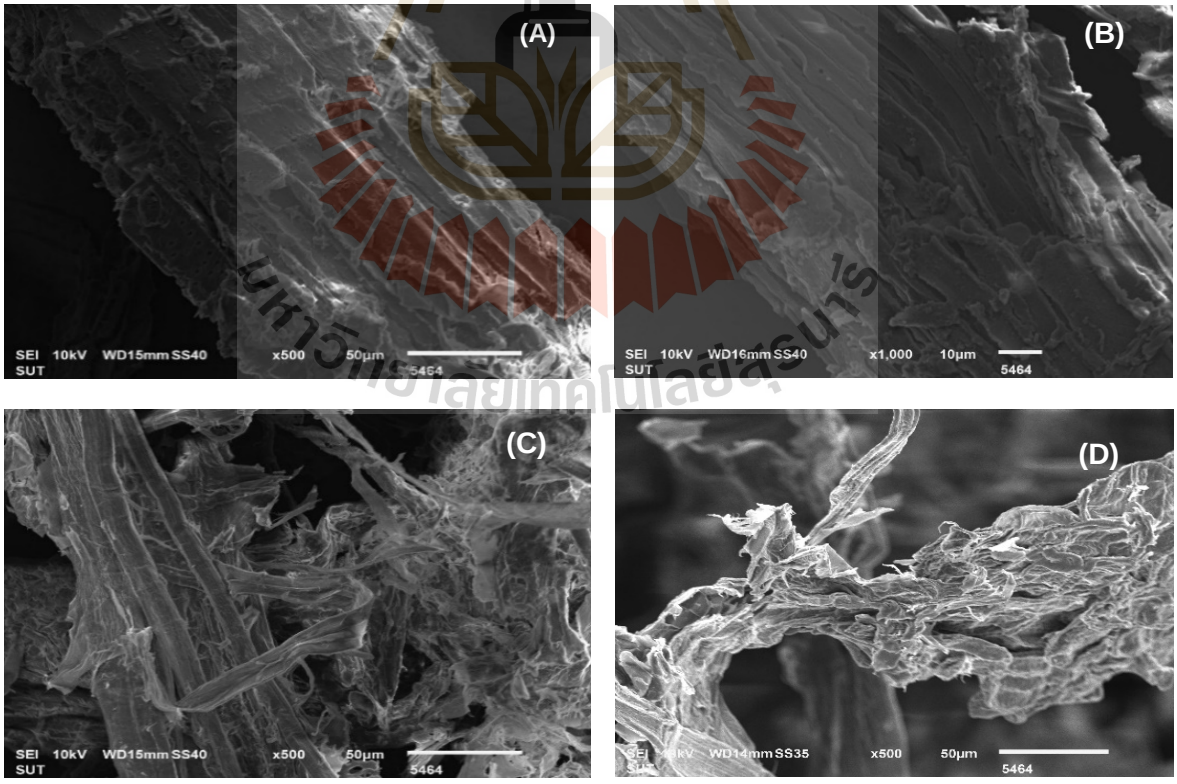


Figure 4.1 Scanning electron microscope of native mulberry stalk (A), mulberry stalk after enzymatic hydrolysis without IL pretreatment (B), mulberry stalk treated with [Ch][Gly] (C) and mulberry stalk treated with [Ch][Ala] (D).

The chemical changes in pretreated softwood MS were also investigated by FTIR spectra (Figure 4.2). Mulberry stalk treated with [Ch][Ala] and [Ch][Gly], the transmittance band at wavenumber of 1728 cm^{-1} (stretching C=O) disappeared after pretreatment, completely disappearance was found from [Ch][Gly]. It was considered with breakdown ester linkage between subunit of lignin complex and sugar residue such as galactose and arabinose on hemicellulose side chain (del Olmo *et. al*, 2016; Lao *et. al.*, 2014; Muhammad *et. al.*, 2016). The peak at wavenumber of 1465 cm^{-1} decreased after pretreatment with [Ch][Ala] and [Ch][Gly]. Intensity peak at this wavenumber correspond with bending alkane (CH-) functional group, decrease in this intensity peak involved with CH- deformation in lignin (Acquah *et. al.*, 2016 and Larsen and Barsberg, 2011). The corresponding to C-O stretching of peak at 1240 cm^{-1} remarkably decrease of intensity. The intensity peak at band between 1240-1210 is in spectra of methylated and acetylated lignin relate to asymmetric vibration of C-O linkage in ether or ester with phenolic hydroxyl group (Larsen and Barsberg, 2010). The decreasing of intensive peak at 1240 cm^{-1}, showing cleavage ether or ester bond between lignin fragment or lignin and saccharide residue in hemicellulose (Ji *et. al.*, 2017). The changing peak at 1215 cm^{-1} was found from both ILs pretreatment. The decreasing intensity peak range 1214-1217 correspond to ring deformation of aryl-O in both of aryl-OH and aryl-OCH_3 stretch (Li *et. al.*, 2007 and Heitner *et. al.*, 2010). FT-IR spectra revealed characteristic alteration of bond in lignin and polysaccharide of pretreated MS residue, these changes show exploitation of cholinium with amino acid ILs on interation of lignin/lignin as well as betweeen lignin and polysaccharide. Several of pretreatment method have promoted enzymatic hydrolysis, but there was lower selective lignin removal than ionic liquid methods. Dilute acid and ionic liquid pretreatment were compared with deligninfication and sugar yields via a model of switchgrass, in which ionic liquid ([C2mim][OAc]) decreased lignin content (16 mg) compare to dilute acid pretreatment (47.7 mg) (Li *et. al.*, 2010). The ionic liquids of 1-

buthyl-3-methylimidazolium [Bmim] cation with anion; acetate ([Bmim]OAc), dimethylphosphate ([Bmim]Me2PO4, [Bmim]DMP), and tosylate [Bmim]OTs can be used to separate >95% lignin fraction from black alder wood (Lauberts *et. al.*, 2017). A type of protic ionic liquid established that higher ionicity in a PIL have the capability for lignin removing and reservation of polysaccharide (Achinivu, 2018). Another [Ch][AA] such as [Ch][AA]-glycerol mixtures removed 67.6% lignin while reserving 95.1% cellulose (90°C, L/S mass ratio of 20:1, 6h) (Zhao *et. al.*, 2017). Cholinium amino acid-glycerol mixture changes major lignin linkages and lignin aromatics units (p-hydroxyphenyl (H), guaiacyl (G), and syringil (S)) occurred during pretreatment by [Ch][AA] (Dutta *et. al.*, 2018). Normally, dissolve of lignin is more challenging than polysaccharide in lignocellulosic materials due to contain of robust covalent bond along with interaction of intra-molecule in complexicity structure (Elgharbawy *et. al.*, 2018). Accordant with the FT-IR spectra of this result, part of lignin and hemicellulose could be miscible in the [Ch][AA], especially remove inter- and intra-molecular bonds between lignin/lignin and lignin-hemicellulose. Cholinium amino acid is the candidate for lignin removing with the high discerning lignin subtraction from lignocellulose. The peak intensity depletion conforms to decrease hemicellulose and lignin content, and remained in polysaccharide residual solid after pretreatment. The chemical changed and the depletion of lignin component after pretreatment showed that both [Ch][AA] ILs possessed a good ability for removing lignin and/or residual xylan from the MS material.

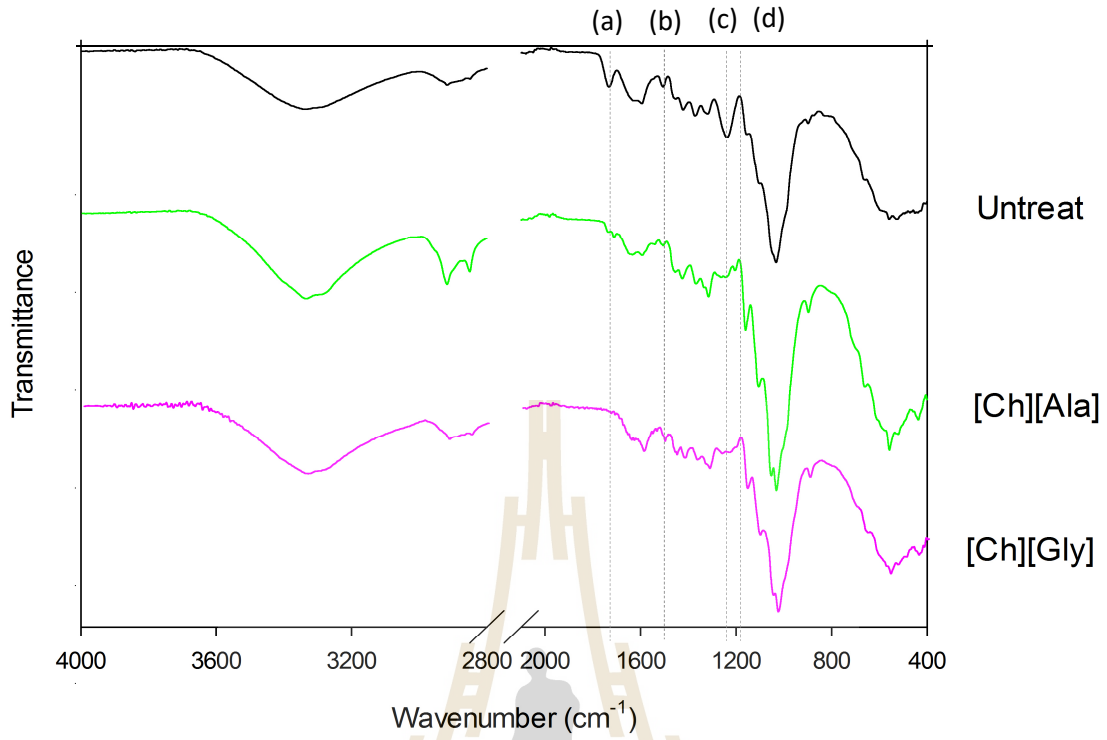


Figure 4.2 FT-IR spectrum of native soft wood mulberry stalk and treated mulberry stalk with [Ch][Ala] and [Ch][Gly]. Regions of the spectra mentioned in the text are annotated as follows: (a) 1728cm^{-1}, (b) 1465 cm^{-1}, (c) 1240 cm^{-1}, and (d) 1214 cm^{-1}

In addition, there was determination of crystalline index (Crl) via XRD spectroscopy (Figure 4.3), crystalline index remarkably increased after pretreatment. Crystalline index of native MS was 54% whereas, after pretreatment MS with [Ch][Gly] and [Ch][Ala] increased to 58.87% and 58.27%. Pretreatment with [Ch][Gly] had a significant effect on increased crystalline index due to removal the amorphous structure such as lignin and hemicellulose residue and Crl may related with an increasing of enzymatic hydrolysis. [Ch][Ala] pretreatment effected on increased Crl however, increment of cellulose amount was lower than comparable with [Ch][Gly]. Crystallinity may relate to released glucose yield due to remove amorphous lignin.

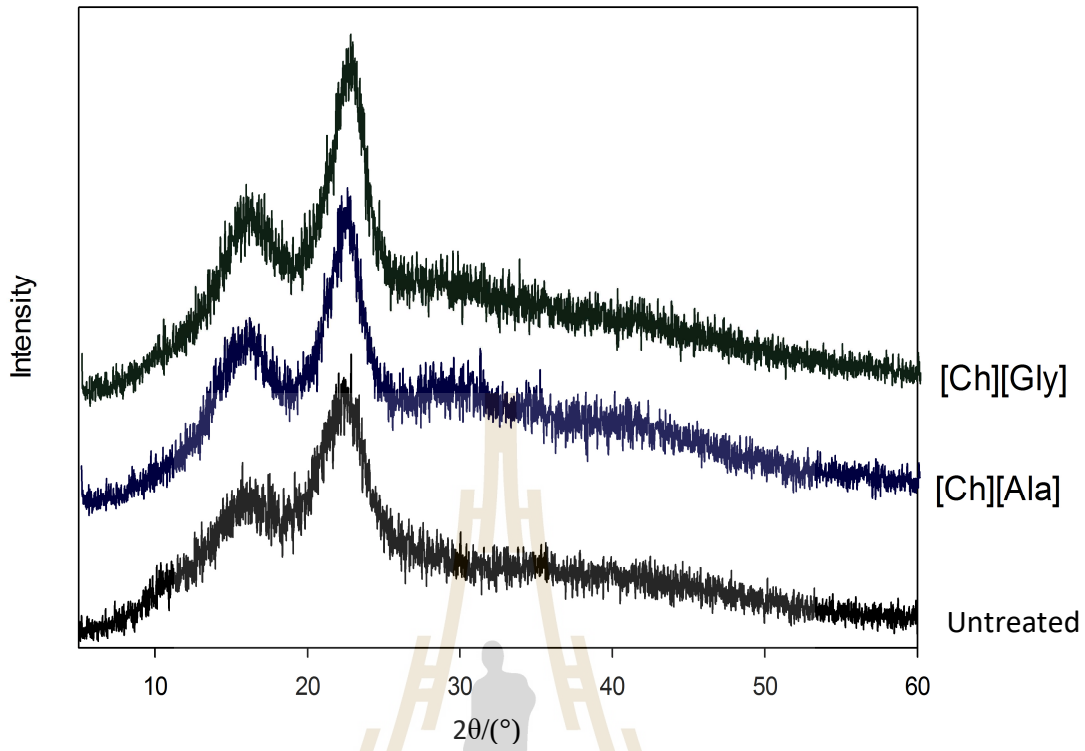


Figure 4.3 X-ray diffraction spectra of native mulberry stalk and pretreated mulberry stalk with [Ch][Gly] at 90 °C 6 h.

The hydrolysate products were examined on thin layer chromatography (TLC silica plate) and analysis the extracted product with H^1NMR and C^{13}NMR. On TLC plate, glucose was the first main product in hydrolysate of untreated MS. Whereas, pretreated MS subsequent enzymatic hydrolysis containing glucose, xylose, and sucrose as main product that demonstrated via qualitative TLC. After 24 h of hydrolysis, cellooligosaccharides are not the dominant products as present on TLC plate (Figure 4.4). The containing of mainly hydrolysate products from [Ch][Gly] pretreatment was glucose and xylose. As comparison hydrolysate of pretreated MS with native biomass, the mixture of homogenization of native MS in water contained main products of glucose and sucrose, cannot detectable xylose. According to product of enzymatic hydrolysis of pretreated MS and mixture of homogenization of native MS, sucrose was found as a product, along with H^1 and C^{13}NMR

spectra, the sucrose spectra was from extracted spot from TLC plate (Fig 4.5). Mulberry fresh fruit contain of total sugar, sucrose, and reducing sugar 12.18–17.02 g/100 g fresh fruit, 1.57–4.36 g/100 g fresh fruit, and 9.42–15.46 g/100 g fresh fruit (Gungor and Sengul, 2008). In photosynthesis, sucrose can be synthesized in leave through carbon fixation lead to accumulation of carbohydrate including of glucose and starch. Sucrose is synthesized in photosynthetic cells and moves cytoplasmically from cell to cell via plasmodesmata until it reaches the phloem parenchyma cells (Baker *et. al.*, 2012; and Slewinski and Braun, 2010). The sugar components of softwood and hardwood biomass differ in sugar content. The main hemicellulose sugar in softwood is mannose, followed by xylose whereas; glucoxylan is a major content in hardwood (Brandt *et. al.*, 2013). Xylose sugar was the second hydrolysis product after glucose and this result corresponding to MS is a group of hardwood that xylose is the main sugar of hemicellulose.

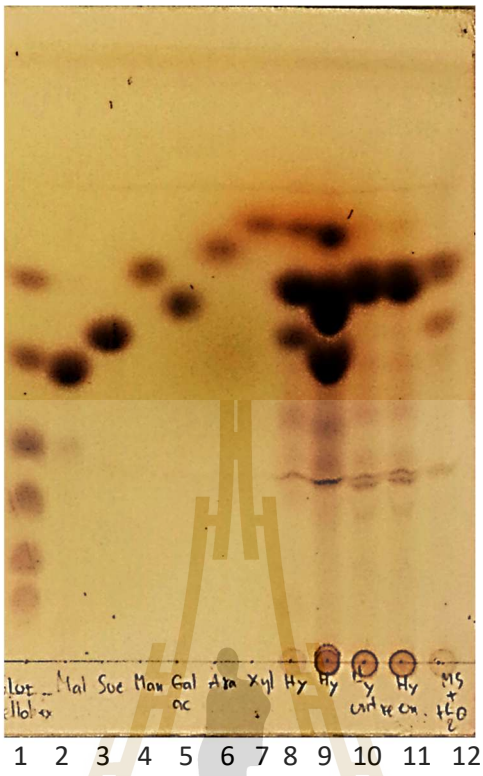


Figure 4.4 Thin layer chromatography of standard solution of sugar, hydrolysate and mulberry stalk powder in water; 1= Glucose to cellohexose, 2=Maltose, 3=Sucrose, 4=Mannose, 5=Galactose, 6=Arabinose, 7=Xylose, 8=Hydrolysate from pretreatment of mulberry stalk at 80°C for 4 h, 9= Hydrolysate of pretreated mulberry stalk with solid loading 8.4%, 10 and 11=Hydrolysate from untreat mulberry stalk, and12=Mixture from homogenization of native mullbery stalk in ultrapure water.

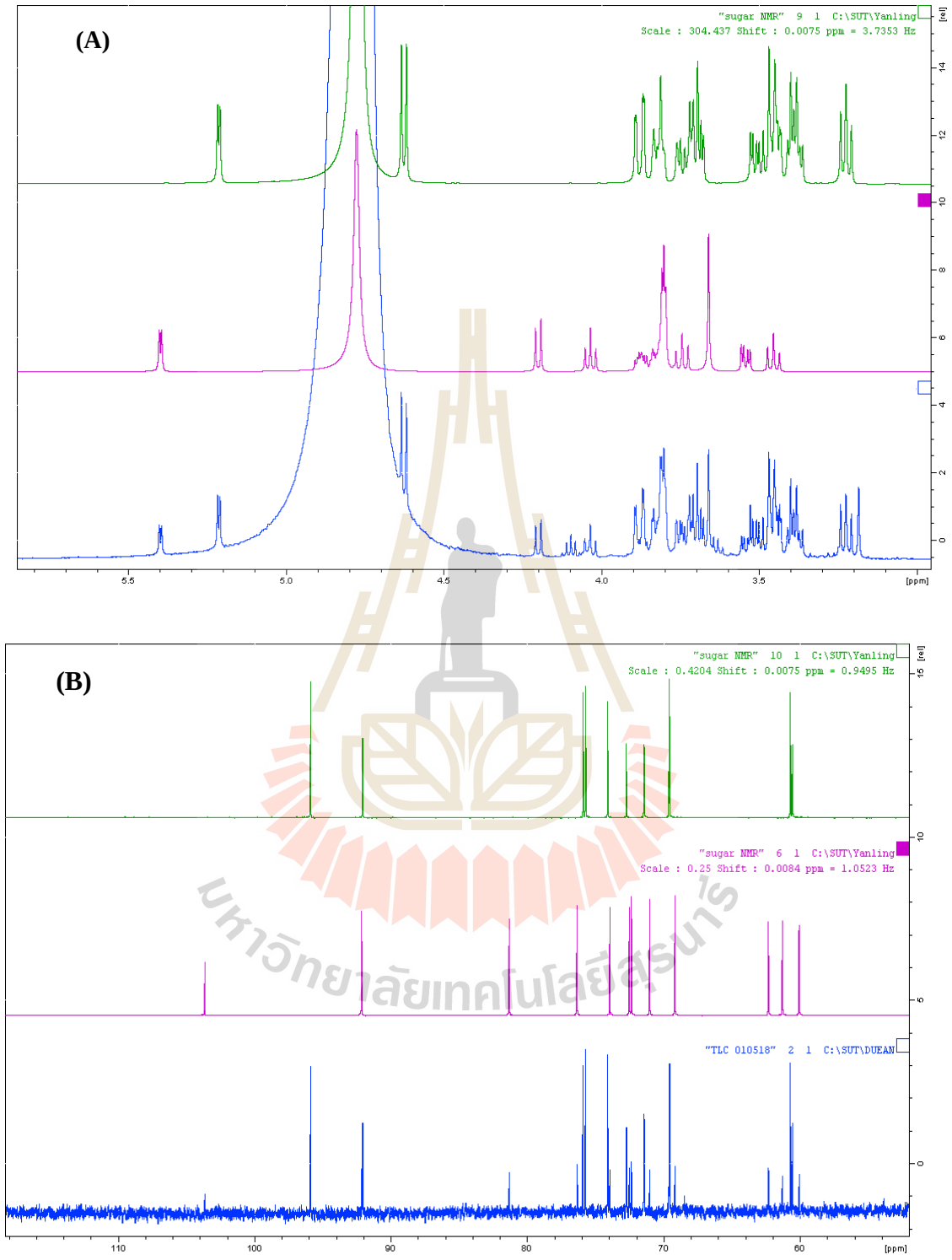


Figure 4.5 ^{1}H **(A)** and ^{13}C **(B)** NMR-spectra of extracted band No.3 (blue line) from TLC as well as standard glucose (pink line) and sucrose (green line).

4.3 Effect of temperature on pretreatment of mulberry stalk and reuse of choinium glycinate

Incubation of mulberry stalk on various temperature (Table 4.3), it was found that glucose yield lower than 60% was obtained at 60 °C reaction temperature. The applying at 60 °C, lignin extractability was lower than 60%, while higher temperature made lignin removal upward trend and including of glucose and xylose yield improvement. At 60 °C pretreatment temperature, the glucose yield was 52.36% (184.32 mg·g substrate^{-1}), which was lower than another pretreatment temperature. Whilst, pretreatment temperature of 70 °C, 66.77% glucose yield or glucose amount was 235.05 mg gsubstrate^{-1}, pretreatment temperature at 80 and 90 °C extracted lignin more than 60%, and >70% (> 260 mg·g substrate^{-1}) of glucose yield was achieved. The result indicating lignin removal tend to increase when extended temperature, and greater glucose and xylose yield can be obtained at 80 and 90 °C. Higher pretreatment temperature led to loss of residual solid recovery, for insistence, there was only 58 and 56% of residual recovery after pretreatment at 80 and 90 °C whereas >60% was found at lower temperature (60 and 70 °C). The loose solid content during higher pretreatment temperature and lignin extractabilites and hemicellulose depletion was quite consistent. At 80 and 90 °C of pretreatment temperature, enzyme efficiently hydrolyzed on polysaccharide structure and liberated monomer of glucose. Temperatures are important impact on enzymatic digestion improvement. Combination of heating and chemical reaction generated structure disruption due to explosion among the lignocellulose particles [Zhao, 2008].

Table 4.3 Effect of temperature on pretreatment of mulberry stalk with [Ch][Gly].

Temperature (°C)	Chemical componennt (% dry weight)[a]			Residue recovery (%)	Lignin extract ability[b] (%)	Sugar yield (%)[c]	
	Cellulose	Hemicellulose	Lignin			Glucose	Xylose
Non pretreatment	31.71	24.29	15.33	100	0	14.93	1.88
60	40.27	7.08	6.32	68	57.80	52.36	24.20
70	41.25	9.05	5.88	62	60.88	66.77	26.18
80	45.25	13.25	5.52	58	63.14	73.29	33.08
90	51.36	11.61	5.20	56	65.28	74.72	33.05

[a]Determined via the NREL procedure. [b]500 mg of Mulberry stalk samples was incubated in 5 g of [Ch][AA] with continuosly magnetic sterring at 90°C for 6 h. [c] Recovered residues or 0.5 g of untreated raw materials were reacted with CTec2 and HTec2 in sodium citrate buffer, pH 5.5 at 55 °C for 24 h.

Pretreatment condition is considered on lignocellulosic materials instance for, hardwood, softwood, type of agricultural waste, and lignin content. It has been demonstrated that lignin could be removed by ionic liquids. Nevertheless, there have been applied some type of ionic liquids instant for 1-Butyl-3-methylimidazolium methyl sulfate, 1-Ethyl-3-methylimidazolium acetate, 1-Butyl-3-methylimidazolium chloride and 1-butyl-3-methylimidazolium trifluoromethanesulfonate at high temperature 120 °C (Brandt *et. al.*, 2011). The application of 1-ethyl-3-methylimidazolium acetate ([EMIM]Ac) for switch grass pretreatment was at 160 °C for 3 h (Heitner *et. al.*, 2010). [EMIM]Ac enhanced sugarcane bagasse enzymatic saccharification and yielded glucose after 60 min pretreatment at 120 °C (da Silva, 2011). On the contrary, the results of this study supported that [Ch][Gly] effective on selective removal lignin and retain carbohydrate structure for

releasing fermentable sugar although, pretreatment reaction was proceed at ambient pressure.

After pretreatment, the recovered [Ch][Gly] was reused (Figure4.6 and Figure4.7) which was found that [Ch][Gly] was still good ability for enhance glucose and xylose yield including of lignin extractability. However, decreasing of glucose and xylose yield was obtained after 4 times of reusing of [Ch][Gly] at 60 and 70 °C whilst at 80 and 90 °C, sugar yield was not different at each times of IL reused. Although lignin accumulation in each batch reaction, lignin extraction by reused [Ch][Gly] was not lower than 60%. Recycle and reusing of IL was regarded to save processing cost and chemical waste management. At 70, 80 and 90 °C of each reusing [Ch][Gly], the glucose yields kept constant higher than 70%, whereas, approximately 65% of glucose yield was obtained from reusable [Ch][Gly] at pretreatment temperature of 60 °C. These results suggested that [Ch][Gly] performed high capacity of lignin extraction, [Ch][Gly] was reused without lignin removing and pretreatment temperature was not factor affecting on [Ch][Gly] reuse.

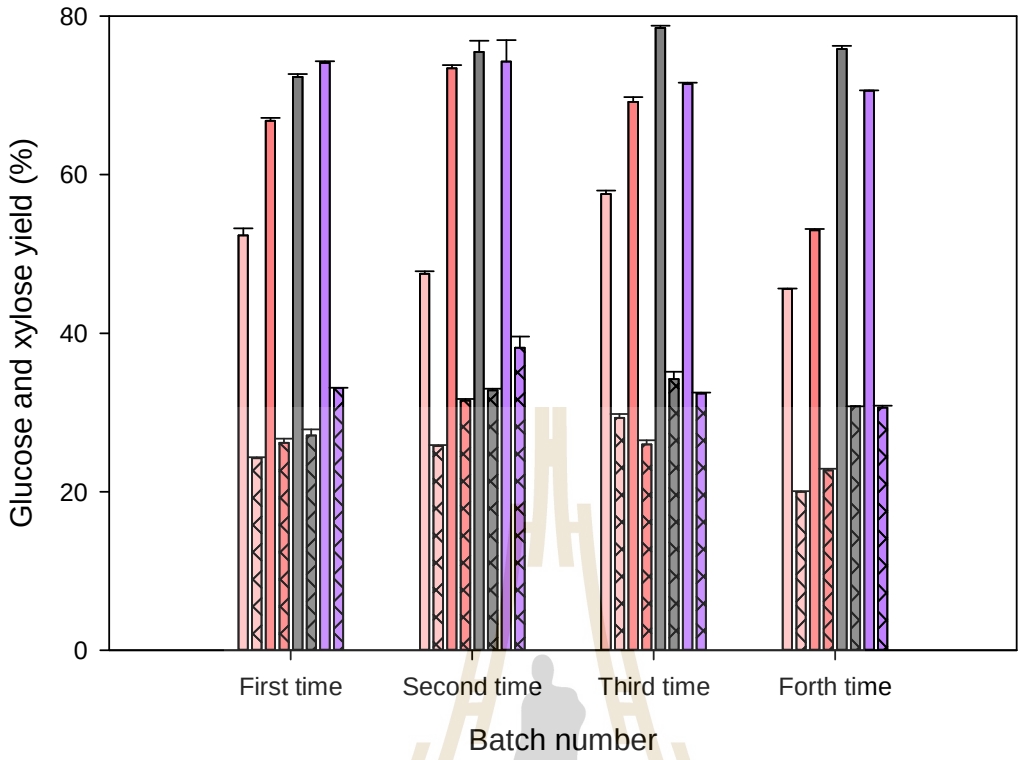


Figure 4.6 Effect of temperature on glucose and xylose yield by [Ch][Gly] reuse on pretreatment mulberry stalk ■ and ▨ represent glucose and xylose yield at 60 °C, ■ and▨ represent glucose and xylose yield at 70 °C, ■ and ▨ represent glucose and xylose yield at 80 °C, and ■ and ▨ represent glucose and xylose yield at 90 °C.

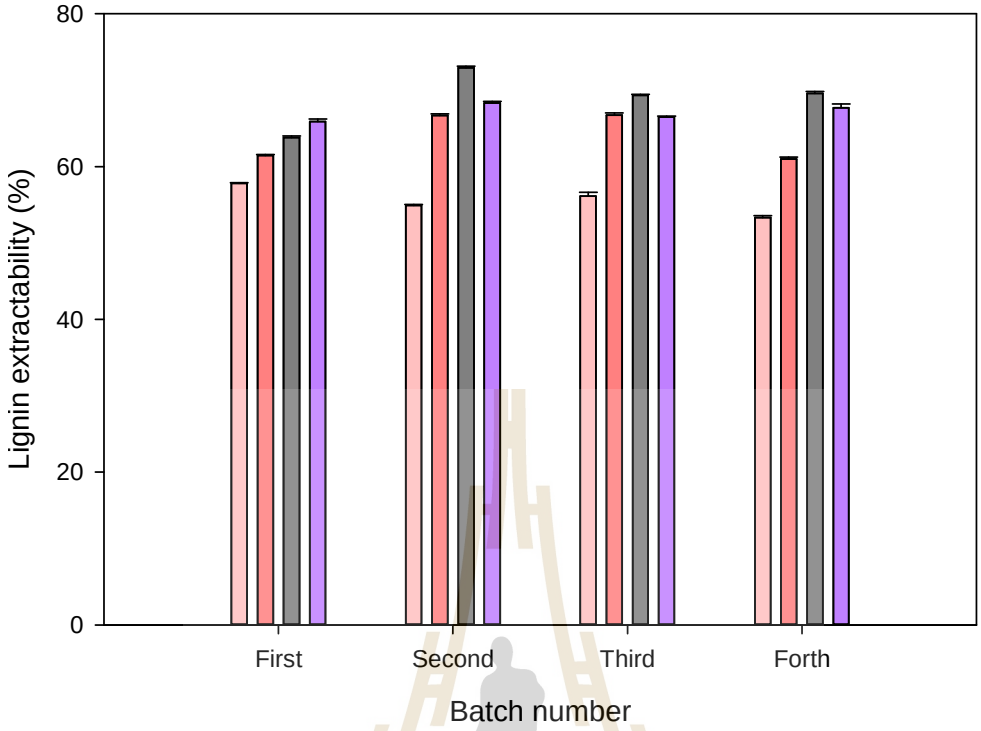


Figure 4.7 Lignin extractability after pretreatment MS with reused [Ch][Gly] at different temperature; ■ = 60 °C, ■ = 70 °C, ■ = 80 °C, and ■ = 90 °C.

4.4 Effect of incubation time on mulberry stalk pretreatment

MS was incubated with [Ch][Gly] by various incubation time at different temperature (Figure 4.8). At 1 h of incubation time from each temperature, glucose and xylose yield were the lowest as compare in same temperature. Pretreatment MS soft wood with [Ch][Gly] at 70 °C for 1 h, glucose yield was <60 and also found at same pretreatment temperature for 3h. At 80 °C, glucose yield had just 61% after pretreatment for 1 h as compare with incubation time 3, 4, and 6 h (>70% of glucose yield). Glucose yield was likewise the lowest at 90 °C for 1 h, when compared with the same pretreatment temperature. On the other hand, prolongation of incubation time had not effect on improvement of structural degradation as a result investigated 63% of glucose yield from 8 h of prolong reaction time. Extend reaction time produced decreasing of cellulose

crystallinity and generating of a few amount of amorphous structure (Auxenfans *et. al.*, 2012). Duration time was an important factor to facilitate enzymatic hydrolysis on carbohydrate fiber by providing amorphous cellulose or remove recalcitrant (Auxenfans *et. al.*, 2012). Nevertheless, long duration time was not always present an increment of released monomeric sugar (Ling *et. al.*, 2017). Glucose and xylose were enhanced when extended incubation time at each temperature. However, extension of reaction time from 4 to 6 h at 80 and 90 °C was not different for the maximum of glucose and xylose yield.

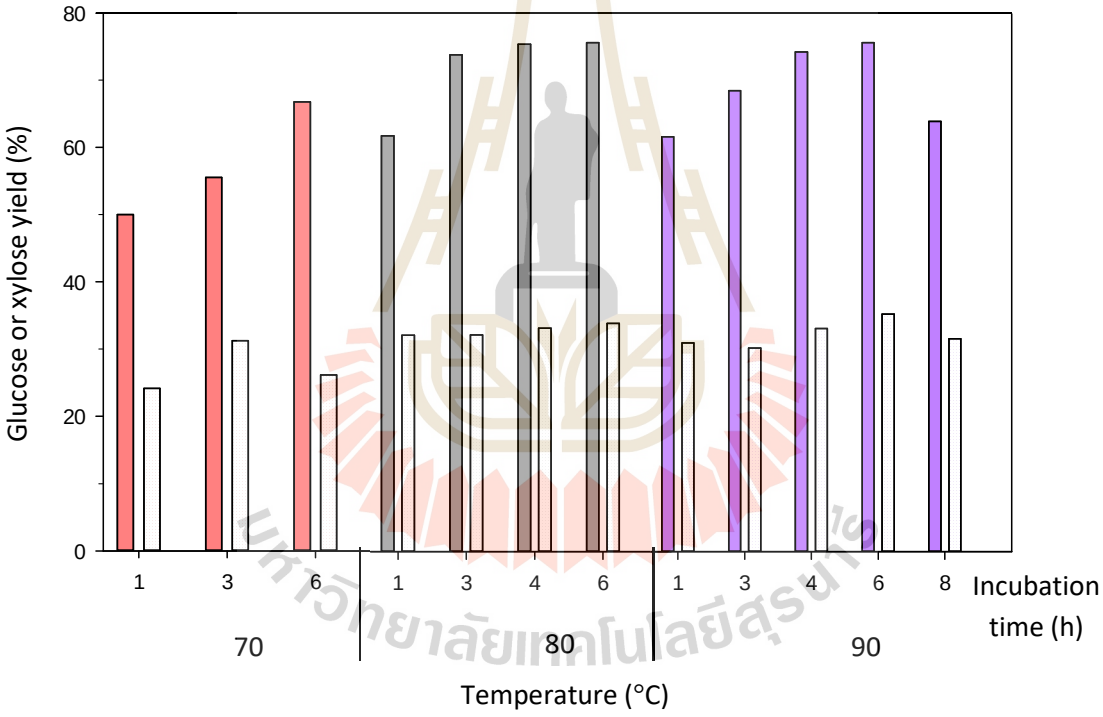


Figure 4.8 Effect of temperature and incubation time on glucose and xylose yield by [Ch][Gly]; ■ and ▨ = glucose and xylose yield at 70 °C, ■ and ▨ = glucose and xylose yield at 80 °C, and ■ and ▨ = glucose and xylose yield at 90 °C.

4.5 Effect of physical sonication on saccharification efficiency

Pretreated MS was subsequently with physical sonication (pretreated MS subsequent sonication; PMsSS) to enhance saccharification (Figure 4.9). Glucose saccharification rate of pretreated MS and PMsSS were 2.51 $gl^{-1}h^{-1}$ and 2.54 $gl^{-1}h^{-1}$, which was not different as compare both of substrate. Rate of enzymatic hydrolysis of pretreated MS and PMsSS was higher than >3 times of untreated MS. Glucose yield from hydrolysis of PMsSS was 81.06% (285.34 mg $gsubstrate^{-1}$) which was higher than yield from pretreated MS (71.55% or 261.49 mg $gsubstrate^{-1}$). Enzymatic saccharification rate rapidly increase until 5h of incubation time. It was found that rate of glucose or xylose saccharification was intent to stationary afterward. This result resembled to increasing sugar yield by ultrasonication of water hyacinth (Harun *et. al.*, 2011). Producing shear force caused greater liberating free sugar due to breaking biomass cell wall but it was not swell as chemical pretreatment (Chukwumah *et. al.*, 2009).

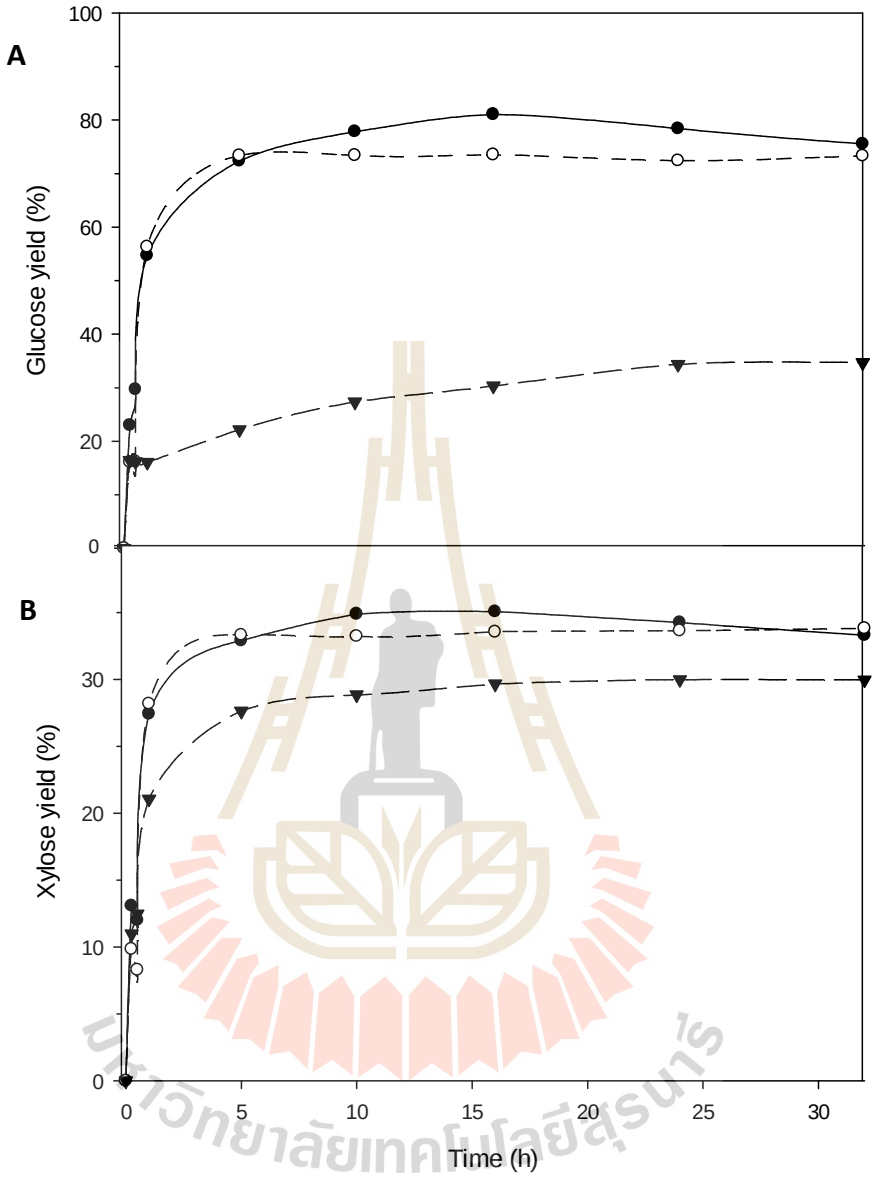


Figure 4.9 Time course of glucose **(A)** and xylose **(B)** yield after enzymatic hydrolysis on untreated mulberry stalk (-▼-), pretreated mulberry stalk (-○-), and treated mulberry stalk and subsequent with homogenization at 9000 rpm for 5 min (-●-).

Table 4.4 Glucose yield and initial saccharification rate from pretreatment of mulberry stem.

Treatment	Residue recovery (%)[a]	Initial saccharification rate of glucose ($gl^{-1}h^{-1}$)[b]
Untreated	100	0.78
Non Homogenizer	54	2.51
Homogenizer	54	2.55

[a]500 mg of mulberry stem sample was incubated in 5 g of [Ch][AA]. [b] Recovered residues or 0.5 g of untreated raw materials were reacted with 20 Unit/ml of CTec2 and HTec2 10% of CTec2 in sodium citrate buffer, pH 5.5 at 55 °C, 200 rpm and time interval of 5h.

Table 4.5 Comparison of pretreatment methods.

Raw materials	Pretreatment methods	Condition of enzymatic hydrolysis	Glucose yield	Ref.
Rice straw	NaOH	2.96%, 81.79 °C and 56.66 min.	254.5 ± 1.2 g kg^{-1} biomass	Kim and Han, 2012
Elephant grass	Stream explosion	10 FPU/g total solids	248.34 ± 6.27 g/kg^{-1} substrate	Scholl *et. al.*, 2015
Mulberry stalk	NaOH Microwave 900 watt, 15 mins; wash with water 2.8 L/g substrate	15 FPU$ml^{-1}$of CTec2 and HTec2 10% of CTec2	83 % Glucose conversion	Chaleamkwan, 2017
Mulberry stalk	[Ch][Gly] IL80°C, 4h; wash with water 250 ml/0.5g substrate	2 FPU$ml^{-1}$of CTec2 and HTec2 10% of CTec2	71 % Glucose conversion or 265 g kg^{-1} biomass	This result, 2018

4.6 Lignin fractionation and properties

[Ch][Gly] performed as the high efficiency of selective dissolving lignin from biomass. Separating of lignin from ionic liquid mixture could be done with the elementary technique. More than 65% of lignin from MS softwood was extracted with [Ch][Gly] (Table 4.2).

Acidification result in H^+ interact with the negatively-charge lignin and neutralize the charge on the molecule surface, zeta-potential on surface of lignin molecule approaches zero. The lignin molecule becomes unstable and favours precipitate (Zhu, 2013).

Considering of lignin recovery, lignin from mixture [Ch][Gly] was recovered 71.25% and 92.80% of lignin content in lyophilized lignin. Lignin contained small amount of carbohydrate (cellulose and hemicellulose) whereas, lignin was the dominant component in precipitated lignin (Table 4.6). An *et. al*., (2015) reported lignin in [Ch][Arg] solvent was afforded 75% of lignin content in LRM from lignin extractability 66%. Normally, lignin in alkaline solution could be precipitated via acidification and increase temperature effect on lignin solubility (Tian *et. al.*, 2015). Lignin in alkaline condition is negatively charge due to dissociation of phenolic and carbonyl groups on the lignin. Lignin molecules repel one another by means of electrostatic interactions. Self-aggregation of kraft lignin macromolecule and the precipitation of lignin have been reported in aqueous solution at room temperature with pH below pk_a of phenolic groups or at high ion strength (Norgren *et. al.*, 2002 A and 2001B).

Table 4.6 Chemical component of precipitated lignin

Raw materials	Solvent	Chemical component (%)			Lignin extractability (%)	Lignin yield (%)	Ref.
		Cellulose	Hemicellulose	Lignin			
Alfalfa	Organic acid/H_2O_2	-	-	-	-	34	Watkins *et. al.*, 2015
Rice straw	[Ch][Arg]	13.8	1.5	74.9	66.1	38.4	An *et. al.*,2015
Mulberry stalk	[Ch][Gly]	0	3.72	92.80	65	71.25	This study, 2018

4.6.1 FT-IR Spectra of precipitated lignin

The FT-IR spectra presented qualities of the extracted lignin and alkaline lignin (commercial lignin from Sigma). The major absorption bands assigned involving functional groups of lignin (Figure4.10 and Table 4.7). The wavenumber of 3392 cm^{-1} relative to OH stretching vibration, cause by presence of alcoholic and phenolic hydroxyl groups involved in hydrogen bonds. Absorption caused by stretching vibrations of C-H on methoxyl group is located at 2920 and 2850 cm^{-1}. Absorption in wavenumber range 2800-1800 cm^{-1} is not appearing in spectra of lignin. Stretching vibration at wavenumber 1733 cm^{-1} involved carbonyl groups normally locate in the 1720-1740 cm^{-1} which contributes to aldehyde (1740-1720 cm^{-1} C=O and 2820-2850 cm^{-1} =C-H), ketone and ester (1735-1750 cm^{-1} C=O and 1000-1300 cm^{-1} C-O) linkage. The intensity peak at this wave number disappeared from the pretreated mulberry stalk residue. The aromatic hydrocarbons show absorption in region 1600-1585 cm^{-1} and 1500-1400 cm^{-1} due to carbon-carbon stretching

vibrations in the aromatic ring and the peak at 1593, 1506 and1460 was assigned to C-C stretches in aromatic ring which is the skeleton of lignin macromolecule. At 1325 and 1235 cm^{-1} indicated C–O vibration in syringyl rings (1325-1330, 1230-1235 cm^{-1} are C-O on syringyl unit). These vibrations of C-O-C on S unit involve 1270-1230 cm^{-1} and 1120-1030 cm^{-1}. The contribution peak at 1025 cm^{-1} is stretching vibration C-O and C-H on aromatic ring and primary alcohol (range of 1025-1035 cm^{-1}). Region of 750-860 contribute to vibration of C-H on aromatic ring. At 842 cm^{-1} is the vibration of C-H out of plane in position 2, 5, and 6 of guaiacyl unit (Boeriu *et. al*., 2004).

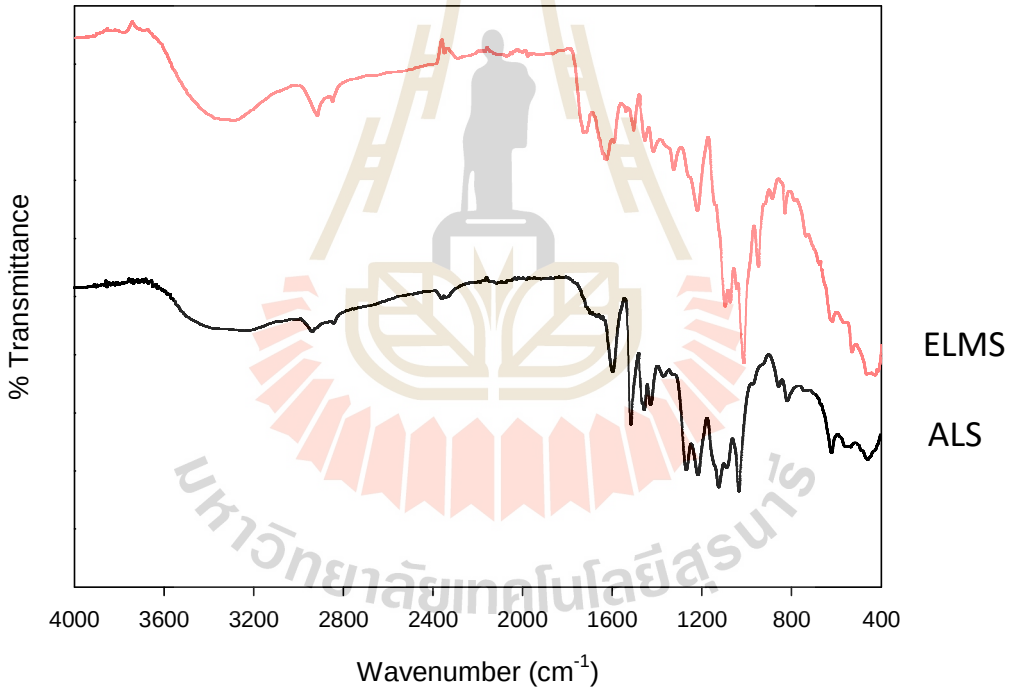


Figure 4.10 FT-IR spectra of extracted lignin from mulberry stalk with cholinium glycinate ([Ch][Gly]) (;ELMS) (-) and alkaline lignin from Sigma (;ALS) (-).

4.6.2 ^{1}H NMR Spectra of precipitated lignin

^{1}H NMR spectra exhibit the chemical shift of functional group of extracted lignin as present on Fig 4.11 and Table 4.6 present the position of signals assigned (Wang,

2014; Zhang, 2014; Jahan and Mun, 2009; and Chen, 1988). The peak signals position at low field 6.8 and 7.2 ppm of both of extracted lignin with [Ch][Gly] and alkaline lignin corresponding to aromatic proton in S and G units. The extracted lignin with [Ch][Gly] showed signal at region 6.8 ppm stronger than 7.2 which was relative to strong vibration of aromatic ring of S unit. The resonance at 4.9 ppm presents the H which is in linkage between β-O-4 ether bonds. Lignin polymer contains the most common linkage of β-O-4 ether bonds. The peak signals at this region is stronger than an alkaline lignin, demonstrating that [Ch][Gly] may not specific react on this linkage. The strong resonance at 3.8 and 3.5 ppm correspond to methoxyl protons ($-OCH_3$). The extracted lignin with [Ch][Gly] and alkaline lignin had the strong resonance peak at 2.2 to 2.5 attributing phenolic hydroxyl groups. The extracted lignin from [Ch][Gly] pretreatment was not contained signals 1.7-2.1 ppm which this resonance peak at this region relative the breaking down aliphatic hydroxyl lignin. The peak at 2.2 to 2.5 of extracted lignin with [Ch][Gly] pretreatment was stronger than alkaline lignin in which this signals involve the aromatic proton. These results correspond to the significant decrease of C=O in carbonyl, ether or ester bond between lignin /saccharide residue and O-C-O in aryl-OH and aryl-OCH_3 unit of pretreated mulberry stalk, indicating that [Ch][Gly] acted on the bond between lignin/saccharide and removed lignin from MS. It is not easy to accurate calculate the quantitative for the peak integration of functional groups from ^{1}H NMR spectra because the overlap by peaks of lignin functional groups. However, FT-IR and ^{1}H NMR spectroscopy revealed the extracted lignin with [Ch][Gly] contained the high content of aromatic, β-O-4 linkage and phenolic-OH indicating that [Ch][Gly] pretreatment method was not specific to degrade the main functional group of natural lignin structure in lignin unit (Figure4.11, Fig 4.12, Fig 4.13 and Figure4.14).

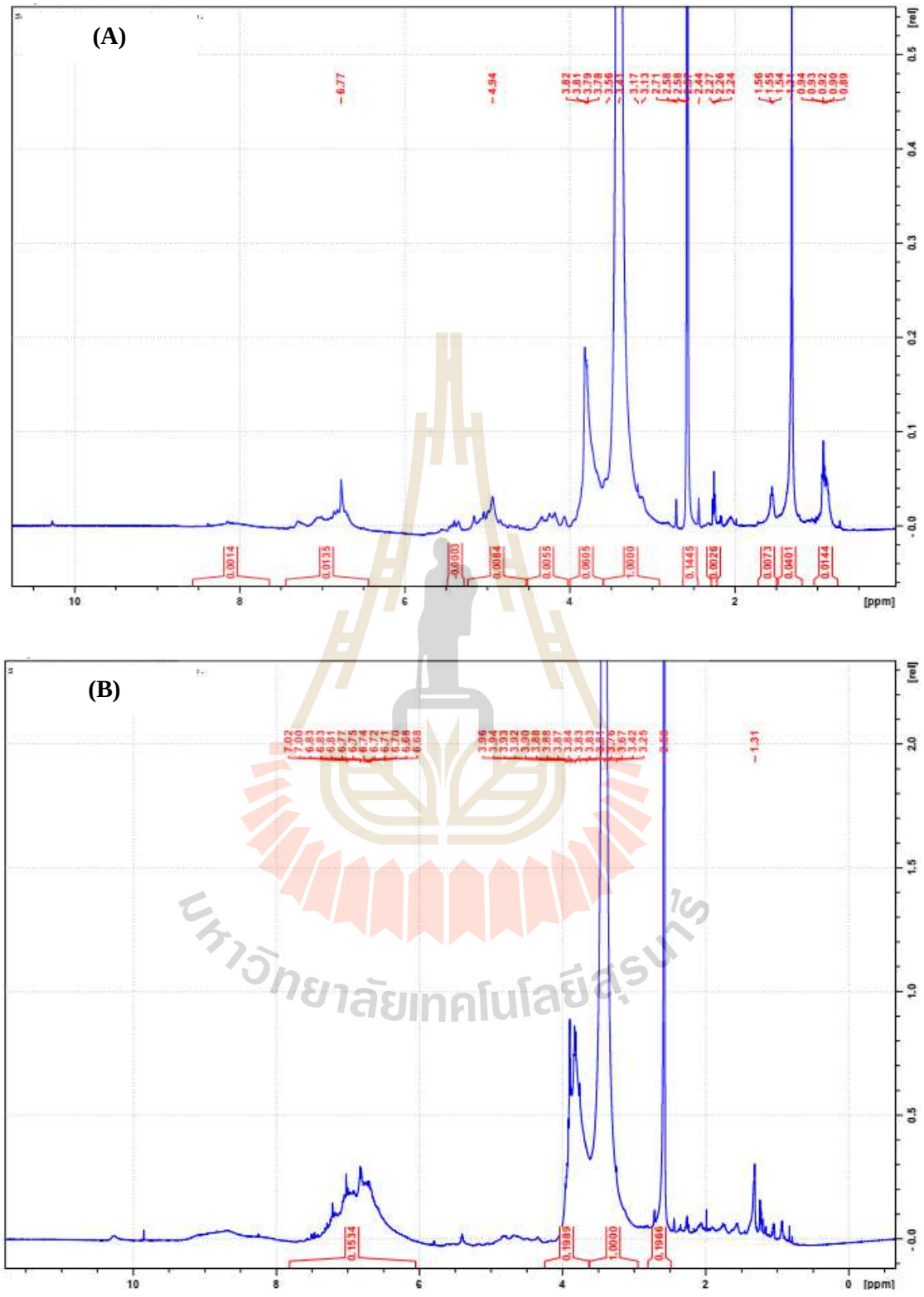


Figure 4.11 ^{1}H NMR spectra of (A) extracted lignin from MS with [Ch][Gly] and (B) commercial alkaline lignin.

Table 4.7 Peak assignments for quantitative ^{1}H NMR spectra.

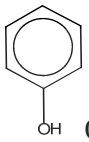
Chemical Shift (ppm)	Assignment
6.25-8.7	Aromatic region
6.8-7.25	Aromatic proton in guaiacyl units
6.8-6.25	Aromatic proton in syringyl units
6.25-5.75	H_α of β-O-4 and β-1 structures
5.2-5.75	H_α of β-5 structure
5.2-4.9	H of xylan residue
4.3-4.9	H_α and H_β of β-O-4 structures
4.0-4.3	H_α of β–β structures, H of xylan residue
4.0-3.5	H of methoxyl group
2.5-3.10	H_β of β–β structures
2.2-2.5	H in acetylated phenolic OH
1.7-2.2	H in acetylated aliphatic OH
0.7-1.6	Hydrocarbon proton in methylene ($-CH_2-$) and methyl ($-CH_3$)

Lignin content of softwood and hardwood biomass differs in guaiacyl (G unit) and syringyl (S unit) ratio. Softwood (for example fir, pine, and spruce) has high G unit to S unit ratio in lignin which is considered to be the reason for its higher resistance to delignification compared to grass or hardwood biomass. Lignin of hardwood is made up of G unit and S unit including of main sugar of hemicellulose is xylose that higher than mannose (Brandt *et. al.*, 2013). The FT-IR and NMR spectra of extracted lignin MS of this study revealed high content of aromatic ring of S unit, moreover glucose and xylose was the main sugar after enzymatic hydrolysis.

Various plants contain lignin with small differences in their chemical structure. Moreover, obtained lignin from the same source but different conditions may also present different structures and properties. However, all kinds of lignin contain similar functional groups. Natural lignin contains the following functional groups: methoxyl, phenolic hydroxyl, primary and secondary aliphatic hydroxyl, ketone and aldehyde groups. The

structure of functional lignin relative NMR or FTIR spectra is presented and summarized in Table 4.8, Figure 4.13, Fig 4.14 and Fig 4.15.

Table 4.8 Principally functional group on lignin with FTIR region

Wavenumber (cm^{-1})	**Functional group**
3392	 O-H stretching vibration in aromatic and aliphatic OH groups
2920, 2850	C-H stretching in methoxyl group (R-OCH_3)
1739	*1735-1750 strong C=O of ester bond
1105,1082	1000-1300 C-O two bonds or more
1600,1506, 1460	 1600, 1580, 1500, 1450 C=C skeleton in aromatic ring
1600-1585, 1500-1400	Aromatic ring vibration
1325	Syringly ring vibration with C-O stretching vibration

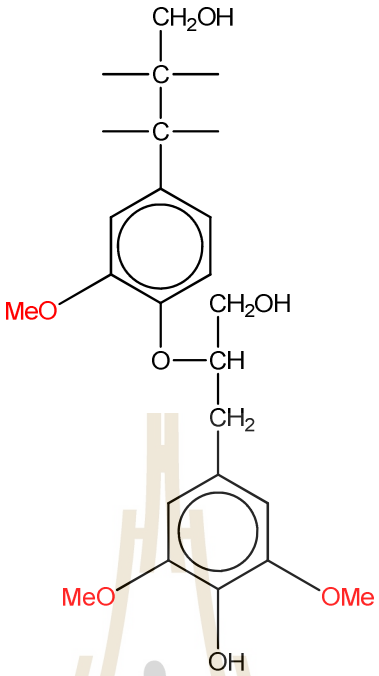


Figure 4.12 Methoxy group on G and S unit.

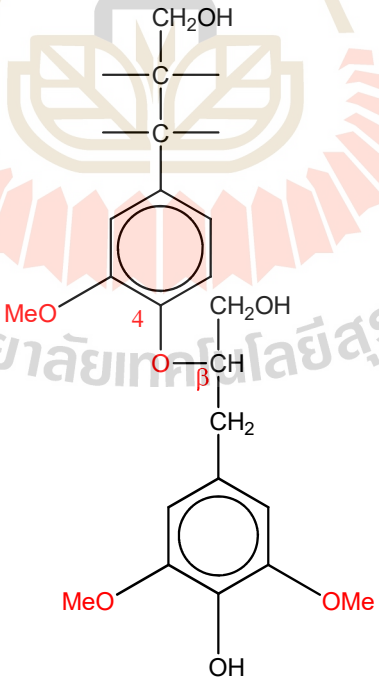


Figure4.13 β-O-4 linkage is mostly found on lignin.

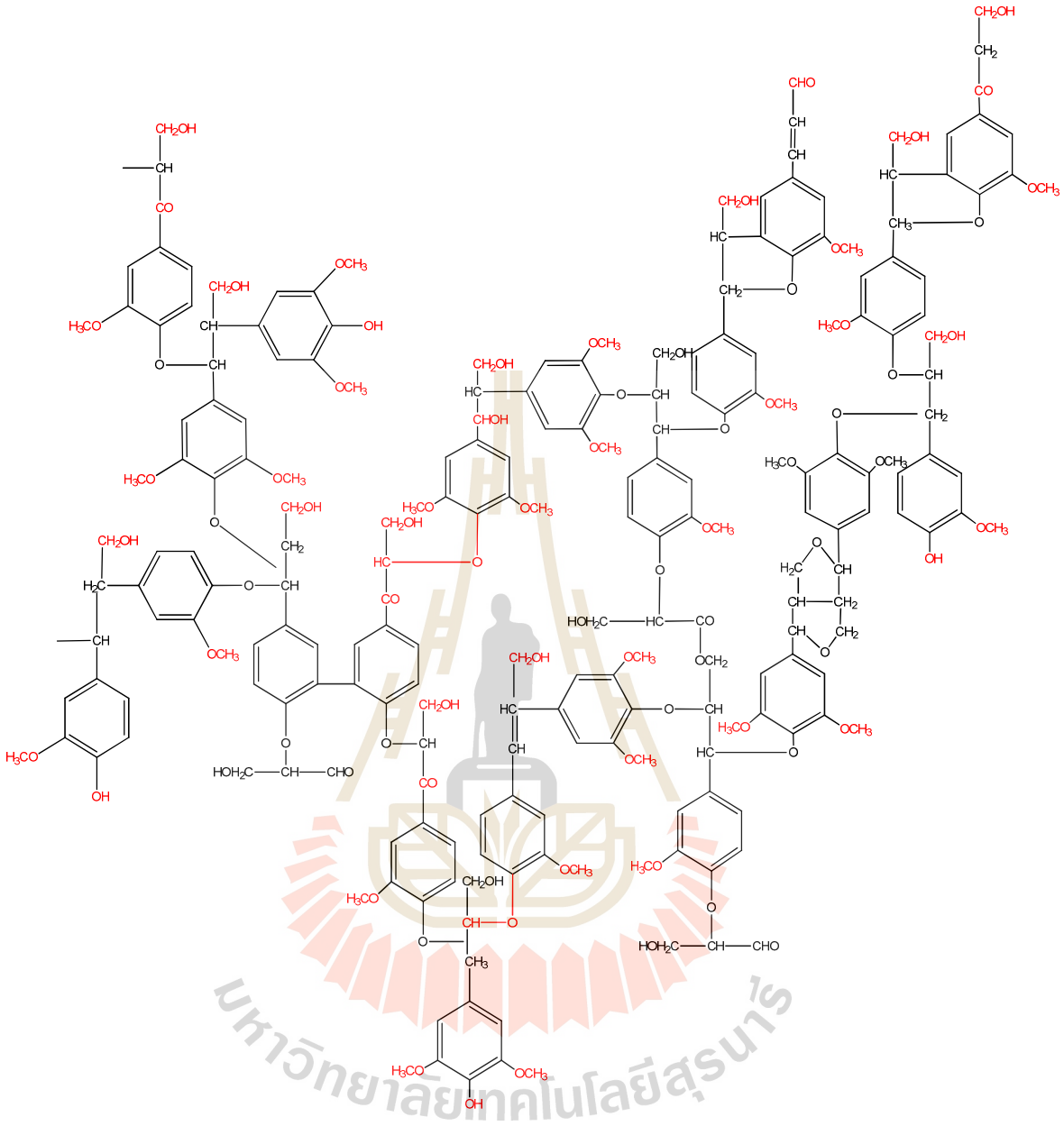


Figure 4.14 lignin structure and functional group from the FT-IR and NMR spectra of LRM precipitation after mulberry stalk pretreatment (Modified from Watkins *et. al.*, 2015)

4.6.3 Thermal property of precipitated lignin

Thermal stability of organic polymers has been commonly determined using thermal gravity analysis (TGA) under N_2 environment. TGA illustrated the relationship between the weight losses and temperature (Figure 4.15). The bond energies are an important factor corresponding to their intrinsic structure and functional groups cause different of thermolysis. The thermal decomposition of purified lignin from MS can be divided into 3 stages. In stage one; the initial stage weight loss step occurred between 30-122 °C due to the evaporation of water and low molecular weight volatile of the lignin. The second stage was around 175-295 °C attributing to the degradation of components of carbohydrates in the lignin samples. The final stage occurred above 300 °C. The decomposition of alcohol, aldehyde acid and phenolics became as a gaseous volatile from lignin at this stage. The extracted lignin by [Ch][Gly] pretreatment on MS had the greatest thermal stability and highest yield char of 29.12%. This result suggested that the extracted lignin can be provided to enhance thermal properties for its applications as thermo-chemical products for examples; bio oil and phenolic resins (Tian *et. al.*, 2015 and Watkins *et. al.*, 2015).

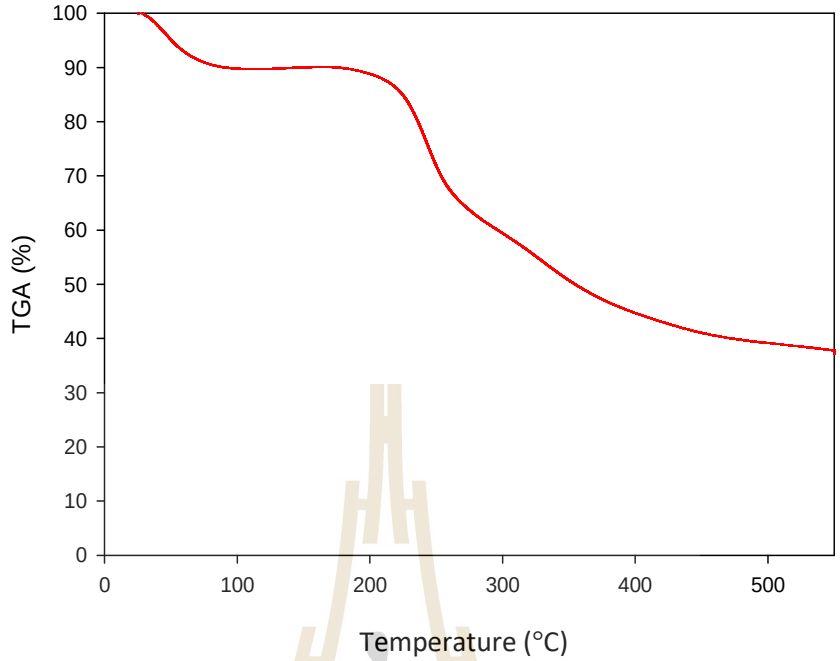


Figure 4.15 TGA curve of precipitated lignin.

Chemical components of each fraction are presented in Figure 4.16. After pretreatment, 44% from initial native biomass was removed from biomass after pretreatment step and this fraction should be retained in liquid part. CRM fraction was recovered of 56%. The cellulose content increased significantly to 51.09% upon pretreatment due to most of lignin (77%) and xylan (68.25%) were removed from native mulberry stalk. The liquid phase was collected to recovery lignin using acidification. The precipitation of lignin from the liquid fraction occurred after acetone/water (1:1 v/v) was added into the mixture, followed by pH adjustment to 2.0, and then let it sediment at 4 °C overnight. LRM precipitation from aqueous [Ch][Gly] was 464 mg·dry weight^{-1}. The LRM contains lignin as dominant component and small amount of hemicellulose. LRM contained >90% lignin, not found cellulose, and <4% hemicellulose and other.

The ILs have been used with the ability of lignin solvent. An *et. al.* (2015) reported the following precipitation of lignin according to Ninomiya *et. al.* (2015) reported the

mixture of IL [Ch][Lys] and biomass was added with acetone:water (1:1 v/v), followed by evaporation of acetone, but no LRM precipitated even overnight at 4 °C. It may involve with ability of solvent of [Ch][Lys] higher than [Ch][AcO). Nevertheless, LRM was recovered in present study with >70% lignin recovery. Furnishing of LRM with acid water is necessary for removing of partial hemicellulose.

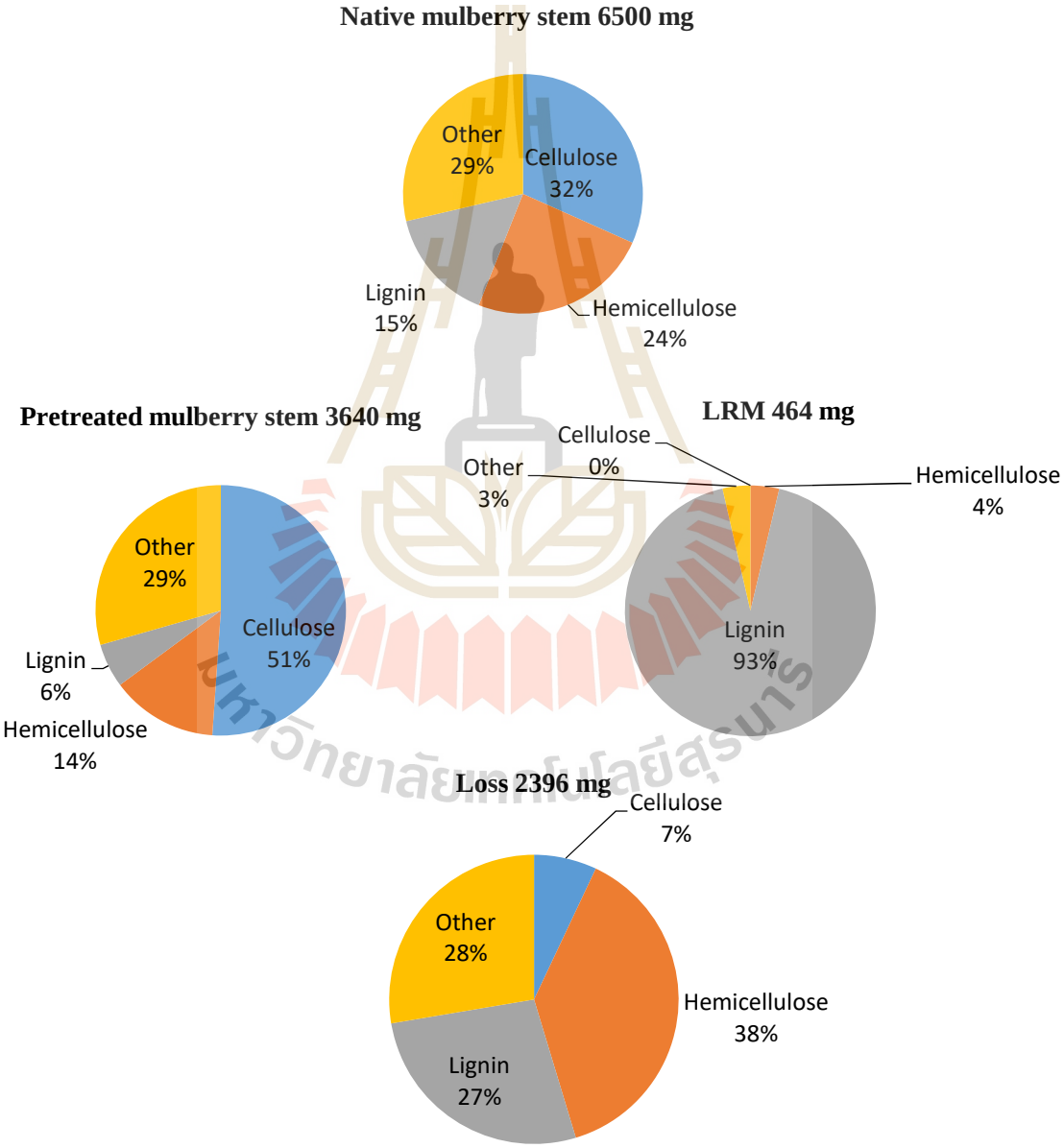


Figure 4.16 Mass balance of recovery lignin and CRM from mulberry stalk pretreatment at 80 °C for 4 h. and composition of the fractionated material.

4.7 Enzymatic hydrolysis in batch and fed batch

The solid residue after pretreatment was fed into enzymatic reaction by increasing of solid loading to investigate efficiency of solid capacity in the reaction mixture of enzyme and increase sugar concentration for further succinic acid fermentation.

4.7.1 Batch enzymatic hydrolysis

The batch hydrolysis was applied by increase solid loading in enzymatic reaction. Various solid loading (5.6, 8.4, 11.2, and 14% w/v) was incubated with different cellulase dosage such as 15, 22 and 40 FPU/g cellulose and hemicellulase 10% of each cellulase dosage. The results suggested that at same enzyme concentration, increasing of solid loading effected on decreasing of sugar concentration. At cellulase dosage of 15 FPU/g cellulose, incubation of 5.6, 8.4, 11.2, and 14% (w/v) solid loading generated the maximum glucose concentration 17.05, 18.95, 27.64, and 29.57 g/l and xylose concentrations were 7.65, 8.38, 10.96, and 12.92 g/l. Glucose yields were 49.48, 35.32, 34.48, and 31.96% and xylose yields were 27.14, 20.44, 19.87, and 18.78%. The initial saccharification rates were 3.35, 3.65, 4.49, and 4.60 $g/l \cdot h^{-1}$ (Figure 4.17).

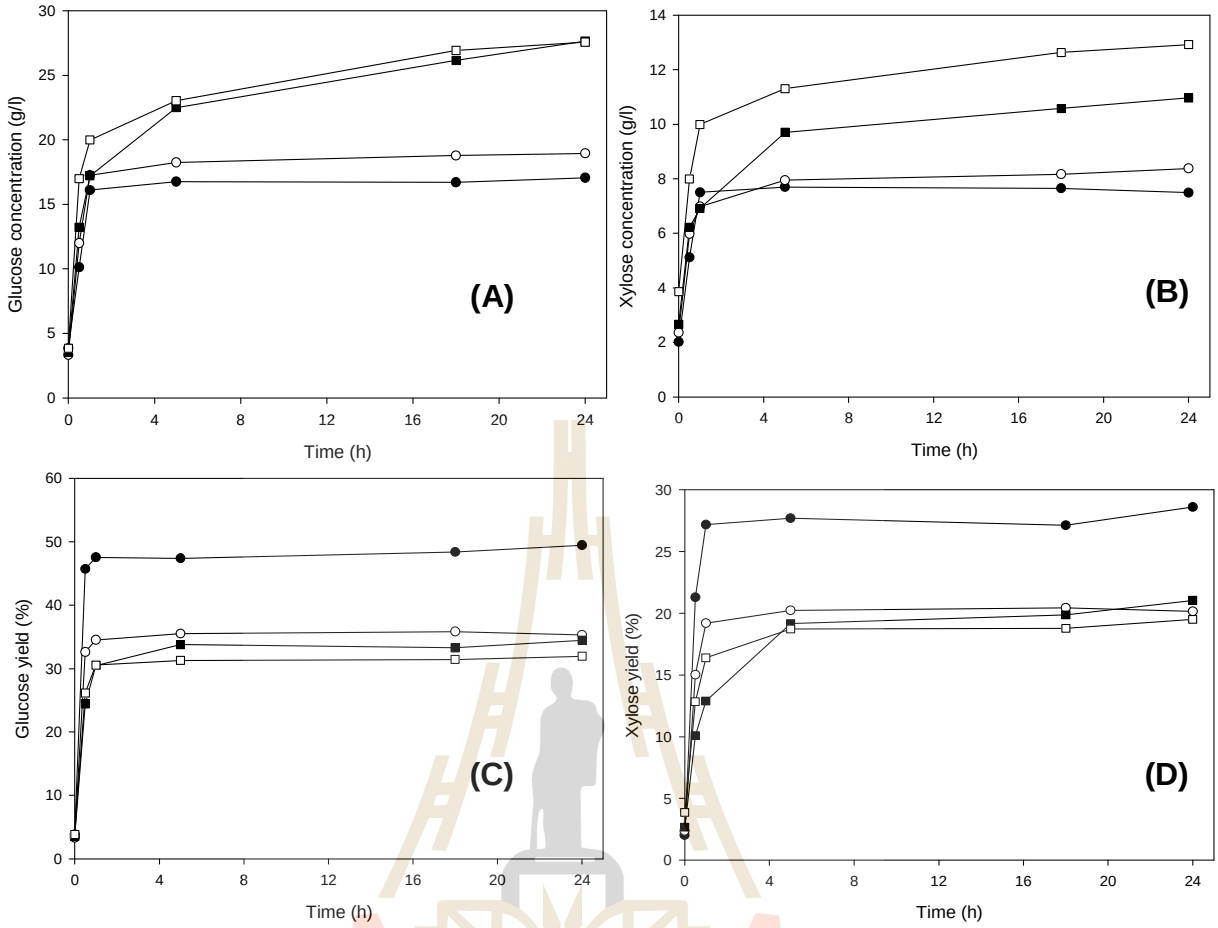


Figure 4.17 Enzymatic hydrolysis profiles at cellulase dosage 15 FPU/g cellulose (hemicellulase 10% of cellulase) with various solid loading of -●- 5.6%, -○- 8.4%, -□- 11.2%, -■- 14%; (A) glucose, (B) xylose concentration, (C) glucose and (D) xylose yield.

At cellulase concentration 22 FPU/g cellulose with various solid loading of 5.6, 8.4, 11.2, and 14% (w/w), glucose concentrations were 33.83, 37.93, 40.89, and 45.71 g/l and xylose concentrations were 10.32, 12.05, 13.82, and 15.56 g/l. Glucose yields were 95.81, 69.32, 58.03, and 51.89% and xylose yields were 37.39, 29.11, 25.05, and 22.55%. The initial saccharification rates were 6.99, 6.96, 7.31, and 8.72 g/l·h^{-1} (Figure 4.18). At the

mixture of cellulase dosage of 40 FPU/g cellulose with 8.4, 11.2, and 14% (w/w) solid loading generated glucose concentration 40.18, 43.34, and 49.05 g/l and xylose concentrations were 17.12, 17.16, and 19.57 g/l. Glucose yields were 76.033, 61.51, and 55.69% and xylose yields were 41.31, 31.09, and 28.36%. The initial saccharification rates were 8.03, 8.56, and 9.32 g/l·h^{-1} (Figure 4.19).

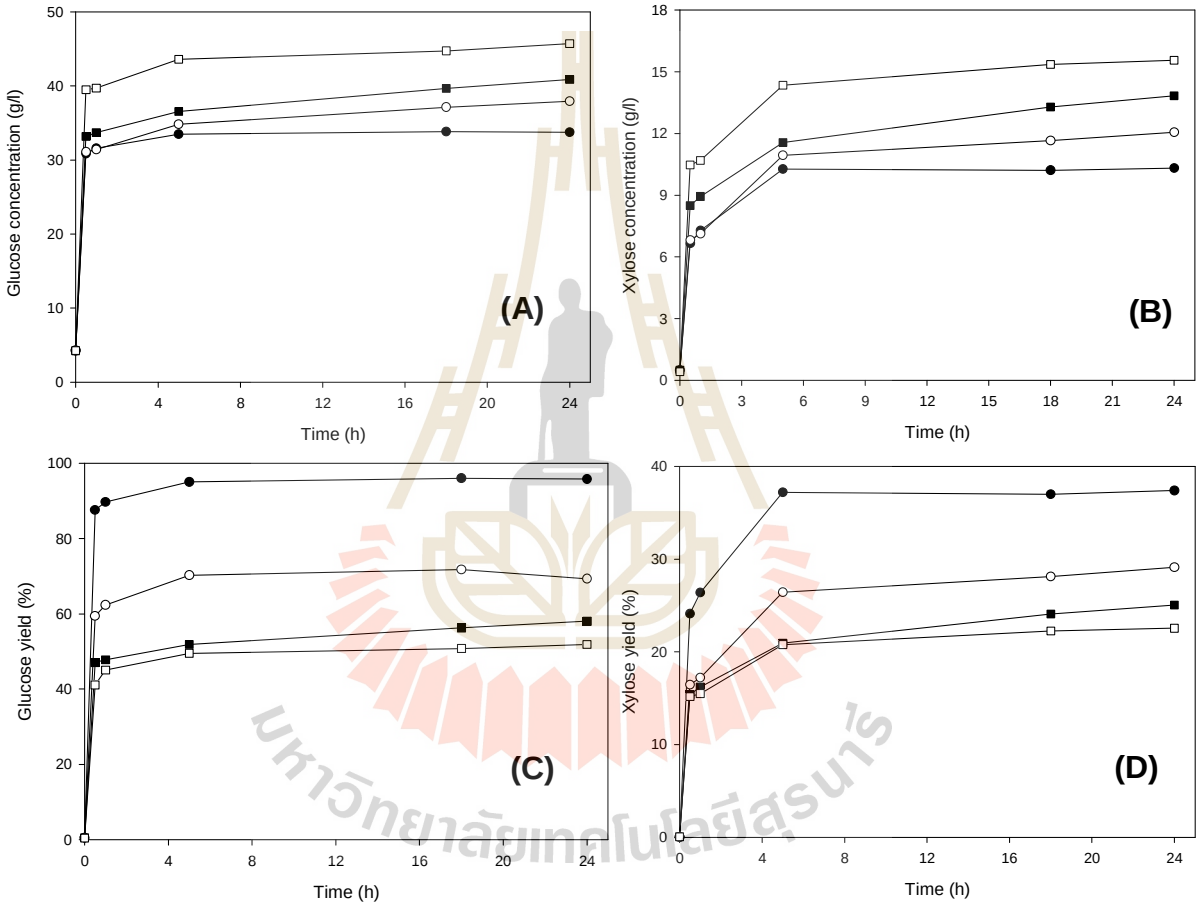


Figure 4.18 Enzymatic hydrolysis profiles at cellulase dosage 22 FPU/g cellulose (hemicellulase 10% of cellulase) with various solid loading of -●- 5.6%, -○- 8.4%, -□- 11.2%, -■- 14%; (A) glucose, (B) xylose concentration, (C) glucose and (D) xylose yield.

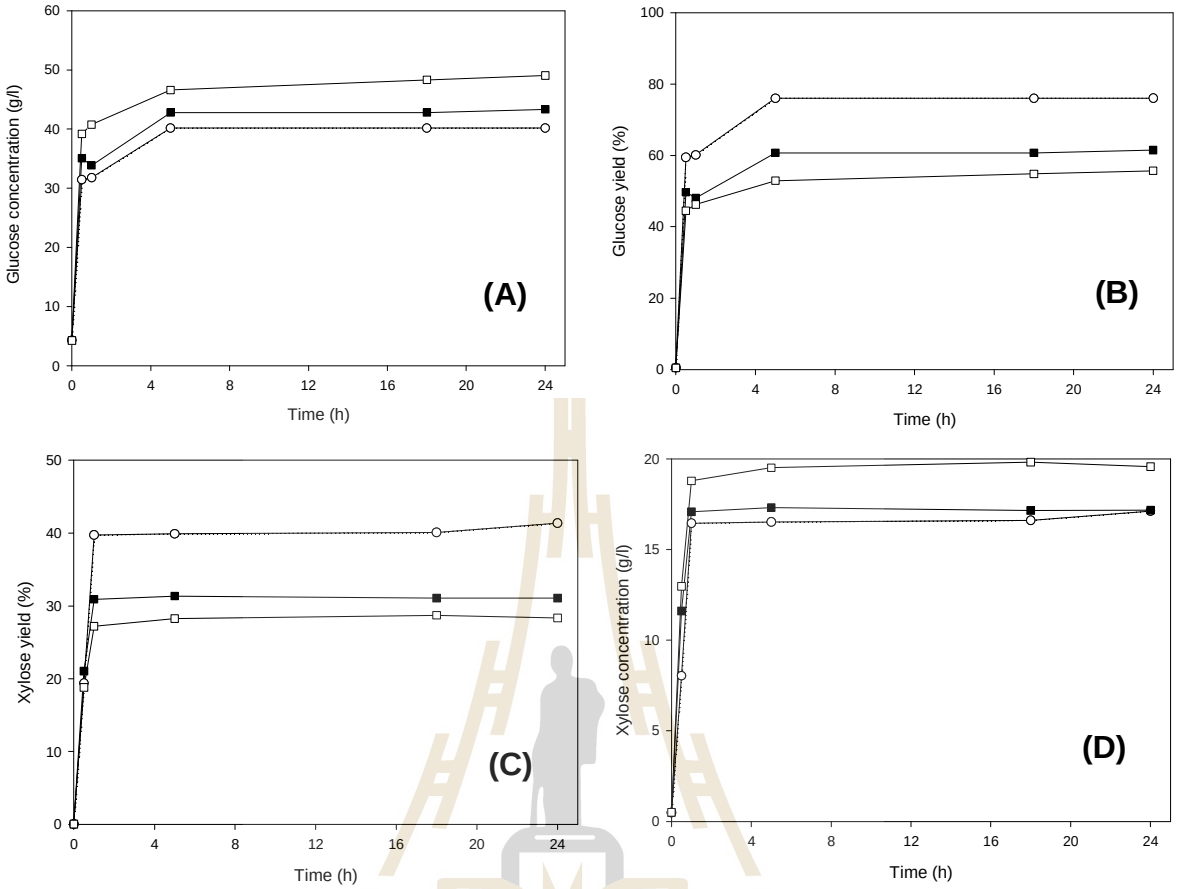


Figure 4.19 Enzymatic hydrolysis profiles at cellulase dosage 40 FPU/g cellulose (hemicellulase 10% of cellulase) with various solid loading of -○- 8.4%, -□- 11.2%, -■- 14%; (A) glucose, (B) xylose concentration, (C) glucose and (D) xylose yield.

At the same enzyme dosage, an increase solid loading affected on rising of both of sugar concentrations, conversely yields of both of sugars were decrease after increasing of solid loading (Figure 4.20). Conventional laboratory enzymatic reaction was carried out at solid loading of ≤ 5% wt. Using of low substrate concentration has the disadvantages; lower obtained sugar amount or sugar concentration than high biomass loading, from this reason leads to low productivity and high cost sugar and its fermentation products

(Ioelovich, 2015). However, high solid biomass in enzymatic reaction cause of low sugar conversion yield. At higher solid concentration in the enzymatic mixture produce more limitation of enzyme working for instance, lack of water available to promote mass transfer, increased substrate viscosity, and increased concentration of inhibitor produced affecting pretreatment. Current technology has allowed the use of up to 30% solid content of starch in reaction whereas, not over 20% solids matter in lignocellulosic materials (Modenbach *et. al.*, 2012).

Increasing of enzyme dosage from 15 to be 22 FPU/g cellulose resulted in improve sugar concentrations and yields. At 5.6% solid loading, glucose concentration was improved from 17.85 to be 33.82 g/l and glucose yield from 49.48 to be 95.81%. At 8.4% solid loading, enhanced glucose concentration from 17.5 to be 36.64 g/l and glucose yield from 35.32 to be 67.17% was found. At 11.2% solid loading, 34.54 g/l improved glucose concentration from 27.58 g/l and enhancing of 57.22% glucose yield from 40.32%. At biomass concentration of 14% dry wt, glucose concentration was improved from 28.15 to be 45.83 g/l and also increased glucose yield was 52.04 from 31.95%. These results showed that increase enzyme from 15 to 22 FPU/g cellulose had affected on enhancing of nearly 2 times sugar concentrations and yields. To enhance sugar concentration and sugar yield for 8.4, 11.2, and 14% solid loading, enzyme concentration was increased from 22 to be 40 FPU/g cellulose. At solid loading 8.4%, glucose concentration was improved from 36.64 to be 40.29 g/l and glucose yield was 76.23 from 69.32%. At solid loading 11.2%, improved 43.39 g/l glucose concentration and 61.58% yield was found (improvement from 34.54 g/l and 57.22%). At biomass concentration of 14%, enhancing of glucose concentration was 49.40 from 45.83 g/l and glucose yield was improved to be 56.09 from 52.04%. These results showed that an enhance enzyme dosage from 22 to be 40 FPU/g cellulose improved sugar concentration and yield 1-0.5 times. An increase enzyme dosage 2 times (from 22 to 40 FPU/g cellulose) can improve slightly sugar concentrations and

yields. There was found that increasing of enzyme concentration effect on rising of sugar concentrations and sugar yields. However, a rise enzyme dosage to 40 FPU/g cellulose had a slightly effect on improvement of sugar conversion. Even though, increase enzyme dosage had not significant raised of glucose or xylose concentration and yield.

The rate and extent of hydrolysis depend on solid loading, enzyme loading, hydrolysis time and structural feature of substrate (Zhu *et. al.*, 2008). Gao *et. al.*, (2014) reported the conversion rate was rapidly in the first 12 h of process due to reduce crystallinity on cellulose structure and prolonging incubation time, the liquefaction rates were decrease which relative to the effect of product inhibition.

The results were opposite with a rise hydrolysis efficiency by an increase cellulase dosage, Gao *et. al.*, 2014 reported more cellulase accelerated enzymatic hydrolysis efficiency and decrease insoluble substrate which caused facilitates heat and mass transfer in whole of process. Present study, increase enzyme dosage from 22 FPU/g substrate to be 40 FPU/g substrate enhance small amount of glucose and xylose. It may be due to the other affecting factor on enzymatic hydrolysis such as product inhibition that is on the above mention.

Fermentable sugar should be as high as possible to save cost of process of bio-ethanol (Puri *et. al*., 2013) including of other fermentation products. At high solid loading, the limitation of enzyme activity occurred for examples; lack of water, viscosity, inhibitor from pretreatment, and product inhibition. The fed-batch hydrolysis was further applied to overcome these problems (Gao *et. al.*, 2014). The condition of cellulase dosage 22 FPU/g cellulose and solid loading of 8.4% were chose for preparation of hydrolysate for succinic acid fermentation. Cellulase dosage of 22 FPU/g cellulose was applied for enzyme loading in fed-batch enzymatic hydrolysis and solid loading was increased from 11.2% (w/v) in fed-batch enzymatic hydrolysis system.

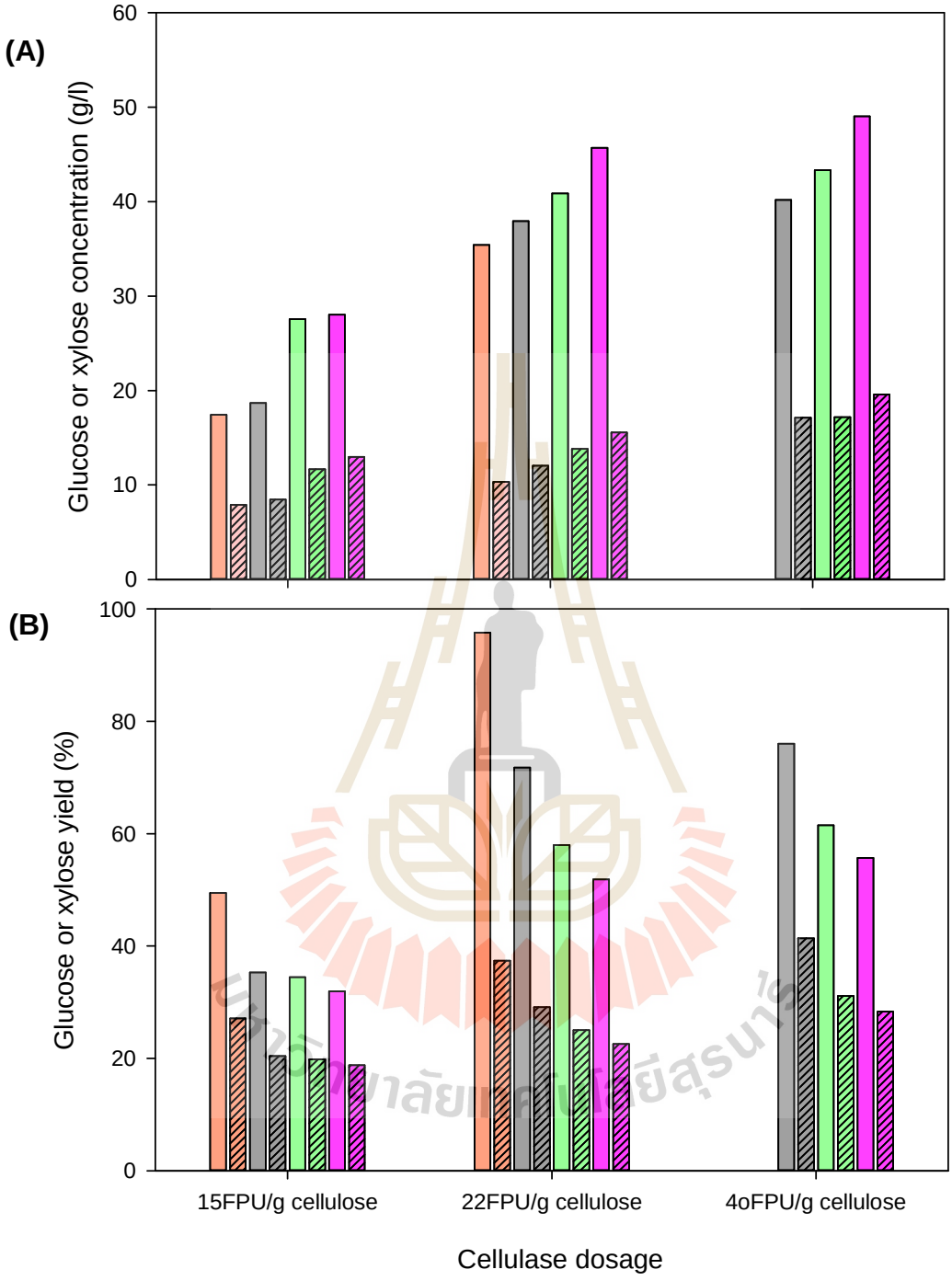


Figure 4.20 Glucose and xylose content of hydrolysate with various solid loading 5.6, 8.4, 10.2, and 14% and enzyme dosages15,22 and 40 FPU/g cellulase (mixture enzyme of cellulase and hemicellulase 10% of cellulase); (A) sugar concentration and (B) sugar yield.

4.7.2 Fed-batch enzymatic hydrolysis

As the batch operation, the sugar yield trended decrease when higher biomass loading in the enzymatic reaction. The fed-batch enzymatic hydrolysis was applied to overcome decreasing of sugar conversion when high solid content in enzymatic reaction.

Fed-batch hydrolysis was operated with various amount of solid loading into enzymatic systems which were coded with operating No. as shown on Table 9 and Figure 4.21. The results suggested that fed batch hydrolysis can improve hydrolysis efficiency as express with increased sugar concentrations and yields of both of sugar (Table 4.9 and Figure 4.21). At total solid loading of 11.2% or operating No.1 (5.6%+5.6% wt), glucose and xylose concentration was 68.63 and 24.29 g/l and glucose and xylose yields was 96.97 and 51.32%. At total 14% dry wt solid concentration (operating No. 2; 5.6%+8.4% wt), obtained glucose and xylose was 77.16 and 25.19 g/l and glucose and xylose was achieved to 86.86 and 42.40%. Moreover, solid loading was improved to total solid 16.8% (operating No.3; 8.4%+8.4%) and 19.6% (operating No.4; 8.4%+5.6+5.6%). At operating No.3, 84.41 and 27.56 g/l of glucose and xylose concentrations were achieved and 78.97 and 38.57% of both of sugar yields were improved. At operating No.4, glucose and xylose concentration was 88.71 and 31.02 g/l and 71.41 and 37.35% of both of sugar yield were achieved.

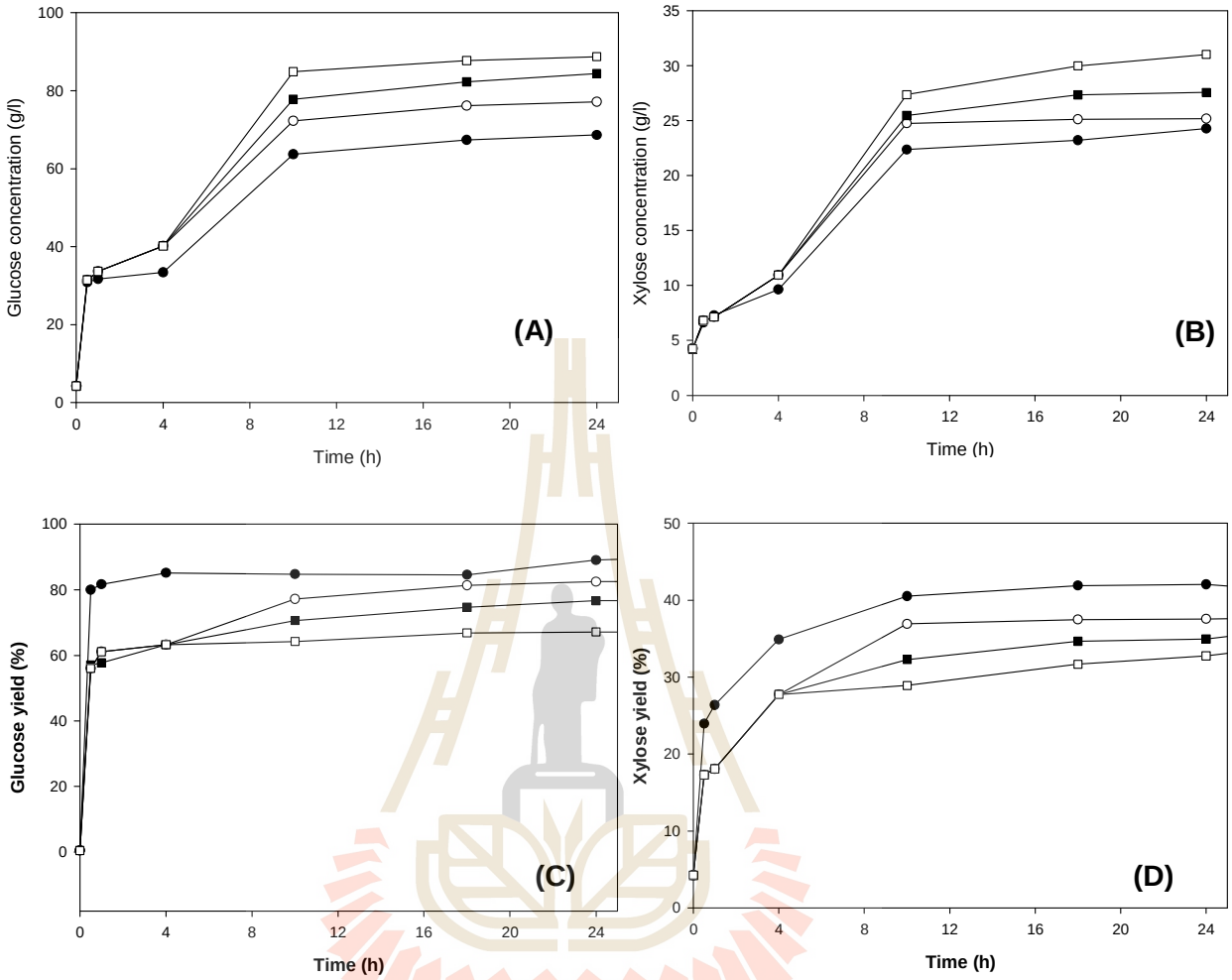


Figure 4.21 Glucose and xylose concentrations (A and B), glucose and xylose yields (C and D) with fed-batch operating; -●- represent 5.6%+5.6% (No.1), -○- represent 8.4%+5.6% (No.2); -■- represent 8.4%+8.4% (No.3), and -□- represent 8.4%+5.6%+5.6% (No.4).

Table 4.9 Sugar concentrations and yields of fed-batch hydrolysis

Operating No.	Solid loading (%)[A]			Sugar concentrations (g/l)		% Yields of sugar	
	1	2	3	Glucose	Xylose	Glucose	Xylose
1	5.6	5.6	-	68.63	24.29	96.97	51.32
2	8.4	5.6	-	77.16	25.19	86.86	42.40
3	8.4	8.4	-	84.41	27.56	78.97	38.57
4	8.4	5.6	5.6	88.71	31.02	71.41	37.35

[A] 22 FPU/g cellulose enzyme dosage for each time of solid adding, solid was added after initiation of 4 h

At solid loading of 11.2% in batch hydrolysis, glucose concentration and yield was 34.54 g/l and 57.22% whereas, 68.63 g/l and 96.97% of glucose concentration and yield was accomplished in fed-batch hydrolysis. Although, cellulase concentration of 40 FPU/g cellulose was added in batch hydrolysis system, sugar yield was slight improved whereas, >1.6 times glucose yield was achieved in fed batch hydrolysis. The results confirm to total solid loading of 14% with enzyme concentration of 40 FPU/g cellulose. At 14% solid loading in batch operation, glucose concentration and yield was 49.40 g/l and 56.09% whereas; fed-batch operation (operating No.2) could achieve to 77.16 g/l and 86.86% of glucose concentration and yield. Theses comparisons suggested that batch hydrolysis operation contain factor that effect on hydrolysis reaction by enzyme. Products inhibition is a limitation for enzyme activity. Fed-batch enzymatic hydrolysis can improve enzymatic efficiency according to results comparisons between batch and fed-batch hydrolysis at solid loading of 11.2 and 14%wt. At total solid loading of 16.8% and 19.6%wt, glucose concentrations and yields were more than 88 g/l and 70%. Again, the results confirm to fed-batch hydrolysis can improve enzyme activity. Moreover, fed-batch hydrolysis can overcome other limitation of enzyme activity in batch process. The fed-batch operations were applied for succinic acid fermentation with high glucose concentration (> 40 g/l initial

glucose concentration).Succinic acid fermentations were operated by various solid loading according to the operation of fed batch hydrolysis.

4.8 Succinic acid fermentation

Pretreatment step can cause of the formation of possibly inhibitor that effect on enzyme activity and organisms in the fermentation processes (Palmqvist and Hahn, 2000). The succinic acid fermentation was used to demonstrate that carbohydrate rich materials (CRM) can be applied for fermentation process. Lignocellulosic conversion to fermentation product can be done by 2 methods; separation hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) process. SHF refers to the physical separation process, in which lignocellulosic bioconversion occurs in two different reactors, while SSF is a one step process that occurs within the same bioreactor (Xu *et. al.*, 2009).

4.8.1 Separation enzymatic hydrolysis and fermentation

The hydrolysates of mulberry stalk were mixtures of sugar, in which glucose and xylose were two major sugars. These sugar contained in CRM of pretreated mulberry stalk, which can be used as co-carbon resources for succinic acid fermentation by *A. succinogenes* ATCC55618 (Figure 4.22). The results suggested that CRM from [Ch][Gly] can be applied for succinic acid fermentation with high yield succinic acid (>0.8 g·g glucose^{-1}). Glucose and xylose from hydrolysate can be utilized by this bacterial strain which there was succinic acid as a dominant product and small concentration of acetic acid and formic acid. Lactic acid was not found from this fermentation. The lag phase was not over 6 h of fermentation. The fermentation profile of hydrolysate of pretreated MS with [Ch][Gly] (HPMS) was similar with pure glucose. Utilizations of glucose and xylose were since 6 h until 24 h of fermentation period and related to succinic acid increased.

At 19 g/l glucose concentration, consumption of glucose from hydrolysate of pretreated MS was 0.78 (g succinic acid/g glucose) and yield of succinic acid on total

consumption of glucose and xylose for succinic acid was 0.63 (g succinic acid/g sugar). Yield of succinic acid on sugar consumption in 19 g/l glucose concentration was smaller than succinic acid yield from 25 g/l glucose concentration. At initial 25 g/l glucose concentration, succinic acid yield on consumption of glucose from hydrolysate of pretreated MS was 0.86 (g succinic acid/g glucose) and yield of succinic acid on total consumption of glucose and xylose was 0.726 (g succinic acid/g sugar). *A. succinogenes* can use both of sugar from 25 g/l until 1.17 g/l residual glucose.

Likely, subtotal consumption of 19.5 g/l initial glucose (1.1 g/l residual glucose). In the case of xylose, this bacterial strain can also utilize almost the whole xylose. The higher concentration of initial glucose and xylose was applied to investigate sugar utilization. At 30 g/l initial glucose concentration, succinic acid was still dominant fermentation product and by products was acetic acid and formic acid. Remain glucose after fermentation was 5.81 g/l and 2.33 g/l xylose residual from 10.15 g/l with succinic acid concentration was 24.81 g/l. Succinic acid yield and productivity from both of sugar utilization was 0.80 and 1.03 $gl^{-1}h^{-1}$. At 25 and 19.5 g/l glucose concentration, productivity of succinic acid from was 0.89 and 0.61 $gl^{-1}h^{-1}$. At lower sugar concentration, sugar is rapidly utilized and gone before higher sugar concentration. The separated CRM after pretreatment MS with an ionic liquid can be used as a substrate for succinic acid fermentation. These CRM will be applied as a material for succinic acid fermentation with high solid loading in the further studies to enhance succinic acid amount from wood mulberry stalk.

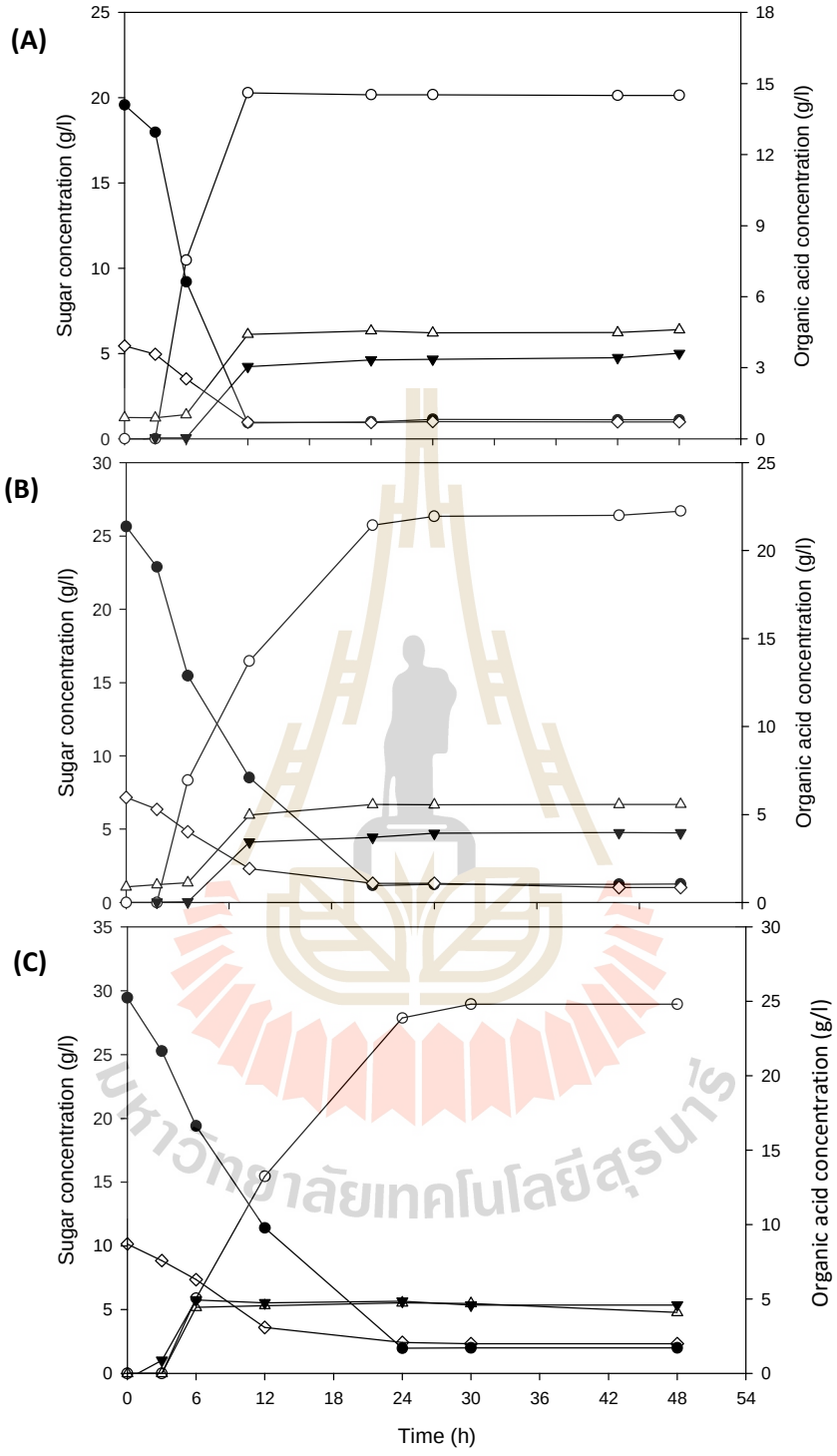


Figure 4.22 Succinic acid fermentation profile in hydrolysate of pretreated MS (batch enzymatic hydrolysis 8.4% solid loading and cellulase of 22 FPU/g cellulose) with an initial glucose concentration of (A) 19 g/l, (B) 25 g/l and (C) 30 g/l; -●- glucose concentration, -◇- xylose concentration, -○- succinic acid, -△- acetic acid, and -▼- formic acid concentration.

The increases content of glucose or xylose in hydrolysates have the effect on succinic acid production. Zheng *et. al.* (2009) reported that succinic acid production increased with increasing ratio of glucose/xylose, however succinic acid from combination of pure glucose and xylose was not greater than that from hydrolysate of corn straw, despite the low ratio of glucose/xylose in this hydrolysate. This suggests the occurrences of other components within the corn straw hydrolysate that promoted succinic acid production. In this result, succinic acid fermentation increased when increase hydrolysates wood biomass in fermentation media which corresponded to fermentable sugar raise in anaerobic condition.

Bacterial strains isolated from bovine rumen have ability to utilize many kinds of sugars. *A. succinogenes* ATCC55618 is one of isolate from cow's rumen that can produced succinic acid from a variety of sugar such as, arabinose, cellobiose, fructose, galactose, glucose, lactose, maltose, mannitol, mannose, sucrose, xylose (Guettler *et. al.*, 1999). In this research, glucose and xylose were the major sugars from wood hydrolysis according to Figure 4.23, which were choose as two major sugars to monitor during fermentation. At 30 g/l glucose and 10 g/l xylose concentration, glucose and xylose were used 78.92 and 76.97% whereas utilization of glucose and xylose were 94.14 and 81.40% when initial glucose and xylose was at 19.5 and 5.4 g/l. Moreover, the consumption of sugar at initial glucose and xylose was 95.18 and 81.82% when initial glucose and xylose was at 25 and 7.1 g/l. Consumption of sugar >75% and succinic acid yield > 0.8 demonstrated that *A. succinogenes* utilize sugar for succinic acid production. The results established that sugar from pretreated mulberry stalk was used by *A. succinogenes* ATCC55618 and notable [Ch][Gly] could improve sugar concentration and yield from wood biomass pretreatment.

4.8.2 Semi-simultaneous saccharification and fermentation

Separation hydrolysis and fermentation (SHF) in batch fermentation was applied for investigation of efficiency of CRM from pretreatment with [Ch][Gly]. The results approved CRM can be applied for succinic acid fermentation after increasing of sugar concentration from hydrolysis reaction. Recently, Simultaneous saccharification and fermentation (SSF) has been wildly interested to decrease step of fermentation using biomass as substrate.

In the Semi-SSF process (10-12 h before 24 h complete hydrolysis), *A. succinogenes* ATCC55618 can also utilize glucose and xylose sugar to generate succinic acid as a dominant product and small amount of byproducts of acetic and formic acid with concentration <5.5 g/l (Figure 4.24). At initial glucose concentration of 30 g/l (biomass loading of 5.6%), succinic acid concentration was 27.59 g/l with yield of succinic acid was 0.88 $g \cdot g^{-1}$sugar consumption and productivity 1.18 $g/l \cdot h^{-1}$. Succinic acid yield on MS substrate was 0.82 $g \cdot g^{-1}$ dry matter from SSF process whereas, succinic acid yield from pretreated MS in SHF was 0.73 $g \cdot g^{-1}$ dry matter and productivity 1.03 $g/l \cdot h^{-1}$.

At 37 g/l glucose concentration (8.4% solid loading), succinic acid concentration was 35.41 g/l with yield of succinic acid 0.77 $g \cdot g^{-1}$ sugar consumption and productivity 1.39 $g/l \cdot h^{-1}$. The result suggested that SSF process could increase succinic acid yield and increase productivity from SHF process. The fed batch hydrolysis had effect on increase sugar concentration and yield. Fed batch hydrolysis and SSF was applied to enhance succinic acid concentration in fermentation.

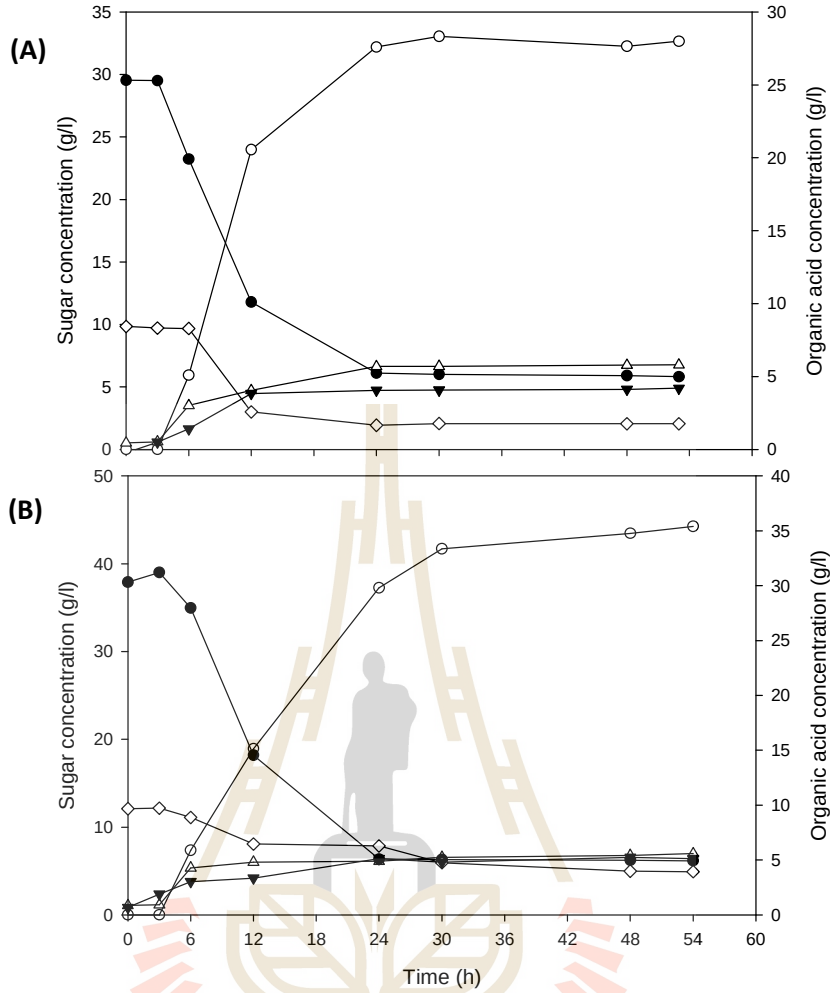


Figure 4.23 Succinic acid fermentation profile in Semi-SSF of PMS with an initial glucose concentration of (A) 30 g/l (5.6% solid loading) and (B) 37 g/l (8.4% solid loading); -●- glucose concentration, -◇- xylose concentration, -○- succinic acid, -△- acetic acid, and -▼- formic acid concentration.

The results showed that Semi-SSF present slightly higher succinic acid concentration and productivity than SHF process. The result similar with succinic acid production from corn straw pretreatment, succinic acid productivity in batch fermentation with SSF (0.99 g/l/h) was higher than SHF (0.95 g/l/h) (Zheng *et. al.*, 2009). Succinic acid has produced via SSF process with temperature for succinic acid production and enzyme activity, 12.5% (w/v) pretreated acid dilute rapeseed meal was applied for succinic acid

production, with the highest concentration and yield was 15.5 g/l and 12.4 g/100 g dry matter or 0.124 g/ g dry matter without yeast extract at 38 °C and pH 6.4 (Chen *et. al.*, 2011).

Optimum pH of commercial cellulase (CTec2) is 45-50 °C (Figure A12), whereas region of optimum temperature for *A. succinogenes* ATCC55618 grow and succinic acid production is at 30-37 °C. At 37°C, relative performance is lower than 45% and succinic acid fermentation could not occur by without initial glucose. From this reason, enzyme was added to generate fermentable sugar before succinic acid fermentation and increase productivity for succinic acid production and temperature of 37 °C was chose for semi-SSF process. Zheng *et. al.* (2010) reported succinic acid concentration and yield decreased when raised temperature 38, 40 and 42 °C, furthermore, the residual sugar concentration in SSF at 40 and 42 °C was higher than that 38 °C. Present study, the pretreated mulberry wood stalk was hydrolyzed at 50 °C until 15 h and was applied for succinic acid production at 37 °C. The mixture of enzymatic reaction was introduced for fermentation before complete hydrolysis reaction and without stop enzyme reaction. As above mention, 50% relative performance commercial CTec2 at 37 °C caused enzyme was still active in the step of fermentation.

Conversion of lignocellulosic material into fermentation products such as ethanol, organic acid can be processed via SHF and SSF (Gao *et. al.*, 2014; Chen *et. al.*, 2011). The advantages of SSF process is elimination of additional steps in the hydrolysis process, efficient and time-saving process. At solid loading of 8.4% (Figure 4.23A and 4.22C), succinic acid concentration with SSF and SHF was 27.59 and 24.81 g/l. The initial sugar concentration of both of process is similar; however succinic acid from SSF process was higher than SHF. Glucose and xylose utilization with SSF process was 80.99 and 80.37% whereas; utilization of both of sugar with SHF was 78.93 and 76.97%. There was sugar consumption by *A. succinogenes* in SSF process greater than SHF. The result implied that

SSF provide environment for sugar utilization of bacteria resulting in improve succinic acid production. Succinic acid concentration and yield with SSF process greater than SHF arise from enzyme is still active even at 37 °C fermentation temperature.

The result of SHF and Semi-SSF processes investigated *A. succinogenes* consume sugar from pretreatment of mulberry stalk by [Ch][Gly]. However, enzymatic hydrolysis at low solid hydrolysis caused of small sugar concentration and productivity of fermentation. According to fed-batch hydrolysis results, high solid content can be applied in enzymatic hydrolysis to increase sugar concentration and yield. The CRMs from mulberry stalk pretreatment were applied as material for succinic acid fermentation with high solid loading in the further studies to enhance succinic acid amount from wood mulberry stalk.

Dry matter biomass in enzymatic reaction was increased by fed-batch enzymatic hydrolysis. Increase dry matter had an effect on enhances fermentable sugar which caused higher succinic acid concentration in batch fermentation (Figure 4.24). At total 11.2% (w/v) solid loading (from fed batch hydrolysis 5.6% + 5.6% dry matter), the initial glucose concentration of 60 g/l produced 41.98 g/l succinic acid, yield succinic acid of 0.82 g/g sugar consumption and productivity 1.39 $g/l \cdot h^{-1}$. At 16.8% total solid dry matter (from fed batch hydrolysis 8.4 % + 8.4% dry matter), 72 g/l initial of glucose concentration produced 41.95 g/l succinic acid or yield of succinic acid from sugar was 0.76 g/g sugar consumption. Yield of succinic acid on dry matter biomass from solid loading of 11.2% and 16.8% was 0.53 and 0.36 g/g substrate, respectively.

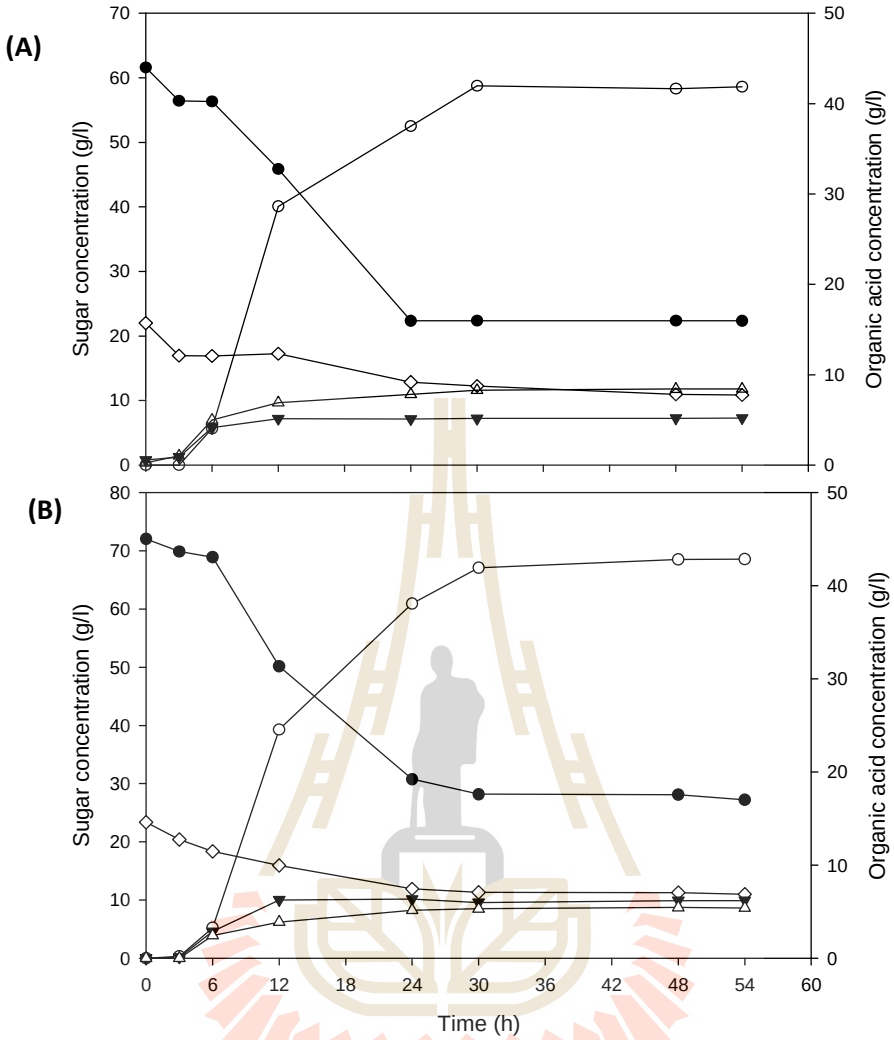


Figure 4.24 Succinic acid fermentation profile in semi-SSF of PMS with an initial glucose concentration of (A) 60 g/l (11.2% total solid loading from 5.6%+5.6% fed-batch hydrolysis) and (B) 72 g/l (16.8% solid loading); -●- glucose concentration, -◇- xylose concentration, -○- succinic acid, -△- acetic acid, and -▼- formic acid concentration.

At solid loading of 11.2%, succinic acid concentration and yield was 41.98 g/l and 0.53 g·g dry substrate^{-1}, which was higher than succinic acid production at 16.8% solid loading. High solid loading caused high glucose and xylose content in fermentation process; however high solid loading had not effect on improve succinic acid production.

An increase substrate is not always enhance succinic acid yield, using 70 g/l cellobiose resulted in 38.9 g/l and 66.6% succinic acid concentration and yield, however 72.2% succinic acid yield was obtained when 30 g/l cellobiose was introduced (Jiang *et. al.*, 2013).

The high initial glucose concentration is important effect on cell growth and metabolite production (Zheng *et. al.*, 2009). The effect of initial sugar on cell growth and succinic acid production by *A. succinogenes* was reported. Lee *et. al.* (1999) reported a longer lag phase was accompanied by lower biomass and succinic acid production at a high initial glucose concentration 80 g/l. Zheng *et. al.*, (2010) reported the initial substrate concentration of 70 g/l, the highest succinic acid concentration of 35.3 g/l, moreover enzyme adding over 20 FPU/g substrate did not improve succinic acid production.

When, consideration of succinic acid yield and amount of succinic acid in SSF batch process, 11.2% solid loading was further applied for enhanced succinic acid production in 2 L bioreactor. Succinic acid fermentation was processed in 2 L stirred fermenter to investigate succinic acid fermentation using pretreated mulberry stalk in SSF process.

Succinic acid was produced in 2 L stirred bioreactor with same condition in 50 ml anaerobic bottle, pH control by 30% $MgCO_3$. In 2 L bioreactor with 500 ml working volume, 46.93 g/l succinic acid concentration was achieved and succinic acid yield was 0.42 g/gsubstrate and 1.89 g/l h^{-1} productivity, in which succinic was still a dominant product (Figure 4.25).

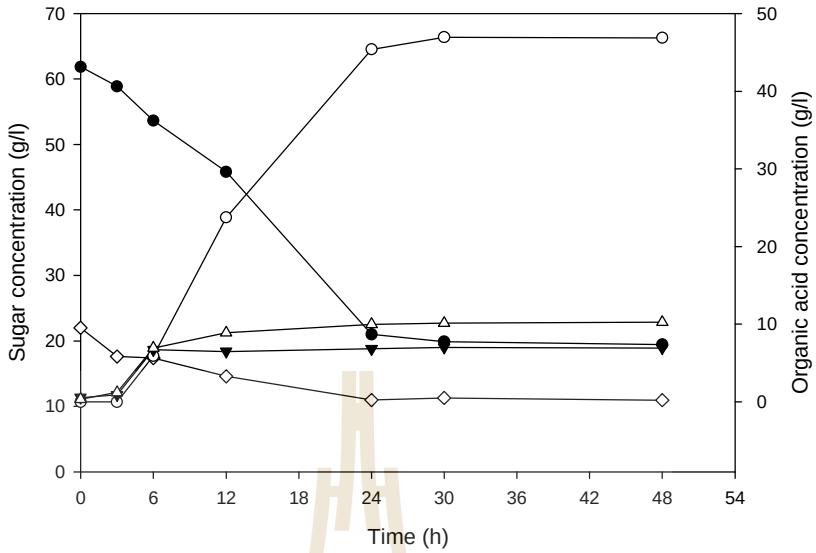


Figure 4.25 Succinic acid fermentation profile in Semi-SSF of PMS with an initial glucose concentration of 60 g/l (11.2% total solid loading from 5.6%+5.6% fed-batch hydrolysis) -●- glucose concentration, -◇- xylose concentration, -○- succinic acid, -△- acetic acid, and -▼- formic acid concentration.

This result exceeded that of the anaerobic bottle fermentation, which might due to the better environment control in the stirred fermenter which can be occurred when proceed in bioreactor, succinic acid concentration and yield in anaerobic bottle was 39.4 g/l and 77.7% and succinic acid reached 45.5 g/l and 80.7% when subjected in 5 L stirred bioreactor (Zheng *et. al.*, 2009). Zheng *et. al.*, (2010) also reported the highest succinic acid concentration of 35.3 g/l and yield of 0.57 g/g substrate in anaerobic bottle and based on the same substrate concentration with anaerobic bottle, succinic acid concentration in 5 l stirred bioreactor was 47.4 g/l and yield reach 0.72 g/g substrate.

Agricultural products have been wildly used for organic acid fermentation including of succinic acid production for instance, tapioca starch (Thuy *et. al.*, 2017). In addition, agricultural wastes have been also introduced for fermentation of many products include among succinic acid such as corncobs, corn stover, and rice straw (Akhtar *et. al.*, 2014).

The technology of conversion of starch into fermentable sugar is not complicated as much as lignocellulosic material technology due to superior recalcitrant and strong phenolic polymer/fiber network in lignocellulosic material than α configuration at the anomeric carbon of starch. However, lignocellulosic material has many advantages such as wastes from agricultural and forest, abandon renewable sources and not competitive with crops for food. Many lignocellulosic wastes have been applied for succinic acid production with different pretreatment methods.

Ionic liquid have been used for fermentation process with lignocellulosic waste for example; 1-allyl-3-methylimidazolium chloride ionic liquid (AmimCl). Combination of AmimCl with steam explosion or hot compressed water was effective in treating efficiency than ionic liquid alone. Pinewood extract produced 20.7 g/l succinic acid with average yield of 0.37 g·g biomass^{-1}. Pretreated pinewood with IL has theoretical yield of succinic acid of 57.1% (Wang *et. al*., 2014).

Among pretreatment methods, succinic acid can be produced with different concentration, yield and productivity. Alkaline and acid pretreatments are the most general method of pretreatment however, these methods required the detoxification and high amount of water to remove inhibitor or reduce alkalinity for instance; furfural, HMF or phenolic which are inhibitory the microbial growth and resulted in low metabolite products (Zheng *et. al.*, 2010).

Cholinium amino acid ionic liquid behaved as a high selective lignin remover from lignocellulosic materials; enhance enzyme accessible on carbohydrate structure, and can be applied for fermentation with not inhibited on organisms. The toxicities of choinium based ionic liquid can be demonstrated by the profile of succinic acid fermentation. ILs pretreatments do not require high amount of water to remove inhibitor substances after pretreatment step (Shi *et al*., 2013).

The entire work flow of succinic acid production and extracted lignin using mulberry wood biomass with [Ch][Gly]IL solvent is illustrated in Figure 4.26. CRM (carbohydrate rich material) and LRM (lignin rich material) from mulberry stalk pretreatment with IL is a representative of biorefinery wood biomass. The 6500 mg initial native mulberry stalk was pretreated with [Ch][Gly] and 56% solid residual after pretreatment or 3640 mg solid remain. About 77% glucose yield of hydrolysis of pretreated MS was achieved. Based on glucose consumption, 76.90% and 84.93% yield succinic acid from SHF and SSF process was obtained.

According to the theoretical yield of succinic acid from 6-carbon sugars and CO_2 via the reductive route is 1.71 mole/mole sugar, or 1.12 g/g sugar, as shown in the following reaction (McKinlay *et. al.*, 2007).

$$7\ C_6H_{12}O_6 + 6\ CO_2 \rightarrow 12\ C_4H_6O_4 + 6\ H_2O\backslash$$

The theoretical yield of succinic acid with IL pretreatment of mulberry stalk is 0.22 g/g substrate. Succinic acid yield in SSF process was 53.08% of theoretical yield. In addition, 71.78% lignin was recovered from the IL/lignin mixture.

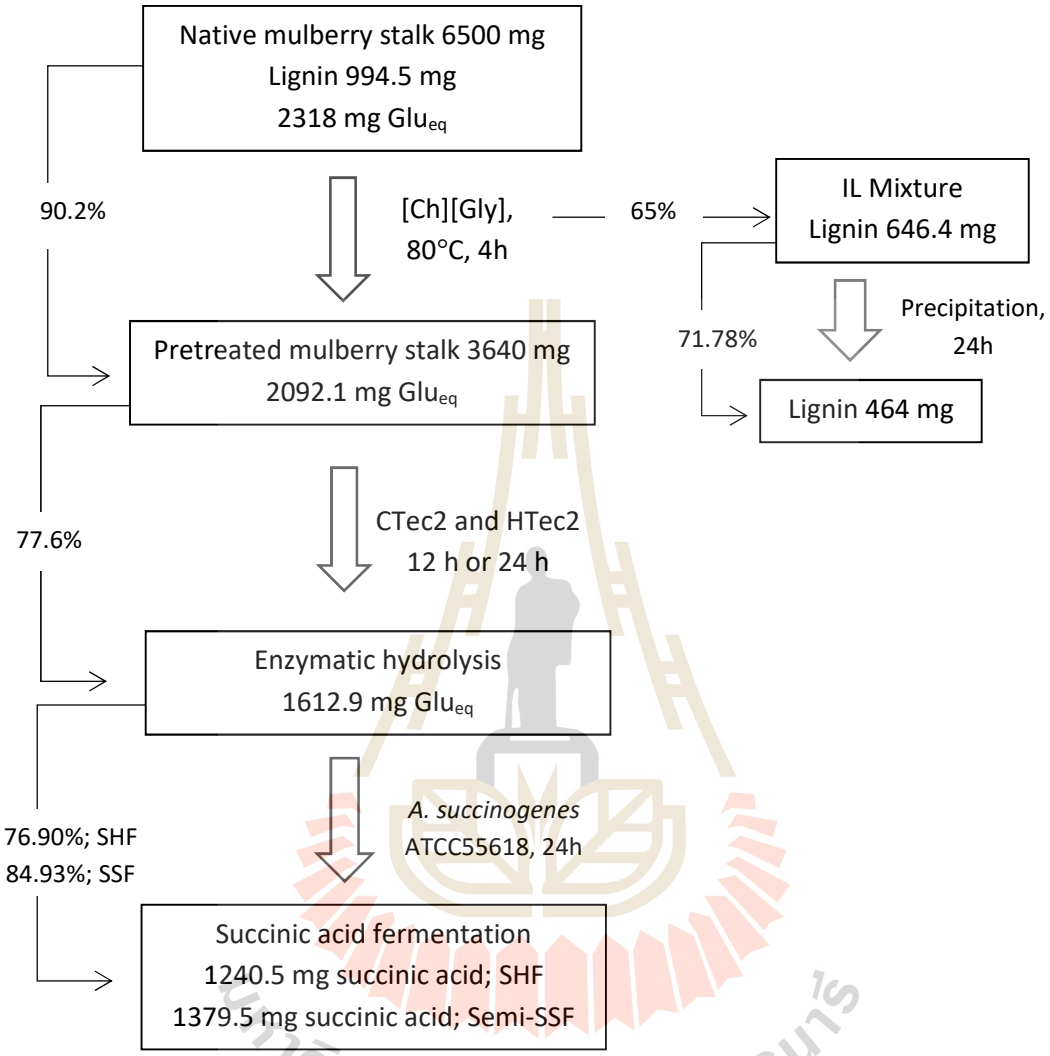


Figure 4.26 workflow of succinic acid production and lignin recovery from mulberry stalk pretreatment with [Ch][Gly].

This workflow is steadily investigate the efficiency of [Ch][Gly] pretreatment of mulberry stalk for biorefinery products. Enhance fermentable sugar as glucose and xylose is obtained by [Ch][Gly] (77.34% glucose and 38.84% xylose yield) whereas glucose and xylose yield of mulberry stalk without pretreatment step was 14.93 and 1.88%. After pretreatment, the fraction of mixture of lignin/IL was collected and >70% yield lignin was recovered, > 90% lignin content in extracted lignin. Furthermore, the fermentation of CRM

fraction produced >0.75 g/g succinic acid yield based on sugar consumption (or >0.7 g/g dry matter substrate succinic acid yield) and >1.8 g/l/h productivity. As above mention, results investigate high efficiency of wood biomass through mulberry stalk and high potential of [Ch][Gly] pretreatment which can be progressed to a pattern of biorefinery product.

CHAPTER V

CONCLUSIONS

5.1 Effect of cholinium based ionic liquid on mulberry stem pretreatment

1. Cholium amino acid ionic liquid ([Ch][Ala] and [Ch][Gly]) pretreatment improved removed of lignin from native mulberry stem (MS) and retained carbohydrate structure. Cellulose content of pretreated MS with [Ch][Ala] and [Ch][Gly] were 41.84 and 51.09% dry wt which increased from native MS (31.71% dry wt). Lignin extractability of [Ch][Ala] and [Ch][Gly] were 65.44 and 65.15 %.

2. Solvent of [Ch][AA] pretreatment improve glucose and xylose yields from the hydrolysis of untreated MS. Glucose and xylose yield from untreated MS were only 14.93 and 1.88 g/l whereas, both of sugar yields from [Ch][Ala] were 66.15 and 24.35 g/l and from [Ch][Gly] were 74.72 and 33.05 g/l.

3. Physical and chemical changes of pretreated MS were demonstrated via SEM, FT-IR and XRD spectra. Feature of pretreated MS was opened and become loosen, the corresponding to C-O stretching of peak at 1240 cm^{-1} remarkably decrease of intensity. The intensity peak at band between 1240-1210 is in spectra of methylated and acetylated lignin relate to asymmetric vibration of C-O linkage in ether or ester with phenolic hydroxyl group. The decrease intensive peak at 1240 cm^{-1} relative to cleave ether or ester bond between lignin fragment or lignin and saccharide residue in hemicellulose. Crystalline index (CrI) of pretreated MS with [Ch][Ala] and [Ch][Gly] was 58.27% and 58.87 % which was higher than untreated MS. These results indicated that

[Ch][AA] improved sugar yield and have the high efficacy remove lignin.

5.2 Effect of temperature on pretreatment of mulberry stem and reuse of choinium glycinate

1. Pretreatment temperature and incubation time were the important factor on sugar yield. The lowest glucose (52.36%) and xylose (24.20%) yields were found at 60 °C pretreatment temperature whereas, both of sugar yield at 80 °C was not lower than pretreatment at 90 °C with an ambient pressure.

2. Solvent of [Ch][Gly] could be recovered and reused, without lignin remove from solvent, this solvent performed the high capacity of lignin and retain carbohydrate structure for the further enzymatic hydrolysis. Pretreatment temperature were not significant effect on sugar yield and lignin extractability of each batch [Ch][Gly] reuse.

3. Glucose and xylose yield at 80 °C for 4 h was >70% glucose yield which was not different from yield at 90 °C 6 h.

5.3 Lignin fractionation and properties

1. Lignin rich material was recovered with 71.25% recovery and 92.80% of lignin content in lyophilized lignin.

2. Chemical properties of LRM such as functional groups and intra-linkage are not different from native wood lignin. The functional groups and main linkage of extracted lignin contained of OH (primary and secondary alcohol), methoxy ($R\text{-}OCH_3$), C=O, C-O, C=C in aromatic skeleton in lignin, C-O in syringly ring, β-*O*-4 linkage.

5.4 Enzymatic hydrolysis in batch and fed batch

1. Batch enzymatic hydrolysis, increase solid loading (5.6, 8.4, 11.2 and 14%) had effect on raise in glucose and xylose concentration but both of sugar yield decrease when high solid content in system even increased enzyme dosage from 22 FPU/g cellulose to be 40 FPU/g cellulose.

2. Fed-batch enzymatic hydrolysis, increase 19.6 % total solid loading can be processed with the highest 88.71 g/l glucose and 31.02 xylose concentration were obtained and glucose and xylose yield was 71.41% and 37.35%.

3. 11.2% and 14% total solid loading was improved sugar yield through the fed batch enzymatic hydrolysis with solid adding in 4 h time interval.

5.5 Succinic acid fermentation

1. Carbohydrate rich materials (CRMs) can be applied for succinic acid fermentation by *Actinobacillus succinogenes* ATCC55618 in the both of SHF and Semi-SSF process.

2. Succinic acid from SSF process was slightly higher than SHF process in 50 ml anaerobic bottle. An increase solid content in SSF process have an effect on enhance succinic acid production in batch fermentation.

3. In 2 l stirred bioreactor, 46.93 g/l succinic acid concentration, 0.42 g/g substrate yield succinic acid and 1.89 g/l/h productivity was achieved.

These conclusion demonstrated high efficiency of [Ch][Gly] solvent for pretreatment of wood biomass with high selective and capacity of lignin extractability. In addition, fractionation after pretreatment (LRM and CRM) can be occurred. The methods of recover LRM have high performance to recover lignin from extraction mixture and the material is in the good quality and quantities of extracted lignin. CRMs are the high effective materials with non-toxicity and facilitate to digest by commercial cellulase. High

concentration and productivity of succinic acid were obtained through fermentation of fermentable sugar from [Ch][Gly] pretreatment. The results establish ability of [Ch][Gly] pretreatment on wood biomass and can be further develop for the bio-chemical production process.

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APPENDIX

APPENDIX

1. Determination of total solids in biomass and total dissolved solids in liquid process samples (Sluiter *et. al.*, 2008)

Biomass samples can contain large and varying amounts of moisture, which can change quickly when exposed to air. To be meaningful, the results of chemical analyses of biomass are typically reported on a dry weight basis. The following procedure describes the methods used to determine the amount of solids or moisture present in a solid or slurry biomass sample. It also covers the determination of dissolved solids in a liquor sample. A traditional convection oven drying procedure is

covered as well as solids determination using an automatic infrared moisture analyzer. The convection oven method is recommended for liquor samples. In this research, conventional method was applied. **Conventional method**;

1. Pre-dry aluminum weighing dishes by placing them in a 105 ± 3ºC drying oven for a minimum of four hours. Cool the dishes in a desiccator. Using gloves or tweezers to handle the dishes, weigh a pre-dried dish to the nearest 0.1 mg. (It may be helpful to place a dry glass fiber pad in the bottom of each pan for liquor samples. Include the weight of the pad with the weight of the pan.) Record this weight.

2. Solid samples usually require 0.5 to 2 grams, slurry samples require 2-5 grams, and liquor samples require 10 ml, per duplicate.

3. Place the sample into a convection oven at 105 ± 3°C for a minimum of four hours. Remove the sample from the oven and allow it to cool to room temperature in a desiccator. Weigh the dish containing the oven-dried sample to the nearest 0.1mg and record this weight.

4. Place the sample back into a convection oven at 105 ± 3oC and dry to constant weight. Constant weight is defined as ± 0.1% change in the weight percent solids upon one hour of re-heating the sample.

5. Calculation

1) Total solids

$$\% \text{ Total solids} = \frac{\text{Weight}_{\text{dry pan plus dry sample}} - \text{Weight}_{\text{dry pan}}}{\text{Weight}_{\text{sample as received}}} \times 100$$

2) Dissolved solids

$$\% \text{ Dissolved solids} = \frac{\text{Weight}_{\text{dry pan plus dry liquor}} - \text{Weight}_{\text{dry pan}}}{\text{Weight}_{\text{sample as received}}} \times 100$$

2. Determination of ash in biomass (Sluiter *et. al.*, 2005)

The amount of inorganic material in biomass, either structural or extractable, should be measured as part of the total composition. Structural ash is inorganic material that is bound in the physical structure of the biomass, while extractable ash is inorganic material that can be removed by washing or extracting the material. Extractable ash can be the result of soil remaining in the biomass. This test method covers the determination of ash, expressed as the percentage of residue remaining after dry oxidation at 550 to 600°C. All results are reported relative to the 105°C oven dry weight of the sample.

1. Using a porcelain marker, mark an appropriate number of crucibles with identifiers, and place them in the muffle furnace at 575 +25 °C for a minimum of four hours. Remove the crucibles from the furnace directly into a desiccator. If using a furnace set to 575 + 25 °C, cool for a specific period of time, one hour is recommended.

2. Place the sample back into the muffle furnace at 575 ± 25oC and dry to constant weight. Constant weight is defined as less than ± 0.3 mg change in the weight upon one hour of re-heating the crucible.

3. Weigh 0.5 to 2.0 g, to the nearest 0.1 mg, of a test specimen into the tared crucible. Record the sample weight.

4. Place the crucibles in the muffle furnace at 575 ± 25 °C for 24 + 6 hours.

When handling the crucible, protect the sample from drafts to avoid mechanical loss of sample.

5. Carefully remove the crucible from the furnace directly into a desiccator and cool for a specific amount of time, equal to the initial cool time of the crucibles.

6. Place the sample back into the muffle furnace at 575 ± 25 °C and ash to constant weight. Constant weight is defined as less than ± 0.3 mg change in the weight upon one

hour of re-heating the crucible. When allowing samples to cool in a desiccator, it is necessary to maintain the initial cool time.

7. Calculation

1) If an air dry sample was used, calculate the oven dry weight (ODW) of the sample, using the average total solids content as determined by the LAP “Standard Method for the Determination of Total Solids in Biomass”.

$$\text{ODW} = \frac{\text{Weight}_{\text{air dry sample}} \times \%\ \text{Total solid}}{100}$$

2) Calculate and record the percentage ash on an ODW basis.

$$\%\ \text{Ash} = \frac{\text{Weight}_{\text{crucible plus ash}} - \text{Weight}_{\text{crucible}}}{\text{ODW}} \times 100$$

3. Determination of extractive in biomass

It is necessary to remove non-structural material from biomass prior to analysis to prevent interference with later analytical steps. This procedure uses a two-step extraction process to remove water soluble and ethanol soluble material. Water soluble materials may include inorganic material, non-structural sugars, and nitrogenous material, among others. Inorganic material in the water soluble material may come from both the biomass and any soluble material that it is associated with the biomass, such as soil or fertilizer.

1. **Prepare the sample for extractive**

The moisture content of a biomass sample can change rapidly when exposed to air. Weigh samples for total solids determination (LAP "Determination of Total Solids and Moisture in Biomass") at the same time as the samples for the extractives determination to avoid errors due to changes in humidity.

2. **Prepare the sample for extractive Extract the sample by Soxhlet method only**

2.1 Prepare the apparatus for extraction.

1) Dry boiling flasks and other relevant glassware in a 105 + 5 °C drying oven for a minimum of 12 hours. Remove the glassware and allow it to come to room temperature in a desiccator. Record the oven dry weight (ODW)

2) Add 2-10 g of sample to a tared extraction thimble. Record the weight to the nearest 0.1 mg. The amount of sample necessary will depend on the bulk density of the biomass. The height of the biomass in the thimble must not exceed the height of the Soxhlet siphon tube. If the biomass height does exceed the siphon height, incomplete extraction will occur.

3) Assemble the Soxhlet apparatus. Add a 250 mL bump trap between the receiving flask and the Soxhlet tube to control foaming if necessary. Insert the thimble into the Soxhlet tube.

2.2 Analyze the sample for ethanol extractives

1) Add 190±5 mL 190 proof ethyl alcohol to the tared ethanol receiving flask. Place the receiving flask on the Soxhlet apparatus. Adjust the heating mantles to provide a minimum of 6-10 siphon cycles per hour.

2) Reflux for 16-24 hours. The reflux time necessary will depend on the removal rate of components of interest, the temperature of the condensers, and the siphon rate.

3) When reflux time is complete, turn off the heating mantles and allow the glassware to cool to room temperature.

4) Remove the thimble and transfer the extracted solids, as quantitatively as possible, onto cellulose filter paper in a Buchner funnel. Wash the solids with approximately 100 mL of fresh 190 proof ethanol. Allow the solids to dry using vacuum filtration or air dry.

2.3 Remove solvent from extractives

1) Combine any solvent from the Soxhlet tube with the solvent in the receiver flask if using Soxhlet method. For automatic extraction method, remove collection vials from instrument.

2) The solvent may be removed from the extract using either apparatus listed or an equivalent device suitable for evaporating water and ethanol.

2.1 To remove the solvent using a rotary evaporator, use a rotary evaporator equipped with a water bath set to 40 + 5 °C and a vacuum source. Transfer the extract into a tared round bottom boiling flask. The vacuum source should be sufficient to remove solvent without extreme bumping. Continue to remove solvent until all visible solvent is gone.

2.2 To remove solvent using a TurboVapII, transfer the extract into a tarred

TurboVap tube if necessary, set the inlet pressure to 15 – 18 psi, and adjust the water bath to 40 °C. Continue to remove solvent until all visible solvent is gone.

2.4 Place the flask or tube in a vacuum oven at 40 + 2 °C for 24 hours. Cool to room temperature in a desiccator. Weigh the flask or tube and record the weight to the nearest 0.1 mg. If necessary, this step may also be performed on the bump trap to quantify any extract remaining in the bump trap.

2.5 Calculation

1) Calculate the oven dry weight (ODW) of the sample

$$\text{ODW} = \frac{\text{Weight}_{\text{air dry sample}} \times \%\ \text{Total solid}}{100}$$

2) Calculate the amount of extractives in the sample, on a percent dry weight basis.

$$\%\text{Extractives} = \frac{\text{Weight}_{\text{flask plus extractives}} - \text{Weight}_{\text{flask}}}{\text{ODW}_{\text{sample}}} \times 100$$

If sucrose measurements were performed, use the second equation to calculate extractives with a correction for the removed sample volume.

$$\%\text{Extractives} = \frac{\text{Weight}_{\text{flask plus extractives}} - \text{Weight}_{\text{flask}}}{\text{ODW}_{\text{sample}}} \times \frac{\text{Weight}_{\text{total}}}{\text{Volume}_{\text{Total}} - \text{Weight}_{\text{flask}}} \times 100$$

4. Determination of structural carbohydrates and lignin in biomass (Sluiter *et. al.*, 2012)

Carbohydrate and lignin make up a major portion of biomass samples. Carbohydrate can be structural or non-structural. Structural carbohydrates are bound in the matrix biomass while non-structural can removed by extraction or washing step. Lignin is a complex of phenolic polymer. This procedure is suitable for samples that do not contain extractives. This procedure uses a two-step acid hydrolysis to fractionate the biomass into forms that are more easily quantified. The lignin fractionates into acid insoluble material and acid soluble material. The acid insoluble material may also include ash and protein, which must be accounted for during gravimetric analysis. The acid soluble lignin is measured by UV-Vis spectroscopy. During hydrolysis the polymeric carbohydrates are hydrolyzed into the monomeric forms, which are soluble in the hydrolysis liquid. They are then measured by HPLC. Protein may also partition into the liquid fraction. A measure of acetyl content is necessary for biomass containing hemicellulose with a xylan backbone, but not biomass containing a mannan backbone. Acetate is measured by HPLC.

Procedure

1. **Prepare the sample for analysis and hydrolysis**

1.1 Place the filtering crucible into muffle furnace at 575°C for a minimum of 4 h. Remove the crucible from furnace directly into desiccator and cool for a specific period of time, one hour is recommended. Weight the crucibles the nearest 0.1 mg. and record this weight.

1.2 Weigh 300±10 mg of the sample or QA standard into tared pressure tube. Record the weight to the nearest 0.1 mg and record this weight.

1.3 Add 3.00 ± 0.01 mL (or 4.92 ± 0.01 g) of 72% sulfuric acid to each pressure tube. Use a Teflon stir rod to mix for one minute, or until the sample is thoroughly mixed.

1.4 Place the pressure tubes in a water bath set at 30 ± 3 °C and incubate the sample for 60 ± 5 minutes. Using the stir rod, stir the sample every 5 to 10 minutes without removing the sample from the bath. Stirring is essential to ensure even acid to particle contact and uniform hydrolysis.

1.5 Upon completion of the 60-minute hydrolysis, remove the tubes from the water bath. Dilute the acid to a 4% concentration by adding 84.00 ± 0.04 mL deionized water. Screw the Teflon caps on securely. Mix the sample by inverting the tube several times to eliminate phase separation between high and low concentration acid layers.

1.6 Prepare a set of sugar recovery standards (SRS) that will be taken through the remaining hydrolysis and used to correct for losses due to destruction of sugars during dilute acid hydrolysis. SRS should include D-(+)glucose, D-(+)xylose, D-(+)galactose, -L(+)arabinose, and D-(+)mannose. SRS sugar concentrations should be chosen to most closely resemble the concentrations of sugars in the test sample. Weigh out the required amounts of each sugar, to the nearest 0.1 mg, and add 10.0 mL deionized water. Add 348 µL of 72% sulfuric acid. Transfer the SRS to a pressure tube and cap tightly.

1.7 Place the tubes in an autoclave safe rack, and place the rack in the autoclave. Autoclave the sealed samples and sugar recovery standards for one hour at 121°C, usually the liquids setting.

2. Analyze the sample for acid insoluble lignin as follows.

2.1 Vacuum filters the autoclaved hydrolysis solution through one of the previously weighed filtering crucibles. Capture the filtrate in a filtering flask.

2.2 Transfer an aliquot, approximately 50 ml, into a sample storage bottle. This sample will be used to determine acid soluble lignin as well as carbohydrates, and acetyl if necessary. Acid soluble lignin determination must be done within 6 hours of hydrolysis. If

the hydrolysis liquor must be stored, it should be stored in a refrigerator for a maximum of two weeks. It is important to collect the liquor aliquot before proceeding to step 3.2.

2.3 Use deionized water to quantitatively transfer all remaining solids out of the pressure tube into the filtering crucible.

2.4 Dry the crucible and acid insoluble residue at 105 ± 3 °C until a constant weight is achieved, usually a minimum of four hours.

2.5 Remove the samples from the oven and cool in a desiccator. Record the weight of the crucible and dry residue to the nearest 0.1 mg.

2.6 Place the crucibles and residue in the muffle furnace at 575 ± 25 °C for 24 ± 6 hours.

2.6.1 A furnace with temperature ramping may also be used.

Furnace Temperature Ramp Program:

- Ramp from room temperature to 105 °C

  Hold at 105 °C for 12 minutes
- Ramp to 250 °C at 10 °C / minute

  Hold at 250 °C for 30 minutes
- Ramp to 575 °C at 20 °C / minute

  Hold at 575 °C for 180 minutes
- Allow temperature to drop to 105 °C

  Hold at 105 °C until samples are removed

2.6.2 Carefully remove the crucible from the furnace directly into a desiccator and cool for a specific amount of time, equal to the initial cool time of the crucibles. Weigh the crucibles and ash to the nearest 0.1 mg and record the weight.

3. Analyze the sample for acid soluble lignin as follows

3.1 On a UV-Visible spectrophotometer, run a background of deionized water or 4% sulfuric acid.

3.2 Using the hydrolysis liquor aliquot obtained in step 2.2, measure the absorbance of the sample at an appropriate wavelength on a UV-Visible spectrophotometer. Refer to section 5 for suggested wavelength values. Dilute the sample as necessary to bring the absorbance into the range of 0.7–1.0, recording the dilution. Deionized water or 4%sulfuric acid may be used to dilute the sample, but the same solvent should be used as a blank.

3.3 Calculate the amount of acid soluble lignin present using calculation section 5.

4. Analyze the sample for structural carbohydrates

4.1 Prepare a series of calibration standards containing the compounds that are to be quantified, referring to Table 1 for suggested concentration range. Use a four point calibration. If standards are prepared outside of the suggested ranges, the new range for these calibration curves must be validated.

Component	Suggested concentration range (mg/ml)
D-cellobiose	0.1-0.4
D(+)glucose	0.1-0.4
D(+)xylose	0.1-0.4
D(+)galactose	0.1-0.4
D(+)arabinose	0.1-0.4
D(+)mannose	0.1-0.4
CVS	Middle of linear range, concentration not equal to a calibration point (2.5 suggested)

4.2 Prepare an independent calibration verification standard (CVS) for each set of calibration standards. Use reagents from a source or lot other than that used in preparing the calibration standards. Prepare the CVS at a concentration that falls in the middle of the validated range of the calibration curve. The CVS should be analyzed on the HPLC after each calibration set and at regular intervals throughout the sequence, bracketing groups of

samples. The CVS is used to verify the quality and stability of the calibration curve(s) throughout the run.

4.3 Using the hydrolysis liquor obtained in step 2.2, transfer an approximately 20 ml aliquot of each liquor to a 50 ml Erlenmeyer flask.

4.4 Use calcium carbonate to neutralize each sample to pH 5–6. Avoid neutralizing to a pH greater than 6 by monitoring with pH paper. Add the calcium carbonate slowly after reaching a pH of 4. Swirl the sample frequently. After reaching pH 5–6, stop calcium carbonate addition, allow the sample to settle, and decant off the supernatant. The pH of the liquid after settling will be approximately 7. (Samples should never be allowed to exceed a pH of 9, as this will result in a loss of sugars.)

4.5 Prepare the sample for HPLC analysis by passing the decanted liquid through a 0.2 µm filter into an autosampler vial. Seal and label the vial. HPLC analysis procedure according to the Appendix section 7.

5. Calculations

5.1 Calculate the oven dry weight (ODW) of the extractives free sample, using the average total solids content as determined by the LAP "Standard Method for the Determination of Total Solids in Biomass."

$$\text{ODW} = \frac{\text{Weight}_{\text{air dry sample}} \times \%\ \text{Total solid}}{100}$$

5.2 Calculate and record the weight percent acid insoluble residue (AIR) and acid insoluble lignin (AIL) on an extractives free basis.

$$\%\text{AIR} = \frac{\text{Weight}_{\text{crucible plus AIR}} - \text{Weight}_{\text{crucible}}}{\text{ODW}_{\text{sample}}} \times 100$$

$$\%\text{AIL} = \frac{(\text{Weight}_{\text{crucible plus AIR}} - \text{Weight}_{\text{crucible}}) - (\text{Weight}_{\text{crucible plus ash}} - \text{Weight}_{\text{crucible}}) - \text{Weight}_{\text{protein}}}{\text{ODW}_{\text{sample}}} \times 100$$

Where; Weight $_{protein}$= Amount of protein present in the acid insoluble residue, as determined in LAP "Determination of Protein Content in Biomass." This measurement is only necessary for biomass containing high amounts of protein

5.3 Calculate the amount of acid soluble lignin (ASL) on an extractives free basis.

$$\%ASL = \frac{UV_{abs} \times Volume_{filtrate} \times Dilution}{\varepsilon \times ODW_{sample} \times Pathlength} \times 100$$

Where;

UV_{abs}= Average UV-Vis absorbance for the sample at appropriate wavelength (see table below)

$Volume_{hydrolysis\ liquor}$= volume of filtrate, 86.73 ml

ε = Absorptivity of biomass at specific wavelength (see table below)

ODW_{sample} = weight of sample in milligrams

Pathlength = pathlength of UV-Vis cell in cm

Absorptivity constants for acid soluble lignin measurement for select biomass types

Biomass type	Lambda max (mm)	Absorptivity at lambda max (L/g cm)	Recommended wavelength (mm)	Absorptivity at recommended wavelength (L/g cm)
Pinus Radiata	198	25	240	12
Bagasse	198	40	240	25
Corn stover-NREL supplied feedstock	198	55	32	30
Populus deltiodes	197	60	240	25

Note: Lambda max values often contain interfering peaks from carbohydrate degradation products. Recommended wavelength values have been chosen to minimize these interferences.

5.4 Calculate total lignin amount on an extractive free basis

$$\%\text{Lignin}_{\text{ext free}} = \%\text{ASL} + \%\text{AIL}$$

5.5 Calculate and record the amount of each calibration verification standard (CVS) recovered following HPLC analysis.

$$\%\text{CVS recovery} = \frac{\text{Conc. detected by HPLC, mg/ml}}{\text{Known Conc. Standard, mg/ml}} \times 100$$

5.6 sugar recovery standards (SRS), calculate the amount of each component sugar recovered after dilute acid hydrolysis, accounting for any dilution made prior to HPLC analysis.

$$\%\text{SRS} = \frac{\text{Conc. detected by HPLC, mg/ml}}{\text{Known Conc. of sugar before hydrolysis, mg/ml}} \times 100$$

5.7 Use the percent hydrolyzed sugar recovery values calculated in step 11.8 to correct the corresponding sugar concentration values obtained by HPLC for each of the hydrolyzed samples ($C_{cor.\ sample}$), accounting for any dilution made prior to HPLC analysis.

$$C_x = \frac{\text{Conc.}_{HPLC} \times \text{Dilution factor}}{\%R_{ave.\ sugar}/100}$$

Where;

C_{HPLC} = Conc. Of sugar as determined by HPLC, mg/ml

$\%R_{ave.\ Sugar}$ = average recovery of a specific SRS component

$C_x = C_{cor.\ Sample}$, concentration in mg/ml of a sugar in the hydrolyzed sample after correction for on 4% hydrolysis

5.8 Calculate the concentration of the polymeric sugar from the concentration of the corresponding monomeric sugars, using an anhydro correction of 0.88 (or 132/150) for C-5 sugars (xylose and arabinose) and a correction of 0.90 (or162/180) for C-6 sugars (glucose, galactose, and mannose).

$$C_{anhydro} = C_{corr} \times \text{Anhydro collection}$$

5.9 Calculate the percentage of each sugar on an extractives free basis.

$$\%\text{Sugar ext. free} = \frac{C_{\text{anhydro}} \times V_{\text{filtrate}} \times \frac{1\text{g}}{1000\text{ mg}}}{ODW_{\text{sample}}} \times 100$$

Where;

V_{filtrate} = volume of filtrate, 86.73 ml

5. Measurement of cellulase activity (Adney and Baker, 1996)

The following method describes a procedure for measurement of cellulase activity using International Union of Pure and Applied Chemistry (IUPAC) guidelines (1).The procedure has been designed to measure cellulase activity in terms of "filter paper units" (FPU) per milliliter of original (undiluted) enzyme solution. The value of 2.0 mg of reducing sugar as glucose from 50 mg of filter paper (4% conversion) in 60 minutes has been designated as the intercept for calculating filter paper cellulase units (FPU) by IUPAC. This procedure is only appropriate for the determination of FPU activity in a cellulase preparation as defined by the IUPAC procedure

Procedure

1. Prepare filter paper 1x6 cm and place a rolled filter paper strip in to each test tube. Add 1.0 ml 0.05 M Na-citrate, pH 4.8 to the tube; the buffer should saturate the filter paper strip. Equilibrate tubes with buffer and substrate to 50 °C.

2. Add 0.5 ml enzyme diluted appropriately in citrate buffer. At least two dilutions must be made of each enzyme sample, with one dilution releasing slightly more than 2.0 mg of glucose (absolute amount) and one slightly less than 2.0 mg of glucose. Target 2.1 and 1.9 mg glucose, respectively, for these two dilutions.

3. Incubate at 50 °C for exactly 60 min.

4. At the end of the incubation period, remove each assay tube from the 50 °C bath and stop the enzyme reaction by immediately adding 3.0 mL DNS reagent and mixing.

5. Blank and controls:

5.1 Reagent blank: 1.5 mL citrate buffer.

5.2 Enzyme control: 1.0 mL citrate buffer + 0.5 mL enzyme dilution (prepare a separate control for each dilution tested).

5.3 Substrate control: 1.5 mL citrate buffer + filter-paper strip.

6. Standard glucose according to DNS method (Appendix section8)

7. After boiling with DNS mixture, let the tubes sit until all the pulp has settled, or centrifuge briefly. Then, determine sugar concentration by DNS method.

8. Calculate enzyme activity

$$\text{Filter paper activity} = \frac{0.37}{[\text{Enzyme}]\text{releasing 2.0 mg glucose}} \text{ units/ml}$$

The numerator (0.37) in the equation is derived from the factor for converting the 2.0 mg of "glucose-equivalents" generated in the assay to mmoles of glucose (2.0 ÷ 0.18016), from the volume of the enzyme being tested that is used in the assay (0.5 mL), and from the incubation time (60 minutes) required for generation of the reducing equivalents. Thus;

$$\frac{\frac{2.0 \text{ mg glucose}}{0.18016 \text{ mg glucose/μmol}}}{0.5 \text{ ml enzyme dilution} \times 60 \text{ minutes}} = 0.37 \text{ μmol/minute-ml}$$

6. Cholinium based amino acid ionic liquid synthesis

Two types of [Ch]AA]ILs had been synthesized using acid-base neutralization reactions. Yields of both of [Ch]AA]ILs were more than 80%. ^{1}H NMR (Bruker Ascend^{TM}500), FT-IR spectra (Bruker Tensor27) characterization for each [Ch][AA] ILs synthesized.

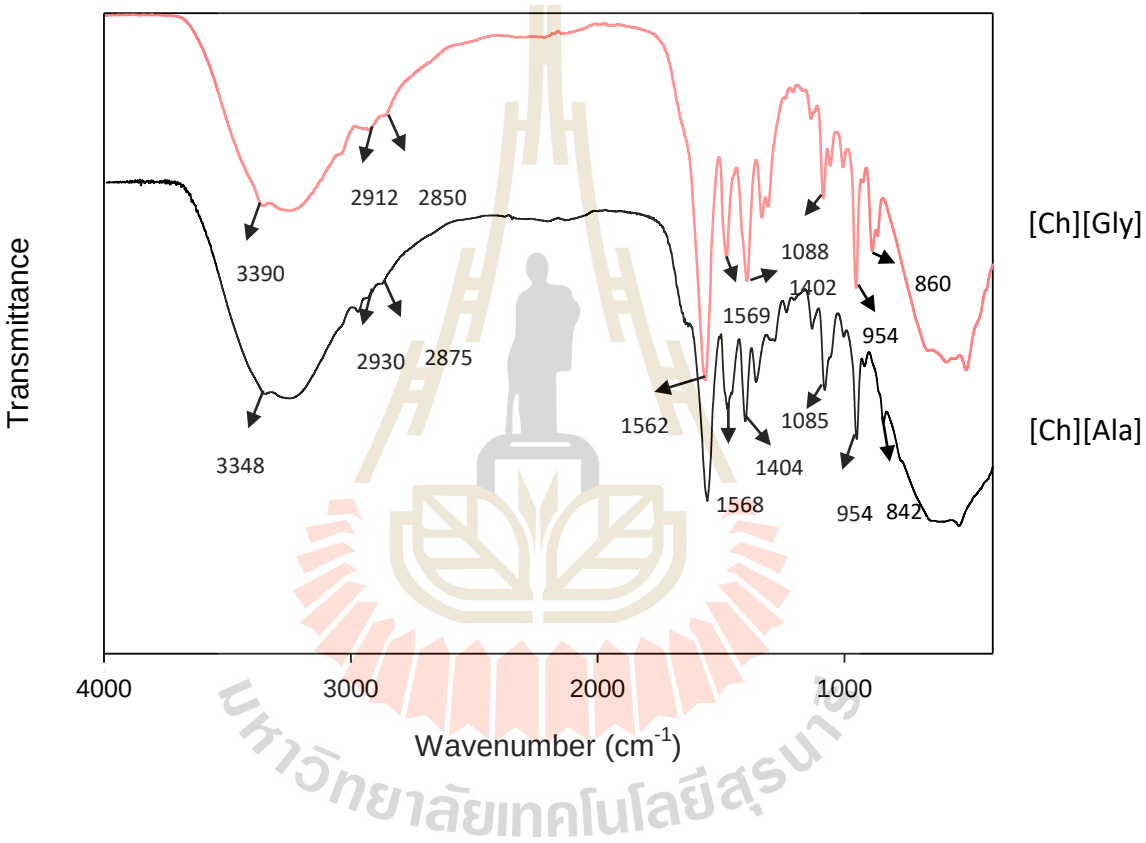

Figure A1 FTIR spectra of [Ch][Gly] and [Ch][Ala]

[Ch][Gly]. ^{1}H NMR (300 MHz, D_2O) δ: 2.946 (2H, s, C*H*$_2$NH$_2$), 2.974 (9H, s (C*H*$_3$)$_3$N), 3.277-3.302 (2H, t, CH$_2$C*H*$_2$OH), 3.813-386 (2H, m, CH$_2$C*H*$_2$N).

[Ch][Ala]. ^{1}H NMR (300 MHz, D_2O) δ: 0.980-0.997 (3H, d, C*H*$_3$CH), 2.963 (9H, s, (C*H*$_3$)$_3$N), 3.059-3.077 (1H, q, C*H*NH$_2$), 3.265-3.290 (2H, t, CH$_2$C*H*$_2$OH), 3.801-3.817 (2H, m, CH$_2$C*H*$_2$N).

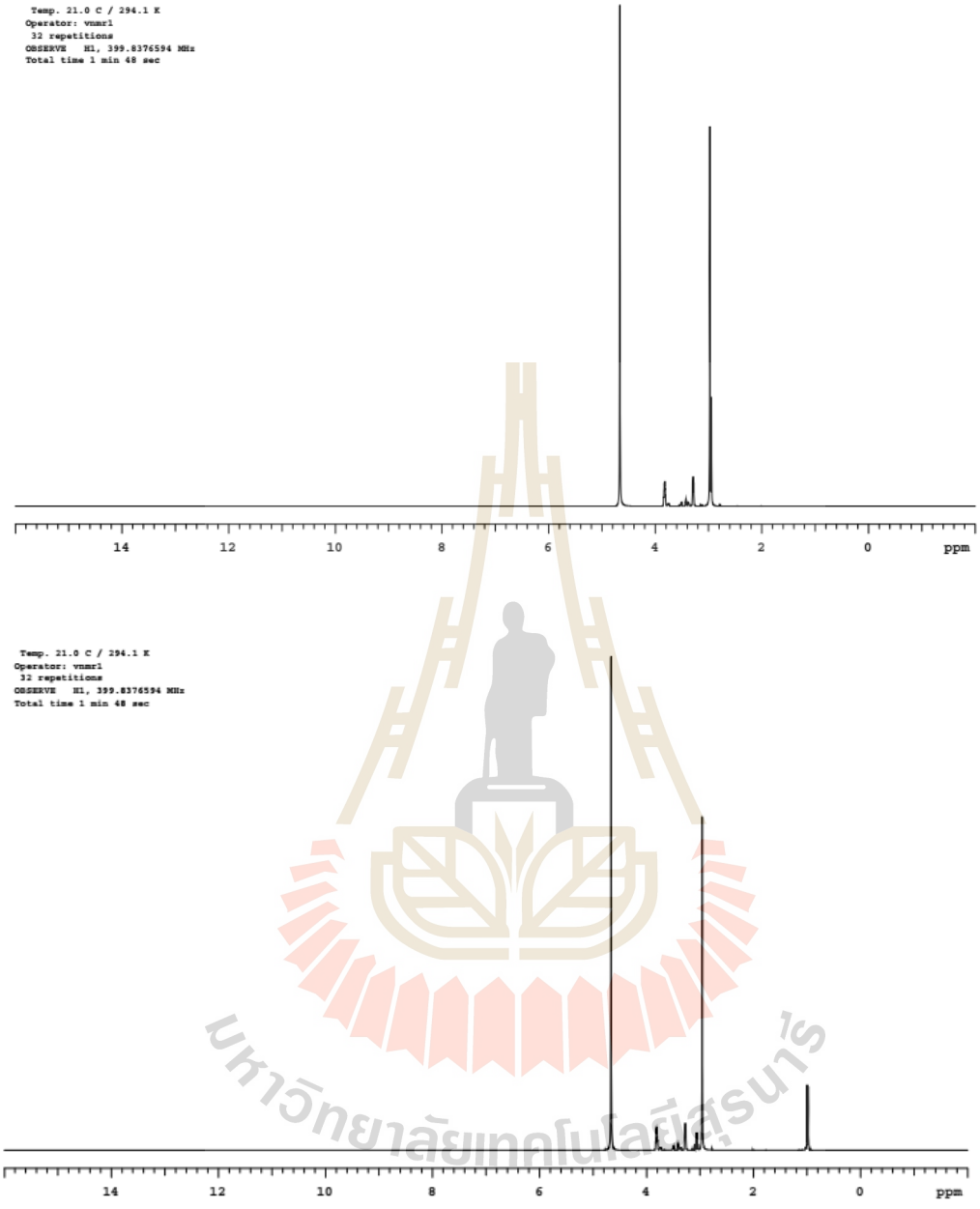

Figure A2 ^{1}H NMR spectra of [Ch][Gly] (a) and [Ch][Ala]

7. High performance liquid chromatography

Principle: depends on interaction of sample analytes with the stationary phase (packing) and the mobile phase to effect a separation. Following are explanations of the separation mechanisms commonly used in HPLC

Procedure:

1. HPLC (Agilent 1260 Agilent technology®) equipped with cation-exclusion column (Aminex HPX-87H; 300 mm × 7.8 mm, 9 µm; Biorad).

2. HPLC condition:

Injection volume: 10 µL, dependent on concentration and detector limits

Mobile phase: HPLC 10 mM H_2SO_4 with HPLC grade water, 0.2 µm filtered and degassed

Flow rate: 0.4 mL/minute

Column temperature: 60 °C

Detector: refractive index for sugar and UV detector

Run time: 40 minutes

3. Mobile phase preparation

H_2SO_4 0.208 ml

Adjust total volume to 1 L with ultrapure water

8. Reducing sugar by DNS method (Miller, 1959)

Principle: 3, 5-dinitrosalicylic acid is a method for estimate reducing sugar. Reducing sugar contains free carbonyl group, have the property to reduce many of reagents. When alkaline solution of 3, 5-dinitrosalicylic acid reacts with the reducing end of sugar, it is converted to 3-amino acid-5-nitrosalicylic acid with orange color.

8.1 Preparation of DNS Reagent

1. Mix distilled water 1416 ml, 3, 5 Dinitrosalicylic acid 10.6 g and Sodium hydroxide 19.8 g, then dissolve mixture

2. Add sodium potassium tartrate 306 g, Phenol (melt at 50°C) 7.6 ml and Sodium metabisulfite 8.3 g

8.2 Procedure

1. Add 1 ml sample solution or standard sugar into tube and 1 ml DNS solution.

2. Boil all tubes (samples and standard sugar) for exactly 5 min in a vigorously boiling water bath containing sufficient water to cover the portions of the tubes occupied by the reaction mixture plus reagent.

3. After boiling, transfer to a cold ice-water bath.

4. Add 2 ml water and determine color formation by measuring absorbance against the reagent blank at 540 nm. With this dilution the glucose standards described above should give absorbance in the range of 0.1 to 1.0 A.

5. Glucose or reducing sugar standard concentrations are 50, 100, 150, 450, 600, and 800 µg/ml.

9. Sodium acetate buffer

0.05 M sodium acetate buffer prepare by dilution of 0.2 M concentration

Solution A: 0.2M CH_3COOH (acetic acid) by add 11.6 ml acetic acid and adjust total volume to 1 l with distilled water

Solution B: 0.2M CH_3COONa (sodium acetate) by weight 16.4 g CH_3COONa or 27.4 g $CH_3COONa \cdot 3H_2O$ and add 1 l distilled water.

Preparation of buffer by mix the solution A and B according to desire pH or following table below

Solution A	Solution B	pH
46.3	3.7	3.6
44.0	6.0	3.8
41.0	9.0	4.0
36.8	13.2	4.2
30.5	19.5	4.4
25.5	24.5	4.6
20.2	30.5	4.8
14.8	35.2	5.0
10.5	39.5	5.2
8.8	41.2	5.4
4.8	45.2	5.6

10. Calculation of glucose and xylose yield

This calculation is based on theoretical sugar in native biomass. The chemical components have changed after ionic liquid pretreatment. Cellulose content have increased to 51.09% (Table 4.2) whereas, hemicellulose content was removed with [Ch][Gly] with hemicellulose remain of 13.77%. Remove of hemicellulose and lignin component resulted in weight loss and solid recovery was 56% wt. Yield of both of sugars have calculated from the native mulberry stem, which has purposed to present the yield of sugar from original biomass without pretreatment. The yield of glucose and xylose was calculated as follows (Hou *et al*., 2012).

$$\text{Sugar yield (\%)} = \frac{\text{Released sugar amount}}{\text{Theoretic sugar amount in native biomass}} \times 100$$

Example of calculation

1. Glucose and xylose concentration from enzymatic hydrolysis was 37.32 and 11.93 g/l and native biomass was 6.5 g in sodium acetate buffer 40 ml, yield of glucose and xylose can be calculated as follow;

1.1 Released glucose amount

$(37.14 \times 43)/1000 = 1.59$ g

Released xylose amount

$(11.93 \times 43)/1000 = 0.51$ g

1.2 Theoretical sugar amount in native biomass

Theoretical glucose amount in native mulberry stem;

$1.1 \times [(6.5 \times 31.71)/100] = 2.26$ g

Where; $1.1 = \frac{180\ (\text{MW of glucose anhydrous;gmol}^{-1})}{160\ (\text{MW of glucose in cellulose;gmol}^{-1})}$

Theoretical xylose amount in native mulberry stem;

$1.14 \times [(6.5 \times 24.29)/100] = 1.79$ g

Where; $1.14 = \frac{150 \text{ (MW of xylose anhydrous;gmol}^{-1})}{132 \text{ (MW of xylose in hemicellulose;gmol}^{-1})}$

1.3 Yield of sugar

Glucose yield

$(1.59 / 2.26) \times 100 = 70.66$ %

Xylose yield

$(0.51 / 1.79) \times 100 = 28.65$ %

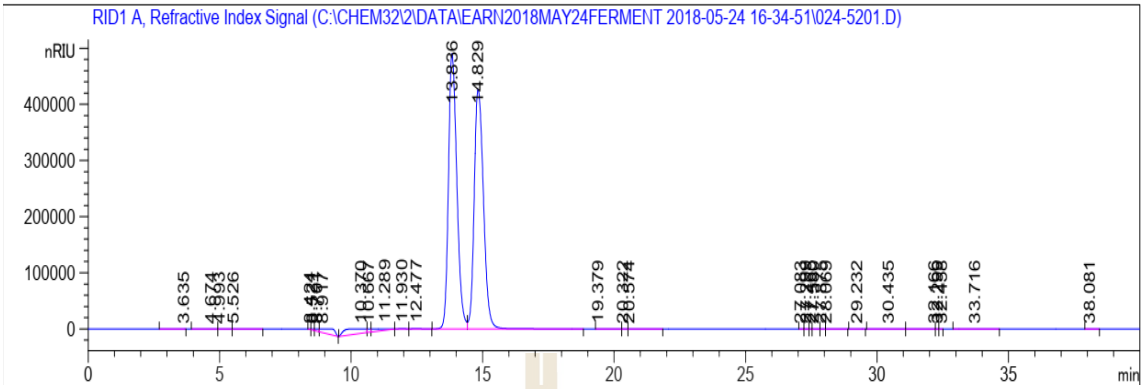

Figure A3 Chromatogram of glucose and xylose; retention time at 13.8 and 14.8 minute.

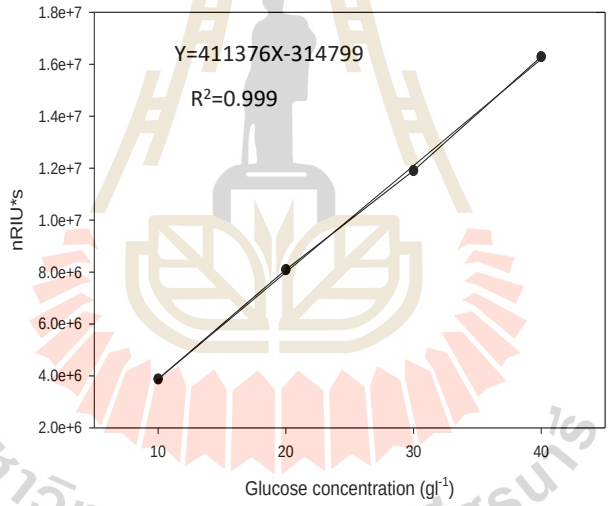

Figure A4 Standard of glucose analyzed by HPLC.

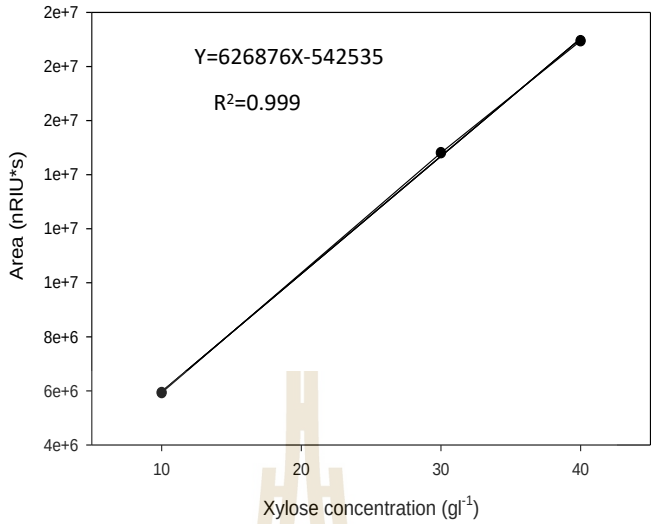

Figure A5 standard curve of xylose analyzed by HPLC.

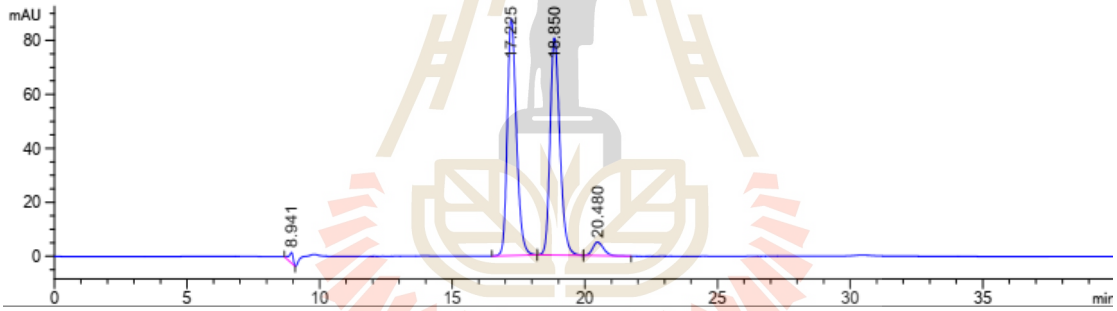

Figure A6 Chromatogram of succinic acid and lactic acid at retention time 17.2 and 19.85.

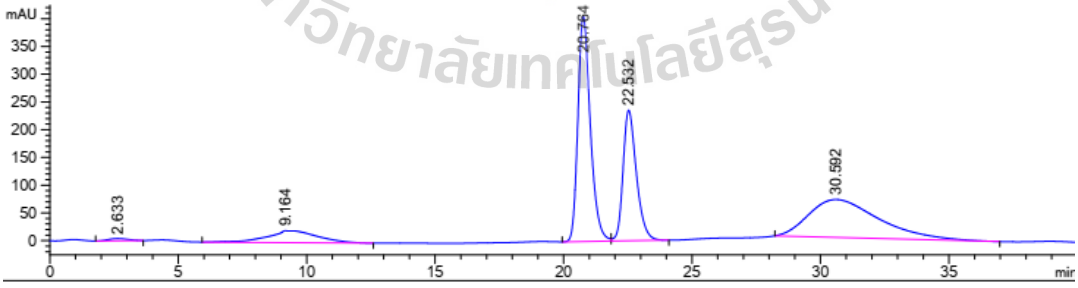

Figure A7 Chromatogram of acetic and formic acid at retention time 20.7 and 22.5 min.

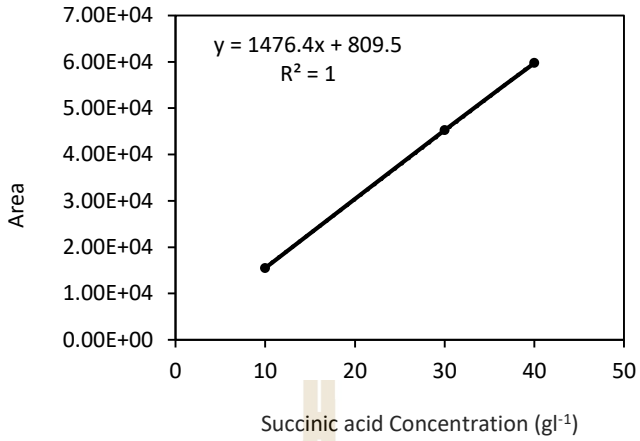

Figure A8 Standard curve of succinic acid.

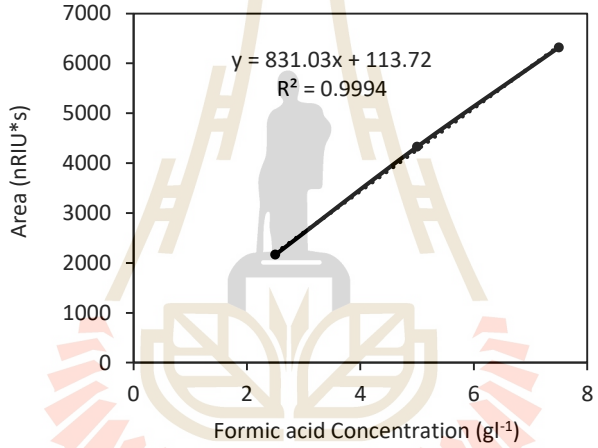

Figure A9 Standard curve of formic acid.

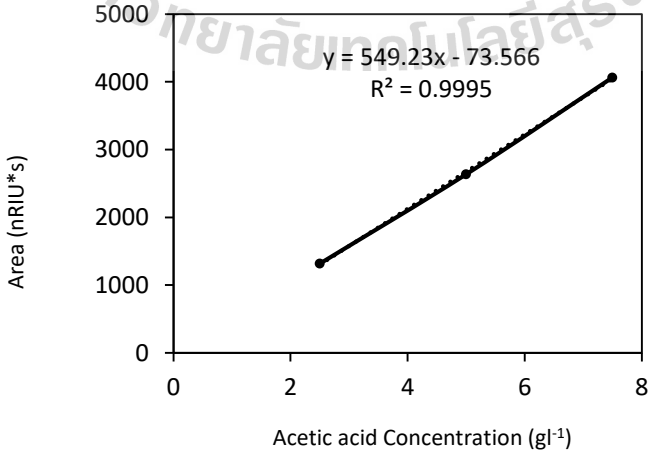

Figure A10 Standard curve of acetic acid

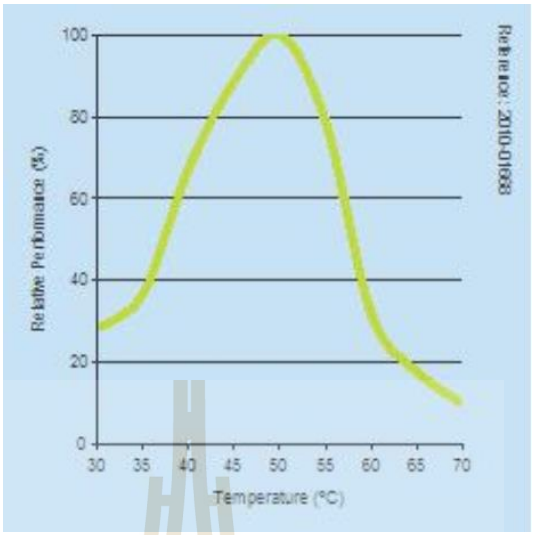

Figure A11 Optimum temperature of CTec2 (Cellulosic ethanol, 2012).

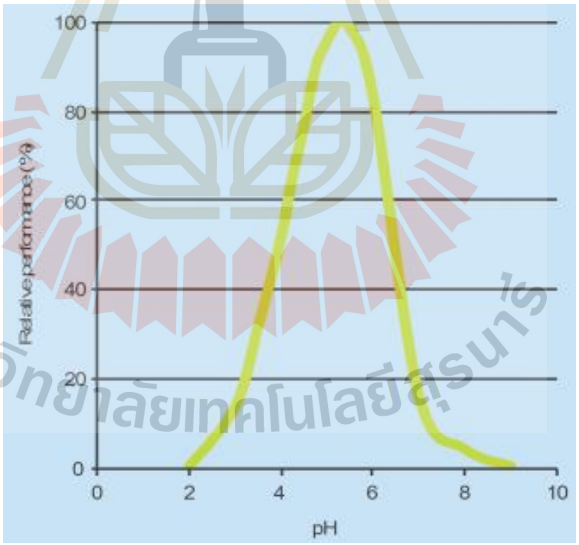

Figure A12 Optimum pH of CTec2 (Cellulosic ethanol, 2012).

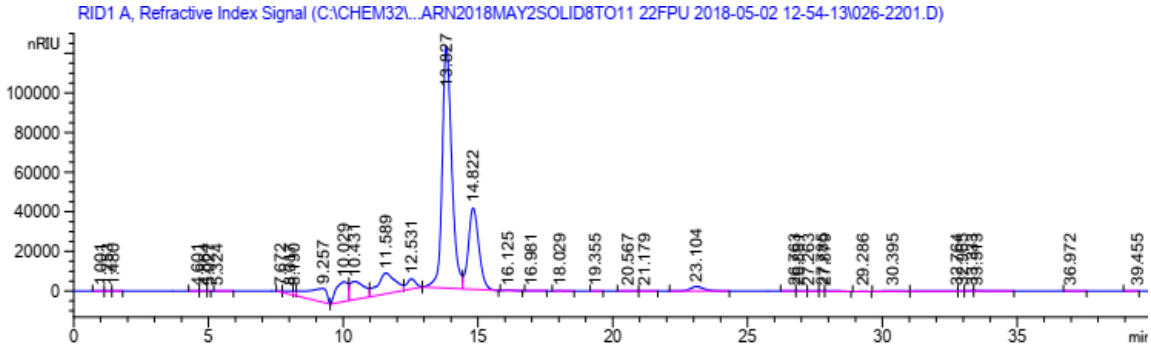

Figure A13 Chromatogram of hydrolysis sample.

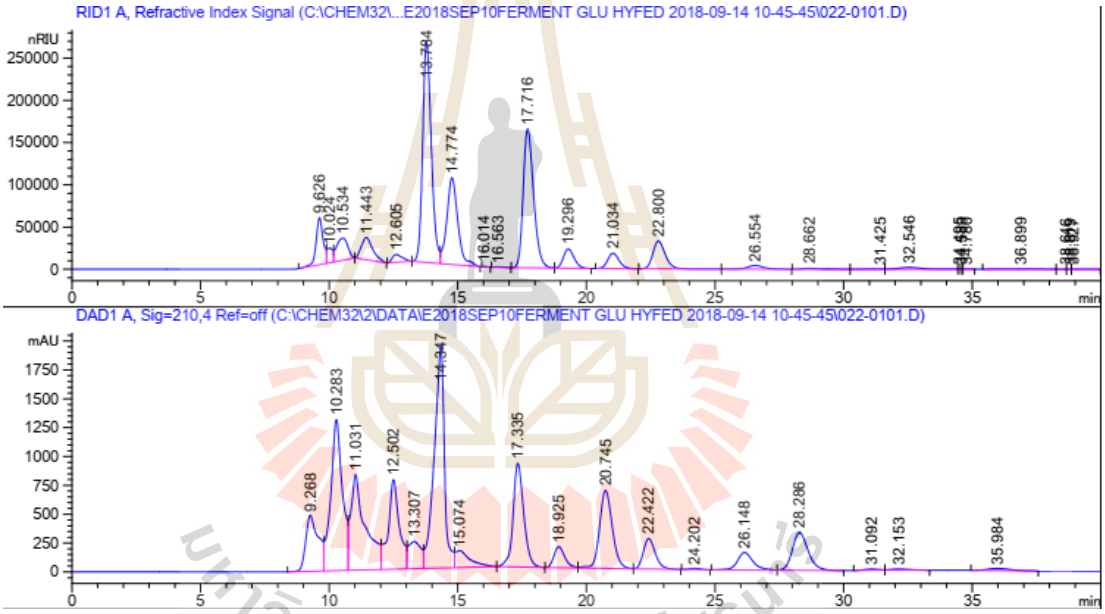

Figure A14 Chromatogram of fermentation broth.

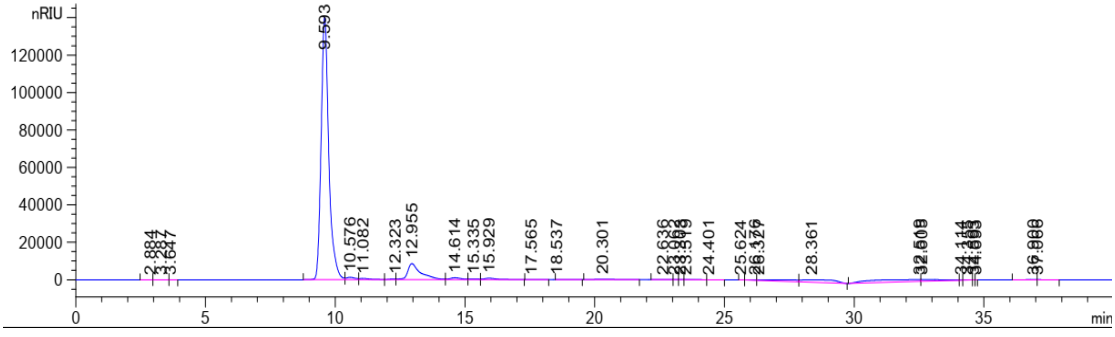

Figure A15 Chromatogram of lyophilized lignin to determine carbohydrate component.

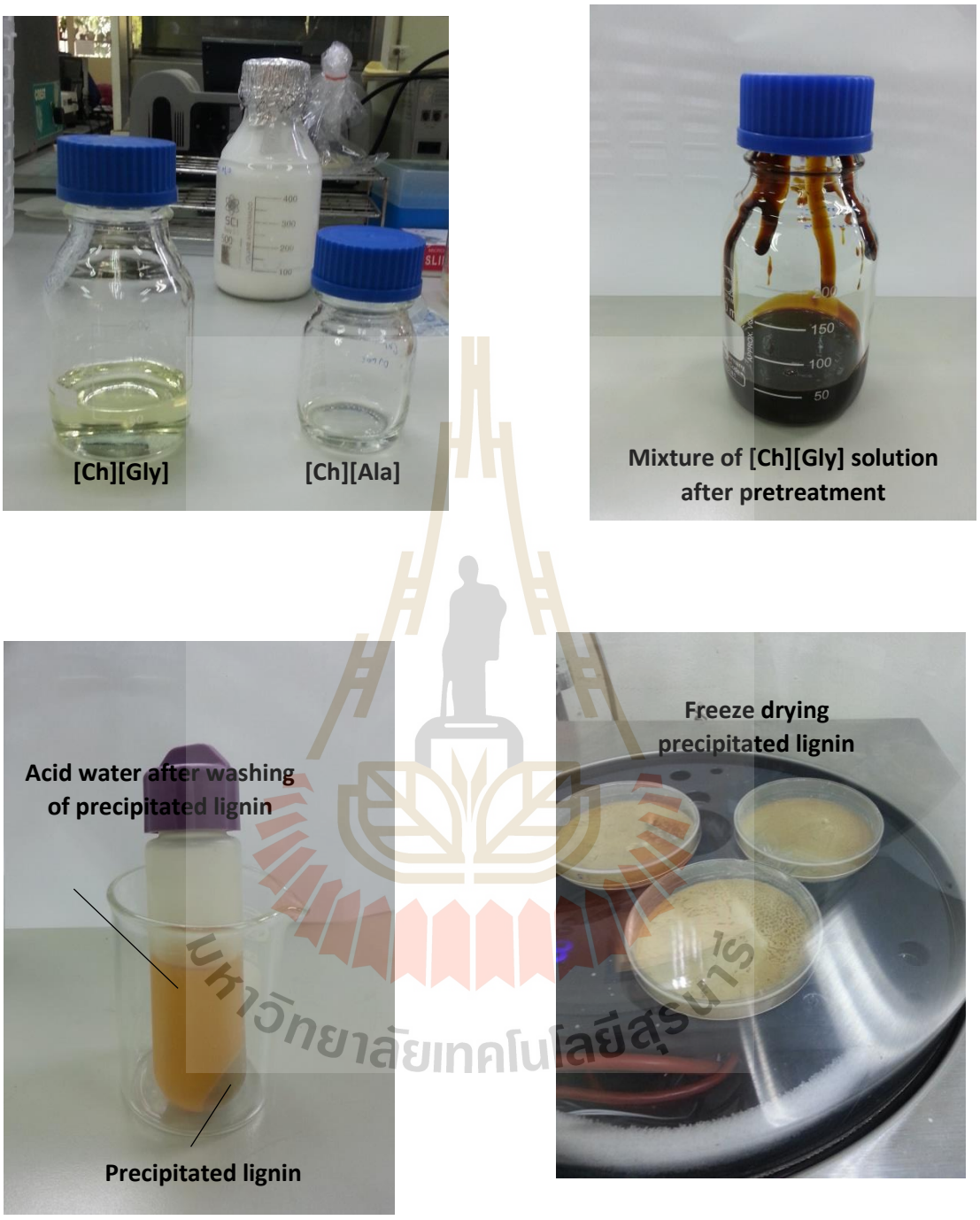

Figure A16 Solution of [Ch][Gly] and [Ch][Ala], the mixture of [Ch][Gly] solution after mulberry stalk pretreatment at 80 °C for 4 h, precipitated lignin after acidification then wash with acid water, and freeze drying the precipitated lignin.

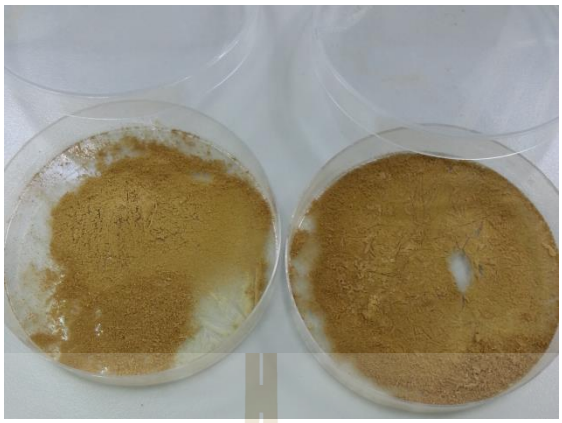

Figure A17 Lyophilized lignin after lignin recovery process.

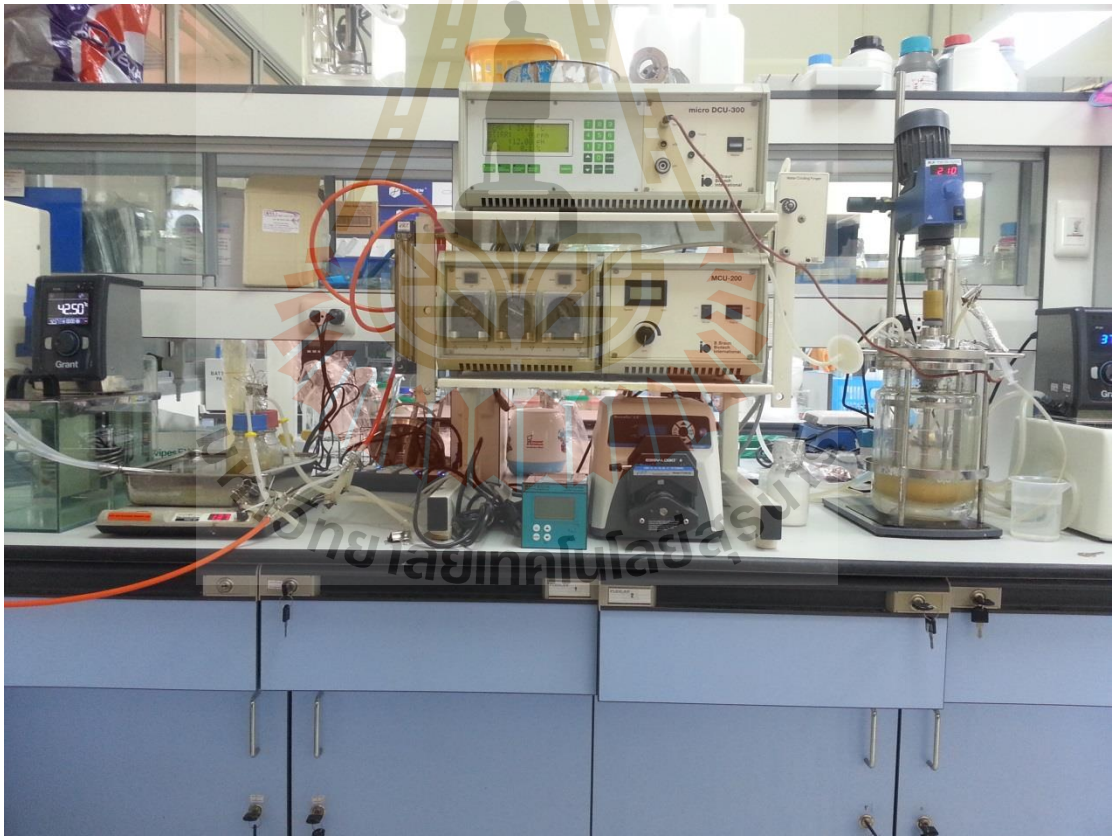

Figure A18 Fermentation of succinic acid 50 ml working volume in the 250 ml anaerobic bottle and 500 ml working volume in 2 l stirrer bioreactor process.

BIOGRAPHY

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