EFFICIENT PRODUCTION OF D-(-)-LACTIC ACID FROM SUGARCANE BAGASSE IN MINERAL SALTS MEDIUM BY METABOLICALLY ENGINEERED *KLEBSIELLA OXYTOCA* KIS004-91T

Sokha Kory

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การผลิตกรดแลคติกชนิดดี (-) ที่มีประสิทธิภาพจากชานอ้อยในอาหารเลี้ยงเชื้ออย่างง่ายด้วยเชื้อ *KLEBSIELLA OXYTOCA* KIS004-91T

นายโสคา โคริ่

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a Master's Degree.

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โสคา โคริ่ : การผลิตกรดแลคติกชนิดดี (-) ที่มีประสิทธิภาพจากชานอ้อยในอาหารเลี้ยงเชื้ออย่างง่ายด้วยเชื้อ KLEBSIELLA OXYTOCA KIS004-91T (EFFICIENT PRODUCTION OF D-(-)-LACTIC ACID FROM SUGARCANE BAGASSE IN MINERAL SALTS MEDIUM BY METABOLICALLY ENGINEERED KLEBSIELLA OXYTOCA KIS004-91T) อาจารย์ที่ปรึกษา : รองศาสตราจารย์ ดร. เขมวิทย์ จันต๊ะมา, 120 หน้า.

งานวิจัยนี้ศึกษาการผลิตกรดแลคติกชนิดดี (-) ที่มีประสิทธิภาพจากชานอ้อย ในขั้นตอนการปรับสภาพนั้นสารละลายเจือจางโซเดียมไฮดรอกไซด์ (ความเข้มข้น 3%, อัตราส่วนของแข็งต่อของเหลวคือ 1 ต่อ 9.1 บ่มที่อุณหภูมิ 50 องศาเซลเซียส เป็นเวลา 4 ชั่วโมง) ถูกใช้เพื่อปรับเปลี่ยนโครงสร้างแบบเมทริกซ์ของชานอ้อย ซึ่งประสิทธิภาพของขั้นตอนนี้แสดงได้จากการแสดงถึงการเอากลับคืนของของแข็งที่ต่ำของชานอ้อย และการเข้าถึงเซลลูโลสในกากชานอ้อยที่สูงขึ้น ของเอนไซม์เซลลูเลสคอมเพล็กซ์ปริมาณเซลลูเลสคอมเพล็กซ์อยู่ในช่วง 5 ถึง 30 ยูนิต ต่อกรัมของชานอ้อยที่ถูกปรับสภาพแล้ว โดยการย่อยนี้ทำในขวดรูปชมพู่ 250 มิลลิลิตร ที่ 50 องศาเซลเซียส และเขย่าด้วยความเร็ว 200 รอบต่อนาที จากผลการทดลอง พบว่าเมื่อใช้เอนไซม์ที่ 25 ยูนิตต่อกรัมชานอ้อยส่งผลให้ได้ปริมาณน้ำตาลสูงกว่าการใช้เอนไซม์ที่ต่ำกว่า 25 ยูนิต ในอัตราที่ใกล้เคียงกัน โดยที่ปริมาณเอนไซน์นี้ค่อนข้างต่ำเมื่อเปรียบเทียบกับการศึกษาก่อนหน้านี้

นอกจากนี้เชื้อ *Klebsiella oxytoca* KIS004-91T ถูกนำมาใช้เพื่อเปลี่ยนน้ำตาลที่ย่อยได้เป็นกรดแลคติกชนิดดี (-) โดยกระบวนการการหมักหลังการย่อย (SHF) และกระบวนการหมักระหว่างการย่อย (SSF) ถูกดำเนินการในถังหมักขนาด 5 ลิตร กรดแลคติกชนิดดี (-) ที่ความเข้มข้น 53.5±3.0 กรัมต่อลิตร ถูกผลิตในระหว่างกระบวนการ SHF แบบกะ สำหรับการใช้ชานอ้อยที่ปรับสภาพแล้วด้วยโซเดียมไฮดรอกไซด์ที่ประมาณ 150 กรัมต่อลิตร โดยมีผลผลิตของการเปลี่ยนน้ำตาลไปเป็นกรดแลคติก ชนิดดี (-) คิดเป็นร้อยละ 95 และ 36 ซึ่งคำนวณจากน้ำตาลที่ถูกใช้ และปริมาณของชานอ้อยที่ปรับสภาพแล้ว ตามลำดับ อย่างไรก็ตามกระบวนการ SSF แบบกะไม่ได้ช่วยปรับปรุงการผลิตกรดแลคติกชนิดดี (-) ของเชื้อสายพันธุ์นี้ ดังนั้นกระบวนการ SHF แบบกึ่งกะถูกนำมาใช้เพื่อเพิ่มประสิทธิภาพการผลิตกรดแลคติกชนิดดี (-) จากการใช้ชานอ้อยที่ถูกปรับสภาพแล้วในปริมาณที่สูงขึ้น ส่งผลให้ได้กรดแลคติกชนิดดี (-) ที่ความเข้มข้น 101.0±0.9 กรัมต่อลิตร จากการใช้ชานอ้อยที่ปรับสภาพแล้วในปริมาณรวม 250 กรัมต่อลิตร นอกเหนือจากนี้พบว่าผลผลิตของกรดแลคติกชนิดดี (-) มีค่าเพิ่มสูงขึ้นเป็นร้อยละ 99 และ 40.4 เทียบกับน้ำตาลที่ถูกใช้และปริมาณของชานอ้อยที่ปรับสภาพแล้ว ตามลำดับ ยิ่งไปกว่านั้นต้นทุนการผลิตของ

กรดแลคติกชนิดดี (-)โดยประมาณยังต่ำกว่าเมื่อเทียบกับการใช้น้ำตาลคุณภาพสูงเป็นแหล่งคาร์บอน จากผลการทดลองนี้แสดงให้เห็นถึงแนวโน้มที่เป็นได้ในการผลิตกรดแลคติกชนิดดี (-) โดยใช้เชื้อ *K. oxytoca* KIS004-91T จากชานอ้อยที่ปรับสภาพด้วยโซเดียมไฮดรอกไซด์ ซึ่งเป็นหนึ่งในชีวมวลลิกโนเซลลูโลสที่ยั่งยืน

สาขาวิชาเทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา_____

ปีการศึกษา 2562 ลายมือชื่ออาจารย์ที่ปรึกษา_____

SOKHA KORY : EFFICIENT PRODUCTION OF D-(-)-LACTIC ACID FROM SUGARCANE BAGASSE IN MINERAL SALTS MEDIUM BY METABOLICALLY ENGINEERED KLEBSIELLA OXYTOCA KIS004-91T. THESIS ADVISOR : ASSOC. PROF. KAEMWICH JANTAMA, Ph.D., 120 PP.

D-(-)-LACTIC ACID/SUGARCANE BAGASSE/NaOH PRETREATMENT/ KLEBSIELLA OXYTOCA KIS004-91T

The efficient production of D-(-)-lactic acid from sugarcane bagasse (SCB) was investigated in this research. In the pretreatment step, a dilute NaOH solution (3% NaOH with a solid:liquid ratio at 1:9.1, incubated at 50°C for 4 h) was used to alter the matrix structure of SCB. The effectiveness of this step was indicated through the low recovery of SCB solid fraction and high accessibility of cellulase complex to the pretreated cellulose residues. Cellulase complex loading in the range of 5 to 30 U/g SCB for enzymatic digestion of the NaOH pretreated SCB was evaluated in 250 mL shaking flask at 50°C and 200 rpm. The result showed that 25 U/g SCB enzyme loading liberated higher sugars levels than those of lower enzymatic loadings with a comparable conversion rate. This loading quantity was quite low compared to those of previous studies.

Further, the mutant *Klebsiella oxytoca* KIS004-91T was used to convert liberated sugars to D-(-)-lactic acid. Separate Hydrolysis and Fermentation (SHF) and Simultaneous Saccharification and Fermentation (SSF) processes were subsequently performed in 5 L bioreactor. For 150 g/L of the pretreated SCB, D-(-)-lactic acid at the

concentration of 53.5±3.0 g/L was obtained during a batch SHF with the conversion yields of 95% and 36% calculated based on liberated sugars consumed and the NaOH pretreated SCB provided, respectively. However, the batch SSF process did not improve D-(-)-lactic acid production by the strain. Therefore, a fed-batch SHF was further investigated to gain more efficiency of D-(-)-lactic acid production from higher solid loading of the NaOH pretreated SCB. As a result, D-(-)-lactic acid at the level of 101.0±0.9 g/L was obtained from 250 g/L of the NaOH pretreated SCB loading. Additionally, D-(-)-lactic acid yields of up to 99% and 40.4% based on liberated sugars consumed and the NaOH pretreated SCB provided were improved. Also, the D-(-)-lactic acid's estimated production cost is lower compared to those produced by high grade sugars as a sole carbon source. These results illustrated a feasible promising of D-(-)-lactic acid production by *K. oxytoca* KIS004-91T from the NaOH pretreated SCB, which is one of the sustainable lignocellulosic biomasses.

School of Biotechnology

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Student's Signature _____

Advisor's Signature _____

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LIST OF ABBREVIATIONS

ackA	=	Acetate kinase
adhE	=	Alcohol dehydrogenase
aq.	=	Aqueous
AM1	=	Alfredo Martinez Mineral Salt Medium 1
ATP	=	Adenosines 5'-triphosphate
bud	=	2,3 butanediol dehydrogenase
BDO	=	Butanediol
cAMP	=	Cyclic adenosine monophosphate
°C	=	Degree Celsius
$CaCO_3$	=	Calcium carbonate
$Ca(OH)_2$	=	Calcium hydroxide
CCR	=	Carbon Catabolite Repression
CDW	=	Cell dried weight
CFU	=	Colony form unit
Cra	=	Catabolite repressor/activator protein
Crp	=	Catabolite receptor protein
DI	=	Deionized
E. coli	=	*Escherichia coli*
Fig	=	Figure
fdh	=	Formate dehydrogenase H
frdABCD	=	Fumarate reductase ABCD

LIST OF ABBREVIATIONS (Continued)

g	=	Gram
g/g	=	Gram per gram
g/L	=	Gram per liter
g/mol	=	Gram per mole
h	=	Hour
HMF	=	Hydroxymethylfurfural
HPLC	=	High-performance liquid chromatography
kg	=	Kilogram
K. oxytoca	=	*Klebsiella oxytoca*
L	=	Liter (s)
LB	=	Luria Bertain
LCC	=	Lignin-carbohydrate complex
ldhA	=	Lactate dehydrogenase A
min	=	Minute
$MgCl_2$	=	Magnesium chloride
NADH	=	Nicotinamide adenine dinucleotide
NaOH	=	Sodium hydroxide
PET	=	Polyethylene terephthalate
pH	=	Potential hydrogen
PLA	=	Polylactic acid
pflB	=	Pyruvate formate-lyase B
pta	=	Phosphate acetyltransferase

LIST OF ABBREVIATIONS (Continued)

SCB	=	Sugarcane bagasse
SHF	=	Separate hydrolysis and fermentation
SSF	=	Simultaneous saccharification and fermentation
w/v	=	Weight per volume

CHAPTER I

INTRODUCTION

1.1 Significant of the study

Lactic acid is a carboxylic acid in the group of hydroxy propionic acid which is a commercially valuable and important platform chemical. It can serve as a precursor for many industrially key compounds: acrylic acid, 1,3-propanediol, methyl acrylate, acrylamide, ethyl 3-hydroxy propionic acid, malonic acid, propiolactone and acrylonitrile (Németh et al., 2017). It is commonly used in cosmetic, food, pharmaceutical, and chemical industries. It is also used in polylactic acid (PLA) production that has a high demand in the current market as it is a safe, biocompatible, and biodegradable polymer (Alsaheb et al., 2015). Chemical production of lactic acid yields racemic mixtures of D-(-) and L-(+)-lactic acid while fermentation by selective microbes can produce high optical purity of D-(-) or L-(+)- lactic acid (Wee et al., 2006). During the fermentation of renewable feedstocks, lactic acid bacteria could provide high yields and titers of lactic acid. However, their demands for complex nutrients during growth and their productions of by-products including acetic acid, acetaldehyde, ethanol, and diacetyl increase production cost, purification, and even waste disposal for the large-scale production. In contrast, many developed metabolically engineered *Escherichia coli* strains may decrease the production cost since they lacked genes responsible for the formation of by-products. Though, they

cannot efficiently utilize a broad range of substrates including xylose, arabinose, cellobiose, and even cellotriose due to the catabolic repression. Therefore, many further genetic modifications and acquired metabolic evolution to enhance rates of substrate utilization were employed thus offering strains to be economically feasible for lactate production (Khunnonkwao et al., 2018; Sangproo et al., 2012; Sawisit et al., 2015a).

Lactic acid production from lignocellulosic materials has gained interest as a possibility to take out obstacles in the production chain as an example reducing the price of input processes, gaining environmental benefits, and adding value to biomass (Oliveira et al., 2019). Sugarcane bagasse (SCB) is one of the lignocellulosic materials that is achieved the point of attraction as it gains more advantages than other carbohydrate substrates due to its abundance, sustainability, non-competitiveness for human consumption and benefits to the environment (Oliveira et al., 2018). SCB consists of cross-linked cellulose, hemicellulose and even lignin which can be hydrolyzed to fermentable sugars including hexoses (D-glucose, D-mannose, D-galactose), pentoses (D-xylose, D-arabinose) (Guilherme et al., 2019; Unrean, 2018) and to valuable by-products such as furfural, HMF, vanillin, etc. Though, there are a variety of processing methods that are required to alter the matrix structure of bagasse and disrupt celluloses and hemicelluloses into fermentative sugars as examples of solvent extraction, steam explosion, thermal pretreatment using bases or acids combine with biological pretreatments and enzymatic hydrolysis (Xin et al., 2019).

Klebsiella oxytoca is a fast-growing bacterium under aerobic and anaerobic conditions. It can use simple nutrients and can utilize many types of carbon substrates, hence avoiding using lots of enzymes for enzymatic digestion of lignocellulosic substrates. Therefore, it may decrease input prices regarding a large-scale production of lactic acid (Sangproo et al., 2012). A metabolically engineered *K. oxytoca* KIS004

in which genes encoding alcohol dehydrogenase (*adhE*), phosphate acetyltransferase (*pta*), acetate kinase (*ackA*), fumarate reductase (*frd*) and pyruvate formate-lyase (*pflB*) were inactivated, allowed it generating high production of D-(-)-lactic acid in low salts medium with a very low concentration of by-products (In, 2019). Using a low salts medium (AM1), the *K. oxytoca* KIS004 able to produce D-(-)-lactic acid around 45.2 g/L from 5% (w/v) glucose and 26.0 g/L from 10% (w/v) glucose while the *K. oxytoca* KIS004-91T (the metabolically evolved strain of *K. oxytoca* KIS004 strain for its utilization of glucose at high concentration) could provide 95.9 g/L of D-(-)-lactic acid from 10% (w/v) glucose. However, this work had been focused on utilizing pure glucose as a sole substrate which is considerably competed as for human food production. Alongside, industrial-scale production of bio-based chemicals or biofuels from clean or pure substrates as feedstock may gain pressure on economic, environmental, and socio-economic implications due to their costs of commodity products, agricultural wastes and competitions for human food and animal feedstocks (Filho et al., 2018). Using lignocellulosic substrates from agricultural or agro-industrial sectors as feedstock materials may reduce 40 to 70 % of the total production cost for lactic acid biosynthesis (Abdel-Rahman et al., 2016).

Thus, this study attempted to use a chemical pretreatment method to extract fermentable sugars from sugarcane bagasse that is the most abundant agricultural waste in tropical and sub-tropical areas including Thailand. The diluted base (NaOH) was used in this pretreatment to breakdown the structure of sugarcane bagasse and to release fermentable sugars. Mixed sugars mainly glucose and xylose (mostly residing in lignocelluloses including SCB) were obtained by the aid of enzymatic hydrolysis by cellulase complex. Hence, the conversion of liberated sugars from SCB into D-(-)-

lactic acid by the mutant *K. oxytoca* KIS004-91T in terms of titers, yields and productivity in batch SHF, batch SSF and fed-batch SHF processes were focused.

1.2 Research objectives

Objectives of this study were to replace the pure carbon substrate (glucose) for D-(-)-lactic acid production with lignocellulosic SCB biomass and to investigate the suitable fermentation operations or parameters for achieving an efficient optically pure D-(-)-lactic acid production by *K. oxytoca* KIS004-91T strain. Thus, this work has focused on:

1. Improving yields of fermentable sugars (mainly glucose) derived from SCB pretreated by a dilute sodium hydroxide (NaOH) pretreatment followed by the cellulase hydrolysis.

2. Comparing D-(-)-lactic acid production efficiency between a batch-separate hydrolysis and fermentation (SHF) and a batch-simultaneous saccharification and fermentation (SSF) from the NaOH pretreated SCB by mutant *K. oxytoca* KIS004-91T in 5 L bioreactor.

3. Evaluating the production of D-(-)-lactic acid in 5 L bioreactor using fed-batch fermentation by a batch separate hydrolysis and fermentation (SHF) process.

1.3 Scope and limitation of the study

Dilute alkaline (3% w/v NaOH solution) was used for the chemical pretreatment of SCB to breakdown its structure and to reveal the cellulose attaching surface. The commercial cellulase enzyme was used for the enzymatic hydrolysis to digest polysaccharide of pretreated SCB into monosaccharides. The low salts AM1 medium containing the NaOH pretreated SCB at a high solid loading of 15% (w/v) or 150 g/L

was used to evaluate D-(-)-lactic acid production by the mutant *K. oxytoca* KIS004-91T in batch SHF, batch SSF, fed-batch SHF processes.

CHAPTER II

LITERATURE REVIEW

2.1 Overview of lactic acid

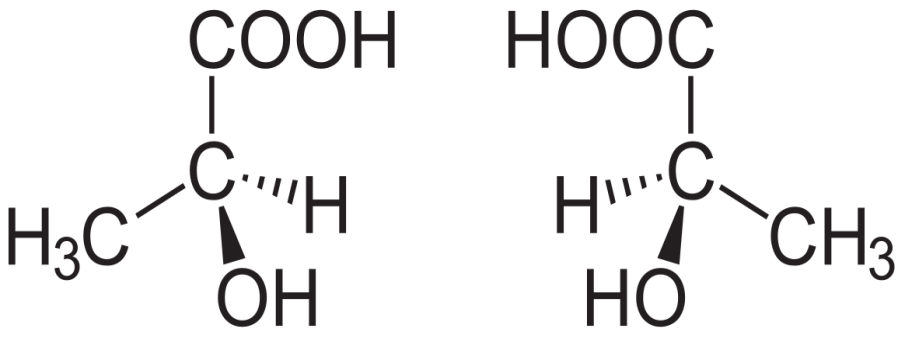
Lactic acid or (S) 2-hydroxy propanoic acid is a three carbons organic acid that has a molecular weight of 90.08 g/mol with the formula: $CH_3CH(OH)CO_2H$ (Razali et al., 2017). Although lactic acid was first discovered in milk during 1780 by Carl Wilhelm Scheele, it has been around since the dawn of life which appears almost in all living organisms (Dusselier et al., 2013). It has a potential role in the anaerobic metabolism of life forms (Sara et al., 2016). Also, it was recognized in 1857 by Louis Pasteur as a fermented metabolite that is produced by specific microorganisms (Wee et al., 2006). Then, it was first produced on the large scale in 1895 by Boehringer Ingelheim company and soon became a major organic acid for many industrial uses because it is in the group of GRAS (Generally Regarded as Safe) referred to FDA (Food and Drug Administration) in USA (Alsaheb et al., 2015).

2.2 Physico-chemical property of lactic acid

Lactic acid is a colorless or yellowish as liquid and white power as the solid form that known as a weak organic acid due to its partial dissociation into the water as showed in the equation: $H_3C\text{-}CH(OH)\text{-}COOH \leftrightarrow H^+ + H_3C\text{-}CH(OH)\text{-}COO^-$ and the correlated acid dissociation constant ($K_a = 1.38\times10^{-4}$) (Ameen et al., 2017). It is a linear, aliphatic thermoplastic polyester with rigidity and clarity as same as

poly-styrene and poly-ethylene terephthalate (Martin et al., 2001). Lactic acid' s chemical structure consists of a chiral carbon atom attached by the hydroxyl group and the other two terminal carbon atoms which are a methyl and carboxylic group (Narayanan et al., 2004). This certain characteristic provides two optical active isomeric forms which are: D-(-)-lactic and L-(+)-lactic acid (Fig. 2.1) that are easily dissolve in water and water-dissolved organic solvents yet insoluble in other organic solvents. Especially, they have an acidic character in an aqueous medium, bifunctional reactivity associated with the presence of a carboxyl and a hydroxyl group that provides a great reaction versatility, and an asymmetric optical activity of the chiral carbon (C2) (Martinez et al., 2013).

Due to the asymmetric structure and multi-functional groups on the carbon molecules, lactic acid is a highly reactive substance which is extremely important in polylactic acid chemistry (Dusselier et al., 2013). Normally, lactic acid releases a proton from the carboxylic group to become ion lactate in solution in which can precipitate with the other salts solution such as $MgCl_2$, $Ca(OH)_2$ and $CaCO_3$ (Benthin, 1995; Narayanan et al., 2004). Moreover, Table 2.1 summarizes the properties of lactic acid and its isomer as well as the mixture of the two isomers.



Isomer D-(-)-lactic acid Isomer L-(+)-lactic acid

Figure 2.1 The isomer forms of lactic acid, D-(-)-lactic acid and L-(+)-lactic acid (Ameen et al., 2017).

Table 2.1 Physico-chemical property of lactic acid, adapted from (Dusselier et al., 2013; Narayanan et al., 2004).

Property	**Unit (conditions)**	**Isomer or concentration**	**Reported range**
Melting point	°C	L-(+) or D-(-)	52.7-53.0
		racemic	16.4-18.0
Boiling point	°C (at 1.87 kPa)	L-(+) or D-(-)	103
		racemic	122
Solid density	g/mL (at 20°C)	-	1.33
Liquid density of aq. Solution	g/mL (at 25°C)	20 wt%	1.057
		88.6 wt%	1.201
pK_a	n/a	L-(+) or D-(-)	3.79-3.86
		racemic	3.73
Heat of combustion, ΔH_C	kJ/mol	-	1361
Specific heat capacity, C_P	J/mol/°C (at 20°C)	-	190

n/a: not available

2.3 Application of lactic acid

Lactic acid is a commercially valuable, important chemical platform and is one of the top 12 building block bio-based chemicals that can be derived from biorefinery selected by the US Department of Energy (Németh et al., 2017). The carboxyl and the β-hydroxyl group have provided lactic acid the high reactivity and possibly undergone hydrogenation, reduction, oxidation, condensation, dehydration, dehydrogenation, decarboxylation, decarbonylation, polymerization and esterification (Dusselier et al., 2013; Liu et al., 2017). As a result, the broad-range chemical intermediates of lactic acid make it become a suitable precursor for synthesizing wide-optically active substances which are applying in many fields including food, cosmetic, pharmaceutical and chemical industries (Fig. 2.2) (Alsaheb et al., 2015; Martinez et al., 2013; Rodrigues et al., 2017).

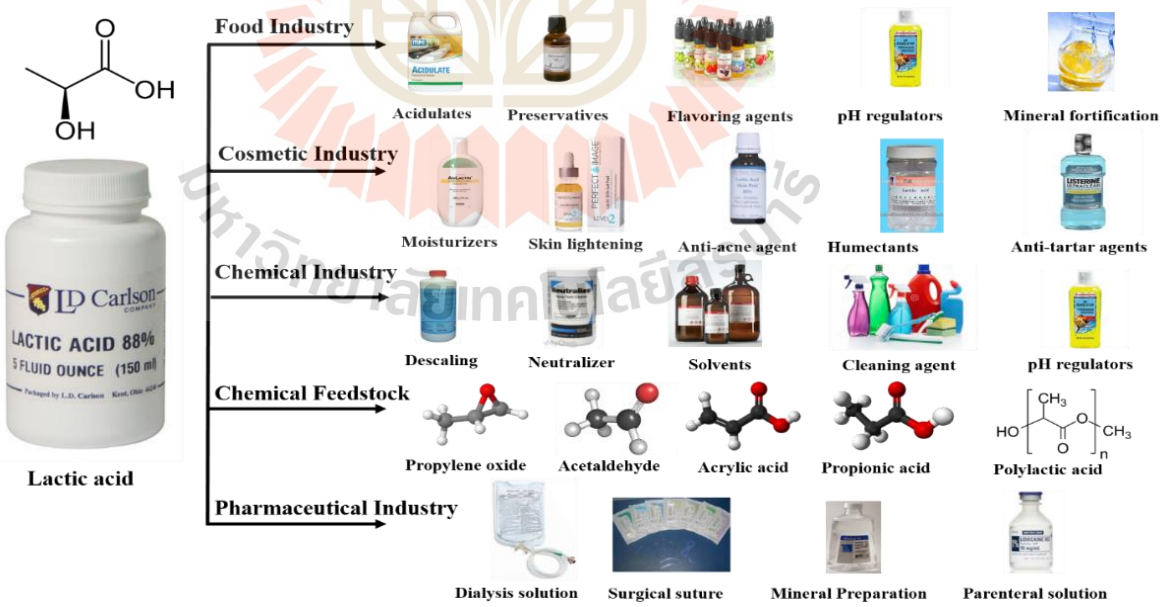


Figure 2.2 Scheme commercial applications of lactic acid and its salt, modified from (Wee et al., 2006).

Since lactic acid is classified as GRAS, it has been extensively applied in almost every part of food production to serve functions either pH regulation, flavoring, microbial quality control, or mineral fortification (Benninga, 1990). In the processing of poultry, fish, and meat industries, lactic acid is applied to enhance flavors as well as increase the product's shelf life by making the acidic condition which is un-favored for the growth of food-borne pathogens and food spoilage microorganisms. Moreover, due to its mild acidic taste and non-volatile odorless, lactic acid is frequently found as an acidulant for preserving and pickling agents in dressings, salads, bakery products, pickled vegetables and beverages. In confectionery products, it is used to increase flavor and pH adjustment. Especially, it helps in lowering the reversed-reaction rate and increasing crystal clear of candies production. Similarly, lactic acid is a major constituent in fermented food as well as yogurt, butter and canned vegetables while its esters including stearoyl-2-lactylate, glyceryl lactostearate, glyceryl lactopalmitate are used as the emulsifying agents in bakery processing (Komesu et al., 2017; Narayanan et al., 2004). Lactic acid is required to improve mineral in food production. As an example, ammonium lactate is the necessary non-protein nitrogen source that preferred adding into the dairy products to increase the nutritive value and decrease the purification cost (Norton et al., 1994).

Lactic acid is also important in cosmetic implementation. It is a natural constituent in cosmetic products that mainly used as moisturizers and pH regulators. During the repression of the tyrosine formation, the lactic acid moisture-detained capability has gained in which it allows skin hydration and skin-lightening. Especially, lactic acid and its salt are the normal constituents of the human body, so they perfectly fit with the new trend for developing safer and natural formularization. Besides this,

they also own many other characterizes including anti-acne and antimicrobial activity that make them interesting as active ingredients in cosmetics (Wee et al., 2006).

Similarly, lactic acid is required in the pharmaceutical field as an electrolyte in many intravenous solutions that are important in restoring stable fluids or electrolytes in human bodies. For example, CAPD or continuous ambulatory peritoneal dialysis solutions as well as dialysis solutions are demanded in conventional artificial kidney machines. Together, it serves as primary functions in the pharmaceutical field such as pH regulator, metal sequestration, and chiral intermediate (Alsaheb et al., 2015). In addition, the biodegradable polymer character of lactic acid has brought it to apply in a wide variety of mineral preparations and medical device creations including surgical sutures, tablets, orthopedic implants, prostheses, and drugs delivery operation systems. For example, calcium lactate is commonly used as an anti-caries agent and calcium deficiency therapy (Datta et al., 2006).

Furthermore, lactate salts are increasingly applied in a different segment of chemical production processes. The main functions of lactic acid in this field including pH regulator, chiral intermediates, descaling agent, solvent, cleaning agent, antimicrobial agent, slow acid-release agent, metal complexing agent, humectant and neutralizer (Wee et al., 2006). Natural lactic acid has a prominent use as an excellent, safe, and environmentally friendly (green) solvent, which is an alternative in many fine mechanical cleaning applications. It is an excellent eraser of polymer and resins due to its high solvency power and solubility. Moreover, oxidized-lactates and lactate esters such as propylene oxide, propylene glycol, acrylic acid and acrylate (Datta et al., 2006) are frequently found as the main particles in pesticides, herbicides and other bioactive

components production. It has low molecular weights, low toxicity and a high ability for increasing the higher yields of various products (Komesu et al., 2017).

The noticeable use of lactic acid in the chemical field is in biodegradable and biocompatible PLA polymers production, which accounts for the huge market of lactic acid utilization (Abdel-Rahman et al., 2013). The resultant of PLA polymer has a broad range spectrum application including protective apparel, food packaging, bottles, mulch film, rigid containers, trash bags, short shelf-life trays and shrink wrap (Rivero et al., 2017). The optically pure lactic acid can be polymerized into a high molecular mass PLA polymer through the serial reactions including polycondensation, depolymerization and ring-opening polymerization (Sodergard et al., 2002). However, the commercial PLA grades are copolymers of poly L-(+)-lactic acid (PLLA) and poly D-(-)-lactic acid (PDLA), which are produced from L-(+)-lactides and D-(-)-lactides (Martin et al., 2001). PDLA is a useful fraction for improving the thermostability of PLLA since the alone use of PLLA provided a low melting point of biopolymer products. During copolymer, PLLA interacts with PDLA in solution to create the stereo complex crystalline structure. Thus, the obtained-products have improved thermal stability, mechanical performance and hydrolysis-resistance than PLLA-based materials (Wang et al., 2008). Especially, the blend of PDLA and PLLA in the ratio of 1:1 has produced a stereo complex structure that has a melting temperature (230°C) higher than a melting temperature of PLLA alone (175°C) (Ikada et al., 1987).

2.4 Lactic acid production routes

The production of lactic acid can perform through microbial fermentation from renewable feedsctocks or chemical synthesis from petrochemical resources including coal, natural gas and petroleum products (Fig. 2.3). Therefore, the microbial

fermentation of lactic acid is preferred since lactic acid obtained by chemical synthesis took a higher price as it needs multiple reactions and production racemate of the DL-lactic acid (Narayanan et al., 2004). Additionally, chemical synthesis of lactic acid has faced the problem of environmental pollution and limited natural supply of petrochemical resources (Daful et al., 2016; Wee et al., 2006). In contrast, microbial biosynthesis generates specific D-(-) or L-(+) of lactic acid once the suitable microorganisms are selected especially lactic acid bacteria (LAB) and *E. coli* strains (Hofvendahl et al., 2000). Further, it offers more benefits including abundant cheap renewable of substrates, low production temperatures, low energy consumption, operational modes and recovery processes (Abdel-Rahman et al., 2011; Rodrigues et al., 2017).

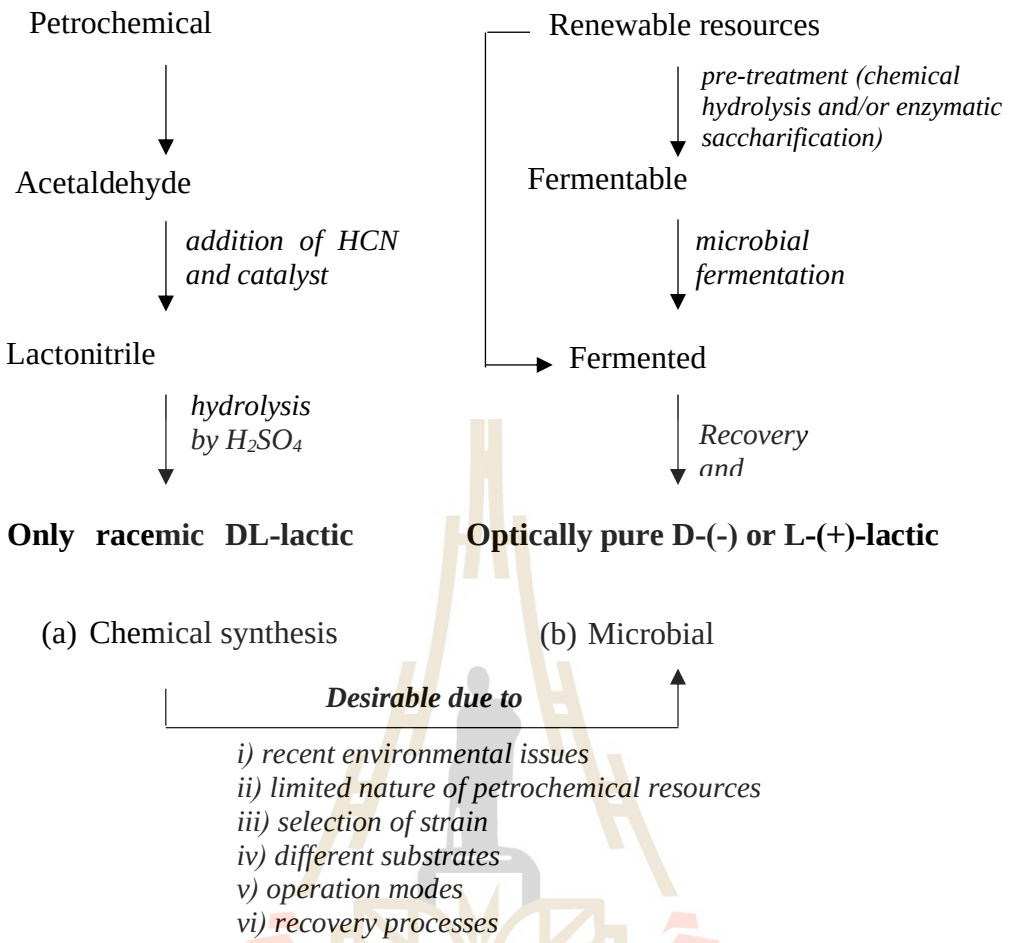


Figure 2.3 Schematic of lactic acid production route. (a) chemical synthesis and (b) microbial fermentation, modified from (Rodrigues et al., 2017; Wee et al., 2006).

2.4.1 Lactic acid production by chemical synthesis

Lactic acid can be created through chemical synthesis from most of its derivatives by the means of specific treatments. There are many available routes for the chemical production of lactic acid including base-catalyzed degradation of sugars, carbon monoxide, water at high temperatures and pressures, oxidation of propylene glycerol, hydrolysis of chloropropionic acid, nitric acid oxidation of propylene and the reaction of acetaldehyde (Gao et al., 2011). In the nitric acid oxidation of propylene, the alkene is oxidized into α-nitro propionic acid by nitric acid in which it is

subsequently converted to lactic acid by hydrolysis reaction (Fig. 2.4). However, it is not a practical production route due to environmental concerns (Vaidya et al., 2005).

(i) Oxidation by O_2

$$\underset{\text{Propene}}{CH_3CH_2H_3} + \underset{\text{Nitric acid}}{HNO_3} \xrightarrow{oxidation/O_2} \underset{\text{α-nitro propionic acid}}{CH_3CHNO_2COOH}$$

(ii) Hydrolysis by H_2O

$$\underset{\text{α-nitro propionic acid}}{CH_3CHNO_2COOH} + H_2O \longrightarrow \underset{\text{Lactic acid}}{CH_3CHOHCOOH} + HNO_3$$

Figure 2.4 Lactic acid production via nitric acid oxidation of propylene pathway (Vaidya et al., 2005).

Furthermore, only the reaction of acetaldehyde using lactonitrile as raw material is technically and economically feasible for lactic acid production among various routes (Datta et al., 2006; Gao et al., 2011). Lactonitrile or 2-hydroxypropanenitrile (CH_3CHOCN) is a by-product of acrylonitrile technology. The chemical synthesis of lactic acid using this route was discovered in 1863 by Wislicenus (Benning, 1990) and the reactions involved in this pathway are described in Fig. 2.5. Firstly, lactonitrile is obtained by the nucleophilic addition of hydrogen cyanide into the liquid phase of acetaldehyde in the presence of a base and high pressure. Then, the crude lactonitrile is recovered and purified by distillation before treating with concentrated HCl or H_2SO_4 in which the resultants are a crude lactic acid with an ammonium salt. After that, the concentrated and purified crude lactic acid is esterified with methanol to produce methyl lactate. Finally, racemic lactic acid was generated through hydrolysis under acid catalyst and distillation (Ameen et al., 2017; Narayanan et al., 2004).

(i) Addition of hydrogen cyanide

$$\underset{\text{Acetaldehyde}}{CH_3CHO} + \underset{\text{Hydrogen cyanide}}{HCN} \xrightarrow{high\ pressure} \underset{\text{Lactonitrile}}{CH_3CHOHCN}$$

(ii) Hydrolysis by H_2SO_4

$$\underset{\text{Lactonitrile}}{CH_3CHOHCN} + H_2O + \tfrac{1}{2}H_2SO_4 \longrightarrow \underset{\text{Lactic acid}}{CH_3CHOHCOOH} + \tfrac{1}{2}\underset{\text{Ammonium salt}}{(NH_4)_2SO_4}$$

(iii) Esterification

$$\underset{\text{Lactic acid}}{CH_3CHOHCOOH} + \underset{\text{Methanol}}{CH_3OH} \longrightarrow \underset{\text{Methyl lactate}}{CH_3CHOHCOOH_3} + H_2O$$

(iv) Hydrolysis by H_2O

$$\underset{\text{Methyl lactate}}{CH_3CHOHCOOH_3} + H_2O \longrightarrow \underset{\text{Lactic acid}}{CH_3CHOHCOOH} + \underset{\text{Methanol}}{CH_3OH}$$

Figure 2.5 Chemical production route of lactic acid using lactonitrile raw material (Narayanan et al., 2004).

In addition, Monsanto (Texas, USA) is the first company that started chemical synthesis of lactic acid during 1963 which represented 40% (4,500 tons) of the lactic acid supplement in the USA. Alongside, Sterling Chemicals also started this technical production and ended the production in the early 1990s, while the Musashino Chemical (in the east) had changed from this technology to fermentation technology (John et al., 2009).

2.4.2 Lactic acid production by microbial fermentation

Biotechnological processes based on fermentation is the flexible solution to deal with problems of lactic acid production by chemical synthesis techniques including the racemic mixture of DL-lactic acid, limited, expensive raw materials and products, temperature and energy consumption (Pal et al., 2009). The industrial microbial fermentation of lactic acid has started during 1856 after the French chemist Loius Pasteur discovered the strain *Lactobacillus* spp. which are able to produce lactic acid from carbohydrates (Carr et al., 2002). Therefore, the biochemical conversion of carbohydrate molecules (glucose) into energy, lactate and other by-products are depending on the type of microorganism used in which their metabolic pathways are illustrated in Fig. 2.6. (John et al., 2007). Lactic acid producing microorganisms catalyze pyruvic acid to lactic acid using two types of enzymes including NAD-dependent L-(+)-lactate dehydrogenase and NAD-dependent D-(-)-lactate dehydrogenase. Thus, the stereospecificity of D-(-) and/or L-(+)-lactic acid obtained from microorganisms depends on the type of enzymes involved (Garvie, 1980). Additionally, Hofvendahl *et al*. (2000) reported that the microbial production route of lactic acid represents 90% of worldwide lactic acid production. Of course, optically pure isomers could predominantly obtain through fermentation processes (Ameen et al., 2017).

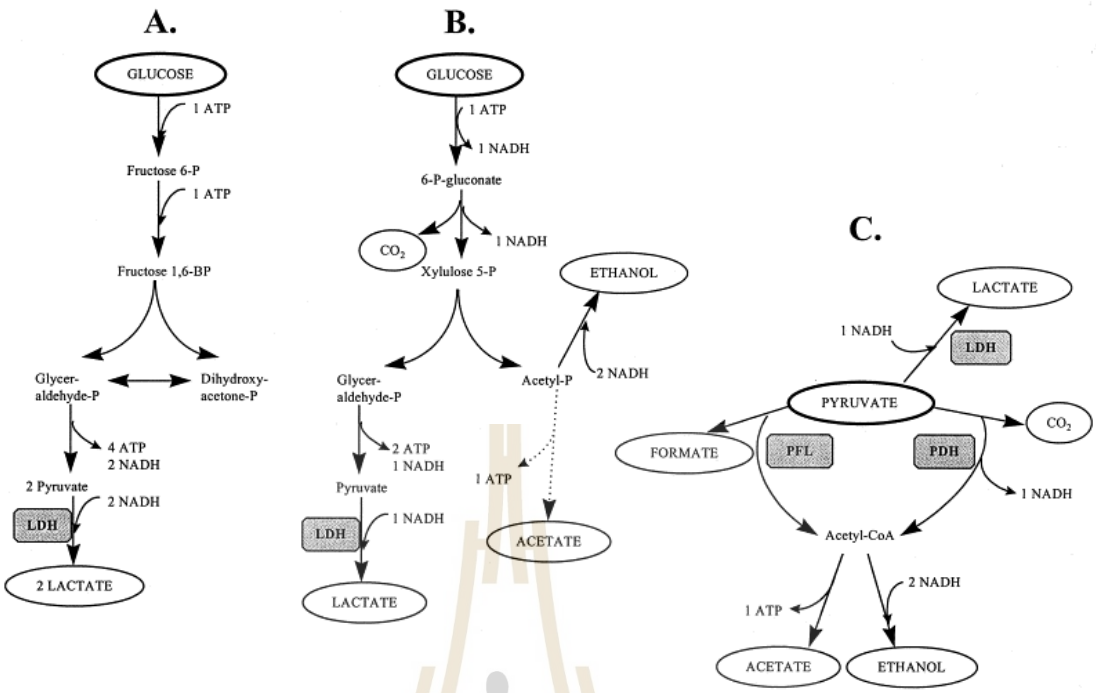


Figure 2.6 Microbial (LAB) production pathway of lactic acid. Homofermentation (A), Heterofermentation (B) and mixed acid fermentation (C). P, phosphate; BP, bisphosphate; *LDH*, lactate dehydrogenase; *PFL*, pyruvate formate lyase and *PDH*, pyruvate dehydrogenase (Hofvendahl et al., 2000).

2.5 Lactic acid producing microorganisms

Various types of microorganisms are able to produce lactic acid including bacteria, cyanobacteria, yeast, fungi and algae. Each of them has their own capability over the other as well as, broad-range substrate utilization, higher yield, higher productivity, reduction of complex nutrient requirements or higher optically pure lactic acid obtained (Abdel-Rahman et al., 2013). Practically, microbial lactic acid producers are divided into two groups which are bacteria and fungi (Litchfield, 1996). Therefore, fungi strains produce only L-(+)-lactic acid in aerobic conditions using starch, glucose or sucrose (Zhou et al., 1999). Differentially, bacterial strains can produce both D-(-)

and L-(+) or a mixture of lactic acid in fermentation (Zhao et al., 2010). Additionally, the gram-positive bacteria, LAB that normally found at plants, fermented foods or terrestrial marine animals mostly produce lactic acid as primary or secondary fermentation products especially the genera *Lactobacillus, Carnobacterium, Lactococcus, Streptococcus, Enterococcus, Vagococcus, Leuconostoc, Oenococcus, Pediococcus, Tetragonococcus, Aerococcus* and *Weissella* (Florou-Paneri et al., 2013). Together, bacterial strains such as *Streptococcus mutans*, *Lactobacillus debrueckii* subsp. *bulgaricus* and *L. sakei* (Dan et al., 2017; Oguro et al., 2017) produce lactic acid as a key end-product of glycolysis under excess sugar or low pH conditions. Hence, bacteria strains are the most favorite microorganisms that researchers used for the production of lactic acid production since they provide higher production yield (Oonkhanond et al., 2017).

E. coli or *Lactobacillus* spp., or fungal organism *Rhizopus* spp. are the frequently used microorganisms in the industrial fermentation of lactic acid (Ameen et al., 2017). Table **2.2** shows the current microorganisms that can generate high concentrations of lactic acid in batch fermentations from the commonly used media and substrates. Additionally, lactic acid producing bacteria are able to utilize sugars, sugar-containing hydrolysates, the single or double-step conversion of starchy or cellulosic wastes by amylolytic direct conversion or the adjoin of enzymes inoculum together. Some of the LAB strains including *Leuconostoc carnosum, Lactobacillus delbrueckii, Leuconostoc mesenteroides* subsp. *dextranicum Lactobacillus coryniformis* subsp. *torquens, Leuconostoc mesenteroides* subsp. *mesenteroides* and *Leuconostoc fallax* are the well-known D-(-)-lactic acid production bacteria (Manome et al., 1998). *Lactobacillus coryniformis* subsp. *torquens* was reported that it could generate D-(-)-lactic acid from

filters paper using the SSF process (Yáñez et al., 2003) while *Lactobacills delbrueckii* JCM1148T could generate lactic acid from sugar beet juice, sugarcane juice and sugarcane molasses (Calabia et al., 2007).

Table 2.2 Microorganisms provided high biotechnological production of D-(-) and L-(+)-lactic acid in various media and substrates.

Lactic acid	Microorganisms	Media/Substrates	Conc. (g/L)	Prod. (g/L/h)	Yield (g/g)	Ref.
D-(-)	*L. delbrueckii*	Molasses	190	4.15	0.95	Dumbepatil et al., 2008
D-(-)	*L. dlbrueckii* NCIM 2025	MRS	150	3.15	0.96	Bhatt et al., 2008
L-(+)	*R. oryzae* NRRL 395	PDA (cassava pulp)	21.82	0.43	0.31	Thongchul et al., 2010
L-(+)	*R. oryzae* NBRC 5378	Glucose and xylose	6.8	0.07	0.23	Saito et al., 2012
D-(-)	*K. oxytoca* KMS004	AM1 (glucose)	34.0	0.35	0.87	Sangproo et al., 2012
D-(-)	*Bifidobacterium* sp.	Simulated fruit vegetable wastes	29.5	1.2	0.68	Wu et al., 2015
D-(-)	*Leuconostoc mesenteroides*	MRS	38.3	0.159	0.99	Mufidah et al., 2016
L-(+)	*Enterobacter aerogenes* ATCC 29007	LB	46.02	0.5	0.41	Thapa et al., 2017
L-(+)	*Bacillus* sp. BC-001	GY agar	108.9	6.1	1.04	Thitiprasert et al., 2017

Table 2.2 (continued).

Lactic acid	Microorganisms	Media/Substrates	Conc. (g/L)	Prod. (g/L/h)	Yield (g/g)	Ref.
D-(-)	*L. delbrueckii spp. delbrueckii*	MRS	201	6.72	0.88	de la Torre et al., 2019
D-(-)	*E. coli*	LB	5	0.25	0.5	Aso et al., 2019
D-(-)	*K. oxytoca* KIS004	AM1 (glucose)	100	2.1	0.96	In, 2019

2.6 The demand and concepts for developing bio-based lactic acid

The global demand for lactic acid production has jumped from 130,000-150,000 (metric) tons per year (Mirasol, 1999) to 714,200 (metric) tons by 2013. Due to the development of new uses and products, global lactic acid requirements annually increase 10-15.5% which are expected to reach 1,960,100 (metric) tons by 2020 (Abdel-Rahman et al., 2016). One of the reasons that expand lactic acid consumption is PLA production which is 18.8% annually grows and accounts for 1,205,300 (metric) tons of worldwide PLA production in 2020. Additionally, PLA polymer is much more environmentally friendly than PET polymer with a simple life cycle (Fig. 2.7) (Avinc et al., 2009). Lactic acid production from renewable resources is another reason that pushes developing bio-based lactic acid. Literally, the microbial fermentation is represented over 90% of total lactic acid production so a replacement of optically pure sugars as substrate by cheap raw materials such as molasses, starchy, wastes of agricultural agro-industrial residues, or lignocellulosic biomass would improve the economical lactic acid fermentation. Alongside, available technologies for the development efficacy of lactic acid producing microorganisms by genetic modifications to gain their abilities utilizing a broad range of cheap substrates or producing the desired optically homonegative pure D-(-) or L-(+)-lactic acid with less or without by-products are another interesting option (Abdel-Rahman et al., 2016; Gao et al., 2011).

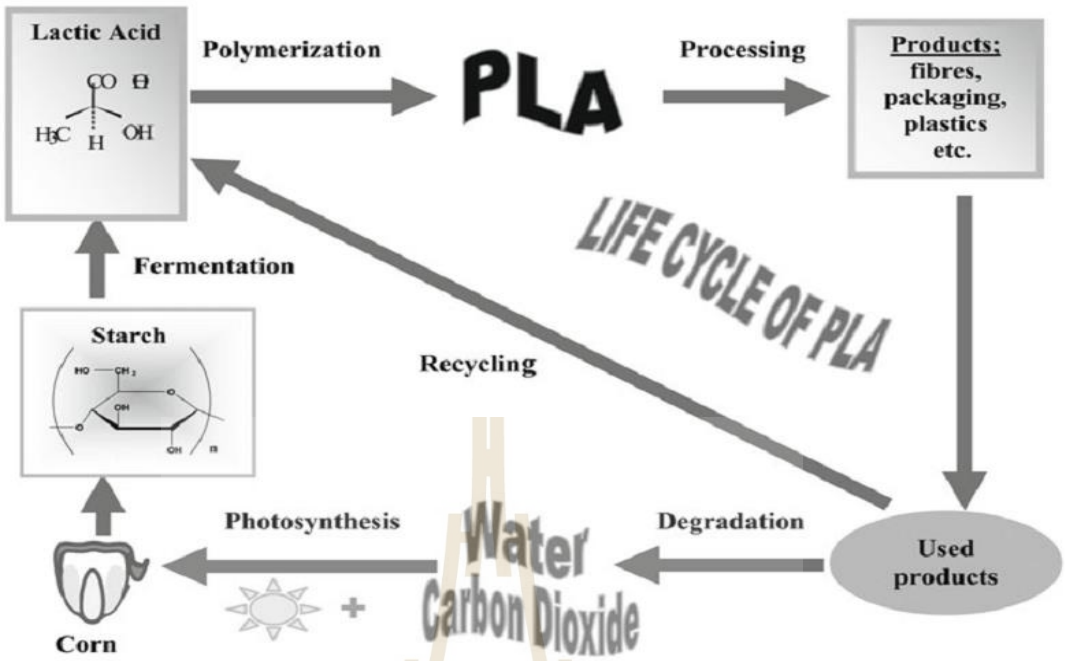


Figure 2.7 The life cycle of PLA polymer which has an initial raw material base as renewable plant stock (Avinc et al., 2009).

2.7 *Klebsiell a oxytoca*

Klebsiella oxytoca is a non-motile, rod-shaped, Gram-negative bacterium in the family *Enterobacteriaceae* (Darby et al., 2014). It has long been used for industrial-scale as its capability to perfectly utilize the wide range of substrates (Jantama et al., 2015). Generally, wild type *K. oxytoca* has four fermentative pathways under anaerobic and microaerobic conditions: the pyruvate formate-lyase pathway utilizing pyruvate to formate as soon as becoming hydrogen and carbon dioxide, acetate, and ethanol. The second is the lactic acid pathway while the third is the succinate pathway and the butanediol pathway is the fourth. Together, *K. oxytoca* M5a1 is a nonpathogenic, safe used bacterium that is normally found in the pulp and paper industry (Wood, 2005). It is an advanced production host that does not need special or high price nutrient sources for growth and has accessible techniques for genetic modification (Pholyiam, 2013; Sangproo et al., 2012). Unlike *E. coli*, *K. oxytoca* M5a1 is able to metabolize soluble

products from lignocellulosic biomass such as cellobiose, cellotriose, xylobiose, xylotriose, arabinosides and products from starch degradation including maltodextrins and cyclodextrins (Wood, 2005).

The developed *K. oxytoca* KIS004 strain (In, 2019) that was eliminated some genes including *adhE, pta-ackA, frd* and *pflB* (Table 2.3), which encode enzymes involving in the production of ethanol, acetate, succinate and formate, respectively, gained higher yield of D-(-)-lactic acid production from optically pure glucose compared to that of its parental strain *K. oxytoca* KMS004 (Sangproo et al., 2012). Additionally, *K. oxytoca* KIS004 initially produced D-(-)-lactic acid at a concentration of 26.0 g/L, yield 0.50 g/g glucose and productivity 0.27 g/L/h from the AM1 medium containing 100 g/L glucose. After performing metabolic evolution in the *K. oxytoca* KIS004 strain, the *K. oxytoca* KIS004-91T could produce D-(-)-lactic acid at the concentration of 95.9 g/L with the yield and productivity of 0.95 g/g of glucose and 1.0 g/L/h, respectively after 96 h incubation in the AM1 medium containing 10% (w/v) glucose under anaerobic conditions. In addition, batch and fed-batch fermentation in 5 L bioreactor revealed that *K. oxytoca* KIS004-91T provided D-(-)-lactic acid at 101 g/L and 129 g/L after 48 h and 68 h, respectively, from 10 % (w/v) glucose (In, 2019).

Table 2.3 Derivations of *K. oxytoca* producing D-(-)-lactic acid strains.

Strain	Relevant	References
K. oxytoca	M5a1	Wild type
KMS002	KMS001, Δ*adhE*	Sangproo et al., 2012
KMS004	KMS003, Δ*adhE,* Δ*pta-ackA*	Sangproo et al., 2012
KIS002	KIS001, Δ*frd*	In, 2019
KIS004	KIS003, Δ*frd*, Δ*pflB*	In, 2019
KIS004-91T	KIS004, metabolic evolution	In, 2019

In Fig. 2.8, four fermentative pathways in *K. oxytoca* KIS004 were inactivated. First, the alcohol dehydrogenase gene (*adhE*) that encodes enzyme converting acetaldehyde to ethanol was knocked-out from wild type *K. oxytoca* M5a1 to conserve NADH for lactate formation and to redirect carbon flux for the lactate production. Then phosphate acetyltransferase (*pta*) and acetate kinase A (*ackA*) genes which produce enzymes transforming acetyl-CoA to acetyl-P to acetate were simultaneously removed from KMS002 to construct KMS004 for reducing the acetate production. After that, the fumarate reductase (*frd*) gene that provides enzyme changing fumarate to succinate was removed from the KMS004 strain thus preventing the strain to re-oxidized NADH via the succinate producing pathway. Finally, the KIS004 strain was constructed by eliminating the pyruvate formate-lyase gene (*pflB*) to remove the full pyruvate formate-lyase pathway for decreasing a carbon flow that is wasted to the by-product's formation.

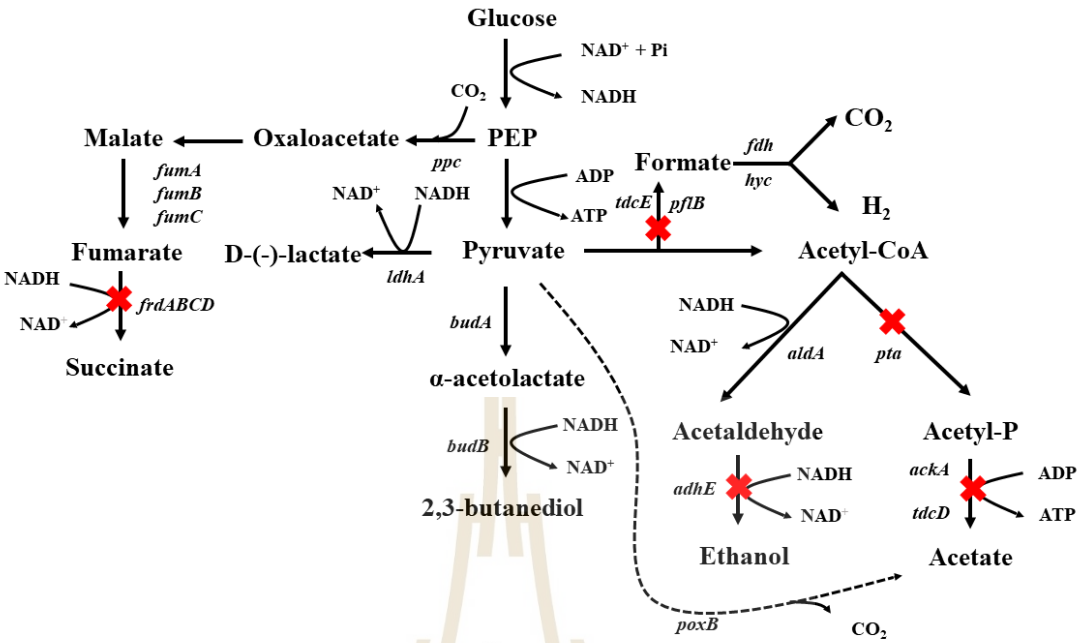


Figure 2.8 Fermentation pathway of *K. oxytoca* KIS004 under anaerobic conditions. Solid arrows represent central fermentative pathways. Dashed arrow represents the alternative acetate-producing pathway via pyruvate oxidase B (*poxB*). The red cross signs represent the inactivate pathways. Gene and enzymes: *ldhA*, lactate dehydrogenase; *pflB*, pyruvate formate-lyase; *pta*, phosphate acetyltransferase; *ackA*, acetate kinase; *tdcD*, propionate kinase; *tdcE*, threonine decarboxylase E; *adhE*, alcohol dehydrogenase; *ppc*, phosphoenolpyruvate carboxylase; *mdh*, malate dehydrogenase; *fumABC*, fumarase isozymes; *frdABCD*, fumarate reductase; *fdh*, formate dehydrogenase; *hyc*, formate hydrogenlyase; *poxB*, pyruvate oxidase; *budA*, α-acetolactate decarboxylase; *budB*, α-acetolactate synthase; *budC*, butanediol dehydrogenase; *aldA*, aldehyde dehydrogenase; *dhaBCE*, glycerol dehydratase; and *dhaT*, 1,3-propanediol oxidoreductase, adapted from (Jantama et al., 2015).

2.8 Lignocellulosic materials

Lignocellulosic materials are the natural carbon and abundant renewable bioresource which accounts for 90% of the global plant biomass production. Approximately, 200 billion tons of global lignocellulosic materials generate annually and 8-20 billion tons are potentially remained (Lin et al., 2006). Therefore, these numerous disposing has harmed the environment due to the inappropriate landfill and their burning that produce ammonia and methane which are the global greenhouse gas. In contrast, most of the lignocellulosic materials can be converted into useful material in industries including enzyme production, enzymatic support, biofuels and fine chemicals (Bilal et al., 2020). It is currently drawing more attention in using it as raw materials in feedstock due to the cheap cost, high sugar content, year-round availability and non-competitive food supply (Abdel-Rahman et al., 2011). Originally, lignocellulosic materials are mainly categorized as four groups including wastepaper, municipal solid waste, forest residues and crop residues. Also, it is commonly categorized as agricultural residues, softwood and hardwood that compose of cellulose, hemicellulose and lignin that link to each other to make the complex matrix structure of the plant cell wall (Fig. 2.9) (Alonso et al., 2012). Moreover, the proportion structure and chemical in lignocellulosic materials vary in or between species due to genetic and environmental effects. The constitutes of cellulose and hemicellulose are higher in hardwood while the softwood has higher lignin (Balat, 2013). Table 2.4 shows the different contents of lignocellulosic biomasses.

Table 2.4 Various cellulose, hemicellulose and lignin composition in lignocellulosic biomasses (% dry weight).

Lignocellulosic biomass	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Aspen hardwood	51	29	16
Birch hardwood	40	39	21
Hardwood barks	22-40	20-38	30-35
Hardwood stems	40-55	24-40	18-25
Willow hardwood	37	23	21
Pine softwood	44	26	29
Softwood	40-44	25-27	25-31
Softwood barks	18-38	15-33	30-60
Softwood stems	45-50	25-35	25-35
Spruce softwood	43	26	29
Coastal Bermuda grass	25	35.7	6.4
Grasses	25-40	25-50	10-30
Switch grass	45	31.4	12
Algae (green)	20–40	20–50	-
Leaves	15-20	80-85	-
Newspaper	40-45	25-40	18-30
Nur shells	25-30	25-30	30-40
Paper	85-99	-	0-15
Primary wastewater solids	8-15	-	24-29
Solid cattle manure	1.6-4.7	1.4-3.3	2.7-5.7
Swine wastes	6	28	-
Waste papers from chemical pulp	60-70	10-20	5-10
Chemical pulps	60-80	20-30	2-10
Sorted refuse	60	20	20
Cottonseed hairs, flax	80-95	5-20	-
Wheat straw	37-41	27-32	13-15
Oat straw	39.4	27.1	17.5

Source: adapted from (Abdel-Rahman et al., 2011).

Table 2.4 (continued).

Lignocellulosic biomass	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Barley straw	33.8	21.9	13.8
Rye straw	37.6	30.5	19
Rice straw	36.2	19	9.9
Rice hull	36	15	19
Sunflower stalks	42.1	29.7	13.4
Soya stalks	34.5	24.8	19.8
Corn cobs	45	35	15
Corn stalks	39-47	26-31	3-5
Cassava pulp	8.1-15.6	2.8-4.6	2.2-2.8
Sugarcane bagasse	40-50	25-35	10-15

Source: adapted from (Abdel-Rahman et al., 2011).

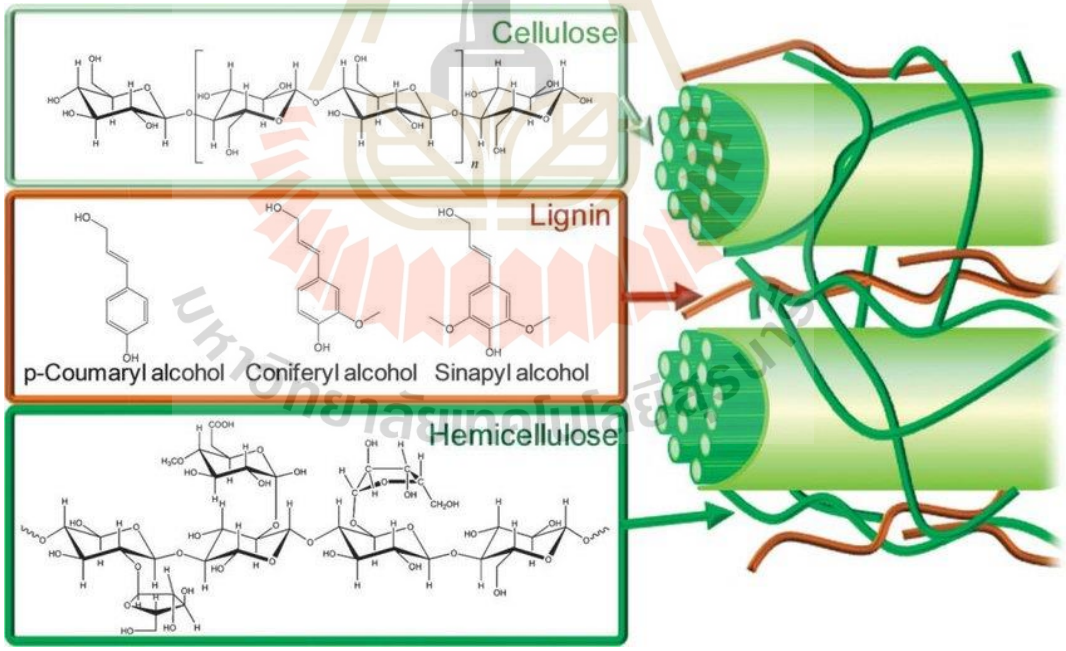


Figure 2.9 The general structure of lignocellulosic materials (Cellulose, Hemicellulose and Lignin as composition) (Alonso et al., 2012).

2.8.1 Cellulose

Cellulose $(C_6H_{10}O_5)_n$ represents approximately 30-60% as a dry matter in lignocellulosic materials. It is a homopolysaccharide composed of β-D-glucopyranose units that covalently link to each other by β-1,4-glycosidic bonds and packed as microfibrils (Brodeur et al., 2011). The presence of multiple hydroxyl groups (OH) in cellulose makes complex intramolecular and intermolecular hydrogen bonds and stronger van der Waals forces that stabilize and crystalize (45-96%) structural microfibrils of cellulose. These crystallite microfibrils are compacted into the fibrils with about 35-40°A width and 500°A length and 7,000-15,000 polymerize degrees. Especially, the crystallite microfibrils compose of three glucose chains including true-crystal chains (core chains) which are the most chemical and biological hydrolysis resistant part of cellulose, sub-crystalline chains (transition chains) and non-crystalline chains (Karimi et al., 2013). The natural crystalline of cellulose needs low pH, high temperature or extended time to make significantly hydrolyzed quantities of cellulose to glucose (Trajano et al., 2013). Moreover, amorphous is another form of cellulosic polymers that are susceptible to enzymatic activities than crystalline form (Pérez et al., 2002). Yet, the cover of hemicellulose and lignin on the microfibrils of lignocellulosic biomasses which gathering as strong seal cover-around cellulose polymer requires intensive treatments before isolation (Chandel et al., 2011).

2.8.2 Hemicellulose

Hemicellulose is the second-main fraction in lignocellulosic materials which typically accounts for 15-35%. It is a short-highly branched heteropolymer of polysaccharides and polyuronides including hexosans polymers (glucosan, galactan, mannan, rhamnan), pentosans (xylan, araban), acetyl, methoxyl, carboxylic group and hexuronic acids. Together, they formed various main polymers such as xyloglucan, glucuronoxylan, galactoglucomanans, arabinoglucuronoxylan, arabinoxylan and glucomannan (Karimi et al., 2013; Trajano et al., 2013). The monosaccharides of hemicellulose link to each other by β-1,4 or β-1,3-glycosidic linkages (Pérez et al., 2002). The major sugar varies from species to species. Mannose is dominant in softwoods hemicellulose with acetyl linked by galactose as side groups while hardwoods and agriculture residues hemicelluloses contain xylose and acetyl linked with arabinose or fucose side groups (Balat, 2013; Karimi et al., 2013; Modenbach et al., 2014). Although lignocellulosic hemicelluloses are more complex structures than cellulose, the amorphous form, short-chain polymers side chains and polyuronides polymers make them more sensitive to hydrolysis by acid as well as hemicellulase enzymes. During acid hydrolysis, it is completely isolated with limited damage to cellulose (Trajano et al., 2013). Hemicelluloses play roles as intermediates link cellulose to lignin making the strong mechanism of the plant cell wall (Pérez et al., 2002).

2.8.3 Lignin

Lignin is another important constituent representing 17-33% of lignocellulosic biomasses. It is a complex of phenol monomers mainly coniferyl, sinapyl and *p*-coumaryl alcohols and their derivatives which connect by cross-linked

C-C and ether (C-O-C) linkages (Bugg et al., 2011). These linkages can interact in the direction β-O-4, α-O-4, β-5, β-1, β-β, 5-5, 4-O-5 to create lignin polymers such as *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S). Lignin links to hemicellulose in a lignin-carbohydrate complex (LCC) structure and acts as a seal enclose cellulose and hemicellulose which strengthens biomasses structure and supporting in water transporting of the plant (Modenbach et al., 2014). The disruption of lignin produces aromatic monomers in the liquid hydrolysate (Trajano et al., 2013). Alongside, the irregular structures, hydrophobicity property and easily formed covalent bonds to nearby molecules (hemicelluloses) of lignin have added a strong surface covered structure to cellulose matrix and strengthened the plant cell wall to resist against external stresses, diseases and biodegradation of microorganisms (Karimi et al., 2013). Generally, lignin in softwoods (28-40%) is higher than hardwoods and agriculture residues (18-25%). It is a barrier in enzymatic hydrolysis since enzymes bind onto its surface thus no activity on the cellulose chains. The presence of lignin in biomass is one of the main drawbacks when applying biomass in fermentation (Brodeur et al., 2011; Karimi et al., 2013) as it seals the chemical and biological degradation of biomasses (Abdel-Rahman et al., 2011). Therefore, lignin and hemicellulose need to be separated to increase cellulose accessible area as well as to alternate its matrix structure (Brodeur et al., 2011).

2.8.4 Extraneous matters

Extraneous materials are a variety compound found in lignocellulosic biomasses. They can be extracted by polar or non-polar solvents which consist of resins (fatty acids, resin acids, fats, phytosterols), terpenes (ketone, isoprene alcohols) and phenols (residue by-product of lignin biosynthesis) as extractives components (Fan et

al., 1982). On the other hand, some extraneous materials which are not dissolved in solvents and cauterized as non-extractives matters. These inorganic components of biomass represent in very low amounts (less than 2 w/w%). They are the resultant of carbonates, metal salts, oxalates, silicates and phosphates deposited in plant cell walls and lumina (Balat, 2013). Therefore, the extraneous parts of biomass do not play a major problem in the treatment since they are in large numbers and frequently show in lower amounts (Karimi et al., 2013).

2.9 Sugarcane bagasse

Sugarcane (*Saccharum* spp.) is a tall perennial grass family that normally grows in tropical and subtropical areas. It is an energy-crop that is commonly used in sugar and ethanol industries (Canilha et al., 2012; Oliveira et al., 2019). The bagasse and the straw are two main wastes that are generated during sugarcane processing, thus SCB is the solid and fibrous remaining waste from sugarcane milling in which it consists of fiber bundles vessels, parenchyma and epithelial cells (Candido et al., 2019; Vieira et al., 2020). Together, the main components in SCB are cellulose 40-50%, hemicellulose 25-35%, lignin 15-20%, ash and wax 2-3% on a dry weight basis (Laopaiboon et al., 2010). Therefore, it contains lesser minerals, waxes and other compounds than many agricultural residues straw. According to the recent data, SCB more than 1.6 billion tons have been produced worldwide annually in which more than 250 kg of SCB waste from 1,000 kg of sugarcane (Schmitt et al., 2020; Vieira et al., 2020). The un-used and some applications of SCB for generating electricity (through burning) have caused the air pollution problem and made much pressure on landfills (Thai et al., 2020). Even though, it has been used in pulp and paper production, SCB is the feasible raw material for creating products based on fermentation including protein-enriched feed, enzymes,

amino acids, an important pharmaceutical compound and organic acids (Pandey et al., 2000). In addition, biochemical conversion of SCB to biochemicals happens in triple steps which are pretreatment, enzymatic or acid hydrolysis and fermentation (Fig. 2.10). Pretreatment is a very critical step in bioconversion of lignocellulose as lignin and hemicellulose are removed or modified and changing in the cellulose structure of SCB (Oliveira et al., 2018). The non-affected pretreatment method on lignocellulosic may lead to struggle saccharification by hydrolytic enzymes and in some cases, it produces inhibitory compounds that act as a barrier to the metabolism of microorganisms (Kodali et al., 2006). Furthermore, it is also one of the potential factors that influent the downstream costs through the fermentation inhibitions, enzymatic hydrolysis rates, enzyme loading, product titers, product purification, mixing power as well as waste treatment requirement (Abdel-Rahman et al., 2011).

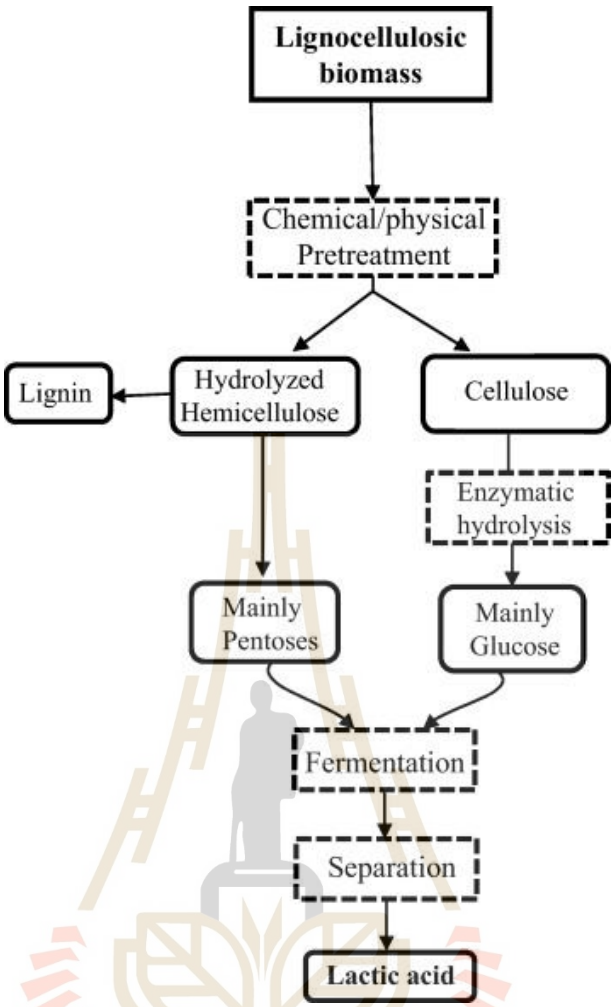


Figure 2.10 The steps in biochemical conversion of lignocellulosic biomasses (sugarcane bagasse) into a chemical product (lactic acid) (Abdel-Rahman et al., 2011).

2.10 Pretreatment methods of lignocellulosic biomasses

Lignocellulosic biomasses including SCB are the abundant and renewable bioresource in which their physical, chemical, and biological can be changed by pretreatment methods (Fig 2.11). During pretreatments, associated structures between cellulose, hemicellulose and lignin are changed thus leading to the fractionation and revealing cellulose structure, increasing the attached-surface area and depolymerizing cellulose (Abdel-Rahman et al., 2011; Pandey et al., 2000). There are four major types

of pretreatment methods that can be apply to remove the lignin and alter the hemicellulose as well as cellulose in lignocellulosic materials including: 1) physical methods consist of fragmentation (hacking, milling, grinding, rolling), microwave radiation, sonication (ultrasounds), spray drying with gamma radiation, mechanical extrusion, pyrolysis, pulse electric field (PEF); 2) chemical methods which are acid pretreatment, alkali pretreatment, organo-solvent, ionic liquids, and ozonolysis; 3) physicochemical methods including ammonia fiber expansion (AFEX), ammonia recycle percolation (ARP), stream explosion, carbon dioxide explosion, liquid hot water (LHW), wet oxidation, sulfite pretreatment to overcome recalcitrance of lignocellulose (SPORL treatment) and 4) biological pretreatment using living microorganisms or their products such as white rot, brown rot and soft rot fungi, bacterial and enzymatic treatment to breakdown the lignocellulosic biomasses (Aftab et al., 2019; Kucharska et al., 2018). Among them, some methods aim to break the lignin complex structure while some alter the strong fibril crystalline structure of cellulose (Sun et al., 1995). Therefore, the suitable pretreatment method should complete the following demands including 1) strongly digestible pretreated solid such as a widely open crystalline surface area of cellulose, partially depolymerized crystalline cellulose and disrupted hemicellulose, altered the lignin structure, 2) less degradation of sugars and high yield desired monosaccharide, 3) low energy input, 4) no or low microbial inhibition, 5) simple and easy to scale up, and 6) economic and environmentally friendly (Abdel-Rahman et al., 2011; Canilha et al., 2012). However, chemical pretreatments including diluted alkali and acid are the common using methods to destruct lignocellulosic biomasses as it is simple, recyclable and do not need high priced equipment (Table 2.5).

Table 2.5 Benefits and drawbacks of pretreatment methods of lignocellulosic biomasses, adapted from (Brodeur et al., 2011).

Pretreatment methods	Benefits	Drawbacks
Alkali	-Efficient removal of lignin -Low inhibitor formation -Recyclable of alkaline	-High cost of alkaline catalyst -Alteration of lignin structure
Acid	-High glucose yield -Solubilizes hemicellulose -Recyclable of acid	-High cost of acids catalyst -Need corrosive resistant equipment -Inhibitors formation
Green solvents	-Lignin and hemicellulose hydrolysis -Able to dissolve high loading of different biomass types -Simple conditions need (low temperature)	-High solvent costs -Complication for solvents recovery and recycle
Stream explosion	-Cost effective -Lignin transformation and hemi-cellulose solubilization -Hight yield of glucose and hemicellulose in two steps process	-Partial hemicellulos degradation -Acid catalyst effective to high lignin content materials -Toxin compound generation
LHW	-Separate of nearly pure hemi-cellulose -No catalyst involves -Hydrolysis of hemicellulose	-High energy and water need -Solid mass contents of cellulose and lignin left over
AFEX	-More accessible of cellulose -Low inhibitors formation	-High effective with herbaceous material and low lignin content biomass -Causes inactivity between lignin and enzymes -Recycling of ammonia needed -High cost of ammonia -Alteration of lignin structure
ARP	-Lignin majority removed -High cellulose content yields	-High effective with herbaceous material -High energy and liquid loading needed
Supercritical fluid	-Low degradation of sugars -Cost effective -More accessible of cellulose	-High pressure needs -Un-affects to lignin and hemi-cellulose

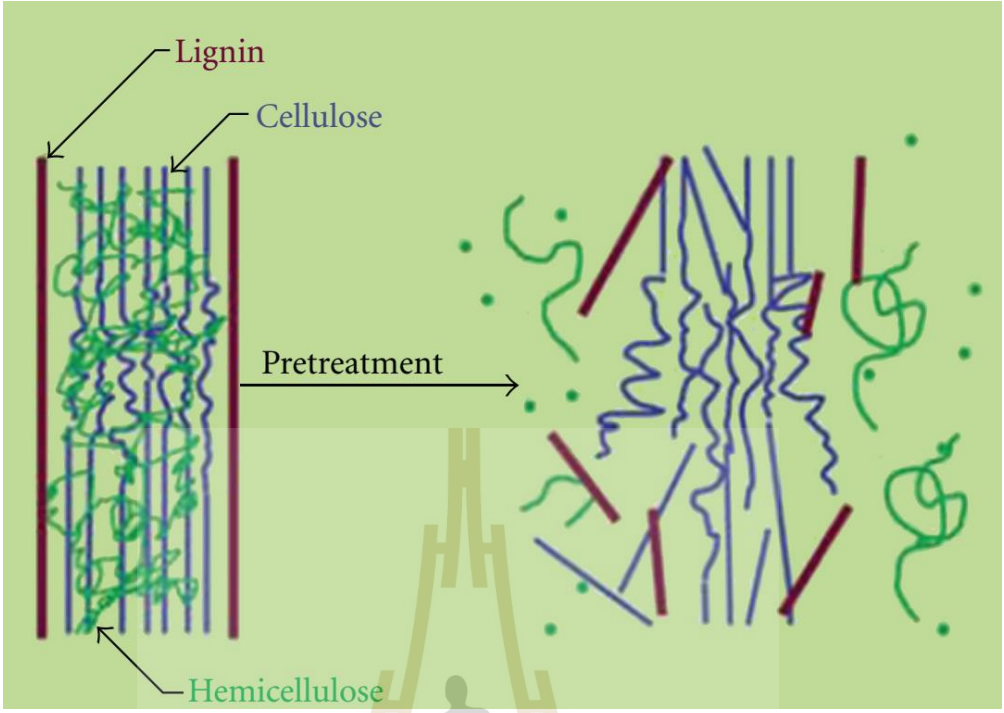


Figure 2.11 The effect of pretreatment methods in destroying lignin, hemicellulose, cellulose matrix structure of bagasse (Brodeur et al., 2011).

2.11 Alkali pretreatment of lignocellulosic biomasses

Alkali pretreatment is a chemical pretreatment method that involves the use of bases including sodium, potassium, calcium, or ammonium hydroxide to alter the chemical structures of lignocellulosic biomasses (Brodeur et al., 2011). Especially, this method provides a significant removal of lignin and a specific number of hemicellulose (Canilha et al., 2012). Alkali pretreatment requires less pressure, ambient conditions, and enzymes thus yielding higher fermentable sugars but fewer sugars degradation, and easily removing or recovering caustic salts (Aftab et al., 2019). Sodium hydroxide is moderate cost as compare to $Ca(OH)_2$ and KOH (Chang et al., 2017). Hence, NaOH is the frequently used alkali agent found to be effective and economical (Pandey et al., 2000). This pretreatment process generates two fractions including liquid which is a mixture of lignin and hemicellulose oligomers, and cellulose residue as a solid fraction

(Sabiha-Hanim et al., 2019). The main parameters effect during NaOH pretreatment including the concentration of NaOH loading, time, temperature, and liquid to solid ratio loading of biomass (Zhao et al., 2009). Sun et al. (1995) reported that bagasse pretreated with 1.5% (w/v) NaOH for 144h at 20°C be able to remove lignin and dissolve hemicellulosic polysaccharides up to 60% and 80%, respectively. Similarly, 40-80% (w/w) of lignin have removed once increasing pH of NaOH pretreated dry wheat straw from 10 to 13 at 140°C (Bensah et al., 2013) while NaOH loading rate 3-9% (w/v, as dry bagasse) had increased delignification of 52.3%-75.5% (Zhao et al., 2009). NaOH pretreatment of lignocellulosic materials at high temperature and higher pH resulted in numerous reactions take place (Hashemi et al., 2016). Additionally, the pretreatment of NaOH is categorized as severe and moderate processes. Normally, the conditions are used in severe processes such as NaOH in the concentration of 0.5-4 % with high temperature and pressure. It is a non-reusable of NaOH. In contrast, a moderate process is a recyclable NaOH which concentration of 6-20% with low temperature and pressure is used (Karimi et al., 2013).

2.11.1 Effect of NaOH pretreatment on lignin and hemicellulose

In NaOH pretreatment of SCB, hydroxyl ion is aimed for delignification which breakdown lignin structure within three reaction groups including fragmentations, condensations, degradation and dissolution (Karimi et al., 2013). Moreover, most of the herbaceous biomasses, their LCC structures composed of phenolic lignin attached to arabinoxylan by the cooperation of ferulic acid (Modenbach et al., 2014). This construction is formed by either ester or ether linkages which make them sensitive destructed by chemical pretreatment (Fig. 2.12). The effectiveness of NaOH pretreatment on lignin removal from SCB is shown in Table 2.6. The

pretreatment of bagasse with the diluted NaOH is also found to increase the cellulose swelling, decrease polymerization and crystallization of cellulose, to efficiently break linkages between lignin and polysaccharides as well as to disrupt lignin structure (Fan et al., 1982). It is found to be effective with hardwood, agriculture residues and herbaceous crops (low lignin content) than softwood (Canilha et al., 2012).

Table 2.6 The previous study related NaOH pretreatment of SCB for lignin removal (% w/w as dry basis) (Sabiha-Hanim et al., 2019).

Lignin in raw SCB	**Linin in pretreated SCB**	**Conditions**	**Equipment**
21.5	10.6	1.0% NaOH, 120°C, 10 min	Autoclave
27.9	9.2	0.9% NaOH, 80°C, 2 h	Autoclave
25.4	7.8	2% NaOH, 121°C, 30 min	Autoclave
18.0	1.8	15% NaOH, 175°C, 15 min	Autoclave
17.8	4.3	4% NaOH, 121°C, 30 min	Autoclave
25.0	9.0	2.5% NaOH, 126°C, 45 min	Autoclave
30.1	18.5	1.0% NaOH, 120°C, 60 min	Autoclave
23.4	5.2	5% NaOH, 121°C, 60 min	Autoclave
25	6	1.0% NaOH, 100°C, 30 min	Steam explosion
34.3	5.7	1.0% NaOH, 100°C, 1 h	Steam explosion
22.0	9.5	2.0% NaOH, 120°C, 40 min	Autoclave

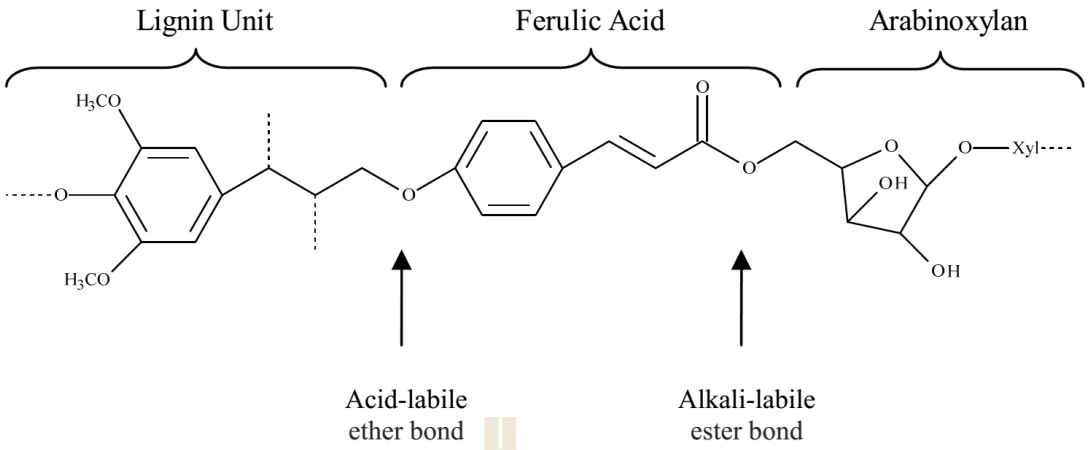


Figure 2.12 The LCC structure of herbaceous biomass that joins by ferulic acid with ester and ether bonds (Modenbach et al., 2014).

The pretreatment with NaOH modified several structures of SCB that increased the availability of enzymatic hydrolysis as a higher delignification ability. During and after pretreatment, lignin is partially or completely disrupted and alters the strength of hemicellulose. Ferulic acid linkage in the LCC structure is the attractive point of the NaOH pretreatment. The mechanism of NaOH pretreatment of SCB is illustrated in Fig. 2.13. Firstly, the hydroxide ion attacks to the carbon of ester bond either the bond between lignin and carbohydrate or between the two lignin components as well as two carbohydrate components. Then, a tetrahedral intermediate form when a negatively charged oxygen atom discharges an alkoxide ($–OCH_3$) from the carboxylic acid. Finally, a fast-irreversible reaction results that alkoxide acts as a base to deprotonate from the carboxylic acid resulting in a degradation of ester and glycosidic bonds of lignocellulosic structures and forcing cellulose swelling, partial recrystallization of cellulose and solubilizing lignin (Modenbach et al., 2014).

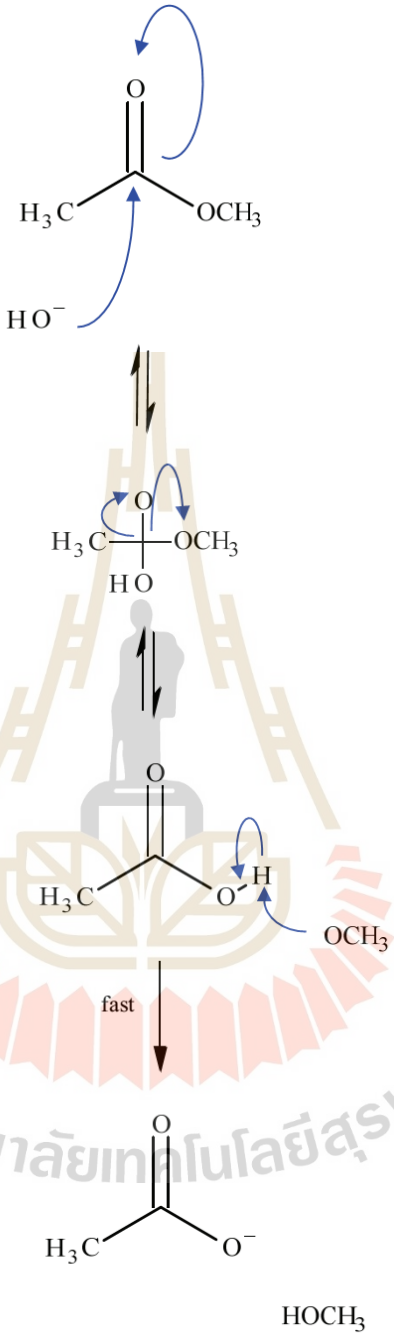


Figure 2.13 The mechanism of NaOH hydrolysis an ester bond. Arrows pointing from molecule components to other components or bonds represent the movements of electrons (Modenbach et al., 2014).

2.11.2 Effect of NaOH pretreatment on polysaccharide structure

During NaOH pretreatment of lignocellulosic materials, partial disruption of cellulose and hemicellulose may happen through the many reactions consisting of dissolution of non-degraded polysaccharides, peeling reaction, glycosidic bonds hydrolysis, acetyl function groups hydrolysis and re-locate of dissolved polysaccharides. Similarly, NaOH pretreatment altered the cellulose fibrils through mercerization, re-ratio crystalline and amorphous regions and depolymerization. Sodium-ion cation is the main parameter in cellulose destruction. It easily moves into the microfibrils of cellulose since it has a smaller size. After entering to internal surface areas of cellulose, sodium ions react to regenerate and mercerize in which significantly cellulose I expanded and recrystallized as cellulose II after water washing (Fig. 2.14) (Karimi et al., 2013). Moreover, the physical structure of lignocellulosic material depends mainly on the external surface area whereas the chemical structure (fiber porosity or capillary structure) is formed through the internal surface area. Naturally, dried raw bagasse is quite small in an internal surface area but yet large in an external surface area. Although the physical pretreatment (size reduction) helps to increase the external surface area, the chemical pretreatment (NaOH) simultaneously reveals both of these surface areas (Fan et al., 1982). Additionally, NaOH pretreatment of lignocellulosic biomasses results in loss less sugars and expels the acetyl group which strengthens the hydrolysis step to obtain higher yield within enzyme and prevents furfural formation (Pandey et al., 2000).

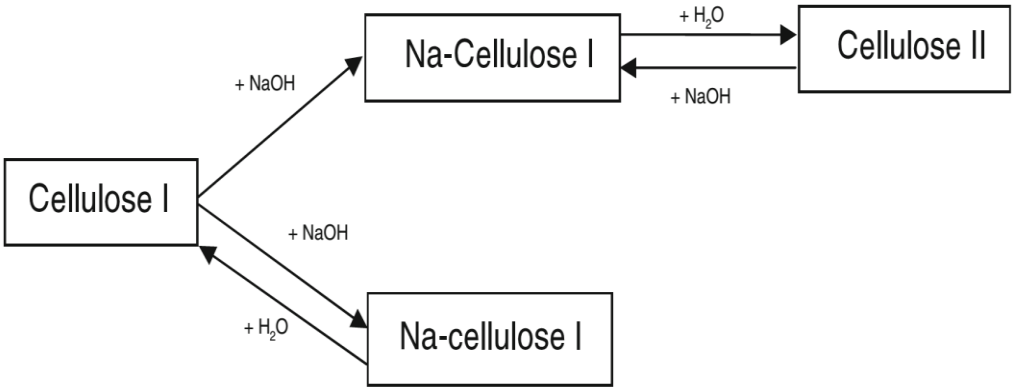


Figure 2.14 The mechanism of sodium ion converting cellulose I into cellulose II in NaOH pretreatment of lignocellulosic materials (Karimi et al., 2013).

2.12 Batch submerged fermentation

Generally, different systems and processes are applied in fermentation such as a batch, a fed-batch, or a continuous flowing process. The batch process is a kind of closed system allowing to add all materials at once to the system during the starting process, except the neutralizing agent for pH control. Then, products can be obtained when the process is completed. Of course, several phases are observed in batch culture including lag phase, acceleration phase, stationary phase and death phase (Fig.2.15). In meanwhile, some factors can affect the D-(-)-lactic acid production rate including pH, temperature, microorganisms, carbon source, nitrogen source and fermentation mode (Ameen et al., 2017; Hofvendahl et al., 2000).

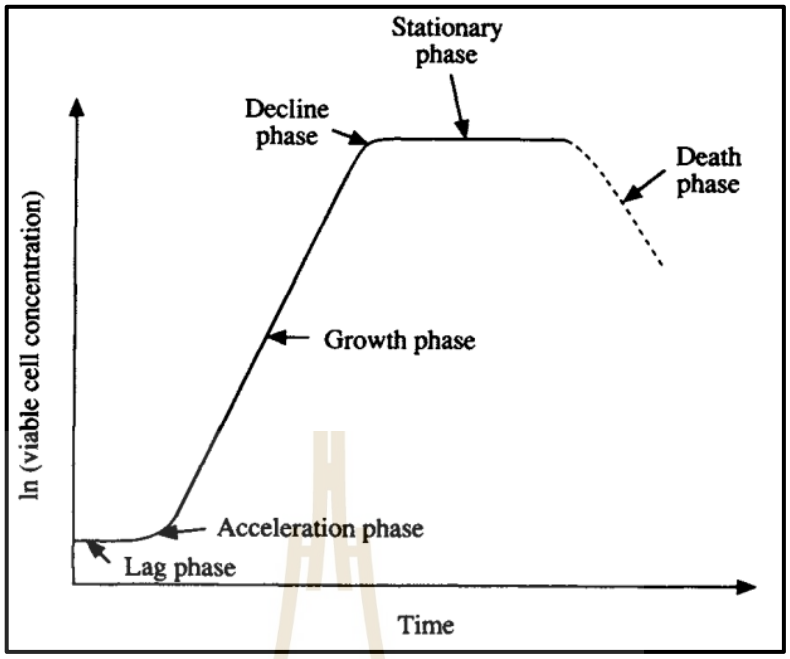


Figure 2.15 Typical growth curve of batch operation system (Doran, 2012).

The batch submerged fermentation is still commonly used in specific industrial processes as an example, brewery industries. The reasons that they still use this operation including easy-going sterilization and feedstock management. Especially, some microbial contaminations are not harmful to human or less property-altered fermented products are acceptable (Sangproo, 2012). Alongside, lactic acid production in batch and fed-batch cultures gain higher concentrations than those of continuous fermentation processes (Ameen et al., 2017; Wee et al., 2006). In somehow, low cell concentration due to the limited level of nutrients, long fermentation time, and low productivity caused by substrate or product inhibition are the drawbacks of the batch fermentation process (Abdel-Rahman et al., 2013; Rodrigues et al., 2017).

2.12.1 Effect of substrate concentration

Normally, microorganisms need carbon sources for cell growth and product formation (Shu, 2007). Most of the bacteria use glucose as carbon sources (Cheng et al., 2015). Glucose provides higher lactic acid concentrations and yields than

those of other sugars including xylose, galactose, arabinose, lactose, fructose and hydrolyzed cellulose (Hofvendahl et al., 2000). However, the growth of bacteria and fermentation decreases during a higher concentration of glucose applied making an imbalance in osmotic pressure (John et al., 2009). Additionally, it is not practically used pure glucose as the carbon source for cultivating microorganisms in large-scale lactic acid production due to the high cost of raw material input (Filho et al., 2018). Thus, industrial and agricultural wastes have been gained interest to be used as raw substrates to provide the sources of carbon including corn stover, cassava starch, cassava pulp, maltodextrin, rice straw, sugarcane molasse, and sugarcane bagasse (Abdel-Rahman et al., 2016; Chan, 2016; Sangproo, 2012; Sawisit et al., 2018; Sawisit et al., 2015a).

Most of the microorganisms cannot utilize polysaccharides directly. Their concentrations also make problems during fermentation. Higher substrate concentrations alter osmotic pressure that leads to a slower proliferation of microbial cells and also alters pH, viscosity and activity of the fermentation medium (Chan et al., 2016). It also prolongs the lag phase, builds up osmotic stress and reduces water activity (Abdel-Rahman et al., 2016). Together, most of the substrates can inhibit enzymatic activities of cells when the concentration is high because substrates bind to the active site of enzymes and block their subsequent activities (Khor, 2014). Fed-batch fermentation is one of the options that can deal with the substrate inhibition as more substrate and nutrients are added during the log phase of microorganisms (Abdel-Rahman et al., 2013). Alongside, the frequently applied initial sugar concentrations are normally in the range of 5–10% (w/v) since lower sugar concentrations lead to be fully utilized in the short fermentation time (Chan, 2016) while sugar concentrations over

150 g/L usually inhibit the lactic acid producers which affect to final concentrations of lactic acid (Abdel-Rahman et al., 2016).

2.12.2 Effect of initial optical density

Optical density (OD) is commonly used to quantify various important culture parameters like cell concentration, biomass production and other changes in cell morphology (Sandnes et al., 2006). High cell density offers high productivity and reduced fermentation time. Moreover, a number of cells are significant in fermentation processes to complete the substrate utilization and suitable for production yields (Agbogbo et al., 2007). Thus, increasing the inoculum size also helps to improve cell viability. It can control the fermentation rate and yield. However, high cell concentration results in the low specific productivity of D-(-)-lactic acid (Souza et al., 2007). Even though, the gain of the initial cell leads to accelerate fermentation rate and reduces the time for glucose utilization, the production yield does not significantly increase (Palmqvist et al., 1996) but drops in the specific cell growth (Khor, 2014). On the other hand, inoculum from 1.0 to 3.0 g/L could increase the lactic acid concentration while the higher inoculum still resulted in substrates remained (Shen et al., 2012). In addition, *Klebsiella oxytoca* KMS004 showed the highest productivity (0.79 g/L/h) when inoculum OD_{550} at 2.0 in AM1 containing 5 % (w/v) glucose while the lower inoculum resulted in the lower lactic acid productivity (Sangproo, 2012).

2.12.3 Enzymatic hydrolysis

Enzyme is a biological catalyst in the form of proteins involving in the chemical reaction of living organisms. Commonly, enzyme reactions often take place under mild conditions to obtain highly specific reaction rates thus the low concentration of enzymes is required even on an industrial scale. Enzymatic hydrolysis is another

important step of the biochemical conversion of lignocellulosic biomass to draw out the fermentable sugars. The purpose of enzymatic hydrolysis is to alter crystalline and depolymerize cellulose structure thus resulting in water-insoluble monosaccharides. The enzymatic hydrolysis rate is mainly affected by the degree polymerization of cellulose (Abdel-Rahman et al., 2011).

Cellulase is used to depolymerize cellulose without the problems associated with the lignocellulosic biomass. The use of cellulase enzyme demands significantly lower temperatures (30 to 60 °C). Therefore, the efficient activities of cellulase enzyme are the combined actions of cellulolytic enzymes as shown in Fig. 2.16 including 1) endo-β-1,4-glucanases that randomly hydrolyze β-1,4-glucosidic bonds of glucose chains and link water molecules to make a new reducing and non-reducing chain end pairs; 2) exo-β-1,4-glucanases or cellobiohydrolases which release soluble cellobioses and glucose subunits by cutting the end of polymer cellulose chains; and 3) β-glucosidases or cellobiases that complete the hydrolysis by cleaving cellobiose into two molecule glucose. (Abdel-Rahman et al., 2011; Wood, 2005). Likewise, the individual cellulases have a limited hydrolysis activity while the cellulase mixture, especially the synergistic cellulases provide higher fermentative sugar yields and less inhibitory by-products if the fermentative sugars are not degraded (Wood, 2005). Alongside, cellulase hydrolysis of lignocellulosic materials at high viscosity (solid loading ≥15% w/v) provides benefits due to high water conservation, lower energy requirement, high yield of fermentative sugars (Xu et al., 2019) which lead to high production of fermentation product (Unrean et al., 2016). However, the possibility of enzymatic saccharification may lower as the substrate increasing due to 1) low heat and mass transfer efficiency by poor mixing performance, 2) hydrolysis reaction-

restriction because of inadequate water content and 3) unfavored enzymatic hydrolysis condition as the spread of lignin and mono-saccharide molecules in the system (da Silva et al., 2016; Liu et al., 2017; Unrean et al., 2016).

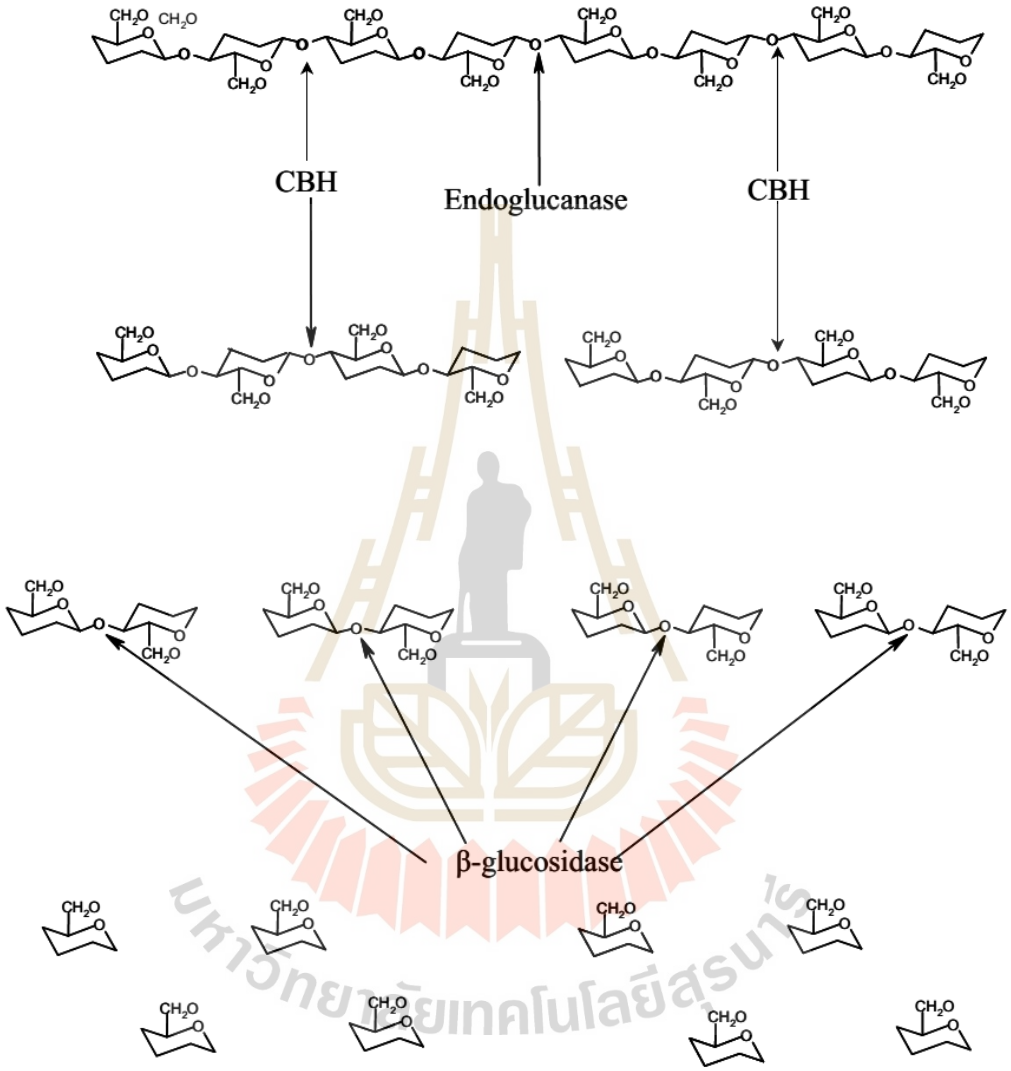


Figure 2.16 The mechanisms of cellulase activity involved the of actions cellulolytic enzymes: endoglucanase, cellobiohydrolase (CBH) and β-glucosidase (Wood, 2005).

2.13 Batch fermentation operational mode

The operational fermentation mode of bagasse with an enzyme can be conducted by either separate hydrolysis and fermentation (SHF) or simultaneous saccharification and fermentation (SSF) (Abdel-Rahman et al., 2013). In the SHF process, enzymatic hydrolysis and fermentation are performed separately so the optimal conditions can be carried out while the SSF process allows both activities happening in the same reactor under the same conditions (Rodrigues et al., 2017). The SHF process demands a smaller quantity of saccharifying enzymes than the SSF process due to the possibility of performing optimal temperature for enzymatic hydrolysis (Ishizaki et al., 2014). In some cases, the SHF process helps in reducing contaminants through sterilization yet it has a limited rate of hydrolysis by the generated sugar inhibits cellulase activity (Xue et al., 2019). Nevertheless, the SSF process offers various advantages over the SHF process including; the use of a single reactor vessel, rapid processing time, reduced end-product inhibition of hydrolysis and increased productivity (Abdel-Rahman et al., 2011). SSF method solves the inhibition of high sugar concentration in the medium as the released sugars by the enzyme from substrates are simultaneously used by microorganisms thus eliminating the chance of substrate inhibition. It also allows high substrate concentration in lower reactor volume but low fermentation cost. Together, it provides less risk of contamination since the contaminating organisms have inadequate time to grow and compete with the substrate utilization to be desired fermenting organisms (John et al., 2009). However, the low conversion rate of enzymatic hydrolysis or long-lag phase of microorganisms due to the differentiation of each optimal condition is the drawbacks of this operational mode (Ghosh et al., 1982; Teter et al., 2014; Wu et al., 1997).

CHAPTER III

MATERIALS AND METHODS

3.1 Microorganism, culture, and fermentation media

K. oxytoca KIS004-91T was previously constructed at Metabolic Engineering Research Unit, School of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology, Nakhon Ratchasima, Thailand. This strain was metabolically evolved in the medium containing 10% (w/v) glucose (In, 2019). The seed culture of *K. oxytoca* KIS004-91T was grown at 37°C, 200 rpm in LB broth containing 10 g of peptone, 5 g of yeast extract and 5 g of sodium chloride per 1 L of reverse osmosis water. The low salt AM1 medium prepared according to Martinez et al. (2007) was used as a fermentation medium (Table 3.1).

3.2 Sugarcane bagasse

3.2.1 Sugarcane bagasse preparation

Sugarcane bagasse was obtained from Suranaree University of Technology`s Farm and was dried by sunlight. The sun-dried SCB was cut into small pieces and ground into 5 mm to 2 cm in length by the grinder machine. The ground SCB was packed in plastic bags and stored in a dry place for further use.

3.2.2 Alkaline pretreatment of SCB

The alkaline pretreatment of SCB was done by a modified method of Wang et al (2019) as follows: The ground SCB was soaked in 3% (w/v) of NaOH solution

with the solid and liquid ratio of 1:9.1 for 4 h at 50 °C. Then, the NaOH pretreated SCB was washed with tap water until the pH of the drained water became neutral. The washed and NaOH pretreated SCB was dried in the oven at 95°C until the weight was constant before storing in sealed plastic bags in a dry place for further use.

Table 3.1 AM1 mineral low salts medium (excluding carbon source).

Component	**Concentration (mmol/L)**
$(NH_4)_2HPO_4$	19.92
$NH_4H_2PO_4$	7.56
Total PO_4	27.48
Total N	47.93
Total K	1.00
$MgSO_4$. $7H_2O$	1.50
Betaine-HCl [(a)]	1.00
Trace Element [(b)]	**(µmol/L)**
$FeCl_3$. $6H_2O$	8.88
$CoCl_2$. $6H_2O$	1.26
$CuCl_2$. $2H_2O$	0.88
$ZnCl_2$	2.20
Na_2MoO_4. 2H2O	1.24
H_2BO_3	1.21
$MnCl_2$. $4H_2O$	2.25
Total Salt	**4.1 g/L**

a: KOH was used to neutralize betaine-HCl stock

b: Trace metal stock (1000x) was prepared in 120 mM HCl

3.3 Enzyme

3.3.1 Cellulase complex enzyme

The commercial enzyme, cellulase complex which contains endoglucanase and β-glucosidase activity with a minor hemicellulase as xylanase activity was purchased from Siam Victory Chemicals Co., Ltd., Bangkok, Thailand. The activity of the enzyme was 8.81 U/mL.

3.3.2 Enzymatic hydrolysis

The enzymatic hydrolysis of the NaOH pretreated SCB was performed in 250 mL anaerobic shaking flasks at 50°C with 200 rpm shaking. The NaOH pretreated SCB was soaked in AM1 medium (AM1 buffer, pH 5.5) at a biomass loading of 10% (w/v on dry basis) (Ong et al., 2019). The NaOH pretreated SCB semi-liquid was subsequently sterilized at 121°C for 20 min. After the temperature reached below 50°C, the cellulase complex enzyme in the range of 5-30 U/g SCB and other AM1 medium components were added into the SCB slurry to evaluate the optimum enzyme loading. The enzymatic digestibility reactions were stopped by placing the slurry at 100°C for 15 min. All saccharification experiments were performed in triplicate.

3.4 Fermentation operational mode and conditions

3.4.1 D-(-)-lactic acid production by the mutant *K. oxytoca* KIS004-91T strain in batch separate hydrolysis and fermentation (SHF)

Batch SHF experiments were carried out in a 5 L bioreactor (New Brunswick™ BioFlowith® /CelliGen® 115 Benchtip Fermentor & Bioreactor, Eppendorf-Germany) with the working volume of 2.5 L. The NaOH pretreated SCB at 15% (w/v as dry basis) was autoclaved at 121°C for 20 min. AM1 medium and cellulase complex at the optimum enzyme loading (15 U/g SCB) were added into the semi-liquid

after sterilization and the enzymatic hydrolysis was allowed for 72 h for a complete hydrolysis. After that, the seed culture was inoculated with the initial OD_{550} of 0.3 (In, 2019). The SHF condition was performed at 50°C, 400 rpm during the enzyme hydrolysis and the temperature of the slurry was reduced to 37°C, 250 rpm with a pH controlled at 7.0 by 6.0 M KOH before inoculating the seed culture. All the batch SHF experiments were performed in triplicate.

3.4.2 D-(-)-lactic acid production by the mutant *K. oxytoca* KIS004-91T strain in batch simultaneous saccharification and fermentation (SSF)

The SSF experiments were performed in a 5 L bioreactor (New Brunswick™ BioFlowith® /CelliGen® 115 Benchtip Fermentor & Bioreactor, Eppendorf-Germany) with the working volume of 2.5 L. The NaOH pretreated SCB at 15% (w/v as dry basis) was autoclaved at 121°C for 20 min. AM1 medium and cellulase complex at the optimum enzyme loading (15 U/g SCB) were added into the slurry after sterilization. The seed culture with the initial OD_{550} of 0.3 was inoculated after the enzymatic hydrolysis for 4 h. The SSF condition was performed at 50°C, 400 rpm during the first 4 h (hydrolysis time) and the temperature was reduced to 37°C, 250 rpm with a pH controlled at 7.0 by 6.0 M KOH before inoculating the seed culture. The batch SSF experiments were performed in triplicate.

3.4.3 D-(-)-lactic acid production by the mutant *K. oxytoca* KIS004-91T strain in fed-batch SHF

The fed-batch SHF experiment was also performed in a 5 L stirred bioreactor (New Brunswick™ BioFlowith® /CelliGen® 115 Benchtip Fermentor & Bioreactor, Eppendorf-Germany). The bioreactor containing the NaOH pretreated SCB at the solid loading of 15% (w/v as dry basis) with an initial working volume of 2 L

was autoclaved at 121°C for 20 min. The AM1 medium components and enzyme loading were provided, and the hydrolysis condition was performed as indicated in the batch SHF condition except the hydrolysis time were reduced from 72 h to 24 h. The NaOH pretreated SCB slurry was intermittently fed to the bioreactor to yield a final concentration of the total solid loading of 25% (w/v, as dry basis) at incubation times from 12 and 20 h. The NaOH pretreated SCB slurry was prepared by the enzymatic hydrolysis (cellulase complex loading 15 U/g SCB) in AM1 medium at 50°C for 24 h to reduce the viscosity of the sample prior to intermittently feeding into the reactor. The fed-batch SHF experiments were performed in triplicate.

3.5 Analytical method

The broths during the enzymatic hydrolysis and fermentation were removed every 24 h and 4 h, respectively, for analyses of sugars, organic acids and cell growth. The sugars (glucose, xylose, arabinose) liberated from the NaOH pretreated SCB and organic compounds (lactic acid, 2,3-BDO, acetate) were measured using HPLC (Agilent Technology) equipped with an ion exchange column (Aminex® HPX-87H, 7.8×300 mm, BioRad) and refractive index detector (RI-150, Thermo Spectra System, USA) with a column temperature of 45°C using 4 mM H_2SO_4 as a mobile phase with a flow rate of 0.4 mL/min. Samples were previously centrifuged to separate solid particles. Then, the supernatant was further filtered through a 0.2 μm filter prior to injecting into HPLC. Also, cell growth was determined by the total plate count technique on LB agar plates which were incubated at 37°C for 24 h before counting the grown cells. The yield of D-(-)-lactic acid production from the NaOH pretreated SCB and its fermentative sugars and productivity were calculated by dividing D-(-)-lactic acid concentration obtained during processes with concentrations of the NaOH

pretreated SCB provided, total sugars consumed and total incubation time, respectively.

3.5.1 Proximal analysis of SCB

The proximal analysis of the NaOH pretreated SCB was performed by Kasetsart Agricultural and Agro-Industrial Product Improvement Institute (KAPI), Kasetsart University (KU). Browning acid chlorite method and Technical Association of the Pulp and Paper Industry (TAPPI) method were used to determine the chemical compositions in this NaOH pretreated SCB.

Browning acid chlorite method was used to determine the holocellulose by weighing 3 g of sample mixed with 160 mL of DI water containing 0.5 mL acetic acid and 1.5±0.1 mL NaOH. Then, this slurry was warmed up to 70-80°C for 1 h with shaking. In this temperature, 0.5 mL acetic acid and 1.5±0.1 mL NaOH were added every 1 h for 3 to 4 times before cooling the slurry down to 10°C and filtrated the solid fraction. The solid fraction was brought to an oven dry (100°C) until constant weight. The holocellulose was calculated as the following equation: % holocellulose = (dry weight of holocellulose × 100)/ weight of pretreated SCB.

TAPPI T203 om-88 was used to determine the α-cellulose in holocellulose. The 1.5 g holocellulose was mixed by shaking with 75 mL NaOH (17.5%) at 2.5°C for a half hour. Then, the solution was adjusted with DI to reach a volume of 100 mL and the reaction was kept for 30 min. After that, the solution was filtrated to recover the solid fraction and the fraction was washed with DI water and 10% acetic acid until pH became neutral before oven drying (105°C) to constant weight.

The α-cellulose was calculated by % α-cellulose = (weight of α-cellulose) × 100/ (weight of holocellulose used) while hemicellulose (β and γ-cellulose) was

calculated by minus the weight of holocellulose used with the weight of α-cellulose found.

TAPPI T222 om-88 was used to find the lignin residue in the NaOH pretreated SCB by weighing the sample of 1.0±0.1 g (without organic compound) and then it was mixed with 15 mL H_2SO_4 (72%) for 15 min. This covered slurry was warmed at 20±1°C for 2 h with shaking. Then, it was adjusted with DI water to reach a volume of 575 mL, refluxed, and kept overnight. After that, the solution was filtrated to recover the solid fraction and washed with hot DI water and over dried (105°C) for 6 h before putting it in desiccator and weight. The lignin content was calculated by % lignin = (weight of dry lignin) ×100/ (weight of the sample used).

3.5.2 Acid digestion of the pretreated SCB

The complete saccharification of the NaOH pretreated SCB by strong acid digestion was adapted from Dunning et al. (1949) method. The NaOH pretreated SCB was weighed 2.0 g, mixed with 10 mL of warm 72% H_2SO_4 (55°C) and agitated at this temperature for 5 min. Then, the slurry was adjusted with DI water to reach the volume of 200 mL (about 4% of H_2SO_4 concentration) before autoclave at 121°C for 25 min. After that, the sample was centrifuged and prepared for HPLC analysis for determined the liberated sugars content.

3.5.3 Calculation method

The cellulose (CS) and hemicellulose saccharification (HS) yields of NaOH pretreated SCB was calculated as the following equations according to Ma et al. (2009).

$$\%CS = \frac{0.9 \times grams\ of\ glucose\ released}{grams\ of\ cellulose\ in\ pretreated\ SCB\ added} \times 100$$

$$\%HS = \frac{grams\ of\ xylose\ and\ arabinose\ released}{grams\ of\ hemicellulose\ in\ pretreated\ SCB\ added} \times 100$$

3.6 Statistical analysis

Analysis of variance (ANOVA) was conducted using SPSS software (IBM® SPSS® Statistics V26.0.0-2019, IBM Corporation). At least triplicate determinations were performed on each test and averages with standard deviation were reported. The differences among mean values were established using Duncan`s multiple range test (DMRT) at 99% significance level ($P < 0.01$).

CHAPTER IV

RESULTS AND DISCUSSION

4.1 The significant changed of NaOH pretreated SCB

Sugarcane is an herbaceous plant thus the structure of raw SCB is built up from cellulose, hemicellulose and lignin residues. Hemicellulose is a bridge connection between cellulose and lignin to make a complex matrix form of SCB`s cell wall. Moreover, hemicellulose and lignin are mainly joined by an LCC complex linkage composed of ester bonds that are quite sensitive to the alkaline pretreatment method. Additionally, hemicellulose and lignin represent over 50% (as dry weight) in raw SCB. Therefore, after the diluted NaOH solution was applied to raw SCB, 63.5±3.9 g of SCB were lost from a total of 100 g of raw SCB used which was 34.5% higher than that of non-NaOH pretreated SCB (Fig. 4.1). The low recovery of the non-NaOH pretreated SCB may be caused by the hot water that acts as a weak acid catalyst to disrupt SCB's matrix cell wall especially at LCC complex bonds during pretreatment (Li et al., 2017; Wyman, 2013). Also, the lower recovery of the NaOH pretreated SCB biomass was considered as the successful destruction of raw SCB structure by the NaOH pretreatment. Irfan at al. (2011) have suggested that the reduction of raw SCB weight is mainly due to the disruption of lignin and partially dissolved of hemicellulose and cellulose into the NaOH solution. Guilherme et al. (2015) also revealed that the lower solid fraction of lignocellulosic materials in the alkaline pretreatment is due to the high conversion of hemicellulose. Based on other previous studies, the more of NaOH

concentration use, the longer incubation time provided and higher temperature applied in the pretreatment had led to even a lower solid recovery of the NaOH pretreated biomasses as the higher delignification was achieved (Silverstein et al., 2007).

Table 4.1 Chemical compositions based on the proximal analysis of SCB after pretreated with 3% NaOH at 50°C, 200 rpm for 4 h.

Chemical components	Values (% w/w, as dry basis)	Compositions in 100 g untreated SCB used[(a)] (g)
α-Cellulose	54.49±0.53	19.9±0.5
Hemicellulose (β- and γ-cellulose)	26.29±0.58	9.6±0.6
Lignin	10.88±0.13	4.0±0.1
Ash	1.38±0.08	0.5±0.1
Extractives	2.20±0.06	0.9±0.1
- Alcohol-benzene solution	0.54±0.04	0.2
- Alcohol solubility	0.13±0.01	0.1
- Hot water solubility	1.54±0.08	0.6

a: These values was calculated based on 100 g of untreated SCB after pretreated with 3% NaOH in which the obtained solid fraction was about 36.5±4.7 g

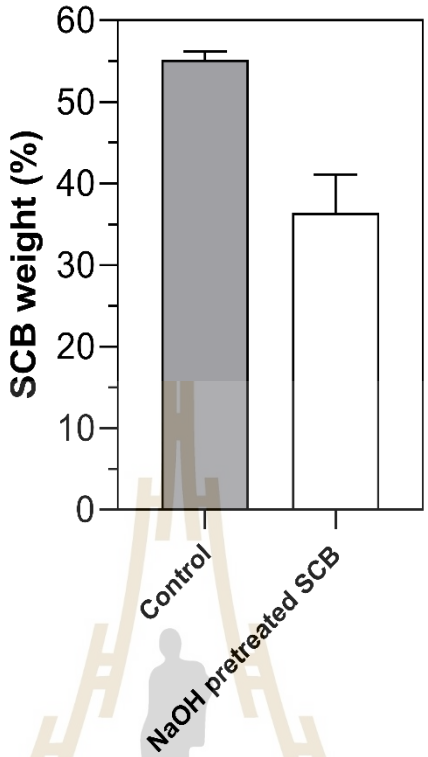


Figure 4.1 Diagram of solid fraction recovery of 3% (w/v) NaOH pretreated and non-NaOH pretreated (replaced by RO water) SCB in the conditioning liquid and solid ratio of 1:9.1 for 4 h at 50°C.

Based on 100 g of untreated SCB provided, the solid fraction of the NaOH pretreated SCB approximately 36.5±4.7 g (Fig. 4.1) was recovered in which it contained cellulose, hemicellulose and lignin about 19.9±0.5, 9.6±0.6 and 4.0±0.1 g respectively (Table 4.1) while the fermentative sugars including glucose and xylose less than 7.0±0.4 g/L were detected in the yellowish liquid fraction of the NaOH pretreated SCB. The yellow color in the liquid fraction of the NaOH pretreated SCB was caused by lignin disruption (Wang et al., 2016). Glucose found in the liquid fraction may be the result of swelling and amorphous region reduction of cellulose by cation sodium ion reacted to microfibril of cellulose in the form of

$(C_6H_{10}O_5)(NaOH)(H_2O)$ (Nosratpour et al., 2018). Table 4.2 shows the significant improvement of released fermentative sugars especially glucose after cellulose digestion from the NaOH pretreated SCB compared to those of the non-NaOH pretreated SCB. From 100 g/L of SCB loading, 49 g/L of glucose was about generated from the NaOH pretreated SCB, which was six folds higher than that of the non-NaOH pretreated SCB after the cellulase complex of 15 U/g SCB was applied. The high release of fermentable sugars from pretreated SCB mainly due to the efficient lignin removal by NaOH which were in agreement with other previous research groups (Prajapati et al., 2020; Wang et al., 2020; Xu et al., 2019b). Jin et al. (2020) used 0.7% of NaOH (70 °C, 3 h) to remove 12.46% of lignin from raw SCB. Also, Irfan et al. (2014) and Liu et al. (2015) reported that up to 77 to 80% of lignin were removed from SCB after pretreatment with 2% NaOH for 2 h at room temperature. Similarly, Wang et al. (2016) revealed that the lignin dissolving capacity of the NaOH black liquor (recycle solution of NaOH pretreatment) was lower in the lignin content in the NaOH pretreated than the non-NaOH pretreated SCB.

Therefore, lignin disruption of raw SCB is the main reason for improving fermentative sugars yield from the NaOH pretreated SCB. During NaOH pretreatment, the cross ester and glycosidic bonds are broken down leading to the destruction of the LCC complex thus increasing the cellulose attaching surface area due to the removal of lignin, ash, and partial hemicellulose (Canilha et al., 2012; Fan et al., 1982; Silverstein et al., 2007). It is a critical step for the cellulase complex effectively to adsorb to the cellulose residue and to provide high saccharification yield (Irfan et al., 2014). Hence, the un-productive bindings in which enzymes bind to lignin or xylooligomers are prevented (Modenbach et al., 2014). The peeling off and hole

formation reactions by NaOH pretreatment destructed raw SCB structure and make more effectiveness of the enzymatic adsorption to the NaOH pretreated SCB (Rezende et al., 2011). Furthermore, cellulose crystalline alteration is another important factor of differentiating yield of fermentative sugars between the NaOH pretreated and the non-NaOH pretreated SCB. Karimi et al. (2013) reported that the aligned mode of molecule in cellulose I was converted into a nonparallel style (cellulose II) which was liable to bioconversion after NaOH pretreatment of lignocellulosic materials. Together, the high degree of polymerization (DP) cellulose, as long chain of glucan and numerous hydrogen linkages (barrier of enzymatic hydrolysis), was reduced once the NaOH pretreatment was introduced. Diluted NaOH pretreatment of SCB helped to increase the internal surface area of cellulose thus decreasing the polymerization and crystalline degree (Sun et al., 2002). Especially, the use of 3% NaOH effectively modified the microstructure of amorphous and crystalline regions of cellulose (Castañón-Rodríguez et al., 2015; Hemansi et al., 2020). Zhang et al. (2018) revealed that the NaOH pretreatment provided higher sugars production and enzymatic digestibility compared to LHW (liquid hot water) and HCl pretreatments and resulted to high glucose and xylose production due to the high lignin removal and partial retaining xylan in the NaOH pretreated SCB.

Table 4.2 Comparison of liberated sugars after an enzymatic digestion by 15 U cellulase/g NaOH pretreated SCB at 50°C, 200 rpm.

Bagasse	Glucose concentration (g/L)			TS concentration (g/L)			CS (%)[a]	HS (%)[a]
	24 h	48 h	72 h	24 h	48 h	72 h	72 h	
Control	5.8±0.6	6.5±0.4	8.2±0.5	8.4±1.0	9.6±0.4	12.3±0.6	13.6±0.8	15.3±0.8
NaOH	36.1±0.9	45.1±0.5	48.7±0.8	47.2±1.1	59.9±1.4	65.7±0.7	80.4±1.2	64.9±0.6

TS: Total fermentative sugars; CS: Cellulose saccharification; HS: Hemicellulose saccharification; Control: the pretreated SCB by RO water; NaOH: the pretreated SCB by NaOH; ±: standard derivation
a: Cellulose and hemicellulose saccharification yield was calculated based on the concentration of liberated sugars at 72 h

4.2 Optimal cellulase complex loading for NaOH pretreated SCB

In enzymatic hydrolysis of lignocellulosic materials, at least two steps are required to obtain monosaccharide sugars. Cellulase needs to attack cellulose surface areas during the first step to depolymerize and hydrolyze cellulose into soluble glucose (Xiao et al., 2004). Thus, the enlarge cellulose surface areas by pretreatment significantly improve hydrolysis yield. Teter et al. (2014) reported that the pretreatment of raw bagasse resulted in a fast conversion rate of enzymatic hydrolysis and rose of fermentative sugars yield yet depended on enzymatic dosages. In Fig. 4.2, soluble glucose and total fermentative sugars were remarkably improved once the cellulase complex increased from 5 to 30 U/g NaOH pretreated SCB. In 100 g/L of the NaOH pretreated SCB used, glucose and total sugars were enhanced from 41.8±1.9 and 52.2±2.5 g/L (5 U/g SCB) to 49.7±1.1 and 72.8±1.9 g/L (30 U/g SCB), respectively after 72 h of hydrolysis. After digestion of 100 g/L of NaOH pretreated SCB, glucose and xylose about 59.1±0.1 and 27.6±0.1 g/L, respectively, were accumulated from 54.49±0.53 g/L of cellulose and 26.29±0.58 g/L of hemicellulose (Table 4.1 and Table 4.3). The cellulose and hemicellulose saccharification yield of the NaOH pretreated SCB digested with 30 U/g SCB were about 82.0% and 87.9%, respectively. However, the saccharification yield obtained by the enzymatic saccharification illustrated slightly lower cellulose and hemicellulose saccharification yields obtained by strong acid digestion (Table 4.3). This may be due to that a minor lignin residue (approximately 10.88% as dry basis) secured some parts of cellulose residing in the pretreated SCB thus preventing the cellulase enzyme to attach surface areas.

Furthermore, the released glucose concentrations (Fig. 4.2) after digestion with 15 to 30 U/g SCB of cellulase complex loading were not statistically different (α =

0.01). Sawisit et al. (2018) found that monosaccharides were released when higher cellulase complex loadings were introduced. However, the more addition of enzyme loadings did not further improve saccharification since the excess of enzyme adsorbed into the bagasse's surface restricted the diffusion process through the cellulose structure. Likewise, enzymatic activities slowed down once the higher concentration of glucose and other soluble sugars were achieved (Pandey et al., 2000). This led to a reduction of glucose yields (da Silva et al., 2016). The higher glucose obtained brought the higher inhibition to β-glucosidase in the cellulase complex and led to increasing cellobiose residues thus inactivation of cellobiohydrolases (Xiao et al., 2004). Yu et al. (2013) also found that 15 U/g of dry solid strengthened the digestibility of the NaOH pretreated SCB up to 99%. However, applying 30 U/g glucan did not show further variations in the conversion rate of glucan compared to those of lower enzyme loadings (Gao et al., 2013).

In addition, the xylose gradually increased along with the higher cellulase complex introduced. This caused by the xylan (hemicellulose) residues in the NaOH pretreated SCB. The xylanase activity in the cellulase complex may not yet inadequate to completely convert xylan to xylose so xylanase may be considered to improve the saccharification of xylose from the hemicellulose part in the NaOH pretreated SCB. Regarding to Table 4.3, higher xylanase activity may be required to make the efficient transformation of hemicellulose into xylose for its microbial consumption. Since the xylose released by 30 U/g NaOH pretreated SCB of cellulase complex represented only 73.7% of the total xylose released or 77.4% of hemicellulose saccharification yield. Based on the glucose utilization behavior of strain and glucose conversion yield, the cellulase complex at the range of 15 U/g NaOH pretreated SCB was economically

selected to saccharify fermentative sugars for D-(-)-lactic acid production in the following experiments.

Table 4.3 Yields of CS and HS and remained fermentative sugars in various cellulase complex digestions 100 g/L of the NaOH pretreated SCB.

Cellulase complex (U/g SCB)	CS (%)[a]	HS (%)[a]	Remained sugars in the pretreated SCB (g/L)[b]			
			Glucose	Xylose	Arabinose	Total
5	69.0	39.4	17.3±1.8	19.4±0.6	3.3±0.1	40.0±2.3
10	72.8	48.0	15.0±0.4	17.6±0.9	2.9±0.1	35.5±1.0
15	80.4	61.5	10.4±0.6	14.1±1.1	3.0±0.1	27.6±1.0
20	80.4	71.4	10.4±1.6	11.4±0.1	3.0±0.2	24.7±1.7
25	80.7	78.4	10.2±0.4	9.6±0.5	2.9±0.2	22.8±1.0
30	82.0	87.9	9.4±1.0	7.3±0.7	2.7±0.1	19.4±1.7
Conc. H_2SO_4	97.6	125.9	59.1±0.1[c]	27.6±0.1[c]	5.5±0.1[c]	92.2±0.2[c]

a: Cellulose and hemicellulose saccharification yields were calculated based on concentrations of liberated sugars in each enzymatic treatment as listed in Table 1A appendix A
b: The remained fermentative sugars was determined by minus optimal digested sugars obtained in strong acid saccharification with liberated sugars in each enzyme loading
c: The concentration of liberated sugars from 100 g/L of NaOH pretreated SCB that completely digested by concentrated H_2SO_4 (72%)

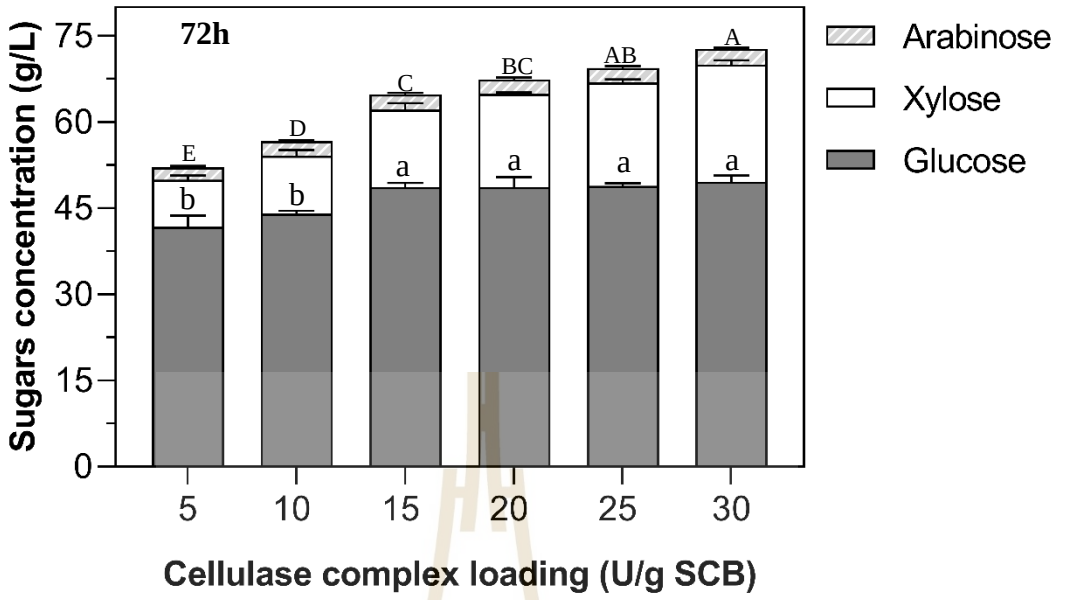


Figure 4.2 Effect of cellulase complex loading on enzymatic hydrolysis of the NaOH pretreated SCB at 50 °C, 200 rpm for 72h. Bars with different capital letters show significant differences between mean values the total sugar concentration ($p < 0.01$) and bars with different lower-case letters show significant differences between mean values the glucose ($p < 0.01$).

4.3 D-(-)-lactic acid production from the NaOH pretreated SCB using separate hydrolysis and fermentation (SHF) process

This experiment aimed to investigate the optimal conditions and efficient fermentation systems for D-(-)-lactic acid production by *K. oxytoca* KIS004-91T from the high solid loading of the NaOH pretreated SCB. As shown in Fig. 4.3 and Table 4.4, glucose, xylose and arabinose at concentrations of 54.2±2.9, 15.1±0.9 and 2.8±0.2 g/L, respectively, were obtained after the enzymatic hydrolysis of 150 g/L of pretreated SCB for 72 h incubation. After that, the seed culture was inoculated into the liquefaction medium. Glucose was completely consumed within 16 h whereas levels of arabinose and xylose were remained at the end of fermentation. Likewise, cell

viability of *K. oxytoca* KIS004-91T reached the highest level at $3.9\pm0.1\times10^9$ CFU/mL within 20 h while D-(-)-lactic acid of 53.5±2.9 g/L with yields of 0.95 g/g sugars consumed and 0.36 g/g pretreated SCB provided. The overall productivity of 1.03±0.06 g/L/h with minor concentrations of 2,3-BDO and acetate was obtained after 52 h of incubation.

Ouyang et al. (2013) and de Olivera et al. (2019) performed the batch SHF processes using *Bacillus* sp. NL01 and *Bacillus coagulans* to convert SCB hemicellulosic hydrolysates for D-(-)-lactic acid with yields of 0.65 g/g and 0.87 g/g sugars consumed, respectively. Comparing with our study, the higher yield obtained indicated that *K. oxytoca* KIS004-91T efficiently utilized liberated sugars from the NaOH pretreated SCB. This was likely that the growth and D-(-)-lactic acid production by the strain were not affected by any inhibitory compounds including acetic acid, hydroxyl acids and furan aldehydes generated during the NaOH pretreatment process of SCB (Niju et al., 2019) due to their completely removal after washing step. Additionally, a high level of substrate concentration did not likely cause an impaired growth of *K. oxytoca* KIS004-91T. Even though the higher solid loading of 15% (w/v, as dry basis) pretreated SCB was provided to accumulate the liberated sugar level up to 80 g/L, an imbalance of osmotic pressure for *K. oxytoca* KIS004-91T as well as fermentation problems including the mixing efficiency were not observed.

Normally, *K. oxytoca* utilizes a wide range of substrates in which its negative CCR (carbon catabolite repression) allows co-utilizing pentose sugars with glucose (Teter et al., 2014). Sangproo et al. (2012) observed that the efficient production of D-(-)-lactic acid from sucrose and fructose by *K. oxytoca* KMS004 (the parental of *K. oxytoca* KIS004-91T stain) was achieved. In addition, *K. oxytoca* KIS004-91T

provided D-(-)-lactic acid production at the level of 66.5±0.4 g/L when 100 g/L of xylose were only provided (data not shown). However, xylose and arabinose less than 2 g/L in total (from approximately 20 g/L) were consumed by *K. oxytoca* KIS004-91T. No simultaneous or gradual consumption of these sugars was observed even the glucose level was depleted or exhausted. It was likely that CCR still governed the utilization of other fermentable sugars rather than glucose in *K. oxytoca* KIS004-91T. The catabolite repressor/activator protein encoded by *cra* gene that dominantly regulates carbon flux in the metabolic network may play significant roles beyond the phosphotransferase systems (PTSs) and cAMP-Crp mechanisms. Ramseier (1996) reported that Cra of bacteria repressed target genes responsible for glycolytic and Entner-Doudoroff pathways which are contrasted with genes in Krebs cycle, glyoxylate shunt and gluconeogenic pathways during inadequate the concentration of fructose-1-phosphate (F1P) or fructose-1,6-bisphosphate (FBP). Further, the mutant *cra* in *E. coli* (Δ*cra*) resulted in faster consumption rates of carbon sources through glycolysis and/or the pentose phosphate pathway(s). Similarly, the *cra* gene knocked out in *Klebsiella* related species allowed the co-utilization of fructose and glucose as well as completely xylose consumption after the hexose sugars depletion in the mixture carbon sources of glucose, fructose and xylose under aerobic conditions. However, the xylose utilization of the mutant strain was not improved in anaerobic conditions (Yao et al., 2013). Although *cra* deletion changed the transcriptional mechanisms in bacteria, the central carbon metabolism activity was not much altering due to the co-operation of Cra and other pleiotropic regulators (Son et al., 2011).

Teter et al. (2014) found that the batch fermentation of mixed sugars medium requires a long time due to a diauxic growth or a prolonged reaction to preferred sugar

(glucose) exhaustion before metabolically switching to utilize other sugars including xylose or arabinose. Additionally, the pentose sugars utilization by microorganisms is sometimes suppressed by a monomer of cellulose or microbial metabolites especially when pentoses are fermented as sole or mixed carbohydrates. Kim et al. (2010) also revealed that a small amount of glucose residue in fermentation media resulted in non-fermented xylose. Moreover, the transport of xylose into the cell usually requires two moles of ATP by the action of the D-xylose ABC transporter in *Klebsiella* and its related species (Khunnonkwao et al., 2018). Our results thus suggested that the ATP supply was not enough for D-xylose transportation via the D-xylose ABC transporter and cell maintenance during mixed sugars fermentation. This was supported by the fact that the cell viability rapidly dropped after glucose was exhausted (Fig. 4.3B) and resulted in no consumption of xylose and even arabinose.

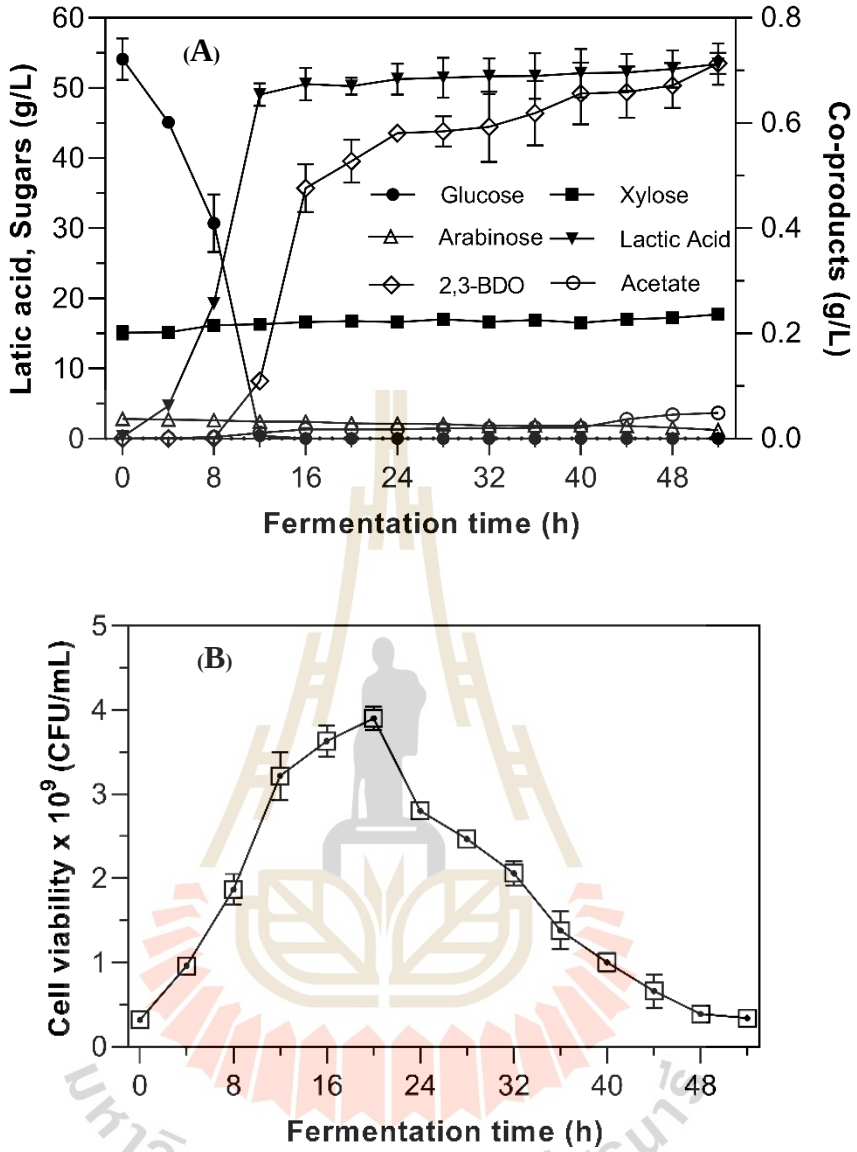


Figure 4.3 D-(-)-lactic acid production from the NaOH pretreated SCB 15% (w/v) as dry matter by *K. oxytoca* KIS004-91T using the SHF process in 5 L bioreactor with 2.5 L working volume at 37°C. (A) Fermentative products and sugars (B) Cell viability.

4.4 D-(-)-lactic acid production in simultaneous saccharification and fermentation (SSF) process

A coupling enzymatic hydrolysis with fermentation can reduce the batch operation time as well as the byproduct formation. However, an adjustment of optimal conditions for this combined step is challenging. Simultaneous saccharification and fermentation (SSF) is performed by fermentation combined with enzymatic hydrolysis to replace the individual step with the aim of reducing operating costs. Therefore, a batch SSF experiment was investigated in 5 L fermentor, with the initial concentration of 150 g/L of the NaOH pretreated SCB. The cellulase enzyme was added together with seed culture at either 15U or 25U per gram of NaOH pretreated SCB. In both conditions, glucose and arabinose were completely consumed after 12 h incubation. In contrast, xylose was gradually accumulated but was not consumed by *K. oxytoca* KIS004-91T throughout fermentation. D-(-)-lactic acid was rapidly produced within 16 h incubation but gradually accumulated until the end of fermentation (Fig. 4.4A and B). The maximum cell viability was observed at 24 h in both conditions (Fig. 4.4C). For 15U cellulase, D-(-)-lactic acid at the level of 47.2±2.5 g/L was produced with a yield of 0.79 ±0.01 g/g sugars consumed and the overall productivity of 0.91±0.05 g/L/h (Fig. 4.4A) while the increasing cellulase loading to 20 U resulted in D-(-)-lactic acid production at the concentration and yield of 48.6±1.6 g/L and 0.80 ±0.01 g/g sugars consumed, respectively. However, the productivity of D-(-)-lactic acid was no significant improved even though the cellulase loading was increased up to 25 U.

Comparing with the SHF process, yield of D-(-)-lactic acid obtained by the SSF process in both conditions was lower about 11.77 % (15U cellulase) and 9.16 % (25U cellulase) (Table 4.4). Similarly, the overall productivity of D-(-)-lactic acid in SSF

process was lower than that of SHF process about 11.65 % (15U cellulase) and 9.71 % (25U cellulase). However, if the total operation time for SHF process was considered, the overall productivity for D-(-)-lactic acid production by SHF process operated for 124 h in total (72 h for the enzymatic hydrolysis and 52 h for fermentation) should be reconsidered. The overall productivity of D-(-)-lactic acid production by SHF process was therefore calculated only at 0.43 g/L/h. In comparison, the overall productivity of D-(-)-lactic acid production by SSF process was yet superior to that of SHF process about 53 % if the total time required for the whole process was reconsidered.

The lower efficiency of D-(-)-lactic acid production during the SSF process by *K. oxytoca* KIS004-91T might be due to that an inadequate level of fermentative sugars, which were slowly released from the pretreated SCB, could not support the fast growth and enhanced D-(-)-lactic acid production. Hama et al. (2015) suggested that the low sugar conversion rate of hardwood pulp by the enzymatic digestion during SSF limited and affected the D-(-)-lactic acid production. Kim et al. (2010) also revealed that the high production of metabolites during the SSF process could be achieved once the interface conditions of a limited and rich substrate were maintained at a suitable level. Additionally, the optimal temperature for the enzymatic hydrolysis by cellulase complex at 50°C was not provided in our condition since the operating temperature during the SSF process was controlled to suit the optimal growth of *K. oxytoca* KIS004-91T at 37°C. Hence, the effectiveness of the cellulose hydrolysis of the NaOH pretreated SCB was reduced.

Furthermore, the NaOH pretreated SCB of 150 g/L in 2.5 L working volume was high enough to increase a medium viscosity thus causing a problematic of mixing capability during the SSF process (Sawisit et al., 2018), to raise an osmotic stress, and

to lessen capacities of mass and heat transfers (da Silva et al., 2020). These conditions hurdle the chemical distribution and block an enzyme binding surface during the SSF process thus yielding the low conversion rate of cellulose to fermentable sugars especially when a conventional stirred tank bioreactor was used (Kristensen et al., 2009). Koppram et al. (2014) also found that the low transforming rate of biomass to fermentable sugars was the major obstacle in an enzymatic digestion of the high solid loading of cellulose to bioproducts. In addition, Unrean et al. (2016) suggested that the effectiveness of SSF process was fallen when the high viscosity from a high concentration of the pretreated SCB slurry was presented during ethanol production. This happened as an inadequate available water for reaction and enzyme degradation. Abdel-rahman et al. (2011) also demonstrated that nutrients as well as concentrations of substrate and metabolites mainly affected the growth of microbes and lactic acid production, therefore, led to lower cell density, titer, yield, and productivity. In summary, the SHF process could be performed using a simpler and easier operation and offered yet more effective than those of SSF mode to produce D-(-)-lactic acid from the high solid loading of the NaOH pretreated SCB by *K. oxytoca* KIS004-91T. So, the SHF process was selected to further perform in a fed-batch system to validate the efficient production of D-(-)-lactic acid at a high concentration of the NaOH pretreated SCB.

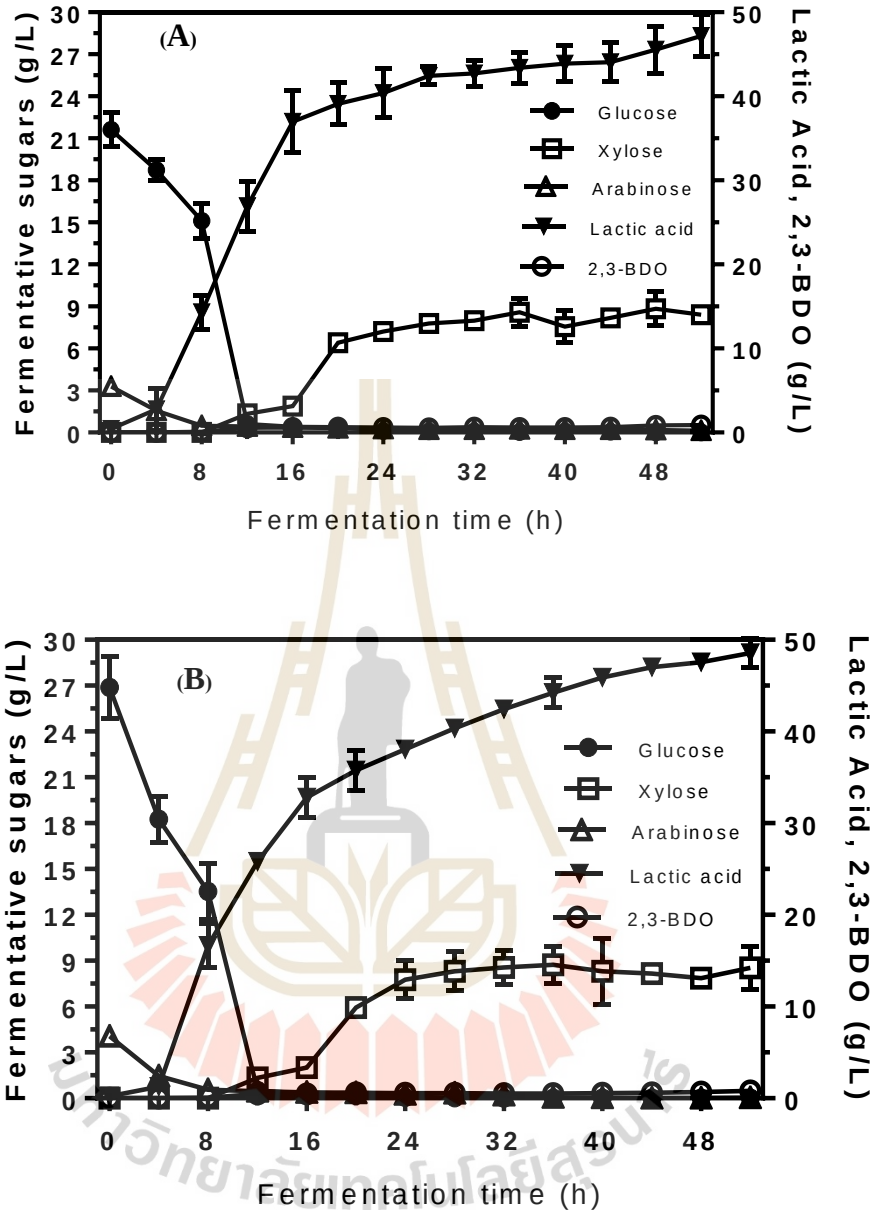


Figure 4.4 Fermentation of the NaOH pretreated SCB of 15% (w/v) as dry matter by *K. oxytoca* KIS004-91T to produce D-(-)-lactic acid in SSF process in 5 L bioreactor with 2.5 L working volume at 37°C. (A) Cellulase at 15 U/g NaOH pretreated SCB (B) Cellulase at 25 U/g NaOH pretreated SCB (C) Cell viability.

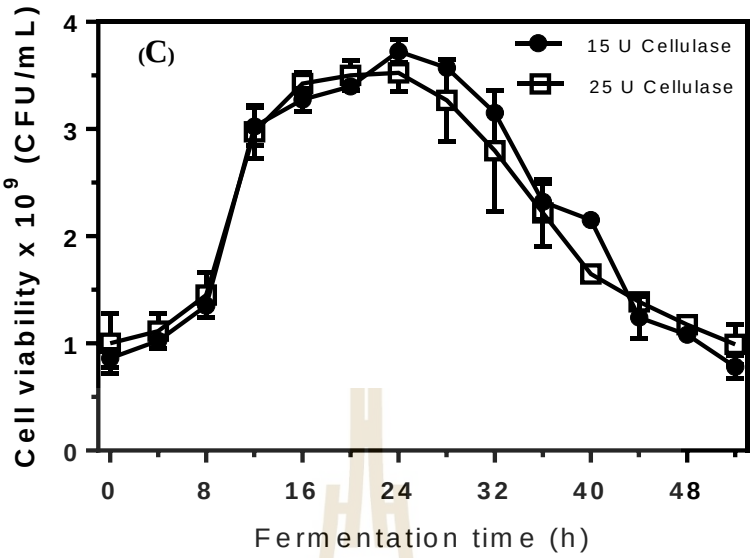


Figure 4.4 (continued).

Table 4.4 Fermentation profile of D-(-)-lactic acid production from the NaOH pretreated SCB by *K. oxytoca* KIS004-91T using SHF, SSF and fed-batch SHF processes.

Process systems	D-(-)-lactic acid				Cell viability (CFU/mL)[c]	Co-products	
	Concentration (g/L)	Yield (g/g)[a]	Yield (g/g)[b]	Productivity (g/L/h)		2,3-BDO (g/L)	Acetate (g/L)
SHF	53.5±2.9	0.95	0.36	1.03±0.06	3.90±0.14	0.71±0.02	0.05
SSF (15U cellulase)	47.2±2.5	0.79	0.32	0.91±0.05	3.73±0.11	0.54 ±0.03	nd
SSF (25U cellulase)	48.6±1.6	0.80	0.32	0.93±0.01	3.53±0.17	0.45 ±0.04	nd
F-SHF	101.0±0.9	0.99	0.40	1.94±0.02	4.45±0.07	0.93±0.01	nd

SHF: Separate Hydrolysis and Fermentation
SSF: Simultaneously Saccharification and Fermentation
F-SHF: Fed-batch Separate Hydrolysis and Fermentation
a: yield of D-(-)-lactic acid from fermentative sugars consumed (sugars consumed in SSF and F-SHF processes were calculated by the theoretical yield of cellulose conversion (0.40) × mass of pretreated SCB provided)
b: yield of D-(-)-lactic acid from the NaOH pretreated SCB provided
c: maximum cell viability in each process system ($\times 10^9$ CFU/mL)
nd: not detected
±: standard deviation

4.5 Efficient production of D- (-) - lactic in fed- batch separate hydrolysis fermentation mode

Fed-batch strategies were previously used to cope with the mixing difficulties causing by the high viscosity of fermentation media in the enzymatic hydrolysis step as well as substrate inhibition. Starting from a low concentration of substrate, the substrate is subsequently or intermittently fed into the reactor vessel to obtain the desired substrate or product concentrations (da Silva et al., 2020). It is an efficient strategy to reduce problems regarding to the high solid loading in simple batch fermentation mode (Gao et al., 2014). Therefore, the fed-batch SHF operation was performed. The digested NaOH pretreated SCB slurries at the solid loading of 5% w/v (as dry basis) was added during 12 and 20 h of fermentation to yield approximately 130 g/L of total sugars or 100 g/L of glucose. As a result, released glucose was rapidly consumed in the first feeding and its consumption was moderately declined in the second feeding. D-(-)-lactic acid significantly accumulated in every addition of the pretreated SCB. Moreover, the cell viability has reached a maximum within 20 h and dramatically decreased after 28 h. However, a rapidly increased xylose after the second feeding indicated the accumulation of xylan within the slurry. Thus, xylose in the concentration of 29.9±0.7 g/L remained non-fermented throughout fermentation (Fig 4.5). D-(-)-lactic acid production of 101.0±0.9 g/L with the yield of 0.99 g/g sugars consumed and the overall productivity of 1.94 g/L/h was achieved. Comparing with the batch SHF process, D-(-)-lactic acid concentration, yield, and productivity using the fed-batch SHF were improved by 88.8%, 4.2%, and 88.3%, respectively. Also, the overall productivity in this fed-batch SHF was improved up to 4.5 folds compared to that of the SHF process if the total operation time was included. Therefore, D-(-)-lactic

acid production using the fed-batch SHF process by *K. oxytoca* KIS004-91T resulted in better titer, yield, and productivity than those of either batch SHF or SSF process (Table 4.4).

The effective production of D-(-)-lactic acid by *K. oxytoca* KIS004-91T was comparable to previous studies in which they conducted the high solid loading of lignocellulosic bagasse to lactic acid (Bai et al., 2016; Hu et al., 2015; Unrean, 2018). Liu et al. (2015) also suggested that fed-batch SHF helped in reducing waste disposal and power supply cost. Together, it provided a higher cell density and prolonged a log-phase lifetime resulting in the higher titer of bioproduct metabolites (Abdel-rahman et al., 2011). During the addition of new substrates, the mixture of enzymes in slurry also assisted recovering an enzymatic activity that lost over time and strengthened shear forces that made the faster release of fermentable sugars. Additionally, the fed-batch strategy provided better benefits once the higher solid loading over 15%, w/v (as dry matter) was applied (da Silva et al., 2020).

D-(-)-lactic acid production by *K.*oxytoca KIS004-91T using NaOH pretreated SCB as a sole carbon source in this study provided similar production yield and overall productivity compared to those of a high grade sugar (optically pure glucose) as a sole carbon source (In, 2019). Also, regarding the D-(-)-lactic acid production in Table 4.5, our results showed comparable or even superior of D-(-)-lactic acid production by *K. oxytoca* KIS004-91T from the waste SCB bagasse or other lignocellulosic biomasses. The higher yield and productivity obtained in this study may be a result of a combined effect of metabolic engineering and evolutionary adaptation previously developed that encouraged carbon flux to produce mainly D-(-)-lactic acid. Nevertheless, the lactic acid titer and productivity achieved in this fed-batch process were below the values of

Lui et al. (2018) due to the higher solid loading of substrate and the enrichment medium used in their study. Therefore, the effective extent from the fed-batch SHF process may indicate industrially feasible production of D-(-)-lactic acid by *K. oxytoca* KIS004-91T from SCB and even other cheap substrate resources.

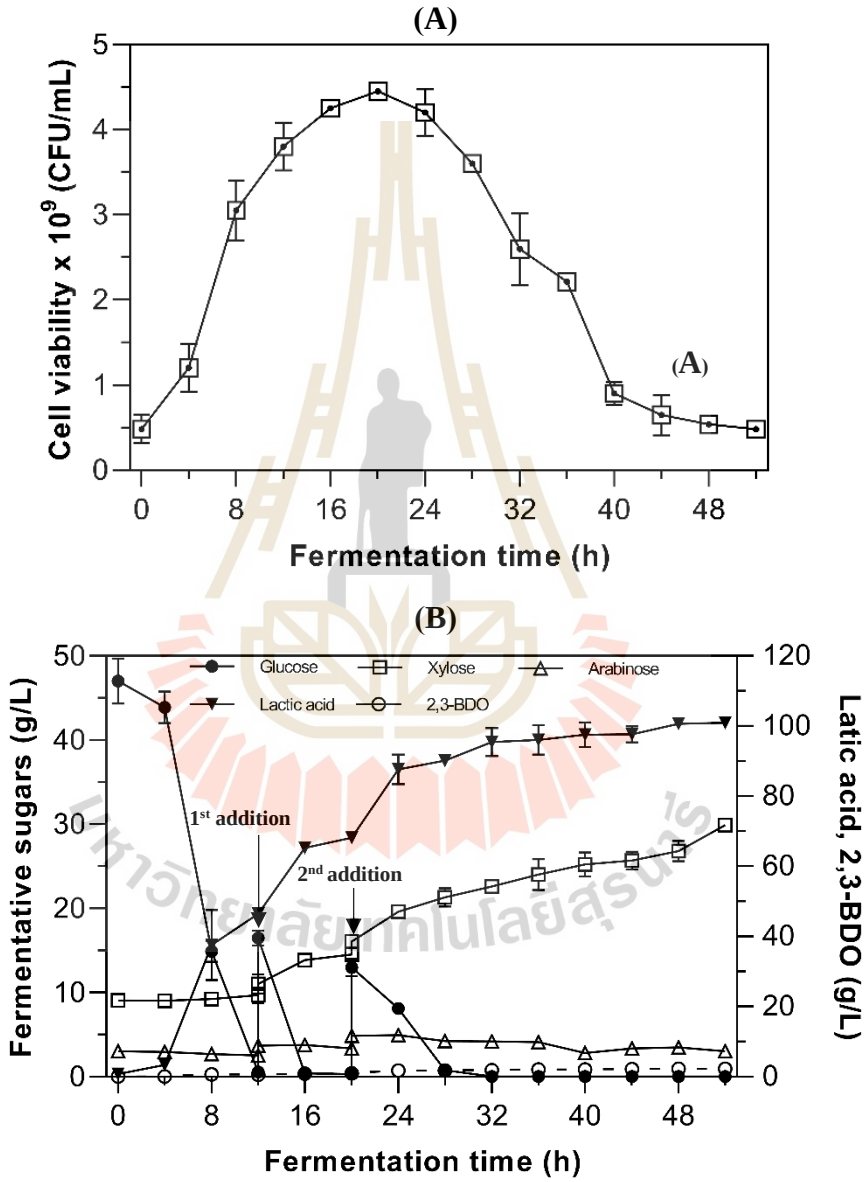


Figure 4.5 D-(-)-lactic acid production from the NaOH pretreated SCB of 15% (w/v) as dry matter by *K. oxytoca* KIS004-91T using fed-batch SHF in 5 L bioreactor with 2.0 L as starting working volume at 37°C. (A) Fermentative products and sugars (B) Cell viability.

Table 4.5 Lactic acid production from lignocellulosic materials in various strains and media by SHF, SSF, and fed-batch fermentation (% dry matter).

Strains	Media	Substrate	Titer[a]	Yield[b]	Pro.[c]	Reference
		Separate Hydrolysis and Fermentation				
K. oxytoca KIS004-91T	AM1	15% NaOH pretreated SCB	53.5	0.95	1.03	This study
Lc. lactis IO-1	NA	3% xylose from SCB hydrolysate	10.9	0.36	0.17	Laopaiboon et al., 2010
B. coagulans NCIM 5648	YE	12.5% NaOH pretreated SCB	51.2	0.81	1.75	Nalawade et al., 2020
L. brevis	MRS	6% sugars from Corncob	39.1	0.70	0.81	Gou et al., 2010
L. lactis RM 2-24	MRS	10% Cellobiose	80.0	0.80	1.66	Singvi et al., 2010
L. rhamnosus ATCC 7469	MRS	8% sugars from recycled paper sludge	61.1	0.81	0.30	Marques et al., 2008
L. delbrueckii sp. *bulgaricus*	MRS	1% corn stover hydrolysate	18.0	-	0.41	Wang et al., 2017
S. inulinus YBS1-5	CSL	15% corncob stover hydrolysate	70.7	0.82	0.65	Bai et al., 2015
L. coryniformis subsp. *torquens*	MRS	14% sugars from pulp mill residue	57.0	0.97	2.80	de Oliveira et al., 2016
L. delbrueckii ssp. *Delbrueckii*	CSL	6% sugars from waste orange peels	-	0.88	2.35	de la Torre et al., 2018
L. rhamnosus LA-04-1	CSL	10% glucose from white rice bran hydrolysate	82.0	0.81	3.73	Li et al., 2012

Table 4.5 (continued).

Strains	Media	Substrate	Titer[a]	Yield[b]	Pro.[c]	Reference
		Separate Hydrolysis and Fermentation				
Bacillus sp. NL01	CSL	9.5% sugars from lignocellulosic hydrolysate	56.4	0.65	1.30	Ouyang et al., 2013
B. coagulans DSM	YE	10% sugars from SCB hydrolysate	56.0	0.87	1.70	de Oliveira, et al., 2019
L. plantarum NCIMB 8826	BE	15% hardwood pulp	63.0	-	0.88	Hama et al., 2015
		Simultaneously Saccharification and Fermentation				
K. oxytoca KIS004-91T	AM1	15% NaOH pretreated SCB	47.2	0.79	0.91	This study
L. delbrueckii subsp. *delbrueckii* UC-3	MRS	10% NaOH pretreated SCB	67.0	0.83	0.93	Adsul et al., 2007
B. coagulans DSM 2314	PYDP	20% (w/w) H_2SO_4 pretreated SCB	64.1	0.80	0.71	van der Pol et al., 2016
L. lactis RM 2-24	MRS	10% bagasse-derived cellulose	73.0	0.73	1.52	Singhvi et al., 2010
L. plantarum ATCC 367	MRS	3% corn stover	21.0	0.70	0.58	Cui et al., 2011
L. rhamnosus ATCC 7469	MRS	8% sugars from recycled paper sludge	73.0	0.97	0.43	Marques et al., 2008
L. coryniformis ATCC 25600	SBME	14% waste *Curcuma longa*	91.7	0.65	2.08	Nguyen et al., 2013
Pediococcus acidilactici TY112	MRS	25% (w/w) corn stover hydrolysate	77.8	0.58	1.02	Yi et al., 2016

Table 4.5 (continued).

Strains	Media	Substrate	Titer[a]	Yield[b]	Pro.[c]	Reference
		Simultaneously Saccharification and Fermentation				
L. plantarum NCIMB 8826	BE	15% hardwood pulp	84.6	-	1.18	Hama et al., 2015
		Fed-batch fermentation				
K. oxytoca KIS004-91T	AM1	25% NaOH pretreated SCB	101.0	0.99	1.94	This study
L. pentosus	YE	30% WIS-SCB	72.8	0.61	1.01	Unrean et al., 2018
B. coagulans LA204	YE	14.4% (w/w) corn stover	97.6	0.68	1.63	Hu et al., 2015
L. pentosus ATCC 8041	CSL	10% corn stover	74.8	0.65	-	Zhu et al., 2007
S. inulinus YBS1-5	CMH	15% corncob residue hydrolysate	107.2	0.85	1.19	Bai et al., 2016
L. plantarum NCIMB 8826	SBME	12% corn stover hydrolysate	61.4	0.77	0.32	Zhang et al., 2016
L. bulgaricus CGMCC	YE	35% cheese whey powder	113.1	-	2.36	Lui et al., 2018
1.6970	CSL	20% cellulose	80.0	0.80	0.30	Ou et al., 2011
B. coagulans 36D1	YE	25% corncob molasses	74.7	0.50	0.38	Wang et al., 2010
Bacillus sp.						

[a] Lactic acid production concentration (g/L)
[b] Yield of total sugars consumed (g/g)
[c] Overall productivity (g/L/h)

4.6 Mass balance and estimated production cost

Mass balance is a powerful tool used for processing analysis (Doran, 2012). Hence, the overall mass balance of D-(-)-lactic acid production from the raw SCB using fed-batch SHF based on this study is illustrated in Fig. 4.6. Based on 2.1 kg of raw SCB applied, the SCB of about 750 g was obtained after the NaOH pretreatment that was further able to turn into 390 g of fermentative sugars by the cellulase digestion. After 52 h of *K. oxytoca* KIS004-91T fermentation, D-(-)-lactic acid of 303 g was accumulated in the fermentation broth along with less than 3.0 g of by-product (2,3-BDO). Therefore, the bioconversion of sugars to 1 kg D-(-)-lactic acid requires 6.93 kg of raw SCB with 1.89 kg NaOH and 37,125 U cellulase complex, respectively. The cost of raw SCB is approximately about 110 bath/ton or 0.0035 US$/kg (https://thailand.tradekey.com/bagasse.htm) and the industrial-grade NaOH is also about 0.35-0.8 US$/kg (www.alibaba.com). The prices of the crude cellulase complex (2,575 U/mL) and AM1 medium were 16.17 US$ (Sawisit et al., 2015b) and 0.43 US$/L (Chan, 2016), respectively. Therefore, the production cost of D-(-)-lactic acid in this study has been estimated at around 1.35 US$/kg. D-(-)-lactic acid production cost by *K. oxytoca* KIS004-91T using the raw SCB as a sole carbon source is about 3 times lower compared to that produced from the optically pure glucose as a sole carbon source (In et al., 2020). Additionally, NaOH is one of the main parameters affecting D-(-)-lactic acid production cost. It may be recycled as a black liquor (BL) solution thus increasing a raw SCB loading in the solid-liquid ratio and therefore reducing the production cost.

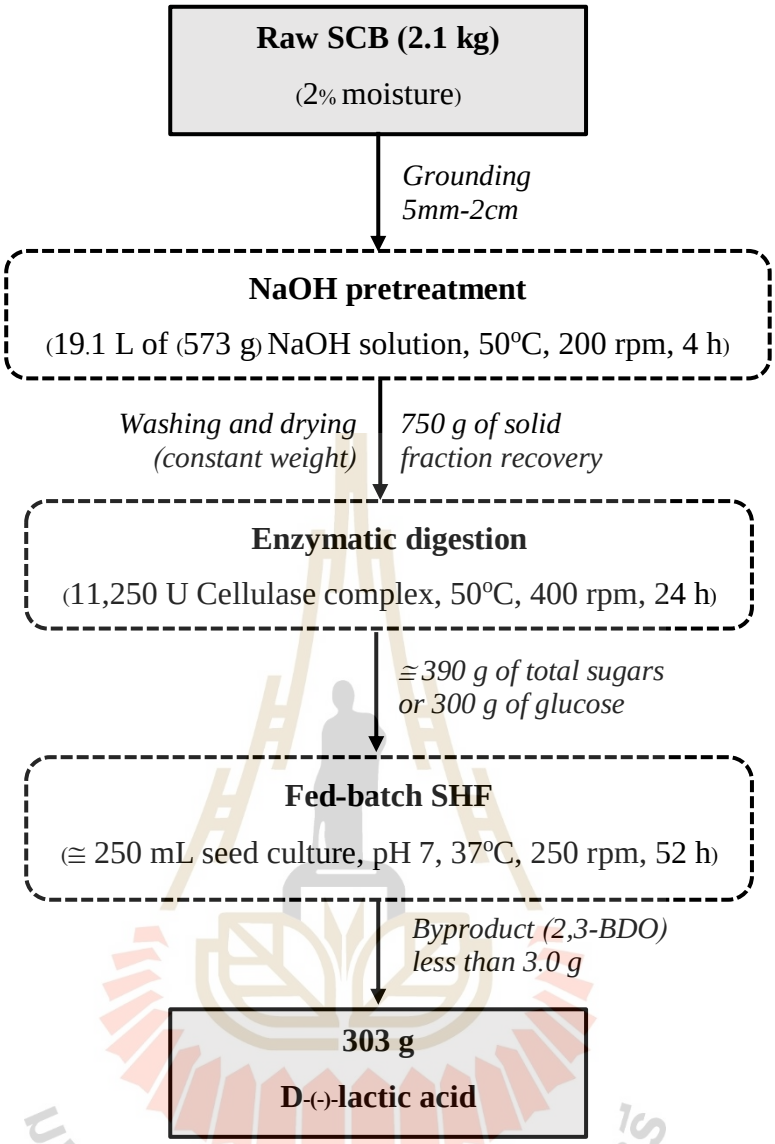


Figure 4.6 Overall mass balance of raw SCB converted to D-(-)-lactic acid based on NaOH pretreatment in the fed-batch SHF process.

CHAPTER V

CONCLUSION

The main purposes of this study were to investigate the effectiveness of the NaOH pretreatment on SCB and optimal condition for its enzymatic hydrolysis as well as the suitable fermentation mode for the high production of D-(-)-lactic acid by *K. oxytoca* KIS004-91T. As a result, applying 3% of NaOH to pretreat SCB in the solid and liquid ratio of 1:9.1 at 50°C for 4 h could remove most of the lignin constituent and a partial hemicellulose compartment as well as could disrupt the cellulose structure. The effective deconstruction of SCB by the pretreatment method in this study could be confirmed by the low recovery of the NaOH pretreated SCB solid fraction, less soluble sugars detected in the liquid fraction and high accessibility of cellulase complex. After the enzymatic hydrolysis by cellulase complex loading of 15 U/g the NaOH pretreated SCB, the total fermentative sugar was liberated at the level of 65.7±0.7 g/L, which was 6 folds higher than that of the non-NaOH pretreated SCB. Furthermore, the optimal enzyme loading of 15 U/g the NaOH pretreated SCB was found to be lower than those from other previously published works (Gao et al., 2013; Ong et al., 2019; Wang et al., 2019; Zhang et al., 2016). This may reflect that the possibility of a cost reduction for D-(-)-lactic acid production from SCB since the high production cost related to enzymes is reasonably concerned.

In the comparison of batch SHF and SSF operations for D-(-)-lactic acid production, the batch SSF process offered lower either titer, yields, productivity or cell

viability than those of the SHF process. This differentiation mainly due to the insufficient carbon source during the SSF process causing by inefficient enzymatic hydrolysis. This led to lower cell growth and D-(-)-lactic acid production. Eventually, fed-batch SHF was validated to improve D-(-)-lactic acid production at the high solid loading up to 25% (w/v) or 250 g/L of the NaOH pretreated SCB. Glucose at 10% (w/v) was generated from the NaOH pretreated SCB and rapidly converted to 101 g/L of D-(-)-lactic acid with conversion rates of 0.99 g/g and 0.40 g/g from sugars consumption and pretreated SCB provided, respectively, and the overall productivity of 1.94 g/L/h. Moreover, the maximum production rate at 4.7 g/L/h was achieved within 8 h with the increase of cell viability up to $4.45\pm0.07\times10^{9}$ CFU/mL while the byproduct of 2,3-BDO less than 1.0 g/L was only detected. Additionally, the estimated D-(-)-lactic acid production cost in this study is about US$1.35/kg, which is considerably lower than those produced from high grade sugars as a sole carbon source.

In conclusion, the main concerns of producing D-(-)-lactic acid from lignocellulosic materials as SCB in this study had been resolved including breaking the natural resistant of bagasse by alkaline pretreatment combined with low enzymatic loading to digest high solid loading biomass. High concentration, production yield and optically pure of D-(-)-lactic acid were obtained by the mutant *K. oxytoca* KIS004-91T using a low-cost and nutrient medium. Therefore, D-(-)-lactic acid production cost related to medium preparation and operation may be reduced due to the lower costs of medium and carbon source as well as the simple fermentation method applied. This observation may reflect the feasible industrial D-(-)-lactic acid production by *K. oxytoca* KIS004-91T from SCB that are abundant in tropical and sub-tropical areas, including Thailand as well as other renewable lignocellulosic materials.

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APPENDICES

APPENDIX A

ANALYTICAL DATA

Table 1A The fermentative sugars released from various cellulase complex loading after 72 h hydrolysis at 50 °C, 200 rpm from 100 g NaOH pretreated SCB.

Cellulase complex (U/g SCB)	**Glucose** (g/L)	**Xylose** (g/L)	**Arabinose** (g/L)	**Total Sugars** (g/L)
5	41.8±1.9	8.2±0.7	2.2±0.2	52.2±2.5
10	44.1±0.5	10.1±1.0	2.6±0.2	56.7±1.2
15	48.7±0.7	13.5±1.2	2.6±0.2	64.8±1.2
20	48.7±1.7	16.2±0.2	2.6±0.3	67.5±1.9
25	48.9±0.5	18.0±0.6	2.6±0.3	69.5±1.2
30	49.7±1.1	20.4±0.8	2.8±0.2	72.8±1.9

Table 2A Duncan`s multiple-range test (DMRT) of Total sugars and Glucose release from NaOH pretreated SCB by various cellulase complex loading (72 h).

Sugars

Duncan[a]

		Subset for alpha = 0.01				
Treatment	N	1	2	3	4	5
5.00	3	52.1661				
10.00	3		56.7054			
15.00	3			64.8440		
20.00	3			67.4651	67.4651	
25.00	3				69.4445	69.4445
30.00	3					72.7783
Sig.		1.000	1.000	.090	.189	.037

Glucose

Duncan[a]

		Subset for alpha = 0.01	
Treatment	N	1	2
5.00	3	41.7962	
10.00	3	44.0897	
15.00	3		48.6769
20.00	3		48.6938
25.00	3		48.8837
30.00	3		49.6585
Sig.		.038	.373

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000

APPENDIX B

ABSTRACT

Proceeding of SUT International Virtual Conference on Science and Technology (SUT-IVCST-2020), 28th August, 2020, Nakhon Ratchasima, Thailand: A potential Use of Sugarcane Bagasse For D-(-)-Lactic Acid Production by *Klebsiella oxytoca*.

Sokha Kory, Panwana Khunnonkwao, Csaba Fehér, and Kaemwich Jantama

Lactic acid is one among the top twelve value-added chemicals that has been used since the 1980s in the fields of pharmaceutical, cosmetic, food, and chemical industries. Recently, poly-lactic acid as a raw material in bioplastics synthesis has increased the high demand for lactic acid production. Normally, a racemic mixture of D-(-) and L-(+)-lactic acid is obtained from the chemical synthesis while the biosynthesis generates optically pure D-(-) or L-(+)-lactic acid. Even though, lactic acid bacteria can provide high lactic acid production, they need special supplements and produce by-products. In contrast, *Klebsiella oxytoca* is a biosafety and simple nutrients-utilizing bacterium that exhibits fast-growing under both aerobic and anaerobic conditions and utilizing broad ranges of carbon sources. Moreover, the production of lactic acid from lignocellulosic materials especially sugarcane bagasse is getting interesting as it is abundance, sustainable, not compete to human consumption, and of course economic and environmental benefits. In this study, *K. oxytoca* KIS004-91T was used to obtain D-(-)-lactic acid from sodium hydroxide pre-treated sugarcane bagasse using minimal low salts medium under simple batch fermentation. After 96 h

of fermentation, D-(-)-lactic acid 59.2 ± 1.4 g/L with 0.40 ± 0.01 g/g, 0.98 ± 0.01 g/g of yield from NaOH pre-treated sugarcane bagasse and total fermentative sugars consumed respectively, and productivity of 0.61 ± 0.02 g/L/h were obtained. The result has illustrated the solid proof of viability fermentation of sugarcane bagasse as a guaranteed method for large-scale production of D-(-)-lactic acid.

Keywords: Lactic acid, *Klebsiella oxytoca*, lignocellulosic biomass, sugarcane bagasse

BIOGRAPHY

Mr. Sokha Kory was born on June 01, 1994, at Kampot province, Cambodia. He obtained the high school certificate in 2013 and subsequently awarded the Cambodia MOEYS-scholarship to continue his Bachelor's degree. He studied Science of Food Processing major from Faculty of Agriculture and Food Processing, University of Battambang, Battambang, Cambodia. During this study, he has awarded "One Asia Foundation Scholarship" and a one-month exchange program scholarship to Kagawa University. After graduation in 2017, he decided to further study for a Master degree in the field of Biotechnology at the school of Biotechnology, Institute of Agricultural of Technology, Suranaree University of Technology, Nakhon Ratchasima, Thailand. During his attendance, he was fully financially funded under the SUT-OROG program. His research topic is "Efficient production of D-(-)-lactic acid from sugarcane bagasse in mineral salts medium by metabolically engineered *Klebsiella oxytoca* KIS004-91T". Some parts of the results from this study had been presented as a visual presentation in the SUT International Virtual Conference on Science and Technology (SUT-IVCST-2020) at Suranaree University of Technology, Nakhon Ratchasima, Thailand during August 28th. This work has been published in the proceeding of SUT-IVCST-2020 in the title of "A potential Use of Sugarcane Bagasse For D-(-)-Lactic Acid Production by *Klebsiella oxytoca*" 2020.