OPTIMIZATION OF D-(-)-LACTIC ACID PRODUCTION FROM PINEAPPLE CROWN USING METABOLICALLY ENGINEERED *KLEBSIELLA OXYTOCA* KIS004-91T

SOCHEATA CHOU

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in Biotechnology

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การหาสภาวะที่เหมาะสมต่อการผลิตกรดแลคติกชนิด D(-) จากจุกสับปะรดด้วยเชื้อที่ผ่านการดัดแปลงพันธุกรรม *Klebsiella oxytoca* KIS004-91T

นางสาวโสะเจียตา จู

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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โสะเจียตา จู: การหาสภาวะที่เหมาะสมต่อการผลิตกรดแลคติกชนิด D(-) จากจุกสับปะรดด้วยเชื้อที่ผ่านการดัดแปลงพันธุกรรม *Klebsiella oxytoca* KIS004-91T (OPTIMIZATION OF D-(-)-LACTIC ACID PRODUCTION FROM PINEAPPLE CROWN USING METABOLICALLY ENGINEERED *KLEBSIELLA OXYTOCA* KIS004-91T) อาจารย์ที่ปรึกษา: รองศาสตราจารย์ ดร. เขมวิทย์ จันต๊ะมา, 82 หน้า

คำสำคัญ: จุกสับปะรด/กรดแลคติก/การปรับสภาพด้วยเบส/การย่อยสลายและหมักแยกกัน/การย่อยสลายและหมักพร้อมกัน

จุกสับปะรดเป็นของเหลือจากกระบวนการแปรรูปสับปะรดในภาคอุตสาหกรรมซึ่งประกอบด้วยลิกโนเซลลูโลส และมีศักยภาพในการนำมาใช้เป็นวัสดุตั้งต้นสำหรับการผลิตสารชีวเคมีโดยจุลินทรีย์ อย่างไรก็ตาม งานวิจัยเกี่ยวกับการเพิ่มมูลค่าของจุกสับปะรดเพื่อวัตถุประสงค์ดังกล่าวค่อนข้างจำกัด งานวิจัยนี้จึงได้มีการพัฒนาแพลตฟอร์มเทคโนโลยีสำหรับการผลิตกรดดี-(-)-แลกติกเพื่อให้ได้ค่าผลผลิต และอัตราการผลิตที่สูงโดยทำการปรับสภาพจุกสับปะรดเป็นแหล่งคาร์บอนในการหมักด้วยเชื้อจุลินทรีย์ *Klebsiella oxytoca* KIS004-91T โดยใช้โซเดียมไฮดรอกไซด์ (NaOH) ความเข้มข้น 0.75 N ซึ่งให้น้ำหนักจุกสับปะรดที่ปรับสภาพแล้ว ร้อยละ 37.8±4.0 ของน้ำหนักต่อน้ำหนักจุกสับปะรดเริ่มต้น น้ำตาลสำหรับใช้หมักได้ร้อยละ 62.5±0.ของน้ำหนักต่อน้ำหนักจุกสับปะรดที่ผ่านการปรับสภาพ สภาวะการย่อยสลายจุกสับปะรถด้วยเอนไซม์ที่ให้ความเข้มข้นของน้ำตาลทั้งหมดสูงสุดที่ 13.69±0.36 กรัมต่อลิตร และน้ำตาลที่ได้จากเซลลูโลสร้อยละ 89.77±1.4 ของเซลลูโลสในจุกสับปะรด คือจุกสับปะรดที่ผ่านการปรับสภาพ 20 กรัมต่อลิตร ใช้เอนไซม์เซลลูเลส 60 พีซียูต่อกรัม ที่อุณหภูมิ 50 องศาเซลเซียส

จากนั้นจุกสับปะรดที่ผ่านการปรับสภาพที่ความเข้มข้น 100 กรัมต่อลิตร ถูกนำไปหมักภายใต้กระบวนการย่อยและหมักแยกกัน (Separate hydrolysis and fermentation) โดยให้กรดดี-(-)-แลคติก 45.69±1.16 กรัมต่อลิตร มีอัตราการเปลี่ยนเป็นผลิตภัณฑ์ 0.81±0.02 กรัมต่อกรัม และอัตราการผลิต 1.92±0.35 กรัมต่อลิตรต่อชั่วโมง ส่วนจุกสับปะรดที่ผ่านการปรับสภาพ 75 กรัมต่อลิตรเพื่อนำไปหมักภายใต้กระบวนการย่อยสลายและหมักพร้อมกัน (Simultaneous saccharification and fermentation) ด้วย ให้ผลผลิตกรดดี-(-)-แลกติก 31.50±1.97 กรัมต่อลิตร ที่อัตราการเปลี่ยนเป็นผลิตภัณฑ์ 0.70±0.03 กรัมต่อกรัม และอัตราการผลิต 1.07±0.03 กรัมต่อลิตรต่อชั่วโมง การเพิ่มประสิทธิภาพด้วยการหมักแบบกึ่งกะด้วยสภาวะกระบวนการย่อยและหมักแยกกัน

(fed-batch SHF) สามารถเพิ่มผลผลิตกรดดี-(-)-แลกติกได้ถึง 62.87±0.42 กรัมต่อลิตร โดยมีอัตราการเปลี่ยนเป็นผลิตภัณฑ์ 0.96±0.07 กรัมต่อกรัม และอัตราการผลิต 1.31±0.01 กรัมต่อลิตรต่อชั่วโมง โดยกระบวนการหมักทั้งหมดดำเนินการด้วยการใช้อาหารเลี้ยงเชื้อเกลือแร่ ให้ผลผลิตกรดดี-(-)-แลกติกที่มีความบริสุทธิ์สูง พร้อมด้วยผลพลอยได้ ได้แก่ 2,3-บิวทานีไดออล และกรดอะซิติก (ประมาณ 3.5 กรัมต่อลิตร) ในระดับต่ำ ผลการวิจัยนี้ชี้ให้เห็นว่าจุกสับปะรดเป็นวัสดุชีวมวลที่มีศักยภาพสำหรับการผลิตกรดดี-(-)-แลกติก ซึ่งสามารถลดต้นทุน เพิ่มมูลค่าของเสีย และส่งเสริมแนวคิดอุตสาหกรรมชีวภาพแบบไร้ของเสียในอนาคต

สาขาวิชาเทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา.....

ปีการศึกษา 2567 ลายมือชื่ออาจารย์ที่ปรึกษา.....

SOCHEATA CHOU: OPTIMIZATION OF D-(-)-LACTIC ACID PRODUCTION FROM PINEAPPLE CROWN USING METABOLICALLY ENGINEERED *KLEBSIELLA OXYTOCA* KIS004-91T. THESIS ADVISOR: ASSOC. PROF. KAEMWICH JANTAMA, Ph.D, 82 PP.

Keyword: Pineapple crown/Lactic acid/Alkaline pretreatment/Separate hydrolysis and fermentation/Simultaneous saccharification and fermentation

Pineapple crowns (PIC) represent a lignocellulosic and industrial waste byproduct from pineapple processing industries, with promising potential as a feedstock for microbial biochemical production. However, limited research has been conducted on the valorization of PIC for this purpose. In this study, a technological platform was developed for high-yield, high-productivity production of D-(-)-lactic acid from PIC using an engineered strain of *Klebsiella oxytoca* KIS004-91T. Pretreatment of PIC was performed using 0.75 N NaOH, resulting in a recovery yield of 37.8±4.0% (w/w) pretreated PIC containing 62.5±0.1% (w/w) fermentable sugars. Enzymatic hydrolysis of 20 g/L of pretreated PIC using an optimal crude cellulase loading of 60 PCU/g at 50°C achieved a maximum total reducing sugar concentration of 13.69±0.36 g/L and a cellulose saccharification efficiency of 89.77±1.41% (w/w). Subsequent fermentation under separate hydrolysis and fermentation (SHF) conditions using 100 g/L of NaOH-pretreated PIC yielded 45.69±1.16 g/L D-(-)-lactic acid, with a conversion yield of 0.81±0.02 g/g and a productivity of 1.92±0.35 g/L/h. Under simultaneous saccharification and fermentation (SSF) using 75 g/L of pretreated PIC, 31.50±1.97 g/L of D-(-)-lactic acid was produced, with a yield of 0.70±0.03 g/g and a productivity of 1.07±0.03 g/L/h. Further optimization using a fed-batch SHF strategy significantly enhanced D-(-)-lactic acid production to 62.87±0.42 g/L, achieving a yield of 0.96±0.07 g/g and a productivity of 1.31±0.01 g/L/h. The fermentation process was carried out in a low-salt medium, producing high-purity D-(-)-lactic acid with minimal by-product formation, including 2,3-butanediol and acetic acid (~3.5 g/L). These findings highlight PIC as a viable alternative biomass for sustainable D-lactic acid production, offering

potential for cost reduction, waste valorization, and advancement toward a zero-waste biorefinery approach.

School of Biotechnology
Academic Year 2024

Student's Signature .....
Advisor's Signature .....

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LIST OF ABBREVIATIONS

ackA	=	acetate kinase
adhE	=	Alcohol dehydrogenase
AFEX	=	Ammonia-based pretreatment
AM1	=	Alfredo Martinez Mineral Salt Medium 1
ATP	=	Adenosine triphosphate
BDO	=	Butanediol
°C	=	Degree Celsius
$C_2H_4O_3$	=	Peracetic acid
CCR	=	Carbon calabolite repression
CDW/L	=	Cell dry weight per liter
CFU/ml	=	Colony forming unit per mililiter
CS	=	Cellulose saccharification
FPU/g	=	Filtrated protein unit per gram
frd	=	Fumarate reductase
g	=	gram
g/g	=	gram/ gram
g/L	=	gram/ liter
g/L/h	=	gram/ liter/ hour
glu	=	Glucose
h	=	Hour
H_2O_2	=	hydrogen peroxide
H_2SO_4	=	Sulfuric acid
H_3O^+	=	Hydronium ions
H_3PO_4	=	Phosphoric acid
HCl	=	Hydrochloric acid
HCN	=	Hydrogen cyanide

LIST OF ABBREVIATIONS (Continued)

HMF	=	Hydroxymethyfurfural
HNO_3	=	Nitric acid
HPLC	=	High-performance liquid chromatography
HS	=	Hemicellulose saccharification
KOH	=	Potassium hydroxide
L	=	Liter
LA	=	Lactic acid
LABs	=	Lactic acid bacteria
LB	=	Luria Bertani
LCC	=	Lignin-carbohydrate bonds
ldhA	=	Lactate dehydrogenase
LHW	=	Liquid hot water
M	=	Molar
mg/g	=	Milligram per gram
min	=	Minute
ml	=	Milliliter
mM	=	Millimolar
mmol/L	=	Millimoles per liter
N	=	Normality
Na_2CO_3	=	Sodium carbonate
NADH	=	Nicotinamide adenine dinucleotide
NaOH	=	Sodium hydroxide
$(NH_4)_2SO_4$	=	Ammonium sulfate
OD	=	Optical density
PCU/g	=	Protein concentration units per gram
pflB	=	Pyruvate formate-lyase
PIC	=	Pineapple crown

LIST OF ABBREVIATIONS (Continued)

PLA	=	polylactic acid
pta	=	Phosphate acetyltransferase
rpm	=	Round per minutes
SHF	=	Separate hydrolysis and fermentation
SSF	=	Simultaneous saccharification and fermentation
SSCF	=	simultaneous saccharification and co-fermentation
U	=	Unit
v/w	=	Volume by weight
w/v	=	weight by volume
w/w	=	weight by weight
xyl	=	Xylose
µm	=	Micrometer
µmol/L	=	Micromoles per liter

CHAPTER I
INTRODUCTION

1.1 Overview

Lactic acid is an organic acid that is known to have two stereoisomers, including L(+) and D-(-) forms. It has been applied in several industries, including foods, pharmaceutical, cosmetic, and chemical industries. It is generally used as a precursor of biodegradable and biocompatible polylactic acid (PLA) polymers for producing packaging, foam, and fibers (Rodrigues et al., 2017). The production of biocompatible PLA products that require D-(-)-lactic acid as raw materials is considered as an early stage of development compared to those derived from petrochemical plastics in industrial scale (Razali and Abdullah, 2017). The market size of D-(-)-lactic acid was estimated at $3.37 billion USD in 2023 and is expected to gain 8.0% of CAGR by 2030. Lactate can be produced by both chemical synthesis and microbial fermentation. Compared to chemical synthesis, biological fermentation of D-(-)-lactic acid is more preferable due to the ability to use renewable resources and lignocellulosic biomass as a feedstock to reduce production costs as well as environment pollution (Yaashikaa et al., 2022).

Considering environmental pollution and production cost savings, biofuel and biochemical production from lignocellulosic biomass have been significantly increased. Lactic acid is so far produced from lignocellulosic biomass generated from agricultural and agro-industrial sectors, including corn stover (Zhang and Vadlani, 2013), waste wood (Hama et al., 2015), and sorghum stalks (Zhang et al., 2016a) using various lactic acid bacteria (LAB). Lactic acid production is performed using different kinds of biomass by various microorganisms, including *Lactobacillus rhamnosu* (Bernardo et al., 2016), *Rhizopus oryzae* (Thongchul et al., 2010), *Enterobacter aerogenes* (Thapa et al., 2017), and metabolically engineered strains of *Escherichia coli* (Utrilla et al., 2009) and

Klebsiella oxytoca (In et al., 2020).

Alternatively, pineapple is known as the most active crop in the global tropical fruit trade, which is mainly distributed in Asia, America, and Africa. With the total global area of pineapple cultivation exceeding 400,000 hectares, pineapple has been produced about 25.4 million metric tons annually (Li et al., 2022). Pineapple is a valuable and non-climacteric tropical fruit and can be eaten fresh or processed as canned, juiced, jams, and dried pineapple based on its maturity (Dhar et al., 2023). Along with pineapple processing and consumption, pineapple crown, peel, core, and stem are left as wastes, with 50% (w/w) of the pineapple weight. Subsequently, the pineapple wastes are disposed of directly in landfills, contributing to environmental pollution due to microbial spoilage and the growing volume of waste associated with increased pineapple production (Hikal et al., 2021). Therefore, among pineapple wastes, pineapple crown is weight about 10-25% of total weight and consists of cellulose and hemicellulose, making it ideal as lignocellulosic biomass (Choquecahua et al., 2020; Prado and Spinace, 2019). To mitigate environment impacts and enhance value creation, the pineapple crown (PIC) presents strong potential for biofuel and biochemical production, owning to its rich carbohydrate content and cost-effectiveness as an agro-industrial waste. To achieve this, the conversion of lignocellulosic fibers in PIC into fermentable sugars requires appropriate pretreatment processes to breakdown the structure of cellulose and hemicellulose, remove lignin, reduce inhibitor accumulation, and increase surface area for subsequent enzyme digestion (Yankov, 2022). However, there are very few research studies on the biochemical conversion of PIC into valuable bioproducts via fermentation due to its complicated lignocellulosic pattern and multiple cellular structure (Arib et al., 2006). Therefore, using pineapple crown as lignocellulosic substrate for D-(-)-lactic acid production is still limited, making its challenges for a proper preparation of PIC to be used for D-(-)-lactic acid production.

Klebsiella oxytoca KIS004-91T strain was previously engineered to efficiently produce high levels of D-(-)-lactic acid from glucose with minimal by-product

formation. This strain achieved a D-(-)-lactic acid concentration of 100 g/L, with a yield of 0.96 g/g and a productivity of 2.1 g/L/h (In et al., 2020). To enhance its industrial feasibility and cost effectiveness for D-(-)-lactic acid production, this study aimed to develop efficient processes for the pretreatment and enzymatic saccharification of PIC. D-(-)-lactic acid production by *K. oxytoca* KIS004-91T strain was optimized using separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) strategies, including batch and fed-batch processes, in a low-cost medium containing fermentable sugars derived from the pretreated PIC.

1.2 Research objectives

This research aims to valorize pineapple crown, which is the waste from dried and canned fruit factories, promoting an effective process of D-(-)-lactic acid production using *K. oxytoca* KIS004-91T strain. This work focused on:

1.2.1 Optimizing the suitable concentration of alkali solution for pretreating pineapple crown into pretreated lignocellulosic structures.

1.2.2 Optimizing the appropriate enzymatic treatment and duration of hydrolysis to obtain the maximum level of sugar conversion from pretreated pineapple crown.

1.2.3 Optimizing the maximum production of D-(-)-lactic acid from pretreated pineapple crown by metabolically *K. oxytoca* KIS004-91T strain.

1.3 Scope and limitations

Fermentation of D-(-)-lactic acid from pineapple waste was conducted under different conditions, including simultaneous saccharification and fermentation (SSF) and separate hydrolysis and fermentation (SHF) processes in batch and fed-batch fermentation by *K. oxytoca* KIS004-91T strain. Accordingly, the concentration of alkaline solution during the pretreatment of pineapple crown and enzymatic loading for enzymatic saccharification of pretreated-pineapple crown were also optimized. Different concentrations of pretreated PIC were investigated to obtain the highest

productivity and yield of D-(-)-lactic acid closed to the theoretical maximum (1 g/g) as appeared using glucose as a substrate.

CHAPTER II

LITERATURE REVIEW

2.1 Lactic acid and its application

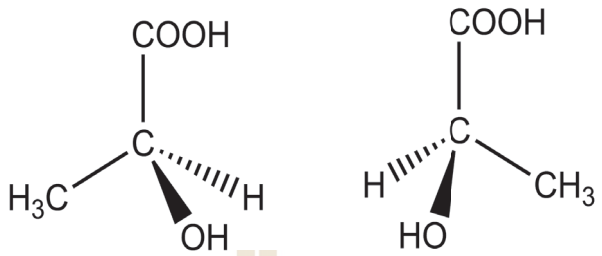
Lactic acid is an organic acid that has been known as milk acid which has a chemical formula as $CH_3CH(OH)COOH$ or $C_3H_6O_3$. It is a colorless to yellowish, odorless hydroscopic crystal or syrupy liquid. Physical and chemical properties of lactic acid are mentioned in Table 2.1 (Dusselier et al., 2013). Lactic acid is a weak acid with pKa 3.86 which partially dissociates in water to release hydrogen ions (eq1) (Ameen and Caruso, 2017).

$$H_3C\text{-}CH(OH)\text{-}COOH \longleftrightarrow H^+ + H_3C\text{-}CH(OH)\text{-}COO^- \qquad \text{(Eq. 1)}$$

Table 2.1 Physical and chemical properties of lactic acid

Lactic acid properties	Details
Compound name	Lactic acid
IUPAC name	2-hydroxypropanoic acid
Appearance	Colorless to yellowish, crystal or syrupy liquid
Solubility	Water, alcohol, ether
Boiling point	122 °C
Melting point	16.8 °C
Molar mass	90.08 g.mol^{-1}
Specific density	1.2
Flash point	113 °C

Lactic acid is composed of a chiral carbon with two terminal carbon atoms consisting of a carboxylic group with a methyl group (Narayanan et al., 2004). It exists in two enantiomeric forms: L-(+)-lactic acid form, also known as (S)-lactic acid and D-(-)-lactic acid, also known as (R)-lactic acid (Ameen and Caruso, 2017).



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

Figure 2.1 Isomer form of L (+) and D-(-)-lactic acid

Lactic acid is recognized as an important precursor in various industries including, food, cosmetics, chemical, and green solvent (Sorensen et al., 2022; Sorensen et al., 2023). While lactic acid is widely used as a food addictive, food preservative, flavoring agent, and scent in food industries, it is also a common ingredient in the cosmetic industry, such as moisturizers, skin-lightening agents, pH regulator, and anti-acne agents. Additionally, in the chemical industry, lactic acid serves various purposes, acting as a descaling agent, pH regulator, neutralizer, chiral intermediate, green solvent, cleaning agent, and slow acid releasing agent. Furthermore, it is an essential chemical feedstock that functions as a precursor to a number of significant chemicals, including propylene oxide, acetaldehyde, acrylic acid, propionic acid, 2,3-pentanedione, ethyl lactate, dilactide, and polylactic acid (Abd Alsaheb et al., 2015). Lactic acid also acts as a precursor to polylactic acid (PLA), a biodegradable polymer widely used in packaging, textiles, and biomedical applications. PLA is eco-friendly and serves as an alternative to petroleum-based plastics, contributing to sustainable practices in the chemical industry (Wu et al., 2023a).

2.2 Lactic acid production

Lactic acid can be produced by chemical synthesis with different pathways and microbial fermentation from renewable resources (Wee et al., 2006). Lactic acid (racemic mixture) can be synthesized by hydrocyanation, followed by cyanohydrin hydrolysis and generating ammonium sulfate ($(NH_4)_2SO_4$) as a byproduct (Figure 2.2). However, hydrogen cyanide (HCN) is known as hazardous chemical, it is challenging for

handling HCN and complicated purification processes are required to obtain food-grade lactic acid. Additionally, in sectors where purity and environmental concerns are considered as crucial, fermentation-based methods are often preferred due to their natural origin and environmentally friendly characteristics. Lactic acid is also produced through the fermentation of carbohydrates or fermentable sugars, typically sugars such as glucose or lactose, by lactic acid bacteria. Lactic acid bacteria (LABs), such as *Lactobacillus* species, naturally produce lactic acid during fermentation (Hoyos et al., 2017).

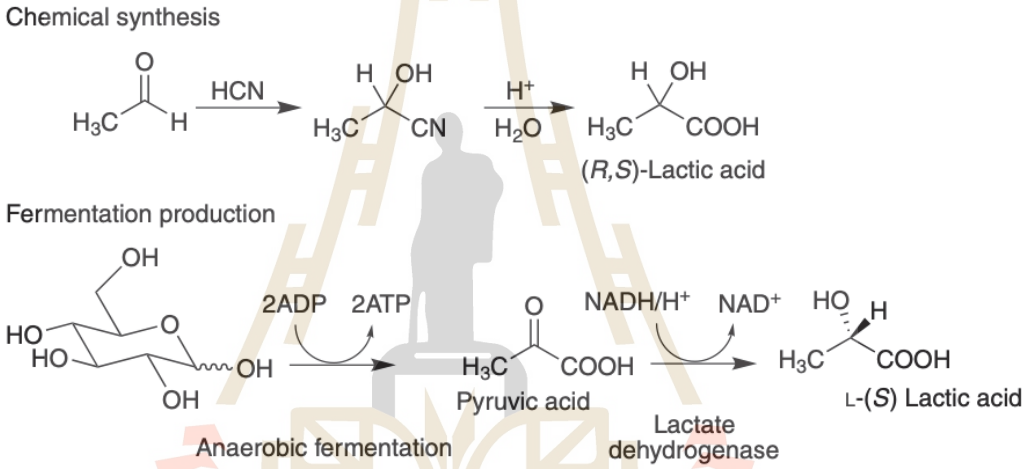


Figure 2.2 Lactic acid production (Hoyos et al., 2017)

2.3 Microbial fermentation of lactic acid

Lactic acid can be produced by various microorganisms, mostly by lactic acid bacteria (LAB). Generally, LAB belong to gram positive bacteria, non-forming spore, and anaerobic fermenting bacteria such as *Lactobacillus, Bacillus, Enterococcus, Vagococcus, Leuconostoc, Streptococcus, and Pediococcus* (Nuraida, 2015; Panagiota et al., 2013). Fungi strains such as *Rhizopus oryzae* could produce L-(+)-lactic acid from starch and glucose (Zhou et al., 1999) while bacteria strains could produce both L-(+)-lactic acid and D-(-)-lactic acid or a mixture of DL-lactic acid (Zhao et al., 2010). The underlying of metabolic pathways of lactic acid production are shown in Figure 2.3 in which 2 molecules of lactic acid are produced from glucose under homofermentative pathway whereas lactic acid and by-products such as ethanol and carbon dioxide (CO_2)

are produced under heterofermentative pathways (Mora-Villalobos et al., 2020). Currently, bacterial strains have remained the preferred choice among researchers for lactate production due to their capability to produce lactic acid at high yield and productivity (Oonkhanond et al., 2017). Table 2.2 shows some examples of D-(-)-lactic acid and L-(+)-lactic acid biotechnological production. Similarly, microorganisms that are targeted for industrial lactic acid production must possess key attributes. These include enhancing productivity to reduce time, improving yield to lower substrate costs, utilizing low-cost media or substrates to achieve high concentrations, minimizing by-product formation to enhance purification efficiency, and demonstrating resilience against contamination and infections (Auras et al., 2010).

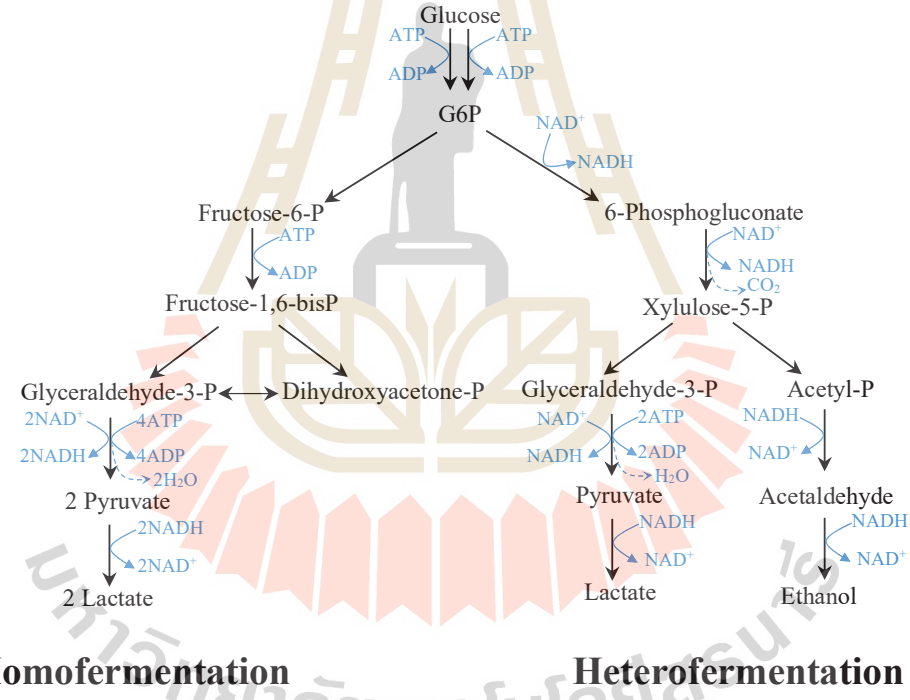


Figure 2.3 Metabolic pathways of lactic acid production by lactic acid bacteria

Table 2.2 D(-) and L-(+)-lactic acid production by microorganisms

Types of lactic acid	Microorganism	Substrate	Fermentation	Lactic acid titer (g/L)	Yield (g/g)	Productivity (g/L/h)	References
L (+)	*B. coagulans* NCIM 5648	Sugarcane bagasse	SHF	69.2	0.76	2.88	(Baral et al., 2020)
D (-)	*L. delbrueckii* subsp. *bulgaricus*	Beechwood	SSF	62	0.69	0.86	(Karnaouri et al., 2020)
D (-)		Pine	SSF	36.4	0.40	1.6	
D (-)	*P.acidilactici ZY15*	Wheat straw	SSCF	128.1	0.69	1.78	(He et al., 2023)
L (+)	*E.mundtii WX1* and *L.rhamnosus*	Corn stover	SHF	34.6	0.9	1.73	(Klongklaew et al., 2023)
L (+)	*E. mundtii* CGMCC 22227	Garden garbage	SHF	59.7	0.62	0.79	(Zhu et al., 2023)

SHF: separate hydrolysis and fermentation, SSF: simultaneous saccharification and fermentation, SSCF: simultaneous saccharification and co-fermentation

2.4 Fermentation modes

The microbial fermentations are carried out in various strategies which plays a crucial role in determining the efficiency, productivity, and yield of the desired fermentation products. The most common fermentation modes are shown in Figure 2.4: batch, fed-batch, and continuous fermentation (Kacaribu and Darwin, 2024). Table 2.3 represents advantages and disadvantages of each process.

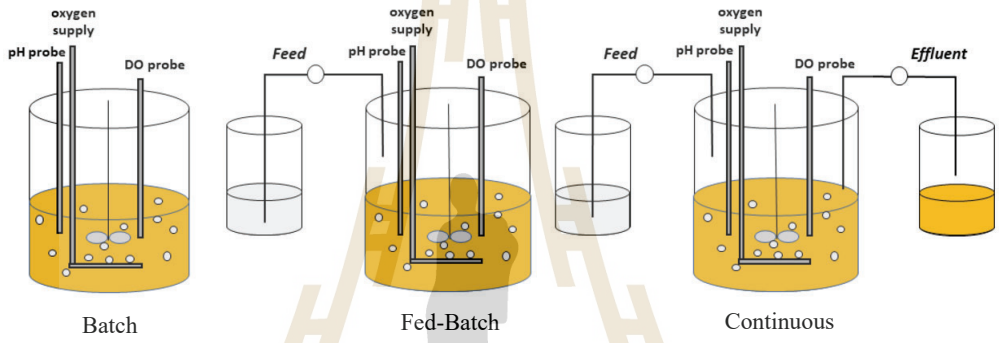


Figure 2.4 The modes of fermentation: batch, continuous, and fed-batch processes (Rajpurohit and Eiteman, 2022)

2.4.1 Batch fermentation

Batch fermentation is simpler and a closed system where all the nutrients, microorganisms, and substrates are all added at the beginning of process, therefore, only acid or alkaline is added during fermentation to control the pH. Likewise, the microorganisms grow through several phases, including the lag phase, exponential or log phase, stationary phase and death phase (Figure 2.5). The desired products are harvested when the operation is completed. Although this mode of fermentation shows lower productivity and inhibition by substrate or product accumulation, batch fermentation is widely used as it minimizes the contamination risk and can be operated easily (Rawoof et al., 2021).

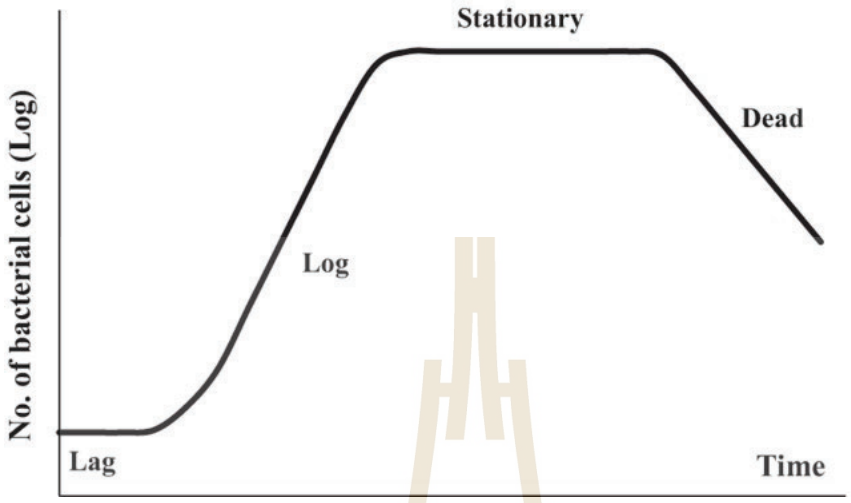


Figure 2.5 The growth curve of bacterial in culture medium (Wang et al., 2015)

2.4.2 Fed-batch fermentation

Fed-batch strategy is conducted to avoid substrate inhibition and prolonged productive phase of microorganism; hence, increment in product titer (Behl et al., 2023). The substrates are added into the fermenter during the operation continuously or in pulses over a period of time without removing the fermentation broth (Mohamed et al., 2021). This method is especially beneficial when substrate can inhibit cell growth or product formation at high concentrations; subsequently, it provides higher productivity and reduces operation times (Tang et al., 2010).

2.4.3 Continuous fermentation

Continuous fermentation is usually performed to extend the exponential phase and enhance productivity without increasing the broth volume. To do so, the fresh medium was continuously added into the fermenter tank while the cell broth is simultaneously removed from the bioreactor at the optimized flow rate and time (Sen and Roychoudhury, 2013). This method is often used in a large scale or industry, while it is not broadly used in laboratories (Mohamed et al., 2021).

Table 2.3 The advantages and disadvantages of each fermentation modes: batch, fed-batch, and continuous processes. Adapt from (Yang et al., 2024)

Fermentation modes	Advantages	Disadvantages
Batch	• Simple to operate • Low risk of contamination and strain mutation	• Relatively slow substrate utilization • Growth of microbes is limited by the nutrient concentration in the medium
Fed-batch	• High cell densities • Minimizes the inhibition of high concentrations of substrate and end-products.	• Longer duration • Slow response of cells to the changes in pH
Continuous	• High productivity and effectiveness • Less sterilization and re-inoculation • Easy to control automatically • Cells can be harvested throughout • Avoids the inhibition caused by a mass of substrate and the cells • Less maintenance cost	• Long-term fermentation may lead to blockages • Risk of contamination

2.5 *K. oxytoca* KIS004-91T

Klebsiella oxytoca is a gram-negative bacterium that is non-motile and rod-shaped in the family *Enterobacteriaceae*. It can be isolated from various tissues of clinically affected individuals and animals, as well as from the skin, mucous membranes, oropharynx, and intestines of both healthy humans and animals (Darby et al., 2014). *K. oxytoca* is extensively utilized on an industrial scale for producing biofuels and biochemicals because of its remarkable ability to efficiently utilize various substrates. *K. oxytoca* KIS004-91T strain was previously engineered to enhance D-(-)-lactic acid production via fermentation in a low nutrient medium. Initially, Sangproo et al., (2012) deleted the *adhE* (alcohol dehydrogenase) gene, responsible for ethanol synthesis, in the *K. oxytoca* M5a1 strain, resulting as the KMS002 strain (Δ*adhE*). Subsequently, the *pta-ackA* (phosphor transacetylase-acetate kinase A) genes, involved in acetyl-CoA and acetate production were deleted, yielding the strain KMS004 (Δ*adhE,* Δ*pta-ackA*). These modifications led to increased D-(-)-lactic acid production rates when using glucose and sugarcane molasses as substrates. Ethanol was not produced while other by-products such as succinate, formate, acetate, and butanediol were still detected in the fermentation broth using KMS002 (Δ*adhE*) and KMS004 (Δ*adhE,* Δ*pta-ackA*) strains. Even though the acetate kinase gene was removed in KMS004 (Δ*adhE,* Δ*pta-ackA*), formate and acetate were still the major by-products formation, likely due to the presence of the *pflB* (pyruvate-formate lyase) gene, which is responsible for converting pyruvate to acetyl-CoA and formate. Consequently, acetyl-CoA may be converted to acetate by alternate acetate kinase isoenzymes. In the anaerobic pathway, *K. oxytoca* KMS004 strain exhibited higher D-(-)-lactic acid titers and yields compared to the aerobic pathway, with reduced by-product formation. Under anaerobic conditions, glucose is converted to pyruvate, which can then be converted to D-(-)-lactic acid via lactate dehydrogenase A by using NADH. Alternatively, pyruvate can be converted to acetyl-CoA in which the wild type strain, can be further converted to ethanol and acetate. However, acetyl-CoA is solely converted to acetate due to deletions of *adhE* and *pta-ackA* genes in KMS004 strain. Additionally, fumarate

reductase plays a crucial role in the anaerobic metabolism of various microorganisms. This enzyme is responsible for catalyzing the reduction of fumarate to succinate, with fumarate acting as the terminal electron acceptor. The *frd* gene, encoding fumarate reductase, is mainly found in microorganisms such as *Escherichia coli* and *Klebsiella oxytoca*. Therefore, three main pathways for NADH oxidation are described, which involve the production of lactate, succinate, and ethanol via lactate dehydrogenase, fumarate reductase, and alcohol dehydrogenase, respectively; in the mixed-acid pathway (Zhang et al., 2009). Therefore, eliminating the *frd* gene could enable direct NADH oxidation towards lactic acid production rather than succinic acid production in the KMS004 strain. Thus, further enhancing D-(-)-lactic acid production in KMS004 strain by eliminating the *pflB* gene, responsible for formate and acetyl-CoA production, could potentially improve D-lactic acid yield by reducing the formation of other by-products. KMS004 (Δ*adhE,* Δ*pta-ackA*) strain was then further deleted *frd* and *pflB* gene to eliminate succinate-producing pathway and to decrease other by-product formation such as acetate and 2,3-butanediol, yielding KIS002 (Δ*adhE,* Δ*pta-ackA,* Δ*frd*) and KIS004 (Δ*adhE,* Δ*pta-ackA,* Δ*frd,* Δ*pflB*) strains, respectively. The fermentation pathway of *K. oxytoca* KIS004 strain are shown in Figure 2.6 (In et al., 2020). Under anaerobic conditions, KIS002 produced 35.4 g/l of D-(-)-lactic acid with yield of 0.75 g/g and productivity of 0.59 g/l/h without succinate, in AM1 medium containing 5% (w/v) glucose. Other by-products such as acetate and 2,3-butanediol, were still produced by KIS002 at concentrations 0.865 g/L and 0.12 g/L, respectively. After deletion of *pflB* gene, 45.2 g/L of D-(-)-lactic acid were produced by KIS004 in AM1 medium containing 5% (w/v) glucose under anaerobic conditions with a yield of 0.96 g/g and productivity of 0.47 g/L/h. Hence, acetate was not produced with KIS004 while lower level of 2,3-butanediol was found compared to KMS004 strain. In order to improve glucose utilization, metabolic evolution (Jantama et al., 2008), was conducted on the KIS004 strain. The KIS004 strain was cultured in LB medium containing 2% (w/v) glucose and transferred to a newly fresh AM1 medium containing 10% (w/v) glucose every 24 h until the mutant strain showed no further improvement. As KMS004 no longer

produces acetate due to the removal of pyruvate formate lyase, it has become auxotrophic for acetate. After the 18th transferred, metabolic evolution was conducted by supplementing 20 mM sodium acetate into the medium. The acetate concentration was gradually decreased along with the growth rate of strain until the strain grew efficiently without the need for external acetate supplementation. Consequently, glucose was completely utilized after the 91th transferred and 98.6 g/L of D-(-)-lactic acid were produced without any by-product formations, resulting as KIS004-91T strain. D-(-)-lactic acid was produced using *K. oxytoca* KIS004-91T strain at 101 g/L and 126 g/L from 10% glucose (w/v) in batch and fed-batch fermentations using glucose as a substrate, and was achieved 98.4 g/L from cassava starch under SHF condition (In et al., 2020). Examples of D-(-)-lactic acid production using *K. oxytoca* are shown in Table 2.4.

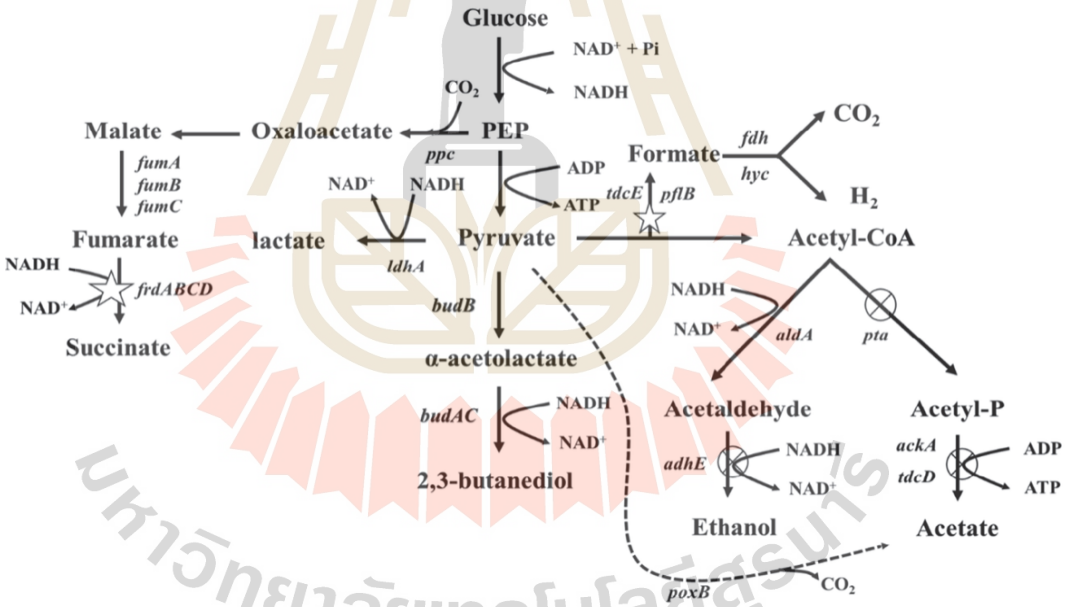


Figure 2.6 Metabolic pathway of *K. oxytoca* strain under anaerobic conditions (In et al., 2020). Solid arrows represent central fermentative pathways. Dot arrow represents an alternative acetate-producing pathway via *poxB*. Cross signs represent gene deletions previously performed in *K. oxytoca* KMS004 (Δ*adhE*Δ*ackA-pta*) strain. Star signs represent deletions of *frd* and *pflB* genes in *K. oxytoca* KIS004 (Δ*adhE*Δ*ackA-pta*Δ*frd*Δ*pflB*) strain.

Table 2.4 D-(-)-lactate production by *K. oxytoca* strain

Types of lactic acid	Microorganism	Substrate	Fermentation	Lactic acid titer (g/L)	Yield (g/g)	Productivity (g/L/h)	References
D-(-)	*K. oxytoca* KMS002	Maltodextrin	SHF	33.6	0.92	0.35	(Sangproo et al., 2012)
D-(-)	*K. oxytoca* KMS004	Maltodextrin	SHF	32.9	0.91	0.34	
D-(-)	*K. oxytoca* KIS004-91T	Sugarcane bagasse	Fed-batch SHF	101.0	0.99	1.94	(Gosalawit et al., 2024)
D-(-)	*K. oxytoca* KIS004-91T	Cassava starch	SHF	98.4	0.93	1.24	(In et al., 2020)

SHF: separate hydrolysis and fermentation

2.6 Lignocellulose biomass

Lignocellulose is an abundant and renewable source for biochemical fermentation due to its composition–cellulose and hemicellulose, about 70% (w/w), were observed. Thus, lignin links cellulose and hemicellulose with covalent and hydrogen bonds. Typically, cellulose is comprised of glucose monomers, which is condensed via ß-(1,4)-glycosidic bonds, forming microfibrils with hydrogen bonds, and linear chains of unbranched polymers. The hydrogen bonds and van der Waals forces in cellulose result in a highly crystalline and rigid structure of lignocellulose (Davison et al., 2013; Gavila et al., 2015). In contrast, hemicellulose is known as short-branched polymers and consists of pentose and hexose, including xylose, arabinose, glucose, mannose, and galactose, respectively. Thus, some uronic acids (acetyl, glucuronic acid, and arabinose side groups) were found in hemicellulose too (Kumar et al., 2020). Unlike cellulose, the crystallinity of hemicellulose is low and more easily hydrolyzed; whereas, it can be removed by sodium hydroxide or precipitated by acidic alcohol (Betts et al., 1991). Otherwise, lignin consists of aromatic polymers such as p-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol, forming p-hrdorxyphenyl (H), guaiacyl (G), and syringyl (S) units, respectively, generating highly branched and cross-linked polymers (Borrero-Lopez et al., 2022). The compositions of these units depend on the type of plants; for instance, hardwoods are mostly built with S and G units, softwoods mainly consist of G units, whereas grasses contain all H, G, and S units (Yankov, 2022). Cellulose and hemicellulose were packed together by hydrogen bonds; thus, lignin cross-linking between these two components via covalent bonds results in a recalcitrant structure and resistance of lignocellulose materials (Ufodike et al., 2020; Vasic et al., 2021). Table 2.5 shows the composition of different types of lignocellulose biomass, including cellulose, hemicellulose, and lignin contents.

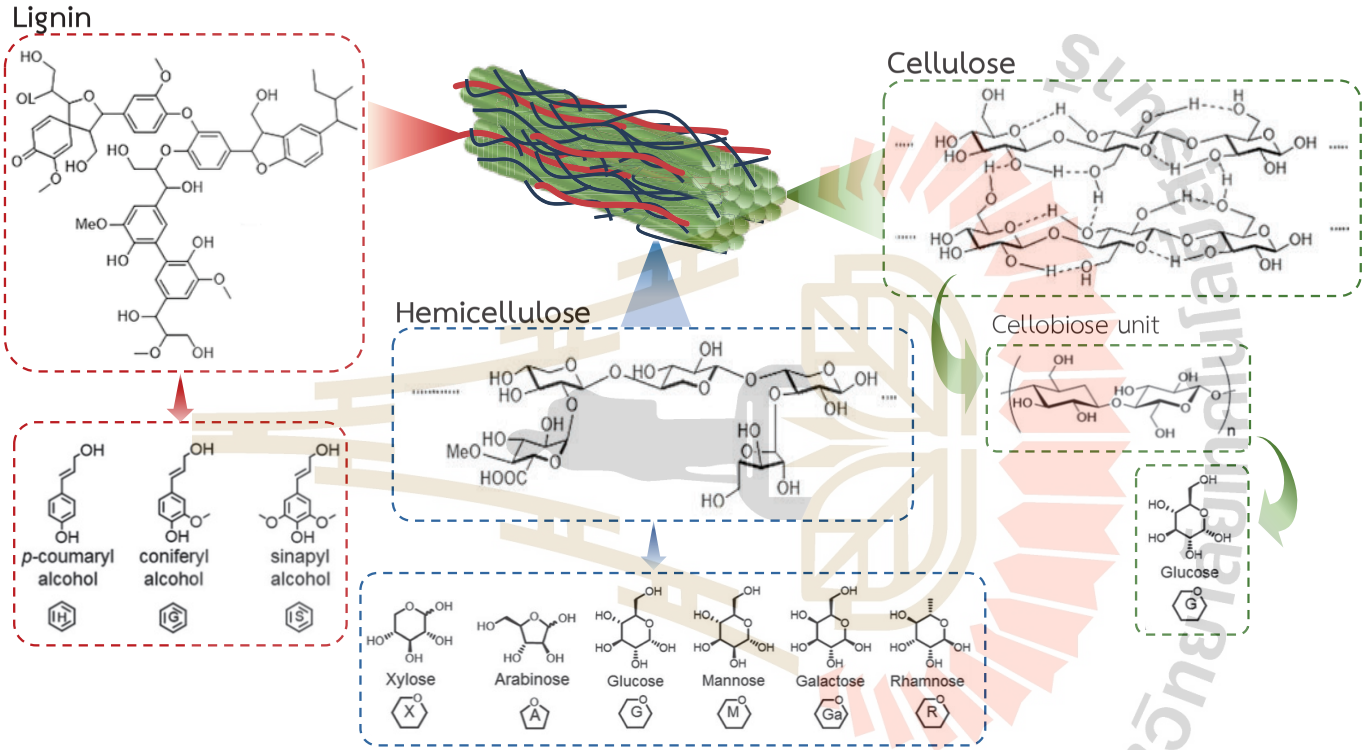


Figure 2.7 Lignocellulose structure and composition including cellulose, hemicellulose, and lignin. Adapted from (Isikgor and Becer, 2015)

Table 2.5 Different types of lignocellulose biomass and its composition

Type of lignocellulosic	Cellulose	Hemicellulose	Lignin	References
Pine sawdust	24.9	31.5	36.6	(Rapado et al., 2021)
Cassava bagasse	13.5	5.8	2.8	(Chen et al., 2020)
Rice husk	47.6	19.1	19.3	(Jaichakan et al., 2021)
Soybean hulls	35.8	23.1	9.1	(Rojas et al., 2014)
Wheat straw	41.1	37.5	13.5	(Grewal and Khare, 2018)
Barley hull	34	36	19	(Kim et al., 2008)
Cotton stover	38	23	20	(Wan and Li, 2010)
Napier grass	47	31	22	(Reddy et al., 2018)
Waste papers	65	13	1	(Chen et al., 2004)
Rice straw	38	32	12	(Lu and Hsieh, 2012)
Corn cob	41	31	12	(Chen et al., 2010)
Brewers spent grains	21.7	19.3	19.4	(Meneses et al., 2013)
Wheat	18.6	34.1	9.5	(Mikulski and Klosowski, 2018)
Pineapple leaves	72.76	17.15	4.76	(Nashiruddin et al., 2020)
Pineapple leaves	66.2	19.5	4.2	(Daud et al., 2014)

2.7 Pretreatment of lignocellulosic biomass

The complex network and resistance of lignocellulosic biomass was naturally attributed to prevent the accessibility of pathogens and enzymes. Therefore, pretreatment was performed to reduce the crystallinity and polymerization degree by breaking the covalent and hydrogen linkages of cellulose, hemicellulose, and lignin,

hence increasing the accessible surface area for further fermentable sugars conversion via enzymatic hydrolysis (Kumar et al., 2009; Vasic et al., 2021). Different methods of pretreatment, including physical, chemical, physiochemical, and biological, were employed to lignocellulose biomass for hemicellulose and lignin removal, thus limiting the degradation of cellulose (Jonsson and Martin, 2016). Generally, physical pretreatment such as chopping, grinding, and milling was conducted to reduce the size of biomass to the particle size as well as the crystallinity, improving digestibility of enzymes (Millett et al., 1976). Meanwhile, chemical pretreatment, including acid, alkaline, oxidative, and organic solvent, was differently applied to enhance enzyme accessibility by removing lignin or dissolving hemicellulose, thus disrupting cellulose structure. Acid pretreatments of lignocellulose are employed for hemicellulose and lignin solubilization, in which either concentrated or diluted acid can be used for acid hydrolysis (Sun and Cheng, 2002). Concentrated acid hydrolysis (30-70%) can be done at low temperature with normal atmosphere, whereas diluted acid hydrolysis (0.1-2%) requires high temperature and pressure to solubilize hemicellulose and lignin (Den et al., 2018). Using acid (HCl, H_2SO_4, H_3PO_4, and HNO_3) for pretreating biomass triggers inhibitors formation (furfural, HMF, and acetic acid) which require subsequent detoxification step (Himmel et al., 1997; Saha et al., 2005). Hence, safety equipment is required during acids pretreatment due to its corrosive and toxicity nature. Unlike acid hydrolysis, alkaline pretreatment using sodium, potassium, calcium, and ammonium are mainly target lignin, breaking ester linkages and disrupting the lignin-carbohydrate bonds (LCC) with partially dissolve hemicellulose while cellulose are remained intact. The solubilization of lignin and hemicellulose and swelling of cellulose offers larger interact surface area for subsequent enzymatic hydrolysis, increasing fermentable sugar formation (Behera et al., 2014). Maurya et al. (2015) mentioned alkaline pretreatment increases accessibility of enzyme to the cellulose due to the removal of acetyl group and uronic acid substitutions in hemicellulose during pretreatment, thus, NaOH was reported as the most effective base among all bases for pretreating lignocellulose (Kim et al., 2016). On the other hand, oxidative pretreatment such as addition of hydrogen

peroxide (H_2O_2) and peracetic acid ($C_2H_4O_3$) remove lignin and hemicellulose from biomass (Garcia-Cubero et al., 2009). Within this pretreatment, alkyl/ aryl ether bonds were cleaved off, disruption of side chained and lignocellulose structure (Maurya et al., 2015; Patel et al., 2023). Similarly, organosolv process could significantly remove lignin and hemicellulose while cellulose remained untouched (Sun and Chen, 2008). Organic solvents such as methanol, ethanol, acetone, and ethylene/ tri-ethylene glycol can be used in this method. Therefore, a proper removal of organic solvent after pretreated is necessary since most of them could inhibit enzyme activity during enzymatic hydrolysis (Mosier et al., 2005). Likewise, physiochemical methods including steam explosion, liquid hot water (LHW), and ammonia-based pretreatment (AFEX), improves enzyme digestibility via combination of physical forces and chemical reactions. These methods promote hemicellulose degradation and lignin disruption (Pan et al., 2005) in which inhibitors are formed accordingly, limiting the enzymatic hydrolysis and fermentation process (Oliva et al., 2003). Normally, biological pretreatment is employed for delignification and hemicellulose degradation using microorganism (mainly fungi). Even though, this method offers lower production cost, the lower hydrolysis rate with longer processing time is considered as disadvantages compared to other methods (Sanchez, 2009; Shi et al., 2008; Sun and Cheng, 2002). The advantages and disadvantages of different methods of pretreatment are shown in Table 2.6.

Table 2.6 The advantages and disadvantages of different methods of pretreatment. Adapted from (Maurya et al., 2015)

Pretreatment method	Advantages	Disadvantages
Milling/Grinding	• Decrease crystallization and degree of polymerization of cellulose • Increase surface area and pore size of biomass	• High power and energy consumption
Alkaline	• Lignin removal • Low inhibitor formation • Decrease cellulose crystallization	• High cost of alkaline catalyst
Concentrated acid	• High glucose yield • Ambient temperature	• High cost of acids catalyst • Corrosion-resistant equipments are required • Inhibitor formation
Diluted acid	• High sugars recovery • Hemicellulose solubilization • Low formation of toxic products	• Inhibitor formation • Detoxification step is required • Need high temperature and pressure

Table 2.6 The advantages and disadvantages of different methods of pretreatment. (continued)

Organosolv	• Hydrolysis of lignin and hemicellulose	• High cost • Solvent recovery and recycling are complicated
Steam explosion	• Cost effective • Hemicellulose solubilization and lignin transformation\ • High yield of glucose and hemicellulose in two-step method	• Generation of toxic compounds • Partial degradation of hemicellulose
Liquid hot water (LHW)	• No chemicals are required • Hemicellulose solubilization	• High energy and water are required • Formation of toxic compound
Ammonia fiber expansion (AFEX)	• Increase surface area • Less inhibitor formation	• Inefficiency of lignin removal • High cost of ammonia • Ammonia recycling is required

Table 2.6 The advantages and disadvantages of different methods of pretreatment. (continued)

Biological	• Cost effective • Delignification • Partial hydrolysis of hemicelluloses • No chemical requirements • Reduction in degree of polymerization of cellulose • Mild environmental condition	• Require amount of time for pretreatment • Lower productivity

2.8 Enzymatic hydrolysis

Enzymatic hydrolysis is a subsequent crucial step after pretreatment process to breakdown polysaccharide of lignocellulose biomass into fermentable sugars to be readily for fermentation step. Generally, this action is carried out under mild conditions in which the optimum temperature ranges between 40-50 °C and pH of 4.5-5.0 (Vasic et al., 2021). During hydrolysis, either cellulose or hemicellulose are cleaved off into simpler sugars such as glucose, cellobiose, and cello-oligosaccharides or xylose, mannose, fructose, and arabinose (Choi et al., 2020; Linton, 2020; Singh et al., 2003). Cellulases play an important role in depolymerization the crystallinity of cellulose by which endoglucanase initiate the hydrolysis of cellulose, randomly split the chains of amorphous region of cellulose by hydrolyzing ß-1,4-glucosidic bond. Meanwhile, exoglucanases cut down the chains of cellulose from the reducing and non-reducing ends of cellulose chains, producing cellobiose and glucose subunits. Then ß-glucosidase or cellobiase breaks cellobiose into two glucose molecules (Mohamed et al., 2011; A. Patel et al., 2019; Srivastava et al., 2018). Figure 2.8 shows the action mode of cellulase enzymes breakdown cellulose.

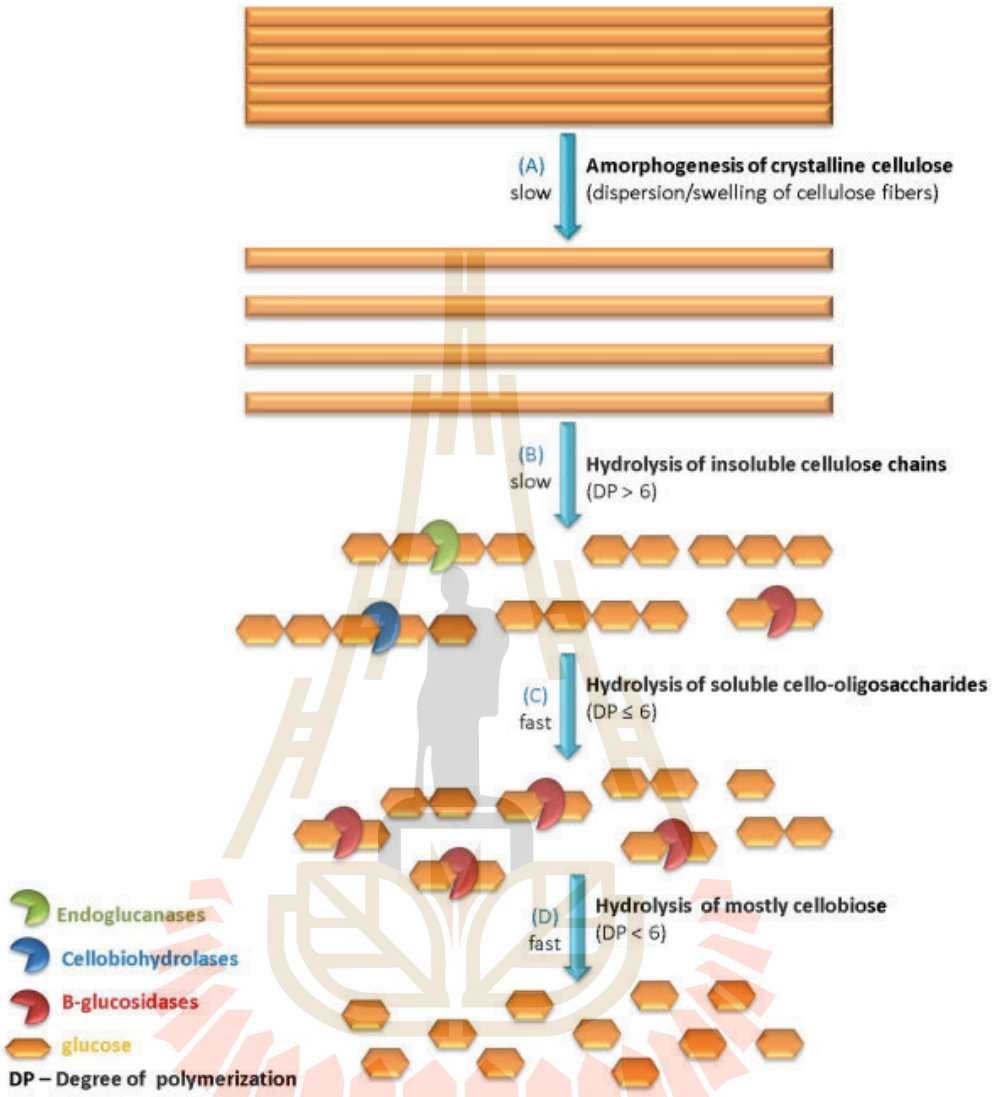


Figure 2.8 The hydrolysis of lignocellulose using cellulase enzymes (Arantes and Saddler, 2010)

2.9 Pineapple crown

Pineapple stands as a leading fruit globally, with Costa Rica, Philippines, Brazil, Thailand, and India serving as major producers. In recent years, the demand for pineapple and its derivatives has surged significantly. Pineapple waste increases not only from the consumption of fresh fruit, commercial juice, but also processed items like canned and frozen products during harvesting and processing methods (Mohd Ali

et al., 2020; Roda and Lambri, 2019). Pineapple crown, the leafy top of pineapple fruits, is considered as a major by-product originating from pineapple processing facilities since its weight around 10–25 percent of the fruit's total weight (Prado and Spinace, 2019). Pineapple crowns are an ideal substrate for lactic acid production owing to their abundance, low cost, and rich carbohydrate content, primarily consisting of glucose and fructose. It contains 79-83%, 19%, 5-15% and 1% of cellulose, hemicellulose, lignin and pectin, respectively (Choquecahua et al., 2020). As waste from pineapple processing industries, they offer a readily available and cost-effective source for fermentation processes. Additionally, pineapple crowns contain essential nutrients and minerals that support the growth and metabolism of lactic acid bacteria, promoting efficient fermentation and higher yields of lactic acid (Byresh et al., 2023). By purposing pineapple crowns for lactic acid production, environmental sustainability is also enhanced, as it helps divert organic waste from landfills, aligning with efforts to minimize resource wastage and promote sustainable practices in industrial processes.

CHAPTER III

MATERIALS AND METHODS

3.1 Strains, media, and growth condition

K. oxytoca KIS004-91T strain was previously engineered to enhance D-(-)-lactic acid production by deleting genes responsible for fumarate reductase (*frdABCD*) and pyruvate formate lyase (*pflB*) activities (In et al., 2020). Cultures were maintained on LB agar composed of 10 g tryptone, 5 g yeast extract, 20 g agar, and 5 g sodium chloride per 1 L distilled water. Seed cultures were prepared by inoculating a full loop of fresh colonies into LB broth supplemented with 2% (w/w) glucose and incubating overnight at 37°C with shaking at 200 rpm until reaching an optical density of approximately $OD_{550} \approx 3.0$. Throughout the fermentation experiments, a low-salts medium known as, Alfredo Martinez medium version 1 (AM1) (Martinez et al., 2007), was used. The AM1 components are shown in Table 3.1.

Table 3.1 Components of AM1 medium and trace metal

AM1	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	1.00
Trace metal	Concentration (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. $2H_2O$	1.24
H_3BO_3	1.21
$MnCl_2$. $4H_2O$	2.25
Total salts	4.1 g/L

KOH will be used to neutralize betaine-HCl stock

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

3.2 Alkaline pretreatment of pineapple crown

Pineapple crown (PIC) was obtained from Chin Huay Public Company Limited, Thailand. It was cut into small pieces (3–5 cm) and dried overnight in an air oven at 80°C. The dried PIC was then soaked in sodium hydroxide (NaOH) solutions of varying concentrations (0.25 N, 0.5 N, 0.75 N, 1 N, and 1.25 N) at 90°C for 90 minutes, using a solid-to-liquid ratio of 1:7 with periodically mixed every 30-minutes interval. Following the alkali treatment, the liquid fraction was discarded, and the solid fraction was thoroughly washed with tap water until the pH of the rinse water reached 7. The

washed, pretreated PIC was subsequently dried in an air oven at 65°C until a constant moisture content of less than 10% (w/w) was achieved. The dried, pretreated PIC was then stored in an airtight bag at ambient temperature until further use.

3.3 Enzymatic hydrolysis of pretreated pineapple crown

The dried pretreated-PIC was milled into particle size less than 40 µm. Therefore, the milled and pretreated PIC at the concentration of 2% (w/w, on dried basis) in AM1 medium was enzymatically hydrolyzed by a sterilized VRE P3 crude cellulase enzyme purchased from Siam Victory Chemicals Co., Ltd (Thailand) with a working volume of 50 ml at 50°C with the agitation speed of 200 rpm for 120 hours at different enzyme loadings (20, 40, 60, 80, and 100 PCU/g). Samples were collected every 12 hours for analyzing released fermentable sugars.

3.4 Total sugar hydrolysis using concentrated acid

The cellulose, hemicellulose, lignin, and ash contents of untreated and pretreated pineapple crown (PIC) were determined according to the method of Sluiter (2012). For total reducing sugar analysis, 4 g of substrate were hydrolyzed with 7 mL of 72% (w/w) sulfuric acid (H_2SO_4) at 55°C for 10 minutes with continuous agitation at 200 rpm. The acid mixture was subsequently diluted with deionized water to a final volume of 200 mL. The resulting solution was autoclaved at 121°C for 35 minutes to complete hydrolysis. After cooling to room temperature, samples were collected for the quantification of total sugars.

3.5 Lactic acid production from pretreated pineapple in mineral salts medium

3.5.1 Lactic acid production using separate hydrolysis and fermentation (SHF) technique

D-(-)-lactic acid fermentation was carried out in a 5 L bioreactor (Infors, Basel, Switzerland) with a working volume of 2 L. Batch separate hydrolysis and fermentation

(SHF) was performed by dissolving pretreated PIC in AM1 medium, followed by sterilization at 121°C for 20 minutes. Hydrolysis was initiated by adding crude cellulase at an enzyme loading of 60 PCU/g (equivalent to 3.75 mL/g substrate) and incubating the mixture at 50°C with agitation at 400 rpm for 24 h. After hydrolysis, the slurry was cooled to 37°C, and inoculation was performed at an initial optical density (OD_{550}) of 0.3 at pH 7.0. Fermentation was carried out at 37°C with agitation at 200 rpm, and pH was maintained at 7.0 by the automatic addition of 6 M KOH. The effect of varying concentrations of pretreated PIC (50, 70, 100, 125, and 150 g/L) on D-(-)-lactic acid production was also evaluated. All experiments were in triplicate.

3.5.2 Lactic acid production using simultaneous saccharification and fermentation (SSF) technique

D-(-)-lactic acid production was assessed under batch simultaneous saccharification and fermentation (SSF) conditions using pretreated PIC concentrations of 50, 75, and 100 g/L. In SSF, crude cellulase and inoculum were simultaneously added to the sterile PIC mixture, and fermentation was conducted at 40°C under the same conditions as SHF. All experiments were in triplicate.

3.5.3 Lactic acid production by fed-batch fermentation

Fed-batch fermentation under SHF conditions was performed to improve D-(-)-lactic acid concentration, yield, and productivity. An initial concentration of 50 g/L of pretreated PIC was used. During fermentation, a concentrated hydrolysate was fed into the bioreactor when the residual glucose concentration in the fermentation broth dropped to 10 g/L, raising the glucose concentration to approximately 25 g/L after feeding. All experiments were conducted in triplicate.

3.6 Analytical methods

3.6.1 Concentration of organic acids, sugars and by-products

Fermentation broth samples were collected every 4 hours to measure cell

biomass, organic acid concentrations, and sugar levels. The samples were analyzed using high-performance liquid chromatography (HPLC) with an ion exchange column (Aminex® HPX-87H, 7.8×300 mm, BioRad) and a refractive index detector (RI-1206, Thermo Spectra System, USA) at 65°C. A 4 mM sulfuric acid solution was used as the mobile phase at a flow rate of 0.6 mL/min. After fermentation, cultures were centrifuged to separate the cells from the supernatant, which was then filtered through a 0.2 µm nylon filter prior to HPLC analysis.

3.6.2 Cell biomass and cell viability

Cell dry weight (CDW) or biomass was determined by measuring the optical density at 550 nm (OD_{550}) using a Bausch & Lomb Spectronic 70 spectrophotometer. The relationship between OD_{550} and biomass was 1 OD_{550} = 0.333 g CDW/L (In et al., 2020). During fermentation, the viable cell count was determined by the spread plate technique on LB agar plates, which were incubated at 37°C for 24 hours. Colonies were counted and expressed as colony-forming units per milliliter (CFU/mL) of culture.

3.6.3 Calculations and statistical analysis

Statistical analysis was performed using GraphPad Prism 10.3 (GraphPad Software, Boston, MA, USA). Data were analyzed by one-way analysis of variance (ANOVA), and all values presented in the figures represent the averages ± standard deviations. Tukey's multiple range test at a 95% confidence level ($p < 0.05$) was used to identify significant differences among the mean values. The percentages of cellulose (%CS) and hemicellulose (%HS) saccharifications of the NaOH-pretreated PIC after the enzymatic hydrolysis were calculated according to equations below, where $C_{cellulose}$ and $C_{hemicellulose}$ are concentrations of cellulose and hemicellulose derived from the NaOH-pretreated PIC, and C_{glu} and $C_{xyl+ara}$ are concentrations of glucose and combined xylose and arabinose released from the NaOH-pretreated PIC during its hydrolysis.

$$\%CS = \frac{0.90 \times C_{glu}}{C_{cellulose}} \times 100 \quad \text{(Eq. 2)}$$

$$\%HS = \frac{0.88 \times C_{xyl+ara}}{C_{hemicellulose}} \times 100 \quad \text{(Eq. 3)}$$

D-(-)-lactic acid production yield (D-LA yield), D-(-)-lactic acid production gross yield (D-LA gross yield), and D-(-)-lactic acid productivity (D-LA productivity) were calculated using equations below, where C_{LA}, $C_{max.\ sugars\ released}$, $C_{sugar\ residue}$, and $C_{substrate}$ were concentrations of D-(-)-lactic acid produced, total sugars released from the NaOH-pretreated PIC digested with 60 PCU/g crude cellulase, sugars residue remained after fermentation, and the NaOH-pretreated PIC provided during fermentation, respectively.

$$\text{D-LA yield} = \frac{C_{LA}}{C_{max.\ sugar\ released} - C_{sugar\ residue}} \quad \text{(Eq. 4)}$$

$$\text{D-LA gross yield} = \frac{C_{LA}}{C_{Substrate}} \quad \text{(Eq. 5)}$$

$$\text{D-LA productivity} = \frac{C_{LA}}{\text{Fermentation duration}} \quad \text{(Eq. 6)}$$

CHAPTER IV
RESULTS AND DISCUSSION

4.1 Alkali pretreatment of pineapple crown

Pineapple crown (PIC), a top section of pineapple fruit (clustered leaves with stem), is an agro-industrial waste generated during pineapple processing that holds significant potential for conversion into high value-added biofuels and biochemicals through microbial fermentation, owning to its high polymeric sugar contents within its lignocellulosic structure. In this study, dried PIC fiber was found to contain 33.12±0.01% (w/w) cellulose, 13.18±0.01% (w/w) hemicellulose, 18.06±0.17% (w/w) lignin, 0.99±0.00% (w/w) ash, and 65.3±0.01% (w/w) extractives. These results indicate that dried PIC contains lower levels of cellulose and hemicellulose but a higher lignin content compared to pineapple leaves, as reported by Sethupathi et al. (2024), who found that pineapple leaves comprise 68.5-82.0% (w/w) cellulose, 18.0-18.8% (w/w) hemicellulose, 4.4-15.4% (w/w) lignin, and 0.9-2.7% (w/w) ash. Therefore, pretreatment is a crucial preparatory step to facilitate the release of the fermentable sugars from PIC by overcoming its lignocellulosic recalcitrance, enabling efficient conversion into D-(-)-lactic acid. An effective pretreatment process should achieve substantial lignin removal while minimizing the degradation of cellulose and hemicellulose and limiting the generation of inhibitory compounds, thereby improving the substrate's digestibility during enzymatic hydrolysis (Shukla et al., 2023). The application of sodium hydroxide (NaOH) as an alkaline solution to lignocellulosic biomass typically reduces the degree of cellulose crystallinity and polymerization, while expanding the surface area through the disruption of ester-linkage of lignocellulose within the lignocellulosic matrix (Ojo and de Smidt, 2023), thus enhancing accessibility during subsequent hydrolysis. In summary, the pretreatment by NaOH is considered as the effective method conferring low cost cooporation compared to those methods pretreating with other bases.

In this study, alkaline pretreatment of dried PIC was performed using sodium hydroxide (NaOH) at varying concentrations (0.25-1.25 N) performed at 90°C for 90 min. The solid recovery of the NaOH-pretreated PIC decreased significantly with increasing NaOH concentration, yielding 50.5±5.73%, 45.53±6.04%, 37.8±4.02%, 32.11±1.26%, and 30.38±1.54% (w/w) for 0.25, 0.5, 0.75, 1.0, and 1.25 N treatments, respectively, compared to 78.92±3.11% (w/w) in the hot water treatment as a control experiment (Figure 4.1). Thus, pretreatment was investigated with the solid to liquid ratio of 1:7 due to the solid recovery from control condition (78.91±3.11%), resulted in no significant difference to those from with solid to liquid ratio of 1:10 (76.48±1.03%). The highest NaOH concentration (1.25 N) led to the greatest solid loss (69.17±1.54 g per 100 g of the NaOH-pretreated PIC), likely due to the disruption of lignin-cellulose-hemicellulose linkages, swelling of cellulose fibers, and solubilization of lignin into the liquid fraction. These also collectively enhanced biomass surface area and reduced its crystallinity and degree of polymerization (Khan et al., 2021). For comparison, hot water pretreatment (90°C for 90 min) as a control experiment resulted in a 21.08±3.11% (w/w) solid loss, possibly due to autohydrolysis caused by the formation of hydronium ions (H_3O^+) at elevated temperatures. This weak acid effect can catalyze glycosidic bond cleavage in cellulose and hemicellulose and partially fragment lignin. Sun et al. (2022a) similarly reported that hot water pretreatment disrupts the lignocellulosic matrix by solubilizing hemicellulose and modifying lignin structure, thereby reducing solid recovery. Likewise, Chen et al. (2018) observed a marked decrease in solid recovery of hydrothermally pretreated wheat straw from 83.53% to 59.77% when pretreatment temperature was increased from 120°C to 200°C.

To evaluate fermentable sugar yields, crude cellulase was applied at a loading of 40 PCU/g to hydrolyze 20 g/L of the NaOH-pretreated PIC samples from each pretreatment condition. As shown in Figure 4.1, the total sugar concentrations released were 9.7±0.28 g/L (48.5±0.01%, w/w), 10.8±0.60 g/L (54.0±0.03%, w/w), 12.5±1.23 g/L (62.5±0.06%, w/w), 12.3±0.39 g/L (61.5±0.02%, w/w), and 12.7±0.37 g/L (63.5±0.02%, w/w) for the PIC pretreated by NaOH concentrations of 0.25, 0.5, 0.75, 1.0, and 1.25 N,

respectively. In contrast, hot water-pretreated PIC (the control condition) only yielded 8.3±0.39 g/L of total sugars, equivalent to a 41.5% (w/w) sugar recovery. Notably, sugar release reached maximum beyond 0.75 N NaOH, indicating that further increases in alkali concentration did not significantly enhance sugar yield. This observation aligns with the findings of Loow et al. (2016), who reported that excessive NaOH concentration or prolonged pretreatment time led to over-disruption of biomass structure, reducing cellulose retention and sugar conversion efficiency. Similarly, Sawisit et al. (2018) reported a decline in cellulose recovery from rice straw pretreated with NaOH concentrations above 1.0 N, while the total reducing sugar yield remained largely unaffected. Han et al. (2012) also reported a decrease in sugar yield from 350 mg/g to 280 mg/g of pretreated wheat straw when 1.0 N NaOH pretreatment was extended beyond 1.5 hours at 121°C. These results highlight the importance of optimizing pretreatment conditions to balance lignin removal with carbohydrate preservation for effective enzymatic hydrolysis. Surprisingly, Nashiruddin et al. (2020) though demonstrated that optimal pretreatment of pineapple leaves was achieved with 2.43% (w/v) NaOH at 87°C for 57.15 minutes, yielding 17.26 g/L of reducing sugars, a 33% improvement over a nonoptimized condition at 1.5 N NaOH pretreatment. This discrepancy may be attributed to differences in the lignocellulosic biomass source.

Based on the results of this study, the optimal pretreatment condition for pineapple crown (PIC) was achieved using 0.75 N NaOH at 90°C for 90 minutes with a solid-to-liquid ratio of 1:7. Under this condition, the NaOH-pretreated PIC exhibited 50.13±0.00% cellulose, 21.08±0.00% hemicellulose, 12.94±0.24% lignin, and 0.33±0.00% ash (w/w). This pretreatment effectively removed 28.35% of lignin and 66.67% of ash, while increasing cellulose and hemicellulose contents by 51.35% and 59.93%, respectively, compared to untreated PIC. Similarly, Asgher et al. (2013) reported a 48.7% lignin removal from sugarcane bagasse using 5% (w/v) NaOH at 121°C for 30 minutes. Additionally, inhibitors including, fufural, 5-HMF, and acetate were observed less than 1 g/L in total analyzed via the complete hydrolysis with 72% H_2SO_4. These results support the role of hydroxide ions (Na^+) in disrupting lignin structures by

cleaving ester and ether linkages in lignin–carbohydrate complexes (LCCs), thereby promoting lignin solubilization. In contrast, Saini et al. (2022) found that hydrothermal pretreatment of pineapple leaves at 150°C for 20 minutes led to increased xylan (19.7±0.42%, w/w) and lignin (19.7±2.16%, w/w) contents, but decreased glucan (33.55±5.10%, w/w), relative to untreated leaves (10.88±0.35% xylan, 14.2±0.42% lignin, and 56.90±2.10%, w/w glucan). This suggests that hydrothermal pretreatment may result in undesirable degradation of cellulose into glucose. Future studies may focus on refining hydrothermal conditions to maximize lignin removal while preserving cellulosic sugars. In comparison, the 0.75 N NaOH pretreatment method presented here appears to be a more effective preparatory step for subsequent enzymatic hydrolysis, especially when targeting glucose as the primary carbon source (Figure 4.1).

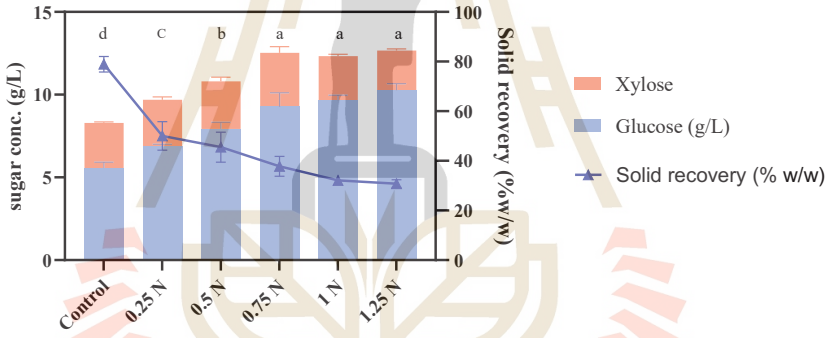


Figure 4.1 Glucose and xylose released from pretreated PIC with different concentration of NaOH (0.25-1.25 N) and hot water pretreatment as a control. Crude cellulase loading at 40 PCU/g was used for enzymatic saccharification of the NaOH-pretreated PIC. The percentage of solid recovery was calculated by dividing gram of the NaOH-pretreated PIC remained after enzymatic hydrolysis with gram of the untreated PIC provided.

4.2 Optimization of enzymatic hydrolysis of pretreated PIC

The enzymatic conversion of NaOH-pretreated PIC (20 g/L) into fermentable sugars was evaluated using varying concentrations (20-100 PCU/g) of crude cellulase

cocktail (VRE P3) at 50°C with 200 rpm agitation over 120 h. Under these conditions, the cellulase complex facilitated the release of glucose and xylose from cellulose and hemicellulose, respectively. Glucose was identified as the predominant sugar released at all enzyme loadings after 120 h of hydrolysis (Figure 4.2A). Total sugar concentrations reached 10.58±0.11 g/L (52.9±0.01%, w/w), 12.50±1.23 g/L (62.5±0.06%, w/w), 13.69±0.36 g/L (68.5±0.02%, w/w), 13.93±0.17 g/L (69.6±0.01%, w/w), and 14.03±0.10 g/L (70.2±0.01%, w/w) at enzyme loadings of 20, 40, 60, 80, and 100 PCU/g, respectively. As shown in Figure 4.2B, the sugar release plateaued after 24 h, with no significant differences at higher enzyme loadings beyond 60 PCU/g. Notably, the total sugar yield at 60 PCU/g was approximately 96.1% of that obtained via complete acid hydrolysis with 72% (w/v) H_2SO_4, which released 14.24±0.07 g/L (71.2±0.00%, w/w). At this optimal enzyme loading, cellulose (%CS) and hemicellulose (%HS) saccharification efficiencies reached 89.77% and 76.81%, respectively. At enzyme loadings of 20, 40, and 60 PCU/g, cellobiose was detected at 12 h hydrolysis and completely hydrolyzed after 72, 36, and 24 h, respectively; suggesting that crude cellulase cocktails contain β-glucosidase activity. However, increasing the cellulase concentration above 60 PCU/g slightly enhanced hemicellulose saccharification but led to a reduction in cellulose saccharification (Table 4.1), likely due to product inhibition, inefficient enzyme utilization, or non-productive enzyme binding.

Kinnarinen and Hakkinen (2014) reported a decline in hydrolysis efficiency when enzyme dosages exceeded the optimal level. Effective cellulose degradation requires the synergistic action of endoglucanase, cellobiohydrolase, and β-glucosidase. While endoglucanase and cellobiohydrolase are prone to feedback inhibition by accumulated cellobiose, β-glucosidase mitigates this by converting cellobiose to glucose. However, elevated glucose concentrations may inhibit β-glucosidase activity (Liu et al., 2023). Furthermore, excessive enzyme loadings can impair substrate accessibility due to enzyme overloading on the biomass surface. Residual lignin in the pretreated PIC may also reduce hydrolytic efficiency by adsorbing enzymes non-productively through hydrophobic or electrostatic interactions (Wu et al., 2023b; Yuan

et al., 2021). Similarly, Sawisit et al. (2018) optimized the enzymatic saccharification of pretreated rice straw using 1–6% (v/w) cellulase complex. Sugar yields significantly increased up to 4% (v/w) enzyme loading, beyond which no further enhancement was observed. The highest total sugar yield of 75.7% (w/w), with 82.0% CS and 35.2% HS, was achieved at 4% (v/w) enzyme loading. Zhu et al. (2023) also demonstrated that enzymatic hydrolysis of pretreated garden waste (grass, leaves, branches) with 10 FPU cellulase/g substrate and 7.5 U β-glucosidase yielded a glucose conversion rate of 60.12% (w/w). However, increasing cellulase loading to 20 FPU/g without adjusting β-glucosidase levels led to a decrease in glucose conversion (56.2% w/w) and a significant drop in cellobiose conversion (39.0% w/w). These findings highlight that cellulase overload, without an adequate β-glucosidase supply, may restrict hydrolysis efficiency due to enzyme overloading, non-productive binding, and progressive inhibition by cellobiose.

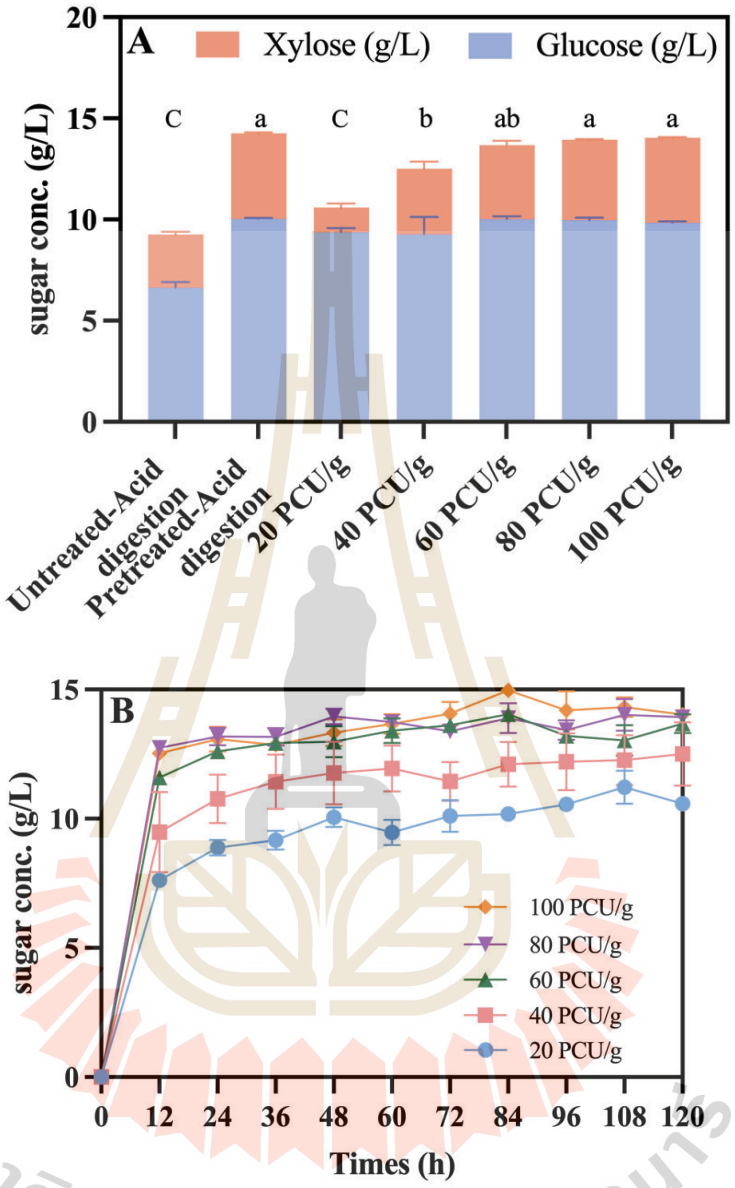


Figure 4.2 **(A)**: Glucose and xylose conversion from 20 g/L pretreated PIC using different crude cellulase loadings (20-100 PCU/g) compared to the acid hydrolysis by 72% H_2SO_4 of untreated and pretreated PIC samples, **(B)** Effect of incubation time on total sugar released from 20 g/L pretreated PIC during hydrolysis with different crude cellulase loadings.

Table 4.1 %CS, %HS, and sugars released from different cellulase concentrations usage during enzymatic saccharification of 20 g/L pretreated-PIC and completed hydrolysis of untreated and pretreated PIC by 72% H_2SO_4

Cellulase cocktail (PCU/g)	%CS	%HS	Glucose (g/L)	Xylose (g/L)	Total sugars (g/L)
20	83.86±2.10 [b]	25.76±4.08 [f]	9.35±0.23 [a]	1.24±0.20 [c]	10.58±0.11 [c]
40	83.01±7.93 [b]	67.83±7.43 [d]	9.25±0.88 [a]	3.25±0.36 [bc]	12.50±1.23 [b]
60	89.77±1.41 [a]	76.81±4.30 [c]	10.0±0.16 [a]	3.68±0.21 [ab]	13.69±0.36 [ab]
80	89.34±1.19 [a]	82.91±0.91 [b]	9.96±0.13 [a]	3.98±0.04 [ab]	13.93±0.17 [a]
100	88.97±0.77 [a]	87.90±0.72 [a]	9.82±0.09 [a]	4.22±0.03 [a]	14.03±0.10 [a]
		72% Conc. H_2SO_4			
Untreated-PIC	59.43±2.47 [c]	54.91±2.65 [e]	6.62±0.28 [b]	2.64±0.13 [c]	9.26±0.22 [c]
Pretreated-PIC	89.97±0.42 [a]	87.93±1.23 [a]	10.03±0.05 [a]	4.22±0.66 [a]	14.24±0.07 [a]

Lower-case letters indicate the significant differences between mean values of three replicates ($p \leq 0.05$) in the same column.

4.3 D-(-)-lactic acid fermentation from pretreated PIC under separate hydrolysis and fermentation

The separate hydrolysis and fermentation (SHF) process was employed to evaluate the effect of varying concentrations of NaOH-pretreated PIC (50, 75, 100, 125, and 150 g/L) on D-(-)-lactic acid production by *Klebsiella oxytoca* KIS004-91Tstrain. The pretreated PIC was initially hydrolyzed using a crude cellulase cocktail (60 PCU/g) at 50°C and 400 rpm. After 24 hours of enzymatic saccharification, the temperature was reduced to 37°C, and a pre-culture of *K. oxytoca* KIS004-91T strain was inoculated into the fermenter. At 50 g/L pretreated PIC (corresponding to initial concentrations of 25.06±0.77 g/L glucose and 7.75±0.49 g/L xylose), glucose consumption immediately started within 4 h after inoculation and was completely depleted by 16 h. D-(-)-lactic acid production rapidly increased from 4 to 12 hours and reached a maximum concentration of 25.36±0.36 g/L within 24 hours. This corresponded to a yield of 0.96±0.03 g/g total fermentable sugars consumed (equivalent to 0.51±0.00 g/g pretreated PIC), with a maximum productivity of 2.00±0.04 g/L/h (Figure 4.3A). By the end of fermentation, only trace amounts of by-products were detected, including 2,3-butanediol (0.91 g/L) and acetate (0.42 g/L) (Table 4.2). Despite complete glucose consumption, xylose remained unutilized, suggesting that carbon catabolite repression (CCR) was in effect where glucose is preferentially metabolized over other sugars. Although *K. oxytoca* M5A1, the parent strain of KIS004-91T strain, is capable of metabolizing various sugars (e.g., glucose, xylose, and fructose), Phosriran et al. (2024) demonstrated that CCR remained active in the KIS004-91T strain. In their study, xylose and arabinose were not utilized even after glucose depletion. Genomic analysis revealed spontaneous mutations in genes related to sugar transport and metabolism, including those involved in CCR, glycolysis, and the hexose monophosphate (HMF) pathway. These mutations likely resulted from adaptive evolution to enhance glucose-based D-(-)-lactic acid production under selective pressure from high-glucose (100 g/L) cultivation conditions. Similar result was found when 75 g/L of pretreated PIC was provided. Xylose utilization remained negligible even when fermentation was

extended to 28 hours while glucose consumption was completed at 20 hours incubation (Figure 4.3B). Although D-(-)-lactic acid production increased by 31.3% to a maximum of 33.53±1.53 g/L, the yield (0.76±0.05 g/g sugars consumed) and gross yield (0.43±0.01 g/g pretreated PIC) declined by approximately 20.8% and 15.7%, respectively, compared to the 50 g/L pretreated PIC process. Nonetheless, the maximum productivity of 1.95±0.05 g/L/h was not significantly different (Table 4.2).

For the 100 g/L NaOH-pretreated PIC, D-(-)-lactic acid production was rapid between 4 and 20 hours of fermentation, gradually increasing until 28 hours (Figure 4.3C). This trend was consistent with glucose utilization, which was completely exhausted within 28 hours. As a result, D-(-)-lactic acid reached 45.69±1.16 g/L, with a yield of 0.81±0.02 g/g sugars consumed, a gross yield of 0.46±0.01 g/g pretreated PIC, and a maximum productivity of 1.92±0.35 g/L/h. Only 2,3-butanediol (0.74±0.10 g/L) was detected as a by-product (Table 4.2). Similar to other conditions, after glucose depletion, D-(-)-lactic acid production became negligible, and xylose remained unconsumed, accumulating at 11.65±0.07 g/L by the end of fermentation. This suggests that the cells entered the death phase due to the depletion of glucose, indicating that *K. oxytoca* KIS004-91T strain utilized glucose as its primary energy source, despite the presence of xylose. Although D-(-)-lactic acid production increased by 42.2% compared to the 75 g/L pretreated PIC condition, the yield and maximum productivity were not significantly different. This phenomenon is consistent with the high glycolytic flux, which maintains a high NADH/NAD+ ratio. D-(-)-lactic acid production is prioritized rather than those of 2,3-BDO and acetate productions because it regenerates NAD+ and produces ATP, ensuring cellular redox balance and avoiding overflow metabolism (In et al., 2020; Jain et al., 2012; Wei et al., 2013).

For 125 g/L pretreated PIC, fermentation was extended to 56 hours, and D-(-)-lactic acid production reached 49.25±2.72 g/L, with a yield of 0.69±0.04 g/g sugars consumed, a gross yield of 0.40±0.01 g/g pretreated PIC, and a maximum productivity of 1.63±0.07 g/L/h (Table 4.2). Glucose consumption was also found to be rapid up to 24 hours and was completely exhausted by 48 hours, while xylose remained

throughout the 56-hour fermentation (Figure 4.3D). Compared to the 100 g/L pretreated PIC, D-(-)-lactic acid production increased by 7.8%, but both maximum productivity and conversion yield decreased by 15.1% and 14.8%, respectively. When the initial pretreated PIC concentration was raised to 150 g/L, fermentation was further extended to 76 hours. Glucose was consumed rapidly up to 44 hours and gradually decreased until 76 hours, leaving 2.70±2.08 g/L glucose residue. D-(-)-lactic acid reached 53.26±4.23 g/L, with a yield of 0.64±0.04 g/g sugars consumed, a gross yield of 0.36±0.03 g/g pretreated PIC, and a productivity of 1.21±0.09 g/L/h (Table 4.2). Although D-(-)-lactic acid production increased by 8.1% compared to 125 g/L pretreated PIC, the maximum productivity decreased significantly by 25.8%. No acetate was detected, but 2,3-butanediol production increased to 1.74±0.09 g/L. This shift in by-product formation indicates metabolic overflow, which is likely a result of high glucose concentrations. Excessive pyruvate and NADH may drive the production of 2,3-butanediol to maintain the NAD+/NADH balance, as *K. oxytoca* KIS004-91T strain lacks the pathways for succinate and acetate production (In et al., 2020; Sun et al., 2022b).

Overall, D-(-)-lactic acid production was 2.10 times higher when pretreated PIC concentrations increased from 50 g/L to 150 g/L; however, maximum productivity dropped by 39.5%. This decrease in productivity likely causes from increased broth viscosity, which impairs mixing efficiency and generates osmotic pressure imbalances, thereby reducing substrate-consumption rates and limiting cell growth during fermentation (Lin et al., 2008; Yankov, 2022). Compared to previous studies, Alrumman (2016) reported a maximum of 27.8 g/L D-(-)-lactic acid produced from the date palm hydrolysate (35 g/L initial sugars) by *Lactobacillus delbrueckii* subsp. *lactis*, with a yield of 0.76 g/g glucose and a productivity of 0.39 g/L/h. Bustamante et al. (2020) produced 45 g/L D-(-)-lactic acid from orange peel waste hydrolysate (70 g/L total sugars) using *L. delbrueckii* ssp. *Delbrueckii* CECT 286, achieving a yield of 0.86 g/g and a productivity of 0.63 g/L/h. However, in both studies, glucose was not fully consumed, even after 72 hours of fermentation. In contrast, the results demonstrate that *K. oxytoca* KIS004-91T can efficiently produce D-(-)-lactic acid, reaching 45.7 g/L from 100 g/L pretreated

PIC with significantly higher productivity and comparable yield.

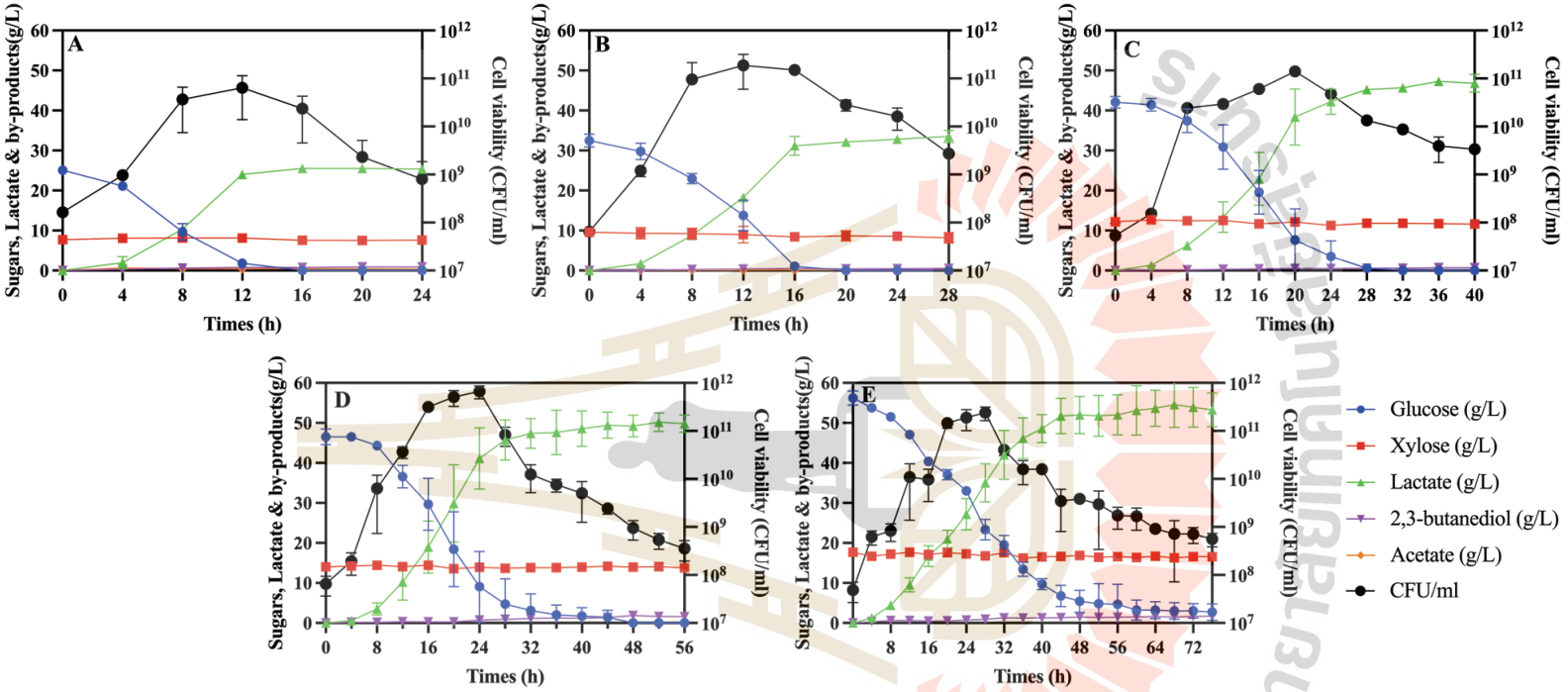


Figure 4.3 D-lactic acid production under SHF with different initial concentration of pretreated PIC. **(A)** 50 g/L, **(B)** 75 g/L, **(C)** 100 g/L, **(D)** 125 g/L, and **(E)** 150 g/L pretreated PIC. Cell viability was determined by spread plate technique.

4.4 D-(-)-lactic acid fermentation from pretreated PIC under simultaneous saccharification and fermentation

Considering production costs, D-(-)-lactic acid production by *K. oxytoca* KIS004-91T strain was evaluated using the simultaneous saccharification and fermentation (SSF) process, aimed at reducing both production time and the impact of glucose inhibition. The process was conducted at 40°C with constant agitation at 200 rpm. The cellulase cocktail and pre-cultured *K. oxytoca* KIS004-91T strain were inoculated simultaneously into the fermentation vessel. The fermentation was maintained at 40°C, and the pH was controlled at 7.0 by automatic addition of 6M KOH. Initial substrate concentrations were varied between 50, 75, and 100 g/L to identify the optimal conditions while minimizing processing time.

As shown in Figure 4.4A, D-(-)-lactic acid was steadily produced from 4 to 16 hours of fermentation, with a slight increase until 36 hours. The final concentration of D-(-)-lactic acid reached 20.73±0.54 g/L, with a yield of 0.67±0.03 g/g sugars consumed (or 0.42±0.01 g/g pretreated PIC) and a productivity of 1.19±0.04 g/L/h (Table 4.2). Glucose was first detected after 4 hours of incubation, rapidly decreasing until 20 hours, while xylose was released and maintained a steady level until 36 hours, reaching 4.53±0.01 g/L. At the end of fermentation, by-products 2,3-BDO and acetate were detected at concentrations of 0.36±0.05 g/L and 0.18±0.04 g/L, respectively. Despite glucose exhaustion, D-(-)-lactic acid production did not significantly increase from 20 to 36 hours, confirming that glucose remained the primary energy source for the strain even in the presence of xylose. For 75 g/L pretreated PIC (Figure 4.4B), both glucose and xylose were detected after 4 hours of incubation. Glucose levels dropped after 8 hours and were completely consumed by 32 hours, while xylose concentration increased rapidly until 60 hours of fermentation. The maximum D-(-)-lactic acid concentration of 34.23±1.01 g/L was reached at 60 hours. By-products, 2,3-BDO (0.57±0.12 g/L) and acetate (0.83±0.25 g/L), were also generated. However, D-(-)-lactic acid production remained stable from 28 to 60 hours, indicating that no further glucose was released after 28 hours. The maximum productivity of 1.07±0.03 g/L/h was

achieved with a yield of 0.70±0.03 g/g sugars consumed and a gross yield of 0.42±0.03 g/g pretreated PIC (Table 4.2). For 100 g/L pretreated PIC (Figure 4.4C), glucose was released at higher levels than in the previous conditions at 4 hours of incubation, but D-(-)-lactic acid production was delayed until 16 hours. D-(-)-lactic acid production sharply increased from 16 to 44 hours, while glucose levels dropped significantly from 24 to 32 hours, indicating simultaneous glucose release and utilization for D-(-)-lactic acid production. After extending fermentation to 60 hours, the maximum D-(-)-lactic acid concentration reached 40.75±3.93 g/L. By-products, acetate (0.68±0.27 g/L) and 2,3-BDO (0.79±0.50 g/L), were also produced. The maximum productivity of D-(-)-lactic acid was 0.87±0.11 g/L/h, with a yield of 0.62±0.08 g/g sugars consumed and a gross yield of 0.38±0.05 g/g pretreated PIC (Table 4.2). In all conditions, the gradual increase of xylose residue (Figure 4.4) indicated that only glucose was utilized for D-(-)-lactic acid production. Feng et al. (2017) suggested that using a mixed sugar source, including xylose or arabinose, leads to decreased D-(-)-lactic acid yield and concentration. Kim et al. (2010) also noted that the carbon catabolite repression (CCR) effect can hinder the co-utilization of sugars, suggesting that glucose released during SSF represses the utilization of other sugars.

In comparison to the SHF process, D-lactic acid concentration in the SSF process was significantly reduced by 18.8%, 2.0%, and 16.4%, while productivity was 40.5%, 45.1%, and 54.7% lower, respectively, for the same initial substrate concentrations. This decrease is attributed to carbon starvation, which results from a lower saccharification rate of cellulose and hemicellulose, causing an imbalance in redox balance and insufficient ATP for cell metabolism and maintenance (Khunnonkwao et al., 2023). The optimal condition for cellulase activity is generally at 50°C and pH 5.0 (Rawoof et al., 2021); however, SSF process was conducted at 40°C and pH 7.0 to support the optimal growth of *K. oxytoca* KIS004-91T strain. This adjustment may have limited the hydrolysis efficiency, thus slowing sugar release and leading to incomplete hydrolysis. The reduction in enzymatic hydrolysis efficiency was reflected in the lower concentration of xylose accumulation at the end of fermentation

in the SSF process (4.53±0.01, 5.53±0.88, and 5.86±2.89 g/L) compared to the SHF process (7.61±0.57, 8.16±1.30, and 11.65±0.07 g/L). Consequently, D-lactic acid production yields were significantly reduced in the SSF process, with decreases of 30.2%, 7.9%, and 23.4% for the initial substrate concentrations of 50, 75, and 100 g/L pretreated PIC, respectively. These results suggest that the increased substrate concentration with simultaneous inoculation of both crude cellulase cocktail and pre-culture in a single step induced high osmotic pressure and shear stress, leading to inefficient mixing due to the high solid-to-liquid ratio (Nguyen et al., 2017; Pratt et al., 2003). Congthai et al. (2025) observed a 33.4%, 66.7%, and 32.6% reduction in succinate production, yield, and productivity, respectively, when acid-pretreated mixed office papers were processed by SSF compared to SHF, due to insufficient sugar availability and inhomogeneous mixing. Even when a 4 h pre-saccharification step was incorporated to reduce shear stress and viscosity, succinate production was still 12.80% lower in SSF compared to SHF, with an initial concentration of 70 g/L of pretreated mixed waste papers, highlighting inefficient hydrolysis. Similarly, the high viscosity of the broth and inefficient saccharification in the biomass slurry contributed to lower SSF process efficiency (Unrean and Khajeeram, 2016). Gosalawit et al. (2024) also reported a decrease of 11.8%, 20.4%, and 12.6% in D-lactic acid concentration, yield, and productivity, respectively, via SSF compared to SHF processes using cassava starch by *Kluveromyces marxianus*. In summary, the results suggest that an initial substrate concentration of 75 g/L pretreated PIC is optimal for the SSF process, as it provided comparable D-lactic acid concentrations and yields to those from SHF, while reducing the total processing time to 32 hours, compared to 44 hours for the SHF process (24 hours for pre-saccharification and 20 hours for fermentation).

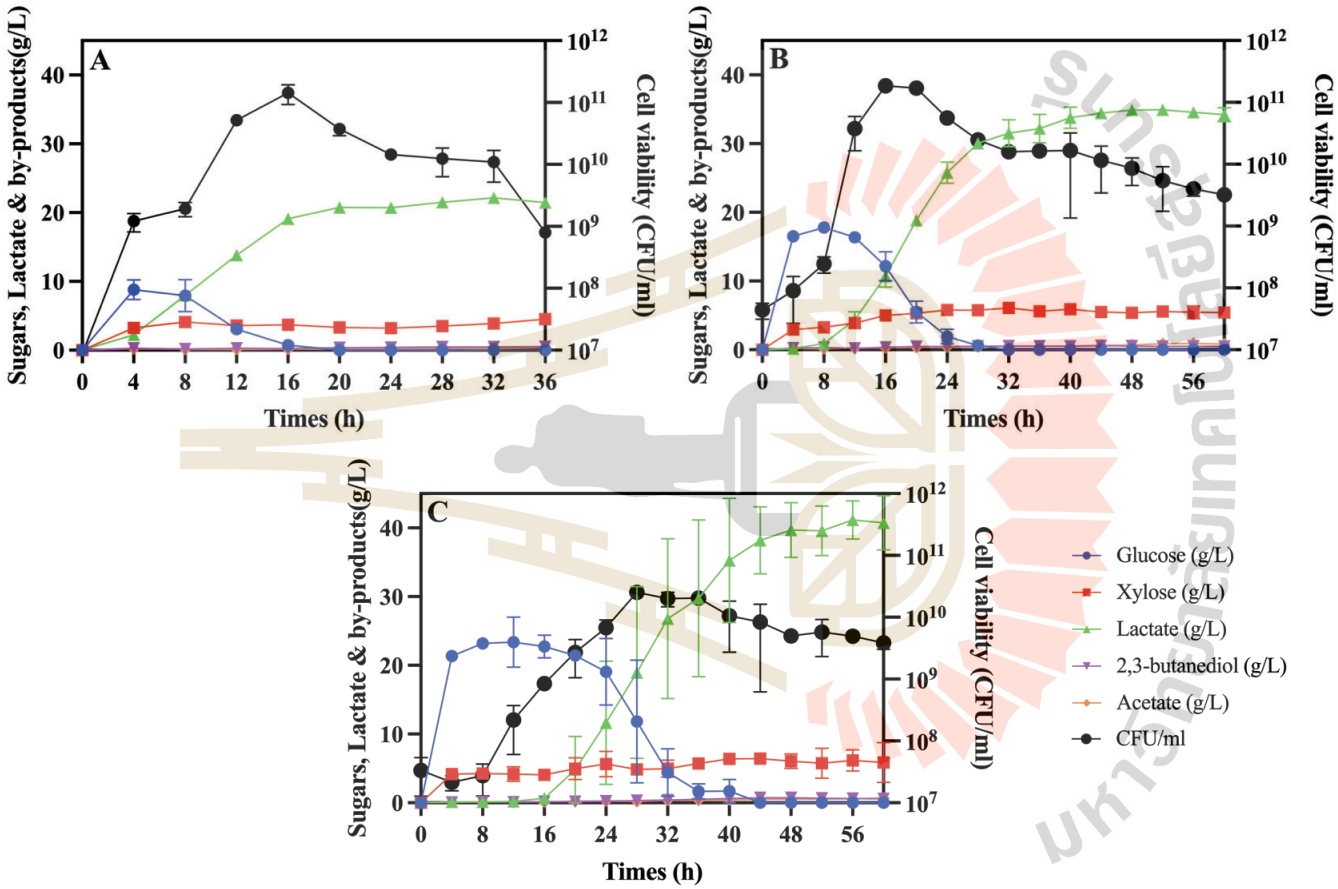


Figure 4.4 D-lactic acid production under SSF with different initial concentration of pretreated PIC. **(A)** 50 g/L, **(B)** 75 g/L, and **(C)** 100 g/L pretreated PIC. Cell viability was determined by spread plate technique.

4.5 D-(-)-lactic acid fermentation from pretreated PIC under Fed-batch SHF

A fed-batch strategy was employed to enhance the concentration, yield, and productivity of D-(-)-lactic acid while mitigating issues related to broth viscosity and substrate inhibition. Initially, 50 g/L NaOH-pretreated PIC was enzymatically hydrolyzed using a cellulase cocktail at 50°C for 24 hours. The resulting hydrolysate was then sterilized at 121°C for 20 minutes and subsequently cooled to 37°C prior to inoculation with the pre-culture strain *K. oxytoca* KIS004-91T strain. During fermentation, the concentrated hydrolysate (120 g/L glucose) was intermittently added to maintain the glucose concentration at approximately 25 g/L, whenever the level dropped to 10 g/L, given glucose served as the principal carbon source for D-(-)-lactic acid production. At the beginning of fermentation, initial sugar concentrations were found at 25.36±0.33 g/L glucose and 8.40±0.32 g/L xylose, totaling 33.75±0.65 g/L. As illustrated in Fig. 4.5, glucose was rapidly consumed, decreasing to 10.90±0.49 g/L within the first 8 hours, allowing the initial feeding to restore glucose levels to 25 g/L. Subsequent feedings were conducted at 12 h and 20 h intervals, contributing a cumulative total sugar addition of 90.28±3.7 g/L. D-(-)-lactic acid production increased sharply between 4 and 20 hours of incubation. However, the production rate gradually declined after 24 hours, reaching a final concentration of 63.14±0.90 g/L at the end of fermentation. Minor by-products were detected, including 2.70±0.69 g/L 2,3-BDO and 0.57±0.01 g/L of acetate. Fig. 4.6 presents an overall mass balance analysis for D-(-)-lactic acid production from NaOH-pretreated PIC using the fed-batch SHF process by *K. oxytoca* KIS004-91T strain. Based on a gross yield of 0.11 g D-(-)-lactic acid per gram of NaOH-pretreated PIC (Table 4.2), approximately 9.1 kg of pretreated PIC is required to produce 1 kg of D-(-)-lactic acid. Given a solid recovery rate of 37.8% following alkaline pretreatment, 24.1 kg of dried PIC is needed to obtain 9.1 kg of NaOH-pretreated PIC. Furthermore, considering that 120 g of dried PIC can be recovered from 1 kg of fresh PIC (equivalent to a yield of 0.12 g/g), an estimated 200.6 kg of fresh PIC is required to produce 1 kg of D-(-)-lactic acid.

Compared to the SSF process, the fed-batch SHF approach demonstrated improved D-(-)-lactic acid production performance, achieving a productivity of 1.31±0.01 g/L/h and a conversion yield of 0.96±0.07 (Table 4.2). When compared to a batch SHF process using 50 g/L of pretreated PIC, the fed-batch mode also achieved a 2.46-fold increase in D-(-)-lactic acid production. These findings are consistent with previous studies. For instance, Vishnu et al. (2020) reported a 3.4-fold enhancement in D-lactic acid production from pretreated rice straw by *Lactobacillus bulgaricus* under fed-batch SSF compared to batch SSF. Similarly, Abdel-Rahman et al. (2021) demonstrated that fed-batch fermentation of sugar beet molasses using *Enterococcus hirae* ds10 resulted in a D-(-)-lactic acid concentration of 61.76 g/L, representing a 1.67-fold increase over batch fermentation. These results collectively support the conclusion that the fed-batch SHF process offers superior performance relative to batch modes of SHF and SSF.

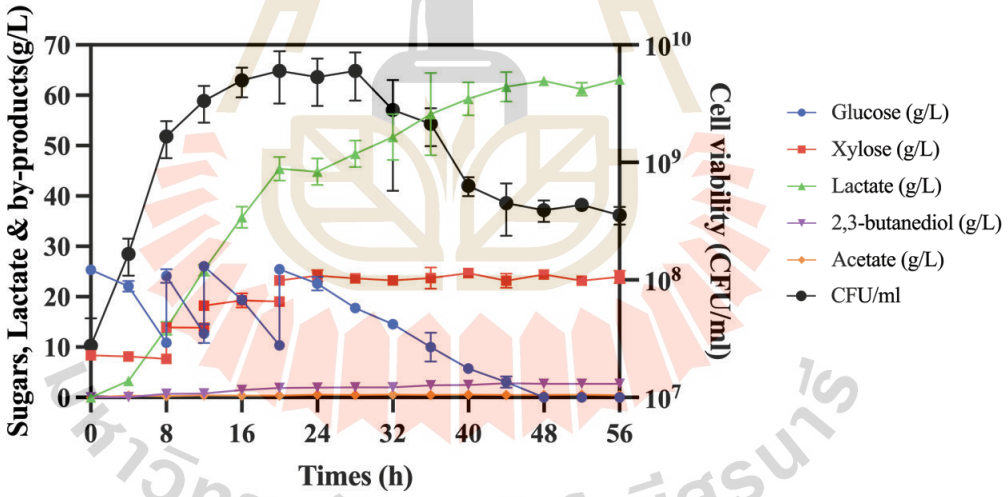


Figure 4.5 D-lactic acid production via fed-batch with initial 50 g/L pretreated PIC. Cell viability was determined by spread plate technique

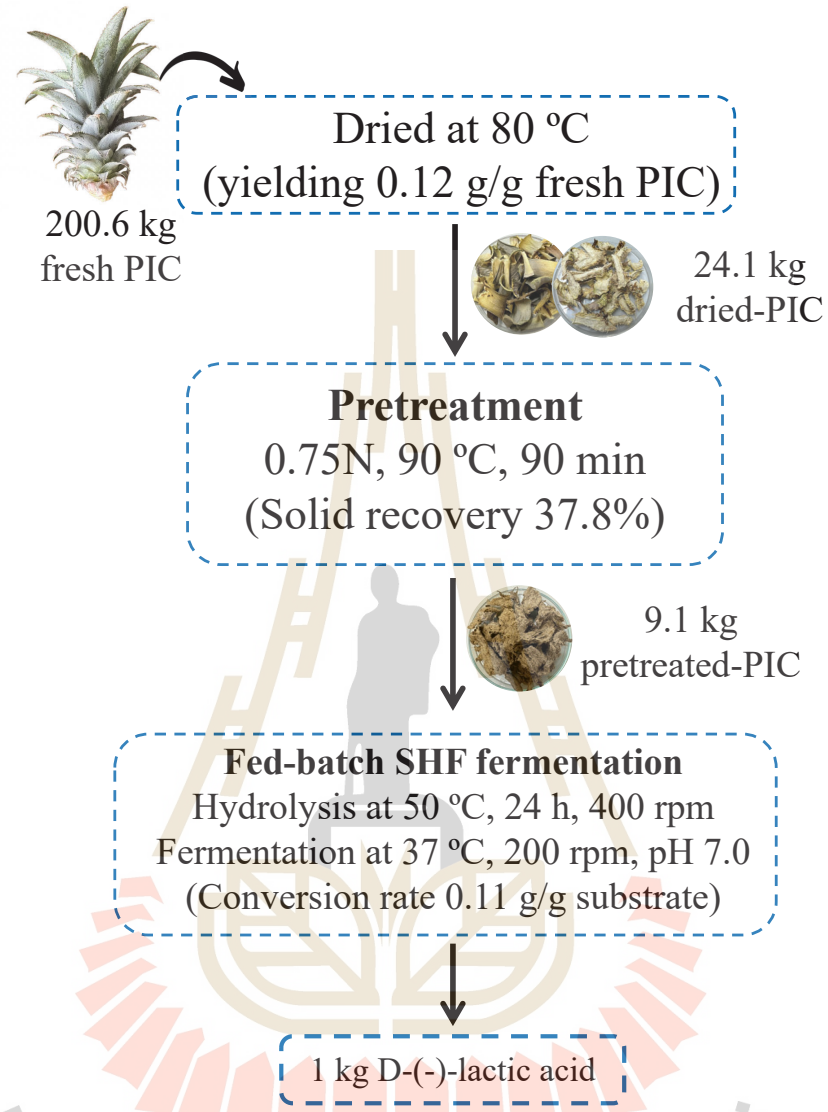


Figure 4.6 Overall mass balance analysis of D-(-)-lactic acid production from fresh pineapple crown using fed-batch SHF.

Table 4.2 The concentration, yield, gross yield, and productivity of D-(-)-lactic acid fermentation from different initial concentration of pretreated PIC via the batch of SHF, SSF and Fed-batch of SHF process.

Substrate Concentration (g/L)	Duration (h)	D-(-)-lactic acid				By-products	
		Concentration (g/L)	Yield (g/g)	Gross Yield (g/g)	Productivity (g/L/h)	2,3-BDO (g/L)	Acetate (g/L)
SHF							
50	16 h	25.53±0.14 ^{ef}	0.96±0.03 ^{a}	0.51±0.00 ^{a}	2.00±0.04 ^{a}	0.79±0.35 ^{ab}	0.41±0.22 ^{a}
75	20 h	32.13±1.04 ^{de}	0.76±0.05 ^{bc}	0.43±0.01 ^{c}	1.95±0.15 ^{a}	0.44±0.16 ^{b}	0.14±0.07 ^{a}
100	28 h	45.69±1.16 ^{bc}	0.81±0.02 ^{ab}	0.46±0.01 ^{b}	1.92±0.35 ^{a}	0.58±0.05 ^{b}	ND
125	48 h	49.25±2.72 ^{b}	0.69±0.04 ^{bc}	0.40±0.01 ^{c}	1.63±0.18 ^{b}	1.49±0.10 ^{ab}	ND
150	76 h	53.26±4.23 ^{ab}	0.64±0.04 ^{bc}	0.36±0.03 ^{cd}	1.21±0.09 ^{c}	1.74±0.09 ^{ab}	ND
SSF							
50	20 h	20.73±0.54 ^{f}	0.67±0.03 ^{bc}	0.42±0.01 ^{c}	1.19±0.04 ^{c}	0.36±0.05 ^{b}	0.18±0.04 ^{a}
75	32 h	31.50±1.97 ^{de}	0.70±0.03 ^{bc}	0.42±0.03 ^{c}	1.07±0.03 ^{d}	0.55±0.06 ^{b}	0.53±0.17 ^{a}
100	44 h	38.19±4.90 ^{ce}	0.62±0.08 ^{c}	0.38±0.05 ^{d}	0.87±0.11 ^{e}	0.74±0.52 ^{ab}	0.51±0.35 ^{a}
Fed batch-SHF							
50	48 h	62.87±0.42 ^{a}	0.96±0.07 ^{a}	0.11±0.00 ^{e}	1.31±0.01 ^{bc}	2.70±0.58 ^{a}	0.52±0.01 ^{a}

- Lower-case letters indicate the significant differences between mean values of three replicates ($p \leq 0.05$) in the same column.
- D-(-)-lactic acid concentration, yield, and gross yield were calculated when glucose was exhausted at the time indicated.
- Productivity was calculated when D-(-)-lactic acid was produced at the maximum rate.
- Yield was calculated based on sugar utilized and gross yield was calculated based on pretreated PIC provided.

Table 4.3 Lactic acid production from different types of substrates and microorganism.

Substrate	Microorganisms	Optical isomer	Fermentation mode	Lactic acid production			References
				Titer (g/g)	Yield (g/g)	Productivity (g/L/h)	
Pineapple crown	*K. oxytoca* KIS004-91T	D-(-)-LA	Fed-SHF	62.9	0.96	1.31	This study
			SHF	45.7	0.81	1.43	
			SSF	31.5	0.70	0.98	
Cassava starch	*K. oxytoca* KIS004-91T	D-(-)-LA	SHF	98.4	0.93	1.43	(In et al., 2020)
Cassava bagasse	*E. coli* JU15	D-(-)-LA	SHF	57.8	1.11	0.98	(Utrilla et al., 2016)
Paper mill sludge	*B. coagulans*	L-(+)-LA	SSCF	82.6	0.83	0.69	(Li et al., 2021)
Wheat straw	*B. coagulans* CC17A	L-(+)-LA	SSCF	26.30	0.71	0.25	(Ouyang et al., 2020)
Spent coffee grounds	*L. rhamnosus* ATCC 10863	N/D	SHF	24.95	0.91	0.54	(Koo et al., 2019)

Table 4.3 Lactic acid production from different types of substrates and microorganism. (Continued)

Cassava bagasse	*L. rhamnosus*	L-(+)-LA	SHF	41.6	0.83	0.87	(Coelho et al., 2010)
Orange peel waste	*L. delbrueckii* ssp. *bulgaricus* CECT 5037	D-(-)-LA	SHF	39	0.84	0.55	(Bustamante et al., 2020)
	L. delbrueckii ssp. *bulgaricus* CECT 286	D-(-)-LA	SHF	45	0.86	0.63	
Broken rice	*L. delbrueckii*	D-(-)-LA	SSF	79	0.81	3.58	(Abdel-Rahman et al., 2013)
Corncob	*R. oryzae* NLX-M-1	L-(+)-LA	SHF	34	0.34	0.71	(Zhang et al., 2016b)

* SHF: Separate hydrolysis and fermentation, SSF: Simultaneous saccharification and fermentation, SSCF: Simultaneous saccharification and co-fermentation.

CHAPTER V

CONCLUSION AND RECOMMENDATION

The main objectives in this study were to optimize the effectiveness strategy of D-(-)-lactic acid production from pineapple crown (PIC) including pretreatment by alkaline, enzymatic hydrolysis using crude cellulase enzyme and the suitable fermentation mode by engineered *K. oxytoca* KIS004-91T. The optimized pretreatment process of PIC was established using 0.75N NaOH at 90°C for 90 min in which 28.35% of lignin and 66.67% of ash was removed compared to the untreated-PIC. Furthermore, cellulose content of PIC was increased to 50.13% after the pretreatment whereas the untreated PIC obtained only 33.12% of cellulose. Fermentable sugar was generated with impressive concentration (13.69±0.36 g/L) from 20 g/L of pretreated PIC by using 60 PCU crude cellulase cocktail /g for hydrolysis for 24 h. Accordingly, D-(-)-lactic acid was produced at the maximum concentration of 45.69±1.16 g/L with a yield of 0.81±0.02 g/g and maximum productivity of 1.92±0.35 g/L/h from 100 g/L pretreated PIC via separate hydrolysis and fermentation (SHF). Under simultaneous saccharification and fermentation (SSF), maximum concentration at 34.23±1.01 g/L of D-(-)-lactic acid was produced, with a conversion yield of 0.70±0.03 g/g and a productivity of 1.07±0.03 g/L/h from 75 g/L of pretreated PIC. The fed-batch separate hydrolysis and fermentation (SHF) process yielded the highest D-(-)-lactic acid concentration of 63.14±0.90 g/L, with a conversion yield of 0.96±0.07 g/g and a productivity of 1.31±0.01 g/L/h. To produce 1 kg of D-(-)-lactic acid, approximately 200.6 kg of fresh PIC was estimated. These findings underscore the potential of PIC as a low-cost, renewable feedstock that supports waste valorization and contributes to the development of a zero-waste biorefinery system. Furthermore, the approach outlined in this study holds promise for broader application in the microbial production of other high-value biochemicals from PIC. Therefore, recycle and reused of alkaline

solution and waste water (water running through pretreated-PIC) shall be optimized, considering of cost saving and water pollution preventative.

REFERENCES

REFERENCES

Abd Alsaheb, R. A., Aladdin, A., Othman, N., Malek, R. A., Leng, O., Aziz, R., & El Enshasy, H. (2015). Lactic acid applications in pharmaceutical and cosmeceutical industries. **Journal of Chemical and Pharmaceutical Research**, 2015, 729-735.

Abdel-Rahman, M. A., Hassan, S. E.-D., Alrefaey, H. M. A., El-Belely, E. F., Elsakhawy, T., Fouda, A., Desouky, S. G., Khattab, S. M. R. (2021). Subsequent improvement of lactic acid production from beet molasses by *Enterococcus hirae* ds10 using different fermentation strategies. **Bioresource Technology Reports**, 13, 100617.

Abdel-Rahman, M. A., Tashiro, Y., & Sonomoto, K. (2013). Recent advances in lactic acid production by microbial fermentation processes. **Biotechnol Adv**, 31(6), 877-902.

Alrumman, S. A. (2016). Enzymatic saccharification and fermentation of cellulosic date palm wastes to glucose and lactic acid. **Brazilian Journal of Microbiology**, 47(1), 110-119.

Ameen, S., & Caruso, G. (2017). **Lactic Acid in the Food Industry**.

Arantes, V., & Saddler, J. N. (2010). Access to cellulose limits the efficiency of enzymatic hydrolysis: the role of amorphogenesis. **Biotechnology for Biofuels**, 3(1), 4.

Arib, R. M. N., Sapuan, S., Megat Ahmad, M. M. H., M. Tahir, P., & Zaman, K. (2006). Mechanical properties of pineapple leaf fibre reinforced polypropylene composites. **Materials & Design**, 27, 391-396.

Asgher, M., Ahmad, Z., & Iqbal, H. M. N. (2013). Alkali and enzymatic delignification of sugarcane bagasse to expose cellulose polymers for saccharification and bio-ethanol production. **Industrial Crops and Products**, 44, 488-495.

Auras, R., Lim, L.-T., Selke, S., & Tsuji, H. (2010). Poly(Lactic Acid): Synthesis, Structures, Properties, Processing, and Applications. **Wiley**, 1-1.

Baral, P., Pundir, A., Kumar, V., Kurmi, A. K., & Agrawal, D. (2020). Expeditious production of concentrated glucose-rich hydrolysate from sugarcane bagasse and its fermentation to lactic acid with high productivity. **Food and Bioproducts Processing**, 124, 72-81.

Behera, S., Arora, R., Nandhagopal, N., & Kumar, S. (2014). Importance of chemical pretreatment for bioconversion of lignocellulosic biomass. **Renewable and Sustainable Energy Reviews**, 36, 91-106.

Behl, M., Thakar, S., Ghai, H., Sakhuja, D., & Bhatt, A. K. (2023). Chapter 21 - Fundamentals of fermentation technology. **Basic Biotechniques for Bioprocess and Bioentrepreneurship**, 313-328. Academic Press.

Bernardo, M. P., Coelho, L. F., Sass, D. C., & Contiero, J. (2016). l-(+)-Lactic acid production by *Lactobacillus rhamnosus* B103 from dairy industry waste. **Braz J Microbiol**, 47(3), 640-646.

Betts, W., Dart, R. K., Ball, A., & Pedlar, S. (1991). Biosynthesis and Structure of Lignocellulose. In (pp. 139-155).

Borrero-Lopez, A. M., Valencia, C., & Franco, J. M. (2022). Lignocellulosic Materials for the Production of Biofuels, Biochemicals and Biomaterials and Applications of Lignocellulose-Based Polyurethanes: A Review. **Polymers**, 14(5), 881.

Bustamante, D., Tortajada, M., Ramón, D., & Rojas, A. (2020). Production of D-Lactic Acid by the Fermentation of Orange Peel Waste Hydrolysate by Lactic Acid Bacteria. **Fermentation**, 6(1), 1.

Byresh, T. S., Malini, B., Kavitha, L., Sunil, C. K., Chidanand, D. V., & Venkatachalapathy, N. (2023). Study of prebiotic effect of pineapple crown powder on development of white finger millet vegan probiotic beverage. **Food and Humanity**, 1, 742-752.

Chen, H., Chen, B., Su, Z., Wang, K., Wang, B., Wang, Y., Si, Z., Wu, Y., Cai, D., &Qin, P. (2020). Efficient lactic acid production from cassava bagasse by mixed culture of *Bacillus coagulans* and *lactobacillus rhamnosus* using stepwise pH controlled simultaneous saccharification and co-fermentation. **Industrial Crops**

and Products, 146, 112175.

Chen, S., Xiaoyu, Z., Deepak, S., Hongbo, Y., & and Yang, X. (2010). Biological pretreatment of lignocellulosics: potential, progress and challenges. **Biofuels**, 1(1), 177-199.

Chen, X., Li, H., Sun, S., Cao, X., & Sun, R. (2018). Co-production of oligosaccharides and fermentable sugar from wheat straw by hydrothermal pretreatment combined with alkaline ethanol extraction. **Industrial Crops and Products**, 111, 78-85.

Chen, Y., Knappe, D. R. U., & Barlaz, M. A. (2004). Effect of Cellulose/Hemicellulose and Lignin on the Bioavailability of Toluene Sorbed to Waste Paper. Environmental Science & Technology, 38(13), 3731-3736.

Choi, K. R., Jiao, S., & Lee, S. Y. (2020). Metabolic engineering strategies toward production of biofuels. *Current Opinion in Chemical Biology, 59*, 1-14.

Choquecahua Mamani, D., Otero Nole, K. S., Chaparro Montoya, E. E., Mayta Huiza, D. A., Pastrana Alta, R. Y., & Aguilar Vitorino, H. (2020). Minimizing Organic Waste Generated by Pineapple Crown: A Simple Process to Obtain Cellulose for the Preparation of Recyclable Containers. **Recycling**, 5(4), 24.

Coelho, L. F., Bolner de Lima, C. J., Bernardo, M. P., Alvarez, G. M., & Contiero, J. (2010). Improvement of L(+)-lactic acid production from cassava wastewater by *Lactobacillus rhamnosus* B 103. **J Sci Food Agric**, 90(11), 1944-1950.

Congthai, W., Phosriran, C., Chou, S., Onsanoi, K., Gosalawit, C., Cheng, K.-C., & Jantama, K. (2025). Exploiting Mixed Waste Office Paper Containing Lignocellulosic Fibers for Alternatively Producing High-Value Succinic Acid by Metabolically Engineered *Escherichia coli* KJ122. **International Journal of Molecular Sciences**, 26(3), 982.

Darby, A., Lertpiriyapong, K., Sarkar, U., Seneviratne, U., Park, D. S., Gamazon, E. R., .Batchelder, C., Cheung, C., Buckyley, E. M., Taylor, N. S., Shen, Z., Tannenbaum, S. R., Wishnok, J. S., & Fox, J. G. (2014). Cytotoxic and Pathogenic Properties of *Klebsiella oxytoca* Isolated from Laboratory Animals. **PLOS ONE**, 9(7), e100542.

Daud, Z., Hatta, M., Kassi, A., & Mohd Aripin, A. (2014). Analysis of the Chemical

Compositions and Fiber Morphology of Pineapple (Ananas comosus) Leaves in Malaysia. **Journal of Applied Sciences**, 14.

Davison, B. H., Parks, J., Davis, M. F., & Donohoe, B. S. (2013). Plant Cell Walls: Basics of Structure, Chemistry, Accessibility and the Influence on Conversion. **Aqueous Pretreatment of Plant Biomass for Biological and Chemical Conversion to Fuels and Chemicals**, 23-38.

Den, W., Sharma, V. K., Lee, M., Nadadur, G., & Varma, R. S. (2018). Lignocellulosic Biomass Transformations via Greener Oxidative Pretreatment Processes: Access to Energy and Value-Added Chemicals. **Frontiers in Chemistry**, 6.

Dhar, P., Nickhil, C., Pandiselvam, R., & Deka, S. C. (2023). Pineapple waste-based-biorefinery for sustainable generation of value-added products. **Biomass Conversion and Biorefinery**.

Dusselier, M., Wouwe, P., Dewaele, A., Makshina, E., & Sels, B. (2013). Lactic acid as a platform chemical in the biobased economy: The role of chemocatalysis. **Energy Environ. Sci.**, 6, 1415-1442.

Feng, X., Jiang, L., Han, X., Liu, X., Zhao, Z., Liu, H., Xian, M., & Zhao, G. (2017). Production of d-lactate from glucose using *Klebsiella pneumoniae* mutants. **Microbial Cell Factories**, 16(1), 209.

Garcia-Cubero, M. T., González-Benito, G., Indacoechea, I., Coca, M., & Bolado, S. (2009). Effect of ozonolysis pretreatment on enzymatic digestibility of wheat and rye straw. **Bioresource technology**, 100(4), 1608-1613.

Gavila, L., Constantí, M., & Medina, F. (2015). d-Lactic acid production from cellulose: dilute acid treatment of cellulose assisted by microwave followed by microbial fermentation. **Cellulose**, 22(5), 3089-3098.

Gosalawit, C., Kory, S., Phosriran, C., & Jantama, K. (2024). Techno-economical valorization of sugarcane bagasse for efficiently producing optically pure D-(−)-lactate approaching the theoretical maximum yield in low-cost salt medium by metabolically engineered Klebsiella oxytoca. **Bioresource technology**, 407, 131145.

Grewal, J., & Khare, S. K. (2018). One-pot bioprocess for lactic acid production from lignocellulosic agro-wastes by using ionic liquid stable *Lactobacillus brevis*. **Bioresource technology**, 251, 268-273. doi: 10.1016/j.biortech.2017.12.056

Hama, S., Mizuno, S., Kihara, M., Tanaka, T., Ogino, C., Noda, H., & Kondo, A. (2015). Production of d-lactic acid from hardwood pulp by mechanical milling followed by simultaneous saccharification and fermentation using metabolically engineered *Lactobacillus plantarum*. **Bioresource technology**, 187, 167-172.

Han, L., Feng, J., Zhang, S., Ma, Z., Wang, Y., & Zhang, X. (2012). Alkali pretreated of wheat straw and its enzymatic hydrolysis. **Braz J Microbiol**, 43(1), 53-61.

He, N., Chen, M., Qiu, Z., Fang, C., Liden, G., Liu, X., Zhang, Bin., & Bao, J. (2023). Simultaneous and rate-coordinated conversion of lignocellulose derived glucose, xylose, arabinose, mannose, and galactose into D-lactic acid production facilitates D-lactide synthesis. **Bioresource technology**, 377, 128950.

Hikal, W., Mahmoud, A., Said-Al Ahl, H., Bratovcic, A., Tkachenko, K., Kacaniova, M. and Rodriguez, R. (2021) Pineapple (*Ananas comosus* L. Merr.), Waste Streams, Characterisation and Valorisation: An Overview. **Open Journal of Ecology**, 11, 610-634.

Himmel, M. E., Adney, W. S., Baker, J. O., Elander, R., McMillan, J. D., Nieves, R. A., Seehan, J. J., Thomas, S. R., Vinzant, T. B., & Zhang, M. (1997). Advanced Bioethanol Production Technologies: A Perspective. **Fuels and Chemicals from Biomass**, 666, 2-45. American Chemical Society.

Hoyos, P., Hernaiz, M. J., & Alcantara, A. R. (2017). 3.28 - Biocatalyzed Production of Fine Chemicals. **Comprehensive Biotechnology (Third Edition)**, 334-373. Oxford: Pergamon.

In, S., Khunnonkwao, P., Wong, N., Phosiran, C., Jantama, S. S., & Jantama, K. (2020). Combining metabolic engineering and evolutionary adaptation in *Klebsiella oxytoca* KMS004 to significantly improve optically pure D-(−)-lactic acid yield and specific productivity in low nutrient medium. **Applied Microbiology and**

Biotechnology, 104(22), 9565-9579.

Isikgor, F. H., & Becer, C. R. (2015). Lignocellulosic biomass: a sustainable platform for the production of bio-based chemicals and polymers. **Polymer Chemistry**, 6(25), 4497-4559.

Jaichakan, P., Nakphaichit, M., Rungchang, S., Weerawatanakorn, M., Phongthai, S., & Klangpetch, W. (2021). Two-stage processing for xylooligosaccharide recovery from rice by-products and evaluation of products: Promotion of lactic acid-producing bacterial growth and food application in a high-pressure process. **Food Research International**, 147, 110529.

Jain, V. K., Divol, B., Prior, B. A., & Bauer, F. F. (2012). Effect of alternative NAD+-regenerating pathways on the formation of primary and secondary aroma compounds in a *Saccharomyces cerevisiae* glycerol-defective mutant. **Appl Microbiol Biotechnol**, 93(1), 131-141.

Jantama, K., Haupt, M. J., Svoronos, S. A., Zhang, X., Moore, J. C., Shanmugam, K. T., & Ingram, L. O. (2008). Combining metabolic engineering and metabolic evolution to develop nonrecombinant strains of *Escherichia coli* C that produce succinate and malate. **Biotechnol Bioeng**, 99(5), 1140-1153.

Jantama, K., Polyiam, P., Khunnonkwao, P., Chan, S., Sangproo, M., Khor, K., Jantama, S. S., & Kanchanatawee, S. (2015). Efficient reduction of the formation of by-products and improvement of production yield of 2,3-butanediol by a combined deletion of alcohol dehydrogenase, acetate kinase-phosphotransacetylase, and lactate dehydrogenase genes in metabolically engineered Klebsiella oxytoca in mineral salts medium. **Metab Eng**, 30, 16-26.

Jonsson, L. J., & Martín, C. (2016). Pretreatment of lignocellulose: Formation of inhibitory by-products and strategies for minimizing their effects. **Bioresource technology**, 199, 103-112.

Kacaribu, A. A., & Darwin. (2024). Biotechnological lactic acid production from low-cost renewable sources via anaerobic microbial processes. **BioTechnologia (Pozn)**, 105(2), 179-194.

Karnaouri, A., Asimakopoulou, G., Kalogiannis, K. G., Lappas, A., & Topakas, E. (2020). Efficient d-lactic acid production by *Lactobacillus delbrueckii* subsp. bulgaricus through conversion of organosolv pretreated lignocellulosic biomass. **Biomass and Bioenergy**, 140, 105672.

Khan, M. F. S., Akbar, M., Xu, Z., & Wang, H. (2021). A review on the role of pretreatment technologies in the hydrolysis of lignocellulosic biomass of corn stover. **Biomass and Bioenergy**, 155, 106276.

Khunnonkwao, P., Phosiran, C., In, S., Kory, S., & Jantama, K. (2023). Valorization of empty oil-palm fruit bunch waste for an efficient improvement of succinic acid production by metabolically engineered *Escherichia coli*. **Biomass Conversion and Biorefinery**.

Kim, J. H., Block, D. E., & Mills, D. A. (2010). Simultaneous consumption of pentose and hexose sugars: an optimal microbial phenotype for efficient fermentation of lignocellulosic biomass. **Appl Microbiol Biotechnol**, 88(5), 1077-1085.

Kim, J. S., Lee, Y. Y., & Kim, T. H. (2016). A review on alkaline pretreatment technology for bioconversion of lignocellulosic biomass. **Bioresource technology**, 199, 42-48.

Kim, T. H., Taylor, F., & Hicks, K. B. (2008). Bioethanol production from barley hull using SAA (soaking in aqueous ammonia) pretreatment. **Bioresource technology**, 99(13), 5694-5702.

Kinnarinen, T., & Häkkinen, A. (2014). Influence of enzyme loading on enzymatic hydrolysis of cardboard waste and size distribution of the resulting fiber residue. **Bioresource technology**, 159, 136-142.

Klongklaew, A., Unban, K., Kalaimurugan, D., Kanpiengjai, A., Azaizeh, H., Schroedter, L., Schroedter, R., Venus, J., & Khanongnuch, C. (2023). Bioconversion of Dilute Acid Pretreated Corn Stover to L-Lactic Acid Using Co-Culture of Furfural Tolerant *Enterococcus mundtii* WX1 and *Lactobacillus rhamnosus* SCJ9. **Fermentation**, 9(2), 112.

Koo, J.-R., Park, H.-M., Kim, S.-K., & Yun, H.-S. (2019). Lactic Acid Fermentation from

Coffee Ground Waste Hydrolysate by *Lactobacillus rhamnosus*. **Journal of Renewable Materials**, 7(4), 365-372.

Kumar, P., Barrett, D. M., Delwiche, M. J., & Stroeve, P. (2009). Methods for Pretreatment of Lignocellulosic Biomass for Efficient Hydrolysis and Biofuel Production. **Industrial & Engineering Chemistry Research**, 48(8), 3713-3729.

Kumar, V., Yadav, S. K., Kumar, J., & Ahluwalia, V. (2020). A critical review on current strategies and trends employed for removal of inhibitors and toxic materials generated during biomass pretreatment. **Bioresource technology**, 299, 122633.

Li, D., Jing, M., Dai, X., Chen, Z., Ma, C., & Chen, J. (2022). Current status of pineapple breeding, industrial development, and genetics in China. **Euphytica**, 218(6), 85.

Li, Y., Bhagwat, S. S., Cortes-Peña, Y. R., Ki, D., Rao, C. V., Jin, Y.-S., & Guest, J. S. (2021). Sustainable Lactic Acid Production from Lignocellulosic Biomass. **ACS Sustainable Chemistry & Engineering**, 9(3), 1341-1351.

Lin, S. K. C., Du, C., Koutinas, A., Wang, R., & Webb, C. (2008). Substrate and product inhibition kinetics in succinic acid production by *Actinobacillus succinogenes*. **Biochemical Engineering Journal**, 41(2), 128-135.

Linton, S. M. (2020). Review: The structure and function of cellulase (endo-ß-1,4-glucanase) and hemicellulase (ß-1,3-glucanase and endo- ß-1,4-mannase) enzymes in invertebrates that consume materials ranging from microbes, algae to leaf litter. **Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology**, 240, 110354.

Liu, S., Zhang, M., Hong, D., Fang, Z., Xiao, Y., Fang, W., & Zhang, X. (2023). Improving the cellobiose hydrolysis activity of glucose-stimulating β-glucosidase Bgl2A. **Enzyme and Microbial Technology**, 169, 110289.

Loow, Y.-L., Wu, T. Y., Md. Jahim, J., Mohammad, A. W., & Teoh, W. H. (2016). Typical conversion of lignocellulosic biomass into reducing sugars using dilute acid hydrolysis and alkaline pretreatment. **Cellulose**, 23(3), 1491-1520.

Lu, P., & Hsieh, Y.-L. (2012). Preparation and characterization of cellulose nanocrystals from rice straw. **Carbohydrate Polymers**, 87(1), 564-573.

Martinez, A., Grabar, T. B., Shanmugam, K. T., Yomano, L. P., York, S. W., & Ingram, L. O. (2007). Low salt medium for lactate and ethanol production by recombinant *Escherichia coli* B. **Biotechnol Lett**, 29(3), 397-404.

Maurya, D. P., Singla, A., & Negi, S. (2015). An overview of key pretreatment processes for biological conversion of lignocellulosic biomass to bioethanol. **3 Biotech**, 5(5), 597-609.

Meneses, N. G. T., Martins, S., Teixeira, J. A., & Mussatto, S. I. (2013). Influence of extraction solvents on the recovery of antioxidant phenolic compounds from brewer's spent grains. **Separation and Purification Technology**, 108, 152-158.

Mikulski, D., & Kłosowski, G. (2018). Efficiency of dilute sulfuric acid pretreatment of distillery stillage in the production of cellulosic ethanol. **Bioresource technology**, 268, 424-433.

Millett, M. A., Baker, A. J., & Satter, L. D. (1976). Physical and chemical pretreatments for enhancing cellulose saccharification. **Biotechnol Bioeng Symp**(6), 125-153.

Mohamed, H., Hassane, A., Atta, O., & Song, Y. (2021). Deep learning strategies for active secondary metabolites biosynthesis from fungi: Harnessing artificial manipulation and application. **Biocatalysis and Agricultural Biotechnology**, 38, 102195.

Mohd Ali, M., Hashim, N., Abd Aziz, S., & Lasekan, O. (2020). Pineapple (Ananas comosus): A comprehensive review of nutritional values, volatile compounds, health benefits, and potential food products. **Food Res Int**, 137, 109675.

Mora-Villalobos, J. A., Montero-Zamora, J., Barboza, N., Rojas-Garbanzo, C., Usaga, J., Redondo-Solano, M., Schroedter, L., Olszewska-Widdrat, A., & López-Gómez, J. P. (2020). Multi-Product Lactic Acid Bacteria Fermentations: A Review. **Fermentation**, 6(1), 23.

Mosier, N., Wyman, C., Dale, B., Elander, R., Lee, Y. Y., Holtzapple, M., & Ladisch, M. (2005). Features of promising technologies for pretreatment of lignocellulosic biomass. **Bioresource technology**, 96(6), 673-686.

Narayanan, N., Roychoudhury, P., & Srivastava, A. (2004). L(+)-Lactic acid fermentation

and its product polymerization. **Electronic Journal of Biotechnology** (ISSN: 0717-3458) Vol 7 Num 2*, 7*.

Nashiruddin, N. I., Mansor, A. F., Rahman, R. A., Ilias, R. M., & Yussof, H. W. (2020). Process parameter optimization of pretreated pineapple leaves fiber for enhancement of sugar recovery. **Industrial Crops and Products**, 152, 112514.

Nguyen, T. Y., Cai, C. M., Kumar, R., & Wyman, C. E. (2017). Overcoming factors limiting high-solids fermentation of lignocellulosic biomass to ethanol. **Proceedings of the National Academy of Sciences**, 114(44), 11673-11678.

Nuraida, L. (2015). A review: Health promoting lactic acid bacteria in traditional Indonesian fermented foods. **Food Science and Human Wellness**, 4(2), 47-55.

Ojo, A. O., & de Smidt, O. (2023). Lactic Acid: A Comprehensive Review of Production to Purification. **Processes**, 11(3), 688.

Oliva, J. M., Saez, F., Ballesteros, I., Gonzalez, A., Negro, M. J., Manzanares, P., & Ballesteros, M. (2003). Effect of lignocellulosic degradation compounds from steam explosion pretreatment on ethanol fermentation by thermotolerant yeast *Kluyveromyces marxianus*. **Appl Biochem Biotechnol**, 105 -108, 141-153.

Oonkhanond, B., Jonglertjunya, W., Srimarut, N., Bunpachart, P., Tantinukul, S., Nasongkla, N., & Sakdaronnarong, C. (2017). Lactic acid production from sugarcane bagasse by an integrated system of lignocellulose fractionation, saccharification and fermentation, and ex-situ nanofiltration. **Journal of Environmental Chemical Engineering**, 5.

Ouyang, S., Zou, L., Qiao, H., Shi, J., Zheng, Z., & Ouyang, J. (2020). One-pot process for lactic acid production from wheat straw by an adapted *Bacillus coagulans* and identification of genes related to hydrolysate-tolerance. **Bioresource technology**, 315, 123855.

Pan, X., Xie, D., Gilkes, N., Gregg, D. J., & Saddler, J. N. (2005). Strategies to enhance the enzymatic hydrolysis of pretreated softwood with high residual lignin content. **Appl Biochem Biotechnol**, 121-124, 1069-1079.

Panagiota, F.-P., Efterpi, C., & Eleftherios, B. (2013). Lactic Acid Bacteria as Source of Functional Ingredients. In K. Marcelino (Ed.), **Lactic Acid Bacteria** (pp. Ch. 25). Rijeka: IntechOpen.

Patel, A. K., Singhania, R. R., Sim, S. J., & Pandey, A. (2019). Thermostable cellulases: Current status and perspectives. **Bioresource technology**, 279, 385-392.

Patel, R., Dhar, P., Babaei-Ghazvini, A., Nikkhah Dafchahi, M., & Acharya, B. (2023). Transforming lignin into renewable fuels, chemicals, and materials: A review. **Bioresource Technology Reports**, 22, 101463.

Phosriran, C., Wong, N., & Jantama, K. (2024). An efficient production of bio-succinate in a novel metabolically engineered *Klebsiella oxytoca* by rational metabolic engineering and evolutionary adaptation. **Bioresource technology**, 393, 130045.

Prado, K. S., & Spinace, M. A. S. (2019). Isolation and characterization of cellulose nanocrystals from pineapple crown waste and their potential uses. **International Journal of Biological Macromolecules**, 122, 410-416.

Pratt, P. L., Bryce, J. H., & Stewart, G. G. (2003). The Effects of Osmotic Pressure and Ethanol on Yeast Viability and Morphology. **Journal of the Institute of Brewing**, 109(3), 218-228.

Rajpurohit, H., & Eiteman, M. A. (2022). Nutrient-Limited Operational Strategies for the Microbial Production of Biochemicals. **Microorganisms**, 10(11), 2226.

Rapado, P., Faba, L., & Ordóñez, S. (2021). Influence of delignification and reaction conditions in the aqueous phase transformation of lignocellulosic biomass to platform molecules. **Bioresource technology**, 321, 124500.

Rawoof, S. A. A., Kumar, P. S., Vo, D.-V. N., Devaraj, K., Mani, Y., Devaraj, T., & Subramanian, S. (2021). Production of optically pure lactic acid by microbial fermentation: a review. **Environmental Chemistry Letters**, 19(1), 539-556.

Razali, N., & Abdullah, A. Z. (2017). Production of lactic acid from glycerol via chemical conversion using solid catalyst: A review. **Applied Catalysis A: General**, 543, 234-246.

Reddy, K. O., Maheswari, C. U., Dhlamini, M. S., Mothudi, B. M., Kommula, V. P., Zhang, J., Zhang, J., & Rajulu, A. V. (2018). Extraction and characterization of cellulose single fibers from native african napier grass. **Carbohydrate Polymers**, 188, 85-91.

Roda, A., & Lambri, M. (2019). Food uses of pineapple waste and by-products: a review. **International Journal of Food Science & Technology**, 54(4), 1009-1017.

Rodrigues, C., Vandenberghe, L. P. S., Woiciechowski, A. L., de Oliveira, J., Letti, L. A. J., & Soccol, C. R. (2017). 24 - Production and Application of Lactic Acid. **Current Developments in Biotechnology and Bioengineering**, 543-556: Elsevier.

Rojas, M. J., Siqueira, P. F., Miranda, L. C., Tardioli, P. W., & Giordano, R. L. C. (2014). Sequential proteolysis and cellulolytic hydrolysis of soybean hulls for oligopeptides and ethanol production. **Industrial Crops and Products**, 61, 202-210.

Saha, B. C., Iten, L. B., Cotta, M. A., & Wu, Y. V. (2005). Dilute acid pretreatment, enzymatic saccharification, and fermentation of rice hulls to ethanol. **Biotechnol Prog**, 21(3), 816-822.

Saini, R., Chen, C.-W., Patel, A. K., Saini, J. K., Dong, C.-D., & Singhania, R. R. (2022). Valorization of Pineapple Leaves Waste for the Production of Bioethanol. **Bioengineering**, 9(10), 557.

Sanchez, C. (2009). Lignocellulosic residues: Biodegradation and bioconversion by fungi. **Biotechnology Advances**, 27(2), 185-194.

Sangproo, M., Polyiam, P., Jantama, S. S., Kanchanatawee, S., & Jantama, K. (2012). Metabolic engineering of *Klebsiella oxytoca* M5a1 to produce optically pure d-lactate in mineral salts medium. **Bioresource technology**, 119, 191-198.

Sawisit, A., Jampatesh, S., Jantama, S. S., & Jantama, K. (2018). Optimization of sodium hydroxide pretreatment and enzyme loading for efficient hydrolysis of rice straw to improve succinate production by metabolically engineered *Escherichia coli* KJ122 under simultaneous saccharification and fermentation. **Bioresource technology**, 260, 348-356.

Sen, S., & Roychoudhury, P. K. (2013). Development of optimal medium for production of commercially important monoclonal antibody 520C9 by hybridoma cell. **Cytotechnology**, 65(2), 233-252.

Sethupathi, M., Khumalo, M. V., Skosana, S. J., & Muniyasamy, S. (2024). Recent developments of pineapple leaf fiber (PALF) utilization in the polymer composites—A Review. **Separations**, 11(8), 245.

Shi, J., Chinn, M. S., & Sharma-Shivappa, R. R. (2008). Microbial pretreatment of cotton stalks by solid state cultivation of Phanerochaete chrysosporium. **Bioresource technology**, 99(14), 6556-6564.

Shukla, A., Kumar, D., Girdhar, M., Kumar, A., Goyal, A., Malik, T., & Mohan, A. (2023). Strategies of pretreatment of feedstocks for optimized bioethanol production: distinct and integrated approaches. **Biotechnology for Biofuels and Bioproducts**, 16(1), 44.

Singh, S., Madlala, A. M., & Prior, B. A. (2003). *Thermomyces lanuginosus*: properties of strains and their hemicellulases. **FEMS Microbiology Reviews**, 27(1), 3-16.

Sluiter, A. (2012). Determination of structural carbohydrates and lignin in biomass: laboratory analytical procedure (LAP): Issue Date, April 2008, Revision Date: August 2012 (Version 08-03-2012): **National Renewable Energy Laboratory**.

Sorensen, H. M., Rochfort, K. D., Maye, S., MacLeod, G., Brabazon, D., Loscher, C., & Freeland, B. (2022). Exopolysaccharides of Lactic Acid Bacteria: Production, Purification and Health Benefits towards Functional Food. **Nutrients**, 14(14), 2938.

Sorensen, H. M., Rochfort, K. D., Maye, S., MacLeod, G., Loscher, C., Brabazon, D., & Freeland, B. (2023). Bioactive Ingredients from Dairy-Based Lactic Acid Bacterial Fermentations for Functional Food Production and Their Health Effects. **Nutrients**, 15(22), 4754.

Srivastava, N., Srivastava, M., Mishra, P. K., Gupta, V. K., Molina, G., Rodriguez-Couto, S., Manikanta, A., & Ramteke, P. W. (2018). Applications of fungal cellulases in biofuel production: Advances and limitations. **Renewable and Sustainable**

Energy Reviews, 82, 2379-2386.

Sun, D., Lv, Z.-W., Rao, J., Tian, R., Sun, S.-N., & Peng, F. (2022). Effects of hydrothermal pretreatment on the dissolution and structural evolution of hemicelluloses and lignin: A review. **Carbohydrate Polymers**, 281, 119050.

Sun, F., & Chen, H. (2008). Organosolv pretreatment by crude glycerol from oleochemicals industry for enzymatic hydrolysis of wheat straw. **Bioresource technology**, 99(13), 5474-5479.

Sun, R., Kang, J., Wang, X., Xiu, B., Ping, W., & Ge, J. (2022). Enhancement of 2,3-Butanediol Production *by Klebsiella pneumoniae*: Emphasis on the Mediation of sRNA-SgrS on the Carbohydrate Utilization. **Fermentation**, 8(8), 359.

Sun, Y., & Cheng, J. (2002). Hydrolysis of lignocellulosic materials for ethanol production: a review. **Bioresource technology**, 83(1), 1-11.

Tang, Y., Wang, X., Li, Y., Lei, M., Du, Y., Kennedy, J. F., & Knill, C. J. (2010). Production and characterisation of novel injectable chitosan/methylcellulose/salt blend hydrogels with potential application as tissue engineering scaffolds. **Carbohydrate Polymers**, 82(3), 833-841.

Thapa, L. P., Lee, S. J., Park, C., & Kim, S. W. (2017). Production of L-lactic acid from metabolically engineered strain of *Enterobacter aerogenes* ATCC 29007. **Enzyme and Microbial Technology**, 102, 1-8.

Thongchul, N., Navankasattusas, S., & Yang, S. T. (2010). Production of lactic acid and ethanol by *Rhizopus oryzae* integrated with cassava pulp hydrolysis. **Bioprocess Biosyst Eng**, 33(3), 407-416.

Ufodike, C. O., Eze, V. O., Ahmed, M. F., Oluwalowo, A., Park, J. G., Okoli, O. I., & Wang, H. (2020). Evaluation of the inter-particle interference of cellulose and lignin in lignocellulosic materials. **International Journal of Biological Macromolecules**, 147, 762-767.

Unrean, P., & Khajeeram, S. (2016). Optimization and techno-economic assessment of high-solid fed-batch saccharification and ethanol fermentation by *Scheffersomyces stipitis* and *Saccharomyces cerevisiae* consortium.

Renewable Energy, 99, 1062-1072.

Utrilla, J., Gosset, G., & Martinez, A. (2009). ATP limitation in a pyruvate formate lyase mutant of *Escherichia coli* MG1655 increases glycolytic flux to d-lactate. **Journal of Industrial Microbiology & Biotechnology**, 36, 1057-1062.

Utrilla, J., Vargas-Tah, A., Trujillo-Martínez, B., Gosset, G., & Martinez, A. (2016). Production of d-lactate from sugarcane bagasse and corn stover hydrolysates using metabolic engineered *Escherichia coli* strains. **Bioresource technology**, 220, 208-214.

Vasic, K., Knez, Z., & Leitgeb, M. (2021). Bioethanol Production by Enzymatic Hydrolysis from Different Lignocellulosic Sources. **Molecules**, 26(3).

Vishnu Prasad, J., Sahoo, T. K., Naveen, S., & Jayaraman, G. (2020). Evolutionary engineering of *Lactobacillus bulgaricus* reduces enzyme usage and enhances conversion of lignocellulosics to D-lactic acid by simultaneous saccharification and fermentation. **Biotechnology for Biofuels**, 13(1), 171.

Wan, C., & Li, Y. (2010). Microbial pretreatment of corn stover with *Ceriporiopsis subvermispora* for enzymatic hydrolysis and ethanol production. **Bioresource technology**, 101(16), 6398-6403.

Wang, L., Fan, D., Chen, W., & Terentjev, E. M. (2015). Bacterial growth, detachment and cell size control on polyethylene terephthalate surfaces. **Scientific Reports**, 5(1), 15159.

Wee, Y.-J., Kim, J.-N., & Ryu, H.-W. (2006). Biotechnological production of lactic acid and its recent applications Food Technol. **Food Technology and Biotechnology**, 44.

Wei, N., Quarterman, J., Kim, S. R., Cate, J. H. D., & Jin, Y.-S. (2013). Enhanced biofuel production through coupled acetic acid and xylose consumption by engineered yeast. **Nature Communications**, 4(1), 2580.

Wu, W., Li, P., Huang, L., Wei, Y., Li, J., Zhang, L., & Jin, Y. (2023). The Role of Lignin Structure on Cellulase Adsorption and Enzymatic Hydrolysis. **Biomass**, 3(1), 96-107.

Wu, Y., Gao, X., Wu, J., Zhou, T., Nguyen, T. T., & Wang, Y. (2023). Biodegradable Polylactic Acid and Its Composites: Characteristics, Processing, and Sustainable Applications in Sports. **Polymers**, 15(14), 3096.

Yaashikaa, P. R., Senthil Kumar, P., & Varjani, S. (2022). Valorization of agro-industrial wastes for biorefinery process and circular bioeconomy: A critical review. **Bioresource technology**, 343, 126126.

Yang, Y., Jalalah, M., Alsareii, S., Harraz, F., Thakur, N., Zheng, Y., Alalawy, A. I., Koutb, M., & Salama, E.-S. (2024). Potential of oleaginous microbes for lipid accumulation and renewable energy generation. **World Journal of Microbiology and Biotechnology**, 40, 337.

Yankov, D. (2022). Fermentative Lactic Acid Production From Lignocellulosic Feedstocks: From Source to Purified Product. **Frontiers in Chemistry**, 10.

Yuan, Y., Jiang, B., Chen, H., Wu, W., Wu, S., Jin, Y., & Xiao, H. (2021). Recent advances in understanding the effects of lignin structural characteristics on enzymatic hydrolysis. **Biotechnology for Biofuels**, 14(1), 205.

Zhang, L., Li, X., Yong, Q., Yang, S. T., Ouyang, J., & Yu, S. (2016). Impacts of lignocellulose-derived inhibitors on L-lactic acid fermentation by *Rhizopus oryzae*. **Bioresource technology**, 203, 173-180.

Zhang, X., Jantama, K., Shanmugam, K. T., & Ingram, L. O. (2009). Reengineering *Escherichia coli* for Succinate Production in Mineral Salts Medium. **Appl Environ Microbiol**, 75(24), 7807-7813.

Zhang, Y., & Vadlani, P. V. (2013). D-Lactic acid biosynthesis from biomass-derived sugars via *Lactobacillus delbrueckii* fermentation. **Bioprocess Biosyst Eng**, 36(12), 1897-1904.

Zhang, Y., Vadlani, P. V., Kumar, A., Hardwidge, P. R., Govind, R., Tanaka, T., & Kondo, A. (2016). Enhanced D-lactic acid production from renewable resources using engineered *Lactobacillus plantarum*. **Appl Microbiol Biotechnol**, 100(1), 279-288.

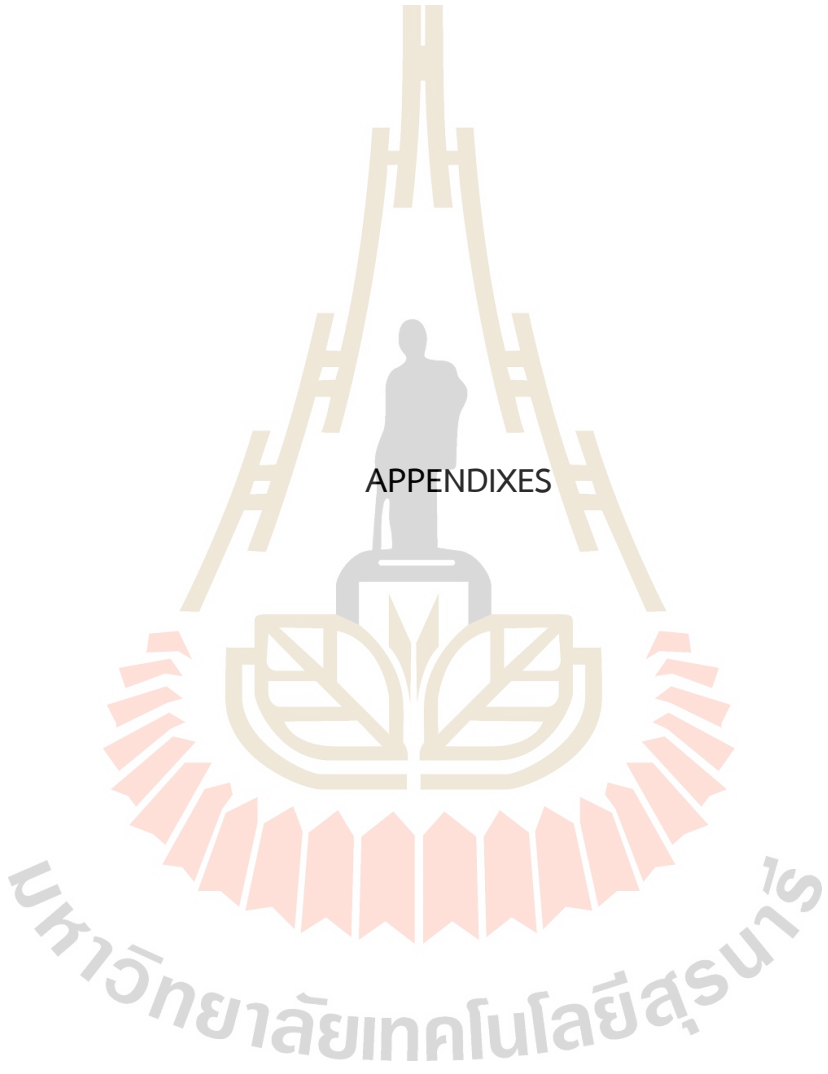
Zhao, B., Wang, L., Li, F., Hua, D., Ma, C., Ma, Y., & Xu, P. (2010). Kinetics of D-lactic acid

production by *Sporolactobacillus* sp. strain CASD using repeated batch fermentation. **Bioresource technology**, 101(16), 6499-6505.

Zhou, Y., Domínguez, J. M., Cao, N., Du, J., & Tsao, G. T. (1999). Optimization of L-lactic acid production from glucose by *Rhizopus oryzae* ATCC 52311. **Appl Biochem Biotechnol**, 77-79, 401-407.

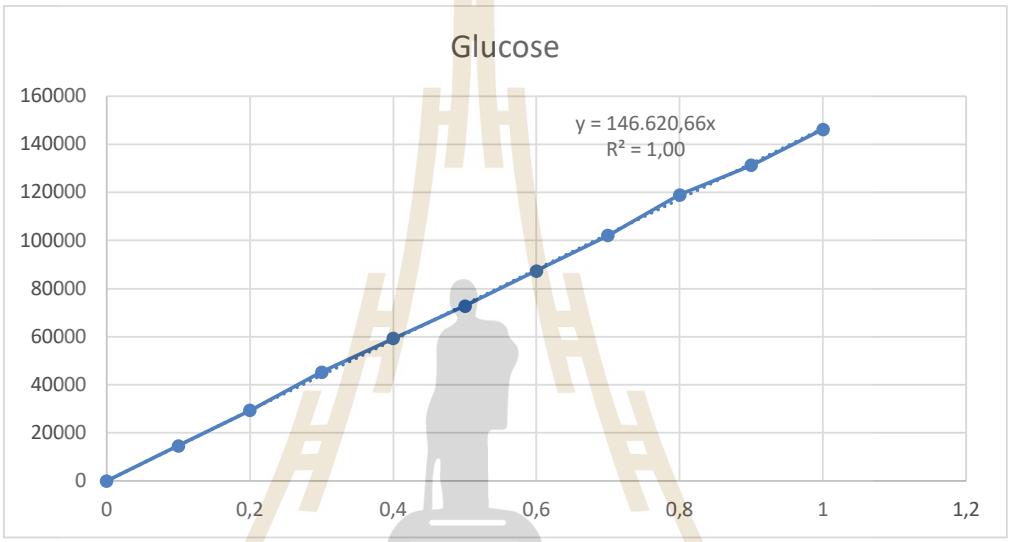
Zhu, W., Sun, H., Zhang, Y., Wang, N., Li, Y., Liu, S., Gao, M., Wang, Y., & Wang, Q. (2023). Improving lactic acid yield of hemicellulose from garden garbage through pretreatment of a high solid loading coupled with semi-hydrolysis using low enzyme loading. **Bioresource technology**, 384, 129330.

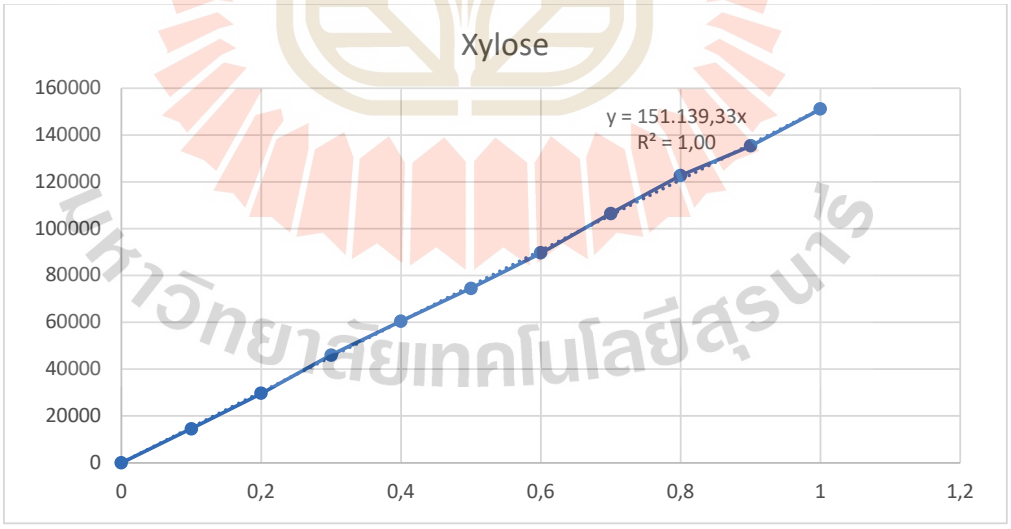
APPENDIXES

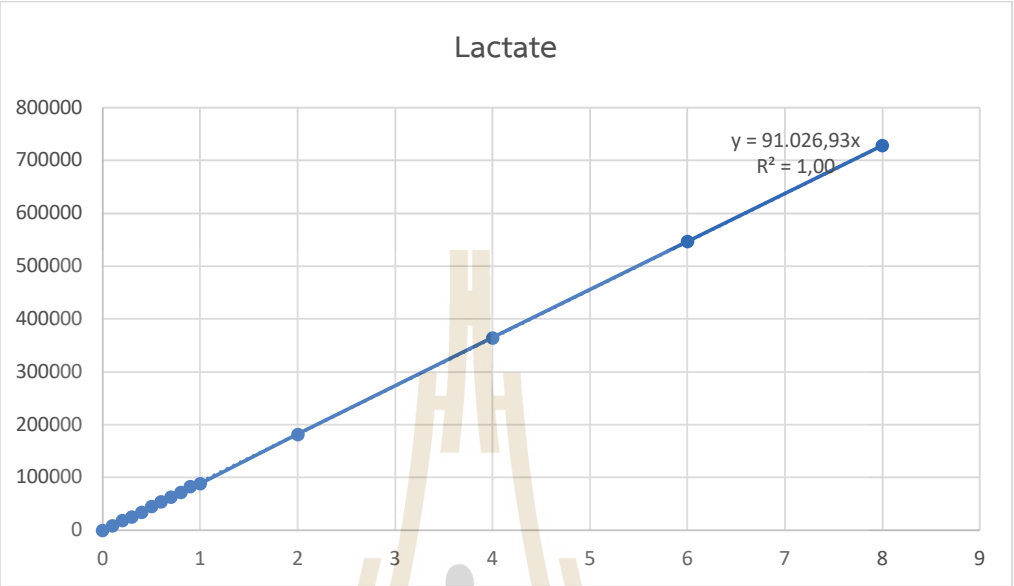


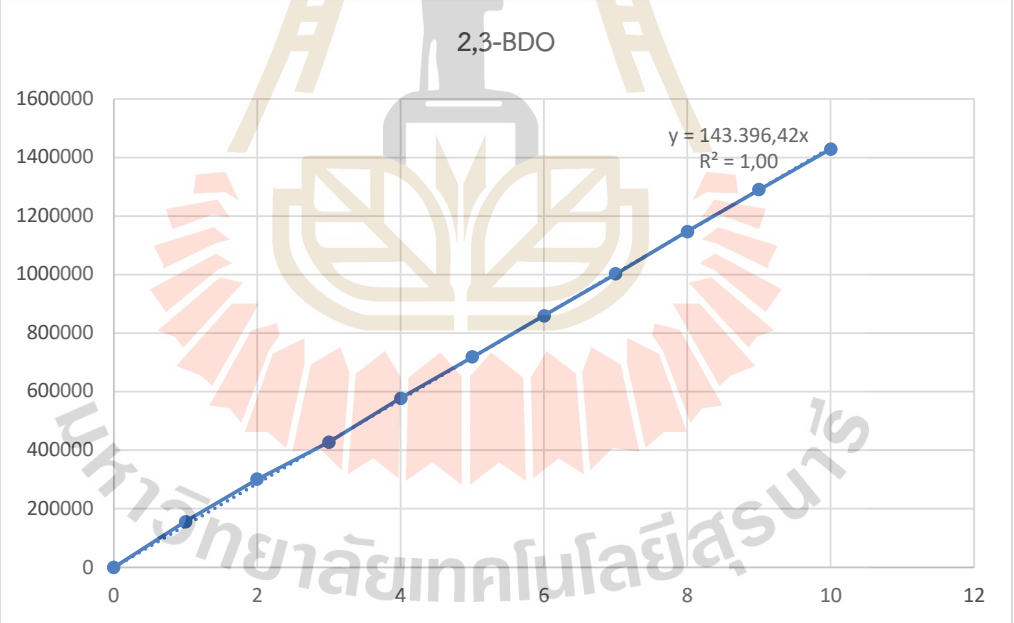
APPENDIX A

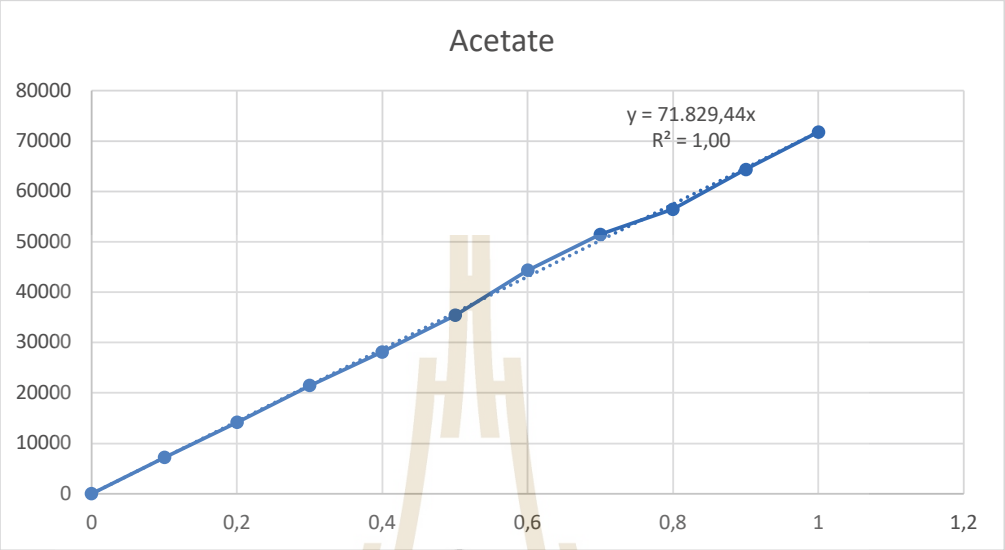
STANDARD ANALYTICAL DATA





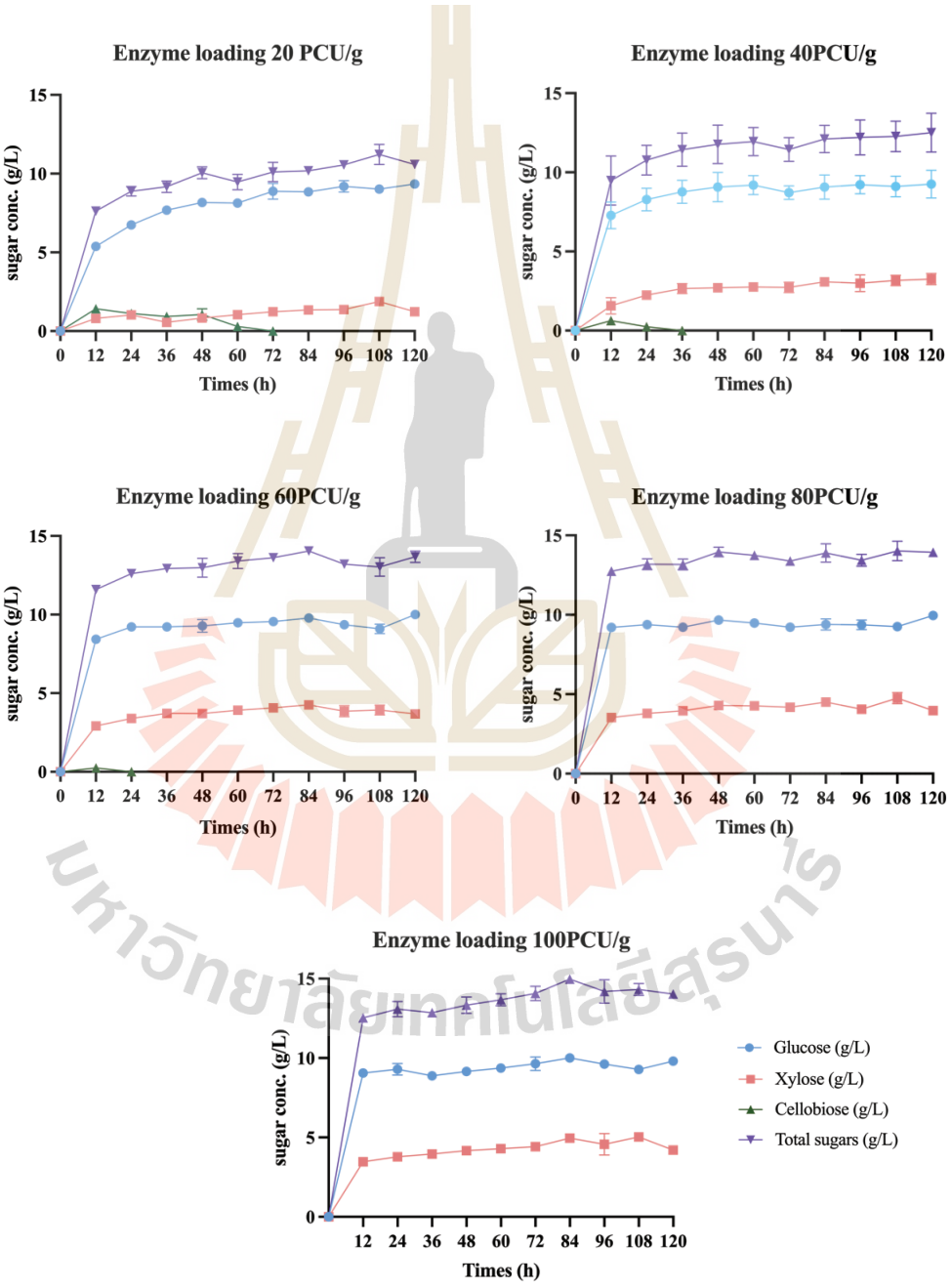






APPENDIX B

SUGAR CONVERSION DURING 120 HOURS



BIOGRAPHY

Miss Socheata Chou was born on January 28, 1999, at Battambang province, Cambodia. She obtained the Bachelor degree of Food Science and Technology (1st Honor), Faculty of Agro-Industrial Technology of Rajamangala University of Technology Tawan-ok, Thailand, in 2021. After graduation, she worked as a deputy-manager of Quality Management (QA & QC) of dried fruit industry, in Cambodia. In 2023, she decided to pursue a Master degree in School of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology, Nakhon Ratchasima, Thailand. Her research project is Optimization of D-(-)-lactic acid production from pineapple crown using a metabolically engineered *Klebsiella oxytoca* KIS004-91T. After she graduates, she plans to work in industry in Cambodia.