SUCCINIC ACID PRODUCTION FROM MIXED WASTE OFFICE PAPER BY *ESCHERICHIA COLI* KJ122

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SUCCINIC ACID PRODUCTION FROM MIXED WASTE OFFICE PAPER BY *Escherichia coli* KJ122

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

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วลัยนุช คงไทย : การผลิตกรดซักซินิกจากกระดาษเหลือทิ้งสำนักงานด้วยเชื้ออีโคไลสายพันธุ์ KJ122 (SUCCINIC ACID PRODUCTION FROM MIXED WASTE OFFICE PAPER BY *ESCHERICHIA COLI* KJ122) อาจารย์ที่ปรึกษา : รองศาสตราจารย์ ดร.เขมวิทย์ จันต๊ะมา, 75 หน้า.

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กรดซักซินิกเป็นสารตั้งต้นที่ใช้ในอุตสาหกรรมซึ่งสามารถผลิตผ่านกระบวนการหมักด้วยจุลินทรีย์โดยวัสดุเหลือใช้ประเภทลิกโนเซลลูโลส ในสภาวะของเศรษฐกิจโลกที่เติบโตขึ้นทำให้เกิดกระดาษเหลือทิ้งจากสำนักงานจำนวนมาก โดยส่วนใหญ่กระดาษเหล่านี้ถูกนำไปแปรรูปเพื่อนำกลับมาใช้ใหม่เป็นกระดาษชำระและกระดาษแข็ง แต่กระบวนการนี้มีต้นทุนที่สูง ดังนั้น การใช้กระดาษเหลือทิ้งจากสำนักงานในการผลิตผลิตภัณฑ์ชีวภาพมูลค่าสูง เช่น กรดซักซินิก จึงเป็นแนวทางที่น่าสนใจอย่างยิ่ง

ในงานวิจัยนี้ พบว่าการปรับสภาพกระดาษเหลือทิ้งจากสำนักงาน โดยใช้กรดซัลฟูริกเจือจางที่ความเข้มข้นร้อยละ 1 ปริมาตรต่อปริมาตร และใช้อุณหภูมิ 121 องศาเซลเซียส เป็นเวลา 20 นาที เป็นเงื่อนไขที่เหมาะสมที่สุดสำหรับการกำจัดหมึกและโทนเนอร์ซึ่งเป็นสารที่สามารถยับยั้งการผลิตกรดซักซินิก จากนั้นทดสอบกระบวนการไฮโดรไลซิสด้วยเอนไซม์ของกระดาษเหลือทิ้งจากสำนักงานที่ผ่านการปรับสภาพด้วยกรดซัลฟูริกเจือจางที่ความเข้มข้น 50 กรัมต่อลิตร ทำการทดสอบภายใต้อุณหภูมิ 50 องศาเซลเซียส โดยการใช้เอนไซม์เซลลูเลสที่ความเข้มข้น 80 พีซียูต่อกรัมของกระดาษเหลือทิ้งจากสำนักงานที่ผ่านการปรับสภาพด้วยกรดเจือจาง ผลลัพธ์ที่ได้เมื่อสิ้นสุดกระบวนการคือ ได้ปริมาณน้ำตาลกลูโคสและน้ำตาลไซโลสสูงสุดที่ 22.46±0.15 กรัมต่อลิตร และ 5.11±0.32 กรัมต่อลิตร ตามลำดับ

สำหรับการผลิตกรดซักซินิกผ่านกระบวนการหมักหลังการย่อยวัตถุดิบด้วยเอนไซม์ (SHF) โดยใช้ *Escherichia coli* KJ122 สามารถผลิตกรดซักซินิกได้ที่ 28.19±0.98 กรัมต่อลิตร โดยมีอัตราการผลิต 1.17±0.04 กรัมต่อลิตรต่อชั่วโมง ในขณะที่กระบวนการย่อยน้ำตาลควบคู่กับกระบวนการหมัก (SSF) ให้ผลผลิตกรดซักซินิกที่ 24.58±2.32 กรัมต่อลิตร และมีอัตราการผลิต 0.82±0.07 กรัมต่อลิตรต่อชั่วโมง

สุดท้ายนี้ ในการหมักแบบกึ่งกะด้วยสภาวะกระบวนการหมักหลังการย่อยวัตถุดิบ (fed-batch SSF) สามารถผลิตกรดซักซินิกได้ความเข้มข้น 51.38±4.05 กรัมต่อลิตร ด้วยผลผลิต 0.75±0.05 กรัมต่อกรัม และอัตราการผลิต 1.07±0.08 กรัมต่อลิตรต่อชั่วโมง งานวิจัยนี้ไม่เพียงเสนอแนวทางเลือกในการใช้กระดาษเหลือทิ้งจากสำนักงานสำหรับการผลิตกรดซักซินิกชีวภาพที่มีมูลค่าสูง แต่ยังให้ข้อมูล

เชิงลึกที่สำคัญต่อการผลิตกรดซักซินิกในระดับอุตสาหกรรมจากของเสีย ซึ่งสนับสนุนความยั่งยืนของสิ่งแวดล้อมและสังคมปลอดขยะ

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ปีการศึกษา 2567 ลายมือชื่ออาจารย์ที่ปรึกษา

WALAINUD CONGTHAI : SUCCINIC ACID PRODUCTION FROM MIXED WASTE OFFICE PAPER BY *ESCHERICHIA COLI* KJ122. THESIS ADVISOR : ASSOC. PROF. KAEMWICH JANTAMA, Ph.D., 75 PP.

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Succinic acid is a precursor used in industrial applications that can be produced through microbial fermentation using lignocellulosic wastes. Mixed waste office paper (MWOP) is widely generated due to economic growth globally. However, most MWOP is recycled into toilet paper and cardboard, but the recovery process by these means is costly. The use of MWOP for bioproducts, including succinic acid, is an alternative and highly attractive approach. In this study, pretreatment of MWOPs with diluted 1% (v/v) H_2SO_4 at 121°C for 20 min was found to be optimal for removing mainly ink and toners as inhibitors. The optimal conditions for the enzymatic hydrolysis of H_2SO_4-pretreated MWOP (AP-MWOP) at a concentration of 50 g/L were also investigated at 50°C, with the crude cellulase loading at 80 PCU/g AP-MWOP. This resulted in the highest glucose and xylose levels of 22.46±0.15 g/L and 5.11±0.32 g/L, respectively. Succinic acid production via separate hydrolysis and fermentation (SHF) by *Escherichia coli* KJ122 reached 28.19±0.98 g/L, with a productivity of 1.17±0.04 g/L/h. For simultaneous saccharification and fermentation (SSF), succinic acid was produced at 24.58±2.32 g/L, with a productivity of 0.82±0.07 g/L/h. Finally, a succinic acid concentration of 51.38±4.05 g/L with a yield of 0.75±0.05 g/g and a productivity of 1.07±0.08 g/L/h was achieved via fed-batch SSF. This study not only offers alternative means to reuse MWOP for the production of high value bio-succinic acid but also provides valuable insights for the industrial production of succinic acid from wastes, supporting environmental sustainability and a zero-waste society.

School of Biotechnology
Academic Year 2024

Student's Signature วลัยนุช วงไทย
Advisor's Signature K. Jantama

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LIST OF ABBREVIATIONS

AP-MWOP	=	Acid pretreated-mixed waste office paper
AM1	=	Alfredo Martinez Mineral Salt Medium 1
°C	=	Degree Celsius
CCR	=	Carbon catabolite repression
CDW	=	Cell dried weight
CFU	=	Colony forming unit
CS	=	Cellulose saccharification
E. coli	=	*Escherichia coli*
g	=	gram
g/g	=	Gram per gram
g/L	=	Gram per liter
g/L/h	=	Gram per liter per hour
h	=	hour
HPLC	=	High-performance liquid chromatography
HS	=	Hemicellulose saccharification
H_2SO_4	=	Sulfuric acid
$KHCO_3$	=	Potassium bicarbonate
KOH	=	Potassium Hydroxide
K_2CO_3	=	Potassium carbonate
L	=	Liter
M	=	Molar
ml	=	milliliter
mM	=	millimolar
mg	=	milligram
min	=	minute
MWOP	=	Mixed waste office paper
rpm	=	round per minute

LIST OF ABBREVIATIONS (Continued)

SSF	=	Simultaneous saccharification and fermentation
SHF	=	Separate hydrolysis and fermentation

CHAPTER I
INTRODUCTION

1.1 Overviews

Succinic acid is considered one of the most important building block chemicals and has been identified as one of twelve potential future chemical structures by the U.S. Department of Energy to be produced from biomasses. It is a valuable chemical with a wide range of industrial applications and is also seen as a potential precursor for the production of other important key chemicals, including food additives, biodegradable polymers, fragrances, flavoring agents, fungicides and herbicides (Nghiem and Kleff, 2017). For this reason, its commercial demand is expected to increase to 768 million metric tons, with a CAR of 27.4% by the year 2025 (Sharma et al., 2020). Currently, succinic acid is produced primarily through chemical processes involving the use of n-butane as the starting material to create maleic anhydride, which is then hydrogenated and hydrated to yield succinic anhydride and finally succinic acid. These processes require high-energy and expensive catalysts, including Ni/Zr/Al/Si alloys, to achieve highly selective conversion of succinic anhydride to succinic acid (Cukalovic et al., 2008). The reactions also generate greenhouse gases, which are known to be environmentally unfriendly. In contrast, the biological process of microbial fermentation is currently an attractive method for producing succinic acid to avoid expensive manufacturing costs and environmental problems from the production of succinic acid by petrochemical processes.

Currently, the paper industry is rapidly expanding due to the enormous demand for papers used in many everyday documents, contributing to an annual waste of approximately 100 million tons (Ma et al., 2021). Wastepaper consists of 50–80% cellulose, 5–15% hemicellulose and a negligible amount of lignin (Zhou et al., 2017). Approximately 400 million tons of wastepaper are produced annually; however, only 65% of the wastepaper is recovered because of the low quality of the paper (Al-Battashi et al., 2022). The remaining waste is inefficiently burned or sent to landfills, causing environmental issues. Alternatively, most waste office paper is

recycled into toilet paper and cardboard, but the recovery process by this means is costly owing to the requirements of a more complex production process and more additional steps than those of brand-new papers (Milbrandt et al., 2018). To reduce expenses, waste paper has been broken into fermentable sugars as substrates for the production of cellulase enzymes and other biofuel and bioproducts instead (Nair et al., 2018). Byadgi and Kalburgi, (2016) also successfully utilized waste newspaper to produce bioethanol with a yield of 6.91% using *Saccharomyces cerevisiae*. Additionally, D-lactic acid was produced from sugars derived from waste office paper and newspaper hydrolysates by *Escherichia coli* JH13, achieving concentrations of 35.86±0.62 g/L and 25.64±0.48 g/L, respectively (Liu et al., 2016).

The challenge for producing succinic acid by microorganisms is the high production costs due to expensive carbon substrates such as glucose, which can counteract the market price of succinic acid. However, succinic acid production through microbial fermentation can be cost effective once renewable feedstocks, including lignocellulosic biomasses, are utilized by microbes as starting materials during the process. Research on improving succinic acid production by renewable feedstocks has focused on *E. coli* strains. *E. coli* KJ122 was developed through a combined strategy of genetic engineering and evolutionary engineering. It has shown the ability to produce high levels of succinic acid, reaching 80 g/L with a molar yield of 1.46 mol/mol glucose consumed and an average volumetric productivity of 0.9 g/L/h (Jantama et al., 2008). Additionally, *E. coli* KJ122 has been shown to produce impressive amounts of succinic acid from various lignocellulosic biomasses, including sugarcane bagasse, rice straw, oil palm empty fruit bunch, and cassava pulp (Phosriran et al., 2024). However, no study has employed waste office paper for succinic acid production. Hence, the feasibility of producing bio-succinic acid through microbial fermentation via waste office paper through appropriate optimization techniques needs to be explored. Therefore, this study developed an efficient process for succinic acid production by the *E. coli* KJ122 strain from abundant waste office paper, a significant global waste material that is often discarded or burned without further use. First, the efficient pretreatment process of mixed waste office paper (MWOP) was investigated to efficiently remove impurities, including inks, which can inhibit microbial growth during succinic acid production. Consequently, the

fermentation process for producing succinic acid by *E. coli* using pretreated MWOP was then optimized to achieve high succinate concentrations, yields, and productivity. This may facilitate further developments to produce succinic acid from MWOP on large industrial scales. In this context, valorizing MWOP to produce succinic acid may be an attractive alternative option for waste management and reducing environmental pollution.

1.2 Research objectives

Succinic acid is a crucial precursor used in the production of various industrial substances. Typically, petroleum oil is the starting material for producing succinic acid through chemical processes. To avoid fluctuations in crude oil prices and promote green and sustainable syntheses, the use of microorganisms in a biological process to produce succinic acid is an alternative option to produce succinic acid. The challenge for producing succinic acid by microorganisms is the high production costs due to expensive carbon substrates such as glucose, which can counteract the market price of succinic acid. Therefore, this study aimed to develop a process for the production of succinic acid by the *E. coli* KJ122 strain from abundant mixed-waste office paper (MWOP), a significant global waste material containing lignocelluloses that is often discarded or burned without further use.

This study focused on investigating the process of MWOP to efficiently remove inks, which can inhibit microbial growth during succinic acid production. The fermentation process for producing high levels of succinic acid via pretreated MWOP was also studied to achieve high succinate concentrations, yields, and productivity. This study also aimed to facilitate further developments to produce succinic acid for large-scale industrial production. The specific objectives included the following:

1.2.1. To examine the lowest concentration of crude cellulase enzyme suitable for hydrolyzing cellulose from pretreated MWOP to a maximum level of fermentable sugars.

1.2.2 To investigate parameters affecting succinic acid production by *E. coli* KJ122, including concentrations of pretreated MWOP and agitation rates during batch fermentation in a 5-liter bioreactor via separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) processes.

1.2.3 To study the potential production of succinic acid by *E. coli* KJ122 via fed-batch SSF processes in a 5-liter bioreactor to increase the succinic acid production rate and yield suitable for industrial application

1.3 Scope and limitations

This research focused on the production of succinic acid by *E. coli* KJ122 via mixed-waste office paper (MWOP) in AM1 media. This study tested the production efficiency of succinic acid by the strain via separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) processes. In addition, factors affecting the fermentation process of succinic acid, including the concentrations of crude cellulase used in hydrolyzing the MWOP to release fermentable sugars and the concentrations of MWOP during the SHF process in both batch and fed-batch operations, were studied

CHAPTER II

LITERATURE REVIEWS

2.1 Succinic acid and its application

Succinic acid is a dicarboxylic acid with the chemical formula $(CH_2)_2(CO_2H)_2$. It is a C4 dicarboxylic acid with significant potential as a platform chemical for the production of various industrial compounds. Figure 2.1 illustrates the numerous derivatives that can be synthesized from succinic acid, highlighting its versatility as a platform chemical in industrial applications. It appears as a white solid with high purity and is odorless. When succinic acid is present in aqueous media, it breaks down into anions called succinate. The physical and chemical properties of this important acid are summarized in Table 2.1 (Saxena et al., 2017).

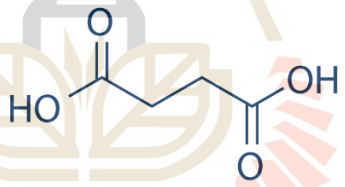


Figure 2.1 Structure of succinic acid.

Table 2.1 Properties of succinic acid.

Succinic acid properties	Details
IUPAC name	Butanediol acid
Boiling	235°C
Melting point	189°C
Molar mass	118.09 g.mol-1
Specific gravity	1.57 $g.cm^{-3}$
Flash point	206°C
Applications	Food products, Pharmaceuticals, and other industrial use.

Succinic acid is a versatile compound widely used in various industries to produce essential products, including surfactants, detergents, flavoring agents, fragrances, biodegradable polymers (such as clothing fibers), food additives, fungicides, and herbicides. It also serves as a precursor for key chemicals used in the pharmaceutical and polymer sectors, such as adipic acid, N-methyl pyrrolidinone, 2-pyrrolidinone, 1,4-butanediol, maleic anhydride, tetrahydrofuran, and gamma-butyrolactone. Furthermore, succinic acid functions as a butanedioic acid, acting as a plant growth regulator. A recent innovation involves the use of succinic acid to produce a new type of biodegradable plastic known as Bionelle, which is an ester formed from succinic acid and 1,4-butanediol (Agarwal et al., 2007).

2.2 Succinic acid production via chemical

To date, succinic acid has been produced primarily through chemical processes. The process involves the use of n-butane as the starting material to create maleic anhydride, which is then hydrogenated and hydrated to yield succinic acid (Figure 2.2). In the first step of the reaction, maleic anhydride is hydrogenated to form succinic anhydride (Reaction 1). The succinic anhydride produced in Reaction 1 can then undergo hydrolysis with water to form succinic acid. Reaction 2 commonly uses a catalyst such as a Ni/Zr/Al/Si alloy, which is highly selective and achieves 98-99% conversion to succinic anhydride (Cukalovic and Stevens, 2008).

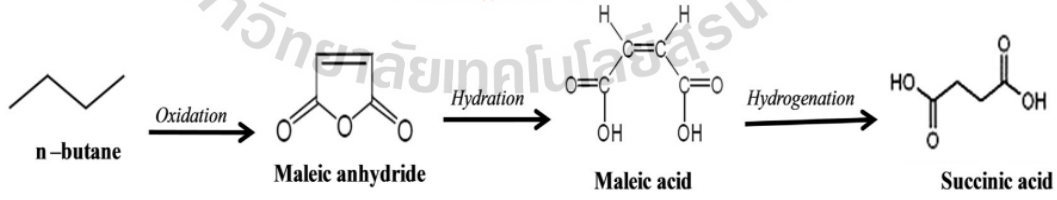


Figure 2.2 Succinic acid production from chemical processes (Saxena et al., 2017).

2.3 Microbial production of succinic acid

Currently, there are numerous microorganisms capable of producing succinic acid through fermentation. These include bacteria such as *Anaerobiospirillum succiniciproducens*, *Lactobacillus sp*., and *E. coli*; yeasts such as *Saccharomyces cerevisiae* (Lee et al., 2008; Li and Xing, 2014; Zhang et al., 2018; Xiberras et al.,

2020), and fungi such as *Aspergillus niger*, *Trichoderma reesei*, and *Phanerochaete chrysosporium* (Alcantara and Mondala, 2021). However, succinic acid production from fungi is challenging due to their spore-forming nature, making fermentation in bioreactors difficult. Advancements in genetics engineering have allowed for the modification of microbial genetic to increase succinic acid yield in various microorganisms. The bioproduction of succinic acid through microbial fermentation is effective because it can utilize renewable feedstock as a starting material. Research has shown that microorganisms can produce succinic acid through the reductive branch of the tricarboxylic acid (TCA) cycle, with succinic acid being an intermediate of this TCA cycle (Liu et al., 2022), which is present in most microbes.

In this pathway, PEP carboxylase (*ppc*) converts PEP and CO_2 into oxaloacetate, which is then converted into malate via the activity of malate dehydrogenase (*mdh*). Malate is further transformed into fumaric acid by fumarate hydratase (*fumABC*), and finally, fumarate and NADH are converted into succinic acid by fumarate reductase (*frdABCD*). Succinic acid is synthesized from PEP and CO_2 at a stoichiometric ratio of 1:1 in this pathway, with PPC playing a critical role in CO_2 fixation. For example, in *E. coli*, PPC is produced during growth on glycolytic substrates (Figure 2.3) (Keseler et al., 2010). Table 2.2 provides examples of wild-type microbial strains and genetically modified strains for producing succinic acid.

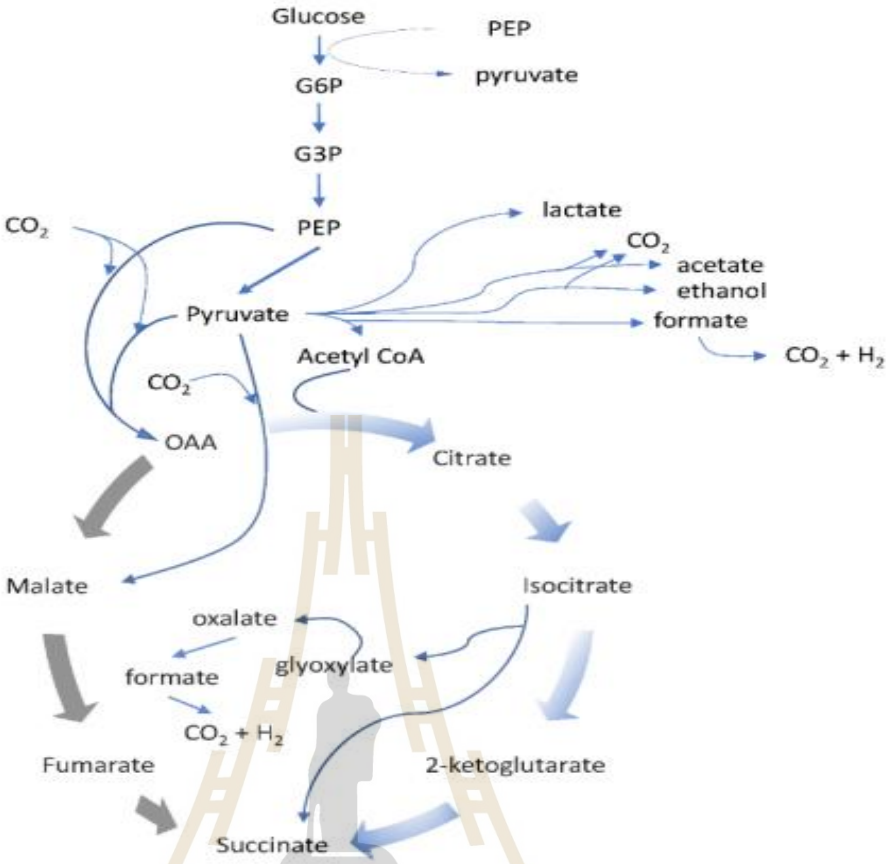


Figure 2.3 Succinic acid pathway in microorganisms (Nghiem et al., 2017).

Table 2.2 Summary of succinic acid production from lignocellulose materials by microorganisms.

Microorganism	Fermentation strategy	Substrate	succinic acid concentration (g/L)	Yield (g/g)	Productivity (g/L/h)	References
Actinobacillus succinogenes ATCC 55,618	SSF	Oil palm empty fruit bunches	33.4	0.47	0.69	(Akhtar and Idris, 2017)
A. succinogenes	SSF	Oil palm empty fruit bunches	42.9	0.61	-	(Akhtar et al., 2020)
Basfia succiniciproducens	Batch	Corn stover	30	0.69	0.43	(Salvachúa et al., 2016)
B. succiniciproducens BPP7	SHF	*Arundo donax* hydrolysate	6.06	0.84	0.14	(Grabar et al., 2014)
S. cerevisiae SHY07-1	SSCF	Sugarcane bagasse	34.84	0.10	-	(Bu et al., 2019)
E. coli FZ661T	Batch	Woody hydrolysate	54.5	-	1.81	(Zhu et al., 2020)

Table 2.2 (continued).

Microorganism	Fermentation strategy	Substrate	succinic acid concentration (g/L)	Yield (g/g)	Productivity (g/L/h)	References
Thermoanaerobacterium thermosaccharolyticum M5+A. succinogenes 130Z	CBP-based microbial cocultivation system	Corn corb	12.51	0.16	0.065	(Lu et al., 2020)
Aspergillus niger and *Trichoderma reesei*	Batch	Coffee husk	19.3	0.95	0.54	(Dessie et al., 2018b)
A. succinogenes	Fed-batch	Sugarcane bagasse	41.0	-	0.30	(Chen et al., 2021)
E. coli MG-PYC	Batch	Herbal extraction residue	35	0.69	-	(Wang et al., 2018)
A. succinogenes 130Z	Batch	Oil Palm Trunk	13.9	0.41	0.23	(Bukhari et al., 2020)

2.4 Succinic acid producing *E. coli* strains

Succinic acid, in particular, has been recognized as a valuable biochemical product and a precursor for many industrial chemicals. Recently, several companies have brought fermentation-derived succinate to the market (Erickson, 2013; Harmsen et al., 2014). *E. coli* has received much attention for its ability to produce various organic acids, including acetic acid, lactic acid, formic acid, and succinic acid under anaerobic conditions. In wild-type *E. coli*, the maximum theoretical yield of succinate under anaerobic conditions is 1 mol per mol of glucose. This yield is limited by the availability of reducing equivalents, specifically NADH (Thakker et al., 2012). As a result, the production of succinic acid from *E. coli* wild-type strains is relatively low. Various metabolic pathways have been explored to increase succinic acid production in *E. coli*. Genetic modifications have been made to increase the production of succinic acid by deleting genes responsible for producing by products during fermentation. However, this approach often leads to an imbalance of redox potentials in the pathway (Zheng et al., 2021) since NADH is a cofactor for succinic acid production. The lack of NADH in cells is a significant reason why succinic acid is not efficiently produced (Okino et al., 2005). Table 2.3 summarizes succinic acid production by metabolically engineered *E. coli* strains.

Table 2.3 Summary of succinic acid production by engineered *E. coli*.

Microorganism	Fermentation strategy	Substrate	Titer (g/L)	Yield (g/g)	References
AFP111	Two-stage electrochemical anaerobic fermentation	Glucose	15.06	0.71	(Zheng et al., 2021)
HD134	Batch fermentations	Glucose	16.30	0.83	(Huang et al., 2019)
MH28	Batch fermentations	Glycerol	84.3	1.00	(Yocum et al., 2018)
ZW333	Fed-batch fermentation	Panax ginseng residue hydrolysate	24.52	0.96	(Su et al., 2021)
KJ122	Batch fermentation	Glucose	81	0.96	(Jantama et al., 2008b)
W1485	Two-stage fed-batch	Glucose	79.8	0.78	(Zhu et al., 2016)
	Aerobic fed-batch	Glucose	36.1	0.37	(Yang et al., 2014)

2.5 *E. coli* KJ122

E. coli C was designed to reduce byproduct accumulation and increase succinate yield close to the theoretical maximum (1.71 mol succinic acid per 1 mol glucose) in minimal salt media (Jantama et al., 2008b). The major pathways by which *E. coli* under fermentative conditions produces lactate, acetate, and ethanol were eliminated from wild-type *E. coli* C since the byproducts greatly influence the high production cost during the of purification of succinic acid. The engineered strain is known as *E. coli* KJ012 (Δ*ldhA::FRT* Δ*adhE::FRT* Δ*ackA::FRT*). Deletions of genes encoding D-lactate dehydrogenase, acetate kinase, and alcohol dehydrogenase are expected to reduce byproduct accumulation during succinic acid fermentation. This strain grows well in mineral-rich media under anaerobic conditions. However, it still produces high amounts of acetate. *E. coli* KJ012 was then transformed into *E. coli* KJ017 (Δ*ldhA::FRT* Δ*adhE::FRT* Δ*ackA::FRT*). The succinic acid yield increased to 0.73 mol/mol glucose in the medium containing 5% (w/v) glucose. The pyruvate formate-lyase gene (*pflB*) and formate transporter (*focA*) genes of KJ017 were then deleted to eliminate formate and acetate, which resulted in *E. coli* KJ032. This strain reduced the production of formate and acetate. *E. coli* KJ032 was developed in media supplemented with 10% (w/v) glucose and 5 mM acetate, generating KJ060 (Δ*ldhA::FRT* Δ*adhE::FRT* Δ*ackA::FRT* Δ*focB-pflB::FRT*) to increase the growth rate and succinic acid production. The succinic acid yield decreased, but the pyruvate and acetate levels increased due to the accumulation of methylglyoxal, which inhibits glycolysis, resulting in a low growth rate of *E. coli* KJ060. *E. coli* KJ060 was then further modified by deleting the gene encoding methylglyoxal synthase (*mgsA*) to obtain *E. coli* KJ070 (Δ*ldhA::FRT* Δ*adhE::FRT* Δ*ackA::FRT* Δ*focB-pflB::FRT* Δ*mgsA*). The *poxB* gene was subsequently deleted in *E. coli* KJ070, which the pyruvate oxidase activity of the strain is expected to be inactivated, thus reducing acetate production. *E. coli* KJ072 (Δ*ldhA::FRT* Δ*adhE::FRT* Δ*ackA::FRT* Δ*focB-pflB::FRT* Δ*mgsA* Δ*poxB*) was then constructed, but deletion of the *poxB* gene did not reduce the acetate yield. The cell yield and succinic acid production were improved during subsequent metabolic evolution to obtain *E. coli* KJ073 (Δ*ldhA::FRT* Δ*adhE::FRT* Δ*ackA::FRT* Δ*focB-pflB::FRT* Δ*mgsA* Δ*poxB*). This strain produced succinic acid titers of 600-700 mM in batch fermentations via mineral salt media supplemented with 10% (w/v)

glucose. *E. coli* KJ073 was engineered to remove the FRT site, which is foreign DNA. The new strain lacking all FRT sites was the *E. coli* KJ091 (Δ*ldhA* Δ*adhE* Δ*ackA* Δ*focB* Δ*pflB* Δ*mgsA* Δ*poxB*) strain. Although both *ackA* and *pflB,* which are involved in acetate production were deleted, a large amount of acetate is produced. It may occur through an alternative pathway that catalyzes the production of acetyl~CoA, acetyl~P, and ATP. Both *tdcD* and *tdcE* within the same operon represent alternatives to *ackA* and *pflB*, respectively (Heßlinger et al., 1998). A new strain known as *E. coli* KJ098 (Δ*ldhA* Δ*adhE* Δ*ackA* Δ*focB-pflB* Δ*mgsA* Δ*poxB*Δ*tdcDE*), lacking *tdcD* and *tdcE* activities, was subsequently developed. Compared with those of the *E. coli* KJ098 strain, the production of acetic acid and malate and the accumulation of pyruvate decreased, whereas that of succinic acid increased. After that, KJ104 (Δ*ldhA* Δ*adhE* Δ*ackA* Δ*focB-pflB* Δ*mgsA* Δ*poxB* Δ*tdcDE* Δ*citF*) was further engineered to eliminate *citF* from the *E. coli* KJ098 strain. This resulted in a significant decrease in cell yield but had no effect on succinic acid yield in the strain. To decrease the diffusion of carbon flux to L-aspartate and increase the yield of succinic acid, the aspartate aminotransferase (*aspC*) gene was subseauently deleted. A new strain, *E. coli* KJ110 (Δ*ldhA* Δ*adhE* Δ*ackA* Δ*focB-pflB* Δ*mgsA* Δ*poxB* Δ*tdcDE* Δ*citF* Δ*aspC*), was obtained. The yield of succinic acid, cell yield, and acetate in KJ110 were not affected even when *aspC* was removed. Next, the *E. coli* KJ122 (Δ*ldhA* Δ*adhE* Δ*ackA* Δ*focB-pflB* Δ*mgsA* Δ*poxB* Δ*tdcDE* Δ*citF* Δ*aspC* Δ*sfcA*) strain was created by deleting both *aspC* and *sfcA* (Figures 2.4 and 2.5). This strain produced the highest succinic acid yield, titer, and productivity. *E. coli* KJ122 produces 1.4-1.5 mol of succinic acid per 1 mol of glucose, which is 85% of the maximum theoretical yield (1.7 mol per 1 mol of glucose).

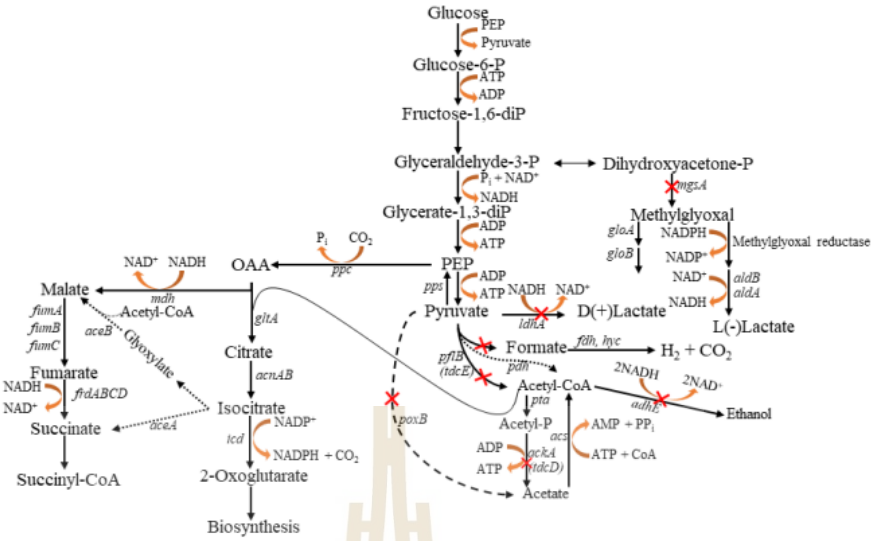


Figure 2.4 Anaerobic metabolism pathway of glucose in *E. coli* KJ122. (Jantama et al., 2008b).

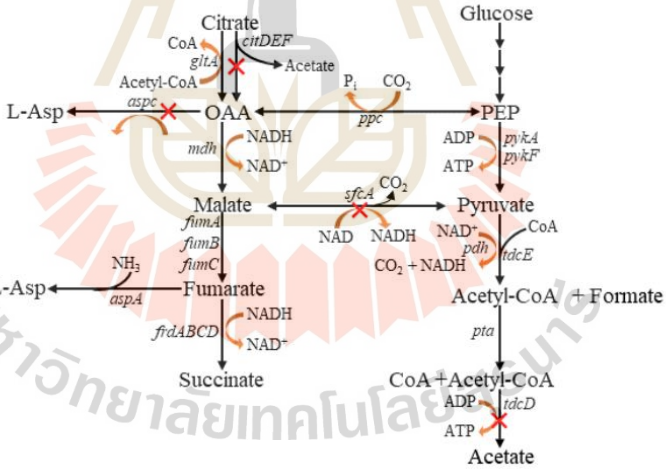


Figure 2.5 Additional genes that are involved in succinic acid production of *E. coli* KJ122 have been deleted. (Jantama et al., 2008b).

2.6 Lignocellulose structure and composition

Lignocellulosic biomass is composed of 33%–55% cellulose, 13%–33% hemicellulose, and 13%–32% lignin by dry weight. These three primary components make up 90% of the total biomass, with the remaining portion consisting of ash, structural proteins, and extractives (Balat, 2011). Cellulose and hemicellulose consist

of C6 sugars (such as glucose) and C5 sugars (such as xylose), both of which can be used in bacterial fermentation. Hemicellulose consists of pentoses, hexoses, small amounts of uronic acids, hydroxyl groups, D-glucuronic acid, and acetylated sugars (Gírio et al., 2010). The hexoses are specifically identified as L-fructose, D-mannose, L-galactose, and D-galactose, while the pentoses include arabinose, L-rhamnose, and xylose (Ojewumi et al., 2022). Cellulose consists of linear chains of β-(1,4)-linked D-glucopyranose units (Vermerris, 2008). Lignin is composed of aromatic units, guaiacyl (G), syringyl (S), and specifically p-hydroxyphenyl (H), with their relative proportions varying depending on the type of biomass (Buranov and Mazza, 2008). The structure and components of lignocellulosic biomass are shown in Figure 2.6.

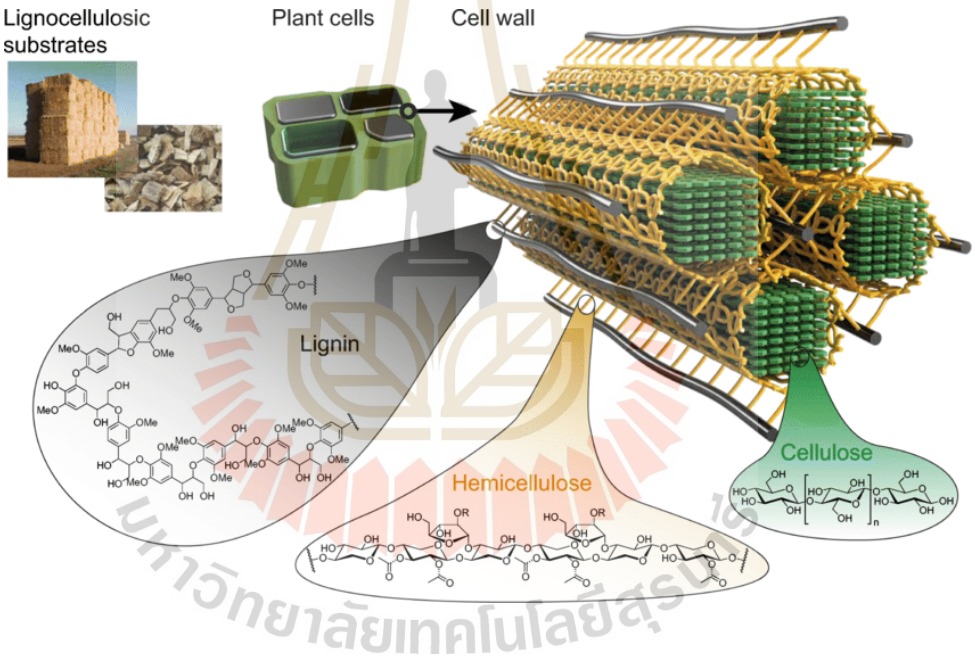


Figure 2.6 Structure and Components of lignocellulosic (Brethauer et al., 2020).

2.6.1 Cellulose

Cellulose is the most prevalent naturally occurring organic, high-molecular-weight polymer in the world. It serves as the main structural component of plant cell walls, making plants stable even without water and allowing them to be renewable and biodegradable (Sanchez and Cardona, 2008). Cellulose is a homopolymer of D-anhydroglucopyranose monomeric units joined by β-(1-4)-glycosidic bonds, forming a linear structure. As shown in Figure 2.7, the typical

structure of cellulose consists long-chain polymers with D-glucose as the repeating unit, forming unbranched chains (Dhawan, 2007; Harun et al., 2013). The degree of plant cellulose polymerization can vary from 7000 to 15000 glucose molecules, depending on the source. Strong hydrogen bonds hold the microfibrils that make up the cellulose chain together, whereas van der Waals forces hold the microfibrils that make up the cellulose fiber together. The crystalline structure of cellulose makes it highly resistant to breakingdown. Natural cellulose loses its mechanical qualities when it absorbs moisture due to its hydrophilic nature, which is important for applications involving paper and board (Fengel and Wegener, 2011; Himmel et al., 2007).

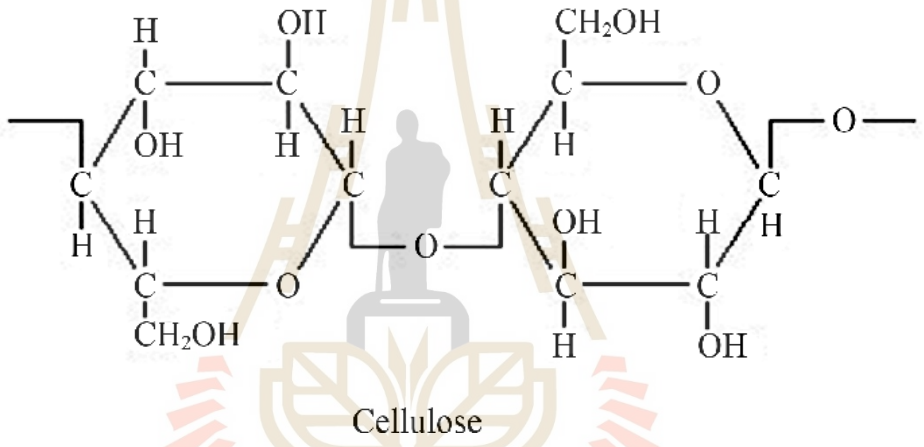


Figure 2.7 Structure of cellulose (Heidi Lynn et al., 2012).

2.6.2 Hemicellulose

Hemicellulose is composed of low-molecular-weight branching polymers. It makes up between 20 and 35% of a plant's dry weight, making it the second most readily available polymer worldwide. Typically, hemicellulose is a collection of homo and heteropolymers. The primary chains or backbones of hemicellulose are anhydrous β-(1-4)-xylopyranose, mannopyranose, glucopyranose, and galactopyranose, which have various substituents. The main sugars found in hemicellulose are D-xylose, L-arabinose, D-glucose, D-mannose, D-glucuronic acid, 4-O-methyl-D-glucuronic acid, and D-galacturonic acid (Latif et al., 2018).

Hemicelluloses with low hydration levels stabilize the cell wall by forming hydrogen bonds with cellulose. Another stabilizing interaction for the cell wall involves covalent bonding with lignin, while water-soluble components help maintain stability in their raw form (Wyman et al., 2005). The chemical structure of hemicellulose is shown in Figure 2.8.

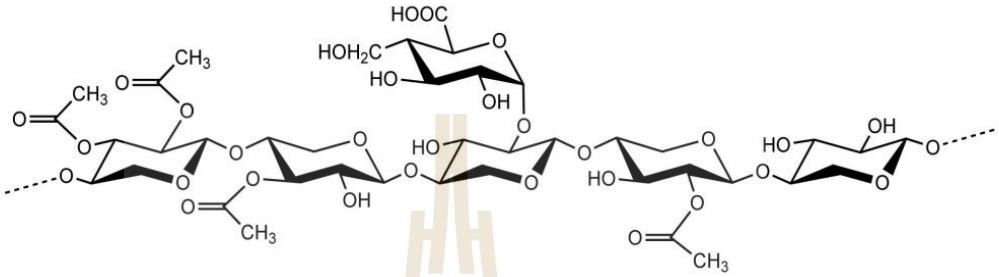


Figure 2.8 The chemical structure of hemicellulose (Hu et al., 2020).

2.6.3 Lignin

Lignin is a complex, high molecular weight polymer composed of phenylpropane units (Figure 2.9). It consists of a diverse group of aromatic polymers formed through oxidative combinatorial coupling of 4-hydroxyphenylpropanoids (Hu et al., 2020). These polymers are primarily deposited in the cell walls of secondary thickened cells, which contributes to cell wall rigidity. This rigidity supports internal nutrient and water transport, and provides an effective defense system against microbial attacks (Latif et al., 2018).

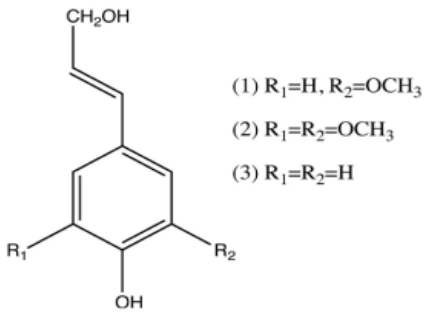


Figure 2.9 Structure of lignin precursors phenylpropanoid units (Jara, 2010).

Table 2.4 Composition of various lignocellulose biomasses

Type of lignocellulose	Cellulose (% w/w)	Hemicellulose (% w/w)	Lignin (% w/w)	Reference
Wheat straw	33-40	20-25	15-20	(Volynets and Dahman, 2011)
Barley straw	34-36	23-29	13-23	(Han et al., 2013; Saha and Cotta, 2010)
Poplar trees	39-46	18-19	20-25	(Wang et al., 2012)
Corn stover	35-37	20-31	18-23	(Li et al., 2012; Saha et al., 2013)
Hardwoods	46-55	13-18	22-27	(Santos et al., 2012)
Paper pulp	60-70	10-20	5-10	(Sun and Cheng, 2002)
Rice straw	36-49	22-23	16-17	(Amiri et al., 2014; Yang et al., 2012)

2.7 Pretreatment with dilute acid

Pretreatment is a crucial process for the production of bioproducts from lignocellulose. It can increase the efficiency of enzyme hydrolysis to produce fermentable sugars. Without pretreatment, more enzymes are required for lignocellulose material. Additionally, after pretreatment, the practical size of pretreated lignocellulose increases the specific surface area and reduces crystallinity (Volynets et al., 2017). Acid pretreatment is one of the most important techniques aimed at achieving high yields of sugars from lignocellulose. Sulfuric acid is widely used for pretreatment, typically at concentrations between 0.2% and 2.5% w/w, and at high temperatures ranging from 130°C to 210°C (Sarkar et al., 2012). During acid pretreatment, hemicellulose is hydrolyzed into monosaccharides, while lignin condenses and precipitates. While acid can breakdown lignocellulose, strongly acidic pretreatment results in the production of inhibitory byproducts, such as furfural and hydroxymethylfurfural (HMF), leading to high operational and maintenance costs, as well as equipment corrosion. Therefore, strong acidic pretreatment is generally avoided, with pretreatment by dilute acids coupled with thermal methods instead (Ariunbaatar et al., 2014; Mahboubi et al., 2017). High temperatures are typically ideal for the hydrolysis of cellulose in diluted acid treatment, which hydrolyzes hemicellulose to produce xylose and furfural, among other sugars (Sassner et al., 2008). Commercial pretreatment using diluted sulfuric acid has been applied to various biomass sources, such as poplar (Wyman et al., 2009), switchgrass (Digman et al., 2010; Li et al., 2010), and corn stover (Du et al., 2010). Factors to consider in pretreatment include high and efficient digestion of cellulose and hemicellulose, minimal loss of feedstock sugars, little to no production of fermentation inhibitors, and a reduction in chemical usage. Adjusting the pH when chemical acids or bases are used is necessary to prevent the production of salts that are harmful to fermenting bacteria (Volynets et al., 2017).

Different lignocellulosic biomasses have been pretreated with acids such as H_2SO_4, HCl, H_3PO_4, and HNO_3. A previous report showed that pretreating maize straw with 1% H_2SO_4 and 2% NaOH increased biomass digestibility to 98% by breaking down cellulose and lignin (Jia et al., 2014). Si et al. (2015) successfully separated hemicelluloses and lignin by pretreating hemicellulose-rich samples with 1% H_2SO_4

and 2% NaOH, resulting in enhanced biomass digestibility to 100%. A diagram of the lignocellulose pretreatment process is shown in Figure 2.10.

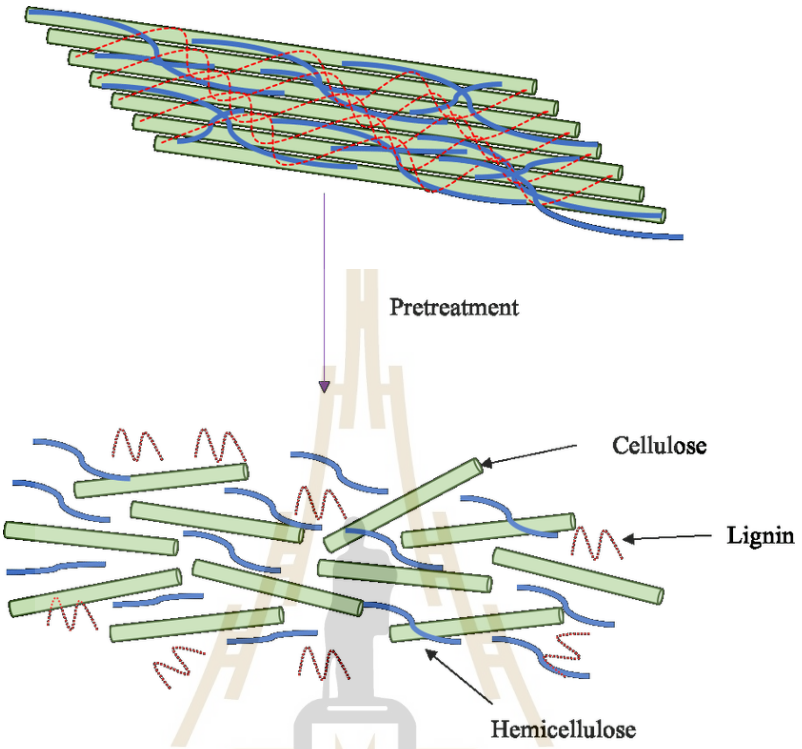


Figure 2.10 Diagram of pretreatment lignocellulose processes (Latif et al., 2018).

2.8 Enzymatic hydrolysis of lignocellulose

Currently, the most commonly studied and industrially utilized cellulase is derived from the fungus *Trichoderma reesei*. Many experimental investigations have been conducted in recent years to provide the groundwork for improving the hydrolysis process to lower costs and increase conversion efficiency. To improve the pretreatment parameters for the enzymatic hydrolysis of lignocellulosic biomass, the reagent concentration, substrate particle size, reaction temperature, and reaction duration, are crucial when high-throughput screening optimization technologies are used (Zhang et al., 2021), are crucial. Ensuring the stability and reusability of enzymes, is essential for large-scale industrial applications.

2.8.1 Mode of action of cellulase

Three essential components make up a cellulose enzyme: *endo*-(1,4)-β-D-glucanase (EC 3.2.1.4), *exo*-(1,4)-β-D-glucanase (EC 3.2.1.91), and β-glucosidases

(EC 3.2.1.21). Endocellulases bind randomly with a glucose chain and hydrolyze one or a few accessible bonds, which are believed to cleave β-1,4-glycosidic bonds only internally and contain cleft-shaped open active sites (Kumar and Murthy, 2013). This enzyme also acts on the crystals of cellulose. Exoglucanase exists in several forms, with β-1,4-glucan glucohydrolase removing one glucose unit at a time from the nonreducing end of the chain (Lee et al., 1995). From the nonreducing end, β-glucosidases hydrolyze soluble cellodextrins and cellobiose into glucose; however, they are inactive with crystalline or amorphous cellulose (Singh et al., 2016). The mode of action of cellulase is shown in Figure 2.11.

In a previous study, cellulase enzymes were used in the pulp and paper industry. Using cellulase in this industry can improve overall performance and enhance the productivity of the paper-making process. In the process of ddeinking waste, cellulases such as Novozyme 342 and Novozyme 613 can be utilized with 95–99% deinking efficiency (Singh et al., 2016). Additionally, *Trichoderma harzianum* was found to produce the enzymes cellulase and xylanase, which were then used to deink photocopier waste paper. They reported that the deactivation effectiveness of enzymes was 23.6% greater than that of the nonenzymatic deactivation process (Pathak et al., 2014).

2.9 Waste paper

Currently, the paper industry is rapidly expanding, leading to waste from production processes such as paper pulp. Paper is also used in many everyday documents, contributing to an annual waste of approximately 100 million tons (Ma et al., 2021). In the United States, only approximately 38% of the 110 million tons of paper and cardboard waste are recycled, while the remaining 62% are inefficiently burned or sent to landfills, causing environmental issues (Milbrandt et al., 2024).

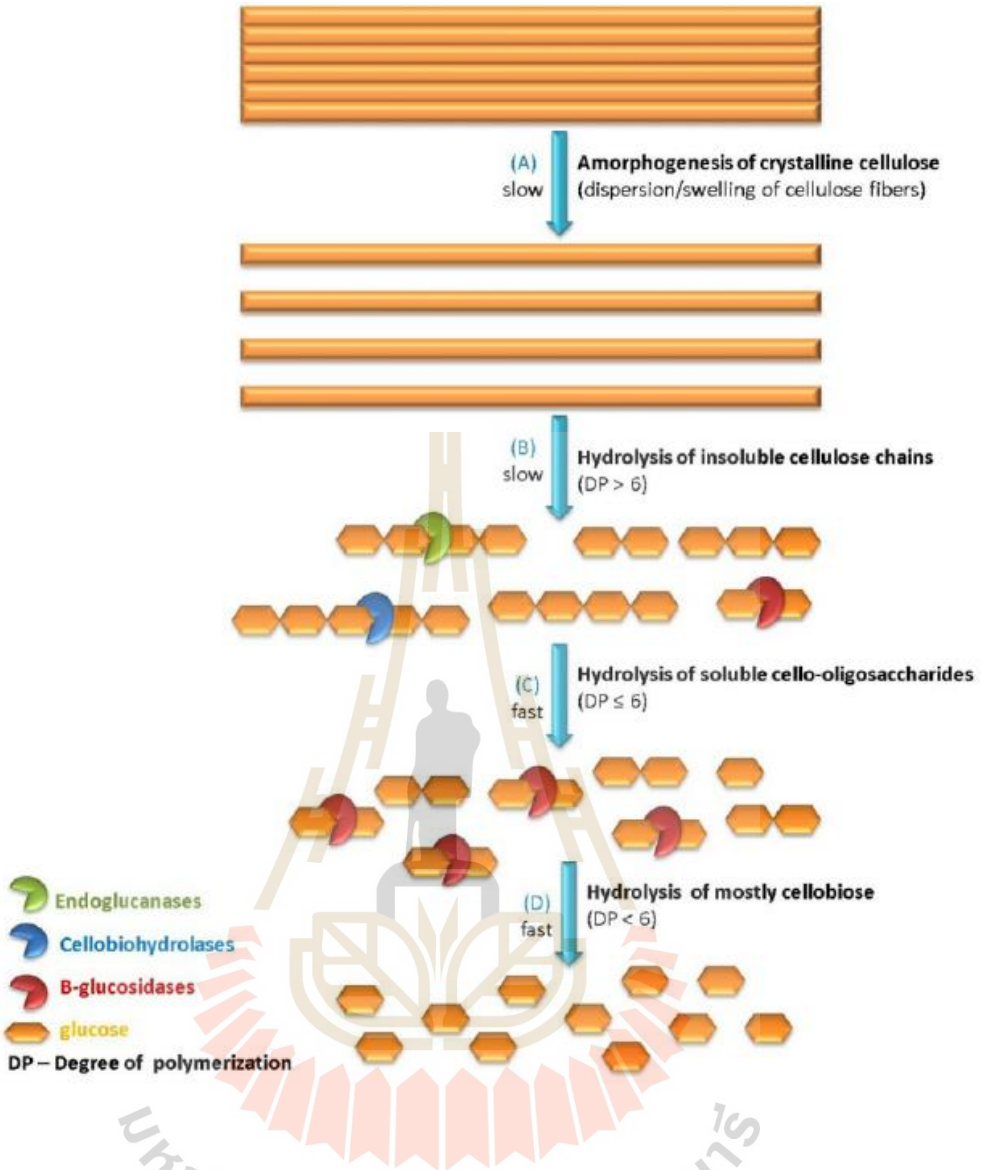


Figure 2.11 The mode of action of cellulase (Arantes and Saddler, 2010).

Waste paper is utilized in the production of many bio-chemicals in the industry. For example, Dubey et al. (2012) produced bioethanol using acid-pretreated waste paper with *Pichia stipitis*. Under optimum conditions, ethanol production reached 3.73 g/L. Additionally, Rocha et al. (2016) produced ethanol using separate hydrolysis fermentation with *Spathaspora passalidarum* HMD, resulting in ethanol production of 13 g/L with a productivity of 0.57 g/L/h. Al Battashi et al. (2021) utilized anaerobic digestion of office paper to produce volatile fatty acids (VFAs), which were then used as a feedstock for polyhydroxyalkanoate (PHA) production by *Cupriavidus necator*. The results showed a PHA yield of 0.31 g/g under optimum conditions. Moreover,

Nair and Sivakumar (2022) reported biodiesel production from waste office paper by *Rhodosporidium toruloides*. Optimization resulted in 10.55 g/L biomass, 6.13 g/L lipid, with a lipid content of 58%. Prasetyo and Park (2013) used waste paper sludge as a feedstock for bioethanol production using *A. cellulolyticus* and thermotolerant *S. cerevisiae* TJ14 via the SSF process. The results showed an ethanol yield of 0.051 g ethanol/g waste paper sludge. Bioethanol production from fermentation with *S. cerevisiae* using waste office paper as a feedstock yielded 0.4 mL bioethanol/g (Awol and Abate, 2021). Thakare and Trivedi (2021) produced bioethanol from newspapers, and after fermentation using *S. cerevisiae*, they obtained a yield of 0.25 mL/g. Nishimura et al. (2016) used waste paper as a feedstock for ethanol production via fed-batch presaccharification and simultaneous saccharification and fermentation (Fed-batch PSSF) by *S. cerevisiae* KF-7. Under these conditions, the waste paper reached a titer of 45.3 g/L and a productivity of 0.54 g/L/h. This demonstrated that fermentation of waste paper can be used to produce a variety of products (Table 2.5). However, no succinate production from MWOP has not been studied.

Table 2.5 Fermentation of waste paper can be used to produce a variety of products.

Products	Microorganism	Fermentation strategy	Titer (g/L)	References
Bioethanol	*Pichia stipites*	Batch fermentation	3.73	(Dubey et al., 2012)
Ethanol	*Spathaspora passalidarum*	SHF fermentation	13	(Rocha et al., 2016)
Ethanol	*P. stipitis* NCIM 3499	S-SSCF fermentation	42.34	(Dey et al., 2021)
Biodiesel	*Rhodosporidium toruloides*	Batch fermentation	6.13	(Nair and Sivakumar, 2022)
L-(+)-Lactic acid	*Rhizopus oryzae*	Batch fermentation	49.1	(Park et al., 2004)
Lactic acid	*Streptococcus thermophiles*	SSF fermentation	39.71	(Yang et al., 2018)
Ethanol	*Saccharomyces cerevisiae* KF-7	Fed-batch PSSF fermentation	45.3	(Nishimura et al., 2016)
PHB	*Ralstonia eutropha* NCIMB 11599	Batch fermentation	4.45	(Neelamegam et al., 2018)
D-lactic acid	*E. coli* JH13	Batch fermentation	35.86	(Liu et al., 2016)

CHAPTER III

RESEARCH METHODOLOGY

3.1 Strains, media, and growth cultivations

The *E. coli* KJ122 strain used in this study was stored at – 80°C at Suranaree University of Technology (SUT), Thailand. Seed cultures were grown in Luria–Bertani (LB) medium containing 20 g/L glucose at 37°C and 200 rpm in a 500 mL small vessel with a working volume of 350 mL for 16 h of incubation. The cultivation was maintained at pH 7.0 by automatically adding a mixture of 3 M K_2CO_3 and 6 M KOH at a 6:1 ratio. The seed culture mixture was then inoculated into fermentation medium at an initial OD_{550} of 0.1 (33.3 mg CDW/l). Anaerobiosis was quickly achieved during growth by the addition of bicarbonate to ensure a CO_2 atmosphere. Low-salt AM1 medium supplemented with 100 mM $KHCO_3$ and 1 mM betaine HCl was used as fermentation broth throughout this study for succinate production and strain maintenance (Phosriran and Jantama, 2024). The composition of the AM1 medium is shown in Table 3.1.

3.2 Pretreatment of mixed waste office paper hydrolysate (MWOP)

The MWOP was cut into pieces 3–5 cm in size and treated with diluted sulfuric acid at concentrations of 1%, 2%, 4%, 6%, and 8% (v/v) with a solid content of 10% (w/v). The mixture was then heated at 121°C for 30 min in an autoclave. The optimal incubation time for autoclaving was also evaluated at different incubation periods of 20, 30, 40, 50, and 60 min.

The acid-pretreated MWOP (AP-MWOP) was washed with tap water until the drain tap water reached a neutral pH. The washed AP-MWOP mixture was subsequently air-dried at 55°C for 24 hours until its moisture content reached approximately 5% (w/w). AP-MWOP pulping was used as a substrate for succinic acid production throughout this study. The total level of sugars released from AP-MWOP pretreated with different concentrations of sulfuric acid was investigated during

enzymatic hydrolysis at a crude cellulase loading of 80 PCU/g AP-MWOP. The optimal incubation time for enzymatic hydrolysis was also evaluated at different incubation periods of 20, 30, 40, 50, and 60 min.

Table 3.1 Composition of AM1 medium.

Component	Concentration (mmol/l)
$(NH_4)_2HPO_4$	19.92
$NH_4H_2PO_4$	7.56
Total PO_4	27.48
Total N	47.39
[a]Total K	1.00
$MgSO_4$.$7H_2O$	1.50
Betaine-HCl	1.00
	Concentration (µmol/L)[b]
$FeCl_3$.$6H_2O$	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
$MnCl_2$.$4H_2O$	2.50
Total salts	4.1 g/L

[a] KOH is used to neutralize betaine-HCl stock.

[b] Trace metal stock (1000X) was prepared in 120 mM HCl.

3.3 Enzymatic and acid hydrolysis of AP-MWOP

AP-MWOP at a concentration of 50 g/L in AM1 medium was hydrolyzed with the crude cellulase enzyme in a 250 mL Erlenmeyer flask with a working volume of 50 mL. The VRE P3 crude cellulase (Siam Victory Chemicals, Thailand) was used to hydrolyze AP-MWOP at enzyme loadings of 60, 80, 100, 120, and 140 PCU/g AP-MWOP for 120 h. The slurry mixture was then incubated at 50°C with shaking at 200 rpm to facilitate saccharification. The cellulase concentration resulting in the

highest level of liberated fermentable sugars released from AP-MWOP was determined to be the most suitable enzyme loading for further experiments.

The complete saccharification of the AP-MWOP by strong acid digestion was adapted from the method of Dunning and Dallas, (1949). The AP-MWOP at a weight of 1.0 g was mixed with 10 ml of warm 72% (v/v) H_2SO_4 (55°C), and the slurry was agitated for 5 min. DI water was added to the slurry, reaching a volume of 200 ml, and the mixture was heated at 121°C for 25 min. The sample was centrifuged, and the supernatant was prepared for HPLC analysis to determine the liberated sugar content.

3.4 Succinic acid production from AP-MWOP via different fermentation modes

AP-MWOP pulping was used as a substrate for succinic acid production in a 5 L bioreactor with a working volume of 2.5 L (Infors, Switzerland). The separate hydrolysis and fermentation (SHF) process was initially applied by hydrolyzing AP-MWOP using the VRE P3 cellulase enzyme at the optimized loading. Saccharification through SHF was carried out at 50°C with agitation at 400 rpm. After 48 h of saccharification, the mixture was cooled to 37°C at 200 rpm, and potassium bicarbonate at a final concentration of 100 mM was added to the fermenter. The seed inoculum of *E. coli* KJ122 was added to the slurry with an initial OD_{550} of 0.5. Fermentation began upon inoculation and was maintained at 37°C. The pH of the fermentation broth was maintained at 7.0 by adding a mixed solution of 6 M KOH and 3 M K_2CO_3 at a 1:6 ratio. Different concentrations of AP-MWOP (50, 70, and 100 g/L) were used to determine the optimal substrate level for succinic acid production. Various agitation speeds (100, 200, and 300 rpm) were also optimized to test the effect of agitation speed on succinic acid production with the appropriate concentration of AP-MWOP.

AP-MWOP was also used as a substrate for succinic acid production during simultaneous saccharification and fermentation (SSF), following the same strategy as the SHF process mentioned above. However, the seed culture and the VRE P3 cellulase were added simultaneously. Fermentation was also performed under the same conditions as the SHF process.

For fed-batch SSF, the optimal conditions from the batch experiments were applied to increase the succinic acid concentration, yield, and productivity in fed-batch mode. The pre-saccharification time was 0, 6, 12, 18, or 24 h before performing the fed-batch SSF process. AM1 medium containing 70 g/L AP-MWOP was initially utilized for succinic acid production. When the total glucose concentration in the fermentation broth decreased below 10 g/L, additional substrates were intermittently fed into the fermenter until the final glucose concentration reached 25–30 g/L. All the experiments were performed in triplicate. The experimental design is shown in Table 3.2.

Table 3.2 Experiment design for batch SHF and SSF experiments with AP-MWOP.

Substrate (g/L)	Partial saccharification before succinate production (h)
	SHF
50	48
70	48
100	48
	SSF
50	18
70	18

3.5 Calculation method

The yield and production of succinic acid from AP-MWOP was calculated via the following equations (Qin et al., 2023).

$$\text{Succinic acid yield (g/g)} = \frac{\text{Succinic acid concentration (g/L)}}{\text{(glucose + xylose) consumed (g/L)}}$$

$$\text{Succinic acid productivity (g/L/h)} = \frac{\text{Succinic acid concentration (g/L)}}{\text{fermentation time (h)}}$$

3.6 Analytical methods

The fermentation broths were collected every 6 h to quantify the cell mass, organic acids, and glucose. Cell growth was measured via a Bausch & Lomb Spectronic 70 spectrophotometer at OD_{550} nm and converted to biomass as the cell dry weight (1 OD_{550} = 0.333 g CDW/L biomass). High-performance liquid chromatography (HPLC) with an Aminex HPX-87H column (7.8*300 mm, Bio-Rad, USA) and refractive index detectors were used to determine the organic acid and glucose contents throughout fermentation (RI-150, Thermo Spectra System, USA). The mobile phase in the HPLC system was sulfuric acid (4 mM) at a flow rate of 0.4 mL/min. The fermentation culture was centrifuged to separate the cells and AP-MWOP pulp from the supernatant. To prepare the sample, a tenfold dilution of the supernatant sample was performed with 20 mM H_2SO_4, and the diluted liquid was filtered through a 0.22 μm nylon filter prior to HPLC injection. The concentrations of sugars and organic acids were used to calculate the sugar consumption rate, succinate yield, and productivity. The number of bacterial cells that survived during fermentation was also measured by spreading the diluted broth on solid LB agar media, and the plates were then incubated at 37°C for 24 h. The number of bacterial colonies that grew on the plates was then counted and reported as the number of colony-forming units per milliliter of culture (CFU/ml).

3.7 Statistical analysis

Analysis of variance (ANOVA) was conducted via GraphPad/Prism 8.0.2 (GraphPad Software, USA). Three independent replications were performed for each test, and average values are reported with standard deviations (SDs). The differences among the mean values were established via Tukey's test at the 95% significance level ($p<0.05$)

CHAPTER IV
RESULTS AND DISCUSSION

4.1 Pretreatment of MWOP with diluted H_2SO_4

Hayat et al., (2021) reported that waste papers usually contain 40% (w/w) cellulose, 32.5% (w/w) hemicellulose, and 22.5% (w/w) lignin. However, MWOPs used in this study contained 81.43 ± 1.90% (*w/w*) cellulose, 2.43 ± 2.27% (*w/w*) hemicellulose, 0.69 ± 0.04% (*w/w*) lignin, and 7.95 ± 0.18% (w/w) ash based on proximal analysis. This suggests that MWOPs contain cellulose and hemicellulose fibers that can be broken down by combined chemical and enzymatic hydrolysis to obtain released fermentable sugars, mainly glucose and xylose, to be fermented to produce valuable biochemicals. Therefore, MWOP may serve as an alternative substance with renewable potential as a feedstock for succinic acid production. Mansy et al. (2024) demonstrated the effectiveness of various chemical pretreatments for waste papers using acids, bases, and hot water. However, they revealed that pretreatment with bases or alkaline agents, in addition to acetic acid and hot water pretreatments, was a noneffective pretreatment for waste office paper because there was no significant increase in the number of released glucose units even after additional physical pretreatments involving autoclaving, microwaving, and sonication. In contrast, strong acid pretreatment, especially with H_2SO_4, is one of the most widely used options because it does not require a recovery step, making it favorable for industrial applications because of its higher monosaccharide conversion yield than those of other strong acids, including HCl and H_3PO_4 (Porninta et al., 2024). The enzymatic hydrolysis of cellulose and hemicellulose fibers in H_2SO_4-pretreated MWOPs can be more effective not only in reducing the recalcitrance of those fibers but also in preventing the formation of microbial inhibitors, including furfural and 5-hydroxymethyl furfural (5-HMF), derived from sugar degradation.

In this study, H_2SO_4 solutions at concentrations of 1%, 2%, 4%, 6%, and 8% (v/v) were used for the pretreatment of MWOP at 121°C for 30 min. Subsequently, the total

amount of sugars released from AP-MWOP was investigated via enzymatic hydrolysis by crude cellulase at 80 PCU/g AP-MWOP. The results indicated that the enzymatic hydrolysis of 50 g/L AP-MWOP pretreated with diluted H_2SO_4 at 1%, 2%, 4%, 6%, and 8% (v/v) resulted in total liberated sugars of 25.32±1.42, 24.56±2.37, 18.07±0.27, 18.28±0.41, and 17.44±0.77 g/L, respectively, with a conversion yield of up to 50.64±0.05% (Figure 4.1A). The pretreatment of MWOP with H_2SO_4 at concentrations greater than 2% (v/v) adversely affected the total number of sugars released. The high percentage of H_2SO_4 solution used for pretreatment likely caused a decrease in the amount of sugar released due to a loss of hemicellulose structures into the black liquor fraction under extremely high acidity during the pretreatment step. This is similar to the findings of Kumar et al., (2009), in which the effectiveness of the diluted H_2SO_4 pretreatment of most lignocellulosic materials was only achieved when its concentration was less than 4% (v/v). Rocha et al., (2016) and Nair et al., (2020) reported that pretreatment of waste office paper with 1% (v/v) H_2SO_4 increased the cellulose content with no detectable levels of furfural or 5-HMF, thus indicating that no detoxification is needed before its subsequent use for bioethanol production by *Spathapora passalidarum* and biodiesel production by *Cryptococcus curvatus*. Da Mota and Gouveia, (2016) also pretreated waste office paper with 1% (v/v) H_2SO_4 at 50°C for 3 h to achieve a maximum glucose concentration of 23 g/L. Alternatively, the use of HCl solution at very high concentrations was also reported to pretreat waste office paper. However, HCl at a concentration of 15% (v/v) was utilized for the pretreatment of 60 g/L waste office paper. The maximum amount of liberated glucose was only 15.7 g/L, with a comparable conversion yield of 26.2%. It is likely that the use of high HCl concentrations for pretreating waste office paper may not be suitable for large-scale applications since highly concentrated acids are hazardous, toxic, and corrosive, thus requiring corrosion-resistant reactors that also increase the capital investment or even operating cost of equipment used for high acid-catalyzed pretreatment. Additionally, NaOH pretreatment was used to demonstrate the pretreatment of waste office paper. On the other hand, Mokatse and van Wyk, (2021) and Ojewumi et al., (2018) reported the utilization of 1.0% (w/v) NaOH to pretreat waste office paper. However, both studies showed that liberated sugar concentrations of 5.7 g/L and 0.37 g/L were achieved only from NaOH-pretreated waste office paper.

Both studies showed that alkaline pretreatment may not be suitable for pretreating MWOP. These data suggested that the 1% (v/v) H_2SO_4 solution was more suitable for pretreating MWOP than the other pretreatment methods were.

H_2SO_4 is typically used for pretreatment at concentrations between 0.2% and 2.5% (v/v) and at high temperatures ranging from 130°C to 210°C to increase the digestibility of pretreated substrates but to reduce the pretreatment time (Sarkar et al., 2012). In this experiment, the utilization of higher temperatures at 135°C for 30 min was therefore investigated with the use of 1% (v/v) H_2SO_4 pretreatment. The results revealed that the total amount of sugar released at the insignificant level of 26.38±4.06 g/L, which was only 4.19% greater than that obtained at 121°C, was observed after enzymatic hydrolysis. An increase in the pretreatment time during autoclaving at 135°C greater than 30 min did not even improve the enzymatic digestibility of AP-MWOP by crude cellulase. The total liberated sugar levels were also comparable even when the pretreatment time was prolonged to 60 min (Figure 4.1B). Pretreatment at 135°C for only 20 min inversely enhanced the effectiveness of enzymatic hydrolysis by increasing the released sugar level to 27.58±0.34 g/L, which was approximately 8.92% greater than that obtained at 121°C for 20 min. However, the levels of the sugars liberated from both conditions were not significantly different. Díaz-Blanco et al., (2018) studied the diluted H_2SO_4 pretreatment of *Agave lechuguilla* at temperatures ranging from 160°C to 200°C with acid concentrations between 0.5% and 1.5% (v/v). The optimal conditions for pretreating agave stems were 180°C and 1.24% (v/v) H_2SO_4 with 10% (w/v) biomass loading. Under these conditions, the recovery rates of hemicellulose sugars and glucose reached maximum at 87% and 68%, respectively. Our results and other findings suggested that the acid concentration and pretreatment temperature had a significant effect on cellulose and hemicellulose recovery and the formation of degradation products during pretreatment, whereas the pretreatment time had a lesser effect. However, these findings contradict those of Martín et al. (2019), who reported that optimal pretreatment conditions for high enzymatic conversion of cellulose in starch-depleted cassava stems were achieved at 195°C with a relatively low concentration of 0.6% (v/v) H_2SO_4 and a pretreatment time of more than 35 min. The maximum cellulose breakdown at 72% was obtained at the pretreatment time of 50 min. However, these authors suggested that H_2SO_4

pretreatment at high temperatures and longer times may have disadvantages in which high energy input is required while high hemicellulose degradation is also acquired.

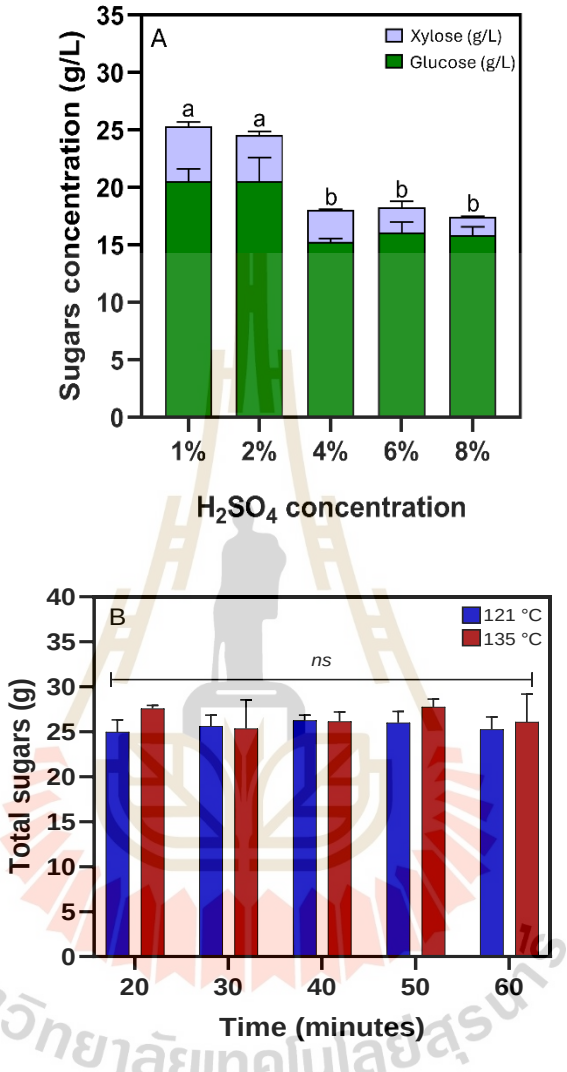


Figure 4.1 Effects of different H_2SO_4 concentrations for pretreatment of MWOP at 121°C for 30 min (**A**) and effect of pretreatment time for pretreatment of MWOP at 121°C and 135°C (**B**) on total released fermentable sugars (glucose and xylose) from the acid-pretreated MWOP after enzymatic hydrolysis by crude cellulase. Each column represents the mean ± SD from three independent replicates. The different alphabet (a or b) above each column indicates statistical significance of differences between the mean values of the total released sugars among different

treatments ($p < 0.05$). Data considered not significant among different treatments are marked *ns* ($p > 0.05$).

Figure 4.2A shows the effects of pretreatment on the total released sugars from 50 g/L untreated-MWOP and AP-MWOP by strong acid digestion and cellulase hydrolysis. The results revealed significant differences in the levels of sugars released from untreated-MWOP (38.21±0.49 g/L with a yield of 0.76±0.01 g sugars/g MWOP) and AP-MWOP (43.47±0.20 g/L with a yield of 0.86±0.00 g/g AP-MWOP) after strong acid digestion. For cellulase hydrolysis, AP-MWOP (27.58±0.34 g/L with a yield of 0.55±0.00 g/g AP-MWOP) offered a substantially higher level of released sugars than did untreated MWOP (6.74±0.61 g/L with a yield of 0.13±0.03 g/g MWOP). This indicated that pretreatment with dilute H_2SO_4 had a greater beneficial effect on the enzymatic hydrolysis of AP-MWOP. On the basis of the results of this study, pretreatment of the diluted H_2SO_4 mixture of MWOP with 1% (v/v) H_2SO_4 at 121°C for 20 min was determined to be the most suitable condition for preparing AP-MWOP for subsequent enzymatic hydrolysis to obtain the highest fermentable sugar level for succinic acid production. This was supported by Guo et al. (2012), who reported that acid pretreatment involves the breakdown or attack of intramolecular and intermolecular bonds between cellulose, hemicellulose, and lignin by hydronium ions, affecting the porosity of the substrate and the accessibility of cellulase.

4.2 Optimal cellulase loading for the enzymatic hydrolysis of AP-MWOP

Enzymatic hydrolysis plays a crucial role in numerous biological and industrial processes, providing a specific, efficient, and environmentally friendly method for breaking down complex molecules. In this study, this process was carried out via the used of a crude cellulase enzyme, which catalyzes the hydrolysis of cellulose and hemicellulose fibers into three types: endoglucanase, cellobiohydrolase, and β-glucosidase (Silvia and Maharani, 2023).

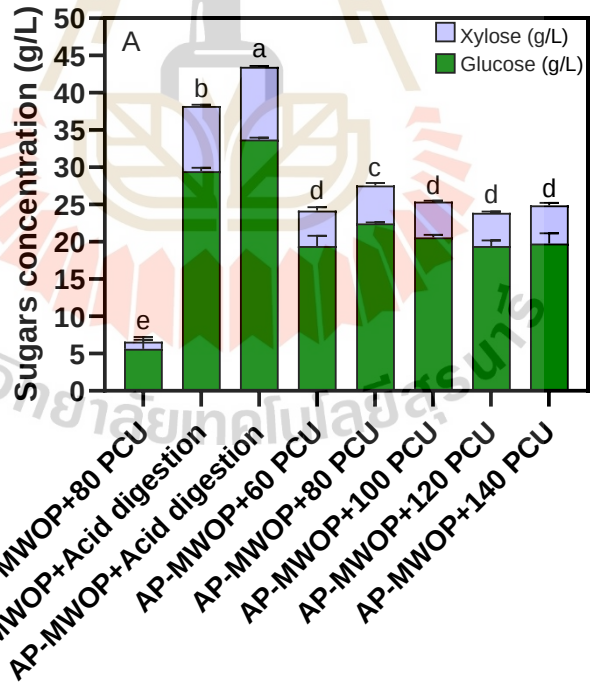
In this study, the effect of enzyme loading on total liberated sugars was assessed to minimize the level of crude cellulase used during the enzymatic

hydrolysis of AP-MWOP. The crude cellulase was used to hydrolyze 50 g/L AP-MWOP at enzyme loadings of 60, 80, 100, 120, and 140 PCU/g AP-MWOP. The enzyme loading analysis revealed total released sugar concentrations of 24.17±1.82 g/L, 27.58±0.34 g/L, 25.40±0.45 g/L, 23.88±0.88 g/L, and 24.89±1.74 g/L, respectively (Figure 4.2A). The results revealed that increasing the enzyme concentration to 80 PCU/g AP-MWOP resulted in significantly greater production of fermentable sugars (glucose and xylose), with the highest sugar yield of 55.16%. However, a further increase in cellulase loading did not increase the total sugar yield. This suggested that the use of higher cellulase loadings rapidly accumulated high amounts of cellobiose and glucose during enzymatic hydrolysis. This caused the inhibition of cellulase activities of both endoglucanase and β-glucosidase Hsieh et al. (2014), thus resulting in the alleviation of total liberated monosaccharides but increasing cellobiose levels instead. Therefore, the lowest level of enzyme loading that sufficiently and efficiently hydrolyzed AP-MWOP to the highest fermentable sugars was preferable. Furthermore, Figure 4.2B shows the hydrolysis rate of AP-MWOP at different cellulase loadings over 120 h. Enzymatic hydrolysis rapidly increased within 12 h of incubation, as reflected by a sharp increase in the total released sugar levels in all cellulase loadings. However, the hydrolysis rates suddenly decreased after 12 to 48 h. No significant increases in total released monosaccharide sugars were noted after 48 h. The utilization of 80 PCU/g AP-MWOP with a sugar yield of 55.16% was thus found to be optimal, as reflected by the highest level of total released sugars and the highest hydrolytic rate compared with those of other cellulase loadings.

Annamalai et al. (2020) demonstrated that the sugar yield substantially increased with 1–3% (w/w) total solid enzyme loading but started to decrease with 4% (w/w) solid enzyme loading for office paper. The maximum glucose yield (23.48 g/L) occurred at 3% (w/w) enzyme loading, and the minimum glucose yield (8.82 g/L) occurred at 1% (w/w) enzyme loading. These findings may suggest that high enzyme loading diminishes the sugar yield as the consistency of the substrates suddenly increases at high enzyme loadings, which influences the mixing homogeneity and mass transfer of the enzymes and causes feedback inhibition via the increased concentration of sugars (Rocha et al., 2016). Al-Battashi and Sivakumar, (2014) demonstrated PHB production by a *Cupriaviduc necator* via the SSF process via the

enzymatic hydrolysis of 100 g/L H_2O_2-pretreated waste office paper (OPHT) with 55.5 FPU cellulase (Sigma, C2730)/g OPHT and 37.5 CBU β-glucosidase (Sigma, 49,291)/g OPHT. However, the maximum PHB concentration of 4.29 g/L was only achieved, with a yield of 0.043 g PHB/g OPHT. This finding implied that the high cellulase loading provided in the study did not guarantee the effectiveness of the enzymatic hydrolysis to achieve high levels of fermentable sugars from OPHT to produce PHB at high concentrations and yields. Brummer et al. (2014) performed enzymatic hydrolysis of waste office paper to achieve a high sugar yield of 18.8% with 2% (w/w) cellulase complex (Novozymes 50013) and 0.2% (w/w) β-glucosidase (Novozymes 50010). Additionally, they recommended the utilization of high enzyme loadings up to 10% (w/w) cellulase complex and 1% (w/w) β-glucosidase for hydrolysis to achieve a twofold higher sugar yield, thus ensuring industrial application for bioethanol production. Park et al. (2001) also demonstrated the use of the commercial Acremonium cellulase AUS0301 for hydrolyzing unpretreated waste office paper. They demonstrated that cellulase at 20.6 FPU/g office paper was required to achieve a hydrolytic yield of up to 50%. However, they also proposed that a higher cellulase loading of 105 FPU/g office paper could be used to obtain a sugar conversion yield of up to 80% to effectively produce bioethanol on a large scale. Hossain et al. (2021) further revealed that the maximum glucose content obtained was 9.75 g/L from acid-treated waste paper (50% glucose yield) after 5 days of enzymatic hydrolysis, with up to 250 FPU/g acid-pretreated waste paper. Mansy et al. (2024) reported that the glucose level increased from 2.98 g/L to 5.01 g/L during the digestion of HCl-pretreated waste office paper when the cellulase loading was increased from 1% to 7% (w/w). When 9% (w/w) cellulase was used, the glucose concentration decreased to 4.07 g/L after 24 h of incubation. Additionally, they reported that the incubation time was 72 h, resulting in the highest release of glucose units at 15.72 g/L when 5% (w/w) cellulase enzyme was used, in which the cellulase loading greater than 5% (w/w) dramatically decreased. Furthermore, Da Mota and Gouveia, (2016) reported that using the lowest cellulase loading (Novozymes 188) of 5.2 FPU/g and 199 CBU/g β-glucosidase with a high solid mass of waste office paper at 75 g/L was optimal for achieving the highest glucose level

among the enzyme loadings tested. These findings confirm that excessive amounts of cellulase enzymes decrease the amount of glucose released. This may imply that an enzyme loading higher than a certain critical value does not improve hydrolysis, since the excess enzyme adsorbed into the substrate restricts the diffusion process through the structure. With the abovementioned information, several previous works showed that high liberated levels of fermentable sugars from pretreated waste office papers were achieved by the use of commercially high-cost cellulase with high enzyme loadings to obtain high levels of fermentable sugars for the subsequent production of bioproducts, but this strategy may increase the cost of the production of bioproducts via fermentation. Compared with our results, the use of crude cellulase at a lower enzyme loading for hydrolyzing MWOP to obtain a relatively high sugar yield compared with those obtained from other previous studies may therefore reduce the cost of succinic acid production at the industrial scale.



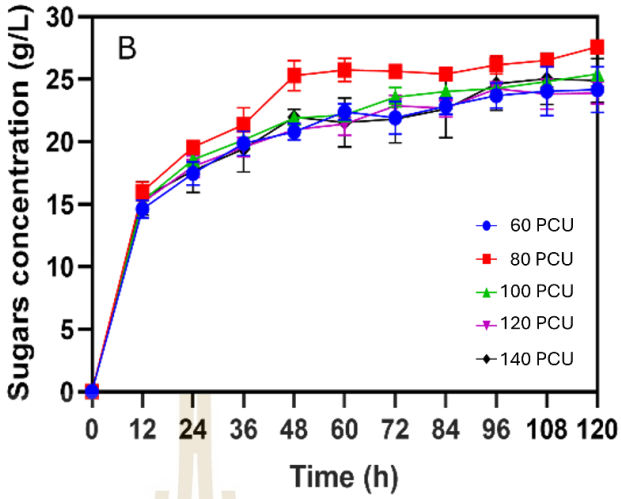


Figure 4.2 Effect of strong acid digestion and crude cellulase loadings (PCU: Protein Centered Unit) used during enzymatic hydrolysis of 50 g/L MWOP (untreated) and AP-MWOP on total released sugars (glucose and xylose) (A), and total released sugars from 50 g/L AP-MWOP hydrolyzed with different crude cellulase loadings at different time intervals (B). Each column represents the mean ± SD from three independent replicates. The different alphabet above each column indicates statistical significance of differences between the mean values of the total released sugars among different treatments ($p < 0.05$).

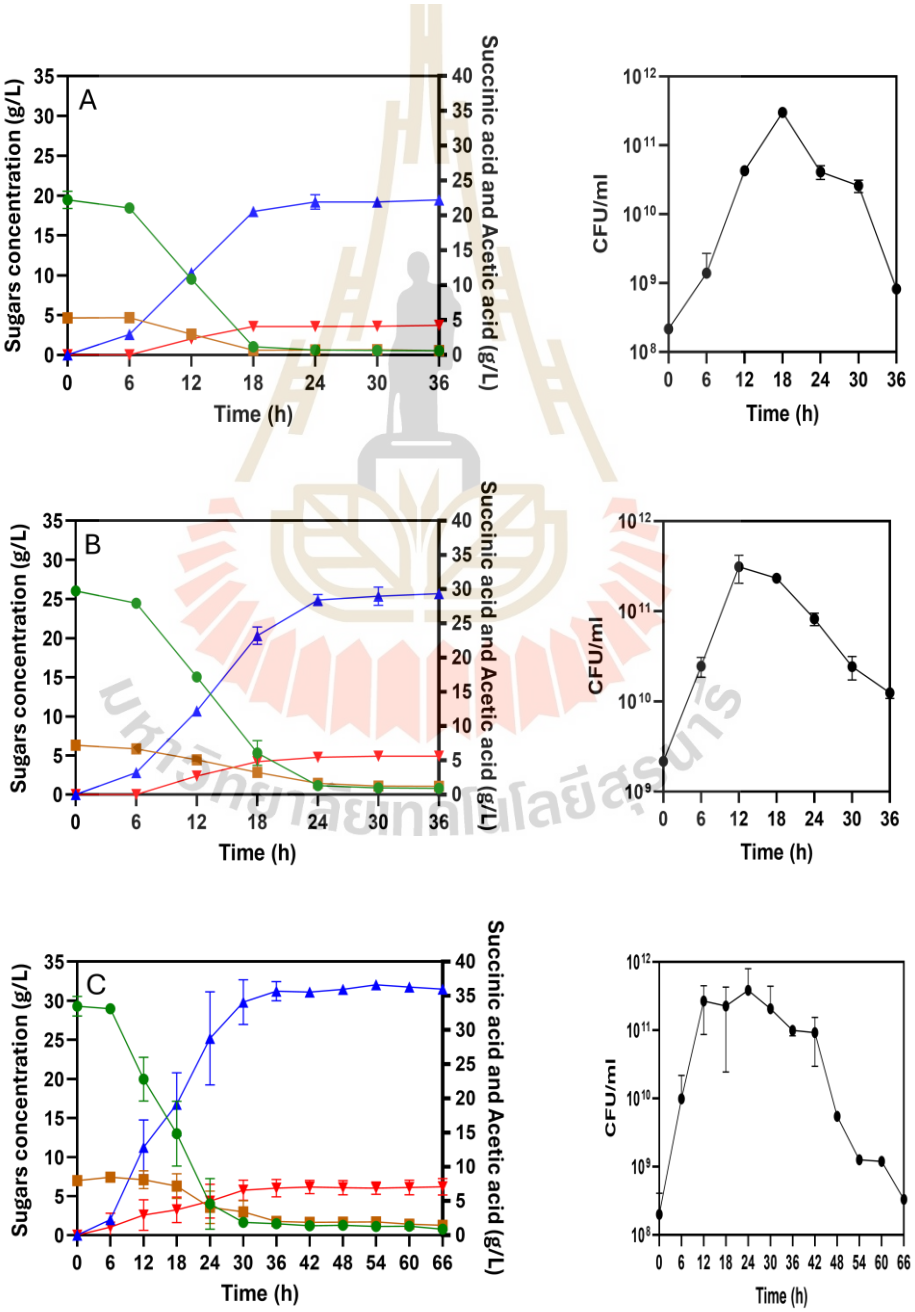
4.3 Succinic acid production from AP-MWOP via SHF fermentation by *E. coli* KJ122

The production of succinic acid from AP-MWOP at different initial concentrations via the SHF process with a 2.5 L working volume in a 5 L fermenter using *E. coli* KJ122 was investigated to evaluate the effects of the AP-MWOP concentration on succinic acid production. For 50 g/L AP-MWOP, initial glucose at a concentration of 19.47±1.06 g/L was slowly consumed in the first 6 h of incubation, whereas no initial xylose at 4.64±0.17 g/L was utilized. After 6 h of incubation, glucose was rapidly consumed by *E. coli* KJ122, while xylose was also co-utilized. Both glucose and xylose were exhausted within 18 h, with consumption rates of 0.79±0.04

and 0.24±0.03 g/L/h, respectively. Succinic acid production increased rapidly between the 6-18 h incubation period, corresponding to rapid glucose consumption, indicating that succinic acid is a growth-associated product. The cell viability approached the exponential phase within 18 h and then entered the death phase because no sugars remained in the fermentation broth to support cell growth and maintenance. After 18 h, succinic acid production slightly increased and did not improve after 24 h because of substrate exhaustion (Figure 4.3A). Succinic acid was produced at a level of 21.79±0.65 g/L, with a yield of 0.90±0.02 g/g sugar utilized, 0.43±0.01 g/g AP-MWOP provided, and a productivity of 0.90±0.02 g/L/h. Acetic acid was produced at a level of 4.10±0.10 g/L. For 70 g/L AP-MWOP (initial contents of 26.03±0.06 g/L glucose and 6.30±0.06 g/L xylose), the production of succinic acid and acetic acid was 28.19±0.98 g/L and 5.36±0.31 g/L, respectively. Glucose was fully consumed at a rate of 0.86 g/L/h within 30 h. Xylose consumption started at 18 h and was complete within 30 h at a consumption rate of 0.21 g/L/h. Succinic acid was substantially produced between 6 h and 24 h, and no increase in succinic acid production was observed after 24 h, even when the incubation time was prolonged to 36 h (Figure 4.3B). Succinic acid production reached its maximum at 24 hours, with yields of 0.87±0.03 g/g sugar utilized, 0.40±0.02 g/g AP-MWOP provided, and a productivity of 1.17±0.04 g/L/h. There was an 8.4% increase in productivity, whereas the yields were not significantly different from those of 50 g/L AP-MWOP (Table 4.1). For 100 g/L AP-MWOP (initial contents of 29.29±1.25 g/L glucose and 6.97±0.34 g/L xylose), glucose consumption started at 6 h and was prolonged until 36 h of incubation. After that, glucose still remained at a concentration of approximately 1 g/L until 66 h without further utilization. Xylose consumption began at 12 h and even slowly continued until 36 h. Xylose at a concentration of 2 g/L remained after 36 h until the end of fermentation. Succinic acid production was quite stable after 36 h but reached the highest level at 54 h, reaching 36.62±0.02 g/L, along with the production of acetic acid at 7.15±1.12 g/L (Figure 4.3C). However, the cells entered the death phase beginning at 24 h, corresponding to the depletion of sugars. Although the succinic acid concentration was greater than those of 50 and 70 g/L AP-MWOP, an overall succinic acid yield and productivity of 0.36±0.01 g/g AP-MWOP and 0.67±0.01 g/L/h, respectively, were achieved (Table 4.1). The overall succinic acid

yield decreased to 10%, while the productivity was also approximately 42.7% lower than that of 70 g/L AP-MWOP. However, the succinate conversion yield of 0.73±0.01 g/g sugar utilized was still comparable. Notably, there was a decrease in the mixing efficiency due to high broth viscosity when the AP-MWOP concentration was increased. The nonhomogeneous mixing resulting from the high solid content, especially at 100 g/L AP-MWOP, caused inefficient and incomplete utilization of sugars, thus adversely affecting succinic acid production in terms of yield and productivity. This was likely due to an abrupt increase in mass and heat transfer during fermentation, reflected by the lack of further utilization of sugars after 30 h, resulting in the sugar remaining in the broth when 100 g/L AP-MWOP was used. Our findings were similar to those of Buyakoztekin and Buyukkileci, (2014). They utilized organosolv-pretreated corncob at a high solid content of 105 g/L for succinic acid production through the SHF process by *A. succinogenes*. Succinic acid at a concentration of 12.7 g/L with a yield of 0.12 g/g pretreated corncob was achieved only because of inefficient and incomplete utilization of sugars with high broth viscosity. Therefore, the optimum concentration of AP-MWOP for succinic acid production via the SHF process is in the range of 50-70 g/L. Increasing solid-substrate loading generally results in elevated amounts of waste office paper-derived glucose for the production of bioproducts. Nair et al. (2018) demonstrated that increasing the level of pretreated waste office paper to 11 g/L significantly increased cellulase production by *Bacillus velzensis* ASN1. However, Mansy et al. (2024) reported that providing the highest tested concentration of waste office paper did not result in the highest glucose concentration but even decreased bioethanol production at higher solid contents. Diaz-Blanco et al. (2018) reported a poorer performance of bioethanol production with acid-pretreated agave stems at higher slurry viscosities. This resulted in a decrease in the glucose conversion yield and even bioethanol production of approximately 16%, even though the solid-substrate loading in the enzymatic hydrolysis of the whole slurry was slightly lower (44 g/L vs 50 g/L). These results implied that a higher solid content of fermentation broth than the critical value unfavored the production of bioproducts by not only causing mixing inhomogeneity but also contributing to the presence of some inhibitory compounds in the slurry, which originated as a consequence of acid pretreatment, since toxic compounds,

such as acetic acid, furfural, 5-HMF, formic acid and, especially, phenolic compounds from lignin, negatively affected enzymatic activity and microbial growth. This finding is supported by the findings of Park et al. (2004), who reported that the production rate of L-(+)-lactic acid by *Rhixopus oryzae* may be inhibited by xylose derived from hemicellulose and that the yield may be inhibited by toxic compounds derived from waste office paper generated during the pretreatment process when higher concentrations of waste office paper are utilized.



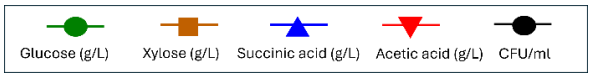


Figure 4.3 Succinic acid production via SHF fermentation by *E. coli* KJ122. (A) 50 g/L AP-MWOP, (B) 70 g/L AP-MWOP, and (C) 100 g/L AP-MWOP. Three independent replications were performed on each test, and the means were reported with the standard deviation (SD).

4.4 Succinic acid production from AP-MWOP via Pre-saccharification batch SSF

For the SSF process, sugar concentrations derived from substrates are usually kept low throughout the procedure. Sugars are slowly released from the pretreated substrate to avoid substrate and product inhibition during enzymatic hydrolysis and fermentation. Figure 4.4A shows the SSF process without pre-saccharification for producing succinic acid from AP-MWOP at 50 g/L AP-MWOP loading. Initially, initial sugar concentrations of 5.02±0.03 g/L glucose and 2.10±0.61 g/L xylose (7.12±0.61 g/L total sugars) were found. Sugar consumption began after 6 h and gradually continued until 30 h, at which point all sugars were exhausted. The rates of glucose and xylose consumption were 0.33±0.07 g/L/h and 0.11±0.03 g/L/h, respectively. After 48 h, succinic acid production reached 14.51±1.15 g/L, with a yield of 0.29±0.02 g/g AP-MWOP provided and a productivity of 0.29±0.02 g/L/h. The cells reached the exponential phase within 18 h, after which the cells entered the stationary phase. The culture eventually entered the death phase after 42 h of incubation. This was likely due to insufficient sugar availability, as cellulase activity was not sufficient to hydrolyze AP-MWOP to release fermentable sugars to maintain microbial growth. Typically, cellulase enzymes exhibit optimal activity at 50°C and pH 5. However, the SSF process requires a lower temperature of 37°C and a neutral pH of 7 to suit the optimal growth of *E. coli* KJ122. This discrepancy between the ideal conditions for cellulase activity (50°C, pH 5) and for microbial fermentation (37°C, pH 7.0) could result in a slower rate of hydrolysis during the SSF process (Suttikul et al., 2016). Under these suboptimal conditions, succinic acid production via the SSF process was less efficient than that via the SHF process in terms of the reduction in the succinic

acid concentration, yield, and productivity by 33.4%, 66.7%, and 32.6%, respectively (Table 4.1). Many studies have attempted to increase the mixing efficiency and enhance enzymatic hydrolysis duringthe SSF process. Wood et al. (1997) reported that intermittent exposure of SSF processes to ultrasonic energy increased ethanol production from mixed waste office paper by 20% compared with nonultrasonic conditions . The ultrasonic energy unblemished the pulp in the waste office paper, thus reducing the viscosity of the slurry and facilitating increased accessibility of cellulase into the fibers, which could reduce the cellulase loadings used during the SSF process by 2-fold. However, they suggested that continuous exposure of the organism to ultrasound was bacteriostatic and decreased ethanol production in the long-term process. Additionally, Yang et al. (2018) added the surfactant *Gleditsia* saponin to fermentation broth for lactic acid production by *Streptococcus* thermophilus via waste office paper with a high solid content via the SSF process to reduce cellulase loading while increasing mixing efficiency and preventing product inhibition. Although they suggested that saponin is a potential additive for developing an effective lactic acid production process, its price is quite high.

To address the issue of suboptimal conditions in SSF, the pre-saccharification SSF process was alternatively introduced to reduce the viscosity of the broth. For the 6 h pre-saccharified SSF, the initial glucose concentration was 7.71±0.32 g/L, and the xylose concentration was 2.33±0.22 g/L, yielding a total sugar concentration of 10.04±0.55 g/L, representing a 29.08% increase in fermentable sugars compared with the non-saccharified SSF process. Glucose consumption occurred rapidly between 6 and 24 h at a rate of 0.49±0.01 g/L/h, whereas xylose was slowly consumed at a rate of 0.15±0.00 g/L/h over 24 h (Figure 4.4B). By the end of the process, the maximum succinic acid concentration was 15.36±1.03 g/L, with a yield and productivity of 0.30±0.02 g/g AP-MWOP provided and 0.42±0.02 g/L/h, respectively. For the 12 h pre-saccharified SSF, the initial glucose concentration was 14.12±1.34 g/L, and the xylose concentration was 3.85±0.30 g/L, resulting in a total sugar concentration of 17.97±1.64 g/L, representing a 60.37% increase compared with that of the non-saccharified SSF process. Glucose consumption was more rapid, particularly between 6 and 18 h, and was complete within 24 h of incubation, with a rate of 0.60±0.04 g/L/h. Xylose consumption was slower at 0.16±0.01 g/L/h but was also complete

within 24 h (Figure 4.4C). This resulted in 17.15±0.03 g/L succinic acid, with a yield and productivity of 0.34±0.01 g/g AP-MWOP provided and 0.47±0.02 g/L/h, respectively (Table 4.1).

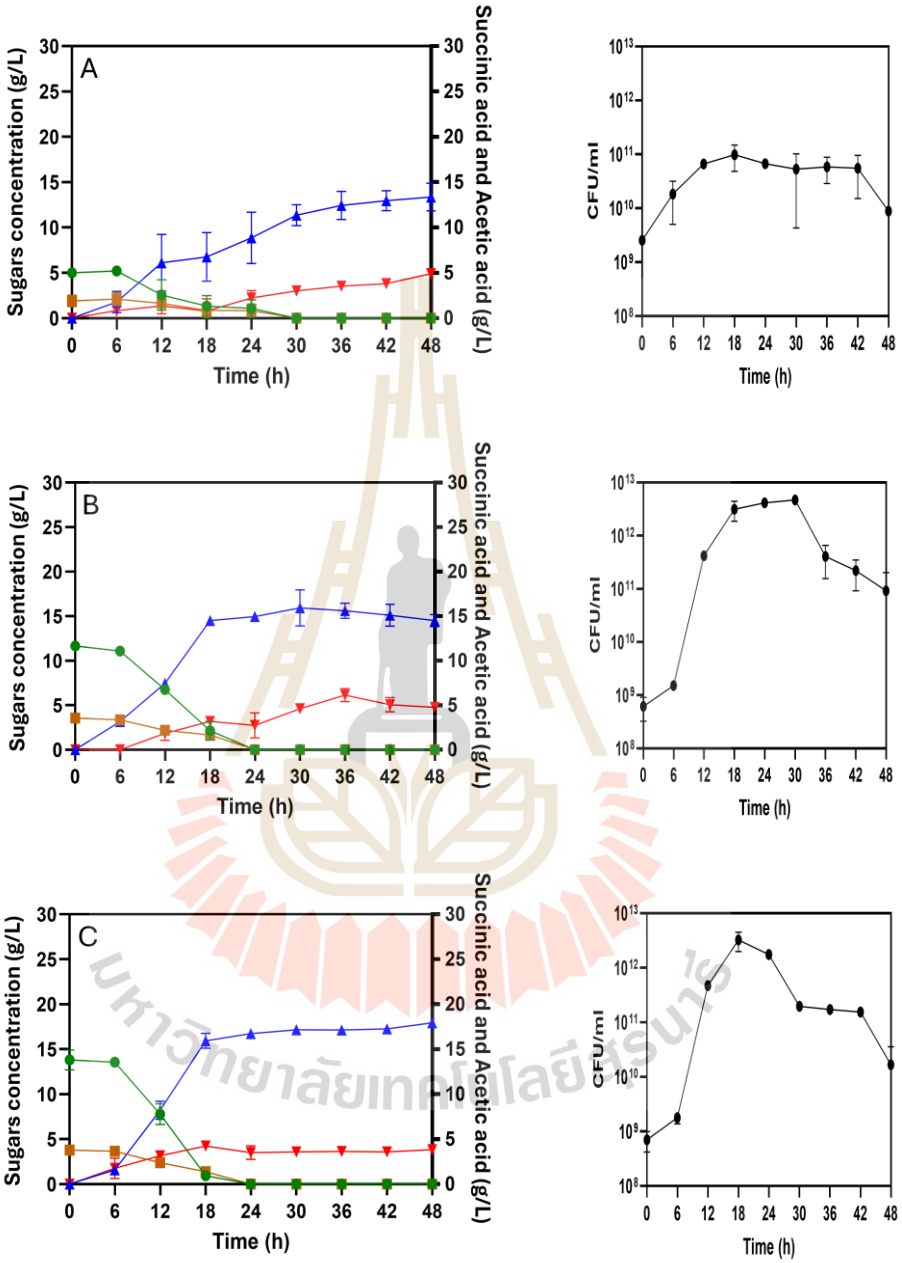
For the 18 h pre-saccharified SSF, the initial glucose and xylose concentrations were 15.60±0.07 g/L and 4.42±0.21 g/L, respectively, which were 64.43% greater than those of the non-saccharified SSF process. Glucose consumption was even faster (0.65±0.00 g/L/h) than that of the 12 h pre-saccharified SSF, occurring within 30 h, whereas xylose consumption was 0.18±0.00 g/L/h (Figure 4.4D). Succinic acid was produced at 22.44±1.93 g/L, with a yield of 0.44±0.03 g/g AP-MWOP, which was comparable to that of the SHF process, and the productivity was 0.75±0.04 g/L/h (Table 4.1). The succinic acid production in this case approached the comparable level observed in the SHF process, suggesting that the pre-saccharification time of 18 h was enough to allow cellulase to release fermentable sugars at a sufficient level to sustain microbial growth and succinic acid production. For the 24 h pre-saccharified SSF, the initial glucose concentration was 15.06±0.96 g/L, and the xylose concentration was 4.48±0.31 g/L, which was 63% greater than that of the non-saccharified SSF but not significantly different from that of the 18 h pre-saccharified SSF. Glucose consumption was similar to that in the 18 h pre-saccharification process, with a rate of 0.64±0.03 g/L/h, thus completing within 30 h. Xylose consumption was 0.20±0.01 g/L/h (Figure 4.4E). The yield and productivity of succinic acid were 21.39±0.01 g/L, 0.42±0.01 g/g AP-MWOP, and 0.71±0.04 g/L/h, respectively (Table 4.1). Cell growth was consistent with sugar availability, showing a gradual decline after the exponential phase at 18 h and eventually entering the death phase due to the depletion of fermentable sugars. Succinic acid production under these conditions was also comparable to that obtained by the SHF process. It may be concluded that pre-saccharifying SSF could reduce the processing time and enhance the effectiveness of enzymatic hydrolysis. However, the rate of succinic acid production may vary depending on the duration of pre-saccharification, and enzyme activity remains a critical factor in determining overall succinic acid productivity.

The 18 h pre-saccharified SSF to produce succinic acid was then applied to 70 g/L AP-MWOP. The results revealed that sugar consumption was similar to that observed for 50 g/L AP-MWOP. Glucose consumption was rapid between 6 and 24 h,

with a glucose consumption rate of 0.78±0.01 g/L/h; thus, it was also complete within 30 h. Xylose consumption was slower, completing at 42 h, with a consumption rate of 0.12±0.00 g/L/h (Figure 4.4F). The maximum level of succinic acid produced at 30 h was 24.58±2.32 g/L, with a yield of 0.35±0.03 g/g AP-MWOP provided and a productivity of 0.82±0.07 g/L/h. Although succinic acid production was 12.80% lower than that of the SHF process using 70 g/L AP-MWOP, it represented an 8.7% increase compared with that of 50 g/L AP-MWOP. The results also suggested that the utilization of a higher concentration of 70 g/L AP-MWOP for succinic acid production via the batch SSF process may not yet improve the process performance in terms of concentration and productivity. A similar result was reported by Jampatesh et al. (2019), who reported that the use of rice straw at concentrations greater than 70 g/L in SSF batches significantly decreased glucose utilization, succinate production and fermentation efficiency in *E. coli* AS1600a due to difficulties in mixing. Additionally, Akhtar et al. (2017) revealed that succinic acid production was even lower in concentration (20.9 g/L) and yield (0.29 g/g) from 100 g/L NaOH-pretreated empty oil-palm fruit bunches than in those obtained from this study when AP-MWOP at 50-70 g/L was used via the SSF process. Sangkharak, (2011) also revealed that ethanol production from pretreated office paper using *Saccharomyces cerevisiae* in the SSF process was only 2.1% (v/v), with a productivity of 0.58 g/L/h. This was due to a low level of liberated glucose at 2184.22 µg/L, even when a high cellulase loading of up to 28.14 FPU/g was provided.

In this study, no significant differences in initial sugar concentrations or succinic acid production were detected between the 18 h and 24 h pre-saccharified SSF processes. Therefore, the 18 h pre-saccharification time was considered the most suitable for the production of succinic acid by *E. coli* KJ122 via the SSF process, as it reduces fermentation time without sacrificing succinic acid production efficiency. At a 50 g/L substrate concentration, the total process time for the 18 h pre-saccharified SSF was 66 h compared with 96 h for the SHF. The energy consumption for the enzymatic hydrolysis was expected to decrease by 62.5% in the 18 h pre-saccharification period, probably reducing the production costs on an industrial scale. This suggests that the overall benefits of the 18 h pre-saccharified SSF, including a reduced fermentation time with energy savings and lower production costs, could

make it more suitable for succinic acid production than the batch SHF process for industrial applications.



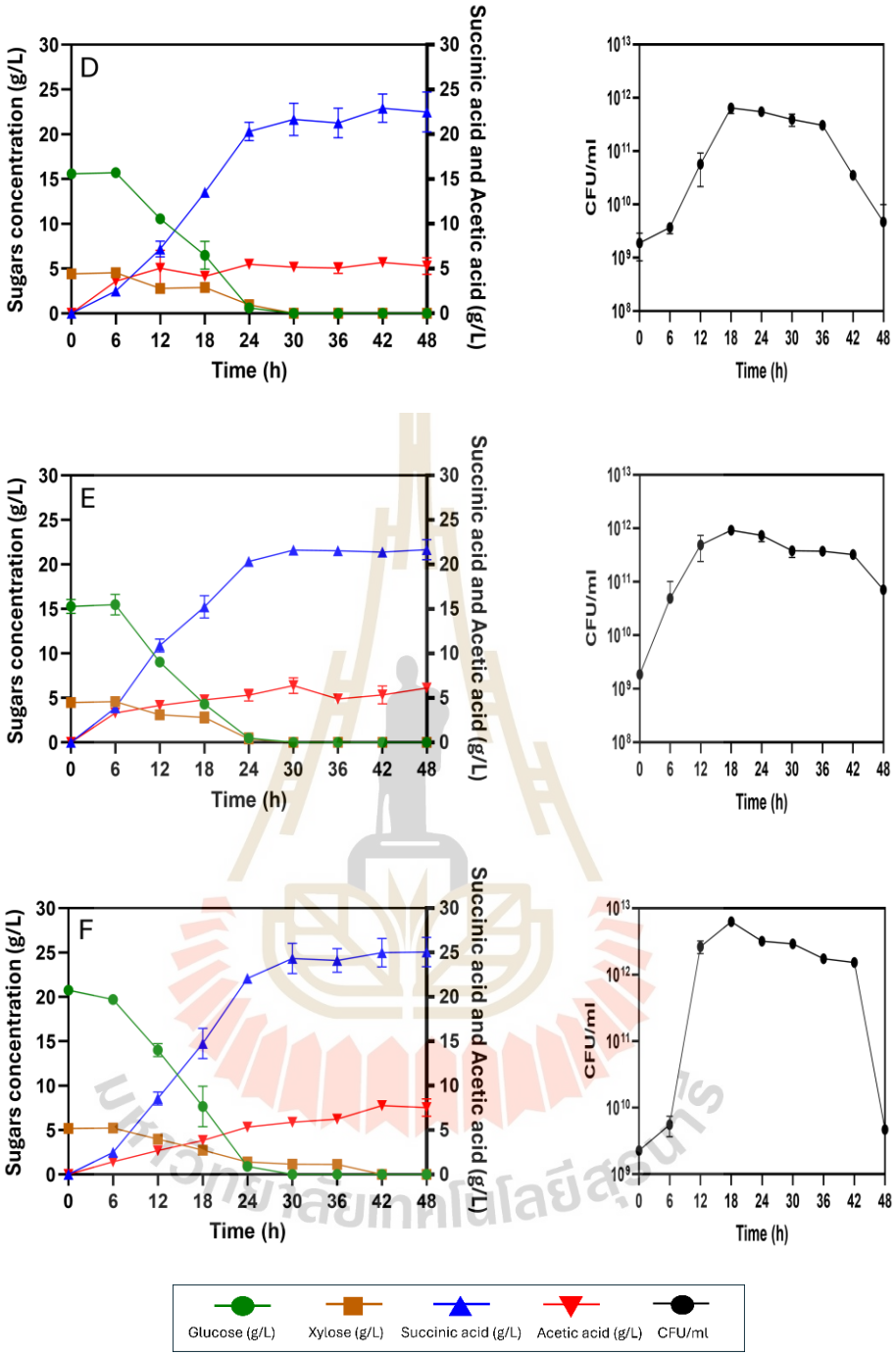


Figure 4.4 Succinic acid production via the SSF process with different pre-saccharification times. (A) 0 h, (B) 6 h, (C) 12 h, (D) 18 h, (E) 24 h with 50 g/L AP-MWOP, and (F) 18 h pre-saccharified batch SSF with 70 g/L AP-MWOP. Three independent replications were performed on each test, and the means were reported with the standard deviation (SD).

4.5 Effects of rpm on succinic acid production

Mixing, an energy-intensive process, becomes particularly challenging in concentrated biomass slurries due to their high viscosity and non-Newtonian characteristics. Insufficient mixing can lead to mass and heat transfer limitations, as well as uneven enzyme distribution. Additionally, energy dissipation from stirring can influence particle size, further impacting enzymatic hydrolysis efficiency, which diminishes hydrolysis yields and decreases the performance of bioproduct production. (Kadić et al., 2014). In this study, the effect of various agitation speeds (100, 200, and 300 rpm) on succinic acid production was investigated.

At 100 rpm, glucose consumption was completed within 36 h at the rate of 0.72±0.00 g/L/h, whereas xylose was slowly consumed at the rate of 0.15±0.01 g/L/h, completing at 36 hours (Figure 4.5A). This agitation speed provided 23.13±0.52 g/L succinic acid with a yield of 0.74±0.03 g/g sugar utilized and the productivity of 0.55±0.01 g/L/h, along with 4.03±0.77 g/L acetic acid. At 200 rpm, sugar consumption was faster, with glucose completely consumed within 30 h at the rate of 0.78±0.10 g/L/h, whereas xylose was fully consumed at 42 h with the rate of 0.12±0.00 g/L/h. The maximum succinic acid production occurred at 30 h, with succinic acid production of 24.58±2.32 g/L, a productivity of 0.82±0.07 g/L/h and an acetic acid concentration of 7.25±1.19 g/L (Figure 4.5B). At 300 rpm, the sugar consumption rates were similar to those observed at 200 rpm, with glucose completely consumed within 30 h at the rate of 0.89±0.10 g/L/h and xylose fully consumed at the same time with the rate of 0.21±0.02 g/L/h. Succinic acid production was highest at 25.85±1.03 g/L with a yield of 0.80±.01 g/g and a productivity of 0.53±0.01 g/L/h, along with 5.28±0.40 g/L acetic acid (Figure 4.5C).

After the end of fermentation, succinic acid production was similar across all agitation speeds (100, 200, and 300 rpm). However, the glucose consumption rate was significantly lower at 100 rpm, likely because of insufficient mixing caused by the high viscosity of the fermentation broth, thus reducing the mass transfer efficiency (Zhou et al., 2018). Comparable succinic acid productivity was observed at 200 and 300 rpm. Therefore, succinic acid production at 200 rpm was considered optimal under these conditions. This result aligns with the shorter lag phase observed at 200 rpm, where succinic acid production was completed in approximately 30 h, compared with

approximately 42 h at lower agitation speeds. Similar findings were reported by Oh et al. (2009). These authors suggested that insufficient CO_2 dissolution in the bioreactor occurred at low agitation speeds, which had a negative effect on microbial cell growth rates. This finding also suggested that agitation speed impacts cell morphology, growth rates, metabolite production, and potential cell damage. Additionally, agitation is essential for ensuring proper mixing, facilitating mass transfer, and maintaining effective heat transfer within the system. Proper agitation promotes a uniform distribution of nutrients and gases, which is critical for optimizing microbial activity and overall fermentation performance. It facilitates mass transfer between different phases in culture and maintains uniform chemical and physical conditions (Rodmui et al., 2008).

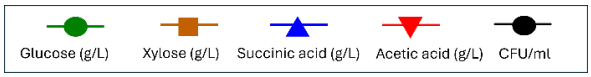


Figure 4.5 Effects of different agitation rates of 70 g/L AP-MWOP with 18 h pre-saccharification time on succinic acid production by *E. coli* KJ122. (A) 100 rpm, (B) 200 rpm, and (C) 300 rpm. Three independent replications were performed on each test, and the means were reported with the standard deviation (SD).

4.6 Succinic acid production from AP-MWOP via pre-saccharified fed-batch SSF

In this study, the strategy used to increase succinate production in *E. coli* KJ122 focused on fed-batch fermentation, a preferred industrial method in which essential nutrients for cell growth and product formation are added intermittently or continuously during batch operation. The fed-batch fermentation began with an 18 h pre-saccharified SSF using an initial substrate concentration of 70 g/L AP-MWOP. After pre-saccharification, the seed culture mixture was added to initiate succinic acid production. Substrates were fed whenever glucose levels dropped below 10 g/L. As shown in Figure 4.6, the initial glucose concentration was 23±2.11 g/L, with 5.04±0.76 g/L xylose. Since succinic acid production primarily utilizes glucose, substrate feeding was focused on maintaining the level of glucose to no less than 10 g/L. After 6 h, glucose was rapidly consumed until 18 h of incubation, and its remaining concentration was 11.05±1.35 g/L. At this point, the sugar stock derived from AP-MWOP was added, thus increasing the glucose concentration to 20 g/L. Additional sugars were fed at 18, 24 and 36 h. At the end of the process, succinic acid at 51.38±4.05 g/L was produced, with a comparable yield of 0.75±0.05 g/g and a productivity of 1.07±0.08 g/L/h. This represented a 52.16% increase in succinic acid production compared with that of batch SSF operation. Cell availability showed a lag phase from 0-6 hours, followed by an exponential phase from 6-18 hours. After 18 hours, cell growth was slow, possibly due to the increasing volume in the bioreactor after the addition of sugar, which reduced the cell density. Cell growth was stable between 36 and 42 h, after which the culture entered the death phase (Figure 4.6). Compared with other studies using fed-batch SSF, Zhong et al. (2024) demonstrated

succinic acid production by *Yarrowia lipolytica* from 65 g/L pretreated corncob. Succinic acid at a concentration of 45.4 g/L with a yield of 0.71 g/g was obtained. Grape stalks were also used to produce 40.2 g/L succinic acid, with a yield of 0.67 g/g produced by *A. succinogenes* (Filippi et al., 2021). Alexandri et al. (2019) also utilized *A. succinogenes* to produce succinic acid from sugar beet pulp. However, only 30 g/L succinic acid was obtained, with yields and productivities of 0.8 g/g and 0.62 g/L/h, respectively. Additionally, carob pods are utilized by *A. succinogenes* 130Z to produce succinic acid (Carvalho et al., 2016). However, the succinic acid concentration of 18.97 g/L was too low to apply in industrial applications. Therefore, the 18 h pre-saccharified fed-batch SSF process using AP-MWOP as a potential and sustainable substrate may be a suitable and cost-effective process for succinic acid production. This process should be further developed to produce succinic acid on an industrial scale.

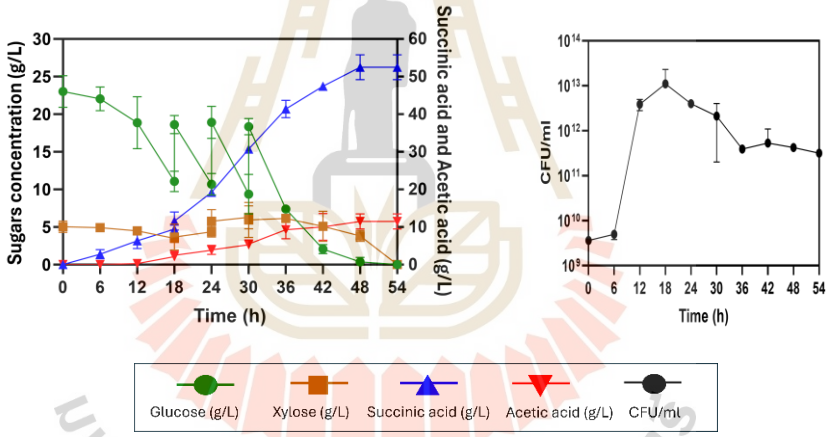


Figure 4.6 Succinic acid production by *E. coli* KJ122 via a pre-saccharified fed-batch SSF process with an initial concentration of 70 g/L AP-MWOP. Three independent replications were performed on each test, and the means were reported with the standard deviation (SD).

Table 4.1 Comparison of succinic acid production from pretreated AP-MWOP under different fermentation conditions.

Fermentation condition	Total time (h)	Succinic acid (g/L)	Conversion Yield (g/g)	Gross yield (g/g)[b]	Productivity (g/L/h)	Acetic acid (g/L)
SHF						
50 g/L	72	21.79±0.65^{d}	0.90±0.02^{a}	0.43±0.01^{a}	0.90±0.02^{ab}	4.10±0.10^{bc}
70 g/L	72	28.19±0.98^{c}	0.87±0.03^{a}	0.40±0.02^{ab}	1.17±0.04^{a}	5.36±0.31^{b}
100 g/L	102	36.62±0.02^{b}	0.73±0.01^{b}	0.36±0.01b	0.67±0.01^{b}	7.15+1.12^{b}
SSF						
50 g/L+0 h pre-saccharification	48	14.51±1.15^{e}	0.58±0.05^{c}	0.29±0.02^{bc}	0.29±0.02^{c}	4.70±0.15^{bc}
50 g/L+6 h pre-saccharification	42	15.36±1.03^{e}	0.61±0.04^{c}	0.30±0.02^{bc}	0.42±0.02^{bc}	5.91±0.91^{b}
50 g/L+12 h pre-saccharification	48	17.15±0.03^{e}	0.68±0.01^{bc}	0.34±0.01^{b}	0.47±0.02^{bc}	3.67±0.14^{bc}
50 g/L+18 h pre-saccharification	48	22.44±1.93^{d}	0.89±0.07^{a}	0.44±0.03^{a}	0.74±0.04^{bc}	5.64±0.07^{b}
50 g/L+24 h pre-saccharification	48	21.39±0.01^{d}	0.85±0.01^{a}	0.42±0.01^{a}	0.71±0.04^{ab}	5.61±1.23^{b}
50 g/L+18 h pre-saccharification	48	24.58±2.32^{d}	0.76±0.07^{ab}	0.35±0.03^{b}	0.82±0.07^{ab}	7.25±1.19^{b}
Fed-batch	66	51.38±4.05^{a}	0.75±0.05^{b}	0.04±0.03^{d}	1.07±0.08^{a}	11.47±1.98^{a}

* Conversion yield is defined as gram succinic acid produced divided by gram total sugars consumed, while gross yield is defined as gram succinic acid produced divided by gram AP-MWOP. Different superscript lower-case letters indicate significant differences between mean values ($p \leq 0.05$) in the same column.

Table 4.2 Succinic acid production from lignocellulose biomass by SHF, SSF, and fed-batch fermentation.

Separate Hydrolysis and fermentation					
E. coli KJ122	Mixed waste office paper	28.19±0.98	0.87±0.03	1.17±0.04	This study
A. succinogenes	Sugar cane bagasse	39.9	0.82	1.37	(Chen et al., 2016)
B. succinicproducens BPP7	Arundo donax	9.4	-	0.14	(Ventorino et al., 2017)
A. succinogenes	corncob	12.7	0.12	-	(Buyukoztekin and Buyukkileci 2024)
A. succinogenes	Duckweed	62.12	0.81	1.04	(Shen et al., 2018)
A. succinogenes 130Z	Corn fiber	27.8	0.61	-	(Vallecilla-Yepez et al., 2021)
A. succinogenes 130Z	carob pods	9.41	0.39	1.67	(Carvalho et al., 2016)
E. coli KJ122	Mixed waste office paper	24.58±2.32	0.76±0.07	0.82±0.07	This study
A. succinogenes	Oil palm empty fruit bunches	49.9	0.61	-	(Akhtar et al., 2020)
A. succinogenes	Duckweed	52.41	0.87	-	(Shen et al., 2018)
A. succinogenes 130Z	Oil Palm Trunk	10.62	0.47	-	(Bukhari et al., 2020)

Table 4.2 (continued).

Fed-batch fermentation					
E. coli AS1600a	Rice straw	78.5	0.86	0.54	(Jampatesh et al., 2019)
A. succinogenes	fruit and vegetable wastes	27.03	1.18	1.28	(Dessie et al., 2018a)
E. coli KJ122	Mixed waste office paper	51.38±4.05	0.75±0.05	1.07±0.08	This study
Y. lipolytica	corncob	45.43	0.71	-	(Zhong et al., 2024)
A. succinogenes	Grape stalk	40.2	0.67	0.79	(Filippi et al., 2021)
A. succinogenes	Sugar beet pulp	30	0.8	0.62	(Alexandri et al., 2019)
A. succinogenes 130Z	Carob pods	18.97	0.94	1.43	(Carvalho et ai., 2016)

CHAPTER V
CONCLUSION AND RECOMMENDATION

A large amount of mixed waste office paper (MWOP) is generated globally due to economic growth in every sector, but recycling it is costly. This study examined the impact of MWOP on high-value bio-succinic acid production by *E. coli* KJ122. The results demonstrated that the most suitable crude cellulase providing the highest levels of liberated glucose and xylose was found at 80 PCU/g AP-MWOP. For the SHF process, the yields of succinic acid produced from 50, 70, and 100 g/L AP-MWOP were 0.90±0.02, 0.87±0.03, and 0.73±0.01 g/g respectively, after 48 hours of saccharification. In the SSF process, the highest succinic acid productivity with a succinic acid concentration comparable to that of the SHF process was obtained via the 18-hour pre-saccharification SSF strategy when 50 g/L and 70 g/L AP-MWOP were applied. Additionally, the fed-batch SSF process significantly improved succinic acid production to 51.3 g/L, with yields of up to 0.75 g/g sugars utilized or 0.04 g/g AP-MWOP provided. This work allows the reuse of MWOP to produce succinic acid as a viable and efficient method. However, these findings also recommend that the 18-hour pre-saccharification SSF process from AP-MWOP may be an ideal lignocellulosic biomass for future experiments on an industrial scale for the production of bio-succinic acid as well as other biochemicals and biofuels. This process is low-cost and energy-efficient, supporting cost-effective and environmentally friendly industries that aim to promote a green and zero-waste society. However, the study found that *E. coli* KJ122 is unable to produce the cellulase complex for the hydrolysis of cellulose and hemicellulose. That means a commercial enzyme needs to be added. To reduce costs, *E. coil* KJ122 expressing genes involved in cellulase production should be developed, potentially decreasing the reliance on commercial enzymes in the future.

REFERENCES

Agarwal, L., Isar, J., Meghwanshi, G.K., Saxena, R.K., 2007. Influence of environmental and nutritional factors on succinic acid production and enzymes of reverse tricarboxylic acid cycle from Enterococcus flavescens. **Enzyme and Microbial Technology**, 40, 629–636.

Akhtar, J., Hassan, N., Idris, A., Ngadiman, N.H.A., 2020. Optimization of simultaneous saccharification and fermentation process conditions for the production of succinic acid from oil palm empty fruit bunches. **Journal of Wood Chemistry and Technology**, 40, 136–145.

Akhtar, J., Idris, A., 2017. Oil palm empty fruit bunches a promising substrate for succinic acid production via simultaneous saccharification and fermentation. **Renewable Energy**, 114, 917–923.

Al-Battashi, H., Sivakumar, H., 2022. High-Solid loading enzymatic hydrolysis of waste office paper for poly-3-hydroxybutyrate production through simultaneous saccharification and fermentation, **Journal of Polymers and the Environment**, 30, 3045–3054.

Al Battashi, H., Al-Kindi, S., Gupta, V.K., Sivakumar, N., 2021. Polyhydroxyalkanoate (PHA) production using volatile fatty acids derived from the anaerobic digestion of waste paper. **Journal of Polymers and the Environment**, 29, 250–259.

Alcantara, J., Mondala, A., 2021. Optimization of slurry fermentation for succinic acid production by fungal co-culture. **Chemical Engineering Transactions**, 86, 1525–1530.

Alexandri, M., Schneider, R., Papapostolou, H., Ladakis, D., Koutinas, A., Venus, J., 2019. Restructuring the conventional sugar beet industry into a novel biorefinery: fractionation and bioconversion of sugar beet pulp into succinic acid and value-added coproducts. **ACS Sustainable Chemistry & Engineering**, 7, 6569–6579.

Amiri, H., Karimi, K., Zilouei, H., 2014. Organosolv pretreatment of rice straw for efficient acetone, butanol, and ethanol production. **Bioresource Technology**, 152, 450–456

Annamalai, N., Al Battashi, H., Anu, S.N., Al Azkawi, A., Al Bahry, S., Sivakumar, N., 2020. Enhanced bioethanol production from waste paper through separate hydrolysis and fermentation. **Waste and Biomass Valorization**, 11, 121–131.

Arantes, V., Saddler, J.N., 2010. Access to cellulose limits the efficiency of enzymatic hydrolysis: the role of amorphogenesis. Biotechnology for Biofuels, 3, 1–11.

Ariunbaatar, J., Panico, A., Esposito, G., Pirozzi, F., Lens, P.N., 2014. Pretreatment methods to enhance anaerobic digestion of organic solid waste. **Applied Energy**, 123, 143–156.

Awol, Z.S., Abate, R.T., 2021. Production of bio-ethanol from solid waste paper using biological activation. Environmental Science, Engineering, Chemistry.

Balat, M., 2011. Production of bioethanol from lignocellulosic materials via the biochemical pathway: a review. **Energy Conversion and Management**, 52, 858–875.

Brethauer, S., Shahab, R.L., Studer, M.H., 2020. Impacts of biofilms on the conversion of cellulose. Applied microbiology and Biotechnology, 104, 5201–5212.

Brummer, V., Jurena, T., Hlavacek, V., Omelkova, J., Bebar, L., Gabriel, P., Stehlik, P., 2014. Enzymatic hydrolysis of pretreated waste paper–Source of raw material for production of liquid biofuels. **Bioresource Technology**, 152, 543–547.

Bu, J., Yan, X., Wang, Y.-T., Zhu, S.-M., Zhu, M.-J., 2019. Co-production of high-gravity bioethanol and succinic acid from potassium peroxymonosulfate and deacetylation sequentially pretreated sugarcane bagasse by simultaneous saccharification and co-fermentation. **Energy Conversion and Management**, 186, 131–139.

Bukhari, N.A., Jahim, J.M., Loh, S.K., Nasrin, A.B., Harun, S., Abdul, P.M., 2020. Organic acid pretreatment of oil palm trunk biomass for succinic acid production. **Waste and Biomass Valorization**, 11, 5549–5559.

Buranov, A.U., Mazza, G., 2008. Lignin in straw of herbaceous crops. **Industrial Crops and Products**, 28, 237–259.

Buyukoztekin, G.K., Buyukkileci, A.O., 2024. Enzymatic hydrolysis of organosolv-pretreated corncob and succinic acid production by *Actinobacillus succinogenes*. **Industrial Crops and Products**, 208, 117922.

Byadgi, S.A., Kalburgi, P., 2016. Production of bioethanol from waste newspaper. **Procedia Environmental Sciences**, 35, 555–562.

Carvalho, M., Roca, C., Reis, M.A., 2016. Improving succinic acid production by *Actinobacillus succinogenes* from raw industrial carob pods. **Bioresource Technology**, 218, 491–497.

Chen, J., Yang, S., Alam, M.A., Wang, Z., Zhang, J., Huang, S., Zhuang, W., Xu, C., Xu, J., 2021. Novel biorefining method for succinic acid processed from sugarcane bagasse. **Bioresource Technology**, 324, 124615.

Chen, P., Tao, S., Zheng, P., 2016. Efficient and repeated production of succinic acid by turning sugarcane bagasse into sugar and support. **Bioresource Technology**, 211, 406–413.

Cukalovic, A., Stevens, C.V., 2008. Feasibility of production methods for succinic acid derivatives: a marriage of renewable resources and chemical technology. Biofuels, **Bioproducts and Biorefining**, 2, 505–529.

Da Mota, H.G., Gouveia, E.R., 2016. Improvement in enzymatic hydrolysis of waste office paper with chemical pretreatment and enzyme loading reduced. **Waste and Biomass Valorization**, 7, 507–512.

Dessie, W., Zhang, W., Xin, F., Dong, W., Zhang, M., Ma, J., Jiang, M., 2018a. Succinic acid production from fruit and vegetable wastes hydrolyzed by on-site enzyme mixtures through solid state fermentation. **Bioresource Technology**, 247, 1177–1180.

Dessie, W., Zhu, J., Xin, F., Zhang, W., Jiang, Y., Wu, H., Ma, J., Jiang, M., 2018b. Bio-succinic acid production from coffee husk treated with thermochemical and fungal hydrolysis. **Bioprocess and Biosystems Engineering**, 41, 1461–1470.

Dey, P., Rangarajan, V., Nayak, J., Das, D.B., Wood, S.B., 2021. An improved enzymatic pre-hydrolysis strategy for efficient bioconversion of industrial pulp and paper sludge waste to bioethanol using a semi-simultaneous saccharification and fermentation process. **Fuel**, 294, 120581.

Dhawan, A., 2007. Synthesis of block copolymers from cellulose nanoparticles via atom transfer radical polymerization. Master Thesis. Graduate Faculty, North Carolina State University, Raleigh, North Carolina, United States of America.

Díaz-Blanco, D.I., de La Cruz, J.R., López-Linares, J.C., Morales-Martínez, T.K., Ruiz, E., Rios-González, L.J., Romero, I., Castro, E., 2018. Optimization of dilute acid pretreatment of *Agave lechuguilla* and ethanol production by co-fermentation with *Escherichia coli* MM160. **Industrial Crops and Products**, 114, 154–163.

Digman, M.F., Shinners, K.J., Casler, M.D., Dien, B.S., Hatfield, R.D., Jung, H.-J.G., Muck, R.E., Weimer, P.J., 2010. Optimizing on-farm pretreatment of perennial grasses for fuel ethanol production. **Bioresource Technology**, 101, 5305–5314.

Du, B., Sharma, L.N., Becker, C., Chen, S., Mowery, R.A., van Walsum, G.P., Chambliss, C.K., 2010. Effect of varying feedstock–pretreatment chemistry combinations on the formation and accumulation of potentially inhibitory degradation products in biomass hydrolysates. **Biotechnology and Bioengineering**, 107, 430–440.

Dubey, A.K., Gupta, P., Garg, N., Naithani, S., 2012. Bioethanol production from waste paper acid pretreated hydrolyzate with xylose fermenting *Pichia stipitis*. **Carbohydrate Polymers**, 88, 825–829.

Dunning, J., Dallas, D., 1949. Analytical procedures for control of saccharification operations. **Analytical Chemistry**, 21, 727–729.

Erickson, B., 2013. Industrial biotechnology's coming of age. **Industrial Biotechnology**, 9, 156–157.

Fengel, D., Wegener, G., 2011. Wood: chemistry, ultrastructure, reactions. Walter de Gruyter.

Filippi, K., Georgaka, N., Alexandri, M., Papapostolou, H., Koutinas, A., 2021. Valorisation of grape stalks and pomace for the production of bio-based succinic acid by *Actinobacillus succinogenes*. **Industrial Crops and Products**, 168, 113578.

Gírio, F.M., Fonseca, C., Carvalheiro, F., Duarte, L.C., Marques, S., Bogel-Łukasik, R., 2010. Hemicelluloses for fuel ethanol: a review. **Bioresource Technology**, 101, 4775–4800.

Grabar, T., Gong, W., Yocum, R.R., 2014. Metabolic evolution of *Escherichia coli* strains that produce organic acids. Myriant Corporation, Quincy, MA (United States).

Guo, F., Fang, Z., Xu, C.C., Smith Jr, R.L., 2012. Solid acid mediated hydrolysis of biomass for producing biofuels. **Progress in Energy and Combustion Science**, 38, 672–690.

Han, M., Kang, K.E., Kim, Y., Choi, G.-W., 2013. High efficiency bioethanol production from barley straw using a continuous pretreatment reactor. **Process Biochemistry**, 48, 488–495.

Harmsen, P.F., Hackmann, M.M., Bos, H.L., 2014. Green building blocks for bio-based plastics. Biofuels, **Bioproducts and Biorefining**, 8, 306–324.

Harun, S., Balan, V., Takriff, M.S., Hassan, O., Jahim, J., Dale, B.E., 2013. Performance of AFEX^{TM} pretreated rice straw as source of fermentable sugars: the influence of particle size. **Biotechnology for Biofuels**, 6, 1–17.

Hayat, A., Rafiq, S., Anwar, P., 2021. Paper waste management: extraction of fermentable sugar from lignocellulosic waste paper. **Pakistan Journal of Analytical & Environmental Chemistry**, 22, 180–189.

Heidi Lynn, R., Priscilla GL, B., Emmanuel, I., 2012. Metal nanoparticle modified polysulfone membranes for use in wastewater treatment: a critical review. **Journal of Surface Engineered Materials and Advanced Technology**, 2, 11.

Heßlinger, C., Fairhurst, S.A., Sawers, G., 1998. Novel keto acid formate-lyase and propionate kinase enzymes are components of an anaerobic pathway in *Escherichia coli* that degrades L-threonine to propionate. **Molecular Microbiology**, 27, 477–492.

Himmel, M.E., Ding, S.-Y., Johnson, D.K., Adney, W.S., Nimlos, M.R., Brady, J.W., Foust, T.D., 2007. Biomass recalcitrance: engineering plants and enzymes for biofuels production. **Science**, 315, 804–807.

Hossain, N., Hoong, L.L., Barua, P., Soudagar, M.E.M., Mahlia, T.M.I., 2021. The effect of enzymatic hydrolysis of pretreated wastepaper for bioethanol production. **Korean Journal of Chemical Engineering**, 38, 2493–2499.

Hsieh, C.C., Cannella, D., Jørgensen, H., Felby, C., Thygesen, L.G., 2014. Cellulase inhibition by high concentrations of monosaccharides. **Journal of Agricultural and Food Chemistry**, 62, 3800–3805.

Hu, L., Fang, X., Du, M., Luo, F., Guo, S., 2020. Hemicellulose-based polymers processing and application. **American Journal of Plant Sciences**, 11, 2066–2079.

Huang, M., Cheng, J., Chen, P., Zheng, G., Wang, D., Hu, Y., 2019. Efficient production of succinic acid in engineered *Escherichia coli* strains controlled by anaerobically-induced nirB promoter using sweet potato waste hydrolysate. **Journal of Environmental Management**, 237, 147–154.

Jampatesh, S., Sawisit, A., Wong, N., Jantama, S.S., Jantama, K., 2019. Evaluation of inhibitory effect and feasible utilization of dilute acid-pretreated rice straws on succinate production by metabolically engineered *Escherichia coli* AS1600a. **Bioresource Technology**, 273, 93–102.

Jantama, K., Haupt, M., Svoronos, S.A., Zhang, X., Moore, J., Shanmugam, K., Ingram, L., 2008a. Combining metabolic engineering and metabolic evolution to develop nonrecombinant strains of *Escherichia coli* C that produce succinate and malate. **Biotechnology and Bioengineering**, 99, 1140–1153.

Jantama, K., Zhang, X., Moore, J.C., Shanmugam, K.T., Svoronos, S., Ingram, L.O., 2008b. Eliminating side products and increasing succinate yields in engineered strains of *Escherichia coli* C. **Biotechnology and Bioengineering**, 101, 881–893.

Jara, R., 2010. The removal of wood components from hardwood by hot water.

Jia, J., Yu, B., Wu, L., Wang, H., Wu, Z., Li, M., Huang, P., Feng, S., Chen, P., Zheng, Y., 2014. Biomass enzymatic saccharification is determined by the non-KOH-extractable wall polymer features that predominately affect cellulose crystallinity in corn. **PloS One**, 9, e108449.

Kadić, A., Palmqvist, B., Lidén, G., 2014. Effects of agitation on particle-size distribution and enzymatic hydrolysis of pretreated spruce and giant reed. **Biotechnology for Biofuels**, 7, 1–10.

Keseler, I.M., Collado-Vides, J., Santos-Zavaleta, A., Peralta-Gil, M., Gama-Castro, S., Muñiz-Rascado, L., Bonavides-Martinez, C., Paley, S., Krummenacker, M.,

Altman, T., 2010. EcoCyc: a comprehensive database of *Escherichia coli* biology. **Nucleic Acids Research**, 39, D583–D590.

Kumar, D., Murthy, G.S., 2013. Stochastic molecular model of enzymatic hydrolysis of cellulose for ethanol production. **Biotechnology for Biofuels**, 6, 1–20.

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, 3713–3729.

Kumar, V., Verma, P., 2024. Pulp-paper industry sludge waste biorefinery for sustainable energy and value-added products development: A systematic valorization towards waste management. **Journal of Environmental Management**, 352, 120052.

Latif, A.A., Harun, S., Sajab, M.S., Markom, M., Jahim, J.M., 2018. Ammonia-based pretreatment for ligno-cellulosic biomass conversion–an overview. **Journal of Engineering Science and Technology**, 13, 1595–1620.

Lee, D., Yu, A.H., Saddler, J.N., 1995. Evaluation of cellulase recycling strategies for the hydrolysis of lignocellulosic substrates. **Biotechnology and Bioengineering**, 45, 328–336.

Lee, P.C., Lee, S., Chang, H.N., 2008. Succinic acid production by *Anaerobiospirillum succiniciproducens* ATCC 29305 growing on galactose, galactose/glucose, and galactose/lactose. **Journal of Microbiology and Biotechnology**, 18, 1792–1796.

Li, C., Knierim, B., Manisseri, C., Arora, R., Scheller, H.V., Auer, M., Vogel, K.P., Simmons, B.A., Singh, S., 2010. Comparison of dilute acid and ionic liquid pretreatment of switchgrass: biomass recalcitrance, delignification and enzymatic saccharification. **Bioresource Technology**, 101, 4900–4906.

Li, Q., Gao, Y., Wang, H., Li, B., Liu, C., Yu, G., Mu, X., 2012. Comparison of different alkali-based pretreatments of corn stover for improving enzymatic saccharification. **Bioresource Technology**, 125, 193–199.

Li, Q., Xing, J., 2014. Microbial succinic acid production using different bacteria species, in: Microorganisms in Biorefineries. **Springer**, 183–206.

Liu, X., Zhao, G., Sun, S., Fan, C., Feng, X., Xiong, P., 2022. Biosynthetic pathway and metabolic engineering of succinic acid. **Frontiers in Bioengineering and Biotechnology**, 10, 843887.

Liu, Y., Fu, X., Wang, Y., Wang, J., 2016. D-lactic acid production from waste paper by recombinant *Escherichia coli* JH13. Presented at the 2016 International Conference on Civil, Transportation and Environment, Atlantis Press, 1054–1057.

Lu, J., Lv, Y., Jiang, Y., Wu, M., Xu, B., Zhang, W., Zhou, J., Dong, W., Xin, F., Jiang, M., 2020. Consolidated bioprocessing of hemicellulose-enriched lignocellulose to succinic acid through a microbial cocultivation system. **ACS Sustainable Chemistry & Engineering**, 8, 9035–9045.

Ma, Z., Yang, Y., Chen, W.-Q., Wang, P., Wang, C., Zhang, C., Gan, J., 2021. Material flow patterns of the global waste paper trade and potential impacts of China's import ban. **Environmental Science & Technology**, 55, 8492–8501.

Mahboubi, A., Ylitervo, P., Doyen, W., De Wever, H., Molenberghs, B., Taherzadeh, M.J., 2017. Continuous bioethanol fermentation from wheat straw hydrolysate with high suspended solid content using an immersed flat sheet membrane bioreactor. **Bioresource Technology**, 241, 296–308.

Mansy, A.E., El Desouky, E.A., Taha, T.H., Abu-Saied, M., El-Gendi, H., Amer, R.A., Tian, Z.-Y., 2024. Sustainable production of bioethanol from office paper waste and its purification via blended polymeric membrane. **Energy Conversion and Management**, 299, 117855.

Mokatse, K.M., van Wyk, J.P.H., 2021. Physical and chemical pre-treatment of waste paper to increase saccharification by *Trichoderma viride* cellulase. **Global Nest Journal**, 23, 195–200.

Martín, C., Peinemann, J.C., Wei, M., Stagge, S., Xiong, S., Jönsson, L.J., 2019. Dilute-sulfuric acid pretreatment of de-starched cassava stems for enhancing the enzymatic convertibility and total glucan recovery. **Industrial Crops and Products**, 132, 301–310.

Milbrandt, A., Zuboy, J., Coney, K., Badgett, A., 2024. Paper and cardboard waste in the United States: Geographic, market, and energy assessment. **Waste Management Bulletin**, 2, 21–28.

Nair, A.S., Sivakumar, N., 2022. Enhanced production of biodiesel by *Rhodosporidium toruloides* using waste office paper hydrolysate as feedstock: Optimization and characterization. **Fuel**, 327, 125174.

Nair, A.S., Al-Bahry, S., Gathergood, N., Tripathi, B.N., Sivakumar, N., 2020. Production of microbial lipids from optimized waste office paper hydrolysate, lipid profiling and prediction of biodiesel properties. **Renewable Energy**, 148, 124–134.

Nair, A.S., Al-Battashi, H., Al-Akzawi, A., Annamalai, N., Gujarathi, A., Al-Bahry, S., Dhillon, G.S., Sivakumar, N., 2018. Waste office paper: a potential feedstock for cellulase production by a novel strain *Bacillus velezensis* ASN1. **Waste Management**, 79, 491–500.

Neelamegam, A., Al-Battashi, H., Al-Bahry, S., Nallusamy, S., 2018. Biorefinery production of poly-3-hydroxybutyrate using waste office paper hydrolysate as feedstock for microbial fermentation. **Journal of Biotechnology**, 265, 25–30.

Nghiem, N.P., Kleff, S., Schwegmann, S., 2017. Succinic acid: technology development and commercialization. **Fermentation**, 3, 26.

Nishimura, H., Tan, L., Sun, Z.-Y., Tang, Y.-Q., Kida, K., Morimura, S., 2016. Efficient production of ethanol from waste paper and the biochemical methane potential of stillage eluted from ethanol fermentation. **Waste Management**, 48, 644–651.

Oh, I.-J., Kim, D.-H., Oh, E.-K., Lee, S.-Y., Lee, J.-W., 2009. Optimization and scale-up of succinic acid production by *Mannheimia succiniciproducens* LPK7. **Journal of Microbiology and Biotechnology**, 19, 167–171.

Ojewumi, M.E., Emetere, M.E., Obanla, O.R., Babatunde, D.E., Adimekwe, E.G., 2022. Bio-Conversion of Waste Paper Into Fermentable Sugars—A Review. **Frontiers in Chemical Engineering**, 4, 926400.

Ojewumi M.E., Obielue B.I., Emetere M.E., Awolu O.O., Ojewumi E.O., 2018. Alkaline pretreatment and enzymatic hydrolysis of waste papers to fermentable sugar. **Journal of Ecological Engineering**, 19, 211–217

Okino, S., Inui, M., Yukawa, H., 2005. Production of organic acids by *Corynebacterium glutamicum* under oxygen deprivation. **Applied Microbiology and Biotechnology**, 68, 475–480.

Park, E.Y., Anh, P.N., Okuda, N., 2004. Bioconversion of waste office paper to L (+)-lactic acid by the filamentous fungus *Rhizopus oryzae*. **Bioresource Technology**, 93, 77–83.

Park, E.Y., Michinaka, A., Okuda, N., 2001. Enzymatic hydrolysis of waste office paper using viscosity as operating parameter. **Biotechnology Progress**, 17, 379–382.

Pathak, P., Bhardwaj, N.K., Singh, A.K., 2014. Production of crude cellulase and xylanase from *Trichoderma harzianum* PPDDN10 NFCCI-2925 and its application in photocopier waste paper recycling. **Applied Biochemistry and Biotechnology**, 172, 3776–3797.

Phosriran, C., Jantama, K., 2024. Systematic reengineering of *Klebsiella oxytoca* KC004-TF160 for enhancing metabolic carbon flux towards succinate production pathway. **Bioresource Technology**, 407, 131137.

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.

Porninta, K., Khemacheewakul, J., Techapun, C., Phimolsiripol, Y., Jantanasakulwong, K., Sommanee, S., Mahakuntha, C., Feng, J., Htike, S.L., Moukamnerd, C., 2024. Pretreatment and enzymatic hydrolysis optimization of lignocellulosic biomass for ethanol, xylitol, and phenylacetylcarbinol co-production using *Candida magnoliae*. **Frontiers in Bioengineering and Biotechnology**, 11, 1332185.

Prasetyo, J., Park, E.Y., 2013. Waste paper sludge as a potential biomass for bio-ethanol production. **Korean Journal of Chemical Engineering**, 30, 253–261.

Qin, Y., Li, Y., Liang, G., Shen, N., Xian, L., Wang, Q., 2023. Efficient Production of Succinic Acid from Sugarcane Bagasse Hydrolysate by *Actinobacillus succinogenes* GXAS137. **Fermentation**, 10, 22.

Rocha, J., Alencar, B., Mota, H., Gouveia, E., 2016. Enzymatic hydrolysis of waste office paper for ethanol production by *Spathaspora passalidarum*. **Cellulose Chemistry and Technology**, 50, 243–246.

Rodmui, A., Kongkiattikajorn, J., Dandusitapun, Y., 2008. Optimization of agitation conditions for maximum ethanol production by coculture. **Agriculture and Natural Resources**, 42, 285–293.

Saha, B.C., Cotta, M.A., 2010. Comparison of pretreatment strategies for enzymatic saccharification and fermentation of barley straw to ethanol. **New Biotechnology**, 27, 10–16.

Saha, B.C., Yoshida, T., Cotta, M.A., Sonomoto, K., 2013. Hydrothermal pretreatment and enzymatic saccharification of corn stover for efficient ethanol production. **Industrial Crops and Products**, 44, 367–372.

Salvachúa, D., Smith, H., John, P.C.S., Mohagheghi, A., Peterson, D.J., Black, B.A., Dowe, N., Beckham, G.T., 2016. Succinic acid production from lignocellulosic hydrolysate by *Basfia succiniciproducens*. **Bioresource Technology**, 214, 558–566.

Sanchez, O.J., Cardona, C.A., 2008. Trends in biotechnological production of fuel ethanol from different feedstocks. **Bioresource Technology**, 99, 5270–5295.

Sangkharak, K., 2011. Optimization of enzymatic hydrolysis for ethanol production by simultaneous saccharification and fermentation of wastepaper. **Waste Management & Research**, 29, 1134–1144.

Santos, R.B., Treasure, T., Gonzalez, R., Phillips, R., Lee, J.M., Jameel, H., Chang, H., 2012. Impact of hardwood species on production cost of second generation ethanol. **Bioresource Technology**, 117, 193–200.

Sarkar, N., Ghosh, S.K., Bannerjee, S., Aikat, K., 2012. Bioethanol production from agricultural wastes: an overview. **Renewable Energy**, 37, 19–27.

Sassner, P., Mårtensson, C.-G., Galbe, M., Zacchi, G., 2008. Steam pretreatment of H_2SO_4-impregnated Salix for the production of bioethanol. **Bioresource Technology**, 99, 137–145.

Saxena, R., Saran, S., Isar, J., Kaushik, R., 2017. Production and applications of succinic acid, in: Current Developments in Biotechnology and Bioengineering. **Elsevier**, 601–630.

Sharma, S., Sarma, S.J., Brar, S.K., 2020. Bio-succinic acid: An environment-friendly platform chemical. **International Journal of Environment and Health Sciences (IJEHS)**, 2, 69–80.

Shen, N., Zhang, H., Qin, Y., Wang, Q., Zhu, J., Li, Y., Jiang, M.-G., Huang, R., 2018. Efficient production of succinic acid from duckweed (Landoltia punctata)

hydrolysate by *Actinobacillus succinogenes* GXAS137. **Bioresource Technology**, 250, 35–42.

Si, S., Chen, Y., Fan, C., Hu, H., Li, Y., Huang, J., Liao, H., Hao, B., Li, Q., Peng, L., 2015. Lignin extraction distinctively enhances biomass enzymatic saccharification in hemicelluloses-rich Miscanthus species under various alkali and acid pretreatments. **Bioresource Technology**, 183, 248–254.

Silvia, S., Maharani, A., 2023. The Effect of Enzyme Concentration and Hydrolysis Time on the Yield of Microcrystalline Cellulose from Rice Straw. **Dinasti Health and Pharmacy Science**, 1, 25–32.

Singh, S., Singh, V.K., Aamir, M., Dubey, M.K., Patel, J.S., Upadhyay, R.S., Gupta, V.K., 2016. Cellulase in pulp and paper industry, in: New and Future Developments in Microbial Biotechnology and Bioengineering. **Elsevier**, 152–162.

Su, X., Xue, Q., Sun, M., Liu, J., Wong, M.H., Wang, C., Chen, S., 2021. Co-production of polysaccharides, ginsenosides and succinic acid from Panax ginseng residue: a typical industrial herbal waste. **Bioresource Technology**, 331, 125073.

Sun, Y., Cheng, J., 2002. Hydrolysis of lignocellulosic materials for ethanol production: a review. **Bioresource Technology**, 83, 1–11.

Suttikul, S., Srinorakutara, T., Butivate, E., Orasoon, K., 2016. Comparison of SHF and SSF processes for ethanol production from alkali-acid pretreated sugarcane trash. Presented at the The 6 th International Conference on Fermentation Technology for Value Added Agricultural Products July 29 th–31st, 2015 Centara Hotel & Convention Centre, Khon Kaen, Thailand, 93.

Thakare, K., Trivedi, M., 2021. Bioethanol Production from Waste Paper. **International Journal of Scientific Research in Biological Sciences**, 8, 2.

Thakker, C., Martínez, I., San, K., Bennett, G.N., 2012. Succinate production in *Escherichia coli*. **Biotechnology Journal**, 7, 213–224.

Vallecilla-Yepez, L., Ramchandran, D., Long, D., Saha, R., Wilkins, M.R., 2021. Corn fiber as a biomass feedstock for production of succinic acid. **Bioresource Technology Reports**, 16, 100868.

Ventorino, V., Robertiello, A., Cimini, D., Argenzio, O., Schiraldi, C., Montella, S., Faraco, V., Ambrosanio, A., Viscardi, S., Pepe, O., 2017. Bio-based succinate production from Arundo donax hydrolysate with the new natural succinic acid-

producing strain *Basfia succiniciproducens* BPP7. **BioEnergy Research**, 10, 488–498.

Vermerris, W., 2008. Miscanthus: genetic resources and breeding potential to enhance bioenergy production, in: Genetic Improvement of Bioenergy Crops. **Springer**, 295–308.

Volynets, B., Dahman, Y., 2011. Assessment of pretreatments and enzymatic hydrolysis of wheat straw as a sugar source for bioprocess industry. **International Journal of Energy & Environment**, 2, 3.

Volynets, B., Ein-Mozaffari, F., Dahman, Y., 2017. Biomass processing into ethanol: pretreatment, enzymatic hydrolysis, fermentation, rheology, and mixing. **Green Processing and Synthesis**, 6, 1–22.

Wang, C., Su, X., Sun, W., Zhou, S., Zheng, J., Zhang, M., Sun, M., Xue, J., Liu, X., Xing, J., 2018. Efficient production of succinic acid from herbal extraction residue hydrolysate. **Bioresource Technology**, 265, 443–449.

Wang, Z., Zhu, J., Zalesny Jr, R.S., Chen, K., 2012. Ethanol production from poplar wood through enzymatic saccharification and fermentation by dilute acid and SPORL pretreatments. **Fuel**, 95, 606–614.

Wood, B., Aldrich, H., Ingram, L., 1997. Ultrasound stimulates ethanol production during the simultaneous saccharification and fermentation of mixed waste office paper. **Biotechnology Progress**, 13, 232–237.

Wyman, C.E., Dale, B.E., Elander, R.T., Holtzapple, M., Ladisch, M.R., Lee, Y., 2005. Coordinated development of leading biomass pretreatment technologies. **Bioresource Technology**, 96, 1959–1966.

Wyman, C.E., Dale, B.E., Elander, R.T., Holtzapple, M., Ladisch, M.R., Lee, Y., Mitchinson, C., Saddler, J.N., 2009. Comparative sugar recovery and fermentation data following pretreatment of poplar wood by leading technologies. **Biotechnology Progress**, 25, 333–339.

Xiberras, J., Klein, M., De Hulster, E., Mans, R., Nevoigt, E., 2020. Engineering *Saccharomyces cerevisiae* for succinic acid production from glycerol and carbon dioxide. **Frontiers in Bioengineering and Biotechnology**, 8, 566.

Yang, J., Wang, Z., Zhu, N., Wang, B., Chen, T., Zhao, X., 2014. Metabolic engineering of *Escherichia coli* and in silico comparing of carboxylation pathways for high

succinate productivity under aerobic conditions. **Microbiological Research**, 169, 432–440.

Yang, L., Cao, J., Jin, Y., Chang, H., Jameel, H., Phillips, R., Li, Z., 2012. Effects of sodium carbonate pretreatment on the chemical compositions and enzymatic saccharification of rice straw. **Bioresource Technology**, 124, 283–291.

Yocum, R.R., Hermann, T., Hu, X., 2018. Fermentation of glycerol to organic acids. U.S. Patent No 10,041,094.

Zhang, H., Han, L., Dong, H., 2021. An insight to pretreatment, enzyme adsorption and enzymatic hydrolysis of lignocellulosic biomass: Experimental and modeling studies. **Renewable and Sustainable Energy Reviews**, 140, 110758.

Zhang, W., Zhang, T., Song, M., Dai, Z., Zhang, S., Xin, F., Dong, W., Ma, J., Jiang, M., 2018. Metabolic engineering of *Escherichia coli* for high yield production of succinic acid driven by methanol. **ACS Synthetic Biology**, 7, 2803–2811.

Zheng, T., Xu, B., Ji, Y., Zhang, W., Xin, F., Dong, W., Wei, P., Ma, J., Jiang, M., 2021. A staged representation electrochemical stimulated strategy to regulate intracellular reducing power for improving succinate production by *Escherichia coli* AFP111. **Biotechnology Journal**, 16, 2000415.

Zhong, Y., Gu, J., Shang, C., Deng, J., Liu, Y., Cui, Z., Lu, X., Qi, Q., 2024. Sustainable succinic acid production from lignocellulosic hydrolysates by engineered strains of *Yarrowia lipolytica* at low pH. **Bioresource Technology**, 408, 131166.

Zhou, S., Zhang, M., Zhu, L., Zhao, X., Chen, J., Chen, W., Chang, C., 2023. Hydrolysis of lignocellulose to succinic acid: a review of treatment methods and succinic acid applications. **Biotechnology for Biofuels and Bioproducts**, 16, 1.

Zhou, Y., Han, L.-R., He, H.-W., Sang, B., Yu, D.-L., Feng, J.-T., Zhang, X., 2018. Effects of agitation, aeration and temperature on production of a novel glycoprotein GP-1 by *Streptomyces kanasenisi* ZX01 and scale-up based on volumetric oxygen transfer coefficient. **Molecules**, 23, 125.

Zhu, F., Wang, C., San, K.-Y., Bennett, G.N., 2020. Metabolic engineering of *Escherichia coli* to produce succinate from woody hydrolysate under anaerobic conditions. **Journal of Industrial Microbiology and Biotechnology**, 47, 223–232.

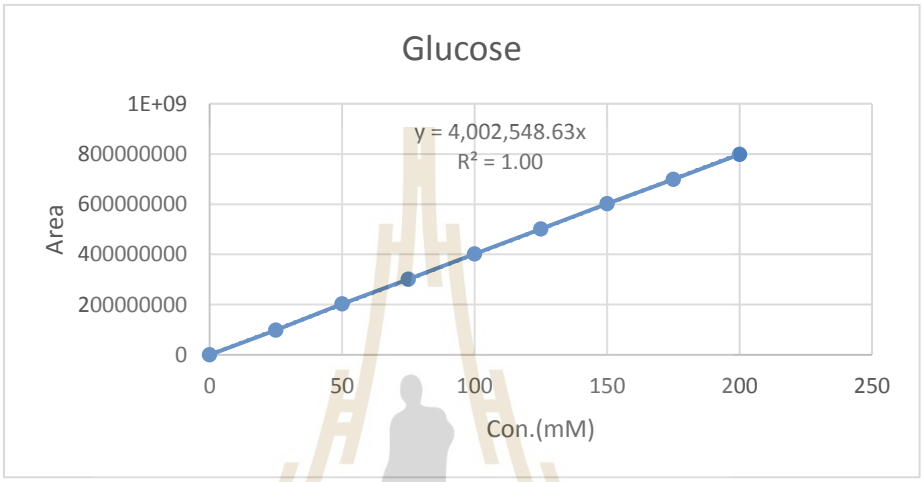
Zhu, L.-W., Xia, S.-T., Wei, L.-N., Li, H.-M., Yuan, Z.-P., Tang, Y.-J., 2016. Enhancing succinic acid biosynthesis in *Escherichia coli* by engineering its global

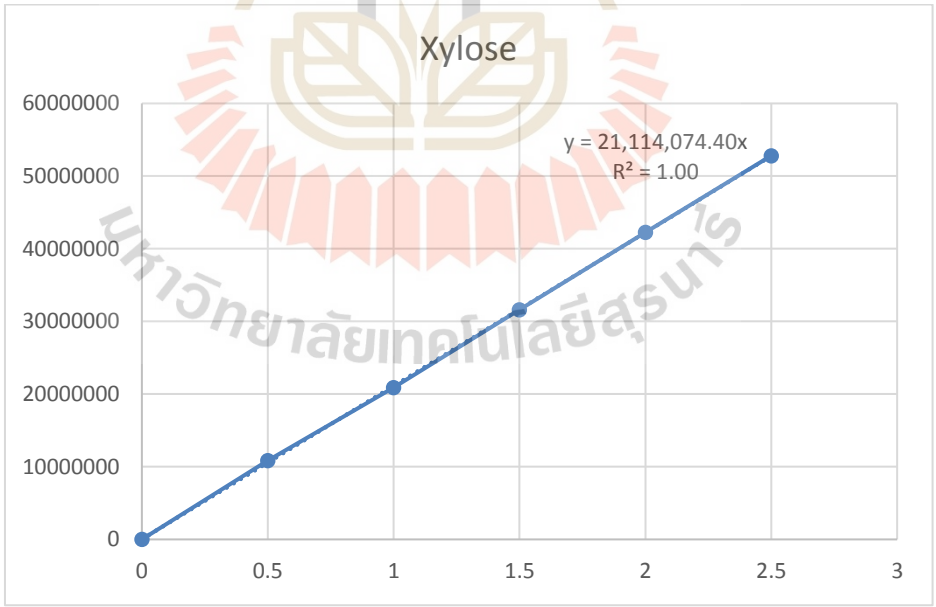
transcription factor, catabolite repressor/activator (Cra). **Scientific Reports**, 6, 36526.

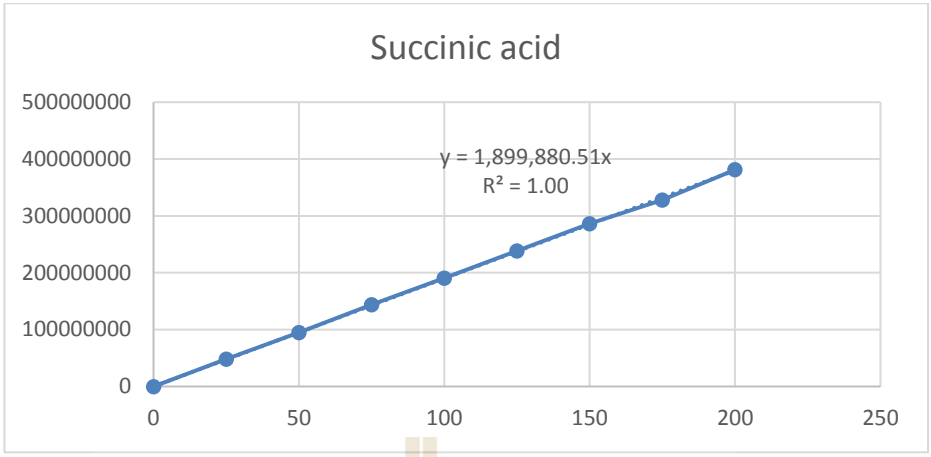
APPENDIX

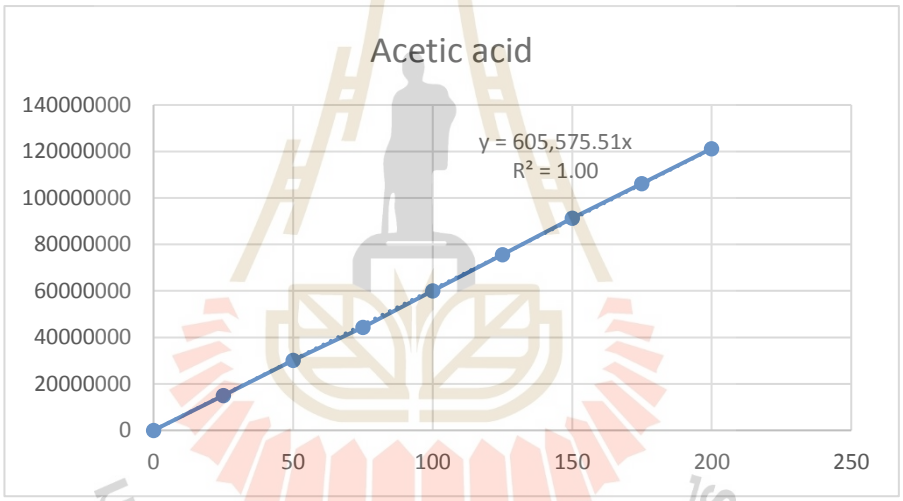
APPENDIX

STANDARD ANALYTICAL DATA









BIOGRAPHY

Miss. Walainud Congthai was born on December 10, 1998, in Nakhon Sawan, Thailand. She obtained her high school certificate in 2016 and continued her study in B. Sc in Biotechnology at Nakhon Sawan Rajabhat University, Thailand. After completing her undergraduate study, she decided to further pursue her study in Master degree in Biotechnology at School of Biotechnology, Institute of Agricultural of Technology, Suranaree University of Technology, Nakhon Ratchasima, Thailand.

Her research topic focuses on succinic acid production from mixed waste office paper by *E. coli* KJ122. Upon graduation from Suranaree University of Technology, she is planning to continue pursuing Ph.D. study in Biotechnology at Institute of Agricultural of Technology, Suranaree University of Technology, Nakhon Ratchasima, Thailand.