กระบวนการหมักและการทำบริสุทธิ์กรดซัคซินิกจากน้ำหมักแป้งมันสำปะหลัง โดยใช้กระบวนการกรองด้วยเมมเบรนและการตกผลึก

นางสาวเหงียน ธิ ฮวง ธุย

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต

สาขาวิชาเทคโนโลยีชีวภาพ

มหาวิทยาลัยเทคโนโลยีสุรนารี

ปีการศึกษา 2559

FERMENTATION AND PURIFICATION OF SUCCINIC ACID FROM CASSAVA BROTH USING MEMBRANE FILTRATION AND CRYSTALLIZATION

Nguyen Thi Huong Thuy

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

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FERMENTATION AND PURIFICATION OF SUCCINIC ACID FROM CASSAVA BROTH USING MEMBRANE FILTRATION AND CRYSTALLIZATION

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

Thesis Examining Committee

_____

(Assoc. Prof. Dr. Montarop Yamabhai)

Chairperson

_____

(Assoc. Prof. Dr.Apichat Boontawan)

Member (Thesis Advisor)

_____

(Prof. Dr. AdrianFlood)

Member

_____	_____
(Prof. Dr. Sukit Limpijumnong)	(Prof. Dr. Neung Teaumroong)
Vice Rector for Academic Affairs and Innovation	Dean of Institute of Agricultural Technology

เหงียน ธิ ฮวง ธุย:กระบวนการหมักและการทำบริสุทธิ์กรดซัคซินิกจากน้ำหมักแป้งมันสำปะหลังโดยใช้กระบวนการกรองด้วยเมมเบรนและการตกผลึก(FERMENTATION AND PURIFICATION OF SUCCINIC ACID FROM CASSAVA BROTH USING MEMBRANE FILTRATION AND CRYSTALLIZATION)อาจารย์ที่ปรึกษา: รองศาสตราจารย์ ดร.อภิชาติ บุญทาวัน,122หน้า.

กรดซัคซินิกเป็นสารเคมีแพลตฟอร์มคาร์บอน 4 อะตอม ที่มีคุณค่านำมาใช้กันอย่างแพร่หลายในงานหลายๆด้าน ในงานนี้ได้ทำการศึกษาการผลิตและการทำให้บริสุทธิ์ของกรดซัคซินิกจากหัวมันสำปะหลังทดแทนการใช้น้ำตาลกลูโคสโดยใช้เชื้อ *Actinobacillus succinogenes* ATCC55168 ทั้งนี้มันสำปะหลังที่ถูกย่อยด้วยเอนไซม์จะถูกนำไปใช้เป็นแหล่งคาร์บอน ซึ่งทั้งนี้ได้มีการศึกษาการใช้มันสำปะหลังเป็นแหล่งคาร์บอนเพียงอย่างเดียวโดยที่ไม่ต้องเติมสารเสริมอื่น ๆ ช่วยในกระบวนการหมักกรดซัคซินิกแบบกึ่งกะ ผลพบว่าสามารถผลิตกรดซัคซินิ กได้ประมาณ 78.33 กรัมต่อลิตร โดยได้ค่าผลผลิต 0.78 กรัมของกรดซัคซินิคต่อกรัมของกลูโคสและค่าผลิตผลการผลิตอยู่ที่4.17 กรัมต่อลิตรต่อชั่วโมงจากนั้นศึกษาการทำบริสุทธิ์ตัวอย่างน้ำหมักด้วยการใช้เทคนิคเมมเบรนแบบ electrodialysis แต่อย่างไรก็ตามผิวหน้าของเมมเบรนจะเกิดการ สะสมอุดตันจากเกลือแมกนีเซียม ส่งผลทำให้ประสิทธิภาพในการแยกสารของระบบลดล งกระบวนการทำบริสุทธิ์ที่มีประสิทธิภาพมากกว่าคือระบบไมโครฟิลเตชั่น นาโนฟิลเตชั่นและเทคนิคการตกผลึกจึงถูกนำมาศึกษา สำหรับไมโครฟิลเตชั่นนั้นจึงเน้นไปที่การแยกเซลล์ออกจากน้ำหมักโดยใช้แบบจำลองอนุกรมความต้านทานจากเมมเบรน รวมถึงความต้านทานของเค้ก การอุดตันของรูและความต้านทานการดูดซับ ประสิทธิภาพการแยกของเมมเบรนนาโนฟิลเตชั่นนั้นทำการศึกษาโดยใช้ส่วนที่ได้จากการกรองผ่านไมโครฟิลเตชั่น หรือเพอร์มิเอทจากไมโครฟิลเตชั่นมาเป็นสารป้อน ทั้งยังศึกษาระบบโดยใช้สารสังเคราะห์ ซึ่งผลที่ได้แสดงให้เห็นว่ามีความสามารถกำจัดสารอื่นๆอาทิ เช่นสารประกอบอินทรีย์ อนินทรีย์ โมเลกุลของสี โปรตีนและแมคโครโมเลกุล โดยเฉพาะอย่างยิ่งพวกไอออนที่มีวาเลนซี่ตั้งแต่ 2 ขึ้นไปได้ นอกจากนี้ยังสามารถเพิ่มการทำบริสุทธิ์จากน้ำหมักขั้นตอนไดอะนาโนฟิลเตชั่นที่มีความสามารถในการแยกสารอื่นๆออกเหลือแต่เพียงผลิตภัณฑ์ที่ต้องการได้อย่างมีประสิทธิภาพ ช่วยส่งผลดีต่อระบบการเก็บเกี่ยวผลิตภัณฑ์โดยรวมสุดท้ายเป็นขั้นการการตกผลึกที่เป็นการทำให้กรดซัคซินิกเกิดเป็นผลึกที่มีความบริสุทธิ์สูงสำหรับเป็นผลิตภัณฑ์

สุดท้ายขึ้นได้ ผลปรากฏว่าได้ผลึกของกรดซัคซินิกมีความบริสุทธิ์ที่ประมาณ 99.35% ซึ่งสอดคล้องกับความสามารถในการตกเป็นผลึกที่ 96.77% ในการทดลองแบบเติมตัวล่อผลึกในอัตราที่สูงจากผลนี้ทำให้สรุปได้ว่าผลึกของกรดซัคซินิกที่มีความบริสุทธิ์สูงสามารถเกิดขึ้นได้จากกระบวนการหมักที่มีการใช้หัวมันสำปะหลังโดยใช้*Actinobacillus succinogenes* ATCC55168

สาขาวิชาเทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา _____

ปีการศึกษา 2559 ลายมือชื่ออาจารย์ที่ปรึกษา_____

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม _____

NGUYEN THI HUONG THUY: FERMENTATION AND PURIFICATION OF SUCCINIC ACID FROM CASSAVA BROTH USING MEMBRANE FILTRATION AND CRYSTALLIZATION. THESIS ADVISOR: ASSOC. PROF. APICHAT BOONTAWAN, Ph.D., 122 PP.

SUCCINIC ACID/CASSAVA ROOTS HYDROLYSIS/MEMBRANE FILTRATION/CRYSTALLIZATION TECHNIQUE

Succinic acid (SA) is a valuable four carbon platform chemical widely applied in many different fields. In this study, the production and purification of SA from cassava roots instead of glucose by using *Actinobacillus succinogenes* ATCC55168 was investigated. Cassava roots were used as the sole carbon source without needing other supplemented-auxiliary components in a fermentation medium. Separate hydrolysis and fermentation was carried out for batch and fed-batch fermentation of SA. The results showed that succinic acid titer of 78.33 g/L with yield and productivity of 0.78 $g_{succinic\ acid}/g_{lucose}$ and 4.17 g/L/h, respectively were obtained for fed-batch fermentation. Subsequently, the broth was purified by membrane techniques. Electrodialysis was investigated; however, membrane fouling from magnesium salt hindered the separation efficiency of the system. A more efficient purification result was obtained by conducting a series of microfiltration (MF), nanofiltration (NF) and crystallization steps. MF was focused on modelling for the separation of bacterial cells using a resistance-in-series model in which membrane resistance, cake resistance, pore-blocking and adsorption resistance were involved. The separation performance of the NF process was examined for the clarified fermentation broth (MF permeate) and re-investigated for a model solution. The

results showed NF process's usefulness for the removal of potential foulants including organic and inorganic substance, colouring molecules, protein and macromolecules, and especially multivalent ions. In addition to enhancing the purity of the fermentation broth, a diananofiltration (DNF) step was conducted which efficiently contributed to a complete recovery of the target product. These advantages assisted invaluably to the downstream processes. Crystallization was investigated as a final step in maximizing the purity of the final crystal product. The succinic acid crystal purity of 99.35% corresponding to the relative crystallinity of 96.77% was obtained for a high seeding-crystallization experiment. These results indicated that a very high quality succinic acid crystal product could be achieved from cassava roots using *Actinobacillus succinogenes* ATCC55168.

School of Biotechnology

Academic Year 2016

Student's Signature_____

Advisor's Signature _____

Co-advisor's Signature _____

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LIST OF ABBREVIATIONS

^{o}C	=	Degree Celsius
%	=	Percent
w/w	=	Weight per Weight
v/v	=	Volume per Volume
g/L	=	Gram per Liter
g/L.h	=	Gram per Liter per Hour
g	=	Gram
g/g	=	Gram per Gram
Da	=	Dalton
µm	=	Micrometer
nm	=	Nanometer
L	=	Liter
mL	=	Mililiter
rpm	=	Roumd per Minute
vvm	=	Volume per Volume per Minute
min	=	Minute
h	=	Hour
et al.,	=	and others
EDBM	=	Electrodialysis bipolar membrane
DI	=	Deionized water
A	=	membrane area (m^2)

LIST OF ABBREVIATIONS (Continued)

A_{fp}	=	filter paper membrane area (m^2)
B	=	constant in equation 2 (s^{-1})
C	=	bacteria concentration in the retentate (kg m^{-3})
C_m	=	bacteria concentration on the membrane wall (kg m^{-3})
C_w	=	bacteria concentration of whole broth
C_f	=	solute concentration in diafiltration feed (g/L)
C_{f0}	=	solute concentration in diafiltration feed at t_0 (g/L)
D	=	shear induced diffusion coefficient ($m^2 s^{-1}$)
J	=	Permeate flux (m s^{-1} or 1 h^{-1} m^{-2})
m	=	weight of bacteria cake (m^{-1})
R_m	=	membrane resistance (m^{-1})
R_c	=	cake resistance (m^{-1})
R_f	=	pore blocking and adsorption resistance (m^{-1})
t	=	Time (s or hour)
M_F	=	mass of the compound in the feed phase (g)
M_P	=	mass of the compound in the permeate phase (g)
α	=	cake resistance coefficient (m kg^{-1} $Pa^{-0.63}$)
α_0	=	specific cake resistance (m kg^{-1})
δ	=	cake thickness (m)
ΔP	=	transmembrane pressure (Pa)
ε	=	relative error
μ_M	=	dynamic viscosity of microfiltration permeate (Pa s)

LIST OF ABBREVIATIONS (Continued)

μ_N	=	dynamic viscosity of nanofiltration permeate (Pa s)
exp	=	experimental
mod	=	modelled
ss	=	steady state
Eq.	=	Equation
VCR	=	volume concentration ratio
NF	=	nanofiltration
DNF	=	diananofiltration
SA	=	succinic acid

CHAPTER I

INTRODUCTION

1.1 Significant of this study

Succinic acid (SA) is an important chemical that can be used in food, pharmaceutical, cosmetic, and chemical industries. Recently, there is an increasing interest in the production of “green” chemicals and biodegradable polymers, such as diethyl succinate (DES), and poly-butylenesuccinate (PBS). In addition, the SA global market demand is also increasingly strongly rising. Extractive fermentation of SA from cheap and readily available agricultural substrates, especially cassava would play a major role in the total production cost. There are other major advantages for using cassava instead of pure glucose such as high productivity and yield, non-competitive with foods supply, and easily to grow on poor-dry and depleted soils as in Nakhon Ratchasima province. It can be expected that succinic acid production cost could be decrease when the cheap carbon source of cassava is used.

The efficiency of fermentation and production is also mainly decided by the product recovery process. Many techniques have been introduced for recovery of SA from fermentation broth including electrodialysis, crystallization, adsorption, direct precipitation, reactive extraction, and membrane separation, respectively. Electrodialysis and crystallization are two of the very promising, and perspective techniques originated bythe rapid development of the membrane processes (Hábová *et al*., 2004). Electrodialysis membrane was effectively used to remove organic acid salts from fermentation broth (Gong *et al.*, 2006). Crystallization itself is a good and

high selectively powerful method in the fermentation industry to produce pure succinic acid-final product from carboxylic acid mixtures (Beierling *et al.*, 2014, Kushiku *et al.*, 2006). Especially, combination simultaneously between two these methods is a fully promising potential to recover high purity SA from fermentation broth that no any research was reported about this issue before. Beside the purification of succinic acid with high purity, production cost should also be considered from an economic point of view in order to be obtained succinic acid product that occupies all the dominances on the global market. All of these are promoting the researchers to develop not only just towards a natural succinic acid production by fermentation, but also purification to high purity and especially decrease processing cost in this bio-based SA production to enhance competitive ability with petrochemical-based production. Those are considered as an important step in order to encourage the commercial production system.

In this work, fermentation of succinic acid was investigated by *Actinobacillus succinogenes* ATCC55168 (*A. succinogenes* ATCC55168) using cassava roots as a main carbon source. The *in situ* succinic acid removal from fermentation broth was carried out by using an electrodialysis system coupled with solution layer crystallization technique. The later technique can separate other organic acid by-products from SA since they cannot crystallize. The main objectives are to alleviate the organic acids toxicity effect, and to obtaine a high purity SA.

1.2 Research objectives

The main objectives of this study are as follows:

1.2.1 To compare SA production in term of titer, yield and productivity between the fermentations from glucose and cassava roots as a carbon source.

1.2.2 To purify SA from the fermentation broth using membrane separation techniques, and crystallization.

1.3 Research hypothesis

1.3.1 SA can be produced from cassava roots by using *A. succinogenes* ATCC55168.

1.3.2 Membrane separation can be used for the removing of impurities in the fermentation broth.

1.3.3 Purification of SA from fermentation broth can be performed by crystallization.

1.4 Scope of thesis

This work consists of two main parts. The first part was SA fermentation by *A. succinogenes* ATCC55168 from glucose and cassava roots as a carbon source. The cassava roots are readily available in Nakhon Ratchasima province, Thailand. The next part was downstream processing of succinic acid from fermentation broth by using electrodialysis (ED), microfiltration (MF), nanofiltration (NF), and crystallization techniques. After finishing the fermentation process, clarified fermentation was produced by the help of microfiltration unit for remove all of the cells and insoluble magnesium carbonate in whole broth. Separation of succinic acid from the clarified fermentation broth was compared by using ED, and NF, respectively. Then, the treated fermentation broth underwent evaporation followed by different crystallization methods including solution layer crystallization, conventional crystallization, and seed loading crystallization.

1.5 Expected results

1.5.1 High fermentation performance with high titer, yield and productivity will be obtained using cassava roots as a main carbon source.

1.5.2 High purity and crystallinity of SA crystals product will be obtained using membrane separation and crystallization method.

1.5.3 This downstream process can be applied to the in the industrial scale production with improving of SA crystal product and reducing of production cost.

CHAPTER II

LITERATURE REVIEWS

2.1 Succcinic acid

SA is a dicarboxylic acid organic compound having chemical formula of $C_4H_6O_4$, molecular weight of 118.09, and CAS No. 110-15-6 with the white crystalline or almost crystalline power (Hu *et al.*, 2013). The chemical structure and physico-chemical properties of succinic acid are shown in Figure 2.1 and Table2.1, respectively.

Figure 2.1 The chemical structure of SA (http://en.wikipedia.org/wiki/succinic_ acid).

SA is considered as a high interest organic acid which could be manufactured from bio-feedstock (Department, 2004). Based on its potentials, succinic acid has also been known as one of the 12 top value precursors announced by Energy Department of United States as a substance that can be wide applied in various industries such as food, pharmaceutical, cosmetic, etc. (Werpy *et al.*, 2004). This is the product that is increasingly being attracting not only the users, but also of course the world-wide researchers to find out new exploitation based on its available application.

Table 2.1 Physico-chemical properties of SA(Ponomarenko *et al.*, 2003).

Properties	
Physical state	Odorless and colorless white crystals
Molar mass	118.09 $g.mol^{-1}$
Density	1.56 $g.cm^{-3}$
Melting point	184 °C (363 °F)
Boiling point	235 °C (455 °F)
Solubility in water	58 $g.L^{-1}$ (20 °C)
Acidity (pKa)	$pK_a1 = 4.2$, $pK_a2 = 5.6$

2.2 Application of succinic acid

SA is a valuable platform chemical with wide applications in many different fields (Li *et al.*, 2010, Sang *et al*., 2005, Song *et al*., 2006). In Figure2.2, principal applications of SA are described (Beauprez *et al*., 2010). SA is applied as the specialty chemicals include detergents, surfactants, corrosion inhibitors. In addition, SA is used as an additive group consists of the applications in pharmaceutical, food, agricultural, and cosmetic. For example, sodium succinate and di-lysine succinate were introduced as flavoring enhancers which could replace monosodium glutamate in low sodium foods (Jain *et al*., 1989). More interestingly, the other multiple outstanding applications in the industries, SA was an important precursor in the production processes for bio-degradable polymers such as 1, 4-butanediol (Beauprez *et al*., 2010), tetrahydrofuran, N-methyl pyrrolidinone, 2-pyrrolidione succinate salts and γ-butyrolactone (Song *et al.*, 2006), and as a monomer for the production of bio-degradable plastics(Zeikus *et al*., 1999) such as poly-butylene succinate (Xu *et al.*, 2009), butylene succinate-co-propylene succinate (Lu *et al.*, 2012), etc.

Figure 2.2 Various chemicals and products that can be synthesized from succinic acid (Beauprez *et al*., 2010).

2.3 Succinic acid market

Currently, the large use of SA and its derivatives is around 20,000-30,000 tons per years with the potential price of $400,000,000 per year (Kidwell, 2008; Zeikus *et al*., 1999).

This rate increases by 10% per year, and the market size is estimated to be more than 270,000 tons per year if SA replaced maleic anhydride for all uses of the latter (Willke *et al.*, 2004). A report showed that, the price of succinic acid that is mainly produced petrochemically from butane through maleic anhydride up to $5.9-8.8/kg depending on its purity (Zeikus et al., 1999). Confronted with the rising price of petroleum and pollution, there are many researchers tending to produceSA from renewable resources using microorganisms and green biotechnology. The large scale of fermentative SA was produced in early 1980 with about 5,000 tons per year, and was sold at $2.2/kg to the food market (Zeikus, 1980). The natural SA sold in the food market is produced by fermentation,and lowering the production cost always attract customers. As expected, natural SA price would be decreased by $0.55/kg at the above level of 75,000 tons/year by utilizing the cheap carbon substrates such as cassava, corn, molasses, sugars (Kidwell, 2008, Xia *et al*., 2014, Zeikus *et al.*, 1999). Hence, such a good economics promise to open new specialty and commodity chemical markets for succinic acid. Moreover, the market of succinic acid is expected to grow at a rate of 18.7% from 2011 to 2016according to a survey report from the Markets and Market. The global for succinic acid in the terms of revenue was estimated to be worth $182.8 million in 2010, and is expected to reach $496.0 million in 2016 (Cao *et al.*, 2013). Specifically, as the Unite State demand for 1,4-Butanediol (BDO) increased from 318,000 tons in 1999 to 408,000 tons in 2004, and it was previously-set out projected 479,000 tons in 2008. BDO's price has also increased from $2.2/kg (1999) to $2.76/kg (2004) (McKinlay *et al.*, 2007). All of these numbers reflected that current succinic acid commercial is rising significantly, and will be continuously more increasing in the next following years.

2.4 Production of succinic acid

SA can be produced in different ways including chemical process, in which petrochemical-based synthesis or liquefied petroleum gas (LPG) is used as a starting material. The latter route is biotechnological process.

2.4.1 Production of succinic acid by chemical process

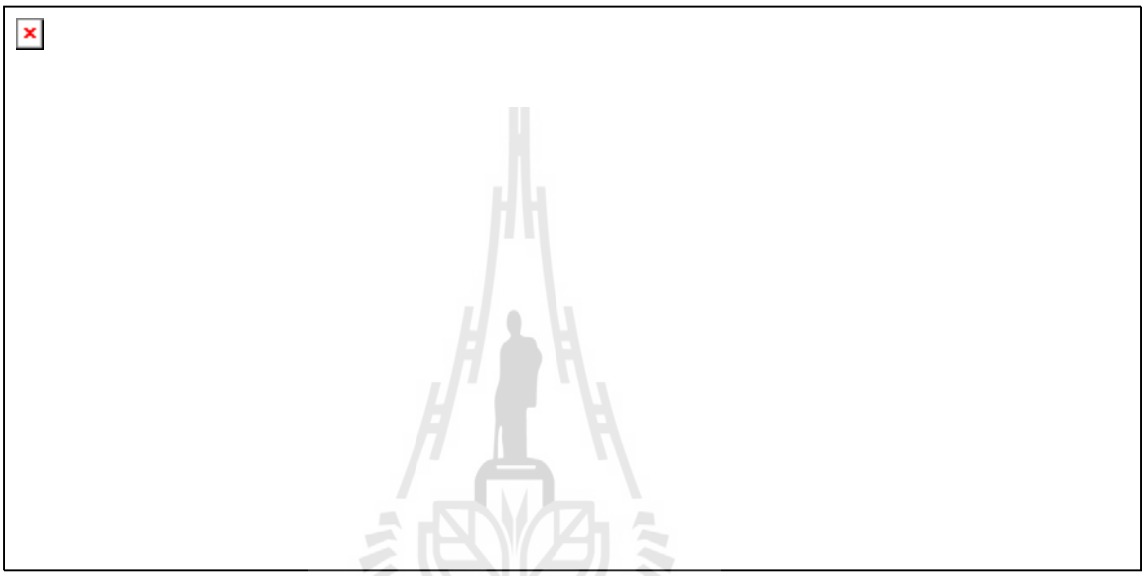


Figure 2.3 Chemical route of the production of SA from maleic anhydride.

SAcan be produced by traditional process using petrochemically derived maleic acid or maleic anhydride as a starting resources, which is produced from n-butane through oxidation over vanadium-phosphorous oxide catalysts. The simplified reaction pathway of n-butane to maleic anhydride is shown in Figure 2.3(Zhang *et al*., 2009). The reaction from maleic anhydride to succinic acid begins by hydrolysis, breaking one of the single bonds between carbon and oxygen, forming maleic acid. The addition of hydrogen breaks the carbon-carbon double bond and completes the reaction, forming SA. Moreover, SA produced from fossil fuels is not being a natural product,and must be carried out at high temperature, high pressure, and high cost for the catalytic system. This was lead to the product cost to be increasing (Li *et al*., 2010).

2.4.2 Production of succinic acid by fermentation process

SA is an intermediate metabolite of the tricarboxylic acid (TCA) or Krebs cycle, and is also one of the fermentation end-products of anaerobic metabolism by the effect of microorganisms (Song *et al.*, 2006). Along with the tremendous unceasing efforts of many researchers towards development of the biological process for succinic acid production, various microorganisms have been reported include both bacteria, and fungi (Agarwal *et al*., 2006, Song *et al.,* 2006). Among them, there are many bacteria which have been found to produce high SA as a major product in fermentation (Guettler *et al.*, 1998). The strains such as *Anaerobiospirillum succiniciproducens* (Glassner *et al*., 1992), *Mannheimia succiniciproducens* (Song *et al*, 2006) were reported. Especially, *A. succinogenes* is considered one of the effective SA producers because they can endure high glucose osmotic pressure, and produce significant amounts of SA with a high volumetric productivity, high SA titer, and less by-product formation (Huh *et al*., 2006). More recently, the SA concentration in fermentation broth was reported to be up to 47.2 g/l corresponding to yield of 0.56 g_{SA}/ $g_{glucose}$ using *A. succinogenes* as a producer (Lubsungneon *et al*., 2014). The most attention here is not only those strains have high potential to produce succinic acid, but also bring the natural product that petrochemical process never. Therefore, this is the strongest interest to the microbial conversion of biomass to SA from the carbon sources such as glucose, arabinose, cellobiose, fructose, etc. The metabolic pathway of SA is illustrated in Figure 2.4. This pathway occurring under the anaerobic condition, and is defined as Phosphoenolpyruvate (PEP) (PEP is intermediate metabolite that is formed from glucose through glycosidic pathway and PEP is also the branch point between the TCA pathways and the fermentative pathways leading to lactic acid and other fermentation products) → Oxaloacetic acid (OAA) → Malic acid (Mal) → Fumaric acid (Fum) → Succinic acid (Suc). Five key enzymes responsible for succinic acid production were identified as PEP carboxykinase

(pck), malate dehydrogenase (mdh), maleic enzyme (me), fumarase (fum) and fumarate reductase (frd) (Song *et al*., 2006). Where, PEP carboxykinase (pck) is the major CO_2 fixing enzyme and responsible for Oxaloacetic acid (OAA) formation, is the crucial step in the formation of succinic acid (Mwakio, 2012). Oxaloacetic acid (OAA) before being converted to succinate by the reductive TCA branch, also called C_4 pathway. The pathway is defined as Phosphoenolpyruvate (PEP) →Oxaloacetic acid (OAA) → Malic acid (Mal) → Fumaric acid (Fum) → Succinic acid (Suc). Also, enzymatic analysis revealed the presence of pyruvate kinase (pyk), pyruvate ferredoxin oxidoreductase (pfo), acetate kinase (ack), alcohol dehydrogenase (aldh) and lactate dehydrogenase (ldh), which affect SA flux in the central metabolic pathways. These enzymes activity is strongly regulated by pH and CO_2 concentration in the fermentation broth. In theory, 1 mole of CO_2 is required to form 1 mole of succinic acid. The higher CO_2 level resulted in an increased succinic acid production at the expense of ethanol and formic acid. If lower CO_2 level, the flux of PEP was observed to favor the C_3 pathway (Mwakio, 2012). The pathway that produces formate, acetate, lactate and ethanol is called the C3 pathway and defined as PEP → Pyruvic acid (Pyr) → Acetyl CoA (AcCoA) → Acetic (Ace) + Ethannol (EtOH), and also include Pyr → Lactic acid (Lac). In addition, the concomitant production of metabolic by-products such as acetic, formic, and lactic acids are also a significantly interest problem because it reduces the SA yield, and makes the purification process difficult and expensive. As a result, the yield of SA can be increased by disrupt the carbon fluxes to lactate, formate, and acetate by inactivating lactate dehydrogenase (LDH), pyruvate formate-lyase (PFL), phosphotransacetylase (PTS), and acetate kinase (AK), respectively.

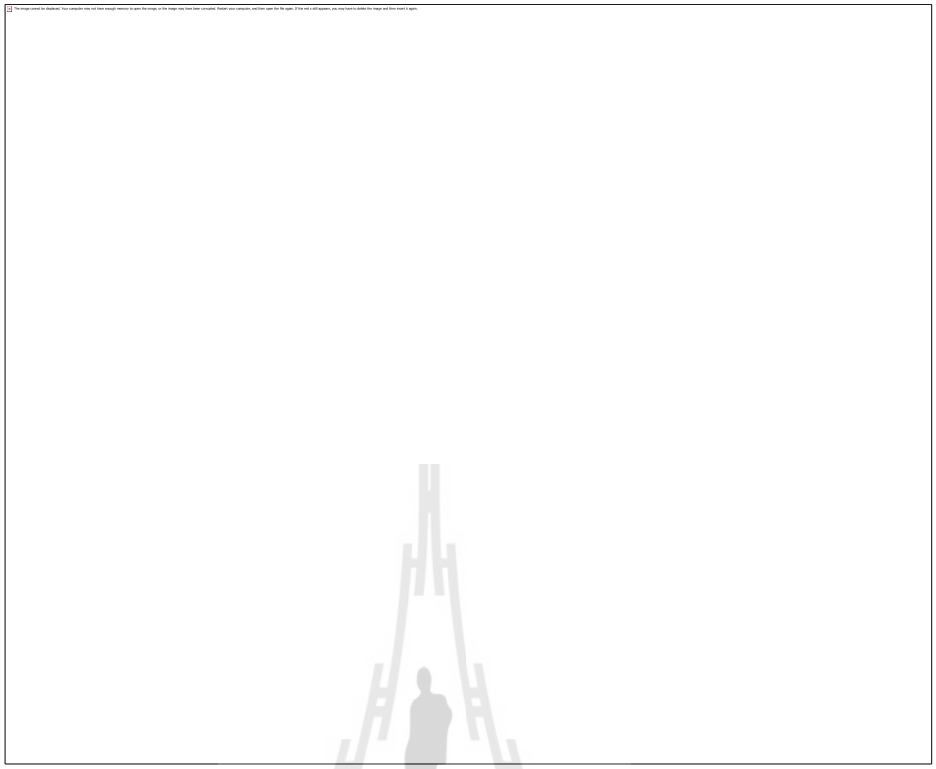


Figure 2.4 Catabolic pathway of SA production of wild-type *A. Succinogenes* (McKinlay et al., 2007).

2.5 Cassava roots

Cassava (*Manihot esculentaCranz.*) is one of the most important available starch substrates for fermentation process (Chen *et al*., 2014), and it is also considered as an important source of food and dietary calories in many countries and regions, including China, Thailand, and Latin America (Pandey *etal*., 2000). Thailand is a world leader in cassava exportation, reached approximately 83% in the worldwide, 2009 (according to the Office of Agricultural Enocomics and Food and Agricultural Organization, 2012). Nakhon Ratchasima province is one of the areas producing fresh cassava reached up to 83.50% of the total production(Poramacom *et al*., 2013). Moreover, according to a recent study, Nguyen *et al* (2014) reported that in Vietnam and in South East Asia, cassava is also considered an attractive raw material for fermentation technology thanks to the advantages such as (i) the

ease of plantation on various soil types and climate conditions; (ii) a very low input and investment for planting; (iii) “all year round” availability feedstock in the form of fresh roots and dry chips; and (iv) a high starch-containing raw materials and a lower proportion of fibers (Nguyen *et al*., 2014). The nature of cassava is starch-rich and robustness, it contains about 50%–70% starch on a dry weight basis and 20%–30% fibers, which are composed mainly of cellulose and other non-starch polysaccharides. Therefore, it has become an important crop that is not only as food, but also as a great potential resource-biomass feedstock for the production of fermentation products (Collares *et al.*, 2012, Xia *et al*., 2014). Thus, this is also a mainly precursor of starch material that will be most interested because it has very high nutrient content, and can be purchased easily.

In fact, cassava is widely and successfully employed as a promising renewable feed stock for many industrial applications; for example, animal feed industry, starch industry (Collares *et al*., 2012), and more recently bio-ethanol production (Li *et al*., 2014, Nguyen et al., 2014), bio-butanol (Li *et al.*, 2012), L-lactic acid (Sobowale *et al.*, 2008; Wang *et al.,* 2010), aromatic compound (Shi *et al.*, 2014) and so on. The result of (Wang *et al.,* 2010) showed that cassava powder was used as a cheap raw bio-resource to ferment lactic acid by *Lactobacillus rhamnosus* strain. The highest lactic acid concentration was obtained up to 175.4 g/l while just using 275 g/l cassava powder concentration (total sugar of 222.5 g/l) in batch fermentation (Wang *et al.,* 2010). Another study reported about succinic acid fermentation from cassava starch with the highest yield and SA concentration of 0.86 g/g and 127.13 g/l, respectively. Therefore, these results suggested for a potential of succinic acid industrial production by using cheap biomass materials like cassava starch (Chen *et al*., 2014).

2.6 Downstream processing of succinic acid

The overall SA production process can be divided into two major parts, namely fermentation of carbohydrates, and purification to produce pure SA. The step of separation and purification poses the most challenges in the overall process due to the presence of impurities after fermentation process, but it is also the best step to improve the economic efficiency of the process (Zeikus *et al*., 1999). Downstream processing is any treatment of culture broth after fermentation to recover and purify succinic acid by removal of impurities especially by-products such as acetic, formic, lactic and pyruvic acids from fermentation broth. Over 50-80% of the production cost in the classical fermentation-based process was generated by downstream processing (Baniel *et al.*, 1995). For these interesting reasons, some new as well as some well-known methods will be presented below for the improvement for recovery of SA.

2.6.1 Crystallization

Crystallization itself is a well and high selectively powerful method in the fermentation industries to produce the pure succinic acid product from the dicarboxylic mixtures (Beierling *et al*., 2014, Kushiku *et al*., 2006). The application of crystallization for downstream processing of SA has been studied in the recently years. In 2008, a direct crystallization method from the traditional calcium precipitation was investigated in order to obtain SA from defined and wheat-based fermentation broths. The result showed that SA crystal were successfully recovered from fermentation broth by using this method. A higher SA crystal purity (95%) and yield (70%) were obtained in the direct crystallization method compared to a slightly modified traditional calcium precipitation method (90% and 24%, respectively) (Luque *et al*., 2009). The principle of this method is based on the concept that carboxylic acids have variously distribution between dissociated and undissociated forms at variously pH values, and the undissociated carboxylic acid has also different the solubility at the varied temperatures. It was found that the solubility of SA was only 3% at 4 ^{o}C and pH 2.0

while other by-acids like lactic acid, acetic acid, formic acid were still exist at soluble form in water (Li *et al*., 2010). Therefore, the SA can be selectively crystallized to bring the pure succinic acid. By this one step recovery method, SA yield was 70% and the purity was 90% which was very high comparedto the 52% using the traditional calcium precipitation only. Thus, crystallization could be regarded as a final purification step for the downstream separation process (Li *et al.*, 2010). In addition, based on above principle of the SA can crystallize at 4 ^{o}C, a model can be developed to crystallize SA using a cooling finger dipped into fermentation broth solution. Under the effect of this cooling temperature, solely SA was crystallized and attached on the crystallizer surface whereas all other impurities still maintained in the solution, and finally moved out. Experimental results showed that up to 99.9% purity of the recovered SA was obtained from fermentation broth containing disodium succinate and sodium hydrogen succinate when using the combination between the crystallization with ion exchange resin to remove the residual ionic impurities (Kushiku *et al.*, 2006). Despite this method has high potential for produce the pure SA; Nevertheless, the product yield can low because much succinate is still residual in the fermentation broth.

2.6.2 Membrane separation process

Membrane separation processes (including microfiltration, ultrafiltration, and nanofiltration) have been studied for the separation and purification of SA. SA in fermentation broth was successively treated by microfiltration, ultrafiltration, and nanofiltration, respectively. The obtained filtrate was acidified to pH 2-3.5 and concentrated under vacuum at 65 °C to remove water and acetic acid. The crystallization of the concentrated succinic acid solution was then carried out, giving a high purity (>99.5 %) of SA with a yield of higher than 75 % (Cheng *et al*., 2012). In addition, the SA fermentation broth from cassava starch was subjected successively to microfiltration, ultrafiltration, and

nanofiltration, removing molecules or particles having a size greater than 0.2 μm, a molecular weight greater than 5,000, and about 150-350 Da, respectively. The final filtrate was then treated by vacuum concentration and crystallization to obtain a high purity (> 99.4 %) of SA(Wu *et al.*, 2007). Nanofiltration is a separation technique whose selectivity is governed both by steric hindrance effects and electrostatic repulsion. In general, nanofiltration membrane could retain the compounds of molecular weight up to 200 Da and charged molecules, especially the multivalent ions. As mentioned above, the SA can be produced by the bioconversion under the effect of the specific strains. However, fermentation process can generate some salts of other organic acids as the by-products (lactate, acetate, formate salts) as well as different impurities like the substrates (i.e. glucose, glycerol) or mineral salts. Thus, further operation of purification, concentration and conversion are needed to obtain the pure SA. Nanofiltration can be considered as an appropriate purification process before conversion to succinic acid. This has been proposed to be applied for the recovery of SA from fermentation broth (Prochaska *et al*., 2014). The fundamental principle of this method is the use of the pressure to separate the soluble ions from aqueous solution through a semi-permeable material. In the recently years, there were many reports concerning the recovery of lactic and fumaric acid by using membrane processes as the purification step (Dey *et al*., 2012, Prochaska *et al.*, 2014, Woźniak *et al*., 2014). In addition, the recovery of SA from fermentation broth using nanofiltration coupled with vapor permeation-assisted esterification was reported. The result of this study showed that removal of both protein and coloring molecules from fermentation broth can be achieved using nanofiltration resulting in a pure and white product. If proteins are not sufficiently removed, the final product might result in a yellowish color. In addition, other macromolecules with a similar size to protein size will be also removed if proteins are removed (Lubsungneon *et al*., 2014).

Nevertheless, the disadvantages of this method are not only high membrane cost, but also high operating cost which requires skilled and intensive labor to perform activities such as chemical cleaning and pretreatment of turbid and suspended solid. In addition, the major concern is the presence of the organic acid by-products that still exit in permeate stream after treatment by membrane processes.

2.6.3 Electrodialysis

Electrodialysis is a membrane separation process that incorporates ion exchange membranes, and electric potential difference to separate ionized or weakly ionized molecules in aqueous solution by transporting through ion exchange membranes under the effect of a direct electric field. It is well known as an interesting technique developed for recovering and purifying salts and organic acids from fermentation broth. Citric, acetic, fumaric, propionic acid have been recovered industrially with this method (Anthony *et al.*, 1992, Lixin *et al*., 2000, Pinacci, 2002; Prochaska *et al*., 2014). Moreover, the recovered lactic acid concentration was reported up to 92.4 g/l when electrodialysis wastewater was used for a fermentation medium (Wee et al., 2005).

In fermentation broth, the dissociated succinate is ionic whilst other components, such as proteins, and carbohydrates are either very weakly ionic or non-ionic. If electrodialysis is used while the fermentation is taking place, liquid from the bioreactor could be run through the electrodialysis system and, as the succinate ions are removed, the remaining fluid including cells and non-ionic or very weakly ionic components can be recycled back to the bioreactor (Lee *et al*., 2008). By the meantime, magnesium succinate will be dissociated, and separated into magnesium ions and succinate ions separately. Magnesium cations will be across cation exchange membrane simultaneously reacts with hydroxyl groups (OH^-) which were dissociated from water forms magnesium hydroxide

($Mg(OH)_2$). Similarly, the succinate anions will be migrated across anion exchange membrane, and simultaneously reacts with the protons H^+ which were also dissociated from water producing SA. One advantage thing is not only separate solely SA stream, but also magnesium hydroxide stream which will be returned into fermenter to maintain optimal pH. More importantly, the nutrient compounds such as glucose, and protein which are not transported through membrane will also be returned into fermenter to continue fermentation process as shown in Figure 2.5

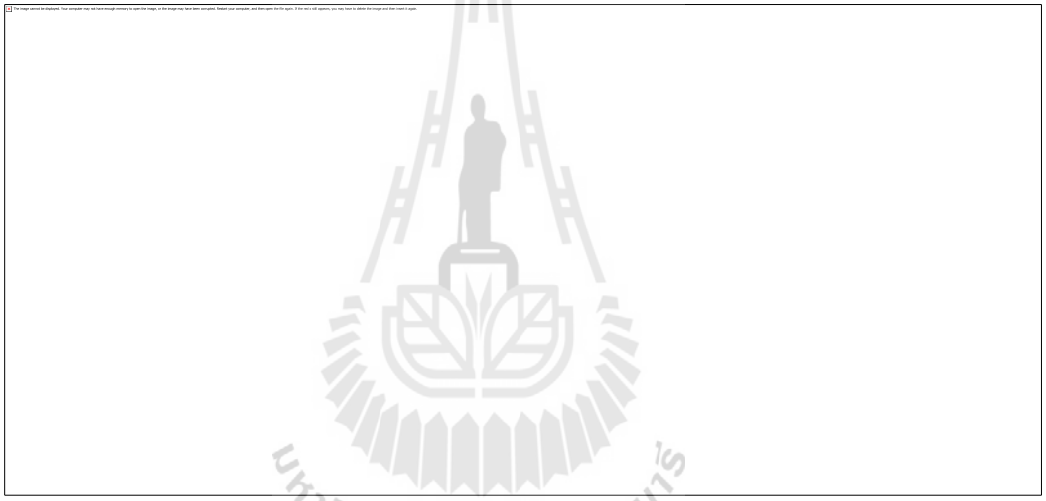


Figure 2.5 Working principle of electrodialysis technique (Hepburn *et al.*, 2012).

This technique can be employed to simultaneously separate SA from fermentation broth during fermentation process. This extractive fermentation shows an opportunity to increase the production yield and volumetric productivity by avoid the product inhibition during fermentation process caused by the acids. This could result in a high effective production in term of a reduction in operating cost, and higher production yield. In 2007, an integrated membrane-bioreactor-electrodialysis system was investigated to achieve SA with high yield, titer and productivity (Meynial-Salles *et al.*, 2008). The succinate ion was concentrated in the concentrate stream, while the depleted diluted stream

was recycledto the fermenter leading to the succinate and biomass concentration in the fermenter to be lower than the measured values when the succinate production was run in a cell recycle membrane bioreactor alone. In contrast, a concentrated solution of 38 g/l of succinate was continuously produced in the concentrate stream generating from the electrodialysis unit. In conclusion, the integrated membrane bioreactor monopolar electrodialysis process developed in this study allowed a decreasing in the level of organic acid in the reactor, and, as a consequence, limits the growth inhibition phenomenon. The result of this system for succinate production at high yield (1.35 mol/mol), high titer (83 g/l), and high volumetric productivity (10.4 g/l.h), respectively. In addition, a very high recovered succinic acid concentration using very low current efficiency and energy consumption was reported with the use of bipolar membrane eletrodialysis to convert succinic acid from sodium succinate solution (Fu *et al.*, 2014).

2.6.4 Reactive extraction

The reactive extraction of SA with amine-based extractant, employing hydrophobic tertiary amines, has been considered as an effective and economical purification method in recent years because the process is operated at normal temperature and pressure (Huh *et al*., 2004). This process is based on reversible reaction between the extractant and the extracted carboxylic acid. The selective separation of specific acid from fermentation broth containing mixed acids can be achieved based on the pKa values of the acids and operating pH (Song *et al.*, 2006).

Amine-based extraction is a method of reactive extraction that separates organic acids based on their pKa values and operating pH as it removes undissociated acids (Hong *et al.*, 2005, Huh *et al.,* 2006). It is a promising method because separation is possible *in-situ* at room temperature and pressure, so no pretreatment is required for this method to function

properly. The focus of much literature in amine-based extraction is the use of tri-n-octylamine (TOA) because previous studies have shown that it extracts succinic acid very well (Huh *et al.*, 2006). TOA for reactive extraction is toxic to cells, because that effect on cell growth and production, other methods of succinic acid extraction needs to be investigated. There are additional steps that must take place to continue the process of separating and purifying SA, such as vacuum distillation and crystallization, but given that the by-product organic acids have been removed, this step becomes easier, reaching a final purity of 99.8% with a yield of 73.1% (Bechthold *et al*., 2008, Lee *et al*., 2008).

2.6.5 Adsorption

Adsorption has shown a good potential and some data have been gathered for the distribution properties of other carboxylic acids, including acetic, lactic, and formic acids. Adsorption phenomenon occurs when components of a gas or liquid are attracted on the surface of solid particles or at fluid interfaces under the effect of a certain force such as electrostatic, or other binding forces between individual atoms, ions or molecules. Many researchers showed that adsorption with weak alkaline anion exchange adsorbents is one of the commonly method to separate organic acids from the fermentation broth such as picric acid (Uslu *et al.*, 2010), formic acid (Uslu, 2009). For example, the NERCB 09 adsorbent was proposed as an effective adsorbent to separate succinic acid from the model solution and fermentation broth because of its high capacity, selectivity, and adsorption rate (Song *et al.*, 2006). In addition, adsorption is also known as a promisingly separation method for recovery of the SA because adsorbents have the advantages of low price, quick recovery, and low regeneration consumption. Ion exchange adsorption has also been widely used in many organic acids separations (Anasthas *et al*., 2001; Shen *et al.*, 2013). However, adsorption is the technique that has been characterized by low separation degrees because other organic

acids can also be absorbed by the adsorbent. Therefore, this step is considered only as a primary recovery of succinic from fermentation broth. Further purification steps are required in order to obtain a high purity SA.

2.6.6 Precipitation

A traditional method for the organic acid isolation from fermentation broth is by precipitation with calcium hydroxide ($Ca(OH)_2$) or calcium oxide (CaO).Precipitating SA out of solution is a separation process that was first proposed by (Datta, 1992). The major role of adding a calcium ion source is in order to neutralizing the fermentation broth, and to precipitating the succinate as calcium succinate because of its low solubility in water. In this process, after the fermentation reaches completion, solids are centrifuged and separated out of fermentation broth. The obtained calcium succinate reacts with concentrated sulfuric acid, which releases free succinic acid. The acid is further purified by active carbon absorption or ion exchange, and then the product is further concentrated and crystallized by evaporation (Cheng *et al.*, 2012).In addition, fermentation process may have produced by-products as calcium lactate. However, all impurities have to be removed in order to make a high purity succinic acid to be used for the bio-plastics industry.

Beside this, the main disadvantage of this method is during the precipitation process, the dosages of $Ca(OH)_2$,CaO, and H_2SO_4 are very large.The calcium in solution reacts with sulfate to produce solid of calcium sulphate, also known as gypsum, which cannot be used commercially due to odor and color impurities. The removal of this by-product must be required by using other separation methods, such as vacuum distillation. In addition, these additives cannot be regenerated or recycled, which results in high process costs. These are the reasons why precipitation with $Ca(OH)_2$,CaO is unlikely to be applied on an industrial

scale for recovery of bio-succinic acid and is generally not considered viable for large-scale production (Kurzrock *et al.,* 2010).

2.6.7 Extractive fermentation

For the production of SA by fermentation, the primary challenges are low product concentration in the fermentation broth, the presence of organic acid by-products, and the requirement of pH control during fermentation that leads to the SA being presented in the salt form. In addition, product inhibition is an important factor for bioprocess development since SA is very toxic to the bacterial cells (Lin *et al*., 2008). In order to increase fermentation performance, SA must be removed from the reaction site as soon as it is produced. This concept is called *in situ* product removal or extractive fermentation. It combines biochemical reaction and product recovery in a single unit operation. It may also be economical to remove the inhibitory product directly from the ongoing fermentation by extraction, membrane, adsorption, or other methods. An ideal *in situ* recovery method for SA would have minimal chemical addition, require no additional energy inputs beyond normal operation, and would leave the biomass unharmed to continue SA production after reducing end-product inhibition. These advantages will lead to reduction of the size of the trash heap down (when compared to the equivalent acid production), can be made to reduce the production cost both variable costs and fixed costs.

An integrated fermentation system for the production of SA by *A. succinogenes* at high titer, yield and productivity was developed by applying *in situ* product removal (ISPR) strategy. This ISPR process was conducted by a coupled expanded bed using anion-exchange resin NERCB 09 adsorption (EBA) system. *A. succinogenes* CGMCC2650 fermented glucose continuously and effectively with the prolonged cell growth cycle from 48 h to 126 h, and significantly increased SA production up to the final titer of 145.2 g/lwith an average yield of

0.52, and productivity of 1.3 g/l.h. The maximum yield and productivity reached 0.76 and 2.58 g/l.h after the first ISPR operation recycle. Compared with fed-batch fermentation process (producing 73.8 g/lSA in 48 h with an overall yield of 0.57 $g.g^{-1}$ glucose and productivity of 1.54 g/l.h), the ISPR technology not only maintained the overall productivity and yield, but also improved the final SA concentration enormously (Li *et al.,* 2011).

Extractive fermentation of succinic acid for the continuous fermentation process using *A. succiniciproducens* coupled with ED technique was also investigated (Meynial-Salles *etal*., 2008). Two types of ion exchange membranes, anion and cation-exchange membrane, allowed the transferring of the ions under a direct electric field through the cathode and anode, respectively. During the separation, the fermentation broth was adjusted the pH to an optimum condition. Because of at each the different pH values will also affect to solubility to remove and separate of these ions. The dissociation constant of succinic acid has two values at pK_{a1} is 4.2 and pK_{a2} is 5.6 which makes acid succinic dissociation into ions of either $succinate^{-}$ or $succinate^{2-}$.

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

All chemicals used throughout in this study were obtained from Himedia (India), Merck (Germany) and Sigma-Aldrich (Canada), except where otherwise specified.

3.1.1 Fresh cassava roots and hydrolysis enzymes

Fresh cassava roots were obtained from a tapioca company (Nakhon Ratchasima, Thailand). The hydrolysis enzymes including α-amylase (Termamyl®), gluco-amylase (Spirizyme®), and cellulase (Vicozyme®) were obtained from Novozymes (Denmark).

3.1.2 Microorganism

A. succinogenes ATCC 55618 was purchased from the American Type Culture Collection (USA) and maintained in 10% skim milk at -70°C. The growth of strain was conducted by the sub-culturing on the agar plates from the skim milk solution to collect the colonies after incubated at 37°C for 24-48 hours as showed in Figure 3.1. The colonies then grew on the agar plates were used for the further fermentation experiments.

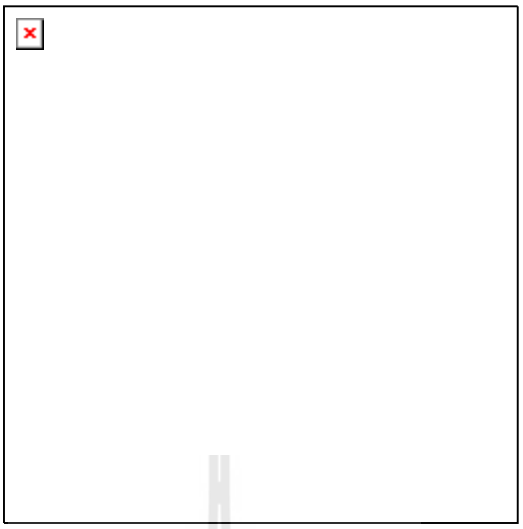


Figure 3.1 *A. succinogenes* ATCC55168 colonies for inoculum preparation.

3.1.3 Electrodialysis bipolar membrane (EDBM) unit

The electrodialysis bipolar membrane (EDBM) DW-Lab unit (Japan) constituted for separation of succinic acid from fermentation broth was shown in Figure. 3.2. The effective surface area of each membrane was 30 cm^2/piece. Platinum and stainless steel was used for anode and cathode, respectively.

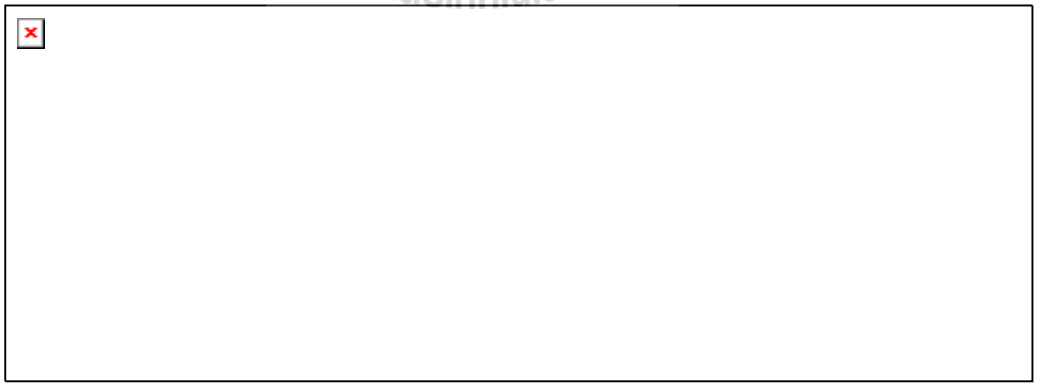


Figure 3.2 Electrodialyer (Selemion^{TM} DW-Lab Specification).

3.1.4 Microfiltration and nanofiltration housing system

The spiral-wound MF (0.1 microns) and NF membrane were made of polyamide and polysulfone, and were purchased from Synder's Filtration, and GE company, respectively. For NF, the molecular weight cut off and effective area was 300 Da and 6.1 m^2, respectively. The two membranes were housed in two stainless steel housing with two separately containers as illustrated in Figure 3.3 andFigure 3.4.

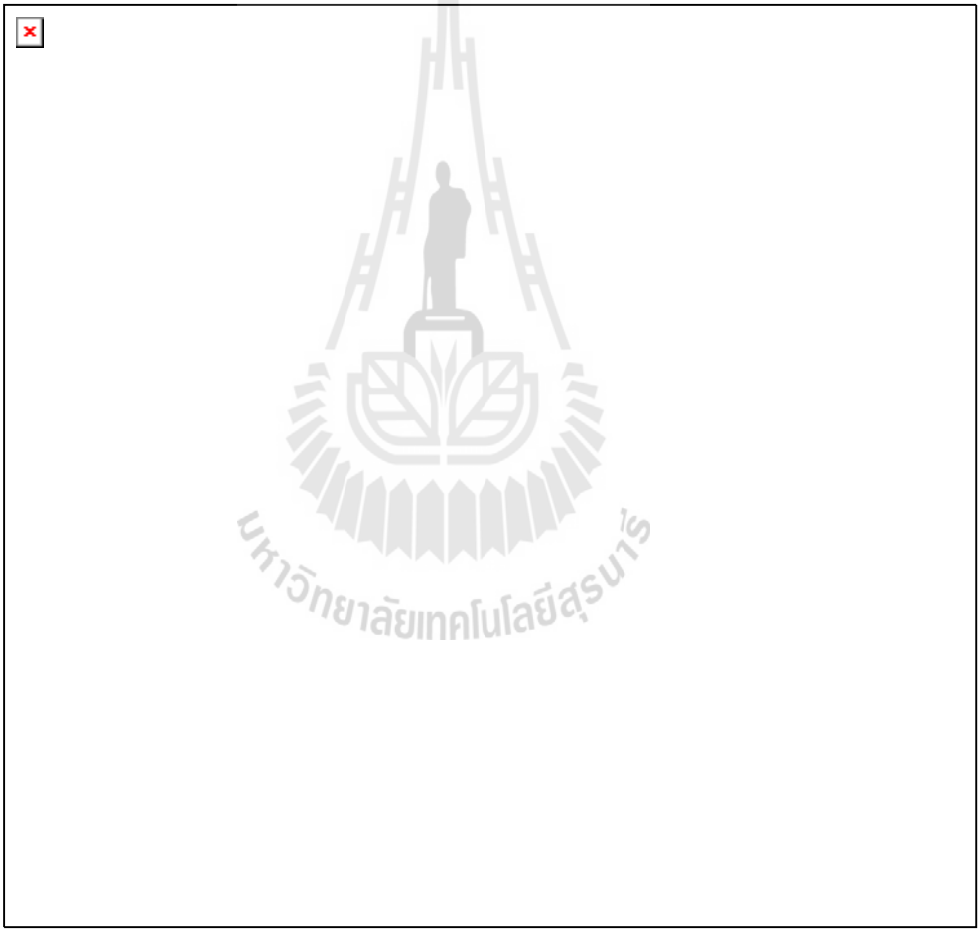


Figure 3.3 The flow diagram of microfiltration and nanofiltration housing system.

Figure 3.4 Experiment of microfiltration and nanofiltration housing system**.**

3.2 Methods

3.2.1 Fresh cassava roots preparation

Fresh cassava roots were washed, cut and crushed using a line system of cutter and grow-mill grinder. During the crushing, DI water was added with the ratio of 1 cassava root mass to 0.5 mass of water. Hydrolysis of cassava roots was performed as follows: 18 g of slurry-milled cassava roots was heated up to 70 °C and simultaneously add 10 ml of cellulase plus with 10 ml of α-amylase enzyme, and incubated at 70 °C for 2 h. Liquefaction was carried out by the addition more of 10 ml of α-amylase enzyme and continue to incubate at 95°C for 2 h. Saccharification was conducted by the addition of 20 ml of gluco-amylase enzyme at 60 °C and remained for 15 h. Only glucose and some other components presented in the enzymatic hydrolysate, and determined by DNS method after got the clear

liquid using a filtration method. This hydrolysate solution was kept in the cold room at 4 °C and used for further experiment. The hydrolyzed cassava roots product was shown in Figure 3.5.

Figure 3.5 Cassava roots solution after hydrolysis.

3.2.2 Succinic acid fermentation process

3.2.2.1 Microorganism and growth condition

From a single colony of *A. succinogenes* ATCC55168 strain was inoculated in 100 mL anaerobic shake flasks with volume of 20 mL, and were incubated at 200 rpm, 37°C for 12 hours using a rotary incubator (New Brunswick Scientific, USA). Subsequently, it was filled into 200 mL in 500 mL anaerobic shake flasks. These flasks were firstly flushed all of oxygen by nitrogen gas followed by adding CO_2 gas and then closed with the butyl rubber stoppers and aluminum caps. The composition of this pre-culture media (TSB media) as followed (per litter): 17.0 g tryptone. 3.0 g soya peptone, 2.5 g dextrose, 5.0 g NaCl and 2.5 g K_2HPO_4. The pH of medium was adjusted to 6.8 by 2 M NaOH solution prior to

sterilization. This 500 mL anaerobic shake flask was also incubated in a rotary incubator, and then was used as an inoculum for the fermentation process.

3.2.2.2 Batch and fed-batch succinic acid fermentation process

The fermentation process was carried out in a 5.0 L fermenter (Sartorius Stedim, Biostat Bplus) with the working volume of 4.0 L. The fermentation process was automatically controlled by the pH and temperature probes. After 12 hours, inoculum were transferred to the medium for the fermentation process with ratio of 10% (v/v) of the fermentation-working volume. The fermentation medium composition consisted of (per liter): 150 g glucose (batch) 70 g glucose (fed-batch), 15 g yeast extract, 3.0 g KH_2PO_4, 1.5 g K_2HPO_4, 3.0 g $(NH_4)_2PO_4$, 1.0 g NaCl, 0.3 g $MnCl_2.6H_2O$, 0.3 g $CaCl_2.2H_2O$, 0.07 g $MnCl_2$, and 0.5 g anti-foam agent. This yeast extract content was replaced by 9 g/L of diammonium phosphate (DAP) for the case of SA fermentation from cassava roots. For fed-batch, the glucose concentration was maintained in the range of 30-40 g/L. Fermentation medium from cassava roots was prepared by the clear liquid of cassava roots hydrolysate was used as the replaced glucose source. After sterilization and before inoculation, the optimum condition was surely controlled such as the fermentation medium anaerobic condition was calibration by oxygen and nitrogen gas followed by carbon dioxide, combined with the automatically adjusting of pH at 6.8 prior to inoculate by the addition of 60 wt% $MgCO_3$ solution. Anaerobiosis was rapidly achieved during the growth to ensure an atmosphere of CO_2. During fermentation process, carbon dioxide was also continuously sparged at 0.1 vvm and pH was maintained at 6.8 by the automatically addition of $MgCO_3$, respectively. The agitation speed was fixed at 200 rpm. Figure 3.6 showed the experimental set up for a fermentation system.

Figure 3.6 Fermentation process of succinic acid production.

3.2.3 Succinic acid purification process

3.2.3.1 Purification of succinic acid from fermentation broth by using electrodialysis bipolar membrane (EDBM) technique

The system for separation of SA fermentation broth by electrodialysis coupled with crystallization comprises of a bioreactor, an EDBM unit and a crystallization system. As experimental design diagram shown in Figure 3.7, the clarified fermentation broth was withdrawn from the bioreactor by using a submerged microfiltration membrane unit causing the bacterial cells to be resided in the bioreactor. This clarified fermentation broth was subsequently introduced into the EDBM unit and re-circulated in feed-dilute compartments inside the EDBM cell by a peristatic pump. Na_2SO_4 2M solution was used as an electrolyte solution, which was circulated along anode and cathode channel during

operation process. A rectifier was constructed in-house to produce direct electric current. It could supply voltage and current density in the range of 0–100 V and 0–1.2 A/cm^2, respectively. The liquid flow rate in the stream was 1.6 L/min.

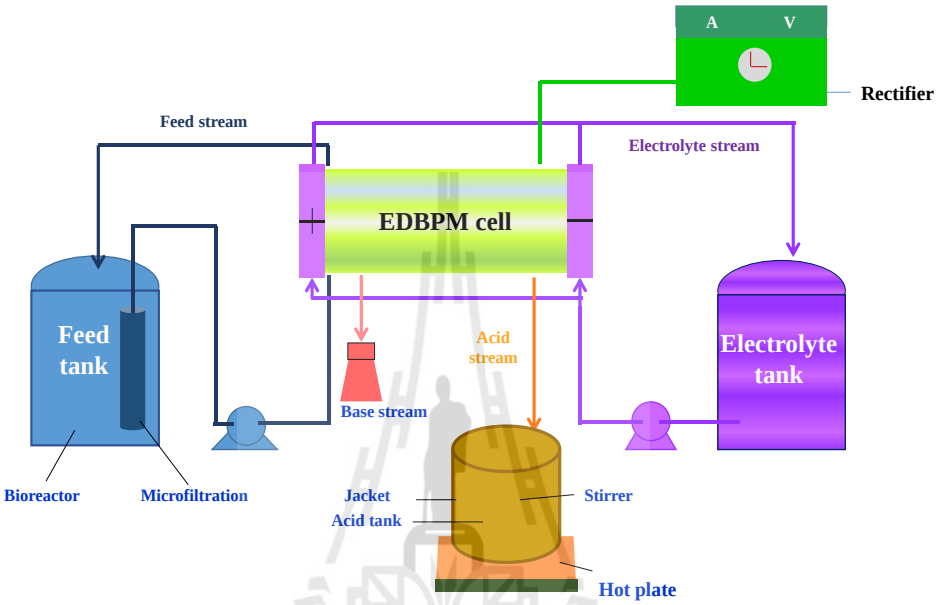


Figure 3.7 The schematic diagram of the membrane bioreactor set up for removal of succinic acid fermentation broth using the EDBM couple with crystallization technique.

3.2.3.2 Purification of succinic acid from fermentation broth by using microfiltration

A large volume of collected fermentation broth was preliminary treated by a filtration bag under a high vacuum pump to remove all of the hardness of magnesium carbonate. The fermentation broth was then fed to in the MF membrane housing to separate the cell debris out. This work also focused on the modeling for clarifying fermentation broth during cross-flow MF via the series model of membrane resistance,

bacteria cell cake resistance, pore-blocking and adsorption resistance to determine the membrane fouling. During the filtration time, the permeate flux (J) is decreased and lower than that obtained from the solvent. In order to understand of the roles of aqueous components in membrane fouling have the different mathematic models have been developed to describe the membrane fouling operation and the most useful tool is the resistance in series model. The permeate flux is thus expressed by:

$$J = \frac{TMP}{\mu . R_t} = \frac{TMP}{\mu(R_m + R_c + R_f)} \quad (1)$$

Where, TMP (ΔP) is the transmembrane pressure, μ is the permeate viscosity, R_m is the hydraulic membrane resistance that obtained when only DI water was filtered. R_c is the cake resistance that caused by the bacteria cells present in the fermentation broth and deposited on the membrane surface. The following expression was expressed:

$$R_c = \frac{m}{A}\alpha_0 = \frac{m}{A}\alpha . \Delta P^n \quad (2)$$

Where, m is the mass of bacteria cake deposited on the membrane surface, A is the membrane area and α_0 is cake specific resistance. This specific resistance is also depended on the transmembrane pressure (ΔP) via a cake resistance coefficient (α) and compressibility index of cake (n). This index was determined by a separate dead-end filtration experiment. In addition of those parameters, the mass m of bacteria cake can be calculated by the integration of mass balance as follow:

$$\frac{d_m}{d_t} = \left(JC - \frac{D}{\delta}(C_m - C)\right)A \quad (3)$$

Where, D is the shear induced diffusion coefficient, is proportional to the wall shear rate and δ is the cake thickness. C and C_m are the bacteria concentration in the retentate and at the membrane wall, respectively.

And R_f is the pore blocking and adsorption resistance that caused by the adsorption and fix of the matters onto the membrane pores. During MF, this resistance will be tend towards a constant steady state, $R_{f.ss}$, corresponding to the adsorption equilibrium as following (Carrère *et al*, 2002):

$$R_f = R_{f.ss}\left(1 - exp(-bt)\right) \quad (4)$$

Besides that, since the permeate flux is known and assuming the completed cell retention, the variation profile in the retentate bacteria cell concentration can be estimated:

$$C = \frac{C_0 V_0}{V_0 - A\int_0^t J\,dt} \quad (5)$$

3.2.3.3 Purification of succinic acid from fermentation broth by using nanofiltration

The MF permeate was continuously collected in NF container. Before starting the NF, the MF permeate was acidified to pH 3.0 by H_2SO_4. The SA separation ability from fermentation broth was evaluated by two different modes that were concentration mode and diananofiltration (DNF) mode in order to recover the filtrated-product. For the concentration mode, the permeate NF was continuously removed to determine flux, rejection and especially for the protein and multivalent ions removal efficiency. When the flux very low, the DNF mode was started and monitored until the remaining succinic acid concentration in the retentate stream was lower than 2.0 g/L. The feed volume was kept

constant by an addition of DI water with pH 3.0 to make up the volume of permeate. The NF process was performed at a constant pressure of 750000 Pa that was tested by deionized (DI) water before at each different pressure in the range between 2.5-10.0MPa at temperature of 20°C and pH 3.0. The separation performance was examined in term of flux, rejection and resistance. The flux of permeate solution was periodically volumetrically measured and the rejection (R%) was calculated as:

$$R(\%) = \left[1 - \left(\frac{C_P}{C_R}\right)\right] \times 100 \qquad 6)$$

Where, C_P and C_R represents the concentration of the components in permeate and retentate stream that were collected after NF process, respectively. In addition, the membrane filtration resistance caused by the different mechanisms was estimated via the fouling behavior by the equation based on the Darcy's law (Ahmed *et al.,* 2007), and the membrane fouling behavior was evaluated by a cleaning procedure by using DI water, NaOH 2% and H_3PO_4 2%. The equation used is:

$$R_{NF} = R_m + R_f + R_C = 3600 \times \frac{TMP}{\mu J} \qquad 7)$$

Where, R_{NF} refers to the filtration resistance (m^{-1}), R_m is membrane hydraulic resistance, R_f is the resistance due to pore blocking and adsorption, R_c is the resistance causes by the cake formation, Jis the permeate flux ($m^3/m^2.h$), TMP is the trans-membrane pressure (Pa), and μ is the viscosity of the permeate (Pa.s), respectively.

Therewith, during NF process, the volume changing profile or volume concentartion ratio (VCR) was also monitored and calcultated as the ratio of initial volume V_0 divided by the retentate volume V_R at a certain time:

For the removal rate of the undesired compounds were calculated as follows:

Where, D: Removal rate (%)

M_F: mass of the compound in the feed phase (g)

M_P: mass of the compound in the permeate phase (g)

The NF membrane separation performance was also confirmed by the re-investigation via the comparison with model solution, which prepared from collected SA-experimental product.

For further purification, the NF permeate was concentrated by using a 100 L reactor-evaporator under the vacuum effect until the brix of solution reached approximately 21. This concentrate SA solution was subsequently used in crystallization experiments.

3.2.3.4 Dead-end filtration experiment

Dead-end filtration experiments were carried out with a Sartorius Milipore laboratory test cell. The membrane was a screen-type filter, and was made of cellulose acetate with a nominal pore size of 0.2 µm, diameter 4 cm (Sartorius Stedim, Germany). Experiments were conducted at each different constant pressure by the setting with compressed nitrogen gas as was illustrated in Figure 3.8. A sample of about 40 ml with

the cell concentration of 1.1 g/L was filtered. Permeate flux was measured a volume cylinder.

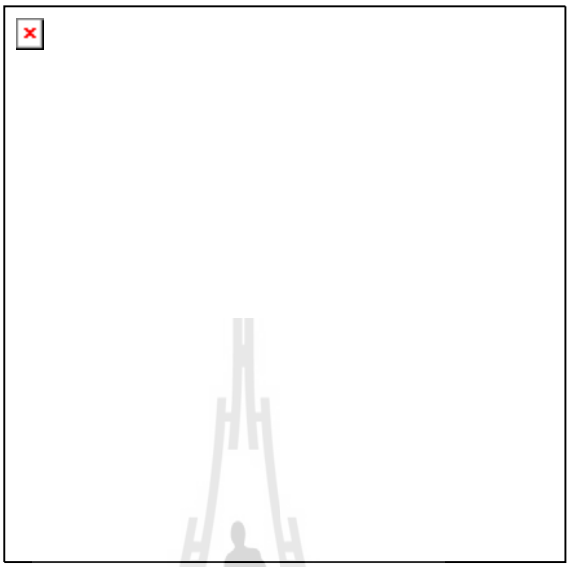


Figure 3.8 Dead-end filtration experiment.

3.2.3.5 Purification of succinic acid from fermentation broth by using crystallization

3.2.3.5.1 Solution layer crystallization-Solution layer crystallization experiments was carried out in a layer crystallizer with a cooling finger as the cooled surface (Beierling *et al*., 2013) as shown in Figure 3.9. The crystallizer is a jacketed-cylindrical glass with an inner diameter of 8 cm, 20 cm height. The cooling finger, made of stainless steel tube with a cylindrical-tube shape, was dipped inside the SA aqueous solution. The temperature of the solution was controlled using a water bath (Julabo, Germany). The cooling water was pumped through inner side of the cooling finger tube by using a water bath with various temperature. The thickness of the crystal line layer was observed online via a camera and image analysis software was utilized to measure the crystal layer thickness. Mass balance was used to estimate the weight of crystal formed by subtracting the remaining succinic acid concentration in solution using a HPLC.

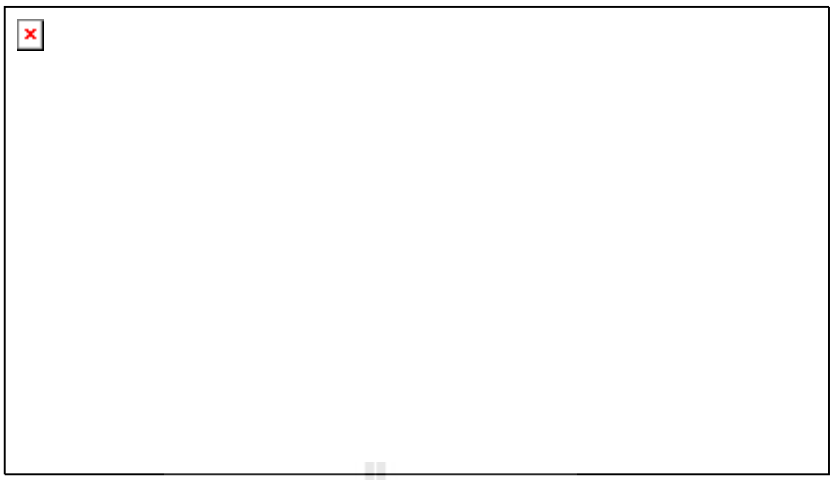


Figure 3.9 Solution layer crystallization experimental diagram.

3.2.3.5.2 Conventional crystallization-After fermentation, the cells in fermentation broth were separated by centrifugation for 20 min at 8000 rpm, and then acidified to pH 3.0 by the addition of 35% (v/v) H_2SO_4. The acidification rate was 1 pH per min. Then, the free acids-released fermentation broth was filtrated one more time to remove all of the residual cell or solid compounds. Then, the clear filtrated acid formed aqueous was evaporated at 80^{o}C to around 21 brix and ultimately cooled to 4^{o}C in the cold room for 25 hours. The SA was crystallized into the crystals, and collected then by washing with cold water. Similary, this experiment was also tested by the SA fermentation broth that was treated by NF membrane (NF permeate).

3.2.3.5.3 Seed loading crystallization-Seed loading crystallization experiments were also performed in a 500 mL jacketed glass vessel with the working volume of 250 ml for each case. The experimental setup is shown in Figure 3.10. The temperature of jacket was controlled with a thermometer using a cooling bath and hot bath. A stirrer with a two-bladed marine type impeller operated at 400 rpm was used to agitate the system. Three series of experiments were conducted in order to characterize the dynamics and the steady

state process by applying seed loading. The seeds with size approximately 650 µm to 800 µm diameter,with seed loads of un-seeding (0%), low seeding (5%), and high seeding (10%) of the final mass at 50°C. Firstly, the obtained material after evaporation process was fully dissolved in the crystallization vessel at 70°C, and then started to cool down to 30°C using linear cooling profile with the rate of 2.5°C/h. Then, the cooling rate was increased faster from 30°C to 4°C just within 1 h, and maintained at 4°C for 4h. The formed SA crystals were washing by using cold water to remove the possible impurities, and then dried at 40°C for 12h.

3.2.3.5.4 Seed preparation-Seed crystal of different sizes were prepared using a standard sieve analysis. The sieve sizes used were 1000, 800, 650, 400, 270, 180 and 100 µm. The sieving time was set to 30 min, the rotation and shaking caused the crystals to distribute throughout the sieve stack pores. The product obtained on the sieve size of 650 µm was collected for seeding (Aamir *et al*., 2010, Gerberding *et al*., 2012).

Figure 3.10 Experiment set up diagram for crystallization operation.

3.3 Analytical methods

3.3.1 Hydrolyzed cassava roots analysis

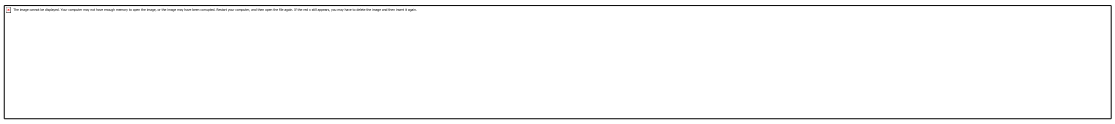
3.3.1.1 Starch content

Starch concentration of the samples was determined using a modified method of (Thang *et al*., 2010). A portion of 20 µL α-amylase was added to 1 mL of culture medium and the mixture was incubated at 90°C for 3 h to hydrolyze starch to soluble dextrin. Then, 8,880 µL of 0.1 M acetate buffer at pH 4.5 and 100 µL of gluco-amylase were added to the solution and the mixture was further incubated at 58°C for 4 h. The solution was allowed to cool down to room temperature and then transferred to a 10 mL volumetric flask followed by filling it with distilled water to the volume. Glucose concentration of this solution was determined using the DNS method. Starch concentration in fermentation broth was calculated as follows:

Where a is dilution factor, and b is correction factor for glucose to starch. The calculation of starch concentration in accordance with glucose concentration is shown in Appendix C.

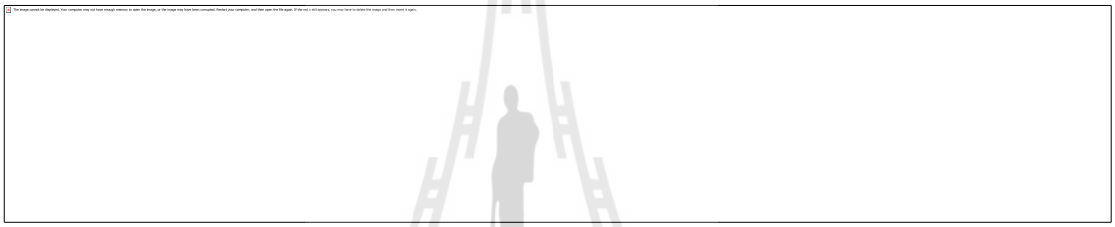
3.3.1.2 Moisture content

The fresh cassava roots sample was dried to a constant weight and moisture content calculated as percentage using the AOAC (1990) method:



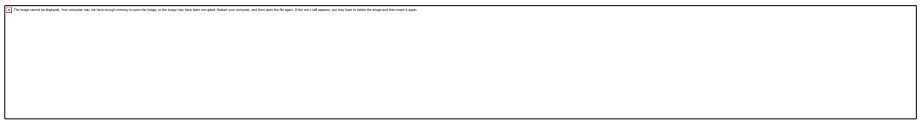
3.3.1.3 Crude protein content

Crude protein content was estimated by titration method in which the value of nitrogen content was determined as:



3.3.1.4 Crude fat content

The fats are extracted from the sample with petroleum ether. The crude fat content was then calculated AOAC (1990), and expressed as percentage of the weight before the solvent is evaporated:



3.3.1.5 Ash content

Ash is considered as the total mineral or inorganic content of the sample. Total ash content was analyzed by the placing 2 g of dried cassava root sample in a crucible previously calcined, and brought to constant weight. That crucible containing sample was then placed in a furnace and heat at 550°C for 12h, leave to cool and transfer to a dryer. The ash content was the calculated AOAC (1990) and expressed as percentage:

Crude fiber content: The crude fiber (%) were estimated with the formula:

3.3.1.6 Nitrogen-free extract

This nitrogen free extract includes all the nutrients not assessed by the prior methods of proximate analysis. There are composed mainly of digestible carbohydrate, vitamins, and other non-nitrogen soluble organic compounds. This content was evaluated by the subtracting the percentages calculated for each nutrient from 100 and expressed as follows:

3.3.1.7 Total sugar

Total sugars were also determined by AOAC method. 10 g of dried cassava root sample was dissolved in 100 mL of distilled water. 10 ml of concentrated HCl was added to the solution and heated in a water bath for 10 minutes. The solution was then neutralized with as base preferably NaOH. The solution was made up to 200 or 300 mL with distilled water and filtered. 10 or 25 mL of mixed Fehling's solution was pipetted into a flask followed by the addition of 15 mL of the solution from the burette. The solution was heated

and on boiling three drops of methylene blue was added. Further quantities of the solution were added from the burette (1 mL at a time) at 10-15 seconds interval to the boiling liquid until the indicator is completely decolourised. The titre values obtained corresponding to mg of invert sugar per 100 ml.

3.3.2 Fermentation broth analysis

3.3.2.1 Organic acids analysis

Succinic acid and organic acids were determined by high performance liquid chromatography (HPLC) (Thermo Scientific, USA). 1.0 mL of the fermentation broth sample was centrifuged at 10×10^3 rpm, 3 min to separate the cell and insoluble $MgCO_3$. The supernatant was adjusted to pH 2 by using H_2SO_4 and then filtered through a 0.2 µm filter analyzed by HPLC using the ZORBEX SB-Aq (4.6 × 150 mm) column. The mobile phase was 1.0% of acetonitrile and 99% of 20.0 mM Na_2HPO_4 (pH 2) and the flow rate was 1.0 mL/min. The detection was UV detector fixed at 210 nm wavelength.

3.3.2.2 Glucose consumption

The amount of glucose consumption will be analyzed by the DNS method. One mL of diluted medium was mixed with 4 mL of DNS solution (10 g of 3, 5-dinitrosalicylic acid, 300 g of $KNaC_4H_4O_6$ in 200 mL of 2N NaOH, and adjusted to 1 L with RO water). The mixture was mixed thoroughly, and the development of color was conducted by boiling the reaction tube for 10 min. This sample was then be measured by a spectrophotometer at wavelength of 540 nm (OD_{540}).

3.3.2.3 Cell concentration

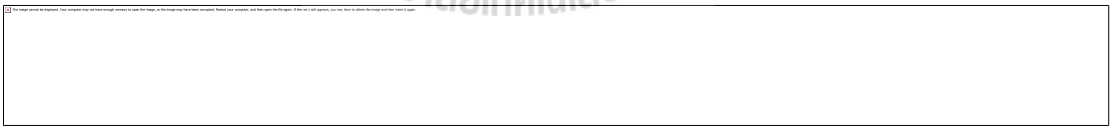
Bacterial growth was determined by optical density (OD) measurements at 660 nm. In order to ensure that none of the $MgCO_3$ remained undissolved, 0.1 mL of the sample was diluted with 21 times by 2M HCl to fully dissolve insoluble $MgCO_3$. The sample was homogenized by vortex mixer at least 20 s before transferring the contentment into a 4.0 mL corvette for optical density measurement at a wavelength of 660 nm.

3.3.2.4 Protein concentration

Protein concentration was analyzed by Bradford method. 100 µl sample solution was mixed well with 5 mL Bradford reagent, and then measured by a spectrophotometer at wavelength of 595 nm (OD_{595}).

3.3.2.5 Succinic acid yield

3.3.2.6 Succinic acid productivity



3.3.3 Electrodialysis analysis

Removal ratio of glucose (D_G) and soluble proteins (D_P) are defined as flows:

Where, M_T and M_W are the mass of glucose or protein in the top phase or still remaining in the obtained crystals (g) and total mass of glucose or protein, respectively.

Recovery rate: The recovery rate (R) of succinic acid was defined by the following equation: (Wang *et al*., 2006).

Where, R was the recovery rate of succinic acid, W_F and W_I were the final and initial amount (unit in gram, g).

Conductivity: Conductivity of the matters before and after treat by ED is measured by an ultra-meter device.

3.3.4 Microfiltration and nanofiltration analysis

3.3.4.1 Permeate flux

3.3.4.2 Organic acids rejection

3.3.5 Crystallization analysis

3.3.5.1 Succinic acid crystals colour

The color of crystalline SA was determined by measuring the optical density of filtered 100 g/L solution of the final product at 420 nm wavelength in a spectrophotometer (Thermo Scientific, GENESYS 10S UV-Vis) (Gerberding *et al.,* 2012).

3.3.5.2 Particle size distribution

The particle size distribution of SA crystals (PSD) was measured by laser scattering via particle size distribution analyzer LA-950V2.

3.3.5.3 Crystal morphology

The crystal morphology characteristic was examined by a Stereoscopic Light Microscope Olympus szx7 with an Olympus SC100 camera directly attached at 1.25 × magnification.

3.3.5.4 Crystalline characterization

The characterization of crystalline products were carried out by a D2 phaser X-ray diffractometer (XRD) from Bruker AXS GmbH, Germanywith CuK_{α} diffraction (λ= 1.5406 ^{o}A) operating at 30 kV and 10 mA with a graphite monochromator in the diffracted beam path. The data were collected in the range of 10-60^{0} 2θ by the step time and step size were selected of 0.2s and 0.02^{0}, respectively. The relative crystallinity of SA crystal products was estimated based on the X-ray diffraction data of the well – defined peak by three most intense peaks at specific 2θ value of 20.0^{o}, 25.5^{o} and 31.5^{o}, respectively. The sample with highest crystallinity was used as the reference to calculate the relative crystallinity as follow: (%) relative crystallinity = (total area of crystalline peaks) / (total area of crystalline peaks for the highest crystallinity sample) (Emrani *et al*., 2011). In addition, the diffracted intensity algorithm, DIFFaX, was used for calculation of phase purity by succinic acid to magnesium sulfate ratio.

3.3.5.5 SEM observing

Scanning electron microscopy (SEM, JSM – 6010LV) was employed to confirm the size and morphology of the crystalline products and also use to check the presence of imperfections (Emrani *et al*., 2011).

CHAPTER IV

RESULTS AND DISCUSSIONS

4.1 Cassava roots preparation

Cassava roots is one of the cheapest carbon source readily available in Thailand. Table 4.1 shows the composition of fresh cassava roots used in this study. Starch was the main component in the dried cassava roots, accounting for 75.4 % (w/w) on dry basic. A previous work also reported that fresh cassava sample generally contained about 77.9 % (w/w on dry basic) starch that was also considered as the main component(Collares *et al*., 2012). This starch component was formed by the connection of the amylose and amylopectin lines, which consist of a large number of the glucose units joined by α, 1-4 and α, 1-6 glycosidic bonds, respectively. Thus, these glucose units were liberated by the hydrolysis using the enzymatic method. The reducing sugar present in hydrolyzed cassava was obtained up to 290 g/L of 12 L volume corresponding to 27% reducing sugar obtained from 1 kg of fresh cassava roots (data not shown). Therefore, fresh cassava root is one of the high potential substrates for low cost bio-based SA fermentation. In addition, the protein content presented in the cassava roots was measuredat 1.54% which was higher than the other parts of cassava, such as 0.8% w/w in cassava starch on wet basic (Olufunmilola *et al*., 2014),1 % w/w in cassava tuber on wet weight (Fakir *et al*., 2012), and 1.77 % w/w in cassava pulp on dry basis (Sawisit *et al.,* 2015). This can also imply that selection of cassava roots was suitable as a substrate for succinic acid production.

Table 4.1 Compositions of whole cassava roots.

Main component	% w/w on wet basic	% w/w on dry basic
Dry matter	35.62	100
Crude protein	1.54	6.8
Total sugar	2.91	8.8
Moisture	65.11	0
Crude fiber	1.14	4.1
Crude lipid	0.09	0.2
Ash	1.05	2.2
Nitrogen free extracts	33.67	86.7
Starch	29.04	75.4

4.2 Succinic acid fermentation process by using *A. succinogenes* ATCC55168

4.2.1 Batch succinic acid fermentation

The utilization of fermentable sugar in medium containing the synthetic glucose, and the cassava roots hydrolysates were evaluated. The results revealed that *A. succinogenes* ATCC55168 strain was able to considerably utilize sugar contained in an enzymatic hydrolysate under the anaerobic condition (Figure 4.1). The SA concentration obtained from the glucose containing medium, and the enzymatic cassava roots hydrolysate medium were obtained at 73.00 g/L and 93.34 g/L with yield of 0.6 and 0.77 $g_{SA}/g_{glucose}$, respectively. The SA productivities obtained from each batch were 4.22, and 6.36 g/L/h. The

above results showed that cassava roots hydrolysate have potential to produce SA with high titter, yield and productivity compared with the synthetic medium containing glucose. The results obtained in this experiment were comparable to those previously research results when used only glucose as a sole carbon source to produce SA(Jantama *et al.,* 2008). The SA production from waste bread by *A. succinogenes* using batch separate hydrolysis fermentation was conducted (Leung *et al*., 2012). The SA concentration of 47.3 g/L with yield of 55 g_{SA}/100 g waste bread were obtained. Similarly,a recent research direction on the different cellulosic substrate source for SA production from corn stover by simultaneously saccharification and fermentation using *A. succinogenes* was also reported. The SA concentration of 47.4 g/L with the yield of 72 g_{SA}/100 $g_{corn\ stover}$ were attained(Zheng *et al.*, 2010). Another work was a study of the SA production from corn stalk hydrolysate product by *E. coli* SD121(Wang *et al*., 2011). The SA concentration of 57 g/L with a yield of 87 g_{SA}/100 $g_{corn\ stalk}$ were reported. The experimental results obtained in this work were able to continue toward the SA production from the cheap cost and high potential material, such as cassava roots used in this study, especially without the need for the supply of expensive yeast extract component in the fermentation medium. Finally, a synthetic medium by glucose as a mainly carbon source and yeast extract as a nitrogen source for SA fermentation was attempted. However, the results of SA concentration production just also in the range of 36.4; 41.7 and 48.4 g/L, corresponding to the ratio of glucose mass (g) and yeast extract mass (g) at 90:4; 85:7 and 80:10, respectively (Zhu *et al*., 2012). This indicated that the SA production decreased with decreasing in the nitrogen source of yeast extract. The nitrogen source, thus, played an important role for the microorganism growth and product formation. Besides that, many other factors also decided to the SA production performance. One of those factors that can't fail to mention that is neutralization reagent. The result in this experiment was obtained maybe due to the efficiency of utilization of $MgCO_3$ as a neutralization reagent source plus CO_2 sparging which simultaneously ensured the anaerobic condition. Because of

the CO_2 can be directly permeated onto the cell membranes, and can be used as an important substrate source for the carboxylation of phosphoenolpyruvate to oxaloacetate, which is converted to SA by the reductive tricarboxylic acid cycle, and menaquinone systems. This characteristic allows high efficient production of SA, an important four-carbon industrial chemical. Or in other hand, CO_2 is not only a necessary substrate for SA metabolism from glucose, but also a good effect for cell growth (Hong *et al*., 2004, Mariet *et al*., 1997, Xi *et al.*, 2011). Especially for the indirectly CO_2 source from the carbonate supplementation as same as the dissolved CO_2 in the culture medium. $MgCO_3$ was considered as the best neutralization reagent for enhancing SA production of *A. succinogenes*(Zou *et al*., 2011). Moreover, $MgCO_3$ still prevented flocculation, prolong the steady phase and improved SA fermentation that others such as NaOH or Na_2CO_3 impossible (Xi *et al*., 2011). In addition, different neutralization reagents were tested for the SA production from glucose as a carbon source using *A. succinogenes*. The result showed that $MgCO_3$ could act as one of the best neutralizers which help to produce SA at high titers and yield. Simultaneously, it can be found CO_2 as useful to increase the titers and yield of product (Li *et al.,* 2010).Thus, an optimized fermentation of *A. succinogenes* could be carried out with $MgCO_3$ as the pH buffer, and continuous CO_2 sparging.

Figure 4.1 Time profile of metabolites production, cell growth, and glucose consumption during batch SA fermentation using *A. succinogenes* ATCC 55618 from cassava roots (A) and synthetic glucose (B) and comparison of fermentation profiles at the end of fermentation (50 h) obtained from enzymatic hydrolysis of cassava roots and synthetic glucose (C).

4.2.2 Fed-batch succinic acid fermentation

The above results showed that substrate (glucose) inhibition was a clear limitation to the fermentation, and the maximum SA production was not more than95 g/L with *A. succinogenes* in batch fermentation. As was demonstrated in a fed-batch polysaccharide and ganoderic acid fermentation by using *Ganoderma lucidum*, the purpose of sugar feeding strategy is to prevent the cell growth limitation and inhibition by the substrate-glucose (Liu *et al*., 2008a). Therefore, in this study, the substrate fed-batch fermentation was investigated to enhance the SA production performance. This fed-batch fermentation was performed both synthetic glucose and cassava roots hydrolysate material aimed to evaluate the efficiency of the different substrate sources. The result in Figure 4.2 showed that, during the first 12 h, the glucose content was utilized very fast from the initial concentration of 70 g/L to approximately 30 g/L. This corresponded toa rapidly growth of cell in comparisonto the batch run. A highly concentrated glucose solution (700 g/L) was intermittently fed into the stirred bioreactor using the peristatic pump during 12-24 h to maintain glucose concentration within the range of 30-40 g/L. Also in this stage just after 15-19 h fermented, the cell concentrations considerably increased, and achieved maximum value of 3.99 and 4.24 g/L for fermentation from synthetic glucose and cassava roots hydrolysate, respectively. The SA concentration reached the maximum value of 151.44 and 100.54 g/L for the fermentation from cassava roots hydrolysate, and synthetic glucose material, respectively. Reversely, other organic acid by-products just slightly increased, especially, lactic acid and formic acid which almost disappeared after the first glucose feeding. A low amount of pyruvic acid was also observed. Productivities were obtained in the fermentation from the synthetic glucose and

cassava roots hydrolysate at 9.67 and 6.1 g/L/h, respectively. SA production in fed-batch fermentation increased 27.39% and 38.37% for the substrates of synthetic glucose and cassava roots hydrolysate compared to the highest SA production in batch fermentation strategy. Thus, the objective of this study strategy for fed-batch fermentation was to enhance the SA production at high titers and productivity. More especially, this experiment result also showed that the SA production using cassava roots hydrolysate was more efficient than that of glucose without yeast extract supplementation. The SA titers and productivitywent up to 150 g/L and 3.2 g/L/h, which was higher than that of glucose 33.61% and 36.92%, respectively. This could be explained that cassava roots hydrolysate product contained some important components of vitamins and minerals which contributed to the cell growth.

The obtained results in this experiment were comparable with the previously reported researchers as presented in Table 4.2. It was illustrated that fed-batch fermentation prevented the limitation and inhibition by the high substrate concentration on the cell membrane osmotic mechanism(Liu *et al*., 2008a). That resulted in SA production of 60.2 g/L resulting in a 32.72% increase in comparison with the highest SA production in batch production at 40.5 g/L. Another work reported about glycerol as a carbon source for SA production in fed-batch fermentation using *A. succiniciproducens* which led to a 2.5-fold increased SA concentration compared with batch run(Bretz *et al*., 2015). SA production in fed-batch fermentation of the *M. succiniciproducens* LPK7 strain was performed to achieve higher SA concentration and productivity than batch fermentation(Lee *et al*., 2006). For fed-batch, the SA concentration and productivity were 52.43 g/L, and 1.8 g/L/h, respectively. For batch fermentation,the values were obtained at just

13.4 g/L, and 0.46 g/L/h, respectively. In summary, fed-batch SA fermentation was developed to enhance high SA product titer, which advantages for the sequentially purification in downstream processing.

Figure 4.2 Time profile of metabolites production, cell growth, and glucose consumption during fed-batch SA fermentation using *A. succinogenes* ATCC 55618 from cassava roots (A) and synthetic glucose (B) and comparison of fermentation profiles at the end of fermentation (50 h) obtained from enzymatic hydrolysis of cassava roots and synthetic glucose (C).

Table 4.2 Comparison of SA production in batch and fed-batch fermentation with other previously published works.

Substrate	Microorganism	Fermentation strategy	Succinic acid production			Reference
			Titers (g/L)	Yield[1] (g/g)	Productivity[2] (g/L/h)	
Glucose	*A. succinogenes*	Batch	38.7	0.82	1.0	(Liu *et al*., 2008)
		Fed-batch	60.20	0.75	1.30	
Glucose	*M. succiniproducens*	Batch	13.40	0.64	1.22	(Lee *et al*., 2006)
		Fed-batch	52.40	0.76	1.80	
Glucose	*A. succinogenes*	Batch	73.00	0.60	1.46	This study
		Fed-batch	100.54	1.00	1.70	
Rapeseed meal	*A. succinogenes*	Batch	15.50	0.12	0.22	(Chen *et al*., 2011)
		Fed-batch	23.40	0.12	0.33	
Raw carob pods	*A. succinogenes*	Batch	9.41	0.54	1.32	(Carvalho *et al*., 2016)
		Fed-batch	18.97	0.94	1.43	
Corn straw	*A. succinogenes*	Batch	45.50	0.81	0.95	(Zheng *et al*., 2009)
		Fed-batch	53.20	0.83	1.21	
Cassava roots	*A. succinogenes*	Batch	93.34	0.77	1.87	This study
		Fed-batch	151.44	1.51	3.22	

[1]The SA yield was calculated as obtained SA concentration divided by utilizing substrate concentration during fermentation.

[2]The SA productivity was calculated as SA obtained concentration divided by overall fermentation time.

4.2.3 Succinic acid fermentation from cassava roots as a sole substrate source

From the above results of both batch and fed-batch fermentation, it showed that use of cassava roots as a carbon source without the need for yeast extract supplementation brought the remarkable result. The result showed higher SA titers, yield, and productivity than that of synthetic glucose as a carbon source. Therefore, this inherently potential of cassava roots as the sole substrate was further examined for SA production in fed-batch process. The fermentable medium was prepared by using only cassava roots hydrolysate product,and simultaneously controlled the pH by $MgCO_3$. The experiment result was presented in Figure 4.3, it showed that the utilizationof glucose was a bit slower than that of fed-batch SA fermentation which used synthetic glucose, and cassava roots as a carbon source supplemented with some other nutrient components. However, the cell concentration achieved in this experiment wasrelatively high corresponding to 3.57 g/L which was higher than that of both batchSA fermentations from glucose and cassava roots. This resulted in the SA titerswas also high with the concentration of 78.33 g/L. The yield and productivity were 0.78 $g_{SA}/g_{glucose}$ and 4.17 g/L/h which were higher than that of result obtained in batch SA fermentation using glucose. The consequence of this experiment revealed that cassava roots possess a valuable potential for SA production without the need for auxiliary components in fermentation medium preparation. In addition, the fresh cassava root is a cheap material resulting in having a great promise in low cost and bio-based economy for SA production. This could be explained that due to some nutrient components presences in cassava roots as already above mentioned. Especially, the protein, vitamin and minerals were found in larger quantities in the cassava root peel than in the peeled cassava root (Julie *et al*., 2009). This is a different point compared with the previously researches who used just cassava starch, or cassava pulp as the substrates. According to FAO/WHO 2001, cassava roots peels occupied a large amount of the minerals and vitamins. The crude

protein gone up to average of 4.9%, which higher than tuber content of 2.3%. In addition, raw cassava roots contain significant vitamin C of 20.6 mg/100 g fresh cassava root (Montagnac *et al*., 2009). Besides that, cassava roots meal still presents some mineral components such as nitrogen (0.73%), phosphorous (0.58%), calcium (0.15%), magnesium (0.12%), postassium (0.82%) (Abu *et al*., 2010).

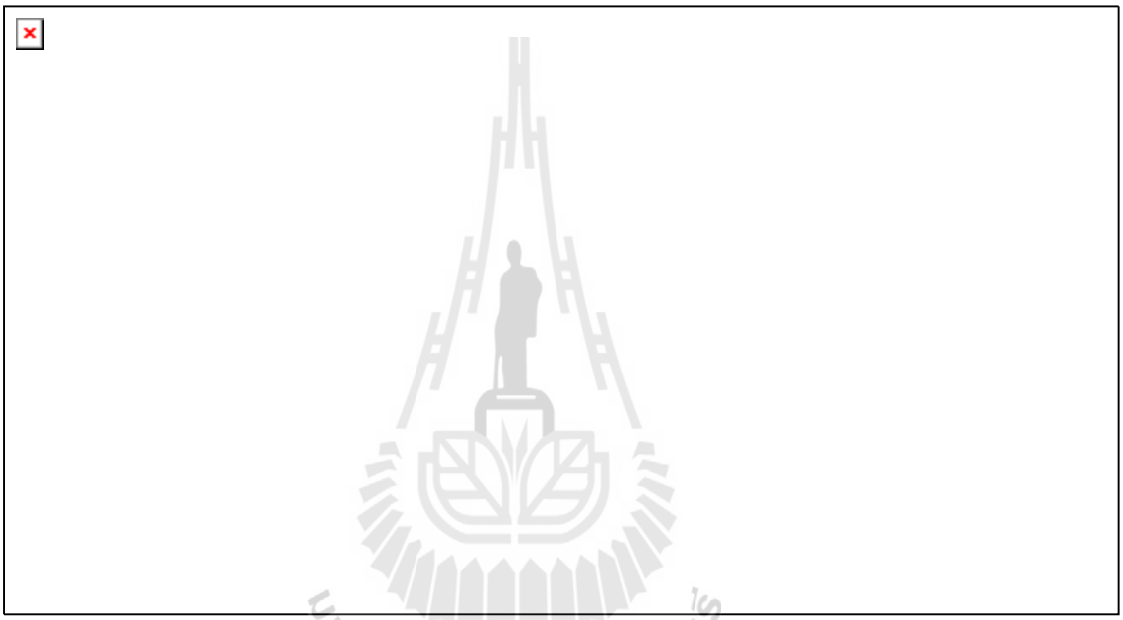


Figure 4.3 Time profile of metabolites production, cell growth, and glucose consumption during fed-batch SA fermentation from cassava only by *A. succinogenes* ATCC55618.

This result was also comparable with some previous research for SA production from different cassava-derived substrates as same as the lignocellulosic such as presented in (Table 4.3). Liu *et al.* studiedthe application of cane molasses for SA production using *A. succinogenes* in fed-batch fermentation. The obtained SA titers after 48 h was just 55.2 g/L corresponded to the obtained productivity of 1.15 g/L/h whereas yeast extract was still added as a nitrogen source for the cell growth (Liu *et al*., 2008a). The price of cassava roots,

yeast extract and glucose were about \$0.06/kg, \$189.39/kg and \$0.39/kg, respectively. The enzyme amount used in this study corresponding to 1 kg of cassava root was calculated at \$0.062. Assuming the succinic acid yield of 0.78 $g_{SA}/g_{glucose}$ on case of cassava roots (containing 27% reducing sugar) (or about $10g_{SA}/(9.87\ g_{glucose}+1\ g_{yeast})$ result of fed-batch SA fermentation from glucose), the raw material cost of the bioprocess was therefore estimated to be \$0.577/kg_{SA} using cassava root or \$19.28/kg_{SA} using glucose and yeast (Baniel *et al*., 1995). Considering the downstream purification process cost for 60-70% of the production cost, the total raw material cost of the SA production from cassava roots (or glucose and supplemented yeast extract as a nitrogen source) would be about \$0.962/kg (or \$32.13/kg). Therefore, cassava roots can be used as an economical and feasible carbon source for SA production by using *A. succinogenes*.

Table 4.3 Comparison of SA production from cassava-derived substrates and others materials from the nature with that in other previously published works.

Substrates	Microorganism	Fermentation strategy	Succinic acid production			Reference
			Titers (g/L)	Yield[1] (g/g)	Productivity[2] (g/L/h)	
Cassava						
- Cassava powder	*E. coliNZN111*	Fed-batch SSF	106	0.66	2.54	(Chen *et al*., 2014)
- Cassava starch	*E. coliNZN111*	Fed-batch SSF	127	0.71	1.77	(Chen *et al*., 2014)
- Cassava pulp	*E. coliKJ122*	Fed-batch SSF	98.63	0.71	1.03	(Sawisit *et al.*, 2015)
Other substrates						
- Cane molasses	*A .succinogenes*	Fed-batch	55.2	0.81	1.15	(Liu *et al*., 2008b)
- Whey	*A. succiniproducens*	Fed-batch	34.7	0.91	1.02	(Samuelov et al., 1999)

- Corn stover	*A .succinogenes*	Batch SSF	47.4	0.72	0.98	(Zheng *et al.*, 2010)
- Waste bread	*A .succinogenes*	Batch SSF	47.3	0.55	1.12	(Leung et al., 2012)
- Wheat	*A .succinogenes*	Batch SSF	64	0.4	1.06	(Du *et al.,* 2008)

[1]The SA yield was calculated as obtained SA concentration divided by utilizing substrate concentration during fermentation.

[2]The SA productivity was calculated as SA obtained concentration divided by overall fermentation time.

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4.3 Purification of succinic acid from fermentation broth

4.3.1 Purification of succinic acid from fermentation broth by using electrodialysis

Theexperimental set up was shown in Figure 4.4. Under a rectifier or DC programmable power supply and simultaneously effect of electrolyte, a direct current was generated across the membrane system. This direct electric current generated the driving force of salt of magnesium of organic acids such as magnesium succinate, magnesium lactate, and magnesium acetate across the feed and permeate side of the membrane. Consequently, these anions and cation will be migrated across the anion-exchange membrane and cation-exchange membrane, and combined with hydrogen ions which produced by bipolar membrane intermediate layer, to form the organic acids and magnesium hydroxide ($Mg(OH)_2$) in acid compartment and base compartment, respectively. Consequently, they were collected at the acid tank and base tank, respectively. By the meantime the non-ionic compounds such as protein, etc. was recycled back to bioreactor such as was expressed in diagram at Figure 4.5.

Experimental run was initially tested using a clarified fermentation broth after the fermentation process. The solution was tested at the constant electrical current density of 0.9 A, and the process was terminated when the electrical current decreased. However, that was not as expected due to the presence of a large amount of magnesium ions, which caused the membrane fouling of $Mg(OH)_2$ just after a short operating time.

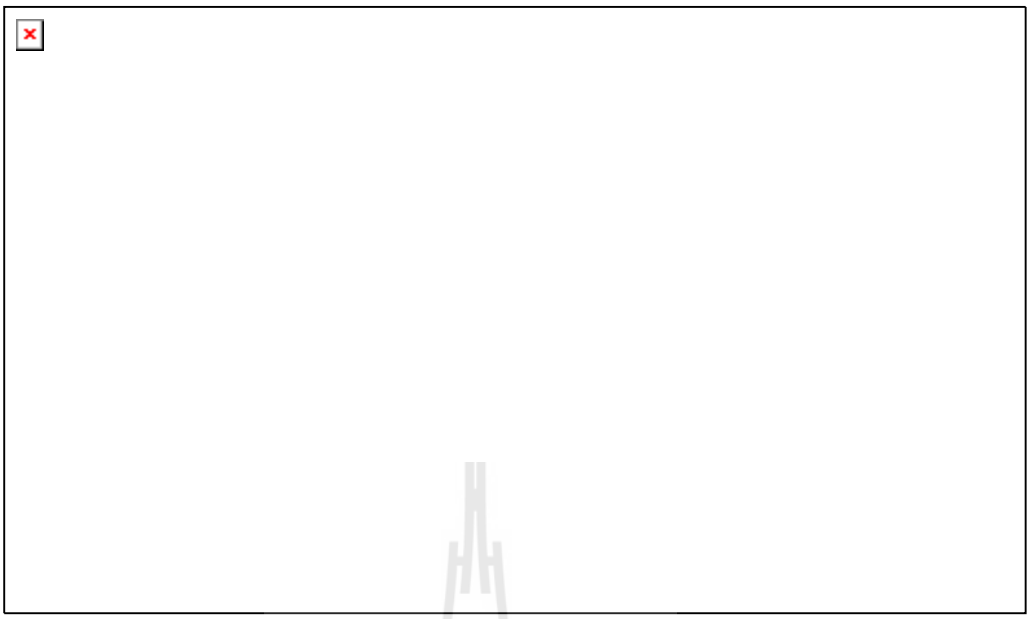
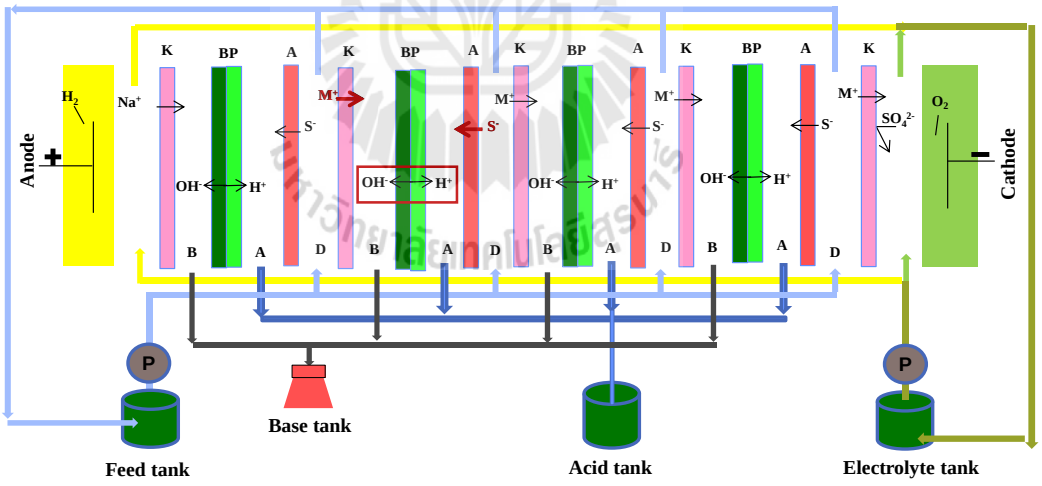


Figure 4.4 Experimental set up for an EDBPM unit.



K: Cation exchange membrane
A: Anion exchange membrane
BP: Bipolar membrane
D: Dilute compartment
B: Base compartment
M^+: Magnesium ion
S^-: Anions was dissociated from fermentation broth that represented succinate
A: Acid compartment

Figure 4.5 Electrodialysis bipolar membrane system constitute.

This phenomenon showed in Figure 4.6.A previous work reported that the efficiency of lactic acid separation from fermentation broth by using bipolar membrane electrodialysis-cation system was low when the feed solutions contain metal ions such as Ca^{2+} and Mg^{2+}(Lee *et al*., 1998). The presence of the metal ions in broth caused the precipitation of metal ions on the membrane surface. In addition, this was also mentioned that longer operating time, higher total current efficiency, energy consumption, and recovery ratiowere obtained when applied to the ammonium lactate fermentation broth compared with ammonium lactate model solution(Timbuntam*et al*., 2008). That was explained by the presence of impurities in the fermentation broth such as the proteins and metal ions that fouled the membrane surface. Due to the poor experimental results of electrodialysis, further downstream of succinic acid from the fermentation broth was focused on more efficient purification process of MF and NF-assisted crystallization.

Figure 4.6 Membrane fouling by magnesium ions.

4.3.2 Purification of succinic acid from fermentation broth by microfiltration

4.3.2.1 Model development

4.3.2.1.1 Membrane resistance - The hydraulic membrane resistance was determined by cross-filtration of DI water at each different transmembrane pressure. Figure 4.7 showed the relationship between water flux ($1/J_w$) and transmembrane pressure (1/TMP) in the linear straight line. The slope was obtained from this line, and helped to find the membrane resistance of 4×10^{11} (m^{-1}).

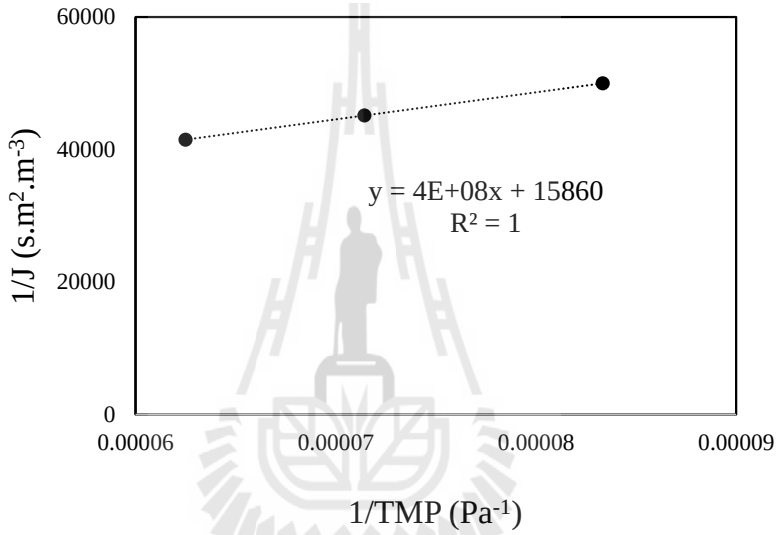


Figure 4.7 MF permeate flux of water at different transmembrane pressures.

4.3.2.1.2 Cake compressibility index - The cake compressibility index was determined from the dead-end filtration experiments. This cakes caused by the deposition of the bacterial cells on the membrane surface during filtration as illustrated in Figure 4.8.

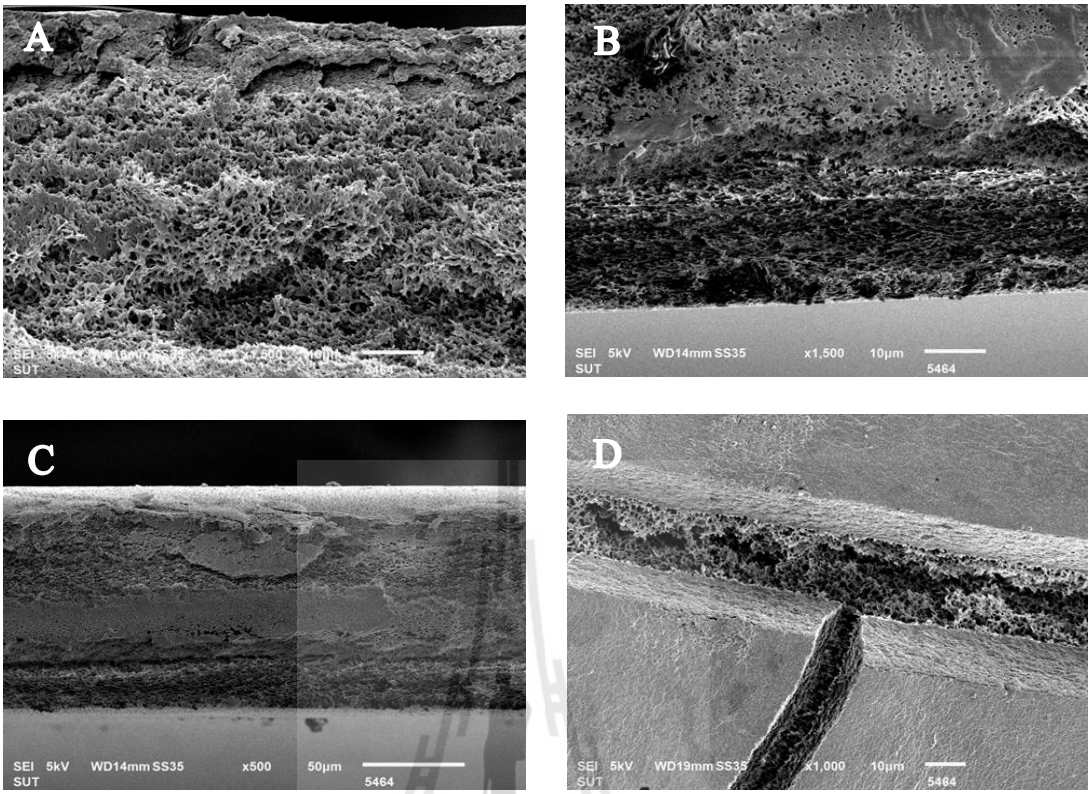


Figure 4.8 SEM images of the cake layer of cellulose acetate filter membrane from dead-end filtration section; (A) cross-section of the cleaned membrane, (B) cross-section of the fouled membrane, (C) cross-section of the fouled membrane showing deposition of a cake layer on the membrane surface, (D) top surface of the fouled membrane.

Different experiments were carried out at each different constant pressure for the whole broth containing bacteria. Specific cake resistance α_0 was calculated by the change of accumulated permeate volume with time at each different operated pressure of dead-end filtration experiment according to equation as followed (Nakanishi*et al*., 1987):

$$\alpha_0 = \frac{\text{Slope}.\ 2A_{fp}^2.p}{} \qquad (24)$$

$$\frac{}{\mu_M C_w}$$

Where, slopes were obtained from the relationship between accumulated permeate volume and time per volume and it was showed in Figure 4.9, 4.10 and 4.11, respectively, A_{fp} was the filter paper membrane area (m^2) used in dead-end filtration experiment, C_w was the feed concentration of the whole broth (kg/m^3). Figure4.12 gave the variation in the specific resistance thus obtained. The cake compressibility index was given by the slope of the straight line in Figure 4.12, and was found to be equal 1.0. This value is in the range of cake formed for the similar-rod shape strains that were reported by (Tanaka *et al*., 1996) such as *B. subtilis,* and*L. delbrueckii*at 0.8, and 1, respectively.

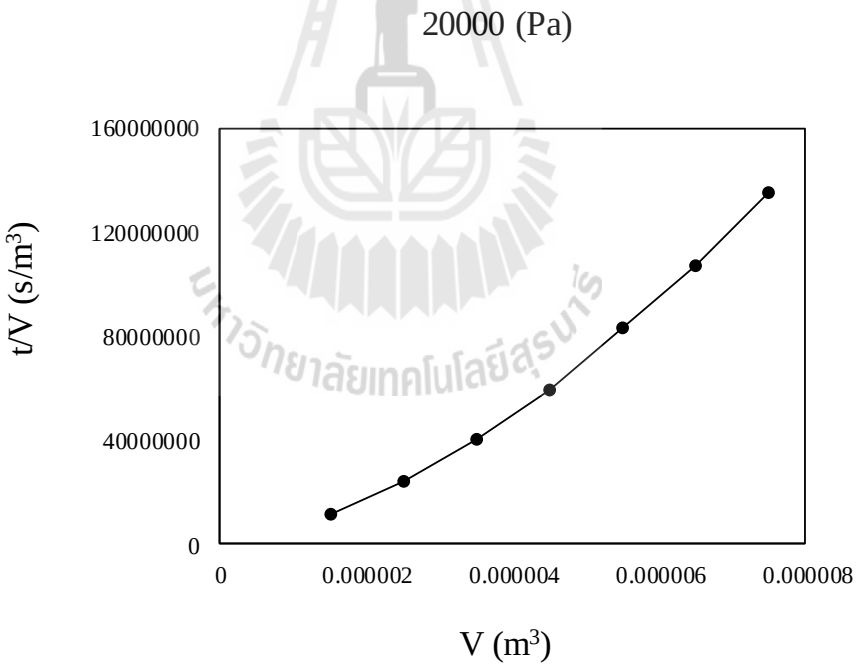


Figure 4.9 Summary of plots of t/V vs. V for whole fermentation broth dead-end filtration under pressure of 20000 Pa.

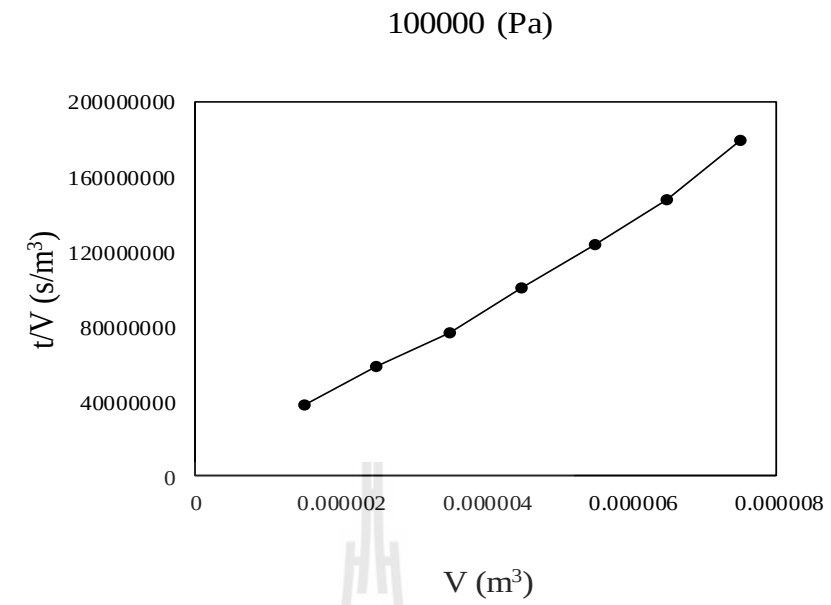


Figure 4.10 Summary of plots of t/V vs. V for whole fermentation broth dead=end filtration under pressure of 100000 Pa.

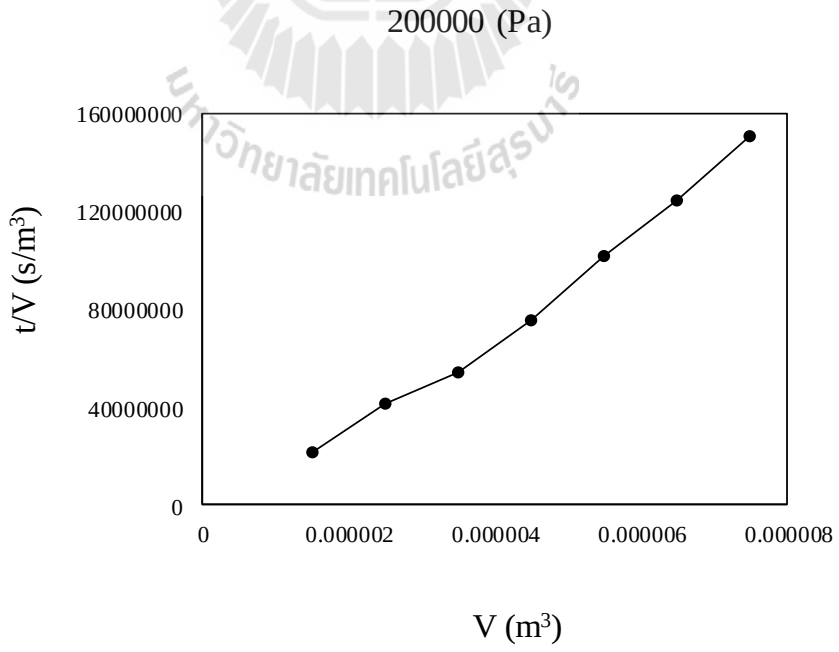


Figure 4.11 Summary of plots of t/V vs. V for whole fermentation broth dead-end filtration under pressure of 200000 Pa.

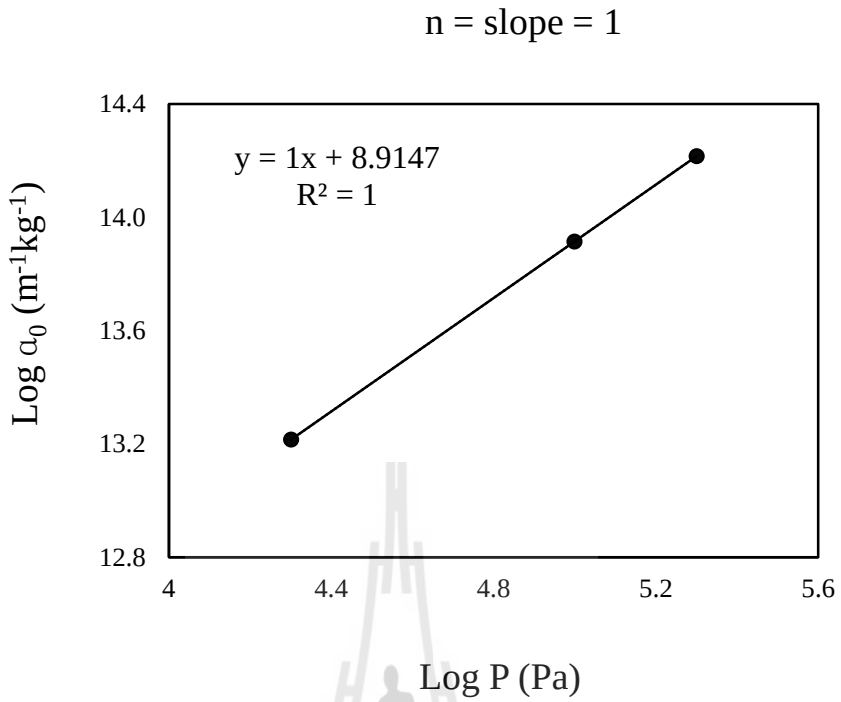


Figure 4.12 Plotting log of specific resistance α_0 vs. transmembrane pressure Pa to get cake compressibility index n of dead-end filtration of succinic acid fermentation broth according to $\alpha_0 = \alpha(\Delta P)^n$.

4.3.2.1.3 Back diffusion coefficient - Normally, model validation of feed broth filtration is understood under constant permeate flux, J_{ss}. Therefore, back diffusion coefficient $\frac{D}{\delta}C_m$, can be used for obtaining J_{ss} of model equation. In addition, when steady state is reached, there is no further net deposition of bacteria on the membrane wall so that Eq. (3) becomes (Hélène *et al.*, 2001):

$$J_{ss}C = \frac{D}{\delta}C_m \qquad (25)$$

The term of $\frac{D}{\delta}C_m$ can be assumed to be constant, was thus calculated from Figure 4.13 and Eq. (3) where J_{ss} was steady state flux obtained during experimental

runs carried out with a constant retentate concentration, C, and expressed by the equation as followed:

$$\frac{D}{\delta}C_m = 1 \times 10^{-5}C - 8 \times 10^{-5} \quad (26)$$

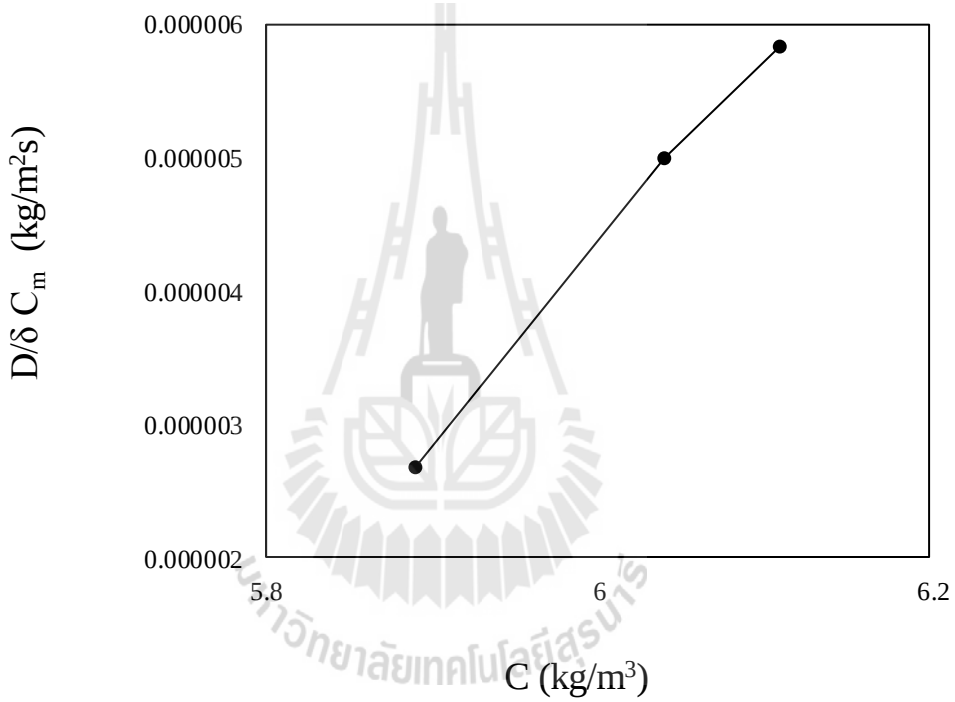


Figure 4.13 Back diffusion coefficient (D/δ)C_m determined from the steady state fluxes obtained during cross-flow microfiltration of succinic acid fermentation broth at constant pressure of 300000 Pa.

4.3.2.1.4 Cake resistance - The cake resistance due to the deposition of the bacterial cells on the membrane surface was determined from Eq. (2) where, the bacterial cake mass, m was calculated by multiplying the bacterial concentration by the permeate volume (Hélène *et al*., 2001). Figure 4.14 showed the change of cake resistance

during the filtration time. The tendency of this curve is as same as already above presented, at the beginning the cake resistance increase very fast and then was already reached to steady state and reached its plateau.

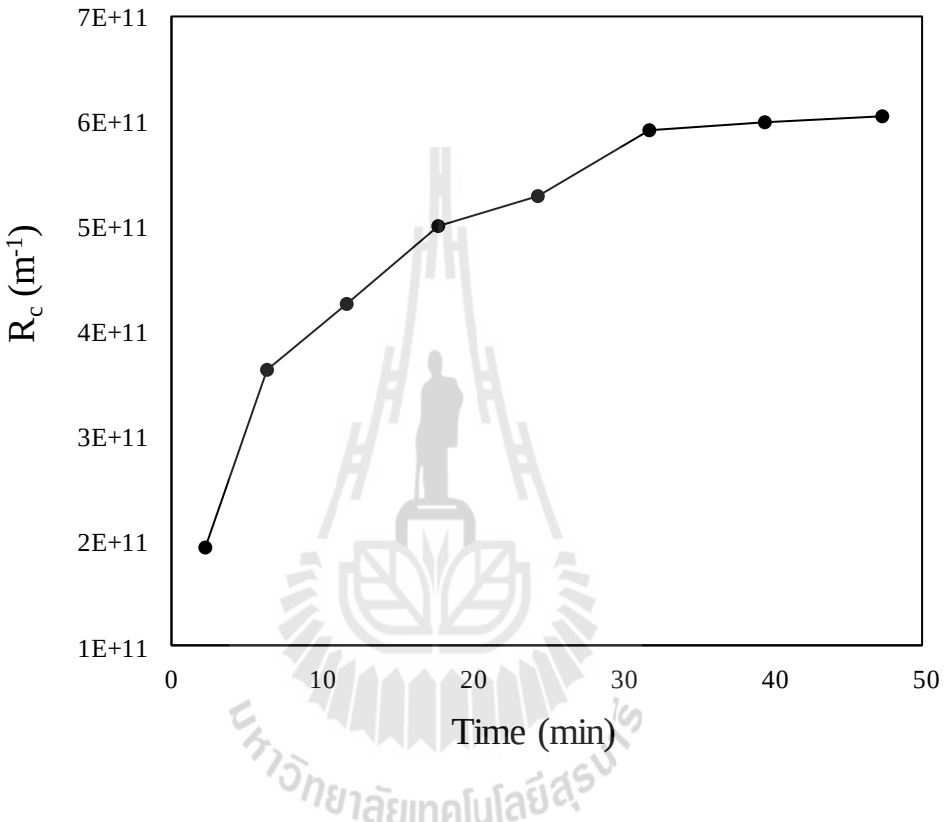


Figure 4.14 Change in the cake resistance during cross-flow microfiltration.

4.3.2.1.5 Pore blocking and adsorption resistance - Besides cake resistance, the pore-blocking and adsorption resistance is responsible for the vast majority of the filtration resistance. The resistance due to pore-blocking and adsorption was assumed by subtracting R_m and R_c from R_t that was obtained from the filtration experiment carried out with SA fermentation broth. Figure 4.15 expressed the variation in resistance over cross-flow MF time. These experimental variations were fitted by Eq. (4).

In addition, based on the equation that was obtained from the diagram of pore-blocking and adsorption resistance in Figure 4.15 (Eq. 26), the average *b* value of 0.052 was found to fit the result of experiment.

$$R_f = 4 \times 10^{11} \ln t + 2 \times 10^{11} \quad (27)$$

From that, pore-blocking and adsorption resistances were modelled according to Eq. (4) and demonstrated as in Figure 4.16.

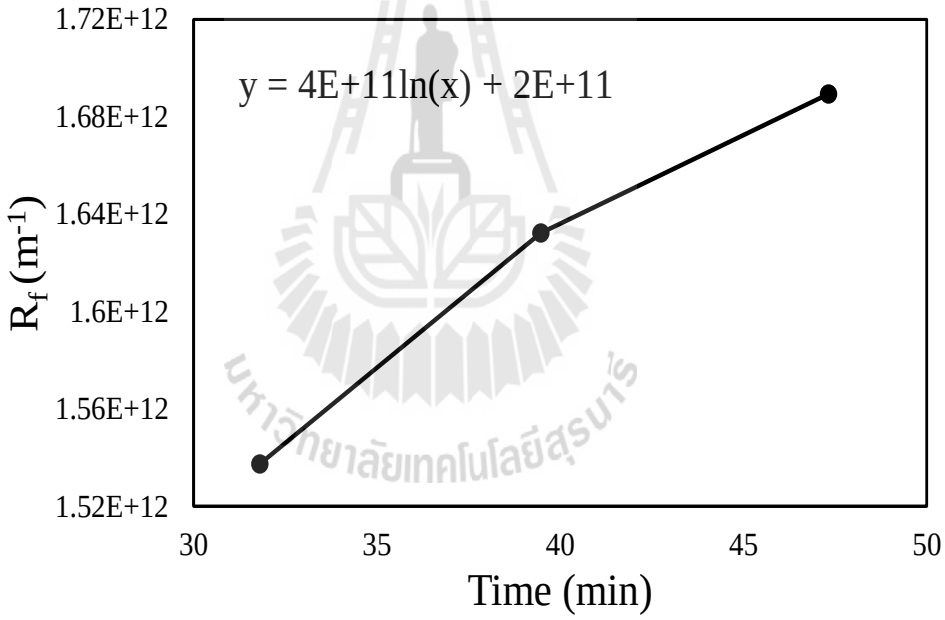


Figure 4.15 Pore-blocking and adsorption resistance over cross-flow microfiltration time of succinic acid fermentation broth from the experiment.

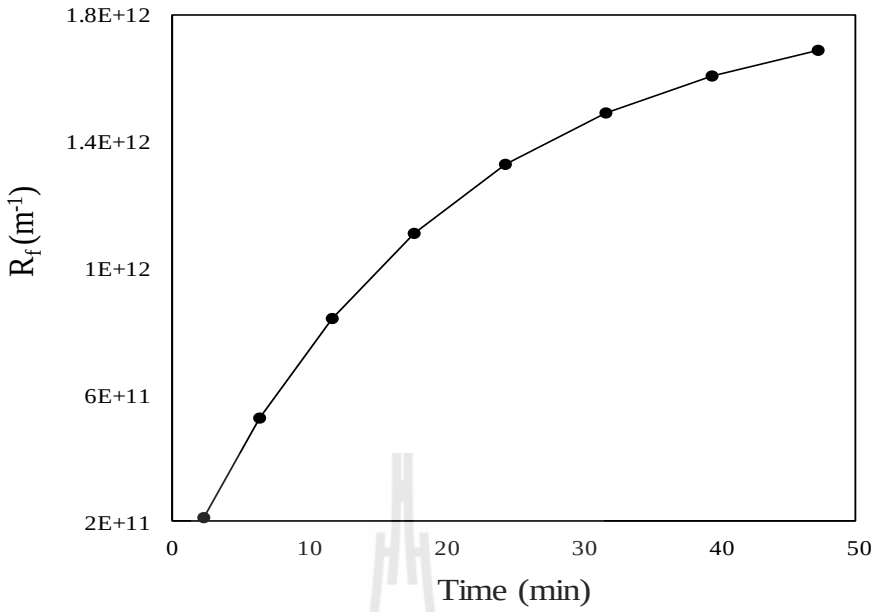


Figure 4.16 Modeled pore-blocking and adsorption resistance over cross-flow microfiltration time of succinic acid fermentation broth.

4.3.2.2 Comparison of modelling constant transmembrane pressure cross-flow microfiltration and operated mode

Mathematical modelling the cross-flow MF at constant transmembrane pressure was determined by fitting the modelled permeate flux, resistances, and bacterial concentration in retentate data to experimental data as showed in Figure 4.17. It was seen that the permeate flux decreased rapidly in the first observed resulting in a rapid increasing in the resistance whilst the bacterial concentration increased slowly. Subsequently, the flux reduction decrease whereas the increase in the bacterial concentration is significantly leaded to the increase in total resistance, and seemly reached steady state then. Thus, bacterial concentration has little effect on the permeate flux and also resistance due to the cross-flow mode of operation. The model was then further validated by comparing the calculated data with the obtained experimental data via mean error. The mean error value ε was used to check the fitting (Hélène *et al*., 2001):

$$\varepsilon = \text{mean}\left(\frac{\left|J_{mod} - J_{exp}\right|}{J_{exp}}\right) \quad 28)$$

The results showed that the ε value lower than 5% was obtained. However, almost the modelled permeate flux was a little bit lower than that of the experimental result. This may due to the experimental start up procedure. At the first time, the system was tested at several times by DI water to find the hydraulic membrane resistance before the introduction of the SA fermentation broth to the system. As a result, the membrane has been already contacted with the DI water before the MF of the real broth. This consequence of the start up process has already been mentioned previously (Dornier *et al*., 1995). In conclusion, the clarification of SA complex fermentation broth was modelled by using resistance-in-series model of membrane, cake, pore-blocking and adsorption. The mathematical modelling was in a good agreement with the experimental result at the mean error of less than 5%. Thus, the data could be very useful for the flux prediction as well as the estimation of membrane area required for a particular MF task at the same operating condition.

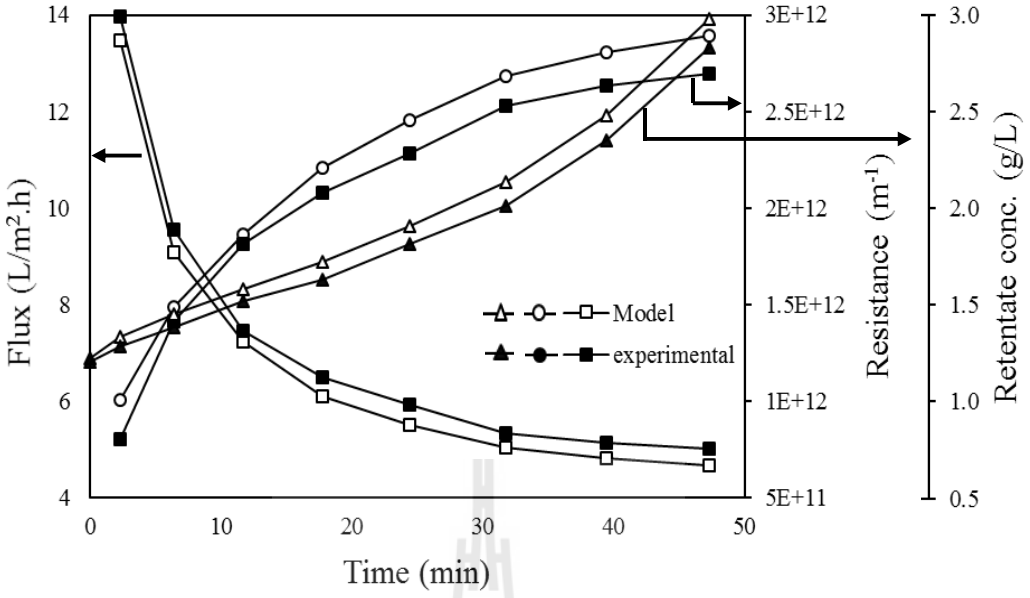


Figure 4.17 Change in permeate flux, membrane resistance and retentate concentration during cross-flow microfiltration. White symbol: modelled; black symbol: experimental.

4.3.3 Purification of succinic acid from fermentation broth by using Nanofiltration

4.3.3.1 Nanofiltration for succinic acid fermentation broth

The value of the membrane hydraulic resistance (R_m) was calculated based on the obtained DI water flux that was already tested by the new membrane at each different pressure as showed in Figure 4.18. From this diagram and Eq. (7), the hydraulic membrane resistance was given equal 0.3×10^{14} m^{-1}.

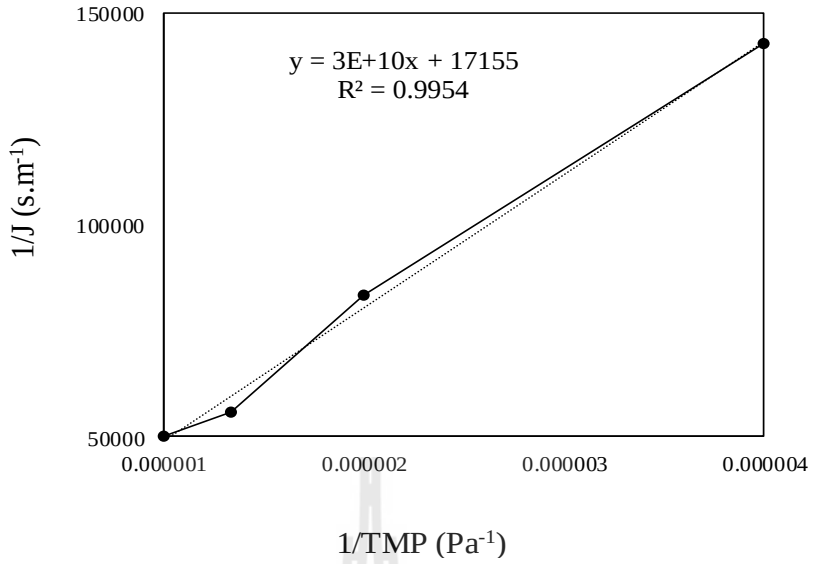


Figure 4.18 NF permeate flux of water at different transmembrane pressure.

4.3.3.1.1 Concentration mode - Based on the experimental results that were demonstrated previously by influence of the operating parameters on the separation performances of organic acid solutions. Organic acids are dissociated according to the pH of the solution, and its rejection was highly pH-dependent. Rejection of SA decreased considerable at pH levels below their dissociation constant pK_a (non-dissociated form) whilst the values significantly increased at pH levels above their pK_a. The lowest rejection was examined at pH 2(Lubsungneon *et al*., 2014). In this work, the clarified SA fermentation broth (MF permeate) was adjusted pH to 3.0 before being introduced to the NF membrane. The broth was already freed from the bacterial cells. However, it still contained some dissolved impurities such as proteins, polysaccharides, colouring molecules, and especially magnesium and sulfate ion. One indispensable aim of the NF process is the removal rates of protein, magnesium sulfate and other metabolites. In this case, the protein and magnesium sulfate removal rate were of interested because the protein compounds can be hydrolyzed into the amino acids which lead to the complex technical problem for the later purification process, especially for the crystallization in this study. If the protein compounds are not

sufficient removed, the final product will be resulted not only in yellowish color, but also stick by the protein membranes on the crystals as same as reduce the process performance and product quality. Moreover, other macromolecules with a similar size to the protein compounds will also be removed if the proteins are removed. In addition, the solubility of magnesium sulfate is also decrease at very low temperature, and especially at high concentration. Therefore, if these compounds are not removed, the final product might also result in their presence. The separation performance of clarified SA fermentation broth and model solution by NF system was compared as shown in Figure 4.19. It expressed the resistance as a very important criteria effecting the permeate flux. In general, the flux tends to decrease with a decreasing in operating pressure. If the pressure is too high, it might result in higher flux at the beginning but causes the rapid deposition of macromolecules on the membrane surface and then the flux will also be reduced. Hence, it is recommended that the operating pressure should be controlled so that is not very high initial permeate flux in which leads to a poor membrane performance (Sang *et al*., 2005). In this experiment, the constant pressure at 750000 Pa, pH 3.0 and temperature 20°C was used to characterize the flux and resistance calculation. In general, the permeate flux tends to decrease very fast at the beginning of the run and gradually reduced in latter time due to mainly the reversible phenomena: concentration polarization, i.e. (Lubsungneon *et al*., 2014, Hélène *et al*., 2002). The initial permeate flux of clarified SA fermentation broth was obtained 1.22 $L/m^2.h$ and then rapidly drop in the first few minutes to 0.2 $L/m^2.h$, followed by a slowly flux decline until the lowest flux of 0.09 $L/m^2.h$ at the end of NF run at 21 hourscorresponding to a decrease in 92.6 % compared to the initial flux. The permeate volumeof 20 L was collected. In addition, the resistance increased and calculated equal 2.7×10^{15} m^{-1} at the end of NF run, this value was then considered as R_{NF}. This caused by the heavily membrane fouling due to

the accumulation of the protein and suspended macromolecule compounds on the membrane surface.

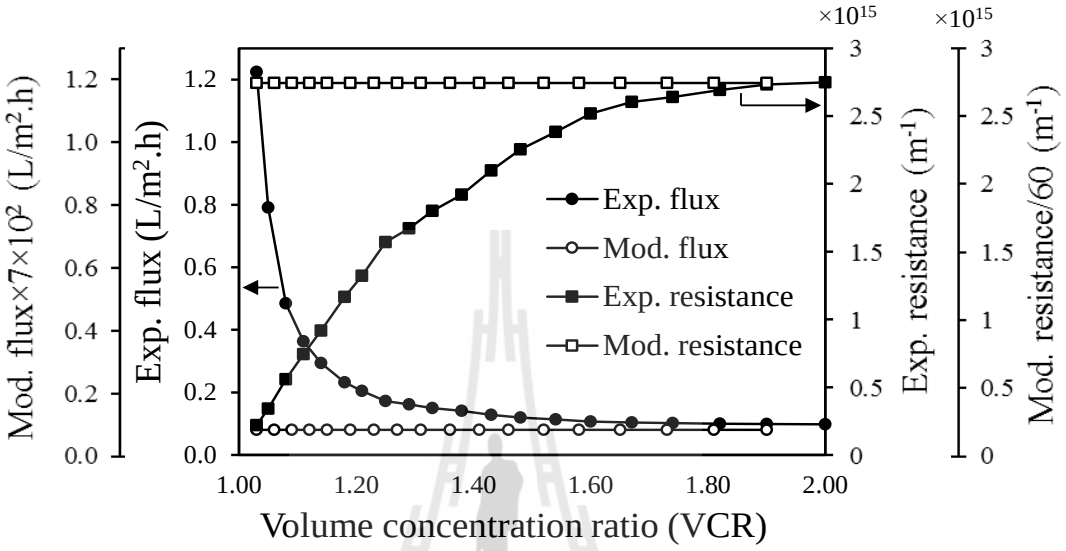


Figure 4.19 Change in the permeate flux and membrane resistance during the NF test for model solution and fermentation broth. Operation condition: feed pressure 750000 Pa; pH 3.0; temperature 20°C.

In order to evaluate the fouling characteristic of NF membrane, a direct procedure of cleaning process was performed by water flux measurements during two cleaning processes of water cleaning and chemical cleaning.Firstly, NF system was washed by DI water until the colourless solution was obtained (R_1), then washed with NaOH 2% for 20 min followed by rinsing with DI water for 20 min and continued washing with H_3PO_4 2%for 20 min and similarly followed by rinsing with DI water until pH was obtained the neutralization (R_2).After washing with water, the permeate flux was sharply increase leaded to the resistance (R_1) also decreased and was calculated at 0.59×10^{14} m^{-1}.

As a result, the resistance due to the cake formation (R_c) is the difference between R_{NF} and R_1 which is 26.41×10^{14} m^{-1}. Besides that, after consequently cleaned by chemical, the permeate flux was increase a little bit more and the resistance (R_2) was calculated at 0.46×10^{14} m^{-1}. That leaded to the resistance due to the pore-blocking and adsorption (R_f) is the difference between R_1 and R_2 which is 0.13×10^{14} m^{-1}. In consequence, the NF membrane fouling characteristic was conjectured by using a cleaning performance. As showed in Figure 4.20, the ratio of the calculated resistances of R_m, R_f and R_c corresponding to 1.12%, 0.48% and 98.4%, respectively. It is interesting to observe that the cake resistance contributed to be a major role on the total resistance of the NF process.

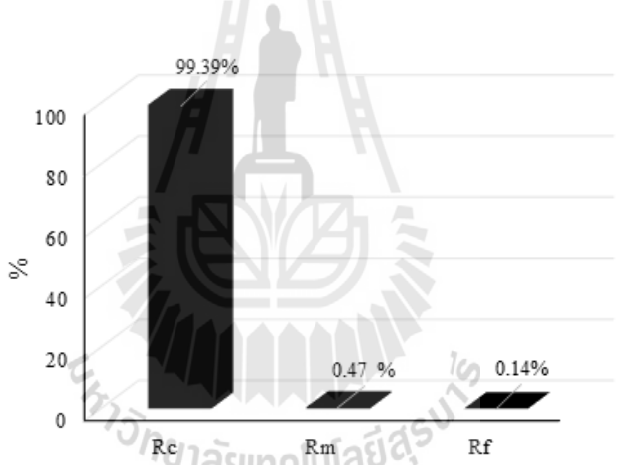


Figure 4.20 Resistance analysis of nanofiltration membrane using a cleaning procedure.

In summary, the cake formation of the protein substance and macromolecule compounds on the membrane surface played an important role in their rejection. On the other hand, the high cake resistance implied that the impurities werealso highly retained. This was furtherly demonstrated by the comparison between the concentrations of the components in feed and permeates solutions as histogram shown in Figure 4.21. It showed the concentration difference of organic acids, protein and the ions between the feed and the permeate (image A & B). An interesting result was observed for

the concentration disparities of protein and multivalent ions in the feed and permeates. The protein concentration in the permeate was only 0.11 g/L whereas its presence in feed was 2.32 g/L. Therefore, the protein removal rate was calculated at 95.3%. Similarly, the rejection of SO_4^{2-}, Mg^{2+}, and Ca^{2+}went up to 99.7% and 99.5%, 95,8%, respectively. Similar to Kang et al., the rejection of $MgSO_4$ over 90% was published in recovery of ammonium lactate,as well as hardness removal from fermentation broth by NF research (Hélène *et al*., 2002). In addition, the colloid and suspended particles in fermentation broth was also significantly eliminated as the NF permeate viscosity reduced 87.5% comparedto the MF permeate viscosity (the result not shown at here). However, the majority of SA and organic acids by-products were still presented in the permeate stream. In conclusion, the most important advantage of NF system was the removal of a larger amount of the potential foulants including dissolved organic and inorganic substances, colloid and suspended matters resulting in the expected decolouration efficiency as showed in Figure 4.21 (C), 95.7% peculiar brown colour in fermentation broth was almost completely deleted. This was also further evidenced at Figure4.21 (D). This step is expected to facilitate for further purification step since the foulants was almost completely removed. This research result was similar to a previous report for the usage of NF membrane as final purification step for lactic acid production process by the efficiently removing of the Mg^{2+}, Ca^{2+}, SO_4^{2-} and PO_4^{3-} ions from a sodium lactate fermentation broth (Antoine *et al*., 2006). In addition, NF led to a nearly total decolouration of brow colour from fermentation broth was also efficiently reported (Lubsungneon *et al*., 2014, Antoine*et al*., 2006). This result can be explained that for these multivalent ions were not only partially higher molecular weights, but also higher charge led to an increased ionic strength in solution resulting in a reduced membrane surface charge also. On the other hand, the electrostatic interaction between membrane and these ions was also decreased. Combined with theirs steric-hindrance affects; therefore,a high rejection was reached. Besides that, the protein molecules and macromolecules are not the uncharged

matter, butpossess larger molecular sizes than membrane pore size.Therefore, it was easily to be eliminated by the NF membrane.

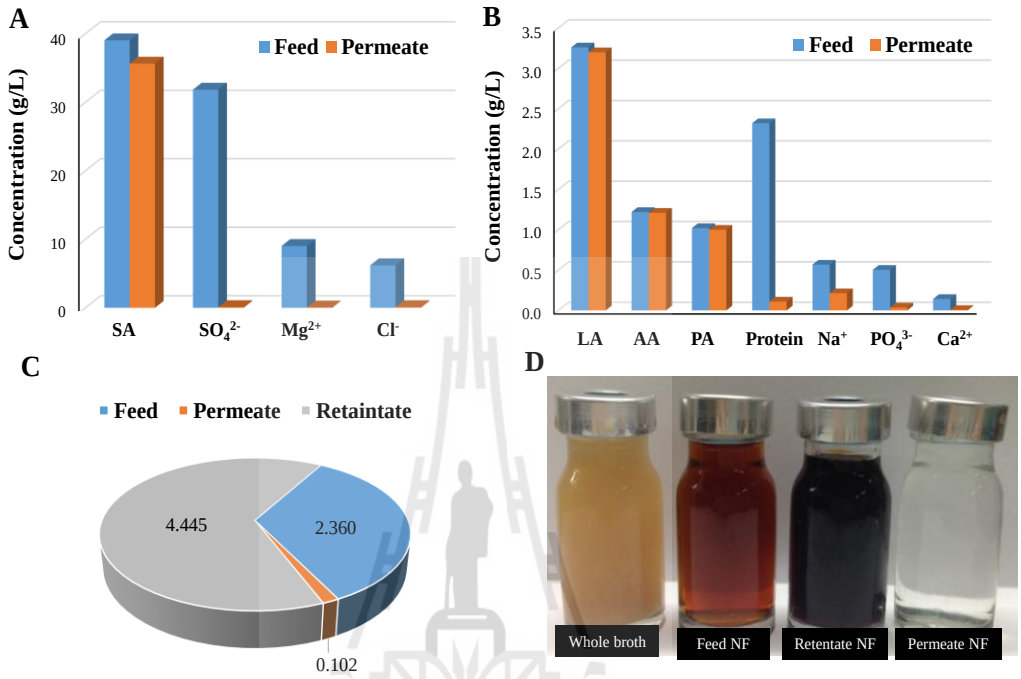


Figure 4.21 Histograms showing the composition of the feed and permeate NF solution. (A) SA, SO_4^{2-}, Mg^{2+}, Cl^-. (B) LA, AA, PA, Protein, Na^+, PO_4^{3-}, Ca^{2+}. (C) The color intensity of the feed, retentate and permeate NF solution at absorbance of 420 nm. (D) Picture showing evidence of the decoloration induced by the NF process.

Figure4.22 also showed the rejection of organic acids in fermentation broth for concentration mode by NF. As already above presented, high rejection for the large components, but low rejection for the target organic acids. The rejection sequence of acids by NF membrane was acetic acid < pyruvic acid < lactic acid < succinic acid. Organic acids are charged weak acids, but their size depend highly on pH, and they are also dissociated according to the pH of solution. The rejection is significant when their pH is greater than pK_a, lower pH is higher proton concentration inferred larger dissociate ability to form the anions

and proton [H^+]. In addition, in this case, low pH was adjusted leading to the membrane was positively charged. Resulting in low rejection of these organic acids by the electrostatic attraction between membrane surface and these anions of organic acids. Besides that, due to SA have molecular weight larger led to higher rejection than the other organic acids. This was recovered by the subsequent DNFoperation mode.

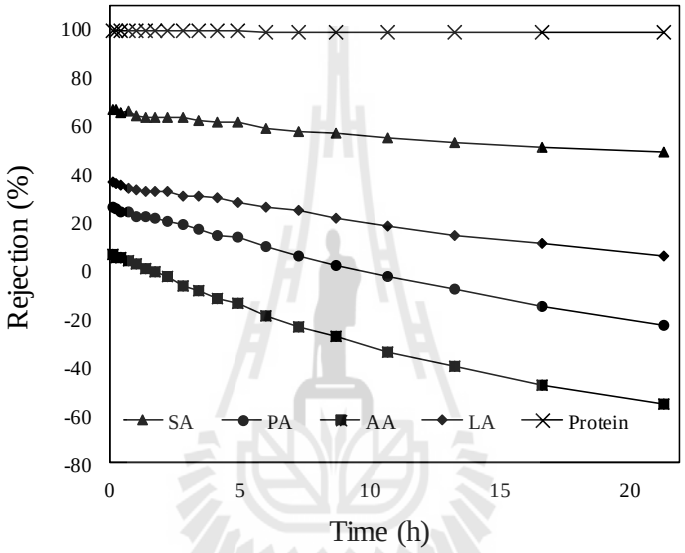


Figure 4.22 Rejection of organic acid salts in fermentation broth via concentration mode by Nanofiltration.

4.3.3.1.2 Dianaofiltration mode - In order to increase the separation process, NF of SA fermentation broth was re-investigated in a sequent DNF mode with the objective to complete recovery of organic acids product. Volume of feed broth was kept constant at 15L by addition of DI water same pH value, and sampling of the feed solution was performed periodically before refill new run. The result obtained was presented in Figure4.23. In plot (A), it can be observed that the permeate flux decreased with increasing VCR and for a VCF in the range from 1.2 to 2.0, volumetric fluxes at higher than 0.7 $L/m^2.h$ were obtained in all DNF steps. The permeate flux decreased very fast at the beginning after refill the DI water. In the latter run of DNF process, the flux increased compared with the

previous run, and it was due to the dilution effect of the feed concentration. In plot (B), it showed the solutes concentration ratios of continuously DNF process as a function of time. The initial concentration of acetic acid, pyruvic acid, lactic acid and succinic acid were 1.88, 3.17, 2.97 and 20.22 g/L, respectively. The reduction of acetic, pyruvic and lactic acids concentration in feed solution was much faster than that of SA because they were not only smaller molecules, but also lower initial concentrations. In contrary, SA deletion in feed solution was taken long DNF time of approximately 24 hours. This was clearly appeared that there were several factors, especially fouling as was above presented, that resulted for a long DNF time. Although it took a long time,theDNF modeproved to be an efficient process to recover SA from the fermentation broth that was not contaminated by the other impurities with the recovery rate of SA approximately 94.9%. Therefore, DNF mode can be considered as a final step of NF process to completely recover SA from fermentation broth.

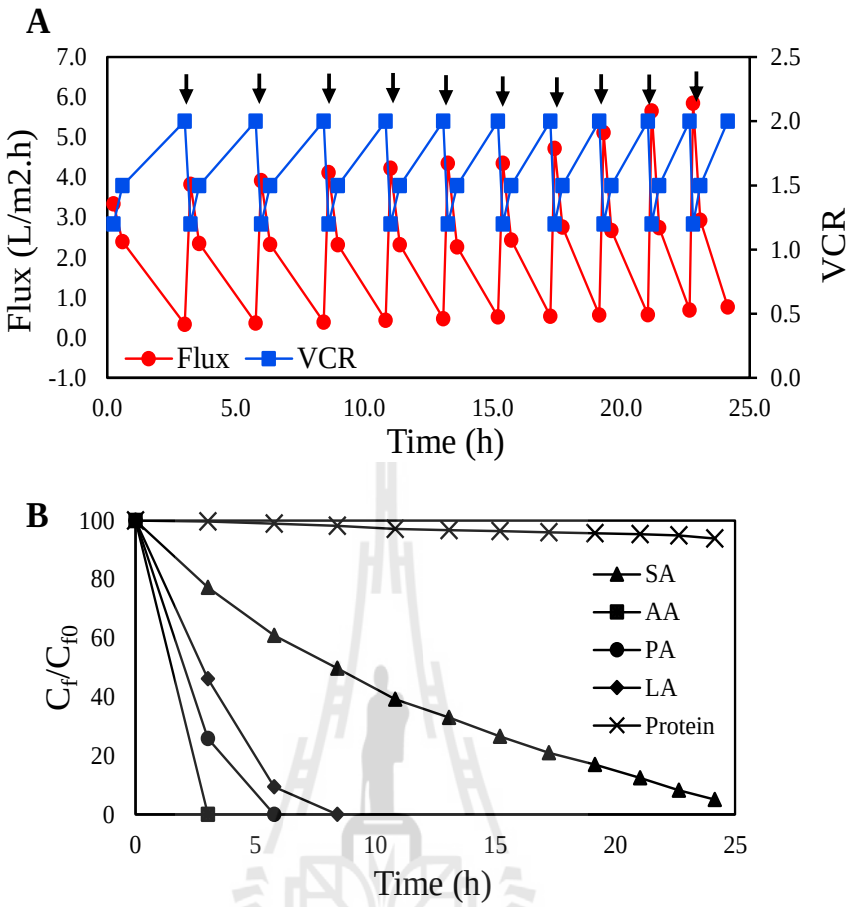


Figure 4.23 (A)Volumetric flux and volume concentration ratio for DNF of fermentation broth (the arrow indicate refilling of the DI water). (B) Solutes concentration profile of fermentation broth during DNF (sampling at before each new refill). Operation condition: feed pressure 750000 Pa; pH 2.0; and temperature 20 °C.

4.3.3.2 Nanofiltration for succinic acid using model solution

The NF process was re-investigated with model solution which mimicked the organic acids obtained from the fermentation broth. This NF process was carried out at the same condition as in the fermentation broth experiment. The initial concentration of acetic, lactic and succinic acids were 2.57, 2.16 and 43.87 g/L, respectively. Experimental results showed that the NF concentration mode time was finished just few minutes. Figure 4.24 showed that the SA rejection reduced to approximately 8% just within 90 seconds

corresponding to 42 times faster than that of fermentation broth. As shown in Figure 4.18,the permeate flux of the solution was constant at 59.02 $L/m^2.h$ corresponding to R_{NF} resistance of 4.5×10^{13} m^{-1}which was very close to the hydraulic membrane resistance of 3×10^{13} m^{-1}. That can be explained by the absence of the foulants, especially for the colloid and suspended dissolved substances. These results further illustrated that NF process was eliminated the foulants to transform the complex form of fermentation broth media to the simple form of synthetic solution. In summary, NF process was successfully used as a powerful assisted tool not only for product recovery, but also for solving the complex problem of fermentation broth media. In addition, NF could also integrated with any other efficient separation methods in order to bring the best results. In addition, this NF system is able to improve for its filtration efficiency if a higher pressure pump is employed which could result in a shorter operation time.

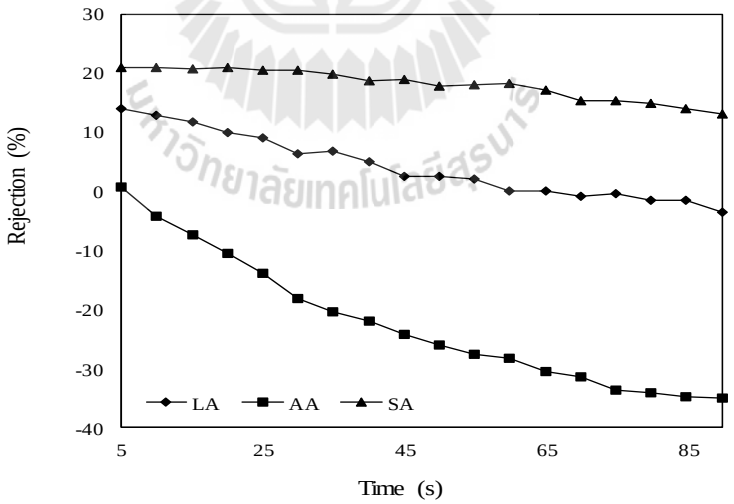


Figure 4.24 Rejection of organic acid salts for model solution by NF process in concentration mode.

4.3.4 Purification of succinic acid from fermentation broth by using crystallization

This experiment was investigated by three different crystallization methods as shown in Figure 4.25. The SA material source used in this experiment consisted of the SA fermentation broth after the end of the fermentation process (direct crystallization), and NF permeate. Each crystallization experiment was expressed below:

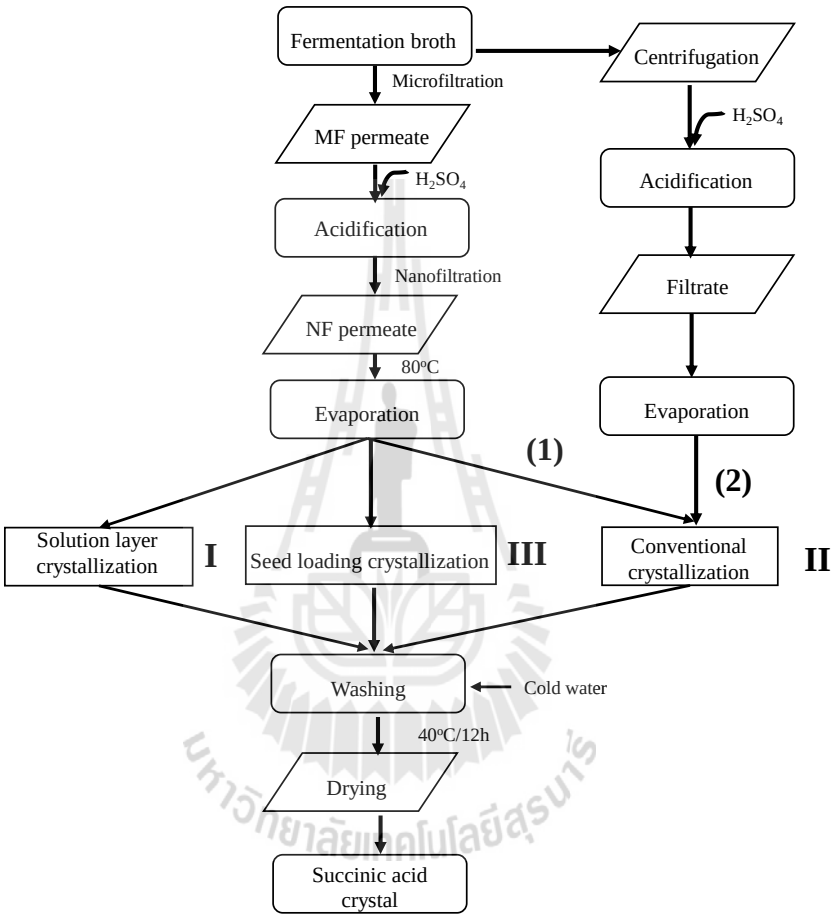


Figure 4.25 Schematic diagram of the three different crystallization processes of succinic acid, (I) Solution layer crystallization from NF permeate, (II) conventional crystallization from NF permeate and fermentation broth untreated by NF membrane (direct crystallization), (III) seed loading crystallization from NF permeate.

4.3.4.1 Solution layer crystallization (Process I)

Solution layer crystallization experiment was tested on the NF permeate as shown in Figure 4.26. Under the effect of cooling temperature, succinic acid was

selectivity crystallized to form the SA crystals on the cooling surface of finger tube. During the crystallization, the formation and growth of SA crystals was higher and higher. This cooling finger was known as a reagent caused cool and, in addition, it was also a stabilizer in order for the succinic acid crystals cling on after the nucleation and crystal growth. However, the SA crystals still continue to growth on the previously formed SA crystals surface, resulted in the formation of a SA solid-block as shown in Figure 4.27. Furthermore, the residual SA crystals still sediment on the crystallizer bottom as seen in Figure 4.26(F). This indicated that the succinic acid crystal was not fully grown on the cooling finger.

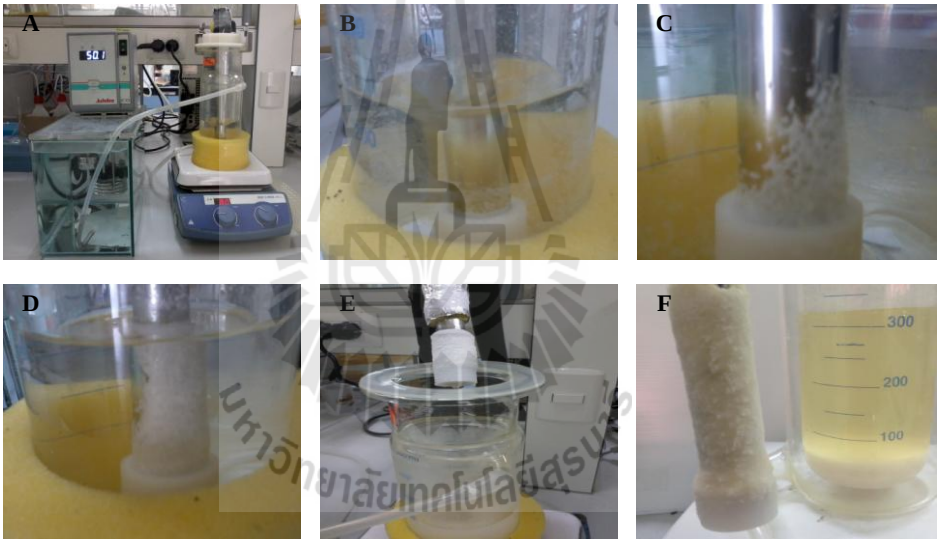


Figure 4.26 (A) Solution layer crystallization experiment. (B) The nucleation of the SA began after 8h. (C) The SA nuclei was growing to the crystals after 14h. (D) The SA crystals formed thicker after 20h. (E & F) The obtained SA crystals thickness layer at the end experiment (after 32h).

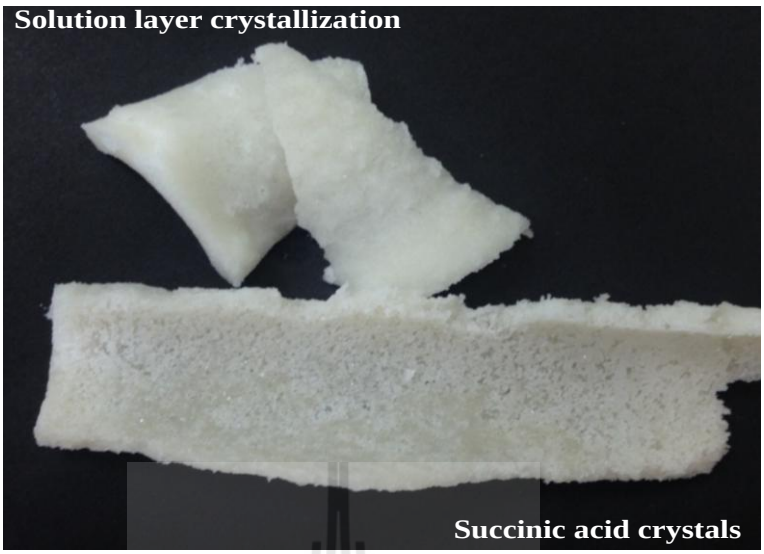


Figure 4.27 SA crystal obtained from the solution layer crystallization.

The obtained SA crystals were observed that was not only a lot of crystals combined and stuck together to a block, but also seemingly crystal size was large. That may be considered as one of the difficult purification problems which caused trouble for the controlling of crystals size and shape distribution. Moreover, it could result in the effect of the solubility when apply commercially. This phenomenon may have to be re-consideredfor the employing of a further method in the SA production process. Normally after crystallization, milling method is commonly employed to reduce the particle size of crystal for the application in the pharmaceutical industry (Zhi *et al*., 2015). In addition, the obtained SA yield was calculated just 57% because some other crystals still suspended in the mother liquor, especially for the small particle crystals.

4.3.4.2 Conventional crystallization method (process II)

The distribution diagram (δ) of carboxylic acid products into acid form/anion form will be different at each different dissociation constant (K_α) of each compound such as acetic acid (K_α=6.2×10^{-5}), lactic acid (K_α=1.37×10^{-4}), pyruvic acid (K_α=2.8×10^{-3}) and succinic acid ($K_{\alpha1}$=2.1×10^{-4}, $K_{\alpha2}$=2.3×10^{-6}). For the monoacid (eq. 29) and di-acid (eq. 30), respectively:

$$\delta_{HA} = \frac{[HA]}{[HA]+[A^-]} = \frac{[H^+]}{[H^+]+[K_a]} \qquad \delta_{A^-} = \frac{[A^-]}{[HA]+[A^-]} = \frac{[K_a]}{[K_a]+[H^+]} \tag{29}$$

$$\delta_{H_2A} = \frac{[H^+]^2}{[H^+]^2 + K_{a1}[H^+] + K_{a1}K_{a2}} \qquad \delta_{HA^-} = \frac{K_{a1}[H^+]}{[H^+]^2 + K_{a1}[H^+] + K_{a1}K_{a2}} \tag{30}$$

$$= 1 - \delta_{HA^-} - \delta_{H_2A} \tag{31}$$

Where, $[H^+]$ was the concentration of proton which controlled by the pH value of solution. Therefore, at each different pH results in each different dissociated proportion. According to a previous research, the result showed that the solubility of succinic acid at pH 2.0 was 73 g/L, and was around 80 g/L at pH 3.0 whereas all other carboxylic acids were still miscible in aqueous phase at pH 1.0-14.0, and temperature above 0 °C (Li *et al*., 2010). Besides that, the solubility curve of succinic acid decreased with a decreasing in temperature, and was only 30 g/L at 4 °C, at pH 2.0. Hence, crystallization of succinic acid could be carried out easily at this condition. As previous presented, normally SA fermentation was operated at pH 6.8 which was above the pK_α of each acid. As a result, the final acid products are almost in their dissociated forms rather than free acid. Thus, when the clarified fermentation broth was adjusted pH to 3.0, SA could be selectively crystallized at 4 °C. This step was carried out by using H_2SO_4 as an agent due to it was diprotic acid it is thus expected to acidify effectively.The conventional crystallization was conducted by two different SA sources as was above presented. The recovery rate and purity of the final

purified crystals from process II_1 and process II_2 were 86.53%; 90.26% and 48.67%; 40.64%, respectively.

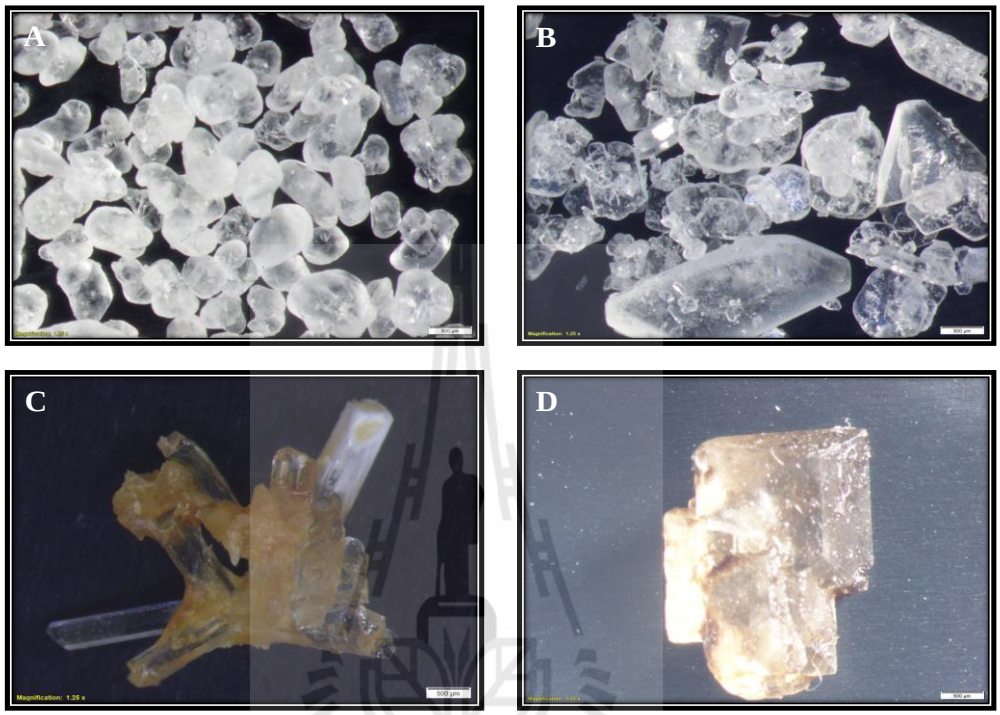


Figure 4.28 Stereomicroscopy images of SA crystals (the scale bar represents 500 µm by the magnification of 1.25x). (A) commercial analytical reagent. (B) product from process II_1; (C & D) product from process II_2.

More specifically, this experiment results were expressed in Figure 4.28 by the stereomicroscopy images with the scale bar represents 500 µm at the magnification of 1.25×. Figure 4.28 (A) was SA crystals image from commercial analytical reagent, Figure 4.28 (B) was an image of SA crystals obtained from process II_1 (NF-treated), and Figure 4.28 (C & D) were the SA crystals images that were obtained by the directly crystallization from fermentation broth (process II_2). These images were observed that the succinic acid crystals obtained from the NF-treated process possessed the morphology very close to that of

commercial analytical reagent. In contrary, the SA crystals obtained from the direct crystallization process have the small-needle-like morphology form with yellow colour. Furthermore, the crystals surface was stuck by the $MgSO_4$ particles, and covered by the colloids.Even few host cell residuals and proteins also cling on the crystals surface both in the corner or gully of crystal as was already clearly shown in Figure 4.28 (C & D).

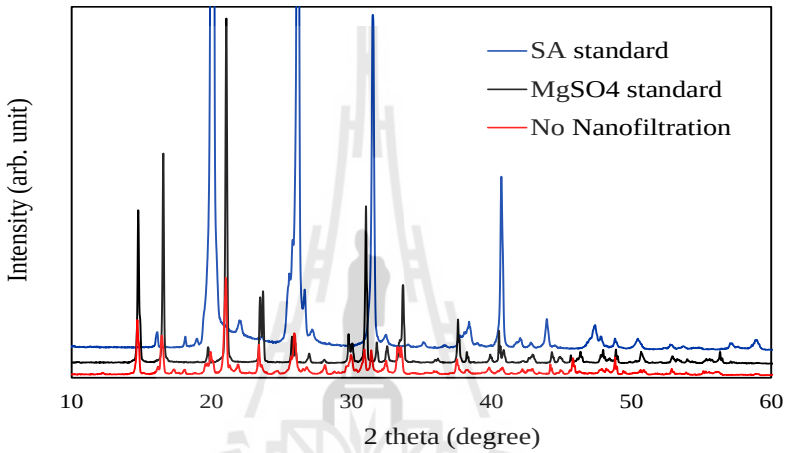


Figure 4.29 XRD pattern of SA crystal by process II_2 (direct crystallization), succinic acid and magnesium sulfate ($MgSO_4$) standard.

These results were also further demonstrated by X-Ray Diffraction (XRD) pattern as shown in Figure 4.29. The XRD image showed the purity of the purified product according to process II_2 by comparing with the standard analytical reagent. Some small peaks coincidence with SA standard peaks whilst others were fitted with $MgSO_4$ standard peaks. Consequently, the result was affirmed that the crystals obtained from the process II_2 contaminated by the $MgSO_4$ particles presence. The diffracted intensity algorithm of phase purity by SA to $MgSO_4$ ratio was calculated at 42.95%-57.05%.

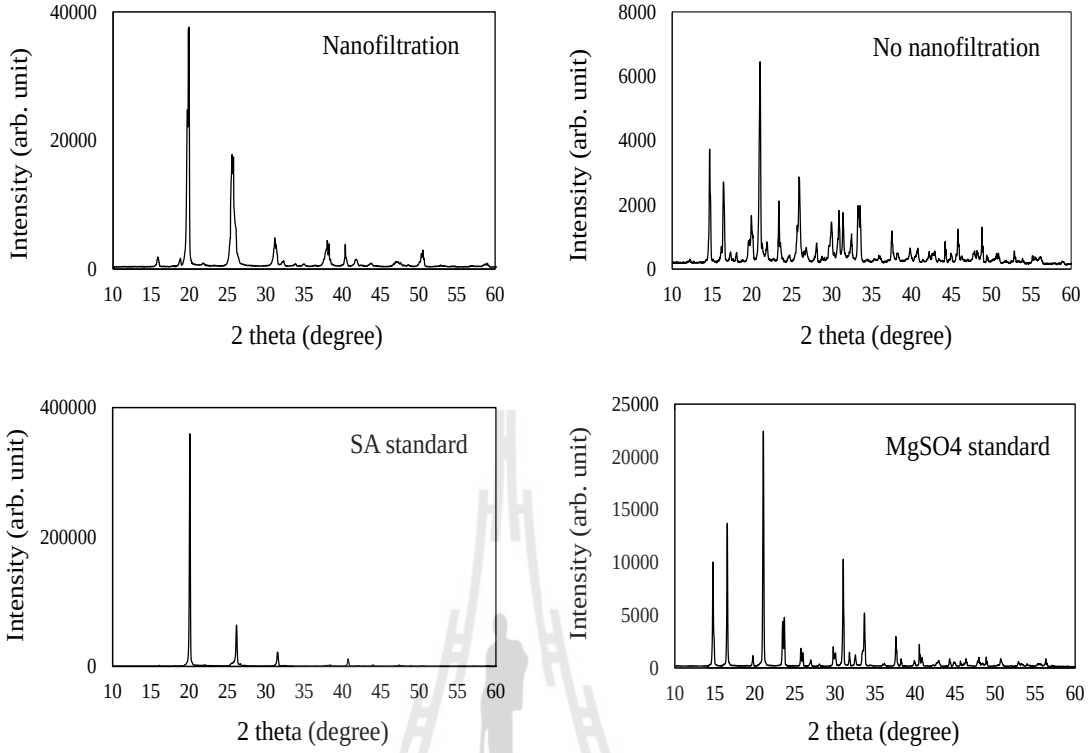


Figure 4.30 XRD patterns of obtained SA crystals after crystallization process compare with SA standard and $MgSO_4$ standard.

Besides that, compared with the standard XRD image of SA, the purity of the final purified SA crystals obtained from the process II_1 was not too different to the standard analytical reagent as shown in Figure 4.30. These could be explained that the process II_1 was treated by NF process resulting in the absence of the foulants. However, the process II still contained a complex mixture of fermentation broth not only just with brown colour, but also especially withthe presence of the $MgSO_4$ and suspended particles at high concentrationafter evaporation. Their presence is one of the main factors which prevents the crystallization and reduce the purity. This can be explained by the crystallization formation mechanism, with the combination of highly solute concentration and rapid cooling, that the supersaturation obtained and this was the driving force for crystal nucleation. When the solution supersaturation raised enough to be in the labile region, the

spontaneously nucleation can begin by the formation of the nucleus with so small size, this was more favorable and stable if have a certain fixture in order for those nucleus cling at the beginning. The presence of the $MgSO_4$ and suspended substances were also become the solid particles at high concentration and cold temperature, resulted in easily for the nucleus cling on them in order to continuously grow into the crystals. In addition, at the first phase of the nucleation or the first phase of the complete order of the fully lattice structure with very precise dimensions and ordered, the edge points usually have only 3 to 5 bonds (corners, edge and sides). This was the greatly chance of the impurities being able to fit in one of these empty places, resulted in low purity and very difficult to separate it out then, typically as the appearance of cells and proteins in the corners and gullies of the crystals in this research result. This was become a quite complexity problem in the purification process, which requiring more further refine treatment for obtaining the purity SA crystals. Moreover, the crystals were covered and immersed in the sticky-protein colloid solution was also one of the trouble problems for washing. The protein removal rate of the products from the process II_1 and process II_2 were calculated at 95.61% and 73.60%, respectively. In conclusion, all of those factors affected to the final SA crystal quality such as relative crystallinity just 3.37% compared with the SA standard. This was also confirmed by SEM image showed that was not only those impurities stuck on the SA crystals surface but also these SA crystals surface had the cracks, that why resulted in very low relative crystallinity in the obtained SA crystals (Figure 4.31).

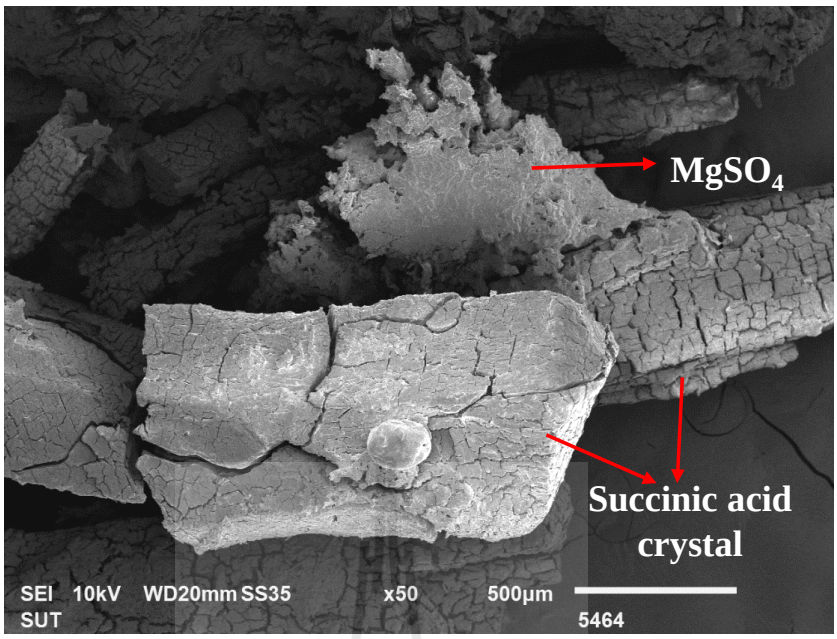


Figure 4.31 SEM image of SA crystals obtained from process II_2.

Table 4.4 Comparison of the purified product by two different crystallization processes.

	Process II_1	**Process II_2**
Recovery rate (%)	86.53	48.67
Purity (%)	90.26	40.64
Protein removal (%)	95.61	73.60
Optical density at 420nm (100 g/L solution)	0.02	0.22
SA-$MgSO_4$ (%)[a]	100-0	42.95-57.05

[a] Calculated by DIFFaX

Besides that, the crystalline crystals colour degree was also investigated by the measurement of optical density of 100 g/L solution of the final product at 420 nm wavelength with the result of 0.02 and 0.22 for the product from the process I and process II, respectively. The comparison of product properties of two these processes was illustrated in

Table 4.4. Conversely, the process I was assisted by the NF membrane resulting in overcame the above disadvantages, and brings the good results of high recovery rate and purity. In addition, the advantages of NF provided high SA product titers in the final broth after evaporation which was very helpful for the crystallization process due to shortened solubility and nucleation distance in order to go to the growth of crystals. Furthermore, the purity of starting materials plays an important role and influential decisions on the growth and crystalline perfection of good quality crystals was also successfully studied on potassium dihydrogen phosphate (Rajesh *et al.*, 2014). These remarkable results showed that crystallization can be considered as a method not only feasible for recovery efficiently final product, but also facilitated for the removing impurities by the selective crystallization This resulted in a highly purified crystalline acid product obtained from the fermentation broth.

4.3.4.3 Seed loading crystallization method (Process III)

The seed loading crystallization experiment was presented in Figure 4.32. The experimental result showed the effect of seed loading to the SA crystallization process. The particle size distribution (PSD) of the final crystal products using 0%; 5% and 10% seed loading were shown in Figure 4.33. Seeding results in an increase in the size of the PSD compare to the un-seeding run where high seeding resulted in the larger particle sizes with a clear distribution curve shift to the right side. For the low seeding experiment, the smaller particle size and narrower distribution close to the commercial succinic acid crystals with the median size of586 µm. In contrast, for the crystals product from un-seeding batch was notonly smaller median size of 260 µm, wider distribution width with span of 1.24 (Figure 4.33), butit also results in the almost thin-glass slice-like morphologyand also have few needles, rhombuses, spherical with low quality crystals was observed by the stereo microscope images as shown in Figure 4.34. The XRD results (Figure 4.35) showed that these

crystals had very low peaks intensity and relative crystallinity (shown in Table 4.6)was just 23.37% compared with the standard succinic acid crystal, which requiring further refine purify method for obtaining the reagent grade SA crystals. That results due to the un-seeding run had a larger metastable zone width implying that it underwent the excessive spontaneously nucleation stage leading to lower crystal purity and quality than seeding cases as presented in Table 4.5. In the other hand, nucleation rate for seeding operation was much lower than for un-seeding operation rate by the growth onto the seed crystals rather than the creation of nuclei (Nagy *et al.*, 2008a).

Figure 4.32 Crystallization experiment.

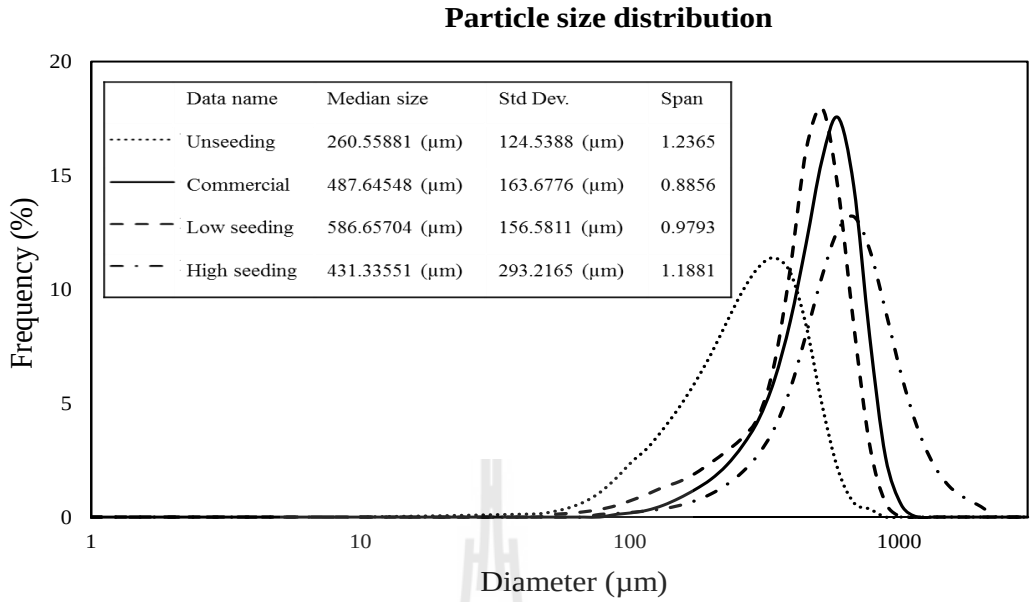


Figure 4.33 Comparisonof particle size distribution of obtained SA crystals after crystallization at each different condition with standard reagent.

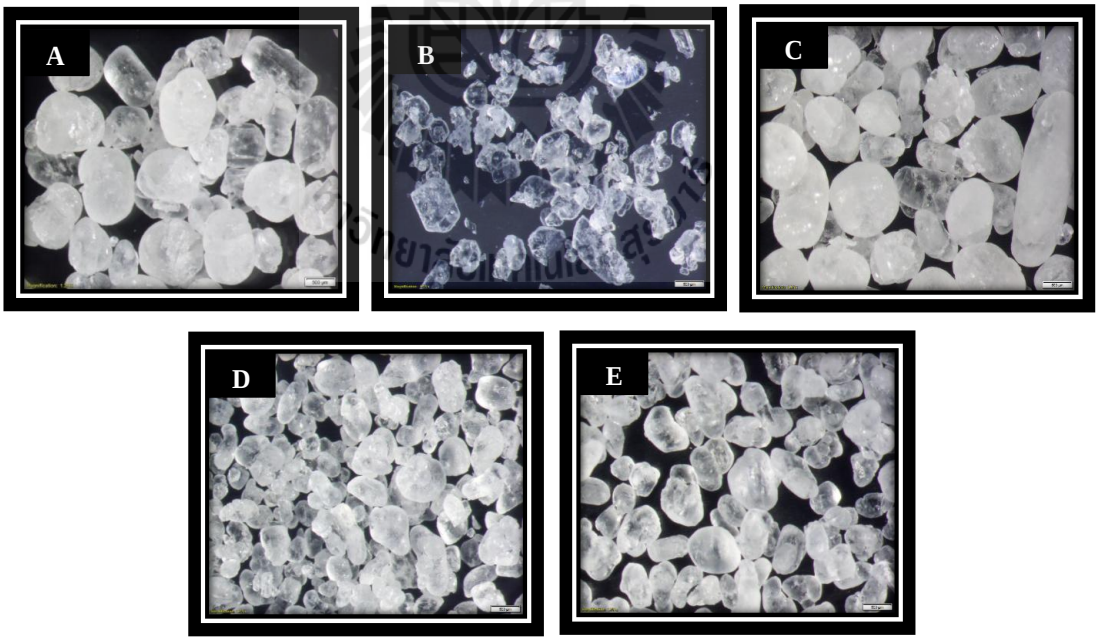


Figure 4.34 Stereomicroscopy images of SA crystals (the scale bar represents 500 µm with the magnification of 1.25×): (A) seed; (B) un-seeding; (C) high seeding; (D) low seeding; and (E) standard reagent.

However, the peak intensity of these crystals was still higher than that of crystals obtained from the process II_1. Although same in the initial SA material (NF permeate), the results were different by the different conducted methods. This could be explained by the effect of mixing during crystallization process. The agitation speed was chosen to be high enough to guarantee that particles were well suspended throughout the process and absorb the solutes, but low enough to avoid attrition or entrainment of bubbles due to vortex formation. At higher mixing rate, metastable zone width will be very narrow and more uniform supersaturation distribution, leading to the suppressed spontaneous nucleation, resulting in bigger SA crystals. Conversely, lower mixing rate, the inhomogeneity of supersaturation field, leading to high local supersaturation and spontaneous nucleation, resulting in the formation of smaller crystals with a large amount of agglomerates. If not mixing such as in the process II_1 case, the crystals are not only small size, but also combine each other becomes a block concluding much of the lamia crystals.

In the spontaneous nucleation, the surface of some substances such as dust particles or the container wall, etc., are regarded as is one of the fulcrums in order for the atoms or molecules of crystals become properly oriented and grip it to steady during the movement of liquid. This was easily observed by the stereo microscope image showed in Figure 4.36 which showed that the SA crystals were also stuck and grip on the impeller surface of stirrer for un-seeding case. This aspect illustrated that the agglomerates still formed. During un-seeding –crystallization, it consist of bigger crystals into the large block (Figure 4.36B), this was similar to the process II_1 as already mentioned and especially in the process II_2, the SA crystals very easy to be agglutinated by the $MgSO_4$ solid. Sander *et al*., 2012 also reported that the agglomerates of Pentaerythritol was formed from the very small particles during the spontaneously nucleation(Sander *et al*., 2012).

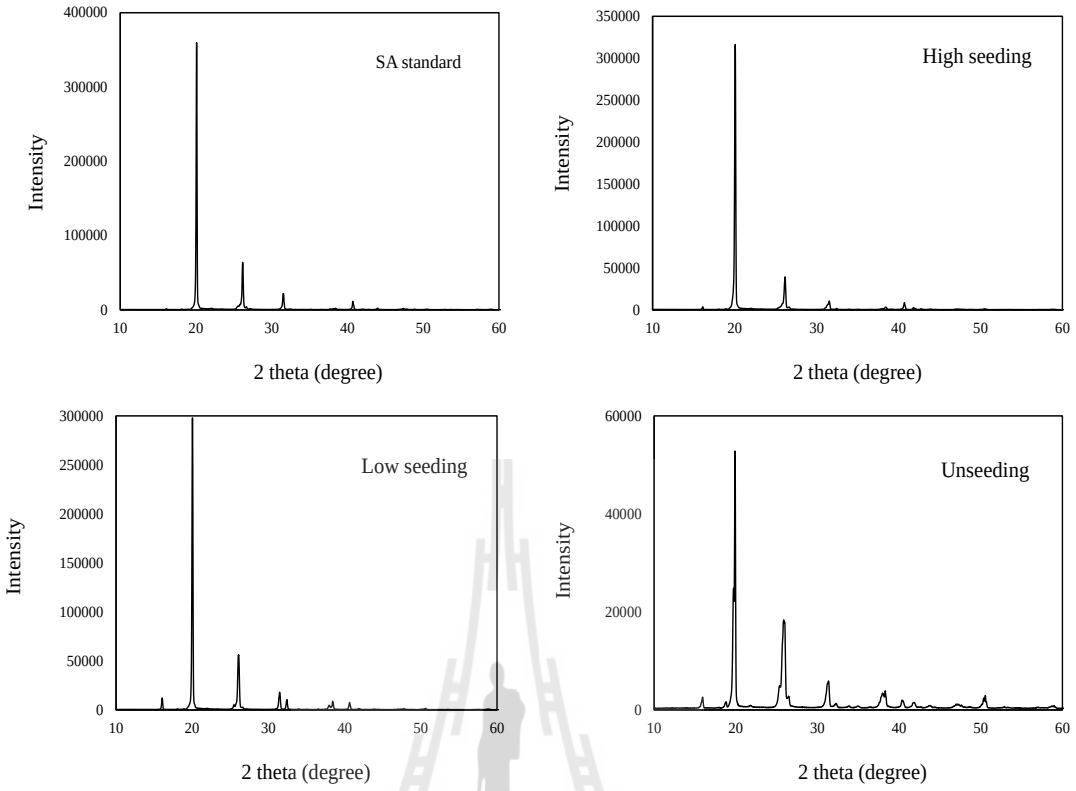


Figure 4.35 XRD pattern of obtained SA crystals after crystallization process and succinic acid standard reagent.

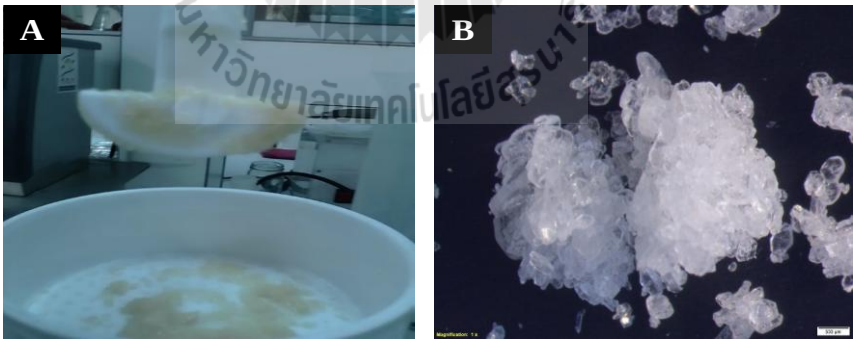


Figure 4.36 Agglomerates formed during un-seeding run (A): the SA crystals stuck on the stirrer from experiment (B): (stereo microscope image with the scale bar represents 500 µm by the magnification of 1X).

This is one of the factors facilitate for contacting with impurities. Crystal with smaller particle size has higher probability of entrapping mother liquor leading to lower

purity (Abidin *et al*., 2009).The smaller crystals size, the higher contacting capacity and the other residual carboxylic acids have stronger affinity leading to easily contaminate toward to the crystal particles surface. Therefore, the seeding strategy was at least partially successful in suppressing the primary nucleation in these experiments and avoids the agglomeration of the crystals (Ferguson *et al*., 2014; Nagy *et al*., 2008; Li *et al*., 2010).

Table 4.5 Result of SA crystallization trials.

	Low seeding (10%)	**High seeding (5%)**	**Unseeding ()%)**
Recovery rate (%)	93.23	92.46	95.02
Purity (%)	99.18	99.35	96.86
Glucose removal (%)	99.65	99.94	98.71
Protein removal (%)	99.85	99.97	99.50

Additionally, the crystals produced by seeding batches were found to have a difference both of cubic-morphology and crystallinity-structural quality to those generated by primary nucleation. In fact, one would expect that a high seeding should yield larger crystals than that with a low seeding. Because of the larger total surface area with a larger seed crystal amount, the secondary nucleation is suppressed. Consequently, the growth rate was dominated than the nucleation (Hermanto *et al*., 2013). This resulted in the larger product size in high seeding than the low seeding product. The result similar to as a publication of (Hermanto et al., 2013) that with the largest seed mean size, product mean size is proportional to the seed loading by the nucleation becomes less dominant than growth. Hence, they dragged on the larger amount of solutes which were adsorbed on the seed crystals surface by the growth. On the other hand, more supersaturation was consumed by crystals growth; therefore, itis not high enough to form the secondary

nucleation. Thus, the lower recovery rate in high seeding case than low seeding case is shown in Table 4.5.

Table 4.6 The effect of different methods on the relative crystallinity, phase purity and morphology of product.

Sample	Relative crystallinity (%)[a]	SA-$MgSO^4$ (%)[b]	Crystal shape[c]
Standard	100	100-0	Hexagonal board, spherical, short cylinder
Low seeding	92.92	100-0	Hexagonal board, spherical, short cylinder
High seeding	96.77	100-0	Hexagonal board, spherical, short cylinder
Unseeding	23.37	100-0	Glass slice, rhombus, needle, spherical

In addition, larger crystals will lead to an increase in purity (Table 4.5) due to their lower available contacted area in comparison tothe smaller crystals. Therefore, it limited entrapping by the impurities from the mother liquor. Moreover, their internal structure was apparent via the XRD patterns from (Figure 4.35) that a higher relative crystallinity of the smaller crystals are produced with lower seed loading than with higher seed loading. As shown in Table 4.5, the relative crystallinity of the high seeding sample was calculated to be 96.77% of the sample standard whichwas higher than the low seeding sample of 92.92%, and unseeding sample of just 23.37%. In addition, these results were also comfirmed by the SEM images as evidenced in (Figure 4.37). SEM pictures showed that the larger crystals from the low seeding (Figure 4.37C) had the rough surface, and much small

pores. This is different from the high seeding sample (Figure 4.37B) which have angurlar and look concreter.

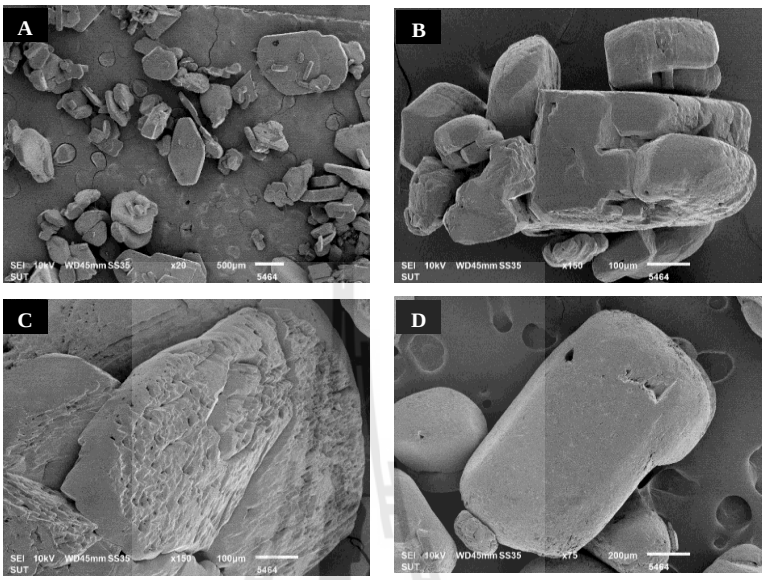


Figure 4.37 SEM image of SA crystals: (A) unseeding; (B) high seeding; (C) low seeding; (D) commercial reagent.

Thus, it could be said that seeding is one of the methods which can be used to control the succinic acid crystallization to generate the crystals more uniform in size, and shape (as the microscope and SEM images were shown in (Figure 4.34 & 4.37), respectively). It is also used to repress the the nucleation rate, especially is the primary nucleation in order to maximine the particle size with best distriction. Since seeded, the seed crystals enter into the growth phase via the absorption the nutrients on the seed crystals surface to grow to the larger crystals. Seeding at a certain time is also play a very important role for the controlling of SA crystallization. This is considered via temperature, and called seed temperature. If high seed temperature, the seed crystals will be easily dissolved and in constract, the new nucleies were formed before if low seed temperature. As shown in (Figure 4.38), the solubility curve of the matters in the broth also decrease with

decreasing temperture. After evaporation at 80^{o}C the solutes concentration was around 21 Brix, and it was also same at 55 ^{o}C. Then, it was start to decrease with decreasing temperature since 50 ^{o}C. At this temperature, the saturated concentration was reached and chosen as seed temperature for the seeding batchs. For low seeding, the superaturation still higher enough to occur the secondary nucleation when the cooling rate increased faster at second stage from 30 ^{o}C down to 4 ^{o}C than high seeding, while the supersaturation degree of high seeding just enough for the available crystals growth. Because of the supersaturation was generated by cooling and consumed by the growth of seed crystals added. This is the reason for the lower recovery rate of high seeding run than that of low seeding, and both were also lower than un-seeding run as presented in Table 4.5.

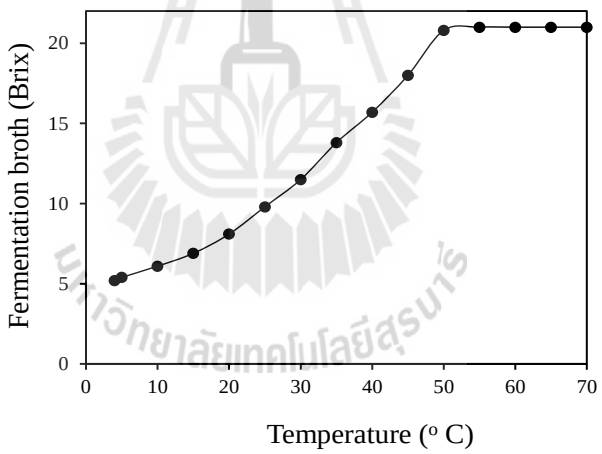


Figure 4.38 Solubility curve of the matter in mother liquor at different temperatures.

If the decreasing in the temperature was too fast since the begin stage or the crystallizing was too fast, the high supersturation maybe also created too fast implying that the impurities can be easily trapped. Nonetheless, in contrary by seeding, un-seeding run had a larger metastable zone width results that it was undergone the excessive spontaneously nucleation stage, and leading to lower purity and quality crystal than seeding cases with small size, wide distribution and un-uniform or more polymorphism (Nagy *et al.*,

2008b). In conclusion, crystallization is not only a widely used separation method for the solid-liquid mixtures based on its capacity to produce high purity product, but it is also the final refine and purify step for the production development of the crystalline compound. The NF system proved to be a valuable tool for the crystallization with the desired crystal properties of purity, shape, morphology and size distribution.

CHAPTER V

CONCLUSION

SA can be produced from cassava roots by *A. succinogenes* ATCC 55168. This strain efficiently produced SA with impressive titer, yields and productivity in an economical and feasible medium using fed-batch separate hydrolysis fermentation, and using $MgCO_3$ as neutralization reagent. A maximal titer, yield and productivity of succinic acid were 151.44 g/L, 1.51 $g_{SA}/g_{glucose}$ and 3.22 g/L.h, respectively which was supplemented by the diamonium phosphate. This fermentation performance at high titers facilitated for the sequentially crystallization step in order to separate, purify and refine for the production development of the high purity crystalline SA at the expected crystal properties of shape, morphology and size distribution.

The two membrane-based processes of MF and NF were successfully employed for the separation and purification of complexity SA fermentation media which are very promising for up-scale application. MF is an efficient operation for the rejection of the bacterial cells and insoluble matters. It was modelled by the resistance-in-series law concluding of membrane resistance, cake resistance, pore-blocking and adsorption resistance. Based on that, it is possible to predict their changing tendency during MF process in order to develop and apply in the up-scale. NF is highly potential as one of the methods used to effectively recover succinic acid from the fermentation broth. In addition, it is also regarded as one of the invaluable support tools not only for crystallization process, but it is also one of the steps use to evaluate final crystal quality. The high degree of purity, and high titer of the starting

material play the extremely important role on the growth and properties of good quality crystals. The highest succinic acid crystal purity of 99.35%, recovery rate of 92.46% and relative crystallinity of 96.77% were obtained in high seeding crystallization.Crystallization process is not only the final refine step to enhance the purity as high as quality of the final product, but also the first recovery step for the downstream separation process, which would be an efficient and economic supplement method for the downstream bioprocessing of the platform chemical SA. The integration of membrane-based process and cooling crystallization might directly provide the SA effectively desire product without the need for any the auxiliary components.

The results obtained in this study might be useful for reducing the cost of SA fermentation process and suggested an industrial potential of succinic acid production from an initial cheap biomass material to form the final bio-succinic acid crystal product.

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BIOGRAPHY

Miss Nguyen Thi Huong Thuy was born on 12th April 1991 in Quang Ngai, Viet Nam. In 2009, she graduated an upper secondary degree at Tran Ky Phong School, Quang Ngai, Viet Nam. In Jun 2013, she graduated a Bachelor degree in Storage and Processing of Agricultural Products at Hue University of Agricultural and Forestry, Hue province,Viet Nam.

In September 2013, she got a scholarship for a Master program from Assoc. Prof. Dr. Apichat Boontawan, Suranaree University of Technology, Thailand. She studied in the field of Bioprocessing engineering at School of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology from September 2013 to August 2016 with the thesis entitled “Fermentation and Purification of Succinic acid from Cassava broth using Membrane Filtration and Crystallization”.