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วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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EXTRACTIVE FERMENTATION OF SUCCINIC ACID USING AN EXTERNAL PRECIPITATION TECHNIQUE

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A Thesis Submitted in Partial Fulfillment of the Requirements for the

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EXTRACTIVE FERMENTATION OF SUCCINIC ACID USING AN EXTERNAL PRECIPITATION TECHNIQUE

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

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กระบวนการดัดแปลงการตกตะกอนของแคลเซียม ควบคู่การตกผลึก และกระบวนการตกผลึกโดยตรง ในการศึกษาครั้งนี้ถูกนำมาใช้ในกระบวนการทำบริสุทธิ์น้ำหมักกรดซัคซินิก และในการศึกษาครั้งนี้ได้มีการติดตั้งไมโครฟิวเตรชั่นเมมเบรนภายในถังปฏิกรณ์ชีวภาพ ซึ่งทำหน้าที่เพื่อขจัดเซลล์ และเพิ่มความเข้มข้นของเซลล์ ในการผลิตกรดซัคซินิคในกระบวนการหมักแบบหมักซ้ำ แคลเซียมคาร์บอเนตถูกนำมาใช้ในการปรับสภาพของน้ำหมัก ซึ่งนำมาแทนที่การใช้แมกนีเซียมคาร์บอเนตที่มีราคาแพง ในกระบวนการผลิตกรดซัคซินิก และใช้ในการตกตะกอนซัคซิเนตในด้วยเช่นเดียวกัน นอกจากนี้การใช้แคลเซียมคาร์บอเนตนั้น สามารถลดการใช้สารเคมีในกระบวนการตกตะกอน แบบดั้งเดิมได้อีกทางหนึ่ง ในกระบวนการทำบริสุทธิ์ขั้นสุดท้ายของผลิตภัณฑ์ในการศึกษาครั้งนี้ กระบวนการกลั่นภายใต้สภาวะสุญญากาศ และกระบวนการตกผลึกถูกนำมาใช้ในการทำบริสุทธิ์ขั้นสุดท้าย โดยค่าความเป็นกรด-ด่าง ของน้ำหมักถูกปรับให้ค่าเท่ากับ 2 ก่อนเข้าสู่กระบวนการกลั่นภายใต้สภาวะสุญญากาศ และกรดที่เป็นผลพลอยได้จากการหมักนั้นจะถูกกำจัดให้หมดไปภายในขั้นตอนนี้ จากนั้นเข้าสู่ขั้นตอนของกระบวนการตกผลึกของกรดซัคซินิกซึ่งจะดำเนินการภายใต้อุณหภูมิ 4°C และในการศึกษาครั้งนี้แสดงให้เห็นว่า การเก็บเกี่ยวกรดซัคซินิกจากน้ำหมัก ด้วยกระบวนการตกผลึกสามารถทำได้สำเร็จ โดยที่ความบริสุทธิ์ของผลึกกรดซัคซินิกสูงถึงร้อยละ 96-97 ในขณะที่ผลผลิตของผลึกกรดซัคซินิกจากกระบวนการตกผลึกโดยตรงให้ผลผลิตที่ร้อยละ 64 ส่วนผลผลิตจากกระบวนการดัดแปลง การตกตะกอนของแคลเซียมควบคู่กับการตกผลึกนั้น ให้ผลผลิตที่ร้อยละ 48 ผลึกของกรดซัคซินิกแต่ละกระบวนการถูกนำมาตรวจสอบคุณสมบัติทางสัณฐานของผลึก ภายใต้กล้องจุลทรรศน์อิเล็คตรอนชนิดส่องกราด โดยผลึกจากกระบวนการดัดแปลงการตกตะกอนของแคลเซียมควบคู่กับการตกผลึกนั้น มีลักษณะพื้นผิวใกล้เคียงกับผลึกของกรดซัคซินิค ที่เป็นสารเคมีเชิงพาณิชย์ จากผลการทดลองดังกล่าว แสดงให้เห็นว่าแคลเซียมคาร์บอเนตสามารถนำมาใช้ ในการปรับปรุงกระบวนการตกตะกอนของกรดซัคซินิกในถังตกตะกอน ภายนอกถังปฏิกรณ์ชีวภาพได้ และกระบวนการดัดแปลงการตกตะกอนของแคลเซียม ควบคู่กระบวนการตกผลึกนั้น สามารถนำมาใช้ในกระบวนการทำให้บริสุทธิ์ หรือกระบวนการหลังการผลิตทางชีวภาพ ของกรดซัคซินิกที่มี

ประสิทธิภาพได้อีกกระบวนการหนึ่งบริสุทธิ์ หรือกระบวนการหลังการผลิตทางชีวภาพของกรดซัคซินิกที่มีประสิทธิภาพได้อีกกระบวนการหนึ่ง

สาขาวิชาเทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา _____

ปีการศึกษา 2556 ลายมือชื่ออาจารย์ที่ปรึกษา _____

JIRAPHORN LUBSUNGNOEN : EXTRACTIVE FERMENTATION OF SUCCINIC ACID USING AN EXTERNAL PRECIPITATION TECHNIQUE. THESIS ADVISOR : ASST. PROF. APICHAT BOONTAWAN, Ph.D., 81 PP.

SUCCINIC ACID/EXTERNAL PRECIPITATION TECHNIQUE/MODIFIED PRECIPITATION/CRYSTALLIZATION

Modified calcium precipitation coupled with crystallization process and direct crystallization process was employed to purify succinic acid from fermentation broth. Microfiltration membrane was set up in bioreactor which was used to remove microbial cells and to increase cell concentration for the production of succinic acid in the repeated batch process. Calcium carbonate was used to neutralize acidity of fermentation broth and to precipitate the succinate. It was used to replace the expensive magnesium carbonate in the succinic acid production. Calcium carbonate was also used to reduce the dosages of chemical in traditional precipitation method. Vacuum distillation and crystallization were used in the final purification step. The pH of fermentation broth was adjusted to 2.0 before vacuum distillation. By-product acids were removed under vacuum distillation. Crystallization of succinic acid from fermentation broth was carried out at 4°C. Succinic acid crystals were successfully recovered from the fermentation broth. The succinic acid crystal was 96% purity and 64% yield in direct crystallization process. In comparison, modified calcium precipitation gave the purity and yield of 97% and 48%, respectively. Scanning electron microscope was used to characterize the succinic acid crystal morphology;

the crystal from modified calcium precipitation process was similar to that of commercial succinic acid reagent with a few impurities near the surface of the crystal. These results demonstrated that calcium carbonate can be used to improve precipitation process in the external precipitation tank. Modified calcium precipitation process coupled with crystallization process would be efficient method for the downstream bioprocessing of succinic acid.

School of Biotechnology Student's Signature _____

Academic Year 2013 Advisor's Signature _____

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Jiraphorn Lubsungnoen

CONTENTS

Page

CONTENTS (Continued)

Page

CONTENTS (Continued)

Page

CONTENTS (Continued)

Page

LIST OF TABLES

Table **Page**

LIST OF FIGURES

Figure **Page**

LIST OF FIGURES (Continued)

Figure **Page**

LIST OF ABBREVIATIONS

$g\ mol^{-1}$	=	Gram per Mole
$g\ cm^{3}$	=	Gram per cubic centimeter
°F	=	Degree Fahrenheit
°C	=	Degree Celsius
%	=	Percent
$ton\ yr^{-1}$	=	Ton per Year
w/w	=	Weigh by Weigh
v/v	=	Volume by Volume
$g\ L^{-1}$	=	Gram per Liter
$g\ L^{-1}\ h^{-1}$	=	Gran per Liter per Hour
$mg\ mL^{-1}$	=	Milligram per Milliliter
g	=	Gram
$g\ g^{-1}$	=	Gram per Gram
Da	=	Dalton
µm	=	Micrometer
nm	=	Nanometer
L	=	Liter
mL	=	Milliliter
rpm	=	Round per Minute
vvm	=	Volume
min	=	Minute

LIST OF ABBREVIATIONS (Continued)

h	=	Hour
et. al.,	=	and others

CHAPTER I

INTRODUCTION

1.1 Significant of study

The development of bio-refineries has recently attracted increasing attention as a means of providing sustainable alternative solutions to convert renewable agricultural materials into numerous economically viable products, and offer competitive performance compared to traditional petrochemical refineries. Many chemicals that could only be produced by chemical processes in the past could potentially be generated biologically from annually renewable resources. Since the global demand for green chemicals increases, succinic acid revealed its large potential for a variety of the applications. Succinic acid is considered as one of the twelve bio-based chemicals by the U.S. department of energy, and has drawn worldwide interest for its industrial application in the foods, fine chemical, pharmaceuticals, and especially biodegradable polymers (bio-plastics).

Currently, there is the most considerable interest on the development of biodegradable polymer since global warming problem has come. Many countries all over the world including Thailand require adding more utilization and market share from bio-plastics. Large succinic acid production companies are interest on the development of manufacturing base in Thailand. BioAmber has partnered with PTT-MCC Biochem, a joint venture between PTT PLC and Mitsubishi Chemical. BioAmber and Mitsui will build a bio-based succinic acid plant in Thailand.

PTT-MCC Biochem selected BioAmber as its partner for the polybutylene succinate (PBS) plant that it plans to commission in 2014. Succinic acid is a raw material used to produce PBS (BioAmber, 2014). Therefore, the country has high potential for development of succinic acid production in order to support bio-plastics industry. Succinic acid is traditionally produced from the petrochemicals through expensive process. Therefore, much attention has been paid to the microbial conversion of the biomass to succinic acid and the recovery of succinic acid from the fermentation broth.

As the end product of energy metabolism, succinic acid can be produced by many anaerobic microbes, such as *Actinobacillus succinogenes*, *Anaerobiospirillum succiniciproducens*, *Mannheinia succinipiproducens*, and *Esheriachia coli* (Li *et al.,* 2012). Typical process for the production of a bio-product like succinic acid by the microbial fermentation consists of seed cultivation, fermentation, product recovery, concentration and purification. The cost of downstream processing can make a very high portion in the total production cost, mounting up to about 50% - 70%. It is necessary to minimize the production cost of the biotechnological process in order to increase its competitiveness with that of petrochemical process (Cheng *et al*., 2012), and it is crucial to develop an economical purification process of succinic acid from fermentation broth. The primary challenges are low product concentration in the fermentation broth, the presence of byproducts including acetic, formic, lactic and pyruvic acids (Song *et al*., 2006), and the requirement of pH control during fermentation that leads to the succinic acid being present in salt form. In addition, product inhibition is an important factor for the bioprocess development since succinic acid is very toxic to the bacterial cells. In order to increase fermentation

performance, succinic acid must be removed from the reaction site as soon as it is produced. This concept is call *in situ* product removal or extractive fermentation. It combines biochemical reaction and product recovery in a single unit operation. Different techniques have been introduced for recovery of succinic acid including direct precipitation, solvent extraction, ion exchange, chromatography, and membrane separation, respectively. Among these processes, precipitation is a very interesting technique due to its simplicity, and no does not require additional chemical or unit operation. If the precipitation of succinic acid can be achieved outside the bioreactor, shifts in the fermentation performance especially yield and volumetric productivity could be obtained.

In this work, fermentation of succinic acid was investigated by using a type strain of succinic acid- producing bacteria. Calcium carbonate was used to control the pH of the broth. A submerge ceramic microfiltration membrane was employed for taking out the clarified broth prior to precipitation by lowering the temperature to an optimum point. As a result, calcium succinate was precipitated out, the precipitate was harvested and further purification by the crystallization method was attempted in order to produce a high purity of succinic acid.

1.2 Research objectives

The main objectives of this study are as follows:

1. To compare succinic acid production performance in term of yield and productivity between the extractive fermentation and the conventional fermentation using calcium precipitation process.

2. To purify succinic acid from the fermentation broth using modified calcium precipitation process.

1.3 Research hypothesis

1. Succinic acid can be produced by using *Actinobacillus succinogenes* ATCC 55618.

2. Precipitation of calcium succinate can be achieved in the external precipitation tank.

3. Purification of succinic acid from the fermentation broth is difficult and expensive.

1.4 Scope of the thesis

This work consists of two main parts. The first part was succinic acid fermentation process that used *Actinobacillus succinogenes* ATCC 55618 as the succinic acid producer. This stain was commercially available, and was purchased from American Type Culture Collection (ATCC, USA). The next part was downstream processing of succinic acid from fermentation broth by using an external precipitation technique. Calcium carbonate ($CaCO_3$) was used to control the pH of the fermentation broth. During the fermentation process, the calcium succinate presented in a soluble form was removed from the bioreactor with the help of a submerge microfiltration (MF) membrane unit. The clarified fermentation broth was transferred into the precipitation tank where the solution temperature was controlled in order to induce precipitation of calcium succinate.

1.5 Expected results

1. High volumetric productivity and the final concentration of succinic acid will be achieved using the repeated batch process with cell re-cycling.

2. High purity and product yield of succinic acid will be obtained using calcium precipitation method and crystallization method.

3. This downstream process can be applied to the industrial scale production with improved of succinic acid and reduced production cost.

CHAPTER II

LITERLATURE REVIEWS

2.1 Succinic acid

Succinic acid (SA) or 1, 4 butanedioic acid (IUPAC systematic name) is an organic acid having the molecular formula of $C_4H_6O_4$ shown in Figure 2.1. Succinic acid can also be converted maleic acid and fumaric acid by oxidation as shown in Figure 2.2. They have the molecular formula: $C_4H_4O_4$. It is a di-carboxylic acid of four carbon atoms. It occurs naturally in plant and animal tissues. It is very important for body because it is used in the Krebs cycle (citric acid cycle), and involved in the intermediary metabolic process. Succinic acid that has become a chemical of the significance in recent years. There is growing interest in the production of succinic acid from renewable resources by microbial fermentation because succinic acid can be used in numerous applications. Application of succinic acid is wildly in many industries such as food industry, pharmaceuticals industry, agriculture industry, cosmetic, photography and textile. Succinic acid was classified by the U.S. Department of energy (2004) as one of the top 12 biomass derived platform chemicals. Physico-chemical properties of succinic acid are shown in Table 2.1.

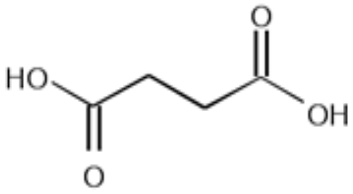

Figure 2.1 The chemical structure of succinic acid (http://chemsynthesis.com).

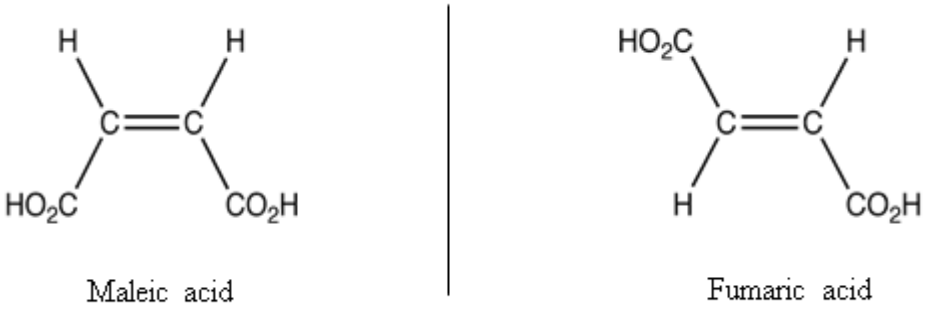

Figure 2.2 The chemical structure of cis and trans-succinic (butanedioic) acid (http://science.uvu.edu).

Table 2.1 Physicochemical properties of succinic acid (http://en.wikipedia.org).

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 (pK_a)	$pK_a1 = 4.2$, $pK_a2 = 5.6$

Succinic acid can be used as a precursor of many industrially important chemicals including adipic acid, 1,4-butanediol, tetrahydrofuran, N-methyl pyrrolidinone, 2-pyrrolidinone, succinate salts and gamma-butyrolactone (Figure 2.3). More importantly, it is used to the synthesis of biodegradable polymers such as polybutyrate succinate (PBS) and polyamide (Nylon®x,4) to produce bio-plastics (McKinley *et al.,* 2007). Nonetheless, the global succinic acid market is much smaller compared with that of the competing chemical, maleic anhydride. This is partially due to the high conversion cost of maleic anhydride to succinic acid by the chemical process, which limits the use of succinic acid for the wide range of applications. On the other hand, recent analysis showed that fermentative production of succinic acid from renewable resources can be more cost-effective than the petroleum-based processes (Song and Lee, 2006).

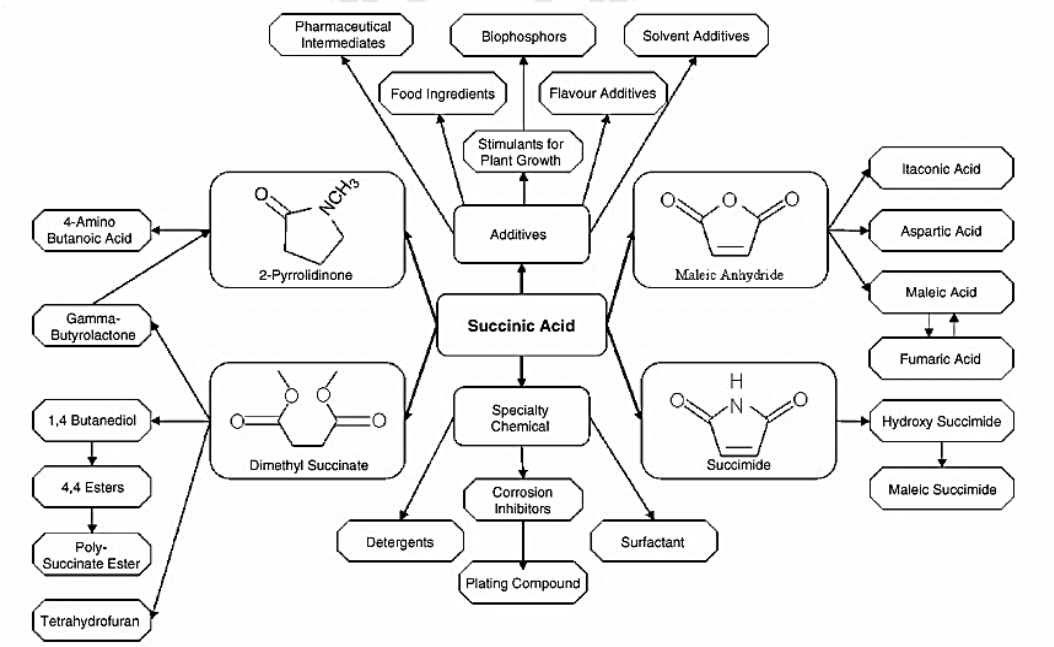

Figure 2.3 Various chemicals and products that can be synthesized from succinic acid (Song and Lee, 2006).

2.1.1 Succinic acid market

At present, succinic acid is commercially produced from the chemical process by the partial oxidation of n-butane, followed by hydrogenation of the maleic anhydride, the cost of butane is approximately 850 – 950 \$ ton^{-1} (McKinley *et al.,* 2007). The price of succinic acid is reporter to be in the range of 5.9-9.0 \$ kg^{-1} depending on its purity. Succinic acid manufacturing cost is affected by several factors including succinic acid productivity and yield, the costs of raw materials, and recovery method. Particularly, the cost of maleic anhydride has been known to contribute most significantly to the overall cost of succinic acid production (Song and Lee, 2006), and the hydrogenation process produces many pollutants by- product that makes the purification of succinic acid costly. The large scale of fermentative succinate was produced in early 1980. Currently, fermentative succinate production is about 5,000 tons yr^{-1} and is sold at 2.20 \$ kg^{-1} to the food market. As expected, biological succinic acid price would be decrease by 0.55 \$ kg^{-1} if production size would be above 75,000 tons due to utilization of the cheap carbon substrates such as corn, starch, molasses and sugars (Zeikus *et al.,*1999). Alternatively, succinic acid can be produced from glucose by fermentation since more than 30,000,000 tons of glucose is produced annually worldwide. The glucose price is approximately 220 - 250 \$ ton^{-1}. Assuming the succinic acid yield of 91% (w/w) on glucose, it is thus clear that fermentative production of succinic acid from the renewable resources can compete with the chemical production process. More importantly, the limited nature of fossil reserves and ever increasing environmental concerns are urging researchers to replace the petroleum-based chemical processes with bio-based processes, and hence there is intense research on the topic in many leading academic and industrial

sectors. Biological succinic acid is poised to replace the petrochemical production of maleic anhydride (2.2 million tons yr^{-1}) (U .S. Department of Energy, 2004), and supply the emerging market for succinic acid and its derivatives (30 million yr^{-1}). BioAmber, Reverdia, Myriant Technologies and DSM-Purac are the companies that are at the forefront of the industrial succinic acid production; their combined production is expected to reach 70,000 tons yr^{-1} by 2013. The projected production is capable of only supplying 0.2 % of the total succinic acid market (McKinley *et al.,* 2007). Although the overall economics still limits the bio-based succinic acid production, the assessment of the raw material cost and the estimation of potential market size clearly suggest that the current petroleum-based succinic acid process will be replaced by the fermentative succinic acid production system in the near future.

2.1.2 Production of succinic acid by chemical process

Succinic acid is commercially produced by the petrochemical resources especially maleic anhydride, which is produced from n-butane through oxidation over vanadium-phosphorous oxide catalysts. Reaction pathway of n-butane to maleic anhydride is shown in Figure 2.4.

Figure 2.4 Reaction pathway of n-butane to maleic anhydride (Hapbern, 2011).

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 succinic acid. However, succinic acid produced from fossil fuels is what gives it the distinction of not being a natural product (Song and Lee, 2006). While this method of the production is currently cheaper than processing by fermentation, there are some very large drawbacks. As the term petrochemical processing implies, succinic acid is made using non-renewable resources such as neutral gas which will become more difficult to find as time passes. As this raw material becomes harder to locate and demand continuous to increase, it will become increasingly expensive. This process is not sustainable in the long term and another solution is required if industries want to continuous producing succinic acid with increasing demand.

2.1.3 Production of succinic acid by fermentation process

The process of fermentation is receiving increasing attention as it can use renewable feedstock as substrates, and is seen as a more green technology compared to chemical production because of its renewable resource consumption and its limited impact on the environment. Alternatively succinic acid can be produced from glucose by fermentation, when produced through fermentation; glucose is converted to succinic acid along a portion of the reductive cycle of the tricarboxylic acid (TCA) cycle (Lee *et al*., 2002). Figure 2.5 demonstrates the reactions and enzymes in typical fermentation process that transforms glucose to succinic acid.

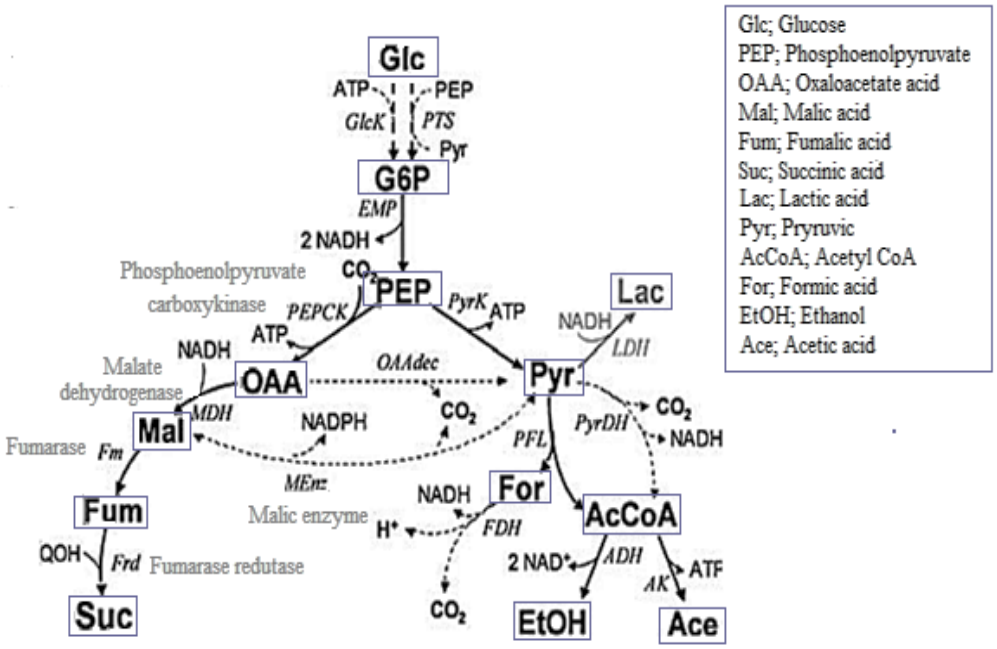

Figure 2.5 Metabolic pathway of typical succinic acid producing microorganism (McKinley *et al.,* 2007).

First, glucose is converted to glucose-6-phosphate by hexokinase, which also adds phosphate to the molecule. Next, three separate enzymes that are part of the Embden-Meyerhoff-Parnas glycolytic pathway lead to the production of phospho-enol-pyruvate (PEP). From PEP, the metabolic pathway can take one of two paths depending on the level of carbon dioxide (CO_2) available to the system. If there is not enough CO_2 present in the system, the preferred metabolic pathway creates end products of formate (For), ethanol (EtOH) and acetate (Ace), as shown on the right side of figure 2.5. With ample supply of CO_2 to the system, the microorganism favours the production of succinic acid, the left half of Figure 2.5 (McKinlay *et al.,* 2007). Through this pathway, PEP is converted to oxaloacetate (OAA) by PEP carboxykinase (PEPCK) with the addition of CO_2. This creates four carbon chains, giving this series of reactions the name ‘C4 pathway’ (Lee *et al.,*

2008). The presence of high levels of carbon dioxide in the system strongly regulates the activity of PEPCK. The next reaction adds hydrogen to oxaloacetate (OAA) to produce malate (Mal), which is converted to fumarate (Fum) by fumarase (FR) with the removal of a water molecule. Finally, with the addition of hydrogen, succinate is formed in its ionic state, which is common as the pH range of production is above the pKa values for succinic acid. The theoretical yield of succinic acid from glucose with carbon dioxide should be 1.17 moles per mole of glucose based on stoichiometry (McKinley *et al.,* 2007). This production method is not without unwanted by-products acid. Values of these are lower than the final concentration of succinic acid, but still make the separation process more time consuming and costly.

For the bio-production of succinic acid to be economical, major areas of biological process improvement need to be addressed, including limiting the use of low-cost amino acids, achieving high yield and concentration as well as using inexpensive carbon sources. Fermentation requires both substrate and media which contain the energy sources, nutrients and minerals needed to ensure optimal productivity rates (Lee *et al*., 2002). Depending on the microorganism chosen for the fermentation process, there are many carbon sources available. In the case of succinic acid production, most of the major sugars present in biomass can be used effectively, including glucose, fructose, arabinose and xylose (McKinley *et al*., 2007). Whilst the cost of these sugars is relatively low, other carbon sources have been examined, in attempts to lower the cost of fermentation. This information makes it clear that the fermentation process offers enormous possibilities for industrial scale production.

2.2 Microorganisms for producing succinic acid

Numerous microorganisms have been isolated and metabolically engineered for the fermentative production of succinic acid. Two main groups involved are fungi and bacteria.

2.2.1 Fungi

Many researchers have made tremendous efforts to develop a biological process for the production of succinic acid by employing fungi such as *Aspergillus niger, Aspergillus fumi-gatus, Byssochlamys nivea, Lentinus degener, Paecilomyces varioti, Penicillium viniferum* and yeast *Saccharomyces cerevisiae*. These organisms produce succinic acid as a metabolic byproduct under aerobic and/or anaerobic conditions. However, the use of fungi has been mostly limited to the manufacture of food and beverages due to the difficulties in fermentation, separation and purification as well as low productivities (Song and Lee, 2006).

2.2.2 *Actinobacillus succinogenes* and other bacteria

The rumen is the primary chamber of the stomach of a ruminant animal; it is here that succinic acid is found (Lee *et al,* 2002). The bacteria that produce succinic acid in greatest concentrations and those that are the focus of the majority of research are *Actinobacillus succinogenes, Anaerobiospirillum succinoproducens,* and *Mannheimia succiniciproducens*. Several *Escherichia coli* strains have been genetically engineered for succinic acid production, but are not natural to ruminant animals (Hapbern, 2011).

The main succinic acid producing bacteria that are the focus of much of literature was evaluated, and the most selected was *Actinobacillus succinogenes*, in particular stain 130Z, ATCC 55618. This bacterium was isolated from bovine rumen at the Michigan biotechnology institute international in Lansing (Guettler *et al.,* 1999). This strain is facultative anaerobic, capnophilic, gram-negative, rod shaped or occasionally filamentous, non-motile bacterium and is considered to have the most potential as a succinic acid producer in an industrial setting (Wan *et al.,*2008). Compared to the other major producers of succinic acid, there are several advantages of this bacterium including tolerance to high level of substrate, high resistance to product inhibition, and high production rate. It also has a high tolerance of oxygen, low production of unwanted acid by-product, and can use a number of substrate in comparison to other succinic acid producing bacteria. Many sources state that *A. succinogenes* is the microorganism of the choice for creating an industrial process, having a high tolerance of succinic acid (Lin *et al.,* 2008).

The Gram-positive bacteria like *Corynebacterium glutamicum* and *Enterococcus faecalis* have been documented to be an excellent succinic acid producer from fumaric acid. However, it should be noted that yield of fumaric acid on glucose was quite low, limiting its commercialization.

The effectiveness of the bio-based succinic acid production process depends on the efficiency of the microorganism to convert the substrate to succinic acid, without excessive by-product formation and with minimal substrate usage toward cell growth and maintenance of cell functions. In theory, 2 mole of succinic acid can be produced from 1 mole of glucose and 2 mole of CO_2 (yield of 1.31 g succinic acid produced per 1 g of glucose consumed, $Y_{P/S}$) provided that all carbon

flux is toward succinic acid production. The use of the vastly different carbon sources, nutrient source and mineral salts affects the microorganism's effectiveness to produce succinic acid. Table 2.2 gives a summary of the component in succinic acid fermentation broth using different bacteria species.

Another factor to consider is how product inhibition affects cell growth. As products accumulate in the aqueous phase of the fermentation, production rates decrease as the cells are forced to spend more energy maintaining the cell rather than fermentation and cell growth. Cells that have a higher tolerance of products can continue producing quickly and give a higher final concentration. The succinic acid production ranges mentioned earlier seem to indicate that there is an upper limit on the final concentration achievable from *A. succinogenes* possibly due to end product inhibition. A previous study showed that *A. succinogenes* could withstand a succinic acid concentration for up to 66.4 gL^{-1} before the production ceased after 84 h, showing that the production can continue up to high concentrations before system reaches end product inhibition (Wan *et al*., 2008). All of the bacteria that can ferment glucose to succinic acid require anaerobic conditions to function properly. This is not the case with *A. succinogenes*, however, and a small amount of oxygen can be tolerated in the headspace of the fermenter (Urbance *et al.,* 2004). This tolerance adds to the list of desirable qualities in a bacterial strain for producing succinic acid as there is less preparation involved in removing oxygen from the system.

Table 2.2 The components in succinic acid fermentation broth using different bacteria species (McKinley *et al*., 2007).

Strains	Components in broth (gL^{-1})													Substrates
	Suc	***Mal***	***Pyr***	***Ace***	***For***	***Lac***	***Cit***	***Eth***	***Gly***	***Glu***	***Xyl***	***Ara***	***Pro***	
A. succinogenes FZ53 (based on ATCC55618)	105.8	-	2.3	18.1	0.7	-	-	-	-	-	-	-	1.9	Glucose
A. succinogenes FZ6 (based on ATCC55618)	70.6	-	2.3	2.8	0.3	-	-	-	-	2.4	1.4	0.9	3	Corn fiber hydrolysate
A. succinogenes CGMCC2650	97.8	-	-	17.4	22.5	5.1	-	-	-	-	-	-	-	Glucose
A. succiniciproducens ATCC53488	50.3	-	-	13.6	1.3	-	-	-	-	1.9	-	-	-	Glucose
A. succiniciproducens ATCC29305	19	-	-	0.6	-	-	-	-	7.5	-	-	-	-	Glucose
M. succiniciproducens KCTC 0769BP	8.8	-	-	3.9	3.6	1	-	-	-	-	-	-	-	Glucose
M. succiniciproducens KCTC 10626BP	52.4	12.3	11.7	0.8	-	0.3	-	-	-	-	-	-	-	Glucose
S. cerevisiae SUC-200 (based on CEN.PK113-6B)	34.5	7.8	-	-	-	-	-	4.5	7.7	-	-	-	-	Glucose
S. cerevisiae SUC-297 (based on CEN.PK113-6B)	43	-	-	-	-	-	-	16.4	14.9	-	-	-	-	Glucose
E. coli AFP111-*pyc* (based on ATCC202021)	99.2	-	-	9.5	-	-	-	4.8	-	4.2	-	-	-	Glucose

Table 2.2 (Continued) The components in succinic acid fermentation broth using different bacteria species (McKinley *et al*., 2007).

Strains	Components in broth (gL^{-1})													Substrates
	Suc	***Mal***	***Pyr***	***Ace***	***For***	***Lac***	***Cit***	***Eth***	***Gly***	***Glu***	***Xyl***	***Ara***	***Pro***	
E. coli KJ073 (based on ATCC8937)	86.5	5.2	-	15	-	0.2	-	-	-	-	-	-	-	Glucose
E. coli KJ060 (based on ATCC8937)	78.8	15.8	4.8	11	-	-	-	-	-	-	-	-	-	Glucose
E. coli SBS550MG-PHL413 (based on ATCC47076™)	45	-	0.1	-	0.8		4.7	-	-	-	-	-	-	Glucose
E. coli SD121 (based on ATCC12435)	57.8	-	-	8.2	-	-	-	1.6	-	-	-	-	-	Corn stalk enzymatic hydrolysate
*C. glutamicumΔldh*A-pCRA717(based on FERMP18976)	146	-	-	16	-	-	-	-	-	10	-	-	-	Glucose

For abbreviations; ***Suc*** succinic acid, ***Mal*** malic acid, ***Pyr*** pyruvic acid, ***Ace*** acetic acid, ***For*** formic acid, ***Lac*** lactic acid, ***Cit*** citric acid, ***Eth*** Ethanol, ***Gyl*** glycerol, ***Glu*** glucose, ***Xyl*** xylose, ***Ara*** arabinose, and ***Pro*** propionic acid.

The use of *A. succinogenes* for the production of succinic acid yields far fewer by-products compared to other bacteria tested, making the separation process simpler and less costly. The microorganism is natural succinic acid producer capable of producing 130 gL^{-1} of succinic acid (Guettler *et al.,* 1996), this concentration shows that *A. succinogenes* is an ideal choice for use in the production of succinic acid. *A. succinogenes* is also a moderate osmophile capable of tolerating high glucose concentration of up to 160 gL^{-1} (Lin *et al.,* 2008), and can grow from a wide variety of carbon sources. In addition, this organism can ferment L-arabinose, cellobiose, fructose, galactose, glucose, lactose, maltose, manitol, mannose, sucrose, D-xylose and salacin (Zeikus *et al.,* 1999).

2.3 Limitations for the fermentation of succinic acid

In a previous study for succinic acid production by *A. succinogenes*, fermentation took places in a defined pH range between 6.0 and 7.2 with the optimal pH for fermentation at 6.8 (Wan *et al.,* 2008). A higher pH will lead to higher cell growth but will increase the amount of the by-products formed. Below a pH of 6.0, little cell growth occurred due to increased cell maintenance demand (Lee *et al.,* 2002). Unfortunately, the pK_a values of succinic acid are 4.20 and 5.61. So when succinic acid is produced it is as a dissociated molecule. This is a problem because most separation methods require the undissociated acid form of succinic acid; hence additional steps are needed to lower the pH to remove products.

Throughout the fermentation process, succinic acid is produced as well as the by-products formic and acetic acid. Because of this, an alkali is required to be constantly added to the fermentation to keep the process operating at or near its

optimal pH. Fermentation reactions also have problems because cells are living organisms and as production take place, product inhibition can occur. Cell growth and synthesis can stop because more energy is required to maintain cells. The formation of by-products can also hinder fermentation because it can have a similar effect to product inhibition at lower concentrations. As is the case with succinic acid fermentation, by-products such as acetate and formate can limit succinic acid production as well as take away from the carbon source used by the bacteria to make the main product, reducing the yield of the desired product (Huh *et al.,* 2006).The idea of the altering organisms in a way to reduce or eliminate by-products has been suggested, some work has been done to alter the genetic code of the bacteria to ensure that only specific genes are expressed, limiting the number of unwanted products (Lee *et al.,* 2008). However, this can lead to decrease in growth and production rates. Because of these limitations, recovery volume and yield are not high enough to warrant fermentation in large-scale applications using these genetically modified strains.

As it stands, current fermentation technology is not yet financially or operationally competitive with the production of succinic acid from butane. The major problem outlined by the US Department of Energy is the cost of fermentation, and significant improvement needs to occur if this process is to be used on an industrial scale. In addition to this production rate, purification stands as the largest expense of the succinic acid production process and is a major area for improvement (McKinley *et al.,* 2007).

2.4 Downstream processing of succinic acid

Succinic acid production can be broken down into two major components, fermentation of the carbohydrates to succinic acid and its separation and purification. The step of separation and purification poses the most challenges in the overall process due to the problems mentioned previously with fermentation, but it is also the most promising step to improve the economics of the process (Zeikus *et al*., 1999). Product recovery, concentration, acidification and purification are the steps necessary to get succinic acid from succinate in the fermentation broth, and currently, there is no single approach that meets all of these requirements. In the case of succinic acid purification, removal of by-product including acetic, formic, lactic and pyruvic acids from fermentation broth is necessary to prevent inhibition, optimize organic acid production. The separation methods studied for succinate recovery include crystallization, precipitation, membrane separation, extraction, chromatography, and *in situ* separation.

The downstream process of the biologically produced succinic acid usually includes three main steps. The first step is the removal of the microbial cells, by using membrane filtration or centrifugation. Next step is the removal of the impurities and primary separation of succinic acid from the fermentation broth, e.g., using evaporation for removal of water or acetic acid, precipitation, electrodialysis, solvent extraction, reactive extraction, and adsorption with ion exchange resin, active charcoal, molecular sieve, or zeolite. The last step is final purification of succinic acid by vacuum evaporation and crystallization (Cheng *et al.,* 2012).

2.4.1 Direct crystallization

A direct vacuum distillation–crystallization was used for succinic acid recovery from fermentation broth by Luque *et al*., (2009). The pH of the aqueous broth was adjusted to 4.2 by addition of hydrochloric acid before vacuum distillation. Some volatile by-product carboxylic acids, such as acetic, formic, lactic acids, in broth were removed under vacuum distillation at 60 °C. The followed crystallization of succinic acid was carried out at 4 °C. When this method was used in a simulated broth, the highest succinic acid yield and purity were obtained at 75 % and 97%, respectively. Direct crystallization method for recovery of succinic acid was also studied. The principle in this method is that carboxylic acids have varied distribution between dissociated and un-dissociated forms at the varied pH, and the undissociated carboxylic acid has different solubility. In this study, the solubility of succinic acid was only 3% at 4°C and pH 2.0 while the other acid by-products were still fully water miscible. Therefore, succinic acid could be selectively crystallized.

By this one-step recovery method, succinic acid yield and purity were 70% and 90%, respectively (Li *et al.,* 2010). Crystallization process could be used usually as the final purification step. Direct crystallization might provide the desired product (in solid or crystal form) without the need for many unit operations. However, the product yield is low because much succinate is still residual in the broth and the low purity product cannot be used as a monomer for polymerization (Cheng *et al.,* 2012).

2.4.2 Membrane separation

Membrane filtration has been tested for the separation and purification of succinic acid. Succinic acid fermentation broth was treated successively by

microfiltration, ultrafiltration, and active charcoal adsorption. 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 succinic acid with a yield of higher than 75% (Cheng *et al.,* 2012). The succinic acid fermentation broth from glucose was subjected successively to microfiltration, ultrafiltration, and nanofiltration with the objective for removing molecules or particles having a size greater than 0.2 μm, molecular weight greater than 5000, and approximately 150 - 350 Da, respectively. The final filtrate was then treated by vacuum concentration, and crystallization to obtain a high purity (> 99.4 %) of succinic acid (Wu *et al.,* 2007). Whilst nanofiltration shows promise, there are still some aspects of the process that are not addressed, such as the price of membranes, membrane fouling and the application of this separation method in real fermenter broth.

2.4.3 Electrodeionization (EDI)

Electrodeionization is a process that incorporates ion exchange technology with membranes and electric potential difference to separate non-ionic or weakly ionic molecules from those that are ionized. In the fermentation broth, the dissociated succinate is ionic while other components, such as proteins, amino acids and carbohydrates, are either very weakly ionic or non-ionic. Electrodeionization targets the dissociated form of succinic acid and removes it whilst leaving behind other compounds. If electrodeionization is used whilst the fermentation is taking place, liquid from the fermenter could be run through the electrodeionization system and as the succinate ions are removed, the remaining fluid including cells can be recycled

back to the fermenter (Hepburn *et al.,* 2012). Electrodeionization is a method that has significant potential, but there are also some shortcomings when using this system on an industrial scale. One of the most glaring problems is that the system requires much electrical energy to function. Given that energy costs are on the rise and electricity may come from non-renewable resources, it would seem counterintuitive to use this method since the intent of succinic acid production via fermentation is to reduce the use of fossil fuels and provide a more environmentally friendly process (Bechthold *et al*., 2008). Glassner and Datta suggested the use of conventional electrodialysis, but then pointed out that the succinic acid purity was much lower than expected at 79.6% in addition to 19.9% of the acetic acid remaining from this process (Lee *et al.,* 2008).

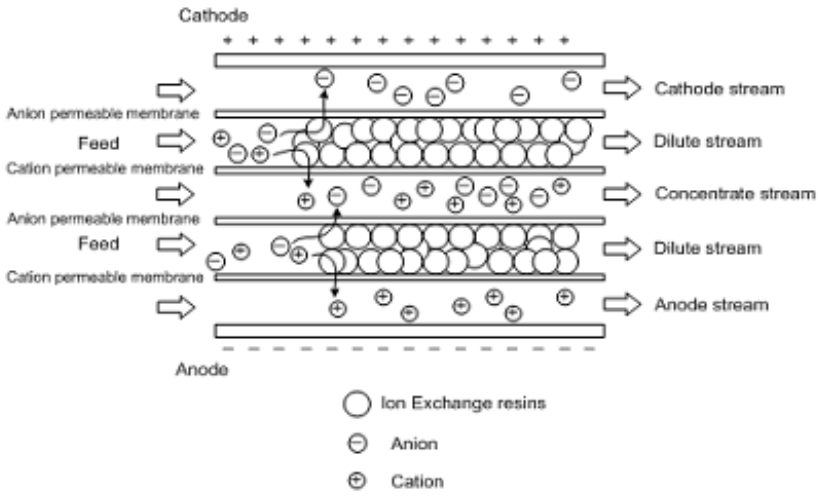

Figure 2.6 Schematic diagram of electrodeionization (EDI) process for recovery of organic acid from fermentation broth (Boontawan *et al.,* 2011).

2.4.4 Reactive extraction

The reactive extraction of succinic acid 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 (Huh *et al.,* 2006, Hong *et al.,* 2005). It is a promising method of separation 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 succinic acid, 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% (Lee *et al.,* 2008, Bechthold *et al*., 2008).

2.4.5 Adsorption

Ion-exchange resins have shown to be promising when used to separate organic acid from fermentation broth. Ion exchange technology involves using a resin that captures cation with an ionic resin. In the case of capturing lactate ion, a cationic

resin is used, meaning that there is no need to alter the pH of the system to remove the molecule (Patel *et al.,* 2008). This method has also been suggested for use with succinic acid as a step prior to crystallization (Song *et al.,* 2007). However, there are very limited research papers that discuss the use of ion-exchange resins with succinic acid purification. The use of a highly acidic ion exchange resin followed by a weak basic exchange resin can remove cations, anions and impurities leaving behind a purified stream with low concentrations of nitrogenous impurities and sulphates. Using ion exchange resins would require a purification step to remove cells from the liquid and the selectivity of these resins is low, leading to additional purification steps after acid removal.

2.4.6 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). Isolation of lactic acid or citric acid using this method has been commercialized. Precipitating succinic acid out of solution is a separation process that was first proposed by Datta, 1992. The major role of adding a calcium ion source is for neutralizing the fermentation broth, and to precipitate 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 the fermentation broth. The calcium succinate is separated from the broth by filtration. The obtained calcium succinate reacts with concentrated sulfuric acid, which releases free succinic acid and generates calcium sulfate (gypsum) as the by-product. Succinic 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 in 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 plastics industry.

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 calcium sulfate, also known as gypsum, which cannot be sold directly as a commodity due to odor and color impurities. This solid can be removed from the solution and the succinic acid, now dissolved in solution, can continue to be removed through other separation methods, such as vacuum distillation. This precipitation method can also take place *in situ* through the addition of a calcium buffer where it helps maintain the pH of the system (Lee *et al.,* 2008).

2.4.7 Esterification

Esterification is an effective downstream process that can remove contaminating organic acids by altering the boiling points of their respective ester compounds. The typical organic acid by-products usually presented are formic acid, acetic acid, and lactic acid, respectively. The esterification of succinic acid involves the chemical reaction with alcohols, such as ethanol, to produce monoethyl succinate (MES) and diethyl succinate (DES). In addition, 2 moles of water are produced. Esterification reactions are characterized by thermodynamic limitations on the conversion yield. Higher DES yields can be obtained by shifting the equilibrium towards products formation by removal of the product, especially water. Dehydration by membrane processes has gained increasing attention in many esterification

processes as an effective energy-saving separation technique. A separate process of esterification-distillation and vapor permeation of the distillate ethanol was successfully introduced for the purification of succinic acid from pre-treated fermentation broth (Lubsungneon *et al*., 2014). Pre-treatment of the broth was necessary in order to remove macromolecule impurities. Vapor permeation (VP) has advantages because the feed can be supplied at high temperature and high pressure resulting in an increase in driving force. After complete the dehydration, most succinic acid was converted into DES. The subsequent step is fractionation followed by hydrolysis of the distilled DES with deionized water to yield ethanol and succinic acid.

2.5 Extractive fermentation

From the each different separation methods, it has positive aspects which can be combined to generate a higher succinic acid recovery than individual processes, but there are some drawbacks associated with them that require a new solution which reduces or eliminates the problems mentioned earlier. An ideal process is one that does not require removal or destruction of the cells requires a minimal amount of the additional chemicals. Key challenge to successful separation of succinic acid from fermentation broth is how to apply separation technology to large-scale industrial processes in a cost and time effective manner that increase productivity and yield. From the aforementioned separation method, it is therefore apparent that there is a need for further research to develop a process which should ideally be simple to carry out and allow the purification of succinic acid directly from the fermentation broth.

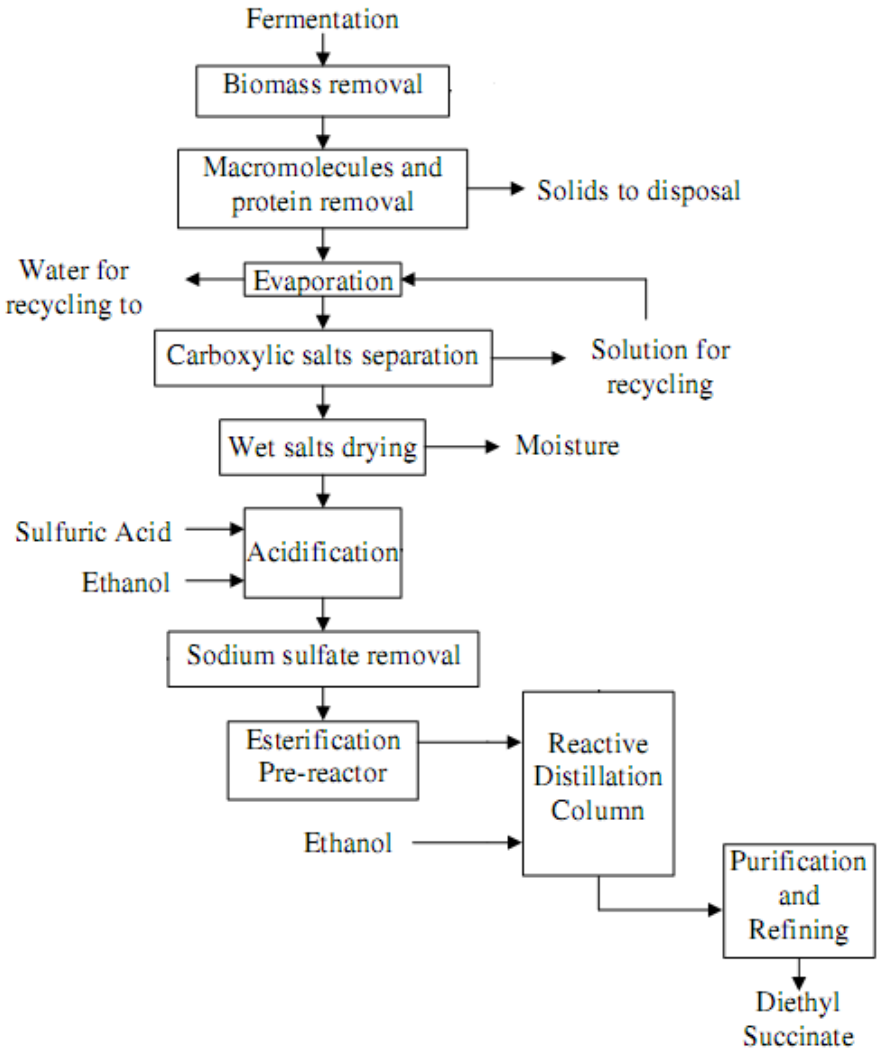

Figure 2.7 Steps in purification of succinic acid from fermentation broth based on esterification and distillation methods (Modified from Londono, 2010).

For future development, traditional separation technique should be improve and coupled with upstream technology. However, product inhibition is an important factor for bioprocess development since succinic acid and organic acids by-products are very toxic to the bacterial cells (Lin *et al*., 2008). In order to increase fermentation performance, succinic acid must be removed from the reaction site as soon as it is

produced. This concept is call *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, or sorption. An ideal *in situ* recovery method for succinic acid would have minimal chemical addition, require no additional energy inputs beyond normal operation, and would leave the biomass unharmed to continue succinic acid 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. Literatures on extractive fermentation of succinic acid are scarce, only two techniques were published using electrodialysis (ED), and expanded bed adsorption.

Extractive fermentation of succinic acid for the continuous fermentation process using *A. succiniciproducens* coupled with ED technique was investigated (Meynial-Salles *et al*., 2008). Two types of charged membranes, anion and cation exchange membrane, allow the ions transfer 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. The dissociation constant of succinic acid has two values at pH 4.2 and 5.6 which makes acid succinic dissociation into ions of succinate ion and calcium ion. The negative charge of succinate ion induces its migration through cation-exchange membrane while sodium or calcium ion will be transported through anion-exchange membrane. The experimental results showed that the concentration of bacterial cells and production rate of succinic acid was 42 g L^{-1}

and 14.8 g L^{-1} h^{-1} which is higher than the batch fermentation up to 28 and 20 times, respectively.

An integrated fermentation process for the production of succinic acid by *A. succinogenes* was developed by applying the extractive fermentation concept. The process was conducted by a coupled expanded-bed adsorption (EBA) with fermentation system. This approach during the product inhibitory period of microbial fermentation enhanced the cell growth of *A. succinogenes* from 48 h to 126 h, and significantly increased succinic acid production up to the final titer of 145.2 g L^{-1} with an average yield of 0.52 g g^{-1}, and productivity of 1.3 g L^{-1} h^{-1}. The maximum yield and productivity reached 0.76 g g^{-1} and 2.58 g L^{-1} h^{-1}, respectively.

CHAPTER III

METERIALS AND METHODS

3.1 Apparatus

1. Bioreactor 5.0 L (UniVessel®, Sartorius, Germany).
2. pH meter (EW-35805-04,Cole Parmer, USA).
3. Thermostat (ED-9118000, Julabo, Germany)
4. Peristaltic pump (Masterflex LS 200-1558,Cole Parmer, USA)
5. Precipitation tank 2.0 L (VN supply, Thailand).
6. Microfiltration membrane (PETT filter, China).
7. Cooling bath (FP90-SL, Julabo, Germany).
8. High performance liquid chromatography (Thermo Scientific, USA).
9. Ultraviolet -visible spectrophotometer (Lambda 2, Perkin Elmer, USA).
10. Rotary Evaporator (RV 10, IKA, Germany).
11. Centrifuge (RC5C plus,Sorvall , United states)

3.2 Materials and methods

All chemicals used throughout this study were obtained from Himedia (India), Merck (Germany), Carlo Erba,(France) and Sigma - Aldrich (Canada), except where otherwise specified. Carbon dioxides were supplied by Linde (Canada).

3.3 Succinic acid fermentation process

3.3.1 Microorganism and growth condition

Actinobaciilus succinogenes ATCC 55618 was obtained from the American Type Culture Collection (Manassasa, VA, USA). The stain was maintained in 5% skim milk at -70°C. The plate was inoculated with the strain and incubated at 37°C for 48 h. Pre-culture medium consisted of the following component (gL^{-1}): tryptone 17.0; soya peptone 3.0, dextrose 2.5, sodium chloride (NaCl) 5.0, dipotassium phosphate (K_2HPO_4) 2.5. The culture was adjusted to 6.8-7.0 prior to autoclave. For the first pre-culture, 50.0 mL of the medium was prepared in an anaerobic bottle, and a colony from a plate culture was incubated for the 12 h at 37°C on the rotary shaker at 120 rpm (New Brunswick Scientific, USA). For the second pre-culture 47.5 mL of the medium was prepared in an anaerobic bottle, incubated with 2.5 mL of the first pre-culture broth, and incubated for 12 h. at 37°C on the rotary shaker at 120 rpm. The second pre-culture was used as an inoculum for the fermentation process.

3.3.2 Batch fermentation process and conditions

The stirred-tank bioreactor used was a 5.0 L agitated bioreactor with single six-bladed turbine. The reactor was aerated through a ring sparger, which was located above the reactor bottom. The bioreactor was equipped with probe of pH and temperature. Fermentation medium was composed of (gL^{-1}): glucose 85.0, yeast extract 15.0, diammonium hydrogen phosphate (DAP) 3.0 monopotassium phosphate (KH_2PO_4) 3.0, K_2HPO_4 1.5, NaCl 1.0, magnesium chloride ($MgCl_2$) 0.3, calcium chloride ($CaCl_2$) 0.3. The pH of the medium was 6.8-7.0 after autoclaving for 20 min

at 121°C. The fermentation medium was inoculated with 10% (v/v) of the second pre culture broth. Batch fermentation was conducted in a 5.0 L bioreactor with the working volume of 3.0 L fermentation medium (Figure 3.1). The optimal temperature was controlled at 37°C, and agitation rate will be controlled at 200 rpm in order to ensure the well-mixed condition throughout the fermentation period. The fermentation broth was initially sparged with 0.5 vvm carbon dioxide (CO_2). The pH was automatically controlled at 6.8-7.0 with the addition of calcium carbonate ($CaCO_3$) 40% wt. The excess carbonate was supplied to buffer the medium during acid production. Fermentation ended when either glucose was completely depleted or no change in glucose concentration.

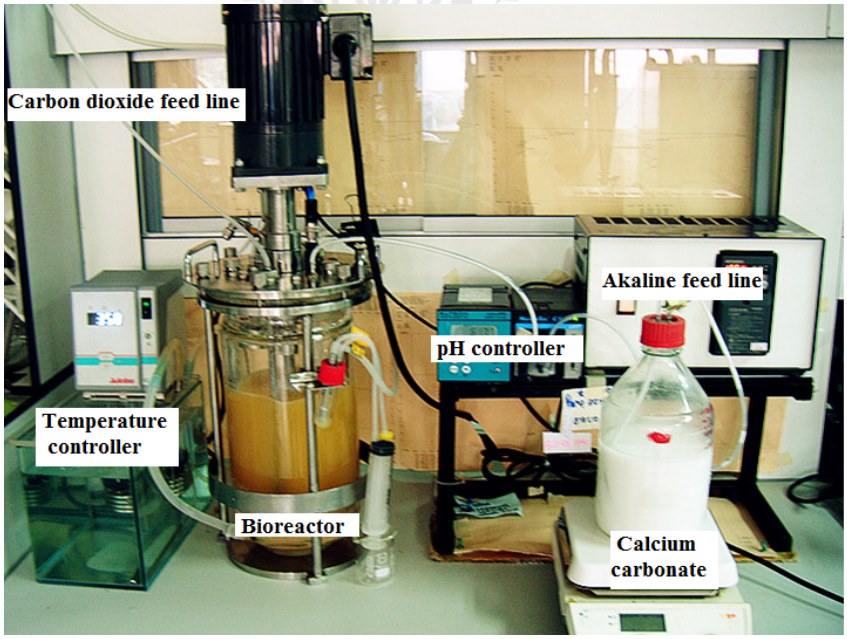

Figure 3.1 Experimental set up for batch fermentation process of succinic acid.

3.3.3 Extractive fermentation using external precipitation technique

Fermentation processes were performed in the same bioreactor as described in the previous section. An external precipitation tank was equipped with the bioreactor for extractive fermentation of succinic acid as shown in Figure 3.2. Initially, fermentation was carried out in conventional batch mode. At the end of the fermentation process, the fermentation broth was removed and the repeated batch mode began. The subsequent culture process used fresh fermentation medium for each cycle. Each fermentation cycle ceased when glucose concentration was depleted and after the completions of each cycle, 80% (v/v) of the broth was removed before being replaced with an equal amount of the fresh fermentation medium. A small volume of the broth at the end of each batch was left within the bioreactor to use as inoculums for next batch culture process. Figure 3.3 shows experimental setup for the extractive fermentation of succinic acid using an external precipitation method. The precipitation tank is 2.0 L jacket glass container equipped with a mixing device in order to ensure homogeneous condition inside the tank. During fermentation, $CaCO_3$ was used to control the pH of the broth. At low calcium succinate ($C_4H_4CaO_4$) concentration, it was presented in a soluble form which can be removed from the bioreactor with the help of a submerge microfiltration (MF) membrane unit. The clarified broth was transferred into the precipitation tank where the solution temperature was controlled using a cooling bath (thermostat 1) in order to induce the precipitation of calcium succinate. The precipitate of calcium succinate was recovered by the centrifugation process.

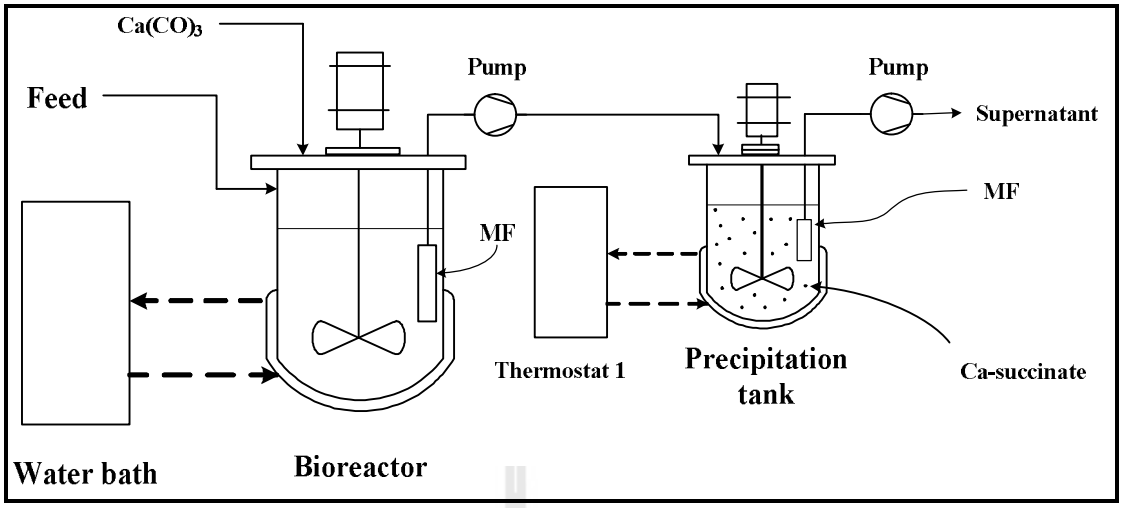

Figure 3.2 Schematic diagrams for extractive fermentation of succinic acid using external precipitation method.

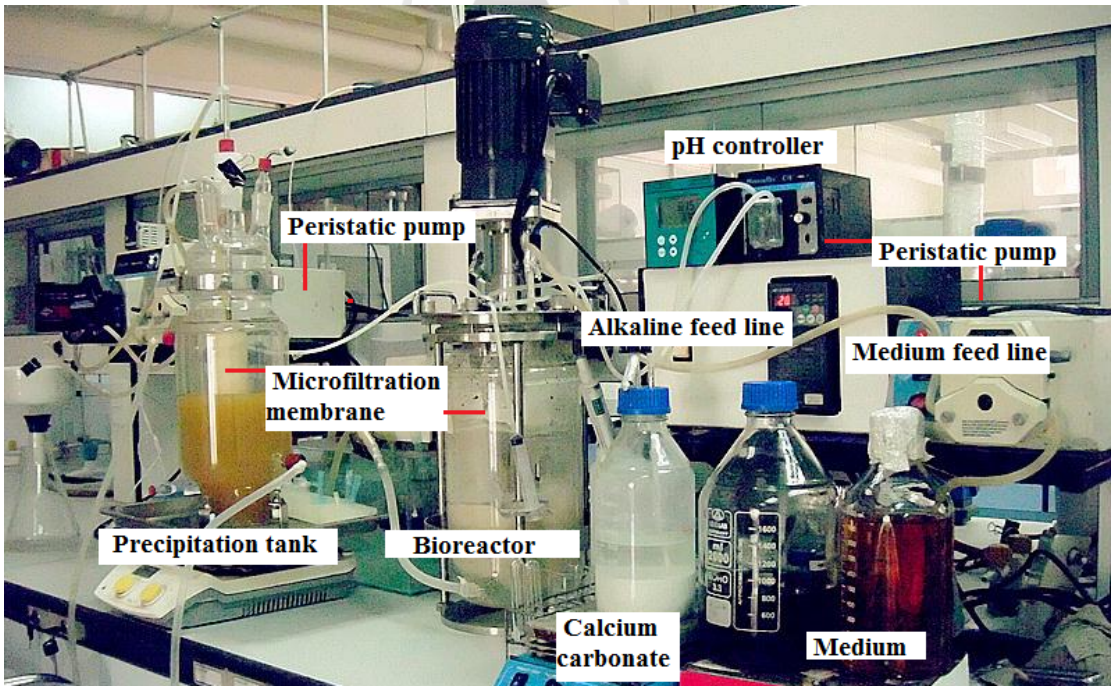

Figure 3.3 Experimental set up for repeat batch fermentation process of succinic acid using external precipitation method.

3.4 Recovery process of succinic acid

For further purification of succinic acid, the organic acid by-product in the fermentation broth has to be completely removed from the final succinic acid product. All of the impurities need to be completely removed during purification steps in order to produced pure succinic acid. The microbial cells from the bioreactor were already separated by a microfiltration membrane, and calcium succinate was precipitate out in the external precipitation tank. Two further purification steps were attempted in this work. The clarified fermentation broth in an external precipitation tank was treated using either the modified calcium precipitation method or the direct crystallization method to recover succinic acid. The schematic diagram was shown in Figure 3.4.

In process I, the filtrate was cooled at 4°C and the impurity was removed by the centrifugation method and then the supernatant was dried by the evaporation method. Free acid in solution of crude calcium succinate was released from calcium succinate by adding 50% (w/v) sulfuric acid (H_2SO_4). The addition of H_2SO_4 was stopped when the pH decrease to around 2.0. H_2SO_4 was added to form calcium sulfate and the precipitate of calcium sulfate was removed by the filtration process. Then, the clear filtrated broth was vacuum distilled at 60°C for 12 h to eliminate residual volatile carboxylic acid, such as acetic acid, lactic acid formic acid. The solution was concentrated to around 50% of its original volumes and the concentrated broth was cooled to 4°C for 24 h and then gathered by the centrifugation process for 10 min at 8000 rpm, 4°C. The succinic acid crystals were dried at 40°C for 24 h.

In the process II, the pH of an aqueous broth was adjusted to 2.0 by the addition of hydrochloric acid (HCl) 35% (v/v) and then was vacuum distilled at 60°C for 12 h to eliminate residual volatile carboxylic acid. The solution was concentrated

to around 50% of its original volumes and the concentrated broth was cooled to 2-4°C for 24 h. The succinic acid was crystallized and then gathered by the centrifugation process for 10 min at 8000 rpm, 4°C, and then the succinic acid crystals were dried at 40°C for 24 h.

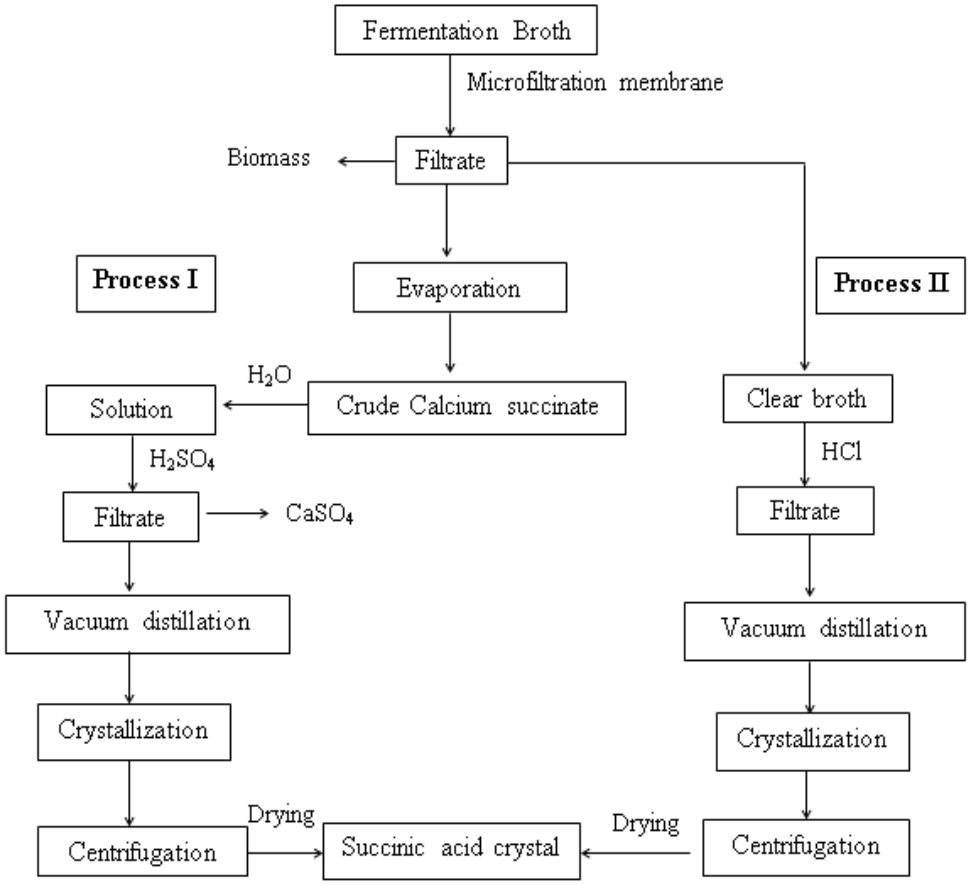

Figure 3.4 Schematic diagram of two recovery processes of succinic acid, (I) modified calcium precipitation method, (II) crystallization method.

3.5 Analytical methods

3.5.1 Organic acids analysis

Succinic acid and the organic acids were determined by high performance liquid chromatography, HPLC (Thermo Scientific, USA). 5.0 mL samples of the bioreactor were collected aseptically every 6 h, and 1.0 mL of the sample was centrifuged at 10×10^3 rpm using centrifuge for 3 min to separate the microbial cell and $CaCO_3$ as pellets at the bottom of the centrifuge tube. The supernatant was withdrawn from the centrifuge tube and filtered through a 0.2 µm filter, and was 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 low rate was 1.0 mL min^{-1}. The detection was UV detector fixed at 210 nm wavelength.

3.5.2 Fermentation broth analysis

Bacterial growth was determined by an optical density (OD) measurement at 660 nm. In order ensure that none of the $CaCO_3$ remained undissolved, 0.4 mL of the sample was diluted with 2.4 mL of 7% (v/v) HCl to form soluble calcium choride ($CaCl_2$) and CO_2. 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 an OD_{660} of 1.0, *A. succinogenes* has concentration of 0.626 g dry weight (DCW) L^{-1}. Glucose was measured by usinga biosensor analyzer. The succinic yield based on the glucose consumption is calculated as followed:

$$\text{yield (g g}^{-1}) = \frac{\text{Succinic acid produced (g)}}{\text{Glucose consumed (g)}}$$

3.5.3 Recovery process of succinic acid analysis

After precipitation with $CaCO_3$ in the external precipitation tank completely, the broth was analyzed protein residual in the broth by using the method of Lowry (Lowry *et al.,* 1951). Purity and yield of the succinic acid crystals in the recovery process were defined as followed:

$$\text{Purity (\%)} = \frac{\text{Succinic concentration in crystals recovered } (gL^{-1})}{\text{Total acid concentration in crystals recovered } (gL^{-1})} \times 100$$

$$\text{Yield (\%)} = \frac{\text{Dry weight of succinic acid in crystals recovered (g)}}{\text{Weight of succinic acid in the initial fermentation broth (g)}}$$

Succinic acid crystals morphological were determined by using the scanning electron microscopy, SEM (JEOL, JSM-6010 LV, Japan).

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Batch and repeat batch fermentation of succinic acid using *A. succinogenes* ATCC 55618

As mentioned previously, *A. succinogenes* ATCC 55618 is the microorganism of choice for creating an industrial process having a high tolerance of succinic acid (Lin *et al.,* 2008). *A. succinogenes* ATCC 55618 was isolated from bovine rumen at Michigan Biotechnology Institute (MBI) International in Lansing, Michigan, USA. It could produce higher concentration succinic acid as a major product than other stains without gene reconstruction and glucose feeding (Zhu, *et al*., 2012). This strain possesses all desired characteristic of the above mentioned, and was used in the fermentation process of this work.

Figure 4.1 *Actinobaciilus succinogenes* ATCC 55618.

4.1.1 Effect of initial glucose concentration on *A. succinogenes* ATCC 55618 growth

The fermentation media were often complication with the addition of high concentration of glucose and ten kinds of slight vitamins. The components were added into fermentation medium for succinic acid production, which led to high cost and complex operation must be paid attention that the complication media are not the assurance of high production of succinic acid. The optimization of the fermentation medium of *A. succinogenes* ATCC 55618 is essential especially initial substrate concentration. Glucose was added into the fermentation medium as carbon source for succinic acid production. A study by Zhu *et al*. (2012) reported that the optimization of fermentation medium for *A. succinogenes* ATCC 55618 and the optimal concentration was 84.6 g L^{-1} of glucose, and 14.5 g L^{-1} of yeast extract (Zhu *et al.,* 2012). Production of succinic acid and yield of succinic acid against glucose were obtained under the optimal medium composition, which was enhanced when compared with the basic medium. The result of this experiment showed that glucose concentration reduced with time which is main characteristic of the batch culture. Initial glucose concentration in which the cells experience during fermentation is very importance. The succinic acid production ranges mentioned earlier seem to indicate that there is upper limit on the final concentration achievable from *A. succinogenes* possibly due to end product inhibition. Urbance *et al*., (2004) reported that *A. succinogenes* could tolerate up to 160 gL^{-1} initial glucose concentration in batch fermentation. A study by Lin *et al*., (2008) showed that the inhibition of the substrate and products on the growth of *A. succinogenes* ATCC 55618 in fermentation using glucose as the major carbon source, *A. succinogenes* ATCC 55618 tolerated up to

143 g L^{-1} glucose and cell growth was completely inhibited with glucose concentration over 158 g L^{-1}. Significant decrease in succinic acid yield and prolonged lag phase were observed with glucose concentration above 100 g L^{-1}. Among the end-product investigated, formic acid was found to have the most inhibitory effect on succinic acid fermentation.

Experimental for determining the effect of initial glucose on *A. succinogenes* ATCC 55618 growth and succinic acid production was carried out in a shake flasks (anaerobic condition), each contained 47.5 mL of the fermentation medium with the initial pH of 6.8. The fermentation medium was incubated with 2.5 mL of the inoculum. The fermentation was incubated at 37°C for 36 h on the rotary shaker at 200 rpm. Table 4.1 presents experimental results for determining the effect of initial glucose on *A. succinogenes* ATCC 55618 growth and succinic acid production. It showed that the maximum succinic acid production of 45.8 g L^{-1} was obtained with 85 gL^{-1} glucose. Too high initial concentration results in the substrate inhibition whereas too low initial concentration might result in low volumetric productivity and succinic acid yield. Production rate decrease as the cells are forced to spend more energy maintaining the cell rather than fermentation and cell growth. Cells that have a higher tolerance of products can continue producing quickly and give a higher final concentration.

So, the optimization of fermentation medium of *A. succinogenes* ATCC 55618 is essential. Accordingly, this study was used the referent of fermentation medium in the same way to that reported by Zhu et al., (2012) and the optimal concentration was located to be 85 g L^{-1} of glucose.

Table 4.1 Effect of initial glucose concentration on *A. succinogenes* ATCC 55618 growth and final succinic acid concentration.

Glucose (gL^{-1})	Dry cell weight (gL^{-1})	Succinic acid production (gL^{-1})	Yield ($g\ g^{-1}$)
40	4.97	28.2	0.70
55	5.03	35.9	0.65
70	5.40	40.0	0.57
85	5.98	45.8	0.53
100	4.37	30.8	0.38
120	3.08	28.0	0.23

4.1.2 Batch fermentation process

Succinic acid fermentation in the batch process was investigated. For the upstream process, 10% (v/v) of an inoculum was grown on tryptic soya broth for 24 h, and was aseptically transferred into a 5.0 L bioreactor containing 3.0 L fermentation medium for the growth of *A. succinogenes* ATCC 55618 using glucose as the main carbon source. The culture was grown anaerobically at 37°C with agitation speed 200 rpm and the fermentation broth was sparged with 0.5 vvm CO_2. The pH was automatically controlled at 6.8-7.0 with the addition of calcium carbonate ($CaCO_3$) solution 40 wt. %. The time profile of glucose consumption and the cell concentrations during the batch fermentation of *A. succinogenes* ATCC 55618 was showed in Figure 4.2. Glucose concentrations rapidly decrease for the first 40 h. Subsequently, the consumption rate gradually decreased and most glucose was

consumed in 58 h. This study, it was observed that *A. succinogenes* ATCC 55618 possessed a relatively short lag phase at approximately 8 h followed by an exponential growth, where the cell concentration reached plateau of approximately 3.8 g L^{-1} until the end of the fermentation process. The residual glucose concentration was 0.6 g L^{-1} after 58 h. The maximum cell concentration was obtained at 3.89 g L^{-1}. During the cells growth, concentration of succinic acid rapidly increased during the first 30 h and then the concentration was constant followed the trend of the cells growth.

Succinic acid is an acid product. Large amount of the alkaline neutralizer are required to maintain the during succinic acid fermentation. In this case the pH was controlled at 6.8 ± 0.2 by an automatic pH controller. Majority of studies on succinic acid production have used $MgCO_3$ as alkaline neutralizer to achieve high product concentration. Nevertheless, the cost of $MgCO_3$ supplementation is not practical for the industrial succinic acid fermentation. In this study was investigated the replacement of $MgCO_3$ with $CaCO_3$ as alkaline neutralizer. The experiment was showed good result of succinic acid concentration, which was almost the same production level as fermentation with $MgCO_3$. The final concentration of succinic acid in the bioreactor was 53.25 g L^{-1} resulting in the conversion yield of 0.62 g g^{-1}. This value is in a good agreement with literature using the same strain (Li *et al.*, 2011). Although succinic acid was the major product, the strain also produced acetic acid, formic acid and lactic acid as the by-product. The final concentrations of formic acid lactic acid and acetic acid were 12.3 g L^{-1}, 2.7 g L^{-1} and 10.5 g L^{-1}, respectively. Succinic acid and organic acids by-product concentration during batch fermentation are shown in Figure 4.3.

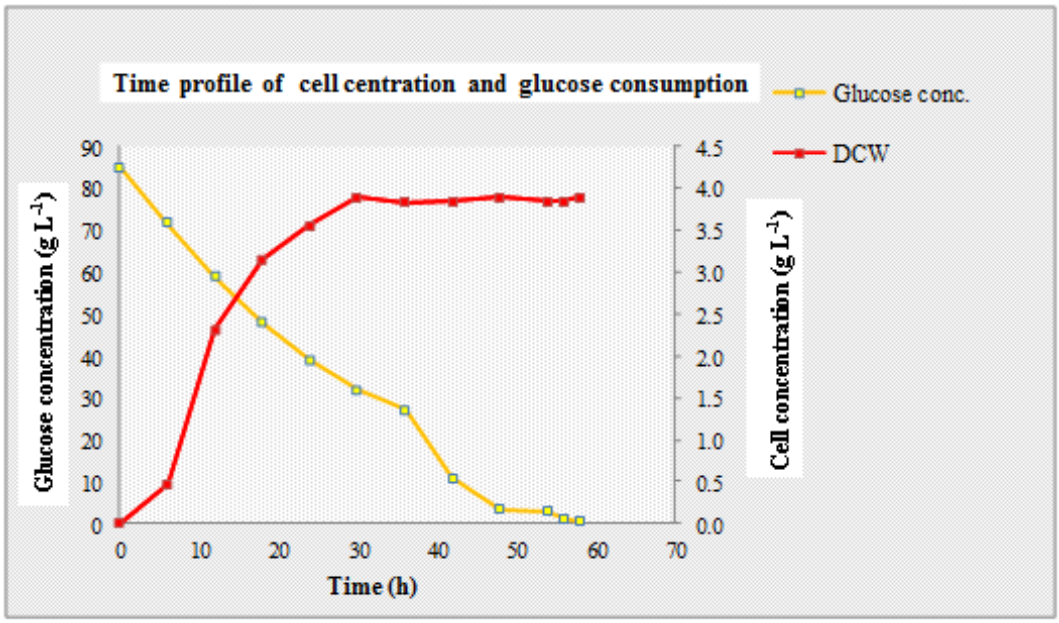

Figure 4.2 The time profile of glucose consumption and cell concentrations during batch fermentation of *A. succinogenes* ATCC 55618.

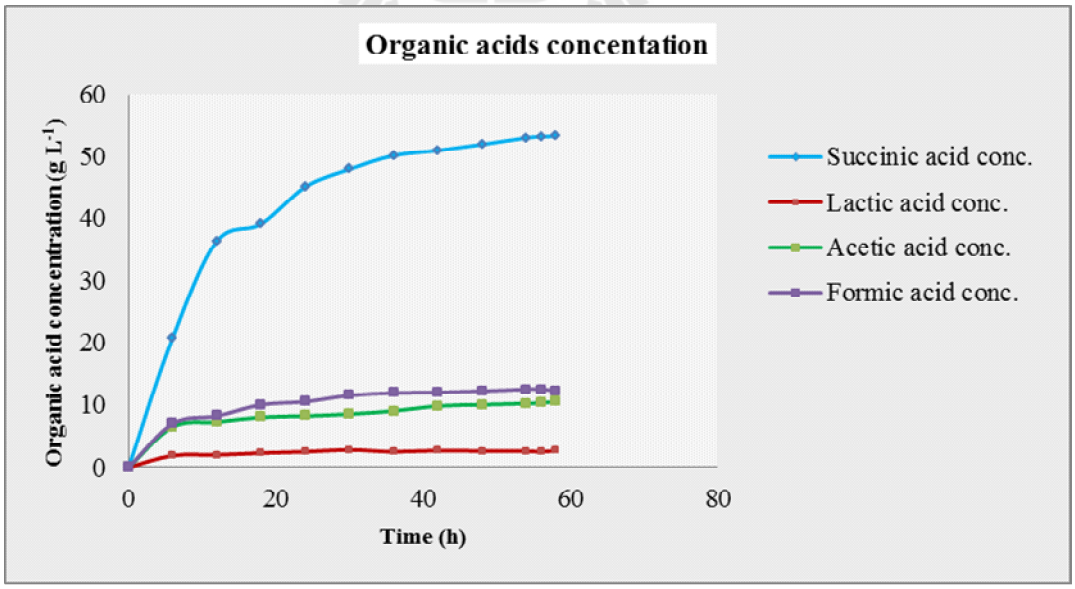

Figure 4.3 The time profile of organic acids concentration during batch fermentations of *A. succinogenes* ATCC 55618.

However, cell growth depends on the nutrient content and the nutrient was limited in the batch fermentation process. For the upstream process, it is favorable to maximize both product yield and volumetric productivity of the fermentation process. Therefore, it is interesting to investigate a repeated batch mode where concentrated substrate solution is constantly added into the bioreactor.

4.1.3 Repeated batch fermentation process

Repeated batch process is batch fermentation process involved repeated cycles of the fermentation by inoculating a part or all of the cells from a previous batch into the next batch. Repeated batch fermentations are performed at the end of the batch fermentation where the fermentation medium is decanted but a small volume of the broth is left within the bioreactor to serve as an inoculum. The batch bioreactor is then refilled with fresh fermentation medium and the batch fermentation is restarted, this process is process is repeated several times. This study, repeated batch fermentation process was conducted in 5.0 L bioreactor with the working volume of 2.0 L fermentation medium. The optimal temperature was controlled at 37°C, and agitation rate will be controlled at 200 rpm. The fermentation broth was sparged with 0.5 vvm CO_2. The pH was automatically controlled at 6.8-7.0 with the addition of calcium carbonate ($CaCO_3$) 40.0 wt. %. Fermentations were performed in the same the bioreactor as the batch fermentations, whereas the repeated batch culture process was used the fresh fermentation medium each cycle. Each fermentation cycle lasted glucose concentration was depleted and after the completions of each cycle, 80% (v/v) of the broth was removed and replaced with an equal amount of the fresh fermentation medium. Bioreactor was used in the repeated batch process which set up

with an external precipitation tank for the extractive fermentation and the recovery of succinic acid. Fermentation broth of each batch was removed via the microfiltration process to the external precipitation tank.

Six fermentation runs were performed. *A. succinogenes* ATCC 55618 was used in repeated fermentation process for succinic acid production. The cell concentrations during the batch cultivation of *A. succinogenes* ATCC 55618 in the repeated batch fermentation process were showed in Figure 4.4. The cell concentrations were increased throughout the fermentation process. The maximum cell concentrations were increased from 4.0 ± 1 gL^{-1} in the first batch to 6.1 g L^{-1} in the second batch and to about 6.6 ± 0.2 g L^{-1} in the four subsequent batches. Cell concentrations in the third batch were showed the highest concentration than other batch; the cell concentration was about 6.8 g L^{-1}. The experimental result was showed that glucose concentration constantly reduced with time. The glucose concentrations during the batch cultivation of *A. succinogenes* ATCC 55618 in repeated batch fermentation process were showed in Figure 4.5. In first batch in repeated fermentation process most glucose was consumed in 54 h and decreased gradually in the second batch to the sixth batch in repeated batch fermentation process.

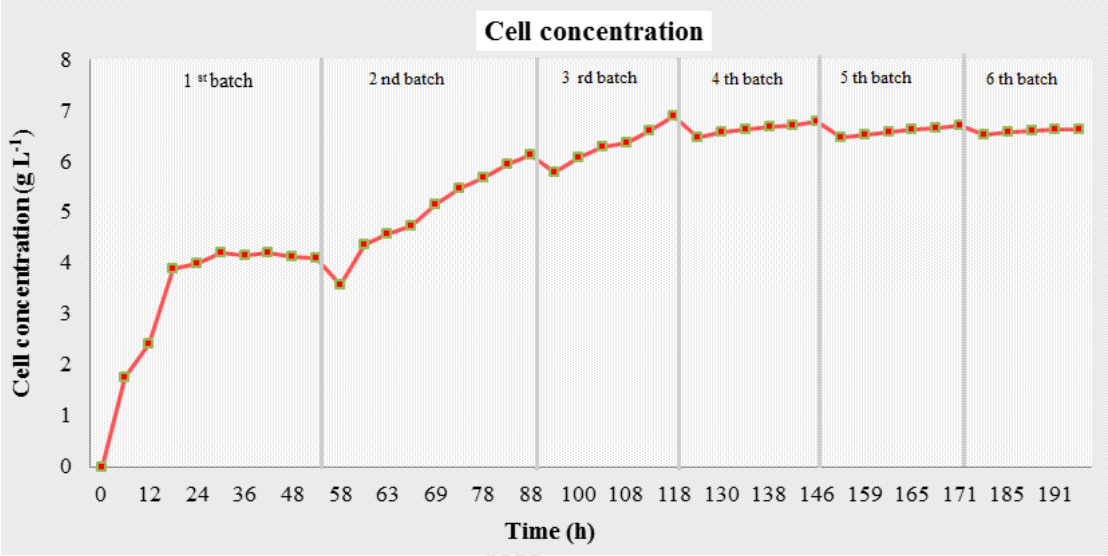

Figure 4.4 The time profile of cell consumptions during repeated batch fermentations of *A. succinogenes* ATCC 55618.

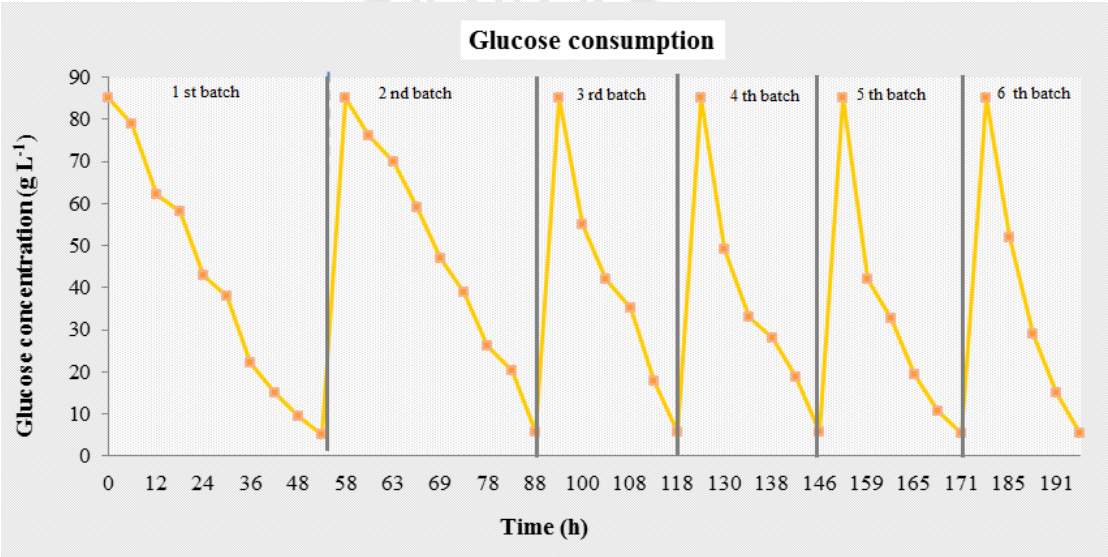

Figure 4.5 The time profile of glucose consumptions during repeated batch fermentations of *A. succinogenes* ATCC 55618.

The final succinic acid concentrations during the batch cultivation of *A. succinogenes* ATCC 55618 in repeated batch fermentation process were showed in Figure 4.6. The final succinic acid concentrations in the sixth batch were slightly lower. The final succinic acid concentration in the first, the second, the third, the fourth, the fifth and the sixth batched were 53.3 g L^{-1}, 58.05 g L^{-1}, 61.38 g L^{-1}, 58.48 g L^{-1}, 57.08 g L^{-1} and 56.08 g L^{-1}, respectively. The final succinic acid concentration in the third batch was showed the highest concentration of succinic acid was compared with each other batch. Nevertheless, the final succinic acid concentration in the fourth, the fifth and the sixth batch was slightly lower which was due to microbial cell might be adhered on the microfiltration membrane surface in the bioreactor. The accessibility of glucose substrate might find difficulties for the inner layer of the cells.

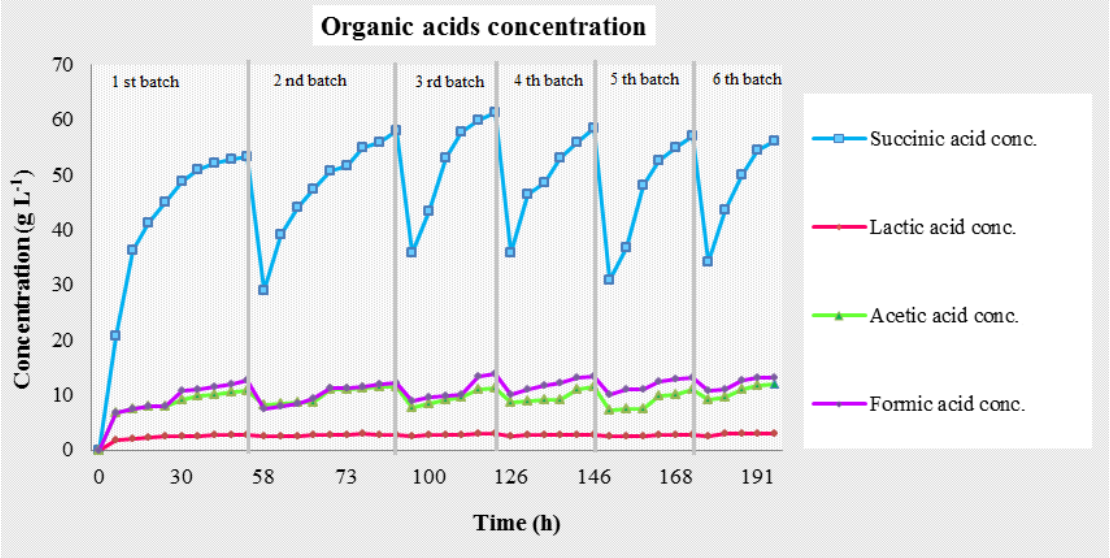

Figure 4.6 The time profile of organic acids concentration during repeated batch fermentations of *A. succinogenes* ATCC 55618.

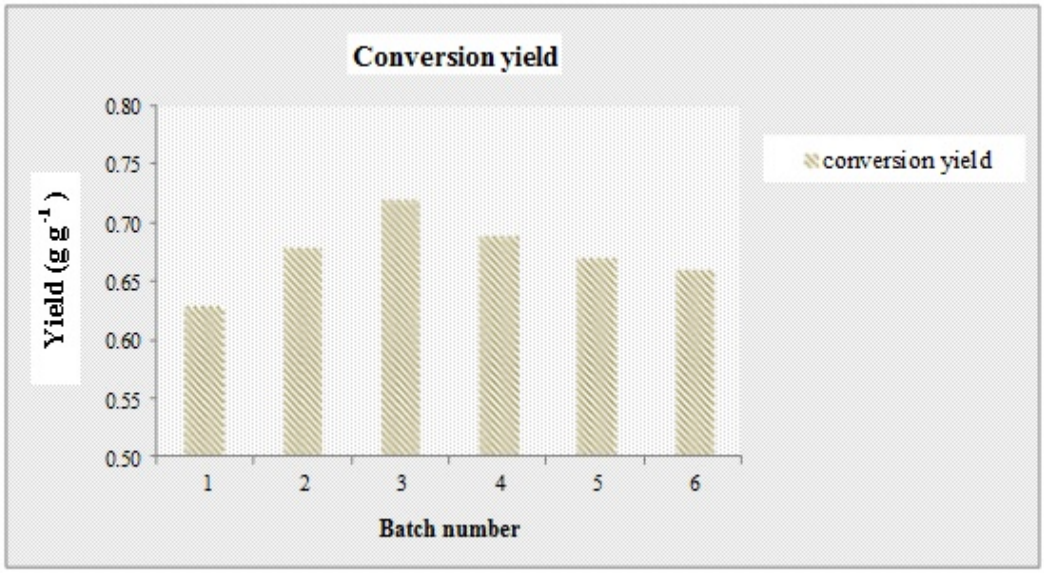

Figure 4.7 The yield of succinic acid based on the glucose consumptions.

In addition, the final organic acid concentration of the by-product in the first to the sixth batch, formic acid concentration was 12.0-13.7 gL^{-1}, lactic acid concentration was 2.7-2.9 g L^{-1} and acetic acid concentration was 10.6-12.0 g L^{-1} for repeated batch of succinic acid fermentation. The yield of succinic acid based on the glucose consumption was shown in Figure 4.7. The conversion yield of glucose to succinic acid was 0.62-0.72 g g^{-1} for the repeated batch succinic acid production. Fermentation time during repeated batch fermentations was generally decreased of the each batch due to an increase in cell concentration. Cell growth and glucose consumption during the repeated batch fermentations of *A. succinogenes* ATCC 55618 were much faster and the final biomass was higher in the second batch to the sixth batch in comparison to the first batch. The result of this study showed that succinic acid concentration of the batch fermentation experiment was 53.25 g L^{-1}. The glucose consumption rate was consumed in 58 h. Repeated batch fermentation experimental was performed six fermentation runs. The final succinic acid

concentrations of an each batch were variously obtained and the final succinic acid concentration in the sixth batch was slightly lower. The highest final succinic acid concentration was showed in the third batch which was 61.38 g L^{-1}.

In comparison to the batch fermentation process, final succinic acid concentration and the product yield increased after the sixth batch fermentation process due to the bioreactor was equipped with the microfiltration membrane allowing a cell-recycle operation mode. The membrane was utilized to remove the fermentation broth into the external precipitation tank for recovery succinic acid in the next step. In addition, the microfiltration membrane was also maintained the microbial cell to exist in the bioreactor which might be increased cell concentration for produced succinic acid in the subsequent batch.

In conclusion, the repeated batch fermentation process has several advantages such as less time required for washing and sterilizing the fermenter, omission of seed preparation time, high growth rates and short main culture time due to the high initial inoculation volume. Theses advantage leads to great saving in term of both time and labor.

4.1.4 Extractive fermentation using external precipitation tank

The classical industrial method for the isolation of the carboxylic acid from fermentation broth is a precipitation with $Ca(OH)_2$ or CaO. Lactic acid and citric acid have been recovered industrially with this method. Moreover, the study by Datta, 1992 was concerned specifically with the precipitation of succinic acid with $Ca(OH)_2$ although these processes are only at lab scale. After additional of $Ca(OH)_2$ or CaO , the calcium salt of succinic acid is filtered off from the fermentation broth.

Nevertheless, $CaCO_3$ was used for neutralization of succinic acid in this experiment, the reagent replaced the using of $Ca(OH)_2$ or CaO in the traditional precipitation method which released calcium succinate. With the help of an internal microfiltration membrane, the clarified broth containing calcium succinate was taken from the bioreactor. Precipitation of the solid calcium succinate was induced by lowering the temperature of the precipitation tank as shown in Figure 4.8. The efficacy of precipitation process was illustrated in term of percentage of precipitation. The value can be calculated by the ratio the precipitate and the initial succinate concentration. Table 4.2 shows experimental result for the percentage of succinic acid precipitation the supernatant at different temperature. At 2.5°C, it was expected that good result of the percentage of succinic acid precipitation could be obtained better than another temperature. However, the experimental result showed that the percentage of the succinate precipitation was achieved only 12.6%. The result of low precipitation with $CaCO_3$ was probably due to the low initial concentration of calcium succinate in the clarified broth. If a solid-liquid separation process is used to collect the precipitate and discard the supernatant, the majority of calcium succinate will be lost resulting in the reduced recovery yield. In order to increase succinic acid precipitation, higher concentration of clarified broth is required. After fermentation process was finished and the calcium salt of succinic acid was filtered off from the bioreactor, the supernatant was concentrated for around 70% of its original volume with the help of a rotary evaporator. The concentrated supernatant was subsequently incubated at 4°C 24 h, and gathered the precipitate by centrifugation at 8000 rpm for 10 min. The solid precipitate of the calcium succinate was further purified.

Table 4.2 The percentage of succinic acid precipitation in supernatant at different temperature. The initial concentration of succinate was 52 gL^{-1}.

Temperature (°C)	(%) of Ca-succinate precipitation
37	0
30	0
20	2.2
10	3.9
5	8.6
2.5	12.6
0.5	12.5

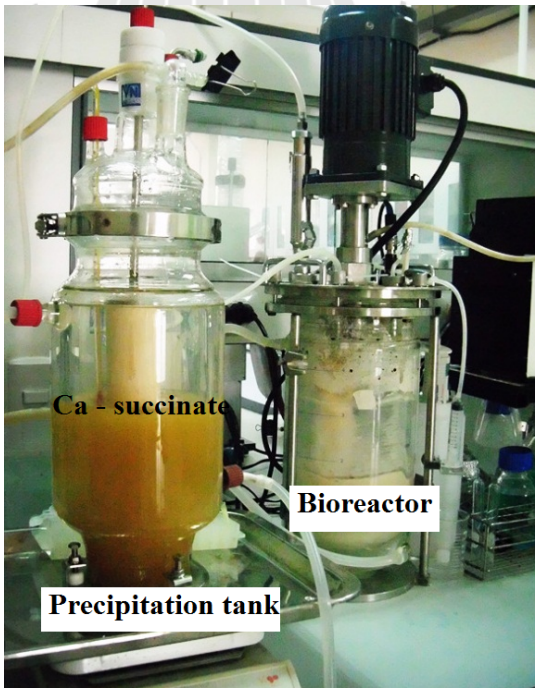

Figure 4.8 The precipitation of calcium carbonate in external precipitation tank.

In purification process, the organic acid by-products have to be completely removed from the final succinic acid product. All of impurity materials need to be completely removed during purification steps in order to produced pure succinic acid. In downstream processing of biotechnological produced succinic acid, no single method has proved to be simple an efficient, and improvements are especially needed with regard to yield, purity and energy consumption. The separation of succinic acid from fermentation broth makes more than 50 % of the total costs in their microbial production. For this reason, some new as well as some well know methods have been investigated for the recovery of succinic acid (Kurzrock and Botz 2010). Generally, the downstream process of the biologically produced succinic acid usually includes three main steps. The first step is the removal of microbial cell, mostly using membrane filtration or centrifugation. The second step is the removal of impurities and primary separation of succinic acid from the fermentation broth, e.g., using, precipitation, electrodialysis reactive extraction, ion exchange resin, the last step is final purification of succinic acid by vacuum evaporation and crystallization.

This study used membrane filtration for removal the microbial cells and removal of the impurities. Primary separation of succinic acid from the fermentation broth employed precipitation process by using external precipitation tank and the final purification of succinic acid was used vacuum distillation method and crystallization method respectively.

4.2 Recovery of succinic acid from fermentation broth.

After the repeated batch succinic acid fermentations of *A. succinogenes* ATCC 55618 finished, the precipitate of the calcium succinate was subjected to further

purification. The by-products of these organic acids (acetic acid, lactic acid and formic acid) in fermentation broth have to be completely removed from the final succinic acid product. Recovery process of succinic acid fermentation, the clear filtrated fermentation broth in external precipitation tank was treated using either the modified calcium precipitation method or the crystallization method to recover succinic acid.

Many succinic acid fermentation processes were often interrupted by the contaminated acid, impurities, carbon sources, protein with crystallization process as the final purification step to refine pure succinic acid. The recovery processes are still not economically feasible because the properties of the carboxylic acid in the acid mixture are similar. According to the physicochemical characteristics, these short chain organic acids have the similar behavior in solution at room temperature. In many fermentation processes, the mixture acids to simplify the process modeling. However, it is not simple to recovery the target acid from the mixture acids effectively in the downstream process because many separation methods do not the perfect selectively toward succinic acid out from the other carboxylic acids. (Li *et al.,* 2010).

Table 4.3 The pK_a values and the solubility in water 20°C of common organic acid produced in *A. succinogenes* fermentation.

Acid	p*Ka*		Solubility in water 20°C	Melting point
	pKa_1	pKa_2		
Succinic acid	5.64	4.21	5.8 -6.8 % (wt)	184 °C
Acetic acid	4.7	-	Miscible	16.5 °C
Formic acid	3.84	-	Miscible	8.6°C
Lactic acid	3.86	-	Miscible	16.8°C

These organic acids have different proportions of dissociated and undissociated forms at different pH value and the solubility of these forms of the acid is different substantially. Li *et al*., (2010) showed the result that solubility of succinic acid was only 3% at 4°C and pH 2.0 while the other acid by-product such as acetic acid, lactic acid and formic acid, were still fully water miscible. In their study, crystallization of succinic acid from fermentation broth was carried out at 4°C and pH 2.0. While acidic by-product remained in the solution, succinic acid could be selectively crystallized (Li *et al.,* 2010).

A direct vacuum distillation-crystallization was used for succinic acid recovery from the fermentation broth by Luque *et al.*, (2009). The pH of the aqueous broth was adjusted by addition of hydrochloric acid before vacuum distillation. The other acid by-product carboxylic acid (acetic acid, lactic acid and formic acid) in the fermentation broth were removed under vacuum distillation at 60°C for 12 h. The followed crystallization of succinic acid was carried out at 4°C. When this method

was applied in fermentation broth produced by *A. succinogenes* ATCC55618. The experimental result was showed good result in the purity of the succinic acid crystals (Luque *et al*., 2009).

In this study, it was reported the recovery process for the effective separation and purification of succinic acid from the mixture carboxylic acid in the fermentation broth was using either the modified calcium precipitation method or the crystallization method to recover succinic acid. The followed the pH of an aqueous fermentation broth was adjusted to 2.0 by addition of hydrochloric acid (HCl) for direct crystallization method and modified calcium precipitation method was adjusted by addition of sulfuric acid. The other acid by-product carboxylic acid such as acetic acid, lactic acid and formic acid, in the fermentation broth were removed under vacuum distillation at 60°C for 12 h in a similar way to reported by Luque *et al,* (2008). The followed crystallization of succinic acid was carried out at 4 °C, which allowed the isolation of highly pure crystals in a similar way to reported by Liu *et al*, (2010).

4.2.1 Modified calcium precipitation method (Process I)

The methodology of the modified calcium succinate crystallization (Figure 4.9). After adding $CaCO_3$, After finishing the fermentation process, the calcium salt of succinic acid was filtered off from the bioreactor and the supernatant was then concentrated for around 70% of its original volume using a rotary evaporator. The concentrated supernatant was subsequently incubated at 4 °C 24 h, and gathered the precipitate by centrifugation at 8000 rpm for 10 min. Re-suspension of calcium succinate was reacted with concentrated H_2SO_4, which release free succinic acid. The

precipitate of calcium sulfate was removed by the filtration process. Succinic acid was purified by vacuum distillation and then the product was concentrated and crystallized. Final step, the succinic crystals were dried by an evaporation method.

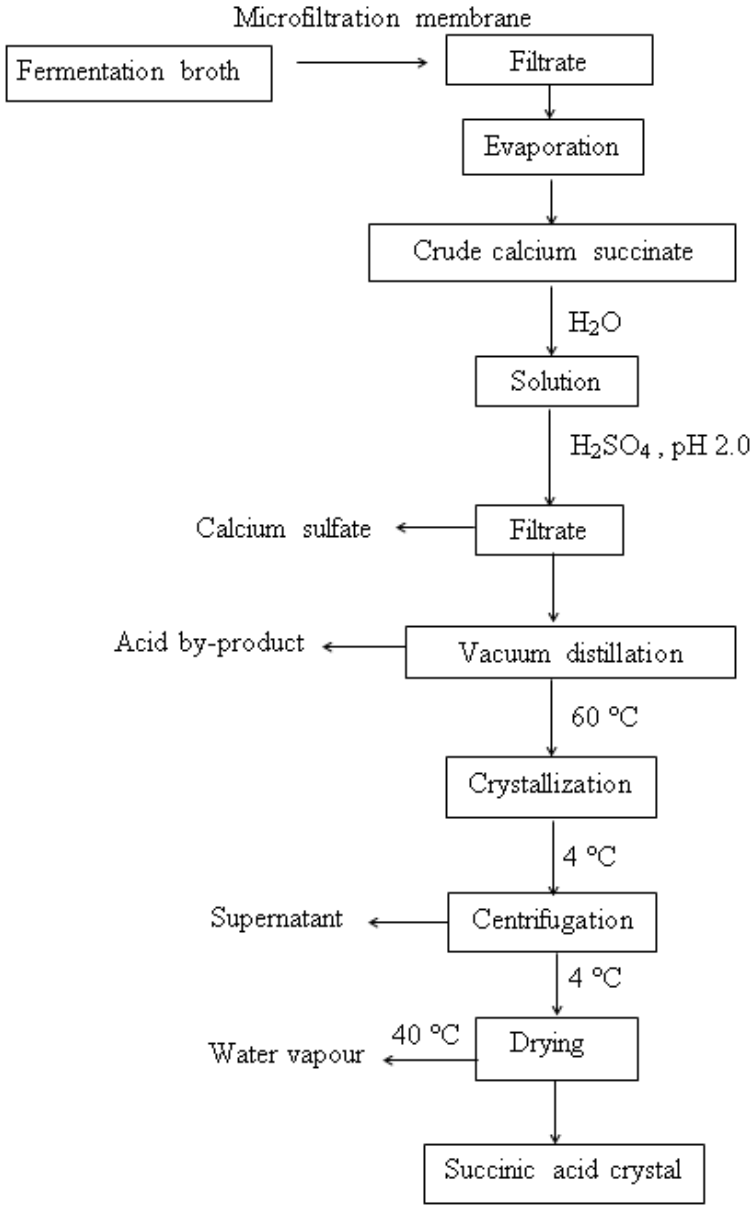

Figure 4.9 Schematic diagram of modified calcium precipitation method (Process I).

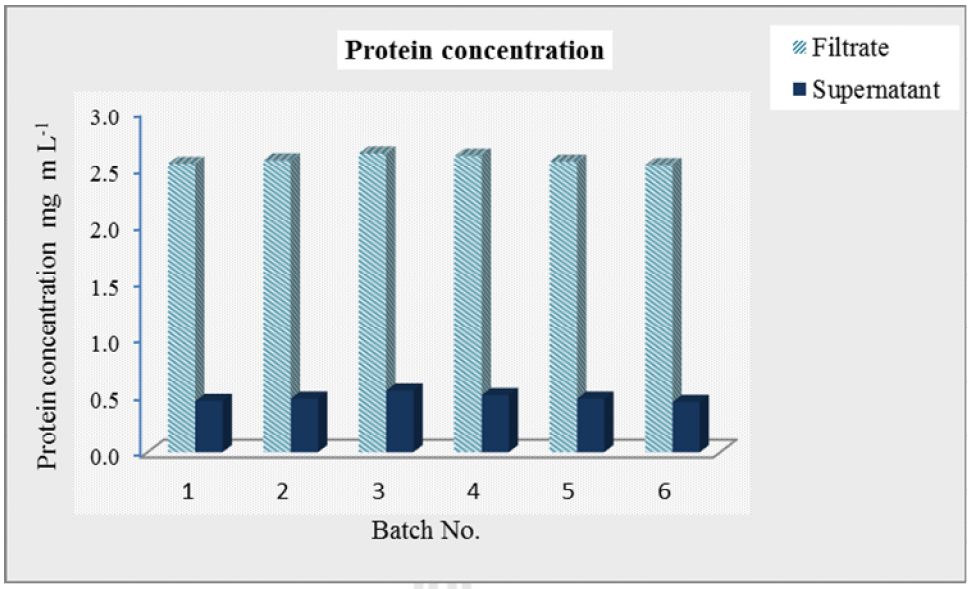

Figure 4.10 Protein concentrations (Process I).

The modified calcium precipitation method was effective for removing proteins, sugar and other by-product including calcium salt in the broth. Protein concentration was analyzed by using Lowry method. As shown in the Figure 4.10., the protein concentrations in the filtrate and the supernatant from centrifugation step was compared. Protein concentrations in the succinic acid crystals were decreased of each batch and the residual protein concentration were 0.45- 0.53 mg mL^{-1} .

The precipitation with $CaCO_3$ in this study, this reagent was replaced the using of $Ca(OH)_2$ or CaO in the traditional precipitation method which released calcium succinate. Addition of the concentrated $CaCO_3$ can be reduced the dosages of $Ca(OH)_2$ or CaO in the traditional precipitation method. The precipitation with $CaCO_3$ can be a viable process for the commercial bio-succinate production with very low technological barriers and risks. However, the calcium succinate was reacted with concentrated H_2SO_4 during the precipitation process which leaded the substantial by-product calcium sulfate or gypsums and solid wastes were caused additional treatment

for this by-product. Moreover, another disadvantage of the modified calcium precipitation method, high consumption of H_2SO_4. This additive cannot be regenerated or recycled, which results in high operation cost (Kurzrock and Weuster-bortz , 2010).

4.2.2 Direct crystallization method (process II)

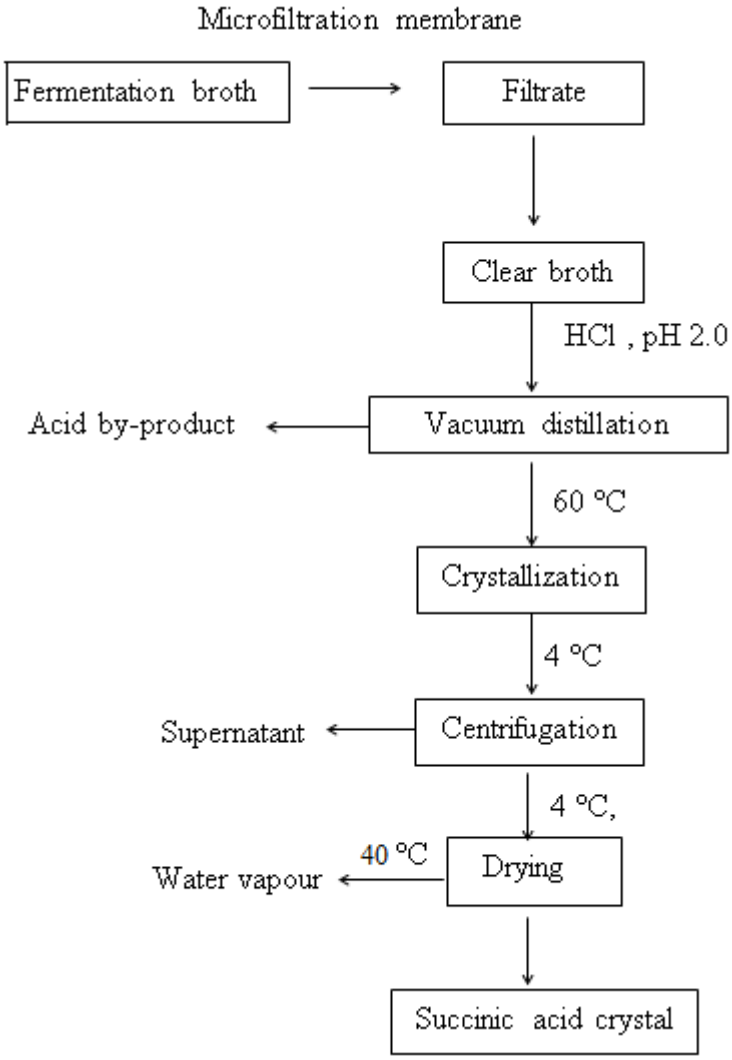

Figure 4.11 Schematic diagram of direct crystallization method (process II).

The methodology of the crystallization process, after adding $CaCO_3$ the calcium succinate was separated from fermentation broth by microfiltration membrane. The pH of the aqueous fermentation broth (Figure 4.11) was adjusted to 2.0 by addition of HCl. It was then followed by vacuum distillation and crystallization as in process of the modified calcium precipitation process for the recovery of succinic acid crystals.

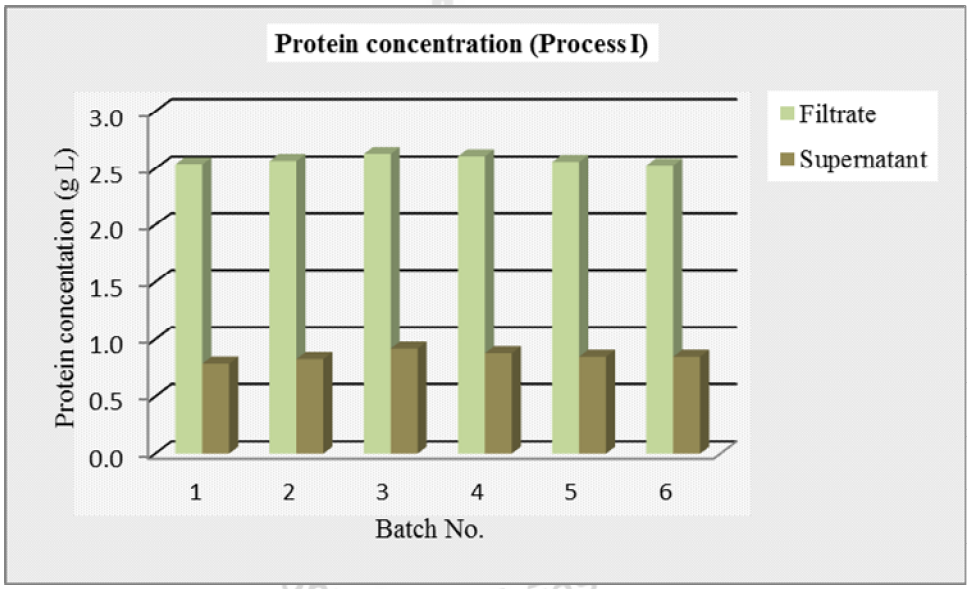

Figure 4.12 Protein concentrations (Process II).

Protein concentration in the clear broth and supernatant was analyzed by using Lowry method. As shown in the Figure 4.12. In the comparison with protein concentration in the filtrate and the supernatant from centrifugation step. Protein concentrations in the supernatant were a few decreased of each batch. The residual protein concentration in the supernatant was 1.75 - 1.88 mg mL^{-1} . In comparison with Process I protein concentration was decreased more than the Process II.

However, the direct crystallization process was simple and environmentally not produced solid waste as the modified calcium precipitation that produced solid waste (calcium sulfate) was difficult to eliminate. This process was effective process for the preparation of succinic acid crystals, Crystallization process could be used usually as the final purification step. Crystallization process might provide the desired product (in solid or crystal form) without the need for many unit operations (Chang *et al.,* 2012).

Generally succinic acid fermentation is operated at pH 6-7 above pK_a of each acid. The final acid products are almost in their salt form rather than free acid. The solubility of succinic acid was only 3% at 4°C, at pH 2.0 (Li *et al.,* 2010). While the other acid by-product were still water miscible. Therefore, when the pH was adjusted to 2.0 with H_2SO_4 or HCl, succinic acid could be selectively crystallized. As the final step of the purification process, the crystallization was carried out at 4°C and the succinic acid crystals were obtained after drying process. The feasibility and effectiveness of the two proposed methodologies was evaluated in the recovery of succinic acid from the fermentation broths.

In this circumstance, the crystallization process could be regards as a powerful tool to separate target acids from the broth. Table 4.4 was presented a summary of the purity and yield of succinic acid crystals recovered from the fermentation broth in the process I (modified calcium precipitation process) and II (direct crystallization process). Overall, the purity of the recovered succinic acid crystal was relatively high (95-97%). However, the yield of the succinic acid crystal by mean of the process I were low (41-48%) compared to those obtained in the process II (55-64%) like other calcium precipitation process (Leque *et al.,* 2009,

Liu *et al,* 2010). The purity of the succinic acid crystal was low or high value which was due to the relatively fast acidification rate and fast cooling rate. If the crystallizing was too fast a high supersaturation maybe created and impurities can be easily trapped (Leque *et al.,* 2009).

Table 4.4 Summary of succinic acid crystals recovered in process I and II.

Batch No.	SA conc. ($g\ L^{-1}$)	Process I		Process II	
		Purity (%)	Yield (%)	Purity (%)	Yield (%)
1	53.30	97	48	96	64
2	58.05	97	44	96	58
3	61.38	96	41	96	55
4	58.48	96	43	96	58
5	57.08	97	43	95	59
6	56.08	97	44	95	60

The succinic acid can also be characterized by XRD or ATR-FTIR to determine optimal cooling curve for the batch crystallization of succinic acid (Feng *et al.,* 2002). Figure 4.13 showed the SEM image 300 × of succinic acid after each the recovery purification process. Figure 4.13(A) was the succinic acid image of the commercial analytical reagent. Figure 4.13(B) was the succinic acid image of the succinic acid crystallized from fermentation broth (Process I) .The morphology of which was similar to that of the commercial reagent with few the impurities and the

protein near the surface of the surface of crystal. Figure 4.13(C) was the succinic acid image of the succinic acid crystallized from the fermentation broth (Process II). In the process II, the main an impurity in the crystal obtained was protein residuals, which requiring future refines treatment for obtaining the reagent grade succinic acid. The different SEM image of succinic acid after each purification process, which might due to the effects of the crystallization temperature or the other carboxylic acid. The other carboxylic acids in the mother liquid could have effects on the crystallization process because carboxylic acids have stronger affinity toward the surface of crystal particles (Wada *et al.,* 2001).

Nevertheless, Crystallization process is one of oldest but effective process for the preparation of the succinic acid crystals, the crystallization process might provide the desire solid products without the need for the unit operation. High purities can be obtained with distinct morphologies properties. Crystallization process could be regarded as not only the final purification step but also the first recovery step for the downstream separation process. In addition, crystallization coupled other separation method will obviously increase the final yield of total recovery process.

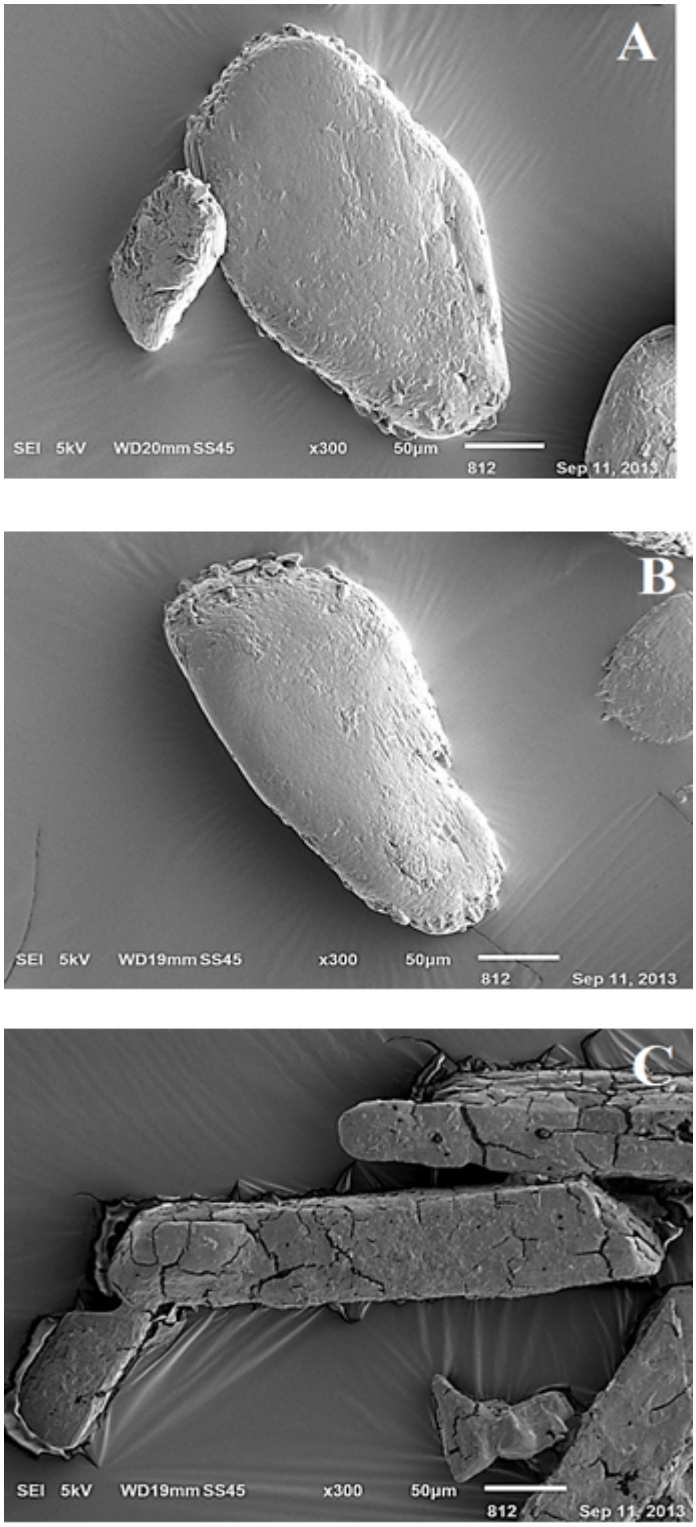

Figure 4.13 SEM image succinic acid: (A) analytical reagent of succinic acid , (B) succinic acid crystals by the process I , (C) succinic acid crystals by the process II.

CHAPTER V

CONCLUSION

Succinic acid can be produced from the optimization fermentation medium by *A. succinogenes* ATCC 55618. This stain was efficiently produced in the repeated batch; a maximal production and yield of succinic acid were 61.38 g L^{-1} and 0.72 g g^{-1}, respectively which was supplied by $CaCO_3$. The repeated batch can be utilized to improve the yield production of fermentation process. Microfiltration membrane was used in this experimental which was set in the bioreactor and the external precipitation tank can be utilized to separate microbial cells and other impurities from fermentation broth which can give high purity of succinic acid. The external precipitation tank can be utilized to obtain the fermentation broth from the bioreactor which can reduce the unit operation of the purification step.

Purification process, modified calcium precipitation couple with crystallization process and direct crystallization process were successfully investigated purification of succinic acid from the fermentation broth. In modified calcium precipitation, precipitation process was primary separation method for removal of the microbial cell and the impurities from fermentation broth and improved recovery of succinic acid. $CaCO_3$ can be used to improve precipitation process in the external precipitation tank. In addition, isolation of succinic acid by precipitation with $CaCO_3$ was also utilized to reduce the dosages of the chemical reagent in traditional precipitation method.

Finally, in this work, the final purification of succinic acid fermentation broth by vacuum distillation and crystallization were studied. These purification processes were simple and effective. Crystallization process was successfully investigated for purification of succinic acid from fermentation broths. Succinic acid crystals were successfully recovered from the fermentation broth. The highest succinic acid crystal was purity 96% and recovery yield was 64% were obtained in direct crystallization process. In comparison, for modified calcium precipitation coupled crystallization process, the highest purity and recovery yield were 97% and 48% respectively. Succinic acid crystals were characterized morphological of succinic acid crystal by scanning electron microscope (SEM). Experimental result showed the different SEM images of the succinic acid crystal after each purification process. Succinic acid crystal from modified calcium precipitation process was similar to that of the commercial reagent with a few impurities near the surface of the crystal.

The results obtained in this study may be useful for reducing the cost of succinic acid fermentation process and suggested an industrial potential of succinic acid production in Thailand that has attracted on development of the bio-succinic acid production.

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APPENDICES

APPENDIX A

MEDIUM

ATCC® 55618 (American Type Culture Collection) Basic fermentation medium composition

To make medium from scratch, follow formulation below (for 1.0 L)

Glucose .....107 g

Yeast extract (YE) .....16 g

Corn steep liquor (CSL) ..... 12 g

KH_2PO_4 .....3.0 g

K_2HPO_4 ..... 1.5 g

NaCl .....1.0 g

$MgCl_2$ .....0.3 g

$CaCl_2$.....0.3 g

$MgCO_3$ ..... 40 g

APPENDIX B

ORGANIC ACID STANDARD CURVES

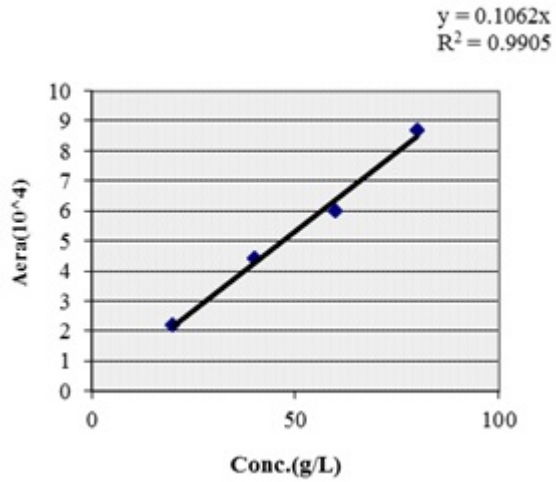

Figure 6.1 Standard curve of succinic acid.

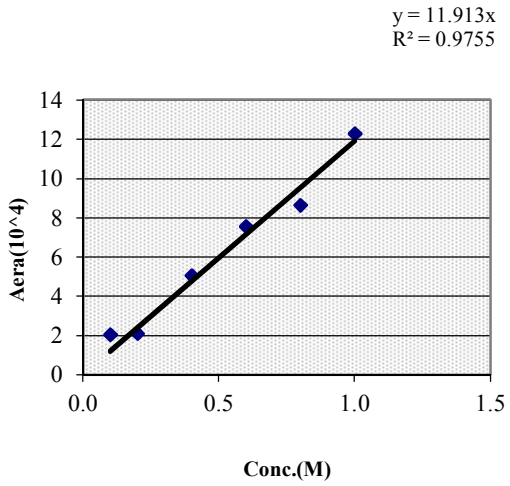

Figure 6.2 Standard curve of lactic acid.

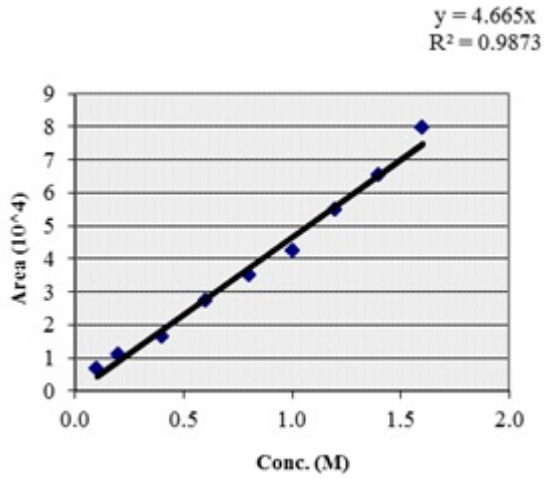

Figure 6.3 Standard curve of acetic acid.

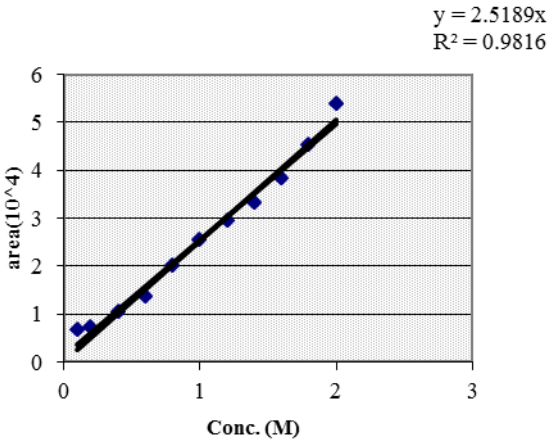

Figure 6.4 Standard curve of formic acid.

APPENDIX C

PROTEIN DETERMINATION

Protein determination (Lowry *et al.*, 1951)

The Lowry protein assay is a biochemical assay for determining the total level of protein in a solution. The total protein concentration is exhibited by a color change of the sample solution in proportion to protein concentration, which can then be measured using colorimetric techniques.

Principle: The –CO-NH- bond (peptide) in polypeptide chain reacts with copper sulphate in an alkaline medium to give a blue colored complex. In addition, tyrosine and tryptophan residues of protein cause reduction of the phosphomolybdate and phosphotungstate components of the Folin-Ciocalteau reagent to give bluish products which contribute towards enhancing the sensitivity of this method.

Reagents Required:

- 2% Na_2CO_3 in 0.1 N NaOH (0.4 g NaOH / 100 ml H_2O + 2 g N_a2CO_3).
- 1 % (w/v) $CuSO_4$. 5 H_2O (1 g/100 ml H_2O).
- 2% (w/v) Sodium tartrate (2 g/100 ml H_2O).
- Folin-Ciocalteu phenol reagent diluted 1:1 with H_2O.
- Bovine Serum Albumin (BSA) (1 mg/ml)

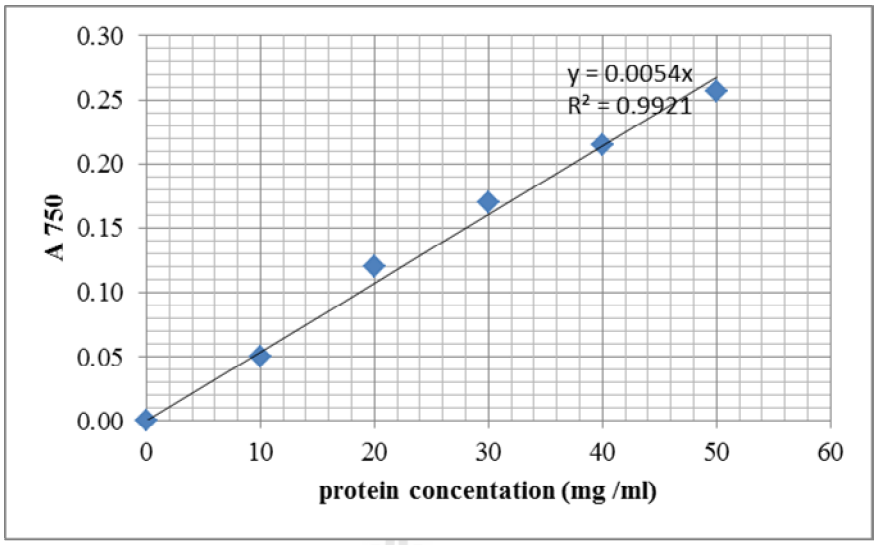

Figure 6.5 Standard curve of protein concentration.

BIOGRAPHY

Miss Jiraphorn Lubsungnoen was born on August 15, 1989 in Nakhon Ratchasima, Thailand. She earned her Bachelor Degree in Science (Environmental Science) from Khon Kean University (KKU) in 2010. She continued with her Master degree in Biotechnology at School of Biotechnology, Institute of Agriculture Technology at Suranaree University of Technology (SUT), Nakhon Ratchasima, Thailand. Her expertise includes the field of downstream fermentation process. Her research topic was extractive fermentation of succinic acid using an external precipitation technique. Her study have been published in journal of membrane science 459 (2014) 132 -142 and the topic was nanofiltration coupled with vapor permeation-assisted esterification as an effective purification step for fermentation-derived succinic acid.