การพัฒนากระบวนการผลิตกรดซักซินิกจากชานข้าวฟ่าง
โดย *Actinobacillus succinogenes* NP9-aA7

นางสาวสุกัญญา พึ่งจะแย้ม

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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PROCESS DEVELOPMENT OF SUCCINIC ACID PRODUCTION FROM SORGHUM STRAW BY *Actinobacillus succinogenes* NP9-aA7

Miss Sukanya Phuengjayaem

A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Biotechnology
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Thesis Title	PROCESS DEVELOPMENT OF SUCCINIC ACID PRODUCTION FROM SORGHUM STRAW BY *Actinobacillus succinogenes* NP9-aA7
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Field of Study	Biotechnology
Thesis Advisor	Assistant Professor Siriluk Teeradakorn, Ph.D.
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สุกัญญา พึ่งจะแย้ม : การพัฒนากระบวนการผลิตกรดซักซินิกจากชานข้าวฟ่างโดย *Actinobacillus succinogenes* NP9-aA7 (PROCESS DEVELOPMENT OF SUCCINIC ACID PRODUCTION FROM SORGHUM STRAW BY *Actinobacillus succinogenes* NP9-aA7) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ดร. ศิริลักษณ์ ธีระดากร, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ศ. ดร. สมบูรณ์ ธนาศุภวัฒน์, หน้า.

จุดประสงค์ของงานวิจัยนี้เพื่อคัดแยก คัดกรอง ระบุสายพันธุ์ และ ศึกษาภาวะที่เหมาะที่สุดต่อการผลิตกรดซักซินิกจากแบคทีเรียที่คัดเลือกได้ จากผลการคัดแยกบนอาหารแข็งขั้นต้นพบว่า 171 ไอโซเลต ให้เคลียร์โซน และเมื่อทดสอบการผลิตกรดเชิงคุณภาพด้วยเทคนิคโครมาโตรกราฟีแผ่นบาง พบว่า 165 ไอโซเลตให้ผลบวก จากนั้นจึงทดสอบการผลิตกรดเชิงปริมาณด้วยเทคนิคโครมาโตรกราฟีเหลวความดันสูงและได้ไอโซเลตที่มีศักยภาพ 58 ไอโซเลต เพื่อศึกษาฟีโนไทป์ ผลการศึกษาพบว่าสามารถแบ่งเป็น 11 กลุ่ม และเลือกไอโซเลตจากตัวแทนกลุ่มไปศึกษาหาลำดับนิวคลีโอไทด์จากยีนส์ 16S rRNA พบว่ามีความใกล้เคียงกับแบคทีเรียจีนัส *Enterococcus* sp. *Streptococcus* sp. *Lactobacillus* sp. *Clostridium* sp. *Lactococcus* sp. *Proteus* sp. และ *Actinobacillus* sp. จาก 11 กลุ่ม ไอโซเลต NP9-aA7 ซึ่งเป็นตัวแทนของกลุ่ม 10 ที่คัดแยกได้จากกระเพาะวัว สามารถผลิตกรดซักซินิกสูงถึง 42.539 กรัมต่อลิตร และผลผลิต 0.709 กรัมต่อกรัมกลูโคส เมื่อศึกษาในระดับยีนส์แล้วมีความใกล้เคียงกับ *Actinobacillus succinogenes* ซึ่งเป็นจุลินทรีย์ที่ไม่มีการเคลื่อนที่ ไม่สร้างสปอร์ แกรมลบ มีลักษณะแท่ง และไม่ก่อโรค ดังนั้นงานวิจัยนี้จึงเลือกไอโซเลตนี้ไปศึกษาต่อการศึกษาภาวะที่เหมาสมต่อการผลิตกรดซักซินิกโดยการใช้สถิติหลายวิธีร่วมกัน ได้แก่ การศึกษาแหล่งอาหารทีละปัจจัยต่อครั้ง การหาปัจจัยที่สำคัญโดยวิธี Plackett-Burman Design (PBD) และ หาความเข้มข้นที่เหมาะสมโดยวิธี Box-Behnken Design (BBD) โดยใช้การแปลผลกราฟแบบ Response Surface Methodology (RSM) ผลที่ได้พบว่า ปัจจัยของแหล่งอาหารที่สำคัญประกอบด้วยกลูโคส 74 กรัมต่อลิตร สารสกัดยีสต์ 30 กรัมต่อลิตร ตัวปรับกลาง 60 กรัมต่อลิตร ($MgCO_3$ 45 กรัมต่อลิตร และ $Mg(OH)_2$ 15 กรัมต่อลิตร) สามารถผลิตกรดซักซินิกสูงสุด 60.087 กรัมต่อลิตร และผลผลิต 0.816 กรัมต่อกรัมกลูโคสที่ 36 ชั่วโมงของการหมัก จากกระบวนการหมักแบบครั้งเดียวในถัง 2 ลิตร *A. succinogenes* NP9-aA7 สามารถผลิตกรดซักซินิกสูงสุด 58.080 กรัมต่อลิตรที่ 27 ชั่วโมงของการหมัก เป็นการยืนยันผลของสมการการผลิตกรดซักซินิกที่ได้จากวิธีทางสถิติ เมื่อเพิ่มก๊าซคาร์บอนไดออกไซด์ลงในอาหารเลี้ยงเชื้อพบว่าซักซินิกสูงขึ้นถึง 72.930 กรัมต่อลิตร และผลผลิต 1.393 กรัมต่อกรัมกลูโคส ที่ความดัน 50.66 กิโลปาสคาลที่ 24 ชั่วโมงของการหมัก เมื่อนำสารละลายย่อยสลายชานข้าวฟ่างมาใช้เป็นแหล่งคาร์บอน *A. succinogenes* NP9-aA7 สามารถผลิตกรดซักซินิกได้ 19.139 กรัมต่อลิตร ใกล้เคียงกับสายพันธุ์ดั้งเดิมของ *Actinobacillus succinogenes* DSMZ 22257 (Type strain) จากงานวิจัยนี้จึงสามารถแนะนำได้ว่า ชานข้าวฟ่างซึ่งเป็นวัตถุดิบเหลือใช้ทางการเกษตรสามารถนำมาใช้เป็นทางเลือกของแหล่งคาร์บอนในการผลิตกรดซักซินิกโดย *A. succinogenes* NP9-aA7 ได้

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต .....

ปีการศึกษา 2559 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

5572882923 : MAJOR BIOTECHNOLOGY
KEYWORDS: SUCCINIC ACID / SCREENING / IDENTIFICATION / ACTINOBACILLUS SUCCINOGENES / STATISTICAL / PLACKETT-BURMAN DESIGN (PBD) / BOX-BEHNKEN DESIGN (BBD) / CENTRAL COMPOSITE DESIGN (CCD) / RESPONSE SURFACE METHODOLOGY (RSM)

SUKANYA PHUENGJAYAEM: PROCESS DEVELOPMENT OF SUCCINIC ACID PRODUCTION FROM SORGHUM STRAW BY *Actinobacillus succinogenes* NP9-aA7. ADVISOR: ASST. PROF. SIRILUK TEERADAKORN, Ph.D., CO-ADVISOR: PROF. SOMBOON TANASUPAWAT, Ph.D., pp.

The aim of this study emphasized the isolation, screening, identification and optimization of the potential bacteria for succinic acid production. From primary screening, 171 isolates exhibited a clear zone on the screening medium. From qualitative analysis, 165 isolates gave positive by TLC. The potential 58 isolates were selected with quantitative analysis by HPLC for phenotypic characteristic. Fifty-eight isolates were divided into 11 groups. Representative isolate from each group have been identified based on its 16S rRNA sequence analysis. They were closely belonged to genus *Enterococcus*, *Streptococcus*. *Lactobacillus*, *Clostridium, Lactococcus*., *Proteus*. and *Actinobacillus*. Among 11 groups, the isolate NP9-aA7 representative from group X was isolated from the bovine rumen. It gave a high succinic acid of 42.539 g/L with a yield of 0.709 g/g glucose. It was identified to *Actinobacillus succinogenes*, non-motile, non-spore-forming, gram-negative rod and non-pathogenic. The statistical method combining a one factor at a time method, a Plackett-Burman Design (PBD) and a Box-Behnken Design (BBD) using a Response Surface Methodology (RSM) were developed to optimize succinic acid production by *A. succinogenes* NP9-aA7. Key medium consisted of 74 g/L of glucose, 30 g/L of yeast extract and 60 g/L of alkaline neutralizer (45 g/L of $MgCO_3$ and 15 g/L of $Mg(OH)_2$) gave a maximum succinic acid to 60.087 g/l with a yield of 0.816 g/g glucose after 36 h of cultivation time. Batch fermentations in a 2 L fermenter, the model was validated with succinic acid of 58.080 g/L after 27 h of cultivation time. Addition of CO_2 partial pressure in the medium had significant improved the production of succinic acid to 72.930 g/l with a yield of 1.393 g/g glucose at the CO_2 partial pressure of 50.66 kPa after 24 h of cultivation time. Using sorghum straw hydrolysate as an alternative carbon source, *A. succinogenes* NP9-aA7 gave a succinic acid of 19.139 g/L closely to type strain of *A. succinogenes* DSMZ 22257. It could be suggested that SSH, a renewable material, could be used as an alternative carbon source for succinic acid production by *A. succinogenes* NP9-aA7.

Field of Study: Biotechnology
Academic Year: 2016

Student's Signature .....
Advisor's Signature .....
Co-Advisor's Signature .....

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CHAPTER I

INTRODUCTION

1.1 Research rationale

Succinic is a dicarboxylic acid having the molecular formula of $C_4H_6O_4$. It is regarded as a precursor for many industrial chemicals and the demand is increasing to continuous. Succinic acid is mostly produced commercially by the petrochemical process from n-butane manufactured via the hydrogenation of maleic anhydride to succinic anhydride, followed by hydration to succinic acid. The manufacturing cost is affected by several factors including productivity and yield of succinic acid, the costs of raw materials and recovery method. Limitation of petroleum resources and the increasing global demands and the emergence of environmental consequences from excessive using fossil fuels is currently being exerted to development of the bio-based succinic acid and its derivatives using renewable biomass as a carbon source to reduce greenhouse gas (Bechthold, Bretz, Kabasci et al., 2008; McKinlay, Vieille and Zeikus, 2007).

Succinic acid is an intermediate of the tricarboxylic acid (TCA) cycle and a fermentative end-product of anaerobic metabolism. Thus, it is synthesized in almost all microbial, plant and animal cells. The organisms suitable for production of effective succinic acid can be divided into fungi and bacteria. Various microorganisms have been screened and studied to produce succinic acid using difference carbon sources. The production of succinic acid by bacteria under the anaerobic condition, just a few Gram-positive bacteria such as *Corynebacterium glutamicum* (Inui, Murakami, Okino et al., 2004) and *Enterococcus faecalis* (Wee et al., 2002) have been reported for succinic acid production. Gram-negative bacteria have been isolated from anaerobic environments such as bark of tree, domestic sludge, cattle waste, rice paddy, marine shipworm, mouth of dog, rumen and gastro-intestines (Song, Alijani, Frank et al., 2006). Gram negative bacteria have been reported succinic acid including *Anaerobiospirillum succiniciproducens*, *Propionibacterium* sp., *Escherichia coli*,

Bacteroides sp., *Ruminococcus flavefaciens*, *Actinobacillus succinogenes*, *Bacteroides fragilis* (Beauprez, De Mey and Soetaert, 2010). Most bacteria, which are isolated from the rumen of ruminants, including *A. succinogenes, A. succiniciproducens* and *M. succiniciproducens* (Song et al., 2006). They are the best candidates for succinic acid production as they produce succinic acid as a major fermentation product. The rumen is a unique microbial ecosystem discover in many species of herbivorous mammals known as ruminants because it has allowed carbon dioxide, methane and traces of hydrogen production (Lee, Lee, Lee et al., 1999b).

The development of succinic acid production, there are many factors involved therefore a great number of experiments should be concurrently run, and the possible interactions between these factors are also needed to be investigated. For the fermentation medium optimization, Plackett-Burman Design (PBD) is a good choice in rapid screening many key medium components, to identify the most significant independent factors (Liu and Tang, 2010). Then the concentration of the key medium is optimized by a Box-Behnken Design (BBD) using a Response Surface Methodology (RSM) for estimation the relationships between the response and the key factors. Compared with the one-factor-at-a-time method, the statistical experimental design has the advantages of reduction the number of experiment numbers and improving statistical interaction analysis (Ren, Lin, Shen et al., 2008).

Theoretically, succinic acid can be formed fermentative from glucose with following stoichiometry (Lee et al., 1999b)

$$Glucose + 2CO_2 + 4H \rightarrow 2Succinic\ acid + 2H_2O$$

Therefore, the supplies of CO_2 and electron donors are necessary to achieve good succinic acid production. The research explained that CO_2 could significantly affect the cell growth of anaerobic bacteria (Dehority, 1971; Ohta, Fukui and Kato, 1989). Also, the level of CO_2 in culture medium could affect the metabolic fluxes and the distribution of fermentation products. In the fermentation of succinic acid under the anaerobic condition, the increasing gas CO_2 has a significant effect on acid production because CO_2 is included into the backbone of three carbon compound to generate four carbon oxaloacetate through PEP carboxylase to increase the

production of succinic acid. Also the effect of CO_2 will be studied in a 2-L fermenter after obtained optimizing medium compositions.

1.2 Research objectives

The overall objectives of this research are as follow:

1. To screen, characterize and identify succinic acid producing bacteria from various sources (CHAPTER III)

2. To optimize succinic acid production by *Actinobacillus succinogenes* DSMZ 22257 (CHAPTER IV)

3. To optimize succinic acid production by the selected bacteria using statistical aspect (CHAPTER V)

4. To develop succinic acid production process using sorghum straw by the selected bacteria and preliminary study of succinic acid production in a 2-L fermenter (CHAPTER VI)

1.3 Scope of dissertation

Currently, succinic acid fermentation process has not been successfully commercialized. Key technical problems blocking rapid advances in developing a bioprocess technology for succinic acid fermentation are of low productivity, multiple product formation and inefficient recovery of product from the fermentation system. This study emphasizes the need to carry out the isolation, screening and identification of potential bacteria for succinic acid production (CHAPTER III). In order to develop the process of succinic acid production with the cost-competitive, strain improvement for the maximum production of succinic acid but minimum of the by-products formation through rational biochemical processes is important. The succinic acid producer; *A. succinogenes* NP9-aA7 is optimized condition using statistical method (CHAPTER V) then scale up to a 2-L fermenter (CHAPTER VI). Besides, a comparison of the results with type strain of *A. sucinogenes* obtained from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cultures (CHAPTER IV). The result from optimization process by *A. succinogenes* NP9-aA7, increased succinic acid production while reduced formation of by-products, such

as acetic and formic. In addition, the possibility of cost-effective succinic acid production by *A. succinogenes* NP9-aA7 from inexpensive and abundant feedstocks using sorghum straw hydrolysate is also investigated. The process of isolation, screening, identification and optimization of promising bacterial strain compared with type strain of microorganism which descripted in four chapters III-VI (Figure 1.1)

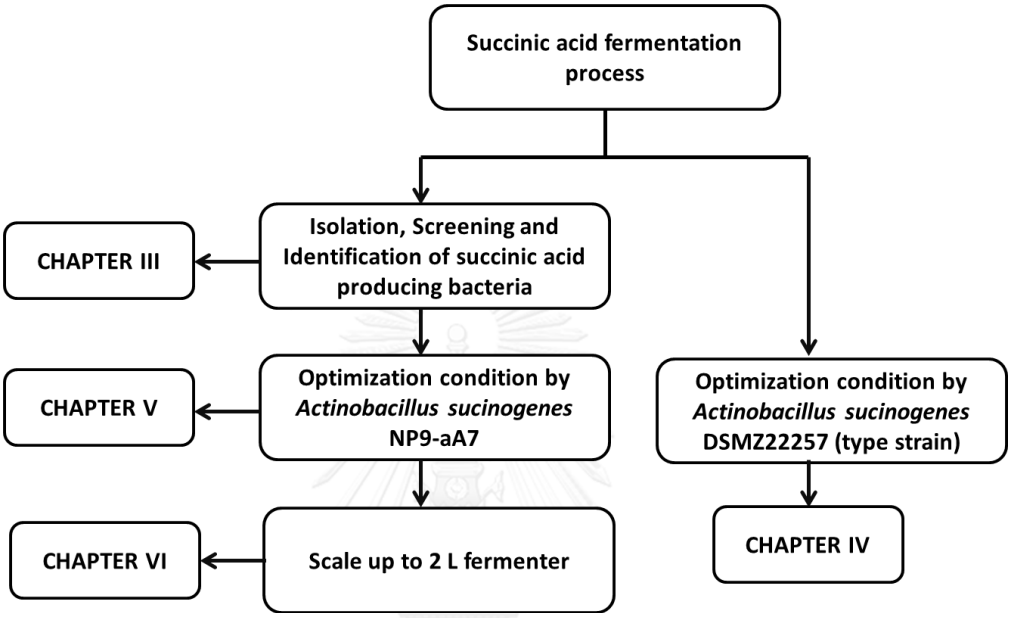

Figure 1.1 Diagram of the scope of this research

1.4 Expected results

Obtain the potential succinic acid bacteria with ability in utilizing sorghum straw hydrolysate. Optimization of succinic medium composition by the selected bacteria and added CO_2 in a 2-L fermenter will improve efficiency of succinic acid production.

CHAPTER II

LITERATURE REVIEWS

2.1 Background of succinic acid

Succinic acid is known as amber acid and synonyms are butanedioic acid, dicarboxylic acid C4, ethylenesuccinic acid. It consists of four carbon aliphatic dicarboxylic acid. It is molecular formula of $C_4H_6O_4$ (Figure 2.1). It has molecular weight: 118.1 g/mol. Physical properties at room temperature is a solid that forms colorless. It is high melting point at 185-187 °C and boiling point at 235 °C.

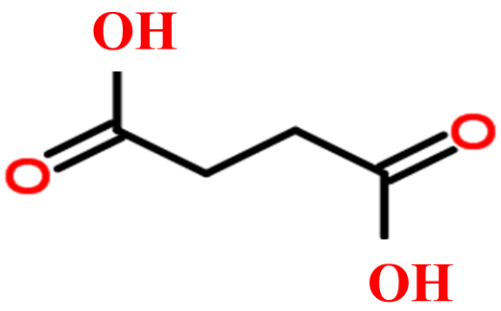

Figure 2.1 Structure of succinic acid

It has molecular weight: 118.1 g/mol. Physical properties at room temperature is a colorless crystalline solid, has negligible vapor pressure. It is high melting point at 185-187 °C and boiling point at 235 °C. It is high water solubility, minimum volatility and non-flammable. Moreover succinic acid is one of the end products of anaerobic fermentation and an intermediate of tricarboxylic acid (TCA) cycle.

2.2 Application of succinic acid

Currently, succinic acid is used or involved in supporting the production of various types of materials that affect everything from pharmaceuticals to food ingredients, flavoring agents, solvent additives, peptide synthesis, biodegradable plastic, fuel additives and many applications are shown in Figure 2.2

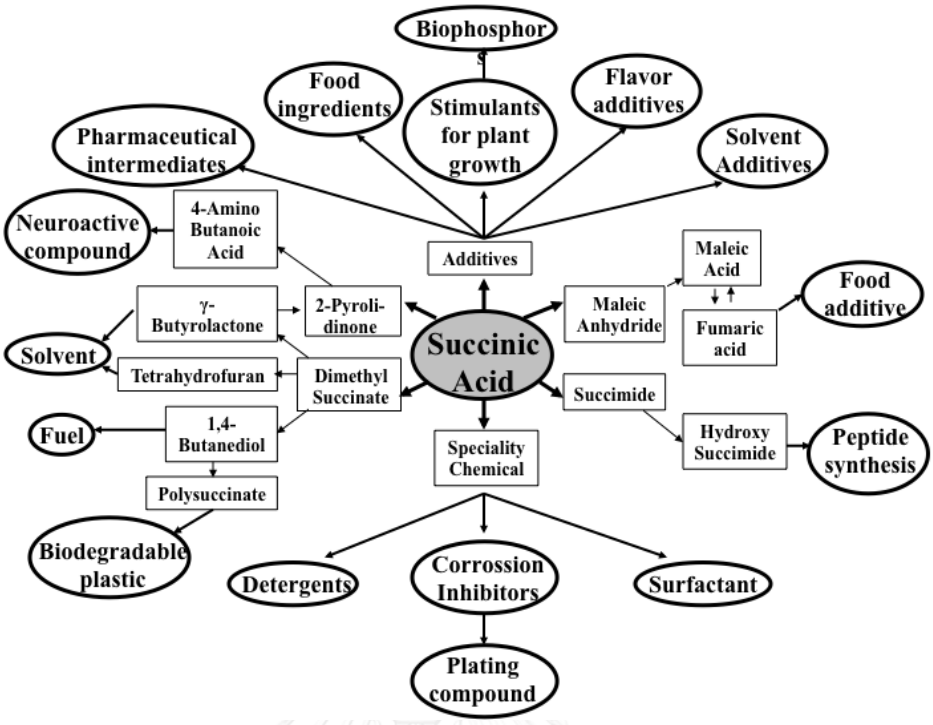

Figure 2.2 Applications of succinic acid.

(http://www.elatewiki.org/index.php/Image:Succinic_acid_uses.png)

Succinic acid can be utilized not only an end product, but also as a precursor for a variety of fine chemicals, including 1, 4-butanediol, tetrahydrofuran, N-methyl pyrrolidinone, 2-pyrrolidinone, succinate salts and γ-butyrolactone for the use in agricultural, food and supplements used in pharmaceuticals industries, antibiotics and vitamins (Van der Werf, Guettler, Jain et al., 1997). Furthermore, the increasing demand for succinic acid is expected as its use is extended to the synthesis of biodegradable polymers such as polybutyrate succinate (PBS) and polyamides (Nylon®x,4) (Willke and Vorlop, 2004) and various green solvents (Rudner, Jeremic, Petterson et al., 2005). Based on the application characteristic above makes succinic acid one of the most attractive green chemicals currently available, and has become the subject of significant concern to bioengineers, as well as chemical engineers.

2.3 Opportunities and Supposition

A new report from Grand View Research Inc., the global succinic acid market is expected to reach USD 237.8 million by 2022, according to rising need in various application segments including 1,4 butanediol (BOD), pharmaceuticals, and polyurethanes, which are key components for the product is expected to result in a greater share for the product over the next seven years. Health sector growth due to increased consumer awareness in emerging Asia Pacific economies is expected to benefit the industry in the forecast period.

Europe dominated the market accounting for over 35.0% of global follow by North America and Asia-Pacific with 31% and 28.0%, respectively. However, the region is expected to testify a sluggish demand for bio-based succinic acid for replacement. The stringent regulatory situation in the European Union due to the implementation of REACH legislation is expected to support the use of biological substitutes in the forecast period. Asia Pacific is expected to be the fastest growing market with Compound Annual growth rate (CAGR) over 10.5% from 2015 to 2022. The lack of strict regulation in the region is expected to be a key driver in the market. Cosmetic industry growth in China, India, and Japan is expected to further augment succinic acid demand over the forecast period. The global succinic acid market was valued at approximately USD 400.0 million in 2014 and is expected to reach approximately USD 1,000.0 million by 2020, growing at a CAGR of over 20% between 2015 and 2020. In case bio based succinic acid in global market is expected to reach market volume of 710.0 kilo tons by 2020, growing at a CAGR of 45.6% during 2013-2020 (Clark, 2014; John, 2015).

2.4 Process of succinic acid production

Currently, commercially succinic acid is mostly being produced by the petrochemical process from n-butane manufactured via the first step in the reaction sequence, maleic anhydride is hydrogenated to succinic anhydride (Figure 2.3A). The catalyst and process conditions for the hydrogenation of maleic anhydride are extremely selective with 98–99% of the maleic anhydride converted to succinic anhydride. The

catalyst typically used is a Ni/Zr/Al/Si alloy. Succinic anhydride can be reacted with water by hydration to succinic acid (Figure 2.3B).

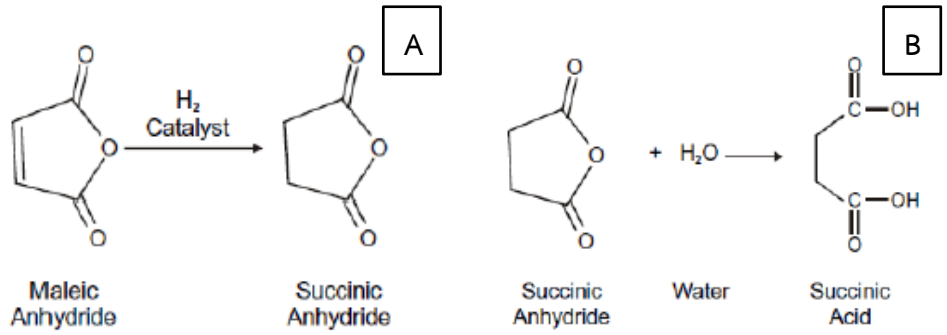

Figure 2.3 The reaction of succinic acid production by the petrochemical process.

Although the overall economics still limits the bio-based succinic acid production, the assessment of raw material cost and recovery method. The estimation of potential market size clearly suggest that the current petroleum based processes will be soon replaced by the fermentative succinic acid production system cause the limited nature of petroleum reserves, the increasing global demands and the emergence of environmental consequences from excessive using fossil fuels is currently being exerted to development of the bio-based succinic acid and It has been produced by microbial fermentation for reduce greenhouse gas (McKinlay, Zeikus and Vieille, 2005; Song and Lee, 2006). 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 (Raja and Dhanasekar, 2011). It is also notable that a greenhouse gas CO_2 is fixed into succinic acid during the fermentation.

2.5 Microorganism for succinic acid fermentation

The production of succinate using microorganisms can be achieved by growing either fungi or bacteria on various liquid or solid nutrient sources. Liquid culture has become the primary means of producing industrial chemical commodities due to its ability to achieve continuous high yields. Depending on the type of microorganism, a liquid fermentation can require some combination of temperature control, pH control, agitation and gas sparging. Under the anaerobic

fermentation, succinic acid is an intermediate in the tricarboxylic acid (TCA) cycle and is one of the end-products found in living organisms. Thus, it is synthesized in almost all microbial, plant and animal cells. Those organisms suitable for the efficient production of succinic acid can be categorized into fungi and bacteria (Song and Lee, 2006).

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 fumigatus* and *Penicillium simplicissimum*. These organisms produce succinic acid as a metabolic byproduct under aerobic and/or anaerobic conditions (Ling, Dibble, Houston et al., 1978; Rossi, Hauber and Singer, 1964). *P. simplicissimum* are known to excrete succinate and citrate co-excretion was studied under anaerobic and aerobic conditions. Increased excretion of succinate was observed when the respiratory function was inhibited using sodium azide or anaerobic conditions. *A. niger* has been recognized as a very important organism for the production of various organic acids, especially citric acid and gluconic acid. This organism produces more than 78 g/L of citric acid with the yield of 65% (w/w) on sucrose (McIntyre and McNeil, 1997). Furthermore, it shows an ability to utilize various carbon sources with a good yield (115%, w/w) on rapeseed oil (Elimer, 1998). Based on genomic, biochemical and physiological information have been reported. it can produce 1.5 mol succinic acid from 1 mol glucose under micro-aerobic condition (David, Akesson and Nielsen, 2003). However, the use of fungi has been mostly limited to the manufacture of food and beverages due to the difficulties in fermentation. They grow preferentially on solid substrates, which are impractical for industrial scale up. They also produce a wider variety of compounds and byproducts which make isolation and purification more complex. Fungi can also form complex morphologies which make it difficult to control what the culture is producing which leads to lower, inconsistent yields (McKinlay et al., 2005; Song and Lee, 2006).

Yeast, *Saccharomyces cereviseae* has been studied to achieve high concentration of succinic acid in the manufacture of wine and liquor manufacturing (Tomizawa M, 1960; Wakai Y, 1980). A series of its mutant strains were developed by the inactivation of undesired genes, and some of them showed the increased levels

of succinic acid compared with the wild type strain (Arikawa, Kuroyanagi, Shimosaka et al., 1999).

A two-step succinic acid production process was developed, in which fumaric acid obtained from the fermentation of glucose and rice bran using *Rhizopus* sp. is subsequently converted to succinic acid by *Enterococcus faecalis* RKY1 (Wee et al., 2002). The yield of fumaric acid conversion to succinic acid was 95% (w/w) and the productivity was 2.2 g/L•h. However, it should be noted that the yield of fumaric acid on glucose and the fumaric acid productivity in the first step were quite low at the levels of 0.5 g/g substrate and 0.21 g/L•h, respectively, limiting its commercialization. This makes it more favorable to use bacteria instead of fungi or yeasts to produce succinic acid. Yeasts and fungi on the other hand grow at rather acidic pH, which would make downstream processing of those processes more favorable (Porro, Bianchi, Brambilla et al., 1999).

Succinic acid is generated by a number of anaerobic bacteria, only few Gram-positive bacteria such as *Corynebacterium glutamicum* (Inui et al., 2004) and *E. faecalis* (Wee et al., 2002) have been studied for succinic acid production. Several engineered *C. glutamicum* strains were created by disruption and replacement of genes, and their optimal culture conditions were developed. It was possible to increase the succinic acid production rate seven times and the glucose consumption rate five times under oxygen deprived condition (Inui et al., 2004).

Gram negative bacteria including; *Anaerobiospirillum succiniciproducens* (Lee, Lee and Chang, 2008), *Propionibacterium* sp., *Escherichia coli*, *Bacteroides* sp., *Ruminococcus flavefaciens*, *Actinobacillus succinogenes* (Guettler et al., 1999), *Bacteroides amylophilus*, *Prevotella ruminicola*, *Succcinimonas amylolytica* (Bryant, Small, Bouma et al., 1958), *Succinivibrio dextrinisolvens*, *Wolinella succinogenes*, *Cytophaga succinicans* (Beauprez et al., 2010), *Mannheimia succiniciproducens* (Lee et al., 2006) and *Klebsiella pneumoniae* MCM B-325 (Thakker et al., 2006). *A. succiniciproducens*, *A. succinogenes* and *M. succiniciproducens* are a non-pathogenic, non-sporeforming capnophilic (CO_2-loving) Gram-negative, are isolated from bovine rumen (Lee, Lee, Hong et al., 2003). *M. succiniciproducens* evidences

CO_2 dependent growth and succinic acid generation characteristics. When the strain was cultured under an atmosphere of 100% CO_2, the strain manifested balanced growth and produced succinic acid, acetic acid, and formic acid at a constant ratio of 2:1:1 with a 0.76 g succinic acid/g glucose succinic acid yield (Lee et al., 2003).

Among them, *A. succiniciproducens* and *A. succinogenes* have been most intensively studied due to their ability to produce a relatively large amount of succinic acid. When continuous cultures of *A. succiniciproducens* have been conducted at a wide range of dilution rates (0.056 to 0.636/h), succinic acid yields were maintained between 0.83 and 0.88 g/g over the entire range of dilution rates, with a maximum volumetric productivity of 6.1 g/L•h (Lee, Lee, Kwon et al., 2000).

A. succinogenes belongs to *Pasteurellaceae* family based on its 16S rRNA sequence analysis. A total of 2115 genes have been identified of which 1768 have a predicted function (Guettler et al., 1999; Liu, Zheng, Sun et al., 2008). It is a capnophilic, facultatively anaerobic, gram-negative bacterium that naturally produces high concentrations of succinate as a fermentation end product in addition to formate, acetate, and ethanol (Van der Werf et al., 1997). It can be use variety of carbon substrates for succinic acid such as arabinose, cellobiose, fructose, galactose, glucose, lactose, maltose, mannitol, mannose, sorbitol, sucrose, xylose or salicin under anaerobic condition (Guettler et al., 1999). Moreover, *A. succinogenes* is a moderate osmophile and has good tolerance to a high concentration of glucose and more resistant to succinic acid than any other previously reported succinic acid producers(Guettler et al., 1999). *A. succinogenes* has recently been used widely for succinic acid production from many renewable resources, such as wheat (Dorado, Lin, Koutinas et al., 2009), straw (Zheng, Dong, Sun et al., 2009), crop stalk wastes (Li, Yang, Wang et al., 2010), corn fiber (Chen, Jiang, Wei et al., 2010) and cane molasses (Liu et al., 2008). *A. succinogenes* 130Z strain and its variant strains (FZ 6, 9, 21, 45 and 53) which were resistant to 1–8 g/L of fluoroacetate were used to evaluate the possibility of commercial succinic acid production. They were able to produce larger amounts of succinic acid and are more resistant to succinic acid than any other previously reported succinic acid producers. Strain 130Z produced 66.4 g/L of succinic acid by consuming 98.3 g/L of glucose after 84 h fermentation. The batch

fermentation was performed in a 1-L fermenter with 15 g/L of yeast extract and corn steep liquor. The concentrations of byproducts including acetic, formic, propionic and pyruvic acids were detected at the values of 12.0, 8.7, 2.5 and 4.3 g/L, respectively. More recently, the continuous and repeat-batch biofilm fermentation of *A. succinogenes* allowed a significant increase in succinic acid productivity (8.8 g/L•h), while the yield of succinic acid was less than 50% (w/w), which is rather low for commercialization (Urbance, Pometto, DiSpirito et al., 2004).

Although the variant strains produced less ethanol, acetic, formic and lactic acids, formation of these byproducts could not be completely avoided. Furthermore, the accumulation of propionic and pyruvic acids, which are not generally detected in the cultivation of other succinic acid producing bacteria, was observed. Considering the costs of separation and purification of succinic acid from fermentation broth containing mixed acids, the formation of byproducts should be minimized, or if possible, completely eliminated by metabolic engineering and fermentation process optimization (Song and Lee, 2006).

2.6 Pathway of succinic acid

In pathway of succinic acid production from *A. succinogenes*, it converts glucose to phosphoenolpyruvate (PEP), at which point metabolism splits into the following two branches: The C4 pathway is defined as: phosphoenolpyruvate-Malate-Fumarate then convert to succinnate. While the C3 pathway is defined as: PEP-Pyruvate-Acetyl-CoA-Acetate and Ethanol (Figure 2.4).

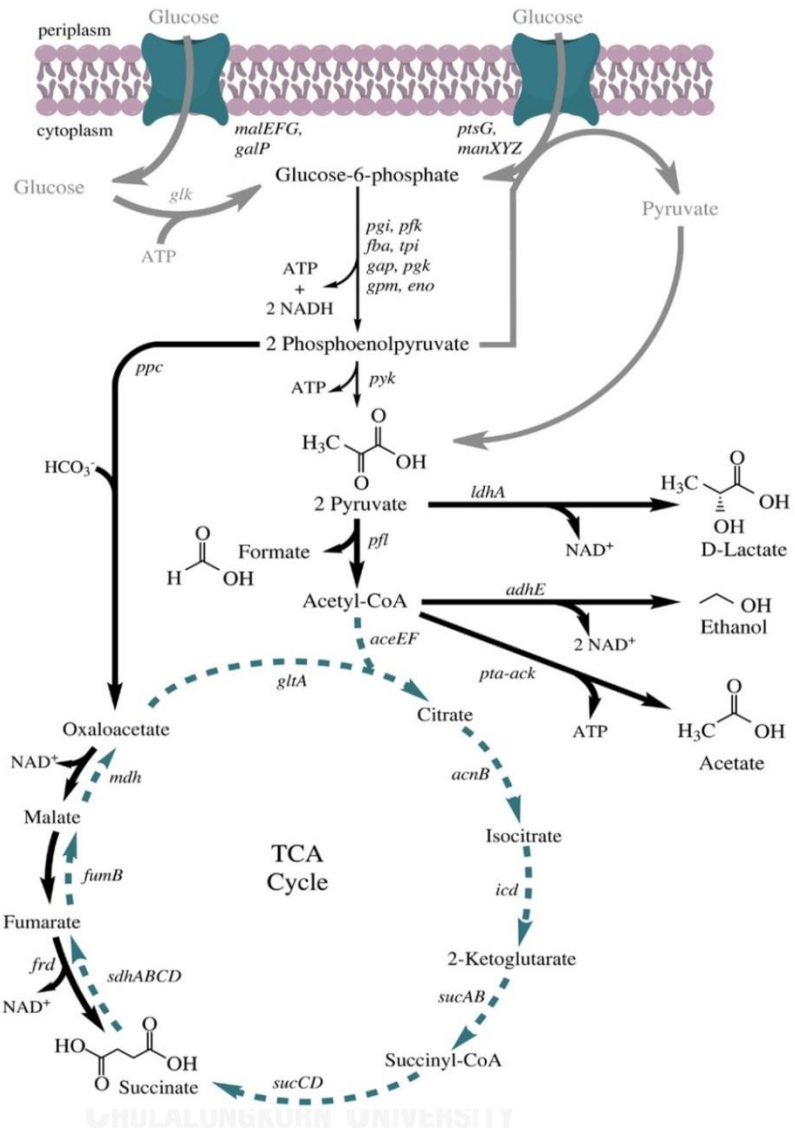

Figure 2.4 The pathway of succinic acid production in bacteria.

Bold gray arrows: glucose transport systems; Solid lines: pathways or reactions for which enzyme activity is detected in vitro; dotted lines: pathways or reactions where no activity or uncertain activity is detected in vitro (Van der Werf et al., 1997). Genes: *malEFG* (maltose ABC transporter), *galP* (galactose:H^+ symporter), *ptsG* (fused glucose-specific PTS enzyme: IIB and IIC component), *manXYZ* (mannose PTS permease), *glk* (glucokinase), *pgi* (glucose-6-phosphate isomerase), *pfk* (6-phosphofructokinase), *fba* (fructose-bisphosphate aldolase), *tpi* (triosephosphate isomerase), *gap* (glyceraldehyde 3-phosphate dehydrogenase), *pgk* (phosphoglycerate kinase), *gpm* (phosphoglycerate mutase), *eno* (enolase), *pyk* (pyruvate kinase), *ppc* (phosphoenolpyruvate carboxylase), *ldhA* (lactate dehydrogenase), *pfl* (pyruvate formate lyase), *aceEF* (pyruvate dehydrogenase complex), *adhE* (alcohol dehydrogenase), *pta* (phosphate acetyltransferase), *ack* (acetate kinase), *gltA* (citrate synthase), *acnB* (aconitase), *icd* (isocitrate dehydrogenase), *sucA* (2-oxoglutarate decarboxylase), *sucB* (2-oxoglutarate dehydrogenase), *sucCD* (succinyl-CoA synthetase), *sdhABCD* (succinate dehydrogenase), *fumB* (fumarate hydratase), *frd* (fumarate reductase), and *mdh* (malate dehydrogenase) (Forster and Gescher, 2014).

Five key enzymes responsible for succinic acid production are identified to be phosphoenolpyruvate carboxylase (*PPC*), phosphoenolpyruvate carboxykinase (*pepck*), malate dehydrogenase (*mdh*), malic enzyme (*sfc*), fumarase (*fum*) and fumarate reductase (*frd*). PEP carboxylation, which is the important committed step for succinic acid production in rumen bacteria. PEPCK converts phosphoenolpyruvate (PEP) with strongly regulated by CO_2 levels and ADP into oxaloacetate and ATP. Theoretically, 1 mol of CO_2 is required to form 1 mol of succinic acid. The higher CO_2 level resulted in an increased succinic acid production at the expense of ethanol and formic acid. This is most likely due to the increased carboxylation of PEP to oxaloacetate rather than PEP conversion to pyruvate. Also, 1 mol of glucose and 2 mol of CO_2 are required to form 2 mol of succinic acid. Synthetic scheme of biobased succinic acid by fermentation with CO_2 is showed in equation 2.1

$$C_6H_{12}O_6 + 2\,CO_2 \rightarrow 2\,C_4H_6O_4 \qquad \text{(Eq 2.1)}$$

Therefore, the addition of extra electron donors including hydrogen and electrically reduced neutral red resulted in the significant increase of succinic acid production (Park and Zeikus, 1999). The increasing of growth rate was thus linked to the increase in substrate level phosphorylation by this reaction. Moreover, the effects of different environmental and nutritional parameters on succinic acid production and on the activities of these TCA cycle enzymes involved in the production pathway (Agarwal et al., 2007). Through pyruvate oxidation, the byproducts of bacteria fermentations are formed: formate, acetate and ethanol. These reactions form extra reductive power under the form of NADH (except for ethanol), which can increase the flux through the reductive TCA branch towards succinate. Their formation is modulated by the presence of carbonate and carbon dioxide in the medium (McKinlay et al., 2005). The addition of extra reductive power by means of hydrogen reduces the fumarate and acetate flux and increases succinate and ethanol formation (McKinlay and Vieille, 2008).

2.7 Screening microorganism for succinic acid fermentation

Many different succinic acid producing Gram-negative bacteria have been isolated in various anaerobic environments such as bark of tree, domestic sludge, cattle waste, rice paddy, marine shipworm, mouth of dog, rumen and gastro-intestines (McKinlay et al., 2005; Song and Lee, 2006). *A. succiniciproducens* isolated from the mouth, the throat and feces of beagle dog. It is a gram-negative obligately anaerobic bacterium that produces succinate, acetate, formate, ethanol, and lactate, from glucose and lactose (Nghiem, Davison, Suttle et al., 1997).

Currently, most bacteria isolated produce succinate naturally in significant titers, have been isolated in the rumen of ruminants. The rumen is a unique microbial ecosystem found in many species of herbivorous mammals known as ruminants because it has provided carbon dioxide, methane and traces of hydrogen production (Kamra, 2005). The structure and composition within the abdomen of is displayed in Figure 2.5.

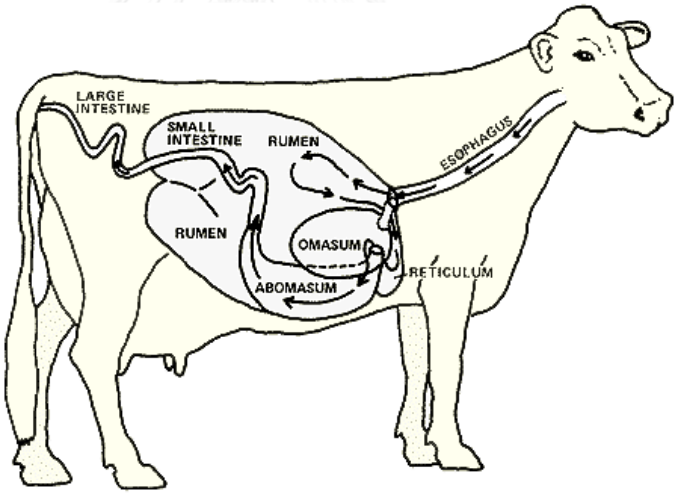

Figure 2.5 The structure and composition within the abdomen of ruminant (https://www.extension.umn.edu/agriculture/dairy/feed-and-nutrition/feeding-the-dairy-herd/img/0469f01.gif)

The primary role of the rumen is to allow pre-gastric digestion of various polysaccharide materials, which is mediated by a great diversity of rumen microorganisms, consisting of 10^{9}–10^{10} bacterial, 10^{5}–10^{6} protozoan and 10^{3}–10^{4} fungal cells/mL of rumen fluid (Orpin and Mathiesen, 1986). The production of C4 dicarboxylic acids in the rumen reduces energy loss associated with methanogenesis

(30–40 mol% of CH_4 is present in the ruminal gas) by increasing the amount of metabolizable energy available to the animal in the form of propionic acid. Although the C4 dicarboxylic compounds, such as oxaloacetic, malic, fumaric and succinic acids are not detected in the ruminal fluid, large amounts of these acids are produced by CO_2 fixation reactions, using 60–70 mol% of CO_2 present in the ruminal gas (Song and Lee, 2006).

A. succinogenes, *M. succiniciproducens* and *B. fragilis* are natural succinate producing strains, which all have been isolated in the rumen. They produce a mixture of volatile organic acids and as capnophiles they can cope with high carbon dioxide and use it as a carbon source together with sugars (Song and Lee, 2006). In some cases carbon dioxide is essential for growth and adapted screening methods have to be employed to isolate novel capnophilic strains (Ueda, Tagami, Kamihara et al., 2008). Most probably these efforts will lead to many more isolates that efficiently produce succinate.

The major C3 compounds in the cell used for carboxylation reaction are PEP and pyruvate. In particular, succinic acid is converted to propionic acid, which can account for 20% (w/w) of total volatile fatty acids (VFAs) in the rumen, by succinic acid utilizing bacteria such as *Veillonella parvula* (Johnston and Goldfine, 1982), *Selenomonas ruminantium* (Linehan, Scheifinger and Wolin, 1978) and *Succiniclasticum ruminis* (van Gylswyk, 1995). Propionic acid produced this way is absorbed through the rumen wall for subsequent oxidation to provide energy and biosynthetic precursors for the animals. Therefore, it is reasonable to think that some microorganisms present in the rumen will be a good succinic acid producer (Song and Lee, 2006). A disadvantage of the same environment is the richness of different substrates. A lot of vitamins and amino acids are abundant in the rumen, which resulted in the loss of biosynthetic routes making the addition of these vitamins and amino acids to minimal medium necessary (McKinlay et al., 2005). The actuality that glutamate is an essential amino acid and α -ketoglutarate can be used instead suggests that isocitrate dehydrogenase and α -ketoglutarate dehydrogenase from the TCA cycle is missing or not active during the growth of glucose.

2.8 Optimization for succinic acid production

The formation of byproducts such as acetic, formic and lactic acids is a major problem that has to be solved because they reduce the succinic acid yield and productivity, while increases the complexity and cost of succinic acid purification and recovery. Additionally, attempts to produce succinic acid by the cost-effective using renewable biomass which is much less expensive than refined carbohydrates.

In addition, not only carbon sources and nitrogen source effect on succinic acid production. The alkaline neutralizers are required to maintain the pH during succinic acid fermentation. The cost of alkaline neutralizer accounts for a significant portion of raw material costs. Majority of studies on succinic acid production have used $MgCO_3$ as alkaline neutralizer to achieve high product concentrations. Nevertheless, the cost of $MgCO_3$ supplementation is not practical for industrial succinic acid fermentation. Also it needs to define alkaline neutralizer for succinic acid production.

The levels of CO_2, culture pH and medium components have been known to be critical factors affecting both cell growth and succinic acid production. The increased CO_2 availability exerted a positive influence on succinic acid yield, while it depressed cell growth resulting in the decreased succinic acid productivity (Lee, Lee, Kwon et al., 1999a).

Zou, Zhu, Li et al. (2011) reported the effect of dissolved CO_2 concentration in the fermentation broth on succinic acid. It could strongly regulate the metabolic flux of carbon and the activity of phosphoenolpyruvate (PEP) carboxykinase, which were the important committed steps for the biosynthesis of succinic acid by *A. succinogenes* that convert a C3 acid to a C4 acid. Both bicarbonate and gaseous CO_2 can be used, and it has been shown that succinate production is greatly affected by supply of CO_2 or its salts. When $MgCO_3$ was used as the only CO_2 donor, a maximal succinic acid production of 56.1 g/L was obtained, which was just decreased by 7.03% compared with that obtained under the supply of gaseous CO_2 and $MgCO_3$.

Li, Zheng, Fang et al. (2011) studied the effect of different alkaline neutralizers on cell growth and succinic acid production by *A. succinogenes*. The use of $MgCO_3$ as alkaline neutralizer resulted in the high cell growth, glucose utilization,

and succinic acid production. Magnesium carbonate was not only control pH and provide CO_2, but also supplies the cofactor Mg^{2+} for PEP carboxykinase, which is the key enzyme used to synthesize succinate during fermentation. Nevertheless, the cost of $MgCO_3$ supplementation is not practical for industrial succinic acid fermentation. A novel method for regulating pH with mixed alkalis ($Mg(OH)_2$ and NaOH) were studied. The total fermentation cost decreased by 55.9%. Optimum succinic acid of 56.4 g/L and yield of 0.73 g/g substrate were obtained.

2.8.1 One-factor-at-a-time

One-factor-at-a-time method is the simplest statistics for study only one factor. It uses to select the source of the production medium and define appropriate the range of concentrations such as nitrogen source, carbon source and alkaline neutralizer then to use other statistical analysis to the next study.

2.8.2 Plackett-Burman design

The fermentation medium optimizations have many factors involved therefore a great number of experiments should be concurrently run, and the possible interactions between these factors are also needed to be investigated. Plackett-Burman Design (PBD) is a good choice in rapid screening many key medium components, to identify the most significant independent factors (Liu and Tang, 2010). To avoid this, the researchers might choose to first use a "screening design" to identify those factors affect the response significantly. In this design, only main effects are estimated; interactions between factors are often considered trivial and neglected.

The Plackett-Burman design type is a two level fractional factorial screening design for studying N-1 variables using N runs, where N is a multiple of 4. A two level fractional factorial design experiments numbers n are in multiples of 4, i. e. n = 8, 12, 20, 24, 28, 32 etc. the number of experiment including; Factors k less than or equal to n–1, For k less than n-1 use dummy factors. The most common used are n=8 and n=12 (Plackett, 1946).

2.8.3 Box Behnken-Design (BBD)

After the key factor was obtained then the key mediums have been optimized by a Box-Behnken Design (BBD) using a Response Surface Methodology

(RSM) for estimation of the relationships between the response and key factors. Compared with the one-factor-at-a-time method, statistical experimental design has the advantages of reducing experiment numbers and improving statistical interaction analysis (Ren et al., 2008). The BBD design is an independent quadratic design in that there are no embedded factorial designs or embedded fractions.

In this design the treatment combinations are at the midpoints of edges of the process space and at the center. These designs are rotatable (or near rotatable) and require 3 levels of each factor. The designs have limited capability for orthogonal blocking compared to the central composite designs. Figure 2.6 illustrates a BoxBehnken design for three factors.

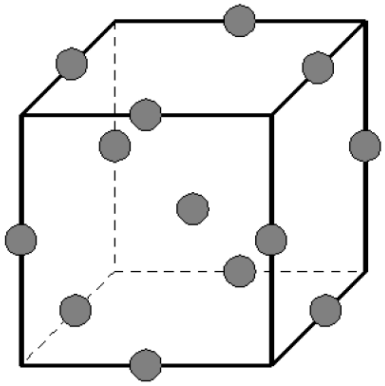

Figure 2.6 A Box-Behnken Design for Three Factors

The geometry of this design suggests a sphere within the process space such that the surface of the sphere protrudes through each face with the surface of the sphere tangential to the midpoint of each edge of the space. The design matrix with three variables and three levels coded as -1, 0, and +1. Experiments are carried out by the conventional 'one-factor-at-a-time' method to select the suitable factors for maximum succinic acid production also the ranges and levels of variables. The test variables are coded according to the following equation (Kilic, Bayraktar, Ates et al., 2002):

$$X_i=(X_i-X_{i*})/\Delta X_i, \qquad \text{(Eq. 2.2)}$$

where X_i is the coded value of the i^{th} independent variable, X_i is the uncoded value of the i^{th} independent variable, X_{i*} is the uncoded i^{th} independent variable at the center point, and ΔXi is the step change value. The statistical software package 'Design-Expert 7.0 (STAT-EASE Inc., Minneapolis, MN, USA, trial version)' is used to analyze the experimental design. The response surface analysis is based on multiple linear regressions that take into account the main, quadratic, and interaction effects in accordance with the following equation:

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_1 X_i + \sum_{i=1}^{3} \beta_2 X_i X_j + \sum_{i=1}^{3} \beta_3 X_i^2 \qquad \text{(Eq. 2.3)}$$

where Y is the predicted response, X_1, X_2 and X_3 are the parameter values for the independent variables. The constants β_0, β_1, β_2 and β_3 are coefficient estimates for succinic acid production. The constants β_0 is intercept term, β_1 is the liner effect, β_2 is the interaction, β_3 is the quadratic effect. In optimization, the response can be related to chosen factors by quadratic models. The experimental data is analyzed using the statistical software to carry out a regression analysis for the equations and for an evaluation of the statistical significance of the different quadratic equation models. The result from statistical including analysis of variance (ANOVA) to obtain the interactive effects between the process variables and the response, the quality of fit of the polynomial model is expressed by the coefficient of determination R^2, and its statistical significance is checked by the *F*-test in the same program.

2.8.4 Central Composite Design (CCD)

Central Composite Design (CCD) is used to develop mathematical models to estimate of the relationships between the response and key factors. Then, to optimize the concentration of the key factors, statistical methods have been done by using response surface methodology (RSM), in which several factors are concurrently identified using fewer experimental (Myers, 1999).

The total number of experiments with three variables are 20 including $2k+2^k+6$, when k=3, where k is the number of variables. At center points had a 2^3 design, which is required for the model; star points or axial points had $2 \cdot k$ design are showed in a random order in which there are six replications at the center points to estimate the pure error (Zhang, Li, Zhang et al., 2012). The star or axial points are, in general, at some value α and $-\alpha$ on each axis. Figure 2.7 illustrates a CCD for three factors.

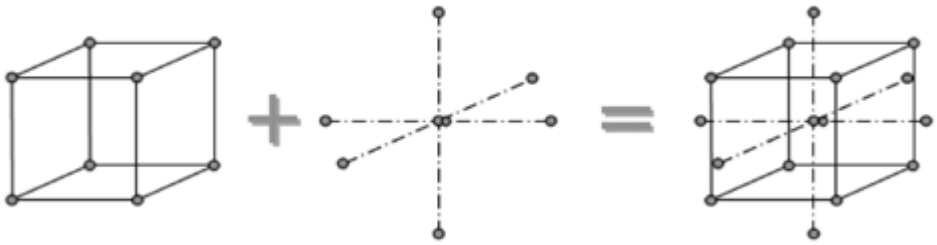

Figure 2.7 Factorial points center points and axial points of Central-Composite Design

In CCD, value of alpha is important to calculate as it could determine the location of axial points in experimental domain. Depending on alpha value, design is spherical, orthogonal, rotatable, or face centered. Practically, it is in between face centered and spherical and is calculated as;

$$\alpha = (2k)^{0.25} \qquad \text{(Eq. 2.4)}$$

Value of α equals 1 is pleasurable because it assure the position of axial point within factorial portion region. It is called face centered design and offers three levels for the factors to be put in the experimental design matrix. The CCD design with three variables at five levels (−1.68, −1, 0, +1, +1.68) is investigated. All the variables are taken to the coded values. In order to control the error bar, 20 runs are showed in a random order in which there are six replications at the center points to estimate the pure error (Zhang et al., 2012). Experimental results obtained are analyzed using response surface regression procedure of statistical analysis system. The optimization of the response could be associated with variables chosen by quadratic models. A quadratic model, which also includes a linear model, is given as Eq. 2.5 (Zhang et al., 2012):

$$Y=\beta_0+\beta_1A+\beta_2B+\beta_3C+\beta_{12}AB+\beta_{13}AC+\beta_{23}BC+\beta_{11}A^2+\beta_{22}B^2+\beta_{33}C^2 \qquad \text{(Eq. 2.5)}$$

where Y is the predicted response; β_0, intercept; β_1, β_2, β_3, linear coefficients; β_{12}, β_{13}, β_{23}, interaction coefficients; β_{11}, β_{22}, β_{33}, squared coefficients. Data are processed for Eq. 2.5 using the Design-Expert program including analysis of variance (ANOVA) to obtain the interactive effects between the process factors and the response. The

quality of fit of the quadratic model is investigated by the coefficient of determination R^2, and its statistical significance is verified by the *F*-test in the same program.

2.9 Renewable resources

Besides, researchers have screened several succinic acid-producing microorganisms. Process developments of fermentative succinic acid for a cost-competitive are strain improvement maximizing the production of succinic acid but minimizing the formation of by-products. To develop the production of succinic acid unit can be improved by using optimum condition, resulting in much increased succinic acid production while reduced formation of by-products, such as acetic, formic, and lactic acids (Song and Lee, 2006). In addition, the utilization of cheap carbon sources; abundant feedstocks, including cane molasses (Liu et al., 2008), wood hydrolysate (Hodge, Andersson, Berglund et al., 2009) and corn fiber (Chen et al., 2010), straw hydrolysate (Zheng et al., 2009) and crop stalk wastes (Li et al., 2010), all of which could be hydrolyzed into mixed sugars.

Agricultural straw, one of the most abundant and renewable sources of lignocellulose biomass, is another potential feedstock for producing succinic acid. It is composed of 35-45% cellulose, 20-30% hemicellulose, and 8-15% lignin (Figure 2.8). Despite its low digestibility, agricultural straw is a good source of fermentable sugars. After pretreatment with dilute acid, alkali, or steam explosion, it can be enzymatically saccharified into fermentable sugars that are mainly a mixture of glucose and xylose. Therefore, straw hydrolysate offers an attractive low-cost feedstock for producing bio-based chemicals, such as ethanol and hydrogen (Saha, Dien and Bothast, 1998).

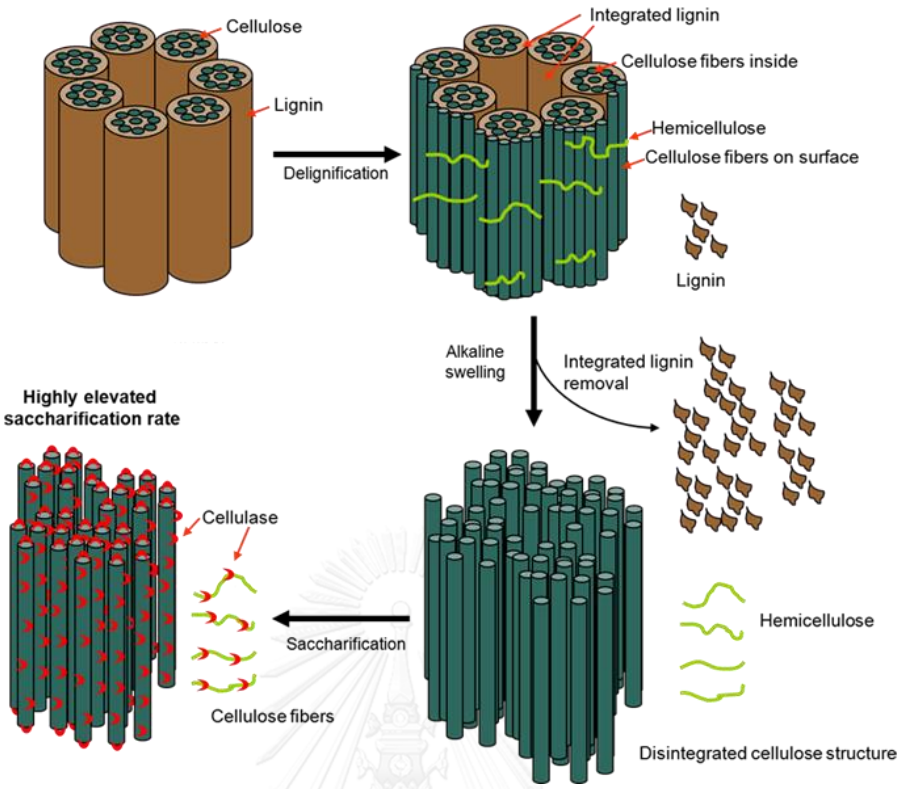

Figure 2.8 The composition, pretreatment and saccharification of lignocellulosic material before used as a carbon source for succinic acid production (Kahar, 2013).

Fortunately, the succinic acid production strains, *A. succiniciproducens*, *A. succinogenes* and *M. succiniciproducens*, have the capability of fermenting both hexose and pentose (Guettler et al., 1999; Liu et al., 2008). Lee et al. (2003) reported *A. succiniciproducens* grew on the medium containing wood hydrolysate was prepared with enzymatic hydrolysis of steam explosive oak wood chips with cellulase enzyme then use as a carbon source for succinic acid production resulting in produced 24 g/L succinic acid with a yield of 0.88 g/g glucose. Kim, Yim, Lee et al. (2004) reported wood hydrolysate was pretreated by NaOH as the carbon source to culture *M. succiniciproducens* MBEL55E, the succinic acid productivity of 1.17 g/L•h and 3.19 g/L•h in batch and continuous fermentation, respectively were obtained. (Zheng et al., 2009) reported that corn stover hydrolysate was used as carbon source in batch fermentation by *A. succinogenes* CGMCC1593. Liu et al. (2008) reported production of succinic acid by *A. succinogenes* CGMCC1593 using cane molasses as a carbon source have obtained 50.6 g/L of succinic acid, resulting in a yield of 79.5 g/g

substrate. Chen et al. (2010) reported the enzymatic hydrolysate of spent yeast cells was evaluated as a nitrogen source for succinic acid production by *A. succinogenes* NJ113, using corn fiber hydrolysate as a carbon source. As a result, succinic acid yield of 67.7% was obtained from 70.3 g/L of total sugar concentration. Yu, Li, Ye et al. (2010) reported corncob hydrolysate was used for succinic acid production. Succinic acids of 23.64 g/L with a yield of 0.58 g/g substrate were produced.

In Thailand, sorghum straw (*Sorghum bicolor* (L.) Moench) is the most abundant and renewable lignocellulose biomass. It is cheap because it is used as animal feed only. It is a cane-like plant with high sugar content. Stem is rich in sugar and juice which brix between 15% and 23%. It is a high photosynthetic efficiency with high biomass yield crop and it is an interesting annual plant because it can adapt to a wide range of climate from the tropics to cool temperate areas. It is drought tolerant and waterlogging resistant, as well as offers salinity and alkalinity resistance properties (Li and Chan-Halbrendt, 2009). This plant mainly composed of soluble (glucose and sucrose) and insoluble carbohydrates (cellulose and hemicellulose). Soluble carbohydrates are easily converted to succinic acid, while insoluble carbohydrates conversion to succinic acid by acid or enzymatic hydrolysis of the biopolymer to soluble oligosaccharides subsequently by fermentation to succinic acid. Sorghum is a potential renewable material for the production of bio-succinate since it is abundant in Thailand. Before using sorghum straw (SS) as a substrate it has been pretreated the lignocellulose. The optimum condition for SS pretreatment at 120 $^{\circ}$C, 3 % H_2SO_4 for 10 min gave a maximum yield of glucose and xylose were 0.234 g glucose/g dry substrate and 0.208 g xylose/g dry substrate, respectively. In this case, a total of 50.04 % of glucan and 76.41 % of xylan were converted to glucose and xylose, respectively (Poonsrisawat, Phuengjayaem, Petsom et al., 2013). The process of succinic acid production from lignocellulosic materials is economically attractive. Despite its potential, the use of sorghum straw for the fermentative production of succinic acid has not been reported yet.

Chapter III

Diversity and Succinic Acid Production of Bacteria Isolated from Thailand

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3.1 Abstract

Currently, the process of fermentation of succinic acid has not been commercially successful because low efficiency, multiple product formation and inefficient fermented product recovery. The aim of this study emphasizes the need to carry out the isolation, screening and identification of potential bacteria for succinic acid production. Bacterial strains were isolated from different sources in Thailand. The result from primary screening, 171 isolates exhibited a clear zone on the screening medium. Secondary screening using TLC for qualitative analysis, 165 isolates were obtained then quantitative succinic analysis using HPLC, the potential 58 isolates were selected for phenotypic characteristic. Fifty-eight isolates were divided into 11 groups. Representative isolate from each group have been identified based on its 16S rRNA sequence analysis. Isolates from group I, II, III and IV were closely related to *Enterococcus* sp. except isolate NP8-aB2 was closely related to *Streptococcus* sp.. Isolates from group V were closely related to *Lactobacillus* sp. Isolates from group VI were closely related to *Enterococcus* sp.*, Clostridium* sp. and *Lactococcus* sp.. Isolate from group VII were closely related to *Enterococcus* sp. and *Lactococcus* sp.. Isolate from group VIII were closely related to *Enterococcus* sp. and *Clostridium* sp. Isolate from group IX was closely related to *Clostridium* sp. Isolate from group X belongs to *Pasteurellaceae* family and were closely related to *Proteus* sp. and *Actinobacillus* sp.. Isolate from group XI were closely related to *Enterococcus* sp. Among 58 isolates, the strain *Actinobacillus succinogenes* NP9-aA7 from group X was selected to further study because it produced high succinic acid of 42.539 g/L with a yield of 0.709 g/g glucose. It was facultative anaerobe and resistant to low pH and non-pathogenic. It could be a promising candidate for further applications.

Key words: soil**,** tree barks, dog saliva, vegetable fermented, buffalo dung, bovine rumen tissue and bovine rumen fluid

3.2 Introduction

Succinic acid, known as amber acid or butanedioic acid, is a dicarboxylic acid having the molecular formula of $C_4H_6O_4$. It is regarded as a precursor for many industrial chemicals including adipic acid, 1, 4-butanediol, tetrahydrofuran, N-methyl pyrrolidinone, 2-pyrrolidinone, succinate salts and gamma-butyrolactone (McKinlay et al., 2007; Song and Lee, 2006). Besides its application in the agricultural, food and pharmaceutical industries, succinic acid can also be used in the synthesis of biodegradable polymers such as polybutyrate succinate (PBS), polyamides and various green solvents (Rudner et al., 2005).

Succinic acid has been synthesized from petrochemical based maleic acid. However, considering the difficulty in obtaining petroleum resources and the volatility of oil prices, succinic acid fermentation is drawing a great deal of attention in response to the current need to develop sustainable processes using renewable resources (Raja and Dhanasekar, 2011). Succinic acid is produced as an intermediate product of the tricarboxylic acid (TCA) cycle and also is a fermentation product of anaerobic metabolism. Thus, it is synthesized in almost all microbial, plant and animal cells. Those organisms suitable for the efficient production of succinic acid can be categorized into either bacteria or fungi (Song and Lee, 2006).

The gram-positive strains of *Corynebacterium glutamicum*, *Enterococcus faecalis* and *Ruminococcus flavefaciens* have been studied for succinic acid production. Several engineered *C. glutamicum* strains were created by disruption and replacement of genes in optimal culture conditions. The rate of succinic acid production was thus increased seven times while glucose consumption increased fivefold under oxygen deprived conditions (Inui et al., 2004). Succinic acids can be produced by gram-negative strains including *Anaerobiospirillum succiniciproducens, Actinobacillus succinogenes* (Caspari and Macy, 1983), *Bacteroides amylophilus* (Caldwell, Keeney, Barton et al., 1973), *Escherichia coli, Mannheimia succiniciproducens, Prevotella ruminicola* (Howlett, Mountfort, Turner et al., 1976), *Succcinimonas amylolytica, Succinivibrio dextrinisolvens, Wolinella succinogenes* and *Cytophaga succinicans* (Guettler et al., 1999). They have been isolated from various anaerobic environments such as domestic sludge, cattle waste, rice paddy, marine

shipworm, mouths of dog, rumen and gastrointestines. But only a few species can produce succinic acid with a high yield. Recently, *A. succinogenes, A. succiniciproducens* and *M. succiniciproducens* have been considered the best candidates for succinic acid production (Song and Lee, 2006). This is most likely due to the fact that the rumen is a highly efficient organ providing an ideal environment to produce succinic acid (Moon, Wee, Yun et al., 2004). The rumen is a unique microbial ecosystem found in many species of herbivorous mammals known as ruminants, caused by carbon dioxide, methane and traces of hydrogen production. Moreover, many vitamins and amino acids are abundant in the rumen resulting in minimal requirements to create a medium (Moon et al., 2004). Although *A. succiniciproducens* is well known as a good succinic acid producer, the fermentation processes which this strict anaerobe is involved in are more difficult to handle than those using facultative anaerobes (Wee et al., 2002) Some facultative anaerobes involved in succinic acid production, such as *Escherichia coli* (Liu, Wu, Li et al., 2011), *A. succinogenes* (Guettler et al., 1999) and *E. faecalis* RKY1(Ryu, Kang and Yun, 1999) have been reported previously. Among these, *E. faecalis* RKY1 were able to produce succinic acid in high yield if cultured anaerobically with glycerol as a hydrogen donor and fumaric acid as a hydrogen acceptor (Ryu, Kang, Pan et al., 2001).

In spite of these points, succinic acid is currently used in the agricultural, food and pharmaceutical industries as a key chemical for the preparation of biodegradable polymers. The first step in the fermentative production of succinic acid is the screening of bacterial strains. To date no process or technology has been successfully commercialized to perform this function. The key problem of the fermentation system was the formation of byproducts such as acetic, formic and lactic acids resulting in reduces the succinic acid yield and productivity, while increases the complexity and cost of succinic acid recovery. Consequently, the objective of this study emphasizes the need to carry out the isolation, identification and screening of the most prominent bacteria with high succinic acid yield and productivity.

3.3 Materials and methods

3.3.1 Chemicals, sources and isolation methods

All chemicals were purchased from Merck (Merck KGaA, Darmstadt, Germany) unless otherwise described. Bacterial strains were isolated from different sources including; soil samples from Suphanburi province of 10 samples, tree barks from Ayutthaya, Nakhonpathom and Suphanburi provinces of 11 samples, dog saliva from Suphanburi provinces of 2 samples, vegetable fermented from Nakhonsawan provinces of 2 samples, Buffalo dung from Surin and Suphanburi provinces of 4 samples, Bovine rumen tissue from Nakhonsawan provinces of 3 samples and bovine rumen fluid from Nakornprathom provinces of 3 samples were collected (Table 3.1). One gram of each sample was enriched in 5 mL of enrichment broth consisting of 20 g/L glucose, 5 g/L polypeptone, 5 g/L yeast extract, 3 g/L K_2HPO_4, 2 g/L NaCl, 2 g/L $(NH_4)_2SO_4$, 0.2 g/L $CaCl_2{\cdot}2H_2O$, 0.4 g/L $MgCl_2{\cdot}6H_2O$, and 15 g/L $MgCO_3$. They were incubated at 37 ºC for 48-72 h under anaerobic conditions using an anaerobic pack (MGC, Japan). Positive tubes were subcultured for the enrichment agar plate diluted to 10^{-6} in Phosphate Buffered Saline (PBS) buffer. The diluted cultures (0.1 mL) were spread onto enrichment agar plates and were incubated in anaerobic conditions at 37 °C. After 24-48 h, visible colonies were picked and re-streaked on fresh enrichment agar plate and incubated overnight at 37 ºC for 24 h under anaerobic conditions.

A single colony extracted from the enrichment agar plates was streaked on screening agar plates consisting of 20 g/L glucose, 1 g/L NaCl, 5 g/L yeast extract, 3 g/L K_2HPO_4, 1 g/L $(NH_4)_2SO_4$, 0.2 g/L $CaCl_2.2H_2O$, 0.2 g/L $MgCl_2.6H_2O$, 15 g/L $MgCO_3$ and 15 g/L agar, pH of the media was adjusted to 6.5 and they were incubated overnight at 37 ºC under anaerobic conditions. Acid-producing isolates exhibited a clear zone around the colonies were selected and purified. They were maintained on a TSA agar plate or slant which consisted of 17 g/L pancreatic digest of casein, 3 g/L soy peptone, 2 g/L glucose, 5 g/L NaCl, and 2.5 g/L KH_2PO_4. Then, positive isolates were stored at -70 °C or lyophilized for further study.

3.3.2 Characterization and Identification of isolates

3.3.2.1 Morphological characteristics

The morphological and cultural characteristics including Gram reaction (Buck, 1982), endospore straining (Schaeffer and Fulton, 1933), cell morphology and colonial appearance of the isolates were determined on the cells grown on a Gifu anaerobic medium (GAM) (Nissui Pharmaceutical Company, Tokyo, Japan) agar plate after incubation under anaerobic conditions at 37 ℃ for 18-24 h.

3.3.2.2 Physiological characteristics

The physiological characteristics included different pH values (3.5-9), temperatures (20-50℃) and NaCl concentrations (6% w/v NaCl) were tested using MRS broth (MRS; de Man, Rogosa and Sharpe) (Appendix A).

3.3.2.3 Biochemical characteristics

Isolates were cultivated at 37℃ under anaerobe for 3 days after that they were diluted by 0.85% NaCl solution (Tanasupawat, Okada and Komagata, 1998) and dropped into medium to test below:

Catalase test, isolates were grown overnight on GAM agar plate and transferred to microscope slide. Then 3% H_2O_2 (Appendix A) was dropped onto colony on the microscope slide. After 5 min, the rapid production of bubbles when colony was mixed with hydrogen peroxide solution was interpreted a positive tests. But no bubble marks have been interpreted a negative tests (Gagnon, Hunting and Esselen, 1959).

Gas production, isolates were examined using Durham tube, a smaller inverted tube which could serve as a trap for gas bubbles generated during fermentation of glucose. A positive test was acidic medium with visible displacement of the gas from the Durham tube (Barrow and Feltham, 1993).

Arginine hydrolysis, isolates were transferred aseptically to a sterile tube of arginine dihydrolase broth (Appendix A). Incubation condition was at 37℃ for up to 24-48 h and the preliminary results were determined. The microbe must first use the glucose present to cause the pH to drop. This was indicated by a change from purple to yellow. Once the medium has been acidified, the enzyme arginine dihydrolase was activated. The culture was incubated an additional 24-48 h at 37 C

to allow the microbe to now use the arginine. The final results are then obtained by observing the tube at 5 days. Change back to purple from yellow indicates a positive test for arginine dihydrolase. Failure to turn yellow at 24 h or to revert back to purple at 5 days indicates a negative result (Niven, Jr and Sherman, 1942).

Nitrate reduction, isolates were dropped into nitrate broth (Appendix A) and inoculated for up to 5 days. Then sulfanilic acid solution (Appendix A) was added 3 drops and followed by 2 drops of *N*,*N*-dimethyl-l-naphthylamine solution (Appendix A). After 3 min a deep, a color change to RED indicates a POSITIVE nitrate reduction test. But the result showed no color change indicates the absence of nitrite. This can happen either because nitrate was not reduced or because nitrate was reduced to nitrite, then nitrite was further reduced to some other molecule. Next step, added a small amount of zinc to each broth. After 5 min a color change to red indicated a negative reaction because nitrate must have been present and must have been reduced to form nitrite while no color change means that no nitrate was present. Thus no color change at this point was a positive result (Conn and Breed, 1919).

Starch hydrolysis, isolates were streaked on starch agar plate (Appendix A). After incubation condition was at 37°C under anaerobe for 24 h, iodine reagent (Appendix A) was added to flood the plate. Clear zone around colonies was positive test ability to digest the starch and thus indicates presence of alpha-amylase. A deep purple to black or bluish color of the agar indicates that starch has not been hydrolyzed and thus a negative test (Iverson and Millis, 1974).

Slime formation, isolates were streaked on 2% sucrose agar plate (Appendix A). Then incubation condition was at 37°C under anaerobe for 24 h. Some bacteria produce a levan as the extracellular polysaccharide. The colonies appear very slimy, mucoidal and runny or as large gum drops on the agar. Some bacteria might produce dextrans in which the colonies were dry and adherent to the plate. A negative reaction was the failure to see extracellular material on the 2% sucrose agar by visual inspection or adherence with a loop (Barrow and Feltham, 1993).

Acid from carbohydrates, isolates were transferred to a sterile tube of medium test (Appendix A). The medium test had carbon sources containing D-amygdalin, L-arabinose, cellobiose, D-fructose, glucose, gluconate, D-galactose,

lactose, maltose, D-mannitol, D-mannose, melibiose, ∞-methyl-D-glucoside, raffinose, rhamnose, ribose, salicin, sorbitol, sucrose, trehalose and D-xylose. Incubation condition was at 37°C for up to 5 days. A positive reaction was recorded when the broth turns yellow. A negative reaction was when no color change occurs. A definite color change that was not quite yellow may be interpreted as a weak positive reaction (Tanasupawat, Chamroensaksri, Kudo et al., 2010).

Statistical Package for the Social Sciences (SPSS) for Windows program version 15 using hierarchical cluster analysis was used to analysis the data from phenotypic characteristics.

3.3.2.4 16S rRNA gene sequence and phylogenetic analysis

Colonies of selected isolates were picked up and transferred to 30 µl sterile distilled water in microcentrifuge tube. After that it was boiled at 95°C for 5 min. Amplification of the 16S rRNA gene was carried out in 50 µl of PCR reaction mixture. The reaction mixtures were shown in Table 3.1 (Tanasupawat et al., 2010)

Table 3.1 Reaction mixtures for PCR

PCR reaction/strain	**Volume (µl)**
Sterile distilled water	30.75
10X PCR buffer	5
$MgCl_2$ (25 mM)	4
Primers (10 *p*mol/µl)	
20F (5'-AGTTTGATCCTGGCTC-3')	2
1530R (5'-AAGGAGGTGATCCAGCC-3')	2
dNTP (2 mM)	1
Taq DNA polymerase (5 U/µl)	0.25

Amplification consisted of 30 PCR cycles. The cycling program was initial denaturation at 94°C for 3 min followed by 30 cycles of denaturation 94°C for 1 min, annealing at 50 °C for 2 min, elongation at 72°C for 2 min. The PCR was ended with a final extension at 72°C for 3 min and amplified product was cool at 4°C. PCR product was checked with agarose gel electrophoresis (Appendix A)

The amplified 16S ribosomal RNA gene sequences were analyzed with Macrogen®, from Korea using primers, 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and

518F (5'-CCAGCAGCCGCGGTAATACG-3'), 800R (5'TACCAGGGTATCTAATCC'3) and 1492R (5'TACGGYTACCTTGT-TACGACTT'3). Sequence alignment was determined by the EzTaxon database (http://www.ezbiocloud.net/eztaxon). Multiple alignments of sequences were performed by the program BioEdit version 7.0.2 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). A phylogenetic tree was constructed by the neighbor-joining method using the program MEGA (version 6), version 6 (Tamura, Stecher, Peterson et al., 2013). A bootstrap analysis of Felsenstein (Felsenstein, 1985) was performed to determine confidence values of individual branches in the phylogenetic tree with 1000 replications.

3.3.3 Succinic acid fermentation of isolates

The ability of isolates to produce succinic acid was investigated by anaerobic fermentation in a medium consisting of 30.0 g/L yeast extract, 2.0 g/L urea, 2 g/L $MgCl_2.6H_2O$, 1.5 g/L $CaCl_2$, 0.07 g/L $MnCl_2$, 4.4 g/L Na_2HPO_4, 3.3 g/L NaH_2PO_4, 30 g/L $MgCO_3$ and the pH was adjusted to 7 (Liang, Liu, Ma et al., 2011b). Glucose was separately sterilized at 121 °C for 15 minutes and added to the medium to maintain the initial concentration of 60.0 g/L. 0.3 µg/L of biotin and 0.2 µg/L of thiamin were prepared by sterile membrane filtration (0.22 µm nylon, Millipore Express, Ireland) and added. The cultivation medium was inoculated with 10% seed inoculum (TSB medium) and incubated at 37 °C, 200 rpm for 48 h under anaerobic conditions.

3.3.4 Analytical method

The culture broth used for succinic acid determination was prepared by centrifuging at 10,000 rpm for 10 minutes at 4 °C. The supernatants were initially analyzed for the presence of succinic acid using thin layer chromatography and the succinic acid was confirmed using high-performance liquid chromatography (HPLC).

3.3.4.1 Cell concentration

The insoluble $MgCO_3$ in the samples was removed by adding 0.2 M of HCl. Then the cell concentration was measured as the amount of absorbance at a 660 nm wavelength using a spectrophotometer (UV160, Shimadzu Corporation, Japan).

3.3.4.2 Glucose concentration

Sugar concentration was measured with the DNS (3, 5-dinitrosalicylic acid colorimetric) method from Miller (1959), with D-glucose as the standard. The mixture contained; 50 µl of sample and 150 µl of DNS reagent were heated in a boiling water bath for 10 min. Then cooled immediately on ice bath and added 1 mL of distilled water. The absorbance was measured by spectrophotometer at 540 nm.

3.3.4.3 Thin layer chromatography (TLC)

Thin layer chromatography is commonly applied as an inexpensive, efficient and fast method for primary detection of succinic acid (Agarwal, Isar and Saxena, 2005). The test samples (10 µL) and 2 g/L of standard succinic acid were spotted onto silica gel TLC plates (Silica gel 60 F254, Merck, Darmstadt, Germany) and resolved using a solvent system comprising ethanol, ammonium hydroxide and water (20:5:3) for 30 minutes . The air dried plates were sprayed with green bromocresol (0.04% w/v in ethanol) and heated at 160 ºC for 5 minutes to reveal the organic acid spots.

3.3.4.4 High-performance liquid chromatography (HPLC)

Fermentation products (succinic, acetic and formic acid) were analyzed with HPLC. Twenty microliter of sample were filtered (0.45 Am, 13 mm membrane disc filters) and loaded on HPLC using a system equipped with a cation-exclusion column (Aminex HPX-87H; 300 mm 7.8 mm; Bio-Rad Chemical) and a refractive index detector (Shimadzu Model RID-6A). The mobile phase was 5 mM of H_2SO_4 solution at a flow rate of 0.6 mL/min with the column operated at 55 ℃.

3.4 Results and discussion

3.4.1 Screening and isolation of succinic acid producing bacteria

3.4.1.1 Primary screening

Two hundred and seven isolates from various sources in Thailand were screened for succinic acid production under anaerobic conditions as below:

Soil samples of 10 samples were collected from upper layer of soils Suphanburi provinces, Thailand. Total 45 isolates were obtained and were represented with SP-(Number of source; 3-10)-(Number of isolate).

Tree barks from of 11 samples were collected from Ayutthaya, Nakhonpathom and Suphanburi provinces, Thailand. They could be isolate to 51 isolates as following;

Nine isolates from 4 samples were obtained from *Ficus religiosa* L. which were collected from Bark of tree in Ayutthaya provinces, Thailand. These Isolates were represented with AY-(Number of source; 1-4)-(Number of isolate).

Five isolates from 1 sample was obtained from *Syzygium cumini* which was collected from bark of tree in Ayutthaya province, Thailand. These Isolates were represented with AY-(Number of source; 5)-(Number of isolate).

Fourteen isolates from 2 samples were obtained from *Samanae saman* which were collected from bark of tree in Bankok, Thailand. These isolate were represented with BK-(Number of source; 1-2)-(Number of isolate).

Seven isolates were obtained from 1 sample of *Sesbania grandiflora* which was collected from bark of tree in Suphanburi province, Thailand. These isolate were represented with SP-(Number of source; 11)-(Number of isolate). Sixteen isolates from 3 samples were obtained from *Musa sapientum* L. which were collected from bark of tree in Nakhonprathom, Thailand. These isolate were represented with NP-(Number of source; 2, 4, 6)-(Number of isolate).

The samples of dog saliva were isolated to 28 isolates from 4 samples of *Canis lupus familiaris* which were collected from dog saliva in Suphanburi provinces, Thailand. These isolate were represented with SP-(Number of source; 14-17)-(Number of isolate).

The samples of vegetable fermented were isolated to 4 isolates from 2 samples of vegetable fermented which were collected from Nakhonsawan provinces, Thailand. These isolate were represented with NS-(Number of source; 17-18)-(Number of isolate).

The sample of buffalo dung of 4 samples could be isolate to 12 isolates including; 2 and 10 isolates were collected from *Bubalus bubalis* in Surin provinces and Suphanburi, Thailand, respectively. These isolate were represented with SR-(Number of source; 1-2)-(Number of isolate) and SP-(Number of source; 1-2)-(Number of isolate).

The sample of bovine rumen tissue of 3 samples could be isolate to 20 isolates were collected from *Bos taurus* in Nakhonsawan provinces, Thailand. These isolates were represented with NS-(Number of source; 13-15)-(Number of isolate).

The sample of bovine rumen fluid of 3 samples could be isolate to 49 isolates were collected from *Bos taurus* in Kasetsart University, Kamphaeng Saen Campus, Nakornprathom provinces, Thailand. These isolates were represented with NP-(Number of source; 7-9)-(Number of isolate).

All of 207 isolates were obtained from various sources in six provinces of Thailand were summarized in Table 3.2

Table 3.2 Collecting the samples from various sources in Thailand.

Isolation Source	Host (Scientific name)	Location (Province)	Collection date	Isolation Name	Total Isolate
Soil sample	-	Suphanburi	27-11-14	SP3-10	38
Soil sample	-	Suphanburi	26-01-15	SP12-13	7
Bark of tree	*Musa sapientum* L.	Nakhonprathom	06-07-14	NP2, NP4, NP6	16
Bark of tree	*Ficus religiosa* L.	Ayutthaya	30-11-14	AY1-4	9
Bark of tree	*Syzygium cumini*	Ayutthaya	30-11-14	AY5	5
Bark of tree	*Samanae saman*	Bankok	26-01-15	BK1-2	14
Bark of tree	*Sesbania grandiflora*	Suphanburi	26-01-15	SP11	7
Dog saliva	*Canis lupus familiaris*	Suphanburi	26-01-15	SP14-17	28
Vegetable fermented	-	Nakhonsawan	26-01-15	NS17-18	4
Buffalo dung	*Bubalus bubalis*	Surin	23-08-13	SR1-2	2
Buffalo dung	*Bubalus bubalis*	Suphanburi	23-08-13	SP1-2	10
Bovine rumen tissue	*Bos taurus*	Nakhonsawan	26-08-14	NS13-15	20
Bovine rumen fluid	*Bos taurus*	Nakornprathom	15-06-15	NP7-9	49
				Total	**207**

Ayutthaya (AY); Bangkok (BK); Nakhonpathom (NP); Nakhonsawan (NS); Suphanburi (SP) and Surin (SR) provinces, Thailand.

3.4.1.2 Secondary screening

The screening medium for succinic acid bacteria that produce organic acid, including acetic acid, lactic acid, formic acid and succinic acid, and they could grow in anaerobic condition. Bacteria produced succinic acid as they exhibited the clear zone around colonies on screening medium. $MgCO_3$ was the key parameter in the agar medium cause magnesium (Mg^{2+}) combined with succinic acid ($C_4H_6O_4$) form to succinate salt ($MgC_4H_4O_4$), so a clear zone was observed (Figure 3.1).

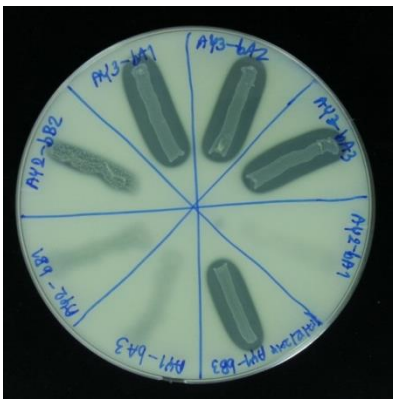

Figure 3.1 Isolates exhibits a clear zone around colonies on a screening medium

All of a total 207 isolates, 171 isolates could produce acid as they exhibited a clear zone around colonies on screening medium plate (Figure 3.1) then these positive isolates were analyzed the qualitative succinic acid by TLC and quantitative succinic acid by HPLC in further study. One hundred and seventy one of isolates included 2 isolates from buffalo dung in Surin, 3 isolates from buffalo dung in Suphanburi, 38 isolates from soil in Suphanburi, 11 isolates from soil in Nakornprathom, 4 isolate from bark of tree in Nakornprathom, 20 isolates from Bovine rumen tissue in Nakhonsawan, 14 isolates from bark of tree in Ayutthaya, 13 isolates from bark of tree in Bangkok, 7 isolates from bark of *Sesbania grandiflora* in Suphanburi, 7 isolates from rice paddy soil in Suphanburi, 24 isolates from dog saliva in Suphanburi, 4 isolates from vegetable fermented in Nakhonsawan and 24 isolates from bovine rumen fluid (Table 3.4).

3.4.1.3 Succinic acid fermentation of isolates

- Thin layer chromatography

The TLC method showed clear yellow spots (Figure 3.2) of different standard organic acids (succinic, lactic, acetic, fumaric, formic and glutamic acid) with distinct retention factor (Rf) values (Table 3.3).

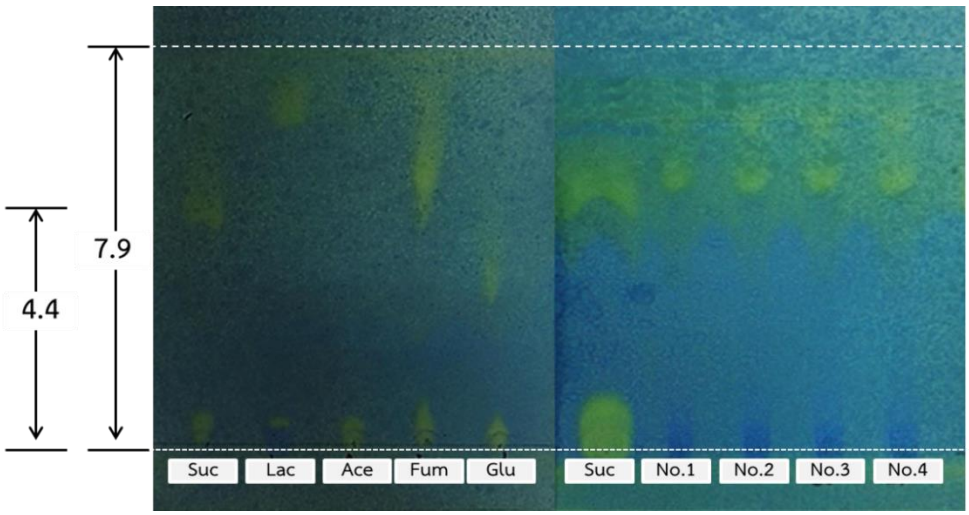

Figure 3.2 Resolution of different standard organic acids on TLC plate

Suc, succinic acid; Lac, lactic acid; Ace, acetic acid; Fum, fumaric acid; Glu, glutamic acid; No.1, NP9-aA3; No.2, NP9-aA7; No.3, NP9-aB1; No.4, NP9-aB2.

Table 3.3 Analysis of different organic acids on TLC plate

Organic acids	Rf values
Succinic acid	0.56
Lactic acid	0.81
Acetic	0.96
Fumaric acid	0.76
Glutamic acid	0.33

Succinic acid was resolved in 30 min and showed a prominent yellow spot with an Rf of 0.56. Among 171 isolates tested, 165 isolates were found to produce succinic acid then these isolates were analyzed the quantitative succinic acid by HPLC in further study.

- High performance liquid Chromatography (HPLC)

Subsequently, 165 isolates from previous studied were analyzed quantitative succinic acid using HPLC, found 165 isolates capable of producing succinic acid and 65 isolates producing succinic acid in excess of 60% yield (Table 3.4). Criteria for selection by high succinic acid production or some outstanding characteristics were selected for further study. The potential 58 isolates were selected for further study.

Table 3.4 Result of primary, secondary screening and succinic acid production by HPLC for screening potential isolates

Isolate	Clear zone on Screening plate	TLC test (RF)[a]	HPLC analysis			
			Succinic acid (g/L)	%Yield	Formic acid (g/L)	Acetic acid (g/L)
SR-II/A1	✓/+	ND	2.026	3.377	0.000	0.000
SR-II/A2	✓/+	ND	1.866	3.110	0.000	0.000
SP-II/A1	✓/+	ND	2.655	4.425	0.000	0.000
SP-II/A2	-	ND	ND	ND	ND	ND
SP-II/A3	✓/+	ND	2.398	3.997	0.000	0.000
SP-II/A4	✓/+	ND	2.015	3.358	0.000	0.000
SP-II/A5	-	ND	ND	ND	ND	ND
SP-II/A6	-	ND	ND	ND	ND	ND
SP-II/B1	-	ND	ND	ND	ND	ND
SP-II/B2	-	ND	ND	ND	ND	ND
SP-II/B3	-	ND	ND	ND	ND	ND
SP-II/B4	-	ND	ND	ND	ND	ND
SP-4/A1	✓/+	0.52	38.445	64.075	0.454	0.823
SP-4/A2	✓/+	0.52	37.189	61.981	0.469	0.773
SP-4/B11	✓/+	0.65, 0.56	35.863	59.772	0.633	0.844
SP-5/A1	✓/+	0.80, 0.55	36.245	60.409	2.778	2.116
SP-5/A2	✓/+	0.79, 0.55	38.252	63.754	26.353	1.877
SP-5/A4	✓/+	0.67, 0.56	37.756	62.926	2.369	1.502
SP-5/A5	✓/+	0.53, 0.50	41.249	68.748	0.398	1.63
SP-5/A7	✓/+	0.68, 0.43	36.955	61.591	1.499	2.038
SP-5/B2	✓/+	0.75, 0.54	37.020	61.700	1.826	1.639
SP-5/B5	✓/+	0.59, 0.45	37.849	63.082	2.415	2.243
SP-5/B9	✓/+	0.66, 0.45	37.929	63.216	2.613	2.114
SP-6/A1	✓/+	0.79, 0.52	36.161	60.268	1.996	1.783
SP-6/A2	✓/+	0.80, 0.50	38.953	64.921	2.500	1.647
SP-6/A3	✓/+	0.82, 0.51	37.807	63.011	0.563	1.293
SP-6/A4	✓/+	0.59, 0.43	37.297	62.162	0.420	0.887
SP-6/A5	✓/+	0.59,0.45	41.050	68.417	0.377	7.129
SP-6/A6	✓/+	0.56,0.43	37.279	62.131	0.000	0.000
SP-6/B3	✓/+	0.52, 0.72	36.920	61.534	2.221	2.173
SP6-B4	✓/+	0.57, 0.66	31.920	53.200	2.277	1.359
SP-8/A2	✓/+	0.59, 0.43	36.868	61.447	1.191	1.439
SP-8/A5	✓/+	0.67, 0.59	37.401	62.335	0.487	0.000
SP-8/A7	✓/+	0.59, 0.45	38.170	63.616	0.243	0.670
SP-8/A8	✓/+	0.59, 0.39	38.480	64.133	0.612	1.210

Table 3.4 Summary of primary, secondary screening and succinic acid production by HPLC for screening potential isolates (continuous)

Sample	Clear zone on Screening plate	TLC test (RF)[a]	HPLC analysis			
			Succinic acid (g/L)	%Yield	Formic acid (g/L)	Acetic acid (g/L)
SP-8/B3	✓/+	0.56, 0.39	37.376	62.293	0.930	0.955
SP-8/B4	✓/+	0.58, 0.72	26.893	44.822	0.089	0.257
SP-8/B5	✓/+	0.61, 0.39	37.417	62.362	0.556	0.766
SP-8/B6	✓/+	0.80, 0.51	40.009	66.681	0.577	1.230
SP-8-A3	✓/+	0.59, 0.51	31.248	52.080	5.866	5.730
SP-9/A3	✓/+	0.59, 0.45	36.987	61.645	5.163	5.717
SP-10/A1	✓/+	0.56, 0.39	37.737	62.894	1.156	1.915
SP-10/A2	✓/+	0.59, 0.39	35.963	59.938	1.205	1.466
SP-10/A3	✓/+	0.72, 0.65	36.410	60.684	1.272	1.688
SP-10/A4	✓/+	0.46, 0.65	37.827	63.045	1.211	0.875
SP-10/A5	✓/+	0.69, 0.43	40.094	66.824	0.000	0.873
SP-10/B3	✓/+	0.82, 0.51	36.899	61.498	1.508	1.430
SP-10/B5	✓/+	0.83, 0.52	38.051	63.419	1.026	0.873
SP-10/B6	✓/+	0.69, 0.64	38.368	63.947	0.566	1.402
SP-10/B7	✓/+	0.69, 0.53	39.402	65.671	0.184	0.924
NP2-A1	✓/+	0.44, 0.81	2.510	4.183	0.217	0.594
NP2-A2	✓/+	0.44, 0.81	3.450	5.750	0.881	1.335
NP2-A3	✓/+	0.53	31.086	51.809	1.194	0.332
NP2-B1	✓/+	0.53	43.112	71.853	1.882	1.646
NP4-A1	✓/+	0.85	ND	ND	ND	ND
NP4-A2	✓/+	0.89	ND	ND	ND	ND
NP4-A3	✓/+	0.58	2.528	4.213	0.654	1.542
NP4-A4	✓/+	0.55	15.424	25.707	12.715	50.325
NP4-B1	✓/+	ND	ND	ND	ND	ND
NP4-B2	✓/+	ND	ND	ND	ND	ND
NP4-B3	-	ND	ND	ND	ND	ND
NP4-B4	✓/+	0.57	13.472	22.453	19.296	43.065
NP6-A1	✓/+	0.78	ND	ND	ND	ND
NP6-A2	✓/+	0.57	44.176	73.627	2.850	0.916
NP6-A3	✓/+	0.58	0.000	0.000	0.000	0.000
NP6-A4	✓/+	0.58	0.000	0.000	0.000	0.000
NS13-aB1	✓/+	0.57	31.178	51.964	0.211	0.773
NS13-aB3	✓/+	0.48	13.620	22.700	0.213	0.052
NS13-bA1	✓/+	0.59	20.198	33.663	0.319	0.26
NS13-cA1	✓/+	0.58	44.037	73.395	0.647	0.220

Table 3.4 Summary of primary, secondary screening and succinic acid production by HPLC for screening potential isolates (continuous)

Sample	Clear zone on Screening plate	TLC test (RF)[a]	HPLC analysis			
			Succinic acid (g/L)	%Yield	Formic acid (g/L)	Acetic acid (g/L)
NS13-cB1	✓/+	0.57	43.637	72.728	0.655	0.265
NS13-dA1	✓/+	0.57	33.738	56.229	0.099	0.172
NS13-dB1	✓/+	0.57	34.786	57.976	0.29	0.144
NS14-aA1	✓/+	0.43	1.091	1.818	0.000	0.080
NS14-aA3	✓/+	0.57	45.554	75.924	0.446	0.185
NS14-aB1	✓/+	0.57	42.319	70.531	0.601	0.320
NS14-aB2	✓/+	0.57	43.998	73.329	0.698	0.269
NS14-bA1	✓/+	0.59	35.898	59.830	1.300	0.648
NS14-bB1	✓/+	0.58	0.229	0.382	0.000	0.000
NS14-cA1	✓/+	0.56	44.938	74.896	0.421	0.519
NS14-cB2	✓/+	0.55	43.888	73.147	0.512	0.376
NS14-dA2	✓/+	0.55	36.252	60.420	0.105	0.181
NS15-aA1	✓/+	0.57	29.002	48.336	0.45	0.252
NS15-aA2	✓/+	0.57	32.324	53.873	0.288	0.291
NS15-bA1	✓/+	0.47	1.282	2.137	0.271	0.000
NS15-bB2	✓/+	0.54	10.069	16.781	0.659	0.413
AY1-bA1	✓/+	0.54	31.261	52.101	0.201	1.503
AY1-bA3	✓/+	0.54	14.412	24.020	2.419	4.928
AY1-bB2	✓/+	0.54	14.569	24.282	2.419	4.628
AY2-bA2	✓/++	0.54	30.934	51.556	2.028	0.634
AY2-bB2	✓/++	0.54	24.097	40.161	1.54	0.542
AY3-bB1	✓/++	0.54	40.558	67.597	0.000	0.000
AY4-aB1	✓/++	0.54	40.498	67.496	0.000	0.000
AY4-bA2	✓/++	0.54	40.357	67.261	0.638	0.402
AY4-bB1	✓/++	0.54	32.026	53.376	1.428	0.911
AY5-aB2	✓/++	0.52	41.868	69.780	0.265	0.334
AY5-bA1	✓/++	0.52	34.362	57.269	0.304	5.581
AY5-bB1	✓/+	0.54	22.454	37.424	0.909	2.527
AY5-bB2	✓/+	0.54	23.326	38.877	0.000	4.406
AY5-bB5	✓/+	0.54	19.021	31.701	2.337	6.526
BK1-A1	✓/++	0.48	23.312	38.853	1.053	0.399
BK1-A2	✓/+	0.49	7.204	12.007	9.005	0.000
BK1-A3	✓/+	0.56	9.558	15.931	11.948	0.000
BK1-B1	✓/+	0.52	35.439	59.065	44.299	0.000
BK1-B2	-	ND	ND	ND	ND	ND

Table 3.4 Summary of primary, secondary screening and succinic acid production by HPLC for screening potential isolates (continuous)

Sample	Clear zone on Screening plate	TLC test (RF)[a]	HPLC analysis			
			Succinic acid (g/L)	%Yield	Formic acid (g/L)	Acetic acid (g/L)
BK1-B3	✓/+	0.56	5.992	9.987	7.490	0.000
BK1-B4	✓/++	0.57	34.041	56.735	42.551	0.000
BK2-A1	✓/+	0.54	3.569	5.948	4.461	0.000
BK2-A2	✓/+	0.52	1.295	2.159	1.619	0.000
BK2-A3	✓/+	0.53	1.190	1.983	8.133	5.390
BK2-B1	✓/+	0.54	24.029	40.049	1.272	4.778
BK2-B2	✓/+	0.52	0.861	1.436	9.721	5.611
BK2-B3	✓/+	0.53	22.770	37.950	2.727	5.342
BK2-B4	✓/++	0.52	32.549	54.248	1.664	1.963
SP11-A1	✓/+	0.57	1.585	2.641	0.706	12.219
SP11-A2	✓/+	0.54	4.153	6.921	5.974	8.400
SP11-B1	✓/+	0.52	0.912	1.519	1.956	12.165
SP11-B2	✓/+	0.51	12.823	21.371	6.862	5.900
SP11-B3	✓/+	0.54	2.059	3.431	6.501	5.162
SP11-B4	✓/+	0.52	17.495	29.159	7.820	6.707
SP11-B5	✓/+	0.53	12.816	21.360	2.333	8.565
SP12-A1	✓/+	0.55	7.646	12.744	7.155	5.082
SP12-B1	✓/+	0.53	6.812	11.353	5.950	8.025
SP13-A1	✓/+	0.52	1.529	2.549	9.809	7.124
SP13-A2	✓/+	0.51	2.072	3.453	7.467	5.734
SP13-B1	✓/+	0.56	30.883	51.472	1.061	2.547
SP13-B2	✓/+	0.57	30.371	50.619	0.875	1.943
SP13-B3	✓/+	0.54	18.953	31.589	4.016	3.090
SP14-A1	-	ND	ND	ND	ND	ND
SP14-A2	-	ND	ND	ND	ND	ND
SP14-A3	✓/++	0.54	22.172	36.953	1.562	0.529
SP14-B1	✓/+	0.52	21.365	35.608	0.0949	7.304
SP14-B2	✓/++	0.53	21.789	36.315	0.566	0.273
SP14-B3	✓/+	0.52	31.036	51.726	0.755	1.329
SP14-B4	✓/+	0.57	30.974	51.624	0.130	0.731
SP15-A1	-	ND	ND	ND	ND	ND
SP15-A2	✓/++	0.52	18.847	31.412	1.400	0.307
SP15-A3	✓/++	0.54	38.784	64.641	0.683	1.848
SP15-B1	-	ND	ND	ND	ND	ND
SP15-B2	✓/++	0.52	19.119	31.865	0.263	0.275

Table 3.4 Summary of primary, secondary screening and succinic acid production by HPLC for screening potential isolates (continuous)

Sample	Clear zone on Screening plate	TLC test (RF)[a]	HPLC analysis			
			Succinic acid (g/L)	%Yield	Formic acid (g/L)	Acetic acid (g/L)
SP15-B3	✓/+	0.53	25.108	41.846	1.863	1.962
SP15-B4	✓/++	0.55	33.284	55.473	2.290	2.336
SP15-B5	✓/++	0.53	36.047	60.078	0.247	0.667
SP16-A1	✓/+	0.52	6.878	11.463	0.850	1.205
SP16-A2	✓/+	0.51	18.271	30.452	0.483	0.340
SP16-A3	✓/+	0.52	2.414	4.024	0.000	0.084
SP16-B1	✓/+	0.53	5.990	9.983	0.084	0.000
SP16-B2	✓/+	0.54	3.669	6.115	0.000	0.000
SP16-B3	✓/+	0.54	2.820	4.700	0.000	0.000
SP16-B4	✓/++	0.54	37.893	63.155	0.000	0.000
SP17-A1	✓/+	0.54	28.619	47.698	0.000	0.000
SP17-A2	✓/++	0.54	36.401	60.669	0.000	0.000
SP17-B1	✓/+	0.54	9.203	15.338	0.000	0.000
SP17-B2	✓/++	0.54	36.946	61.577	0.000	0.000
SP17-B3	✓/+	0.52	0.186	0.310	0.000	0.000
SP17-B4	✓/+	0.52	0.264	0.440	0.000	0.000
NS17-B1	✓/+	0.52	2.099	3.498	7.174	6.567
NS17-B2	✓/+	0.52	0.991	1.651	9.285	7.872
NS18-A1	✓/+	0.54	24.065	40.109	0.737	0.462
NS18-B1	✓/+	0.54	19.520	32.534	0.803	0.541
NP7-aA1	✓/+	0.43	0.525	0.876	0.000	0.000
NP7-aB1	-	ND	ND	ND	ND	ND
NP7-aB2	✓/++	0.57	41.540	69.233	0.284	1.489
NP7-aB3	-		ND	ND	ND	ND
NP7-bA1	✓/++	0.57	43.591	72.652	3.692	3.670
NP7-bA2	✓/++	0.57	40.737	67.895	1.159	2.486
NP7-bB1	X	ND	ND	ND	ND	ND
NP7-bB2	✓/+	0.67	ND	ND	ND	ND
NP7-cA1	-	ND	ND	ND	ND	ND
NP7-cA3	-	ND	ND	ND	ND	ND
NP7-cB1	-	ND	ND	ND	ND	ND
NP7-cB2	✓/-	0.90	ND	ND	ND	ND
NP7-cB3	✓/++	0.58	36.178	60.298	0.438	0.000
NP8-aA1	✓/-	0.43	ND	ND	ND	ND
NP8-aA2	✓/++	0.59	30.912	51.519	0.000	10.296

Table 3.4 Summary of primary, secondary screening and succinic acid production by HPLC for screening potential isolates (continuous)

Sample	Clear zone on Screening plate	TLC test (RF)[a]	HPLC analysis			
			Succinic acid (g/L)	%Yield	Formic acid (g/L)	Acetic acid (g/L)
NP8-aB1	✓/+	0.81	ND	ND	ND	ND
NP8-aB2	✓/++	0.51	40.838	68.063	0.559	0.000
NP8-aB3	✓/++	0.59	39.128	65.214	0.704	0.262
NP8-bA1	✓/++	0.58	36.250	60.417	1.783	2.239
NP8-bB1	-	ND	ND	ND	ND	ND
NP8-bB2	✓/-	0.90	ND	ND	ND	ND
NP8-cA1	✓/+	0.58	0.775	1.292	0.165	3.966
NP8-cA2	✓/-	0.90	ND	ND	ND	ND
NP8-cA3	✓/-	0.71	ND	ND	ND	ND
NP8-cA4	✓/+	0.39	45.462	75.769	0.000	0.000
NP8-cB1	✓/-	0.81	ND	ND	ND	ND
NP8-cB2	✓/-	0.81	ND	ND	ND	ND
NP8-cB3	✓/+	0.49	2.153	3.589	0.000	2.988
NP8-cB4	✓/-	0.51	ND	ND	ND	ND
NP9-aA1	✓/++	0.51	39.474	65.790	0.000	0.000
NP9-aA2	-	ND	ND	ND	ND	ND
NP9-aA3	✓/++	0.56	40.806	61.010	2.282	3.046
NP9-aA4	-	ND	ND	ND	ND	ND
NP9-aA5	-	ND	ND	ND	ND	ND
NP9-aA6	-	ND	ND	ND	ND	ND
NP9-aA7	✓/++	0.57	42.539	70.898	2.921	1.859
NP9-aB1	✓/+	0.56	1.768	2.947	0.000	3.275
NP9-aB2	✓/++	0.56	43.358	72.264	0.057	0.255
NP9-bA1	✓/++	0.56	32.566	54.276	0.572	0.644
NP9-bA3	✓/++	0.54	36.435	60.725	0.259	0.910
NP9-bB1	-	ND	ND	ND	ND	ND
NP9-cA1	✓/+	0.36	0.186	0.310	1.743	3.270
NP9-cA2	✓/-	0.64	2.099	3.498	ND	ND
NP9-cA3	✓/+	0.81, 0.44	0.991	1.651	0.249	0.290
NP9-cA4	✓/++	0.36	ND	ND	3.976	4.475
NP9-cB1	✓/-	ND	19.520	32.534	ND	ND
NP9-cB2	✓/++	0.54	2.099	3.498	0.261	0.000
Total of positive test (isolates)	171	165	165	65	-	-

[a], Standard succinic acid showed an R_f of 0.51-0.59; -, Negative result; + Possitive result; ND, not detected

From Table 3.4 showed summary of producing succinic acid bacterial strain using primary, secondary screening then they were analyzed the qualitative succinic acid by TLC and quantitative succinic acid by HPLC. From this result could be summarized that succinic acid producing bacteria were screened from 7 sources and 6 provinces in Thailand.

Two hundred and seven isolates from various sources in Thailand were screened for succinic acid production under anaerobic conditions.

The result from primary, 171 isolates exhibited a clear zone on the screening medium. Secondary screening, 165 isolates were found by TLC analysis to produce succinic acid. Subsequently, quantitative succinic acid using HPLC, found 165 isolates capable of producing succinic acid. The potential 58 isolates were selected for phenotypic characteristic.

3.4.2 Characterization and Identification of isolates

3.4.2.1 Morphological characteristics

All isolates with succinic acid production ability were studied for morphological characteristics. The results of morphological characteristics of 165 isolates were shown in Table 3.5. The most isolates were Gram-positive. Only two isolates were Gram-negative. Six isolates were Corynebac, 24 isolates were short rod, 25 isolates were rod shape and other isolates were shown in Table 3.5.

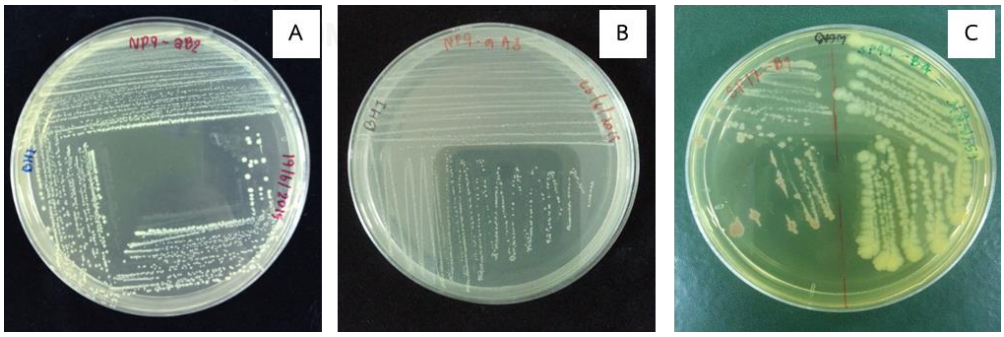

Figure 3.3 The morphology of colony on TSA agar plate. A, NP9-aB2; B, NP9-aA3; C, SP17-B1 and SP11-B4

Figure 3.3 showed representative of isolate for the morphology of colony including; NP9-aB2 was white, circular, convex, entire, and optical property was opaque (Figure 3.3A), NP9-aA3 was white, punctiform, convex, entire and optical property was translucent (Figure 3.8B), SP17-B1 was purple, circular, convex, entire and optical property was translucent (Figure 3.3C), SP11-B4 was white, irregular, convex, curled and optical property was opaque (Figure 3.3C)

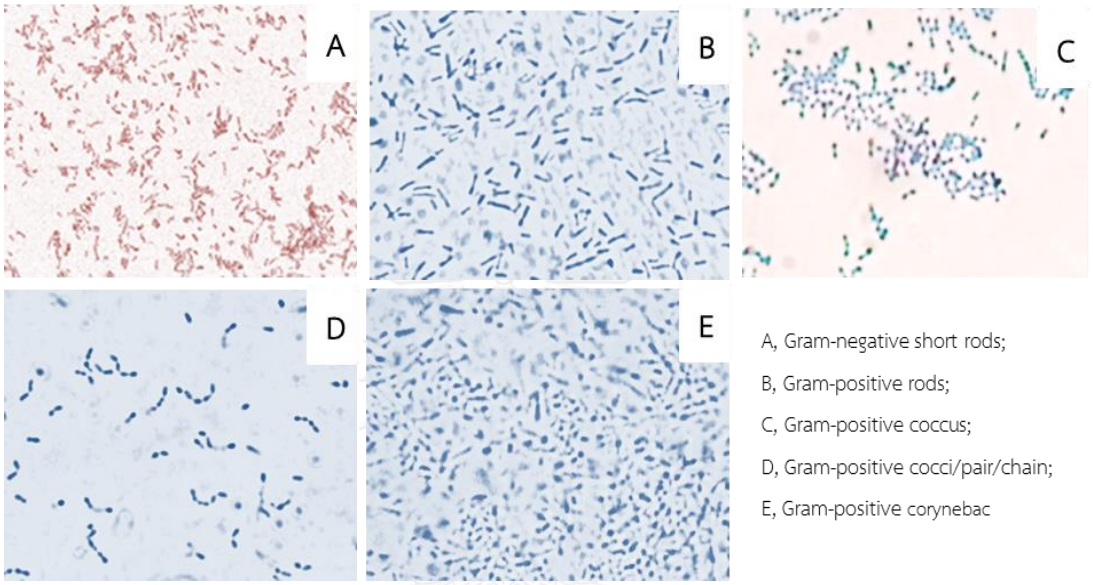

Figure 3.4 The morphological of cell and gram stain under microscopic was expanding 100x. A, NP9-aA7; B, SP12-A1, C, AY1-bA1; D, SP5-B2 and E, BK1-A1

All results from microscopic observation were shown in Table 3.5 and the examples of bacteria shape of some isolates were shown in Figure 3.4. NP9-aA7 was Gram-negative short rod, SP12-A1 was Gram-positive rod, AY1-bA1 was Gram-positive coccus, SP5-B2 was Gram-positive cocci pair/chain and BK1-A1 was Gram-positive corynebac. All isolates was non spore-forming.

Table 3.5 Morphological characteristics of 165 isolates

Isolate	Morphology of colony					Morphology of cell	
	Color	Form	Elevation	Margin	Optical property	Gram strain	Shape
SR-II/A1	White	Circular	Convex	Entire	opaque	+	Coccus
SR-II/A2	White	Circular	Convex	Entire	Opaque	+	Coccus
SP-II/A1	White	Circular	Convex	Entire	Opaque	+	Coccus
SP-II/A3	White	Circular	Convex	Entire	Opaque	+	Coccus
SP-II/A4	White	Circular	Pulvinate	Entire	Opaque	+	Coccus
SP-4/A1	White	Punctiform	Convex	Entire	Opaque	+	Cocci pair
SP-4/A2	White	Circular	Convex	Entire	Opaque	+	Cocci pair
SP-4/B11	White	Punctiform	Convex	Entire	Opaque	+	Cocci pair
SP-5/A1	White	Circular	Convex	Entire	Opaque	+	Cocci pair
SP-5/A2	White	Circular	Convex	Entire	Opaque	+	Cocci pair
SP-5/A4	White	Circular	Flat	Curled	Opaque	+	Coccus
SP-5/A5	White	Circular	Convex	Entire	Opaque	+	Cocci pairs
SP-5/A7	White	Circular	Convex	Entire	Opaque	+	Cocci pairs
SP-5/B2	White	Circular	Convex	Entire	Opaque	+	Rod, cocci pair
SP-5/B5	White	Punctiform	Convex	Entire	Opaque	+	Rod chain
SP-5/B9	White	Circular	Convex	Entire	Opaque	+	Cocci chain
SP-6/A1	White	Circular	Convex	Entire	Opaque	+	Coccus, Cocci chain
SP-6/A2	White	Circular	Convex	Entire	Opaque	+	Coccus, Cocci chain
SP-6/A3	White	Irregular	Convex	Entire	Opaque	+	Cocci pairs, chain
SP-6/A4	White	Circular	Convex	Entire	Translucent	+	Cocci pairs, chain
SP-6/A5	White	Circular	Convex	Entire	Translucent	+	Cocci pairs, chain
SP-6/A6	White	Circular	Convex	Entire	Opaque	+	Cocci pairs, chain
SP-6/B3	White	Punctiform	Convex	Entire	Opaque	-	Cooci pair
SP6-B4	White	Circular	Pulvinate	Entire	Opaque	+	Coccus, rod
SP-8/A2	White	Circular	Convex	Entire	Opaque	+	Cocci pairs, chain
SP-8/A5	White	Circular	Convex	Entire	Opaque	+	Cocci pairs, chain
SP-8/A7	White	Circular	Convex	Entire	Opaque	+	Coccus
SP-8/A8	White	Circular	Convex	Entire	Opaque	+	Coccus
SP-8/B3	White	Circular	Convex	Entire	Opaque	+	Cocci pairs, chain
SP-8/B4	White	Punctiform	Convex	Entire	Opaque	-	short rod
SP-8/B5	White	Circular	Convex	Entire	Opaque	+	Cocci pair, chain

Table 3.5 Morphological characteristics of 165 isolates (continuous)

Isolate	Morphology of colony					Morphology of cell	
	Color	Form	Elevation	Margin	Optical property	Gram strain	Shape
SP-8/B6	White	Circular	Convex	Entire	Opaque	+	Cocci pair, chain
SP-8-A3	White	Circular	Convex	Entire	Opaque	+	coccus
SP-9/A3	White	Circular	Convex	Entire	Opaque	+	Cocci pairs, chain
SP-10/A1	White	Circular	Convex	Entire	Opaque	+	Cocci pairs, chain
SP-10/A2	White	Circular	Convex	Entire	Opaque	+	Cocci pairs, chain
SP-10/A3	White	Circular	Convex	Entire	Opaque	+	Cocci pairs, chain
SP-10/A4	White	Circular	Convex	Entire	Opaque	+	Cocci pairs, chain
SP-10/A5	White	Punctiform	Convex	Entire	Opaque	+	Cocci paris
SP-10/B3	White	Circular	Convex	Entire	Opaque	+	Cocci pairs, chain
SP-10/B5	White	Circular	Convex	Entire	Opaque	+	Cocci pairs, chain
SP-10/B6	White	Circular	Convex	Entire	Opaque	+	Cocci pairs, chain
SP-10/B7	White	Circular	Convex	Entire	Opaque	+	Cocci pairs, chain
NP2-A1	White	Punctiform	Convex	Entire	Opaque	+	Coccus
NP2-A2	White	Punctiform	Convex	Entire	Opaque	+	Coccus
NP2-A3	White	Circular	Convex	Entire	Opaque	+	Coccus
NP2-B1	White	Punctiform	Convex	Entire	Opaque	+	Coccus
NP4-A3	White	Circular	Convex	Entire	Translucent	+	Rod
NP4-A4	White	Punctiform	Convex	Entire	Opaque	+	Rod
NP4-B4	White	Circular	Pulvinate	Entire	Opaque	+	Cocci chain
NP6-A2	White	Punctiform	Convex	Entire	Opaque	+	Cocci chain
NP6-A3	White	Punctiform	Convex	Undulate	Opaque	+	Coccus
NP6-A4	White	Punctiform	Convex	Entire	Opaque	+	Cocci pair
NS13-aB1	White	Punctiform	Convex	Entire	Translucent	+	Cocci pair
NS13-aB3	yellow	Irregular	Convex	Undulate	Translucent	+	Cocci chain
NS13-bA1	yellow	Irregular	Flat	Undulate	Opaque	+	Rod
NS13-cA1	White	Punctiform	Convex	Entire	Translucent	+	Cocci pair
NS13-cB1	yellow	Irregular	Convex	Undulate	Translucent	+	Cocci chain
NS13-dA1	White	Punctiform	Convex	Entire	Opaque	+	Cocci pair
NS13-dB1	yellow	Punctiform	Convex	Entire	Translucent	+	Cocci pair
NS14-aA1	White	Irregular	Umbonate	Undulate	Translucent	+	Cocci chain
NS14-aA3	White	Irregular	Convex	Undulate	Translucent	+	Cocci chain

Table 3.5 Morphological characteristics of 165 isolates (continuous)

Isolate	Morphology of colony					Morphology of cell	
	Color	Form	Elevation	Margin	Optical property	Gram strain	Shape
NS14-aB1	White	Punctiform	Convex	Entire	Translucent	+	Cocci chain
NS14-aB2	White	Punctiform	Flat	Undulate	Translucent	+	Cocci chain
NS14-bA1	Yellow	Irregular	Convex	Undulate	Opaque	+	Rod
NS14-bB1	White	Circular	Convex	Undulate	Opaque	+	Rod
NS14-cA1	White	Irregular	Convex	Undulate	Translucent	+	Cocci pair
NS14-cB2	White	Irregular	Convex	Undulate	Translucent	+	Cocci pair
NS14-dA2	Yelloiw	Irregular	Convex	Undulate	Opaque	+	Cocci chain
NS15-aA1	White	Irregular	Convex	Undulate	Opaque	+	Coccus
NS15-aA2	White	Circular	Convex	Entire	Opaque	+	Cocci chain
NS15-bA1	White	Irregular	Convex	Undulate	Opaque	+	Cocci pair
NS15-bB2	White	Irregular	Umbonate	Undulate	Opaque	+	Rod
AY1-bA1	White	Circular	Convex	Entire	Translucent	+	Rod
AY1-bA3	White	Circular	Convex	Entire	Translucent	+	coocus
AY1-bB2	White	Circular	Convex	Entire	Translucent	+	coocus
AY2-bA2	White	Punctiform	Convex	Entire	Translucent	+	coocus
AY2-bB2	White	Punctiform	Convex	Entire	Translucent	+	Short rot
AY3-bB1	White	Circular	Convex	Entire	Opaque	+	Coccus
AY4-aB1	White	Circular	Convex	Entire	Opaque	+	Coccus
AY4-bA2	White	Circular	Convex	Entire	Opaque	+	Coccus
AY4-bB1	White	Irregular	Convex	Irregular	Opaque	+	Coccus
AY5-aB2	White	Circular	Convex	Entire	Translucent	+	Coccus
AY5-bA1	White	Circular	Convex	Entire	Translucent	+	Rod
AY5-bB1	White	Irregular	Convex	Irregular	Opaque	+	Coccus
AY5-bB2	White	Circular	Convex	Entire	Translucent	+	Coccus
AY5-bB5	White	Circular	Convex	Entire	Translucent	+	Rod
BK1-A1	White	Circular	Convex	Entire	Opaque	+	Corynebac
BK1-A2	White	Irregular	Convex	Irregular	Opaque	+	Corynebac
BK1-A3	White	Irregular	Convex	Irregular	Opaque	-	Corynebac
BK1-B1	White	Circular	Convex	Irregular	Opaque	+	Coccus
BK1-B3	White	Irregular	Convex	Irregular	Opaque	+	Rod
BK1-B4	White	Circular	Convex	Entire	Opaque	+	Cocci pair

Table 3.5 Morphological characteristics of 165 isolates (continuous)

Isolate	Morphology of colony					Morphology of cell	
	Color	Form	Elevation	Margin	Optical property	Gram strain	Shape
BK2-A1	White	Irregular	Convex	Irregular	Opaque	+	Coccus
BK2-A2	White	Irregular	Convex	Irregular	Translucent	-	Rod/corynebac
BK2-A3	White	Irregular	Convex	Irregular	Opaque	+	Coccus
BK2-B1	White	Circular	Convex	Entire	Opaque	-	Coccus
BK2-B2	Grey	Circular	Convex	Entire	Translucent	-	Rod
BK2-B3	Grey	Circular	Convex	Entire	Translucent	+	Rod/corynebac
BK2-B4	Yellow	Irregular	Convex	Irregular	Opaque	+	Rod/cocci bacilli
SP11-A1	White	Circular	Convex	Entire	Translucent	-	Coccus
SP11-A2	White	Circular	Convex	Entire	Opaque	+	Coccus/short rod
SP11-B1	White	Circular	Convex	Entire	Opaque	+	Coccus
SP11-B2	White	Circular	Convex	Entire	Opaque	+	Coccus
SP11-B3	White	Irregular	Convex	Irregular	Opaque	+	Rod
SP11-B4	White	Irregular	Convex	Curled	Opaque	+	Rod/Corynebac
SP11-B5	White	Irregular	Convex	Irregular	Opaque	+	Rod
SP12-A1	White	Irregular	Convex	Irregular	Opaque	+	Rod
SP12-B1	White	Irregular	Convex	Irregular	Opaque	+	Coccus
SP13-A1	White	Irregular	Convex	Irregular	Opaque	+	Rod
SP13-A2	White	Irregular	Convex	Irregular	Opaque	+	Rod
SP13-B1	White	Circular	Convex	Entire	Translucent	+	Cocci in chain
SP13-B2	White	Irregular	Convex	Irregular	Opaque	+	Rod
SP13-B3	White	Irregular	Convex	Irregular	Opaque	+	Rod/corynebac
SP14-A3	White	Punctiform	Convex	Irregular	Opaque	+	Short rod
SP14-B1	White	Circular	Convex	Entire	Opaque	+	Coccus
SP14-B2	White	Circular	Pulvinate	Entire	Opaque	+	Short rod
SP14-B3	White	Circular	Convex	Entire	Opaque	+	Coccus
SP14-B4	White	Circular	Convex	Entire	Opaque	+	Short rod
SP15-A2	White	Circular	Convex	Entire	Translucent	+	Short rod
SP15-A3	White	Circular	Convex	Entire	Opaque	+	Coccus
SP15-B2	White	Circular	Convex	Entire	Translucent	+	Short rod
SP15-B3	White	Irregular	Convex	Irregular	Opaque	+	Short rod
SP15-B4	Yellow	Circular	Convex	Entire	Opaque	+	Short rod

Table 3.5 Morphological characteristics of 165 isolates (continuous)

Isolate	Morphology of colony					Morphology of cell	
	Color	Form	Elevation	Margin	Optical property	Gram strain	Shape
BK2-A1	White	Irregular	Convex	Irregular	Opaque	+	Coccus
BK2-A2	White	Irregular	Convex	Irregular	Translucent	-	Rod/corynebac
BK2-A3	White	Irregular	Convex	Irregular	Opaque	+	Coccus
BK2-B1	White	Circular	Convex	Entire	Opaque	-	Coccus
BK2-B2	Grey	Circular	Convex	Entire	Translucent	-	Rod
BK2-B3	Grey	Circular	Convex	Entire	Translucent	+	Rod/corynebac
BK2-B4	Yellow	Irregular	Convex	Irregular	Opaque	+	Rod/cocci bacilli
SP11-A1	White	Circular	Convex	Entire	Translucent	-	Coccus
SP11-A2	White	Circular	Convex	Entire	Opaque	+	Coccus/short rod
SP11-B1	White	Circular	Convex	Entire	Opaque	+	Coccus
SP11-B2	White	Circular	Convex	Entire	Opaque	+	Coccus
SP11-B3	White	Irregular	Convex	Irregular	Opaque	+	Rod
SP11-B4	White	Irregular	Convex	Curled	Opaque	+	Rod/corynebac
SP11-B5	White	Irregular	Convex	Irregular	Opaque	+	Rod
SP12-A1	White	Irregular	Convex	Irregular	Opaque	+	Rod
SP12-B1	White	Irregular	Convex	Irregular	Opaque	+	Coccus
SP13-A1	White	Irregular	Convex	Irregular	Opaque	+	Rod
SP13-A2	White	Irregular	Convex	Irregular	Opaque	+	Rod
SP13-B1	White	Circular	Convex	Entire	Translucent	+	Cocci in chain
SP13-B2	White	Irregular	Convex	Irregular	Opaque	+	Rod
SP13-B3	White	Irregular	Convex	Irregular	Opaque	+	Rod/corynebac
SP14-A3	White	Punctiform	Convex	Irregular	Opaque	+	Short rod
SP14-B1	White	Circular	Convex	Entire	Opaque	+	Coccus
SP14-B2	White	Circular	Pulvinate	Entire	Opaque	+	Short rod
SP14-B3	White	Circular	Convex	Entire	Opaque	+	Coccus
SP14-B4	White	Circular	Convex	Entire	Opaque	+	Short rod
SP15-A2	White	Circular	Convex	Entire	Translucent	+	Short rod
SP15-A3	White	Circular	Convex	Entire	Opaque	+	Coccus
SP15-B2	White	Circular	Convex	Entire	Translucent	+	Short rod
SP15-B3	White	Irregular	Convex	Irregular	Opaque	+	Short rod
SP15-B4	Yellow	Circular	Convex	Entire	Opaque	+	Short rod

Table 3.5 Morphological characteristics of 165 isolates (continuous)

Isolate	Morphology of colony					Morphology of cell	
	Color	Form	Elevation	Margin	Optical property	Gram strain	Shape
SP15-B5	White	Circular	Convex	Entire	Opaque	+	Coccus
SP16-A1	white	Irregular	Convex	Irregular	Opaque	+	Coccus
SP16-A2	White	Circular	Convex	Entire	Translucent	+	Coccus
SP16-A3	White	Circular	Convex	Entire	Translucent	+	Short rod
SP16-B1	Yellow	Irregular	Convex	Irregular	Opaque	+	Short rod
SP16-B2	White	Punctiform	Convex	Entire	Translucent	-	Short rod
SP16-B3	White	Circular	Convex	Entire	Opaque	+	Short rod
SP16-B4	White	Circular	Convex	Entire	Opaque	+	Short rod
SP17-A1	Grey	Circular	Convex	Entire	Translucent	+	Short rod
SP17-A2	White	Circular	Convex	Entire	Opaque	+	Short rod
SP17-B1	Purple	Circular	Convex	Entire	Translucent	+	Short rod
SP17-B2	White	Circular	Convex	Entire	Opaque	+	Short rod
SP17-B3	White	Circular	Convex	Entire	Opaque	+	Cocci in chain
SP17-B4	White	Circular	Convex	Entire	Opaque	+	Coccus
NS17-B1	White	Circular	Convex	Entire	Translucent	+	Short rod
NS17-B2	White	Circular	Convex	Entire	Translucent	-	Rod
NS18-A1	White	Circular	Convex	Entire	Opaque	+	Rod
NS18-B1	White	Circular	Convex	Entire	Opaque	+	Rod
NP7-aA1	White	Punctiform	Convex	Entire	Translucent	+	Rod
NP7-aB2	White	Circular	Convex	Entire	Opaque	+	Rod
NP7-bA1	White	Circular	Convex	Entire	Opaque	+	Short rod
NP7-bA2	White	Punctiform	Convex	Entire	Translucent	+	Cocci in chain
NP7-cB3	White	Punctiform	Convex	Entire	Translucent	+	Rod, cocci in pair
NP8-aA2	White	Punctiform	Convex	Entire	Opaque	+	Coccus
NP8-aB2	White	Irregular	Umbonate	Irregular	Translucent	+	Cocci in pair
NP8-aB3	White	Irregular	Convex	Irregular	Translucent	+	Cocci in pair
NP8-bA1	White	Punctiform	Convex	Entire	Translucent	+	Cocci in pair
NP8-cA1	White	Punctiform	Convex	Entire	Translucent	+	Cocci in pair
NP8-cA4	White	Punctiform	Umbonate	Entire	Translucent	+	Short rod
NP8-cB3	White	Punctiform	Convex	Entire	Opaque	+	Rod
NP9-aA1	White	Irregular	Convex	Irregular	Translucent	+	Cocci in pair

Table 3.5 Morphological characteristics of 165 isolates (continuous)

Isolate	Morphology of colony					Morphology of cell	
	Color	**Form**	**Elevation**	**Margin**	**Optical property**	**Gram strain**	**Shape**
NP9-aA3	White	Punctiform	Convex	Entire	Translucent	-	Short rod
NP9-aA7	Gray	Punctiform	Convex	Entire	Translucent	-	Short rod
NP9-aB1	White	Irregular	Convex	Irregular	Translucent	+	Cocci in chain
NP9-aB2	White	Circular	Convex	Entire	Opaque	+	Rod
NP9-bA1	White	Circular	Convex	Entire	Translucent	+	Short rod
NP9-bA3	White	Punctiform	Convex	Entire	Translucent	+	Cocci in pair
NP9-cA1	White	Circular	Convex	Entire	Opaque	+	Short rod
NP9-cA3	White	Punctiform	Convex	Entire	Translucent	+	Rod
NP9-cA4	White	Punctiform	Convex	Entire	Translucent	-	Coccus
NP9-cB2	White	Circular	Convex	Entire	Translucent	+	Cocci in pair

Of the total number of isolates, fifty-eight isolates were selected for phenotypic characteristics in further study. Reasons to choose these isolates because high yield (more than 60% yield), moreover, some isolate have the appearance of a new base contract.

3.4.2.2 Physiological characterizations

All of a 58 isolates consisted of 13 isolates were rods, 15 isolates were short rod, 8 isolates were coccus, 5 isolates were cocci in chain and only 4 isolates were corynebac. Gram negative rod of 5 isolates and other isolate were Gram-positive.

The results from morphological, physiological and biochemical characteristic were grouped using a hierarchical cluster in the statistical package for the social sciences for windows (SPSS) program. The isolates were divided into 11 groups by dendrogram using SPSS the result of dendrogram was shown in Figure 3.5. The physiological characteristics of 58 isolates were shown in Table 3.6

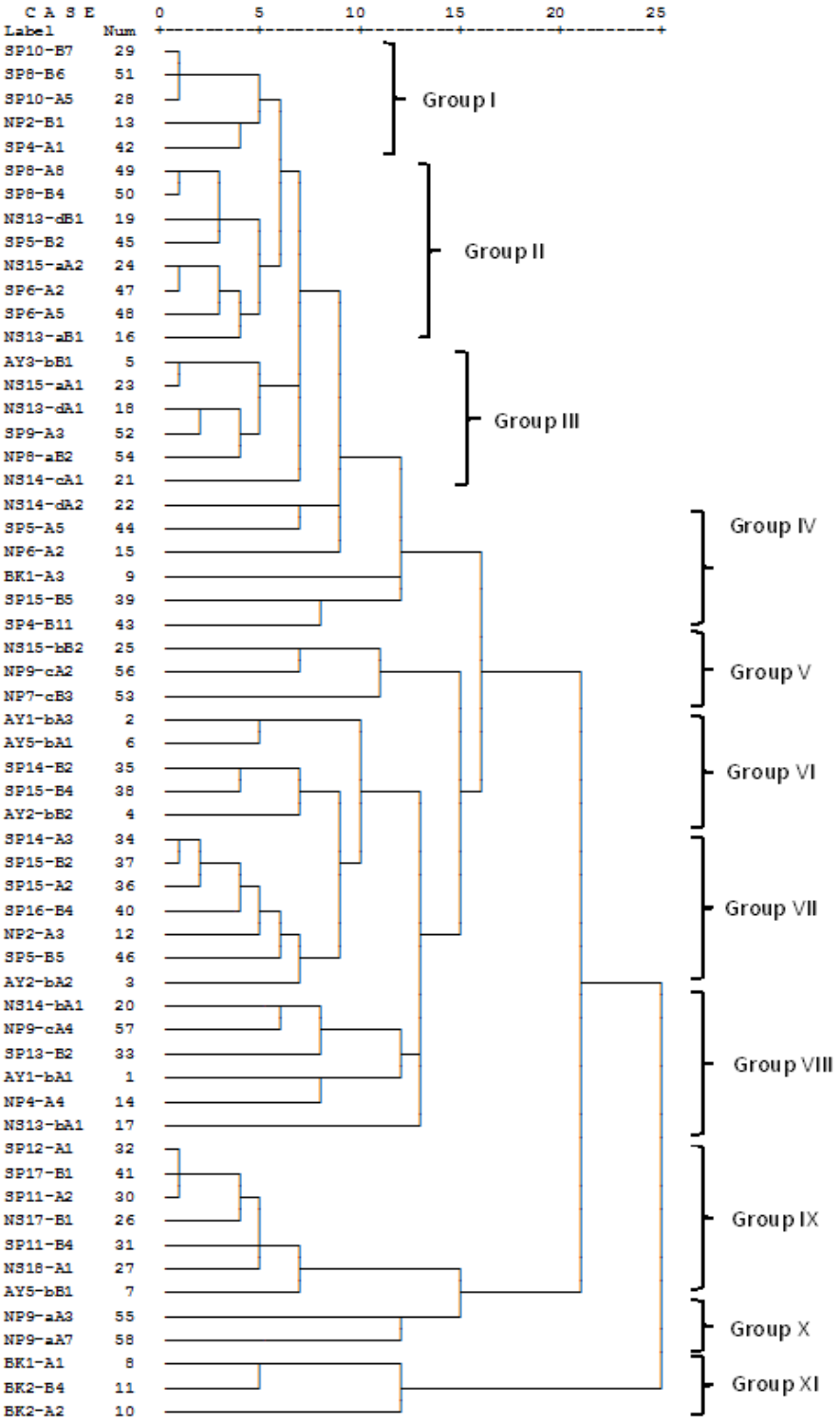

Figure 3.5 Dendrogram of the hierarchical cluster in SPSS program of 58 isolates based on morphological, physiological and biochemical characteristics

Table 3.6 Physiological characteristics of 58 isolates

Group	Isolates	pH 3.5	pH 5.0	pH 9.0	20 °C	40 °C	50 °C	6% NaCl
1	NP2-B1	-	+	+	+	-	-	+
1	NS13-dA1	-	+	+	+	+	+	+
1	SP10-A5	-	+	+	+	-	-	+
1	SP10-B7	-	+	+	+	-	-	+
1	SP8-B6	-	+	+	+	-	-	+
2	NS13-aB1	-	+	+	+	+	+	-
2	NS13-dB1	-	+	+	+	+	+	+
2	NS15-aA2	-	+	+	+	+	-	+
2	SP5-B2	-	+	+	+	+	-	-
2	SP6-A2	-	+	+	+	+	-	-
2	SP6-A5	-	+	+	+	+	-	-
2	SP8-A8	-	+	+	+	+	-	+
2	SP8-B4	-	+	+	+	-	-	+
3	AY3-bB1	-	+	+	+	+	+	+
3	NS14-cA1	-	+	+	+	-	-	+
3	NS15-aA1	-	+	+	+	+	+	+
3	SP9-A3	-	+	+	+	+	+	+
3	NP8-aB2	+	+	+	+	+	+	+
4	BK1-A3	-	+	+	+	-	-	+
4	NP6-A2	-	+	+	+	+	+	+
4	NS14-dA2	-	+	+	+	-	-	+
4	SP15-B5	-	+	+	-	+	+	+
4	SP4-A1	-	+	+	+	-	-	+
4	SP4-B11	-	+	+	+	+	-	-
4	SP5-A5	-	+	+	+	-	-	-
5	NS15-bB2	+	+	-	+	-	-	+
5	NP7-cB3	+	+	-	-	+	-	-
5	NP9-cA2	+	+	+	+	+	-	+
6	AY1-bA3	-	-	+	-	-	-	-
6	AY2-bB2	-	-	+	+	+	-	+
6	AY5-bA1	-	+	+	+	-	-	-
6	SP13-B2	-	+	+	+	+	+	+
6	SP14-B2	-	-	+	+	-	-	+
6	SP15-B4	-	-	+	+	-	-	+

Table 3.6 Physiological characteristics of 58 isolates (continuous)

Group	Isolates	pH 3.5	pH 5.0	pH 9.0	20 °C	40 °C	50 °C	6% NaCl
7	AY2-bA2	-	-	+	+	+	+	+
7	NP2-A3	-	+	+	+	-	-	+
7	SP14-A3	-	+	+	+	-	-	+
7	SP15-A2	-	+	+	+	-	-	+
7	SP15-B2	-	+	+	+	-	-	+
7	SP16-B4	-	+	+	+	+	+	+
7	SP5-B5	-	+	+	+	+	-	-
8	AY1-bA1	-	-	+	+	-	-	-
8	NP4-A4	-	-	+	+	-	-	+
8	NS13-bA1	-	+	+	+	-	-	-
8	NS14-bA1	-	+	+	+	+	+	+
8	NP9-cA4	+	+	+	-	+	-	+
9	AY5-bB1	-	+	+	+	-	-	-
9	NS17-B1	-	-	+	-	-	-	-
9	NS18-A1	-	+	-	-	-	-	-
9	SP11-A2	-	+	+	+	-	-	-
9	SP11-B4	-	+	+	+	-	-	-
9	SP12-A1	-	+	+	+	-	-	-
9	SP17-B1	-	+	+	+	-	-	-
10	NP9-aA3	-	-	+	+	+	-	+
10	NP9-aA7	+	+	-	-	+	-	+
11	BK1-A1	-	-	+	+	-	-	+
	Total Positive/Negative	6/52	46/12	54/4	51/7	27/31	13/45	39/19

From the result of physiological characteristics only 6 isolates including; NP8-aA2, NP7-cB3, NP9-aA7, NP9-cA2, NP9-cA4 and NS15-bB2 were grow in pH 3.5 which, benefit of acid tolerant property suitable to apply in acid fermentation process.

3.4.2.3 Biochemical characteristics

The results of biochemical characteristics were shown in Table 3.7. All 58 isolates showed negative reactions to slime formation. Only 4 isolates including; NS18-A1, SP11-A2, SP12-A1 and SP17-B1 were positive reaction to starch hydrolysis. But catalase, gas production, arginine hydrolysis, nitrate reduction and acid from carbohydrates showed different reaction in Table 3.7.

Table 3.7 Biochemical characteristics of 58 isolates

Isolates	NP2-B1	NS13-dA1	SP10-A5	SP10-B7	SP8-B6	NS13-aB1	NS13-dB1	NS15-aA2	SP5-B2	SP6-A2	SP6-A5	SP8-A8	SP8-B4	AY3-bB1	NS14-cA1	NS15-aA1	SP9-A3	NP8-aB2	BK1-A3	NP6-A2
Group	1					2								3					4	
Shape	2	3	3	3	3	3	3	4	3	4	4	2	2	2	3	2	3	3	5	4
Amygdalin	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	+	+
Arabinose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+
Cellobiose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Fructose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Galactose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Gluconate	+	+	-	-	+	-	+	-	+	-	-	-	+	-	-	+	-	+	-	+
Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Lactose	+	+	+	+	+	+	-	+	+	+	+	+	+	+	-	+	+	+	+	+
Maltose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+
Mannitol	+	-	-	-	-	+	+	+	+	+	-	+	+	-	-	-	-	+	+	-
Mannose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Melibiose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Methyl-D-glucoside	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
Raffinose	+	+	+	+	+	-	-	-	-	-	+	-	-	-	-	-	+	+	+	-
Rhamnose	-	-	-	-	-	-	+	+	+	+	-	+	+	-	-	-	-	-	-	+
Ribose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Salicin	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Sorbitol	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
Sucrose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
Trehalose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+
Xylose	+	-	+	+	+	-	+	+	+	+	+	+	+	-	-	-	-	-	+	+
Arginine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
Catalase	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
CO_2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-
Nitrate	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-
Starch hydrolysis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Slime	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 3.7 Biochemical characteristics of 58 isolates (continuous)

Isolates	NS14-dA2	SP15-B5	SP4-A1	SP4-B11	SP5-A5	NS15-bB2	NP7-cB3	NP9-cA2	AY1-bA3	AY2-bB2	AY5-bA1	SP13-B2	SP14-B2	SP15-B4	AY2-bA2	NP2-A3	SP14-A3	SP15-A2	SP15-B2	SP5-B5
Group	4					5			6						7					
Shape	4	2	3	3	3	0	0	0	2	1	1	0	1	1	1	2	1	1	1	0
Arginine	+	+	+	+	+	-	+	-	-	+	-	+	+	+	+	+	+	+	+	+
Catalase	-	-	-	-	-	-	+	-	-	-	-	+	-	-	-	+	-	-	-	-
CO_2	-	-	-	-	-	+	+	+	-	-	+	-	-	-	-	-	-	-	-	-
Nitrate	-	-	-	-	+	-	-	-	-	+	+	-	-	+	-	-	-	-	-	-
Starch hydrolysis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Slime	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Amygdalin	+	+	+	-	+	-	-	-	+	-	+	+	-	-	+	+	+	+	+	+
Arabinose	+	-	+	-	+	+	+	+	-	+	-	+	-	-	-	-	-	-	-	-
Cellobiose	+	+	+	-	+	-	+	+	+	+	+	+	-	+	+	+	+	+	+	+
Fructose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Galactose	+	+	+	+	+	-	+	+	+	-	+	+	-	-	-	+	+	+	+	+
Gluconate	+	-	+	-	-	+	+	+	-	+	-	-	+	+	+	+	+	-	+	+
Glucose	+	+	-	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+
Lactose	-	-	+	-	+	+	-	+	+	-	+	+	-	-	-	-	-	-	-	-
Maltose	+	-	+	-	+	+	+	+	+	-	+	+	-	-	+	+	+	+	+	+
Mannitol	+	-	+	-	+	-	-	-	+	-	+	+	+	-	+	+	+	+	+	+
Mannose	-	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+
Melibiose	-	-	+	-	-	+	+	+	+	-	+	+	-	-	-	-	+	-	-	-
Methyl-D-glucoside	+	-	+	-	+	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-
Raffinose	+	-	+	+	-	+	-	+	-	-	-	+	-	-	-	-	-	-	-	-
Rhamnose	+	-	+	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
Ribose	+	-	+	-	-	+	-	+	+	-	+	+	+	+	-	+	+	+	+	+
Salicin	+	+	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Sorbitol	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+
Sucrose	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	-	-
Trehalose	+	+	+	+	+	-	+	+	+	+	-	+	-	+	+	-	+	+	+	-
Xylose	+	-	+	-	+	+	-	-	-	-	-	+	-	-	+	-	-	+	-	-

Table 3.7 Biochemical characteristics of 58 isolates (continuous)

Isolates	AY1-bA1	NP4-A4	NS13-bA1	NS14-bA1	NP9-cA4	AY5-bB1	NS17-B1	NS18-A1	SP11-A2	SP11-B4	SP12-A1	SP17-B1	NP9-aA3	NP9-aA7	BK1-A1	BK2-A2	BK2-B4
Group	8					9							10		11		
Shape	0	0	0	0	1	0	1	0	1	0	0	1	1	1	5	5	5
Arginine	-	+	+	+	-	-	+	-	+	-	+	+	-	-	-	+	+
Catalase	+	+	+	-	-	+	+	+	-	-	-	-	+	+	-	+	-
CO_2	+	-	-	-	-	-	+	+	+	-	+	+	+	+	-	-	-
Nitrate	-	+	-	+	+	-	-	-	-	-	-	-	+	+	-	+	-
Starch hydrolysis	-	-	-	-	-	-	-	-	+	-	+	+	-	-	-	-	-
Slime	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Amygdalin	+	-	+	+	+	-	-	-	-	-	-	-	-	+	-	-	+
Arabinose	-	-	-	+	+	-	-	-	-	-	-	-	-	+	-	-	-
Cellobiose	+	-	+	+	+	-	-	-	-	+	-	-	-	+	-	-	+
Fructose	+	+	+	+	+	+	-	-	-	-	-	-	-	-	+	+	+
Galactose	+	+	+	+	+	-	-	-	-	-	-	-	+	-	-	+	-
Gluconate	-	-	-	+	-	-	-	-	-	-	-	-	+	+	-	-	-
Glucose	+	+	+	+	+	+	-	-	+	-	-	-	+	+	+	-	+
Lactose	+	+	-	+	+	-	-	-	-	-	-	-	-	+	-	-	-
Maltose	+	+	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-
Mannitol	+	-	+	+	+	-	-	-	-	-	-	-	-	+	-	-	-
Mannose	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	+
Melibiose	+	-	+	+	+	-	-	-	-	-	-	-	-	-	-	+	-
Methyl-D-glucoside	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
Raffinose	+	+	+	-	+	-	-	-	-	-	-	-	-	-	-	+	-
Rhamnose	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
Ribose	+	-	+	+	+	-	-	-	-	-	-	-	+	+	-	+	-
Salicin	+	+	-	+	+	-	-	-	-	-	-	-	-	+	-	+	+
Sorbitol	+	+	+	+	+	-	-	-	-	-	-	-	-	+	-	+	-
Sucrose	+	+	-	+	+	+	-	-	-	-	-	-	-	-	+	+	+
Trehalose	+	+	-	+	+	-	-	-	-	-	-	-	+	-	-	-	-
Xylose	-	-	+	+	+	-	-	-	-	-	-	-	+	+	-	+	-

0, Rod shape; 1, Short rod; 2, Coccus; 3, Cocci in pairs, 4, Cocci in chain; 5, Corynebac; +, Positive reaction; w, weakly positive; -, negative reaction.

From Figure 3.5, Table 3.6 and Table 3.7 showed that all of 58 isolate were divided into 11 groups. The results could be described as bellow.

Group I of isolates

Group I consisted of 6 isolates, namely, NP2-B1 SP-A1, SP8-B6, SP10-A5 and SP10-B7. Group I was isolated from bark of tree and soil (Table 3.2). They were gram positive and cocci in pairs, only isolate NP2-B1 was coccus. They grew at a pH of 5-9 in 6% NaCl and at 20 °C but did not grow at pH 3.5. Some isolate in this group could grow in the range 40-50 °C (Table 3.6). They were fermented in D-amygdalin, arabinose, cellobiose, fructose, galactose, glucose, lactose, maltose, mannose, melibiose, raffinose, ribose, salicin, sucrose and trehalose. They were not able to ferment ∞-methyl-D-glucoside and rhamnose. The ability of group I isolates in sugar fermentation were variable as shown in Table 3.7. They showed positive reactions of arginine hydrolysis while negative reactions of catalase, gas production, nitrate reduction (except SP10-A5), starch hydrolysis and slime formation.

The ability to produce succinic acid of the isolate Group I was in the range 39.400-43.112 g/L. NP2-B1 produced the highest succinic acid of 43.112 g/L follow by NS13-dA1 and SP10-A5 gave a succinic acid of 42.172 and 40.094 g/L, respectively. Succinic acid production of these three isolates were similar also concern by product in fermentation. The isolate SP10-A5 gave a little acetic acid of 0.172 g/L moreover, it not produced formic acid. Therefore, isolate SP10-A5 was representative from this group for further study using 16S rRNA gene sequence analysis.

Group II of isolates

Group II consisted of 8 isolates, namely, NS13-aB1, NS13-dB1, NS15-aA2, SP5-B2, SP6-A2, SP6-A5, SP8-A8 and SP8-B4. Group II were corected from bovine rumen and soil sample. They were gram positive while morphology of each isolate was different (coccus, cocci in pairs and cocci in chain) (Table 3.5). They grew at a pH of 5-9 in 6% NaCl and at 20 °C but did not grow at pH 3.5. Some isolate in this group could grow in the range 40-50 °C (Table 3.7). They were fermented in D-amygdalin, arabinose, cellobiose, fructose, glucose, lactose (except NS13-dB1), maltose, mannose, melibiose, raffinose, ribose, salicin, sucrose and trehalose. They were not able to ferment ∞-methyl-D-glucoside, raffinose (except SP6-A5) and sorbitol. The

ability of group II isolates in sugar fermentation were variable as shown in Table 3.7. They showed positive reactions of arginine hydrolysis while negative reactions of catalase, gas production, nitrate reduction (except SP5-B2), starch hydrolysis and slime formation.

The ability to produce succinic acid of the isolate Group II in the range 26.893 – 43.482 g/L. Isolate NS13-dB1 produced the highest succinic acid of 43.482 g/L so isolate NS13-dB1 was representative for further study using 16S rRNA gene sequence analysis. In addition isolate NS13-aB1 was a different apparent the morphology of colony from other isolate in this group (irregular, translucent) (Table 3.6) so it was attractive for study using 16S rRNA gene sequence analysis.

Group III of isolates

Group III consisted of 5 isolates, namely, AY3-bB1, NP8-aB2, NS14-cA1, NS15-aA1 and SP9-A3. Group III were corected from bovine rumen and soil sample. They were gram positive while morphology of each isolate was different (coccus and cocci in pairs) (Table 3.6). They grew at a pH of 5-9 in 6% NaCl and at 20 ℃ but did not grow at pH 3.5 (except NP8-aB2). Some isolate in this group could grow in the range 40-50 ℃ (Table 3.6). They were fermented in cellobiose, fructose, galactose, glucose, mannose, melibiose, ribose, salicin, and sucrose. They were not able to ferment ∞-methyl-D-glucoside, rhamnose, sorbitol and xylose. The ability of group III isolates in sugar fermentation were variable as shown in Table 3.8. They showed positive reactions of arginine hydrolysis while negative reactions of catalase, gas production (except NP8-aB2), nitrate reduction, starch hydrolysis and slime formation.

The ability to produce succinic acid of the isolate Group II was in the range 36.252-40.838. Isolate NP8-aB2 produced the highest succinic acid of 40.838 g/L so isolate NP8-aB2 and NS15-aA1 were representative for further study using 16S rRNA gene sequence analysis.

Group IV of isolates

Group IV consisted of 7 isolates, namely, BK1-A3, NP6-A2, NS14-dA2, , SP4-A1, SP4-B11, SP5-A5 and SP15-B5. Group I was isolated from tree bark, bovine rumen, siol sample and dog saliva. They were gram positive while morphology of each isolate was different (coccus, cocci in pairs and corynecac) (Table 3.5). They grew at a pH of

5-9 in 6% NaCl at 20 °C (except SP15-B5) but did not growth at pH 3.5 and at 50 °C (Table 3.6). They were fermented in fructose, galactose, trehalose. The ability of group IV isolates in sugar fermentation were variable as shown in Table 3.8. They showed negative reactions of catalase (except NP6-A2), gas production, nitrate reduction, starch hydrolysis and slime formation (Table 3.7).

The ability to produce succinic acid of the isolate Group IV in the range 9.560-41.249 g/L. Isolate SP5-A5 produced the highest succinic acid of 41.249 g/L. In addition the morphology of colony of NS14-dA2 was a different apparent the morphology of colony from other isolate in this group (yellow, irregular and translucent) (Table 3.6). Therefore, isolate SP5-A5 and NS14-dA2 were representative for further study using 16S rRNA gene sequence analysis.

Group V of isolates

Group V consisted of 3 isolates, namely, NP7-cB3, NP9-cA2 and NS15-bB2. Group III was isolated from bovine rumen fluid and bovine rumen tissue. They were Gram-positive rod. They grew at a pH of 3.5-5.0 in 6% NaCl (except NP7-cB3) but did not grow at pH 9 (except NP9-cA2) and at 50 °C. They were fermented in arabinose, fructose, gluconate, glucose, maltose, mannose, melibiose and sucrose. They were not able to ferment D-amygadalin, mannitol and sorbitol. The ability of group V isolates in sugar fermentation were variable as shown in Table 3.7. They showed positive reactions of gas production while negative reactions of nitrate reduction, starch hydrolysis and slime formation (Table 3.7).

The ability to produce succinic acid of the isolate Group V was in the range 12.586-36.179. Isolate NP1-A2 produced the highest succinic acid of 36.179 g/L. In addition, the morphology of NS15-bB2 was rod shaped and was different shape from other isolate in this group (Table 3.5). Therefore, isolate NP1-A2 and NS15-bB2 were representative for further study using 16S rRNA gene sequence analysis.

Group VI of isolates

Group VI consisted of 6 isolates, namely, AY1-bA3, AY2-bB2, AY5-bA1, SP13-B2, SP14-B2 and SP15-B4. Group VI was isolated from bark of tree and dog saliva. They were gram positive while the morphology of each isolate was different such as isolate AY1-bA3 was coccus, AY2-bB2, AY5-bA1, SP14-B2 and SP15-B4 were short rod

and SP13-B2 was rod. They grew at a pH of 9 at 20 °C (except AY1-bA3) but did not grow at pH 3.5-5. They were fermented in and salicin but not able to ferment rhamnose and sorbitol. The ability of group VI isolates in sugar fermentation were variable as shown in Table 3.7. They showed positive reactions of arginine hydrolysis (except AY1-bA3 and AY5-bA1) while negative reactions of catalase (except SP13-B2), gas production (except AY5-bA1), nitrate reduction, starch hydrolysis and slime formation (Table 3.7).

The ability to produce succinic acid of the isolate Group VI in the range 21.789-34.362 g/L. Isolate AY5-bA1 produced the highest succinic acid of 34.362 g/L. Therefore, isolate AY5-bA1, AY2-bB2, SP13-B2 and SP14-B2 were representative for further study using 16S rRNA gene sequence analysis.

Group VII of isolates

Group VII consisted of 7 isolates, namely, AY2bA2, NP2-A3, SP5-B5, SP14-A3, SP15-A2, SP15-B2 and SP16-B4. Group VII was isolated from bark of tree, soil and dog saliva. They were gram positive while the morphology of each isolate was different such as isolate NP2-A3 was coccus, AY2bA2, SP14-A3, SP15-A2, SP15-B2 and SP16-B4 were short rod and only SP5-B5 was rod. They grew at a pH of 5-9 in 6% NaCl at 20 °C but did not grow at pH 3.5 and at 50 °C (except SP16-B4). They were fermented in D-amygdalin, cellobiose, fructose, galactose, glucose, lactose, maltose, mannitol, mannose, melibiose, ribose and salicin. They were not able to ferment arabinose, lactose, raffinose, ∞-methyl-D-glucoside and rhamnose. The ability of group VII isolates in sugar fermentation were variable as shown in Table 3.7. They showed positive reactions of arginine hydrolysis while negative reactions of catalase (except NP3-A2), gas production, nitrate reduction, starch hydrolysis and slime formation.

The ability to produce succinic acid of the isolate Group VII in the range 18.847-38.857 g/L. Isolate NP2-A3 produced the highest succinic acid of 38.857 g/L follow by AY2-bA2 gave a succinic acid of 38.667 g/L. In addition the morphology of colony of SP14-A3 was a different apparent the morphology of colony from other isolate in this group (white, irregular and opaque) (Table 3.5). Therefore, isolate NP2-A3, AY2-bA2 and SP14-A3 were representative for further study using 16S rRNA gene sequence analysis.

Group VIII of isolates

Group VIII consisted of 5 isolates, namely, AY1-bA1, NP4-A4, SP13-bA1, NS14-bA1, and NP9-cA4. Group VIII was isolated from bark of tree, dog saliva and and bovine rumen. They were gram positive rod. They grew at a pH of 9 and at 20 °C but did not grow at pH 3.5 and at 50 °C (except NS14-bA1). They were fermented in cellobiose, galactose, glucose, rhamnose and sorbitol. The ability of group VIII isolates in sugar fermentation were variable as shown in Table 3.7. They showed positive reactions of catalase (except NS14-bA1 and NP9-cA4) while negative reactions of starch hydrolysis and slime formation.

The ability to produce succinic acid of the isolate Group VIII in the range 15.424-40.917 g/L. Isolate NP1-A2 produced the highest succinic acid of 40.917 g/L. In addition isolate AY1-bA1 gave a high succinic acid (31.264 g/L) and isolate NS13-bA1 was a different apparent the morphology of colony from other isolate in this group (yellow, irregular and opaque) (Table 3.5). Therefore, isolate NP1-A2, AY1-bA1 and NS13-bA1 were representative for further study using 16S rRNA gene sequence analysis.

Group IX of isolates

Group IX consisted of 7 isolates, namely, AY5-bB1, NS17-B1, NS18-A1, SP11-A2, SP11-B4, SP12-A1 and SP17-B1. Group IX was isolated from bark of tree, ferment fruit and dog saliva. They were gram positive rod. They grew at a pH of 9 (except NS18-A1) but did not grow at pH 3.5 and at 40-50 °C. They were not able to fermented in D-amygdalin, arabinose, galactose, glucose, lactose, maltose, mannose, melibiose,∞-methyl-D-glucoside, raffinose, ribose, salicin, sorbitol trehalose and xylose. The ability of group IX isolates in sugar fermentation were variable as shown in Table 3.7. Isolate NS18-A1, SP11-A2, SP12-A1 and SP17-B1 showed positive reactions of starch hydrolysis while all isolate in group IX were negative reactions of gas production and slime formation.

The ability to produce succinic acid of the isolate Group IX in the range 2.099-24.065 g/L. Isolate NS18-A1 produced the highest succinic acid of 24.065 g/L while isolate NS17-B1 and SP17-B1 produced succinic acid of 2.099 and 9.202 g/L, respectively. However they were a different apparent the morphology of colony from

other isolate in this group (white and gray, circular and translucent) (Table 3.5). Therefore, isolate NS18-A1, NS17-B1 and SP17-B1 were representative for further study using 16S rRNA gene sequence analysis.

Group X of isolates

Group X consisted of 2 isolates, namely, NP9-aA3 and NP9-aA7. Group I was isolated from bovine rumen. They were gram negative rod. They grew in 6% NaCl and at 20 ℃ but did not grow at 50 ℃. They were fermented in gluconate, glucose, ribose and xylose. They were not able to ferment fructose, maltose, mannose, melibiose, ∞-methyl-D-glucoside, raffinose, rhamnose and sucrose. The ability of group X isolates in sugar fermentation were variable as shown in Table 3.7. They showed positive reactions of catalase gas production and nitrate reduction while negative reactions of starch hydrolysis and slime formation.

The ability to produce succinic acid of the isolate Group X in the range 40.806-42.539 g/L. Isolate NP9-aA3 and NP9-aA7 were high succinic acid similar furthermore, they were Gram-negative rod. Therefore both isolate were representative for further study using 16S rRNA gene sequence analysis.

Group XI of isolates

Group XI consisted of 3 isolates, namely, BK1-A1, BK2-A2 and BK2-B4. Group XI was isolated from tree bark. They were gram positive and the morphology of isolates were corynebac. They grew at a pH of 5-9 and in 6% NaCl but did not grow at pH 3.5 and at 50 ℃. They were fermented in fructose. They were not able to ferment arabinose, gluconate, lactose, mannitol, rhamnose and sucrose. The ability of group XI isolates in sugar fermentation were variable as shown in Table 3.7. They showed negative reactions of catalase, starch hydrolysis and slime formation (Table 3.7).

The ability to produce succinic acid of the isolate Group XI in the range 1.619-23.312 g/L. Isolate BK1-A1 produced the highest succinic acid of 23.312 g/L therefore isolate BK1-A1 was representative for further study using 16S rRNA gene sequence analysis.

3.4.3 16S rRNA gene sequence and phylogenetic analysis

Twenty-four isolates were represented of 58 Isolates from phenotypic characterization, which were studied 16S rRNA gene sequence using universal primer (20F (5'-AGTTTGATCCTGGCTC-3') and 1530R (5'-AAGGAGGTGATCCAGCC-3')). The PCR products (1500 base pairs) of each isolates were run gel electrophoresis. For example of some isolates; NS13-aB1, NS13-dB1 and NS15-aA1 were shown in Figure 3.6.

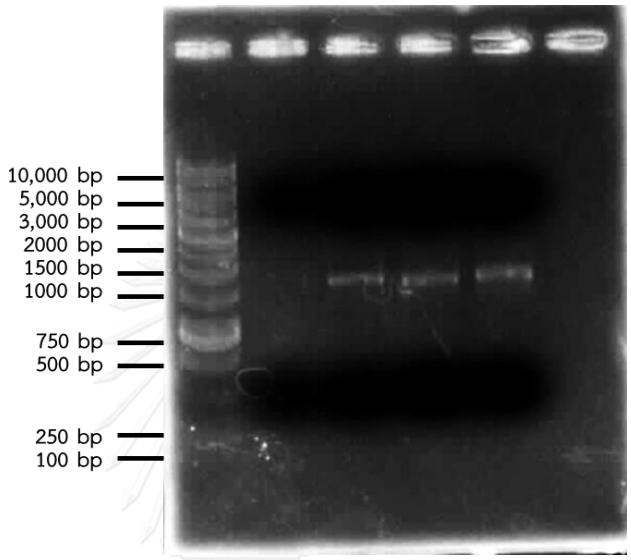

Figure 3.6 Result of gel electrophoresis on 1% agarose gel based on 16S rRNA gene sequence

A genes sequencing of representative isolate have been identified based on its 16S rRNA sequence analysis. Results occur Isolates from group I, II, III and IV were closely related to *Enterococcus* sp. except isolate NP8-aB2 was closely related to *Streptococcus* sp.. Isolates from group V were closely related to *Lactobacillus* sp. Isolate from group VI were closely related to *Enterococcus* sp., *Clostridium* sp. and *Lactococcus* sp.. Isolates from group VII were closely related to *Enterococcus* sp. and *Lactococcus* sp.. Isolates from group VIII were closely related to *Enterococcus* sp. and *Clostridium* sp.. Isolates from group IX were closely related to *Clostridium* sp.. Isolates from group X belonged to *Pasteurellaceae* family based on its 16S rRNA sequence analysis and closely related to *Proteus* sp. and *Actinobacillus* sp.. The last one, isolates from group XI were closely related to *Enterococcus saccharolyticus* sub sp.. The results of percentages similarities and the DDBJ accession number for the 16S rRNA gene sequence of isolate were shown in Table 3.8. The phylogenetic

relationships between representative isolate from each group and other closely related members of the genus was shown in Figure 3.7 and Figure 3.8.

Table 3.8 Isolate number, sources, identification and 16s RNA gene sequence similarity (%) and accession number of isolates.

Group	Isolate	Isolation Source	Identification	% similarity	Length (bp)	Aceccession No.
1	SP10-A5	Soil	*Enterococcus hirae* ATCC 9790 (T)	100	1356	LC192797
2	NS13-aB1	Bovine rumen tissue	*Enterococcus hirae* ATCC 9790 (T)	100	1349	LC122276
2	NS13-dB1	Bovine rumen tissue	*Enterococcus faecium* ATCC19434 (T)	99.93	1376	LC122274
3	NS15-aA1	Bovine rumen tissue	*Enterococcus hirae* ATCC 9790 (T)	100	1333	LC122278
3	NP8-aB2	Bovine rumen	*Streptococcus lutetiensis* CIP 106849 (T)	99.84	1262	LC192791
4	NS14-dA2	Bovine rumen tissue	*Enterococcus faecium* ATCC19434 (T)	99.93	1346	LC192795
5	NS15-bB2	Bovine rumen tissue	*Lactobacillus fermentum* NBRC 3956	99.78	1391	LC122284
5	NP7-cB3	Bovine rumen	*Lactobacillus ruminis* NBRC 102161 (T)	100	1321	LC192790
6	AY2-bB2	Bark of Ficus religiosa L.	*Enterococcus casseliflavus* ATCC49996(T)	99.69	1295	LC120365
6	AY5-bA1	Bark of Ficus religiosa L.	*Clostridium tertium* DSM 2485 (T)	100	6501225	LC192789
6	SP14-B2	Dog mouth	*Lactococcus formosensis* 516 (T)	99.83	1398	LC122286
6	SP13-B2	Soil	*Clostridium butyricum* DSM10702 (T)	98.87	1190	LC192798
7	AY2-bA2	Bark of Ficus religiosa L.	*Enterococcus casseliflavus* ATCC49996 (T)	99.73	1383	LC122272
7	NP2-A3	Soil	*Enterococcus duran* CECT411 (T)	99.68	1285	LC122273
7	SP14-A3	Dog mouth	*Lactococcus garviae* ATCC 49156 (T)	99.85	1368	LC122287
8	AY1-bA1	Bark of Ficus religiosa L.	*Clostridium bifermentans* ATCC638 (T)	99.85	1293	LC192840
8	NS13-bA1	Bovine rumen tissue	*Lactobacillus oris* DSM 4864 (T)	99.85	1359	LC122285
8	NP9-cA4	Bovine rumen	*Lactobacillus reuteri* (T)	99.7	1319	LC192794
9	NS17-B1	Fruit fermented	*Clostridium indolis* DSM 775 (T)	99.84	1238	LC192800

Ayutthaya (AY), Nakhonpathom (NP), Nakhonsawan (NS), Suphanburi (SP) and Surin (SR) provinces, Thailand.

Table 3.8 Isolate number, sources, identification and 16s RNA gene sequence similarity (%) and accession number of isolates (continuous).

Group.	**Isolate**	**Isolation Source**	**Identification**	**% similarity**	**Length (bp)**	**Accession No.**
9	NS18-A1	Fruit fermented	*Clostridium amygdalinum* BR-10 (T)	97.82	1491	LC192796
9	SP17-B1	Dog mouth	*Clostridium amygdalinum* BR-10 (T)	97.84	1398	LC192799
10	NP9-aA3	Bovine rumen	*Proteus mirabilis* ATCC 29906 (T)	99.92	1257	LC192792
10	NP9-aA7	Bovine rumen	*Actinobacillus succinogenes* 130z (T)	99.86	1440	LC192793
11	BK1-A1	Bark of *Samanae saman*	*Enterococcus saccharolyticus sub sp.*	100	1364	LC122283

Ayutthaya (AY), Nakhonpathom (NP), Nakhonsawan (NS), Suphanburi (SP) and Surin (SR) provinces, Thailand.

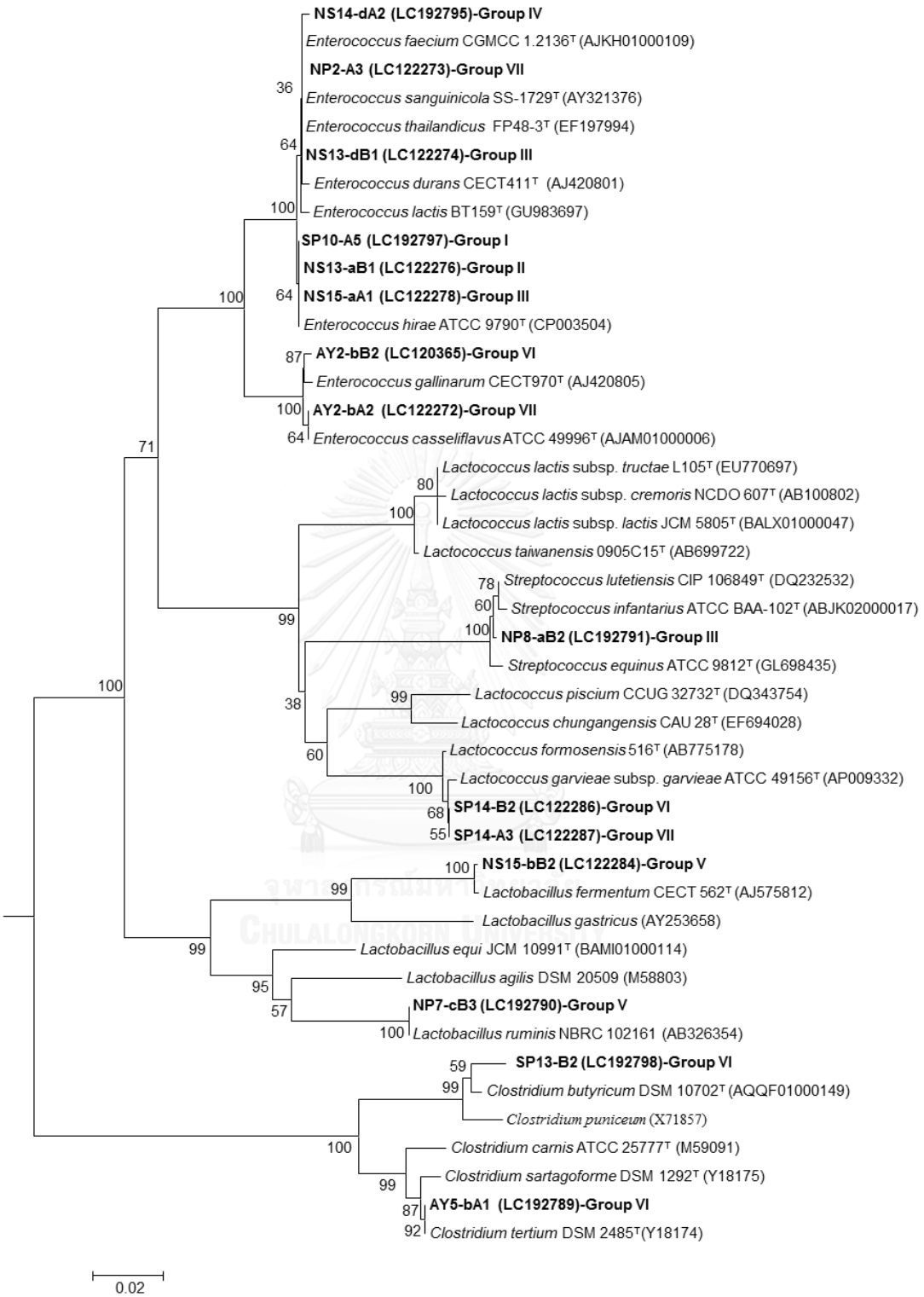

Figure 3.7 Neighbor-joining phylogenetic tree of isolates in Group I to Group VII based on 16S rRNA gene sequences. Bootstrap values were expressed as percentages of 1000 replications. The accession numbers were shown in parentheses.

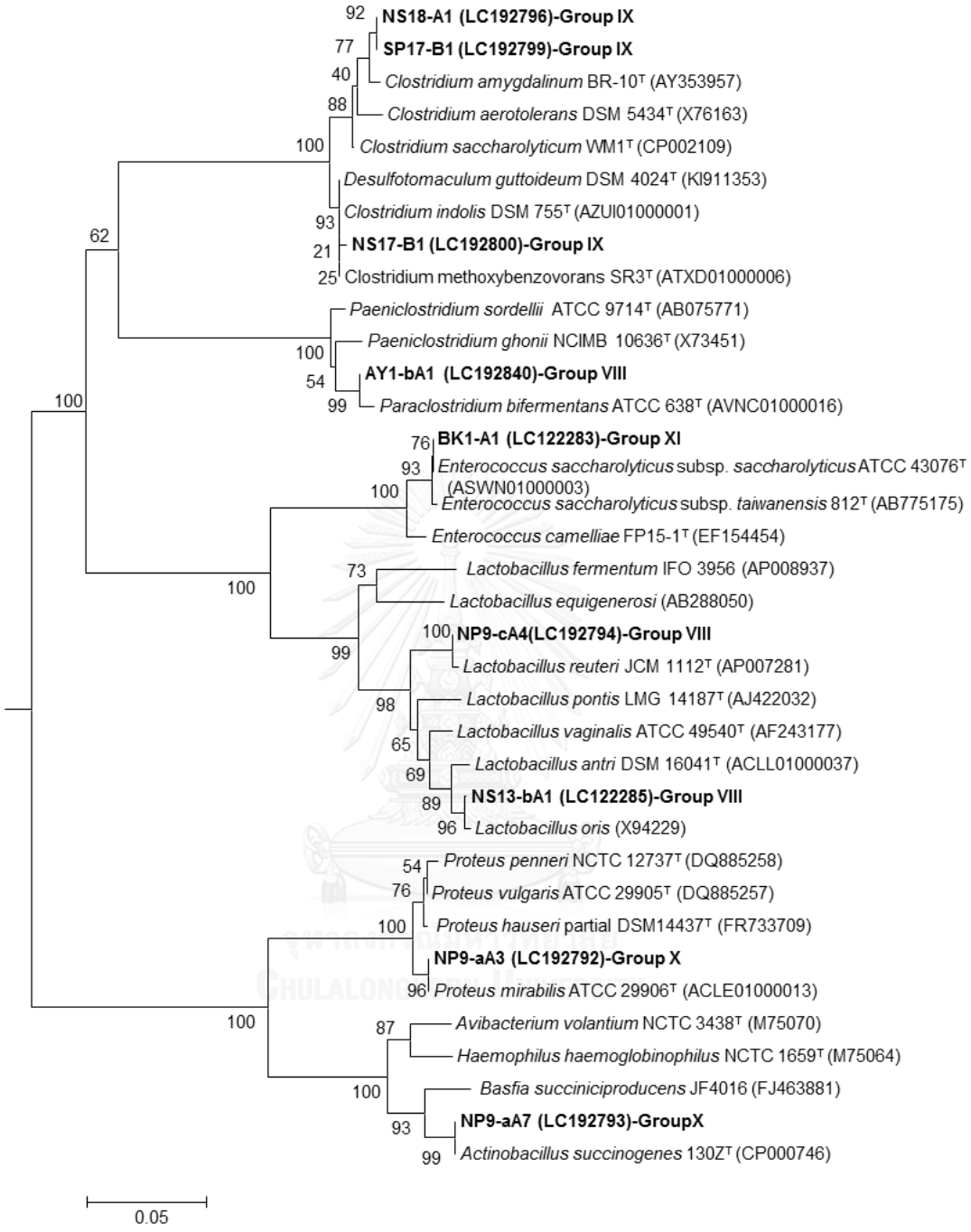

Figure 3.8 Neighbor-joining phylogenetic tree of isolates in Group VIII to Group XI based on 16S rRNA gene sequences. Bootstrap values were expressed as percentages of 1000 replications. The accession numbers were shown in parentheses.

From these results, 24 isolates were closely related to *Enterococcus* sp., *Streptococcus* sp, *Lactobacillus* sp., *Clostridium* sp., *Lactococcus* sp. *Proteus* sp. and *Actinobacillus* sp.

Concerning the properties of all isolates were obtained in this study such as acidity resistance, optimum temperature, ability to use a variety of carbohydrate. The most important property was produced high value of succinic acid however we get many isolates that have similar properties. Therefore, one of the important property and should not be ignored was pathogenicity (Table 3.9) for safety and could be a promising candidate for further applications. In addition, other previous research about succinic acid production were summarized in Table 3.10.

Consequently, all of reason was an encouragement to use the strain *Actinobacillus succinogenes* NP9-aA7 for optimization of succinic acid production because it was high ability to produce succinic acid of 42.539 g/L with a yield of 0.709 g/g glucose closely to succinic acid from *A. succinogenes* NJ 113 gave a succinic acid of 45.2 g/L however the succinic acid productivity of 0.886 g/L·h was obtained from *A. succinogenes* NP9-aA7 higher than the succinic acid productivity of 0.646 g/L·h was obtained from *A. succinogenes* NJ 113 (Xi et al., 2012). *A. succinogenes* NP9-aA7 was a facultative anaerobe and resistant to low pH and was non-pathogenic. It could be a promising candidate for further applications.

Table 3.9 The advantage and disadvantage of bacteria were isolated.

Microorganism	Group	Isolate	Advantage	Disadvantage	Succinic acid (g/L)	Formic acid (g/L)	Acetic acid (g/L)	Reference
A. succinogenes 130z (T)	10	NP9-aA7	- Non-pathogenic, - High succinic acid	- Already reports	42.539	2.921	1.859	(Guettler, Rumler and Jain, 1999)
C. amygdalinum BR-10 (T)	9	NS18-A1	- Able to utilized C5 sugar - High production of hydrogen	- Strictly anaerobic - Isolated from patients	24.065	0.737	0.462	(Carlier, Manich, Loiez et al., 2006; Jayasinghearachchi and Lal, 2011)
C. amygdalinum BR-10 (T)	9	SP17-B1			9.202	0.000	0.000	
C. bifermentans ATCC638 (T)	8	AY1-bA1	- Non- pathogenic	- Already reports - Strictly anaerobic	31.264	0.201	1.503	(Leja, Myszka and Czaczyk, 2013)
C. butyricum DSM10702 (T)	6	SP13-B2	- Non-pathogenic - Probiotic - Inhibit Clostridium difficile	- Strictly anaerobic	30.371	0.875	1.943	(Yamaoka, Imaeda, Yoneno et al., 2015)
C. indolis DSM 775 (T)	9	NS17-B1	- Non-pathogenic - New species (discovered in 2014)	- Low succinic acid production	2.099	7.174	6.567	(Biddle, Leschine, Huntemann et al., 2014)
C. tertium DSM 2485 (T)	6	AY5-bA1	- Nonpathogenic but effect on neutropenic and nonneutropenic patients	- Strictly anaerobic - β-lactam resistant (cephalosporin; clindamycin and metronidazole)	34.362	0.304	5.581	(Miller, Brazer, Murdoch et al., 2001)
E. casseliflavus ATCC49996 (T)	6 7	AY2-bB2 AY2-bA2	Non-pathogenic	- Vancomycin-resistant	30.121 38.667	1.54 2.028	0.542 0.634	(Agarwal, Isar, Meghwanshi et al., 2007)

Table 3.9 The advantage and disadvantage of bacteria were isolated (continuous)

Microorganism	Group	Isolate	Advantage	Disadvantage	Succinic acid (g/L)	Formic acid (g/L)	Acetic acid (g/L)	Reference
E. duran CECT411 (T)	7	NP2-A3	- Antimicrobial activity in fermented food - Antioxidant ability - Probiotic and high succinic acid production	- Vancomycin-resistant	38.857	1.194	0.332	(Pieniz, Andreazza, Anghinoni et al., 2014)
E. faecium ATCC19434 (T)	2	NS13-dB1	- Antimicrobial activity in fermented food - Probiotics and high succinic acid production	- Vancomycin-resistant	43.482	0.29	0.144	(Kang and Lee, 2005; Mascini, Troelstra, Beitsma et al., 2006)
E. faecium ATCC19434 (T)	4	NS14-dA2			36.252	0.105	0.181	
E. hirae ATCC 9790 (T)	1	SP10-A5			40.094	0.000	0.873	
E. hirae ATCC 9790 (T)	2	NS13-aB1	- Coper absorption control - High succinic acid	- Vancomycin-resistant - Pathogen in poultry and mammal	38.973	0.211	0.773	(Solioz and Stoyanov, 2003; Vardanyan and Trchounian, 2015)
E. hirae ATCC 9790 (T)	3	NS15-aA1			36.252	0.450	0.252	
E. saccharolyticus sub sp.	11	BK1-A1	- Non-pathogenic - Able to utilized various carbon source for organic acid production	- Low succinic acid production	23.312	1.053	0.399	(Chen, Lin, Pan et al., 2013)

Table 3.9 The advantage and disadvantage of bacteria were isolated (continuous)

Microorganism	Group	Isolate	Advantage	Disadvantage	Succinic acid (g/L)	Formic acid (g/L)	Acetic acid (g/L)	Reference
L. fermentum NBRC 3956	10	NS15-bB2	- Probiotic - Cholesterol reducing - Used in the supplementary food industry	- Low succinic acid	12.586	0.659	0.413	(Lye, Khoo, Karim et al., 2012; Tomaro-Duchesneau, Saha, Malhotra et al., 2015),
L. oris DSM 4864 (T)	9	NS13-bA1	- Probiotic - Cholesterol reducing - Used in the supplementary food industry.	- High lactic acid	25.247	0.319	0.260	(Anandharaj and Sivasankari, 2014)
L. reuteri (T)	9	NP9-cA4	- Probiotic - Cholesterol reducing, - Used in the supplementary food industry	- High lactic acid	40.917	3.976	4.475	(Kaneuchi et al., 1988)
L. ruminis NBRC 102161 (T)	8	NP7-cB3	- Probiotic - It administered to treat Helicobacter pylori and cholesterol reducing	- High lactic acid	36.179	0.438	0.000	(O' Donnell, Harris, Lynch et al., 2015; Yun, Yim, Kang et al., 2005)
L. formosensis 516 (T)	6	SP14-B2	- Probiotic - Non-pathogenic - A new species (discovered 2015)	- High lactic acid	21.789	0.566	0.273	(Chang, Chen, Lee et al., 2015)
L. garviae ATCC 49156 (T)	9	SP14-A3	- Facultative anaerobic	- Fish pathogen affecting saltwater fish in the Far East - Rare pathogen in human - Low virulence	22.172	1.562	0.529	(Morita, Toh, Oshima et al., 2011)
Proteus mirabilis ATCC 29906 (T)	6	NP9-aA3	- Facultative anaerobic	- Produced high urease resulting in kidney stones.	40.806	2.282	3.046	(Ranjbar-Omid, Arzanlou, Amani et al., 2015)
S. lutetiensis CIP 106849 (T)	6	NP8-aB2	- Non-pathogenic	- Clindamycin-resistant	40.838	0.559	0.000	(Almuzara, Bonofiglio, Cittadini et al., 2013)

Table 3.10 Comparison of bacterial strains with succinic acid ability

Strain	Gram	Succinic acid (g/L)	Substrate	Time (h)	Productivity (g/L•h)	Succinic acid yield (g g^{-1})	By product	Reference
A. succiniciproducens	-	17.8	20 g/L glucose	13.5	1.310	0.890	4.45 g/L of Ace	(Lee, Lee, Lee et al., 2000)
Bacteroides fragilis	-	12.5	15 g/L glucose	30	0.417	0.833	ND	(Isar, Agarwal, Saran et al., 2006)
M. succiniciproducens LPK7	-	13.4	20 g/L glucose	24	0.558	0.630	0.53 g/L of Ace, 0.27 g/L of Lac, 2.62 g/L of Mal and 2.47 g/L Pyr	(Lee, Song and Lee, 2006)
Klebsiella pneumonia SAP	-	2.1	10 g/L glucose	24	0.088	0.210	0.9 g/L of Lac, 0.83 g/L of For, 0.2 g/L of Ace and 0.5 g/L of Pro	(Thakker, Chandresh, S. et al., 2006)
K. pneumoniae MCM B-325	-	7.0	10 g/L of fumarate	24	0.291	0.973	ND	(Chandresh, BHOSALE and Ranade, 2006)
Basfia succiniciproducens	-	0.5	2 g/L glucose	19	0.026	0.250	ND	(Kuhnert, Scholten, Haefner et al., 2010)
A. succinogenes NJ 113	-	45.2	30 g/L glucose	70	0.646	0.904	2.9 g/L of Ace	(Xi, Chen, Xu et al., 2012)
A. succinogenes CGMCC 1593 mutant F3-II-3-F	-	95.6	130.8 g/L glucose	48	1.990	0.790	6.2 g/L of Ace	(Zheng, Zhang, Yan et al., 2013)
L. reuteri M9	+	1.46	2 g/L fumarate	48	0.030	0.730	ND	(Kaneuchi, Seki and Komagata, 1988)
E. faecalis RKY1	+	72.0	80 g/L of fumarate	-[a]	17.100	0.900	ND	(Wee, Yun, Kang et al., 2002)
		95.6	100 g/L of fumarate	24	4.3	0.956	ND	
E. flavescens	+	14.25	30 g/L sucrose	30	0.475	0.475	ND	(Agarwal et al., 2007)
A. succinogenes NP9-aA7	-	42.539	60 g/L glucose	48	0.886	0.709	2.921g/L of For and 1.859 g/L of Ace	This study

[a], continuous; ND, not detected; For, formic acid; Ace, acetic acid; Lac, lactic acid; Mal, malic acid; Pry, pyruvic acid; Pro,

3.5 Conclusions

In this study, the isolation, screening, characterization and identification of succinic acid producing bacteria were screened from 7 sources and 6 provinces in Thailand. The result from primary screening, 171 isolates exhibited a clear zone on the screening medium. Secondary screening, 165 isolates with succinic acid ability were obtained by TLC. Then confirm the quantitative analysis, concentration of succinic acid was in the range of 0.186-45.554 g/L. Subsequently, 58 isolate with 60% yield were further characterized including morphological, physiological and biochemical characteristics.

Fifty-eight isolates were divided into 11 groups. Representative isolate from each group have been identified based on its 16S rRNA sequence analysis. Isolates from group I, II, III and IV were closely related to *Enterococcus* sp. except isolate NP8-aB2 was closely related to *Streptococcus* sp.. Isolates from group V were closely related to *Lactobacillus* sp. Isolates from group VI were closely related to *Enterococcus* sp., *Clostridium* sp. and *Lactococcus* sp.. Isolate from group VII were closely related to *Enterococcus* sp. and *Lactococcus* sp.. Isolate from group VIII were closely related to *Enterococcus* sp. and *Clostridium* sp. Isolate from group IX was closely related to *Clostridium* sp. Isolate from group X belongs to *Pasteurellaceae* family and were closely related to *Proteus* sp. and *Actinobacillus* sp.. Isolate from group XI were closely related to *Enterococcus* sp.

Among 58 isolates, the strain *Actinobacillus succinogenes* NP9-aA7 from group X was selected to further study because it produced high succinic acid of 42.539 g/L with a yield of 0.709 g/g glucose. It was facultative anaerobe and resistant to low pH and non-pathogenic. The potential isolate NP9-aA7 needs to be integrated with the fermentation process by optimizing the medium composition for cell growth and promote the succinic acid production.

Chapter IV

Succinic acid production from by *Actinobacillus succinogenes* DSMZ 22257 (type strain)

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4.1 Abstract

Production of succinic acid by *Actinobacillus succinogenes* DSMZ 22257using sorghum straw hydrolysate (SSH) as a low cost carbon source was developed. In anaerobic fermentation, the maximum succinic acid concentration of 52.180 g/L, corresponding to a yield of 0.870 g/g glucose was obtained from 60 g/L of glucose and faster cells growth was also observed. When using 40 g/L of SSH as a carbon source, succinic acid of 16.671 g/L, corresponding to yield of 0.777 g/g substrate was achieved after 24 h of cultivation. Statistical method: Plackett-Burman Design (PBD) was applied for a preliminary optimization of succinic acid fermentation medium by *A. succinogenes* DSMZ 22257. The highest succinic acid of 15.746 g/L was obtained with fermentation medium contained 50.0 g/L of yeast extract, 5.0 g/L of urea, 5.0 g/L of $CaCl_2$, 0.25 g/L of $MnCl_2$, 2.50 g/L of Na_2CO_3 and 50 g/L of $MgCO_3$. The results from PBD, yeast extract and $MgCO_3$ were identified as the key medium components. Then key medium were optimized by Central Composite Design (CCD) using a Response Surface Methodology (RSM). The regression equation showed that the R^2 was 0.9751 and non-significant lack of fit indicated the model was a good fit. The optimized concentrations of SSH, yeast extract and $MgCO_3$ were 45 (1), 34.55 (0.91) and 29.25 (-0.15) g/L, respectively. From statistical analysis, the concentration of succinic acid 19.059 g/L was obtained. This was a 17.85% improvement over that attained with the one-factor-at-a-time method. The present study suggested that the renewable sorghum straw could be utilized as an alternative carbon source for succinic acid production.

Keywords *Actinobacillus succinogenes,* Hydrolysate, Sorghum straw, Succinic acid, Plackett-Burman Design (PBD)**,** Central Composite Design (CCD) and Response Surface Methodology (RSM)

4.2 Introduction

Succinic acid, a dicarboxylic acid with the molecular formula of $C_4H_6O_4$, was regarded as one of the most important platform chemicals. It can be used as a precursor for many chemicals of industrial importance, including adipic acid, 1, 4-butanediol, tetrahydrofuran, N-methyl pyrrolidinone, 2-pyrrolidinone, succinate salts, and gamma-butyrolactone (McKinlay et al., 2007; Song and Lee, 2006). In addition its application in agricultural, food, and pharmaceutical industries, succinic acid could also be used in the synthesis of biodegradable polymers such as polybutyrate succinate (PBS), polyamides, and various green solvents (Rudner et al., 2005). Presently, succinic acid is produced commercially by catalytic hydrogenation of petrochemical derived maleic acid or maleic anhydride. Because of rising global requirements for oil and occurrence of the environmental impact of fossil fuel use excessive, the production of sulfuric acid fermentation from renewable biomass by anaerobic bacteria has become increasingly attractive economically. Using renewable resources carbon and other greenhouse gases as substrates for bio-based succinic acid has outstanding environmental benefits. (Bechthold et al., 2008; McKinlay et al., 2007) Some bacteria are likely candidates for use in the production of bio-based succinic acids include *Anaerobiospirillum succiniciproducens*, *Actinobacillus succinogenes* (Guettler et al., 1999; Liu et al., 2008), *Mannheimia succiniciproducens Prevotella ruminicola* (Howlett et al., 1976), *Escherichia coli* AFP111 (Stols and Donnelly, 1997) and *Corynebacterium glutamicum* (Okino, Inui and Yukawa, 2005). However, the cost of a bio-based succinic acid fermentation is key aspect for competitive with petroleum-based succinic acid. Therefore, the utilization of cheap carbon sources instead of glucose is important for the cost-efficient production of succinic acid. The renewable biomass for producing industrial chemicals are oil plants (oil, fat, glycerol, celluloses), starch plants (starch, inulin, carbohydrates, celluloses), sugar beets and sugar cane (sucrose), wood (lignocellulose, cellulose), and waste residues from agriculture and industry (biomass, fats, oils, whey, glycerol) (Willke and Vorlop, 2004). Some economical biomass feedstocks such as whey, wood hydrolysate and cane molasses have been reported for the production of bio-succinic acid.

Agricultural straw, one of the most abundant and renewable lignocellulose biomass in the world, is composed of 35–45% cellulose, 20–30% hemicellulose, and 8–15% lignin. Agricultural straw is a good source for fermentable sugars despite of its low digestibility. After pretreatment with dilute acid, alkali or steam explosion, it can be enzymatically saccharified to fermentable sugars that are mainly a mixture of glucose and xylose. Therefore straw hydrolysate can also serve as an attractive low-cost feedstock for producing bio-based chemicals such as ethanol, hydrogen, or other higher value products (Hawkes, Dinsdale, Hawkes et al., 2002; Saha et al., 1998). It has been demonstrated that *A. succinogenes* could utilize various carbon sources including xylose with yeast extract as complex nitrogen source to produce succinic acid (Liu et al., 2008). Therefore, the utilization of cheap carbon sources instead of glucose was important for the cost-efficient production of succinic acid. In Thailand, sorghum straw (*Sorghum bicolor* (L.) Moench) is renewable lignocellulose biomass. Generally, it was used as animal feed only. It consist of soluble (glucose and sucrose) and insoluble carbohydrates (cellulose and hemicellulose) (Li and Chan-Halbrendt, 2009).

However, the use of sorghum straw as carbon source for the fermentative production of succinic acid by *A. succinogenes* has not been reported yet. Also present study, for the first time, the economical production of succinic by *A. succinogenes* from pretreated sorghum straw hydrolysate (SSH). The effects of initial sugar concentration and complex nitrogen sources on cell growth and succinic acid production from sorghum straw hydrolysate was investigated.

Consideration the components in the fermentation medium, a great number of experiments should be concurrently run and the interactions between these components were necessary to be investigated. Statistical approach to applied, Plackett-Burman design (PBD) was a good choice in rapid screening many factors to identify the most significant independent factors (Liu and Tang, 2010). Compared with the one-factor-at-a-time method, statistical experimental design has the advantages of reducing experiment numbers and improving statistical interaction analysis (Ren et al., 2008). Central Composite Design (CCD) was used to develop mathematical models to estimate of the relationships between key variables and the

response. Response Surface Methodology (RSM) was following studied to optimize the concentration of the key variables , statistical methods have been done by using, in which several factors were concurrently identified using fewer experimental (Myers, 1999). Furthermore, there are no reports on statistical optimization of succinic acid production using sorghum straw hydrolysate (SSH) by *A. succinogenes*. Also the present study, a central composite design (CCD) was employed for optimization succinic acid production by *A. succinogenes* DSMZ 22257 using SSH as a low cost carbon source was developed.

This present study, we developed fermentation medium using SSH as a carbon source for succinic acid production.

4.3 Material and methods

4.3.1 Sources and Microorganisms

4.3.1.1 Lignocellulosic material

Sorghum straw used in this experiment was obtained from the Suphanburi Field Crop Research Center, Suphanburi province, Thailand. Sorghum straw consisted of 44.51% cellulose, 38.62% hemicellulose, 6.18% lignin and 10.69% ash. The chopped sorghum straw was dried in oven at 70 $^{\circ}$C to a constant weight. Thirty grams of chopped sorghum straw were suspended in 300 mL of 3% aqueous solution of H_2SO_4 at 120 $^{\circ}$C for 10 minutes and 170℃ with for 10 min (Poonsrisawat et al., 2013). After pretreatment step, the hydrolyzates were neutralized with 40% NaOH, centrifuged and filtered through 0.45 μm filter papers before analysis of total reducing sugars by DNS method and monomeric sugars (glucose, xylose, galactose, arabinose and mannose) by HPLC.

4.3.1.2 Microorganisms

A. succinogenes DSMZ 22257 was used for succinic acid fermentations. It was obtained from Leibniz Institute DSMZ-German Collection of Microorganisms and Cultures was used as a representative for investigating the succinic acid production from agricultural material. Bacteria strain was maintained on TSA (Tryptice Soya Agar) agar slants containing: pancreatic digest of casein 17 g/L, soy peptone 3 g/L, glucose 2 g/L, NaCl 5 g/L, KH_2PO_4 2.5 g/L and agar 15 g/L.

4.3.2 Medium and succinic acid fermentation

Succinic acid production was investigated by anaerobic fermentation in the medium consisted of yeast extract 30.0 g/L, urea 2.0 g/L, $MgCl_2.6H_2O$ 2 g/L, $CaCl_2$ 1.5 g/L, $MnCl_2$ 0.07 g/L, Na_2HPO_4 4.4 g/L, NaH_2PO_4 3.3 g/L, $MgCO_3$ 30 g/L and adjust pH to 7 (Li et al., 2011). Glucose was separately sterilized at 115 ℃ for 20 min and added to the medium to maintain the initial concentration of 60.0 g/L. Biotin 0.3 µg/L and thiamin 0.2 µg/L are prepared by sterile membrane filtration (0.22 µm nylon, Millipore Express, Ireland) and added. It was incubated at 37 $^{\circ}$C with 200 rpm for 48 with 10% seed inoculum (TSB medium). The medium for seed culture contained pancreatic digest of casein 17 g/L, soy peptone 3 g/L, glucose 2 g/L, NaCl 5 g/L, KH_2PO_4 2.5 g/L. and incubated at 37 $^{\circ}$C with 200 rpm for 24 h.

4.3.3 Effect of the sugar concentration on succinic acid production by *A. succinogenes*

The fermentative process was done using different carbon sources (20-60 g/L of glucose and SSH) under anaerobic conditions and incubated at 37 $^{\circ}$C with 200 rpm for 24 h.

4.3.4 Optimization of medium composition for succinic acid production using Plackett-Burman Design (PBD)

The first optimization approach using Plackett and Burman Design (PBD) with 12 experiments and 6 variables (yeast extract, urea, $CaCl_2$, $MnCl_2$, $NaCO_3$ and $MgCO_3$) was carried out. The experimental was designed with two levels, namely, minimum and maximum, coded as “-1” and “+1,” respectively, as shown in Table 1. The statistical software package “Minitab 17” was used for analysis of the experimental data.

Table 4.1 Experimental design using Plackett–Burman methodology for succinic acid production by *A. succinogenes* DSMZ 22257.

Factor	A	B	C	D	E	F	G[a]	H[a]
	Yeast extract	Urea	$CaCl_2$	$MnCl_2$	$NaCO_3$	$MgCO_3$	D1	D2
Low(-1)	5	0.5	0.5	0.25	0.25	5	-1	-1
High(+1)	50	5	5	2.5	2.5	50	+1	+1

[a] Dummy variable

* Cultivation condition; anaerobe at 37°C, 200 rpm for 24 h

4.3.5 Optimization fermentative medium for succinic acid production using Central Composite Design (CCD) and Response Surface Methodology (RSM)

In order to evaluate the effect of variables on the response surface in the region of investigation, a three-variables-five-level CCD was performed. The previous report of the one-factor-at-a-time method the concentration of three variables; SSH (A), yeast extract (B), and $MgCO_3$ (C) were 40, 30 and 30 g/L, respectively as a center point (Phuengjayaem and Teeradakorn, 2016) showed in Table 4.2.

Table 4.2 The code level and concentration of the key variables used in CCD.

	A	B	C
	SSH	Yeast extract	$MgCO_3$
+1.68	48.4	38.4	38.4
+1	45	35	35
0	40	30	30
-1	35	25	25
-1.68	31.6	21.6	21.6

The test variables were coded according to the following equation (Kilic et al., 2002):

$$x_i = (X_i - X_i^*)/\Delta Xi \qquad \text{(Eq. 4.1)}$$

where x_i is the coded value of the i^{th} independent variable, X_i is the uncoded value of the i^{th} independent variable, X_i^* is the uncoded i^{th} independent variable at the center point, and ΔX_i is the value of the variable has changed. The statistical

software package 'Design-Expert 6.0 (trial version)' was used to identify the experimental design. The total number of experiments with three variables were 20 including $2k + 2^k + 6$, when k=3, where k is the number of variables.

The CCD design with three variables (SSH, yeast extract, and $MgCO_3$) at five levels (−1.68, −1, 0, +1, +1.68) was presented in Table 4.2. All the variables were taken to the coded values. In order to control the error bar, 20 runs were showed in a random order in which there were six replications at the center points to estimate the pure error (Zhang et al., 2012). Concentration of succinic acid was analyzed after 24 h of cultivation time. The optimization of the response could be associated with variables chosen by linear or quadratic models. A quadratic model, which also includes a linear model, was given as following (Zhang et al., 2012):

$$Y=\beta_0+\beta_1A+\beta_2B+\beta_3C+\beta_{12}AB+\beta_{13}AC+\beta_{23}BC+\beta_{11}A^2+\beta_{22}B^2+\beta_{33}C^2 \qquad \text{(Eq. 4.2)}$$

where Y was the predicted response; β_0, intercept; β_1, β_2, β_3, linear coefficients; β_{12}, β_{13}, β_{23}, interaction coefficients; β_{11}, β_{22}, β_{33}, squared coefficients. Data were processed for Eq. 4.2 using the Design-Expert 6.0 program including analysis of variance (ANOVA) to obtain the interactive effects between the process variables and the response. The quality of fit of the quadratic model was investigated by the coefficient of determination R^2, and its statistical significance was verified by the F-test in the same program.

4.4 Analytical methods

4.4.1 Cell concentration

The insoluble $MgCO_3$ in the sample was removed by adding 0.2M of HCl (Zheng et al., 2009). Then the cell concentration was measured as the absorbance at wavelength 660nm using a spectrophotometer (UV160, Shimadzu Corporation, Japan).

4.4.2 Reducing sugars

The reducing sugars in the sorghum straw hydrolysate (SSH) were measured by DNS (3, 5-dinitrosalicylic acid colorimetric) method applied from Miller (1959) with D-glucose as the standard. The mixtures containing 50 µl of sample and 150 µl of DNS reagent were heated in a boiling water bath for 10 minutes and were cooled

immediately in an ice bath. Afterwards, one milliliter of distilled water was added and the absorbance at wavelength 540 nm was measured using spectrophotometer.

4.4.3 Fermentation products

Fermentation products (succinic, acetic and formic acid) were analyzed using high performance liquid chromatography (HPLC). The supernatants were filtered through a cellulose acetate membrane filters pore sized 0.45 μm. The inject volume was 20 μl. The HPLC system was equipped with a cation-exclusion column (Aminex HPX-87H; 300 mm 7.8 mm; Bio-Rad Chemical) and a refractive index detector (Shimadzu Model RID-6A). The mobile phase consisted of a 5 mM H_2SO_4 solution at a flow rate of 0.6 mL/min and the column was operated at 55 $^{\circ}$C.

4.5 Results and discussion

4.5.1 Effect of the glucose concentration on succinic acid production by *A. succinogenes* DSMZ 22257

The results showed fermentation cultivation using glucose as a carbon source with various concentrations in the range 0-60 g/L. The time course of cell growth and residual reducing sugars were shown in Figure 4.1A and Figure 4.1B, respectively.

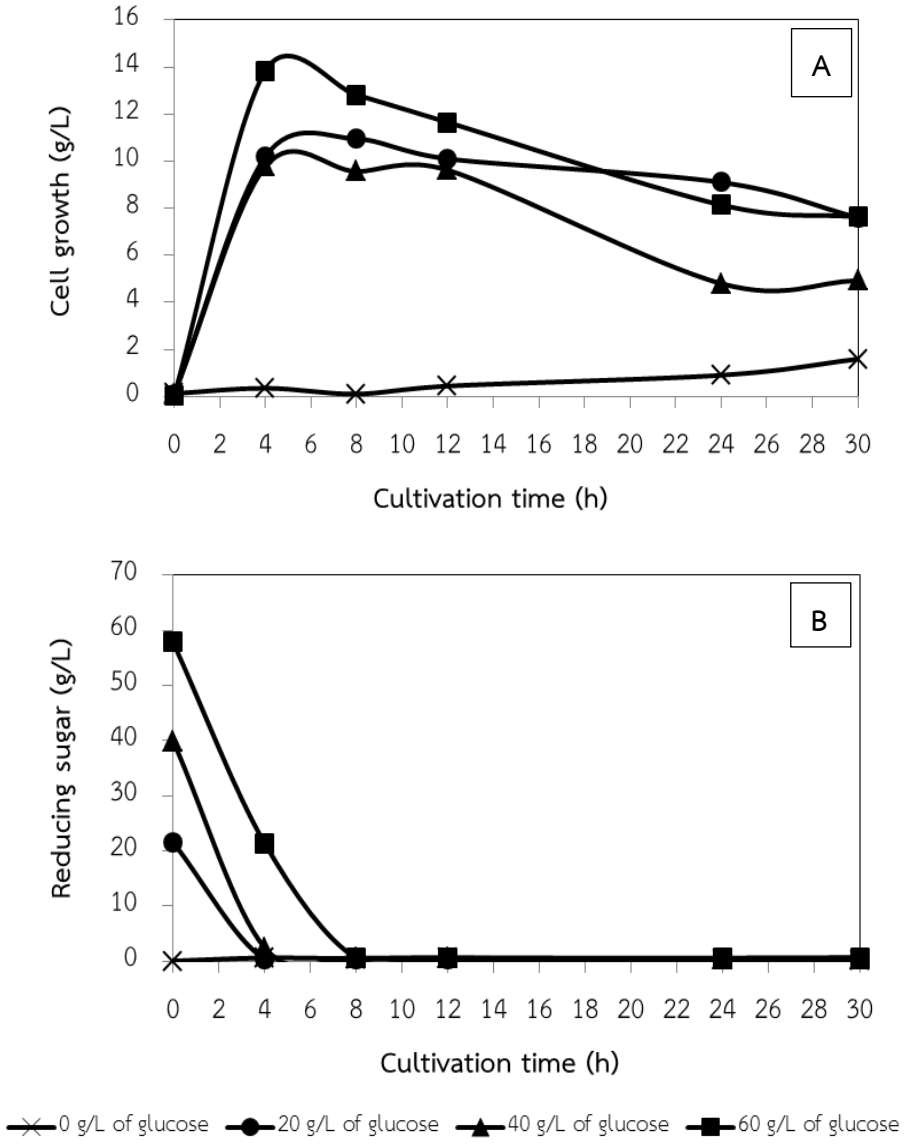

Figure 4.1 Time course of cell growth (A) and residual reducing sugars (B) in the succinic acid fermentation by *A. succinogenes* DSMZ 22257 using glucose as a carbon source.

The cell growth of *A. succinogenes* was increased rapidly and reached a maximum cell growth of 12.81 g/L after 4 h of cultivation (60 g/L of glucose as a carbon source). The lag phase of cell growth was not observed in this case. Figure 4.2 showed that increasing the initial glucose concentration from 0 to 60 g/L gave the maximum succinic acid concentration (52.180 g/L), corresponding to a yield of 0.870

g/g glucose (60 g/L of glucose). The residual reducing sugars were diminished after 8 h of cultivation time.

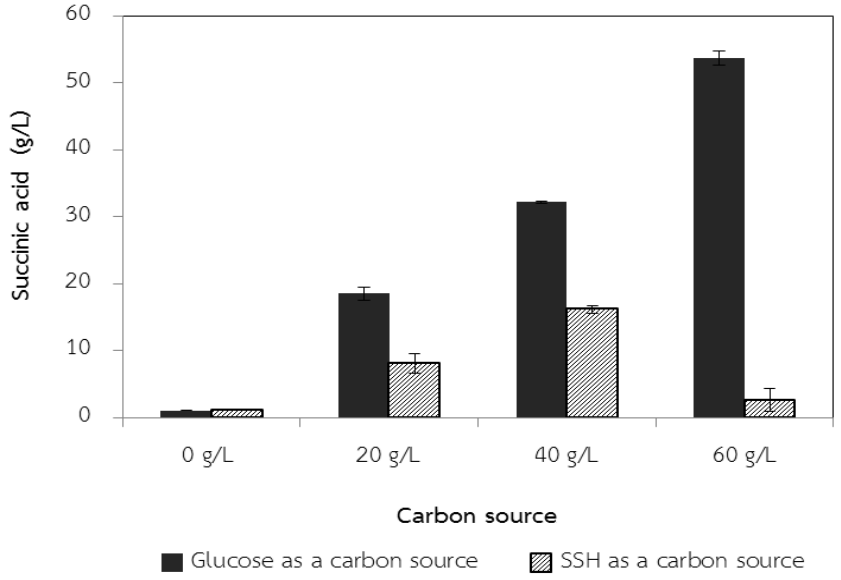

Figure 4.2 Succinic acid produced by *A. succinogenes* DSMZ 22257 using glucose or SSH as a carbon source.

4.5.2 Effect of the SSH concentration on succinic acid production by *A. succinogenes*

The fermentation cultivation used SSH as a carbon source with various concentrations in the range 0-60 g/L (equivalent to glucose concentration). The maximum succinic acid concentration of 16.671 g/L from 40 g/L of SSH was shown in Figure 4.2, corresponding to a yield of 0.777 g/g substrate at 24 h of cultivation times.

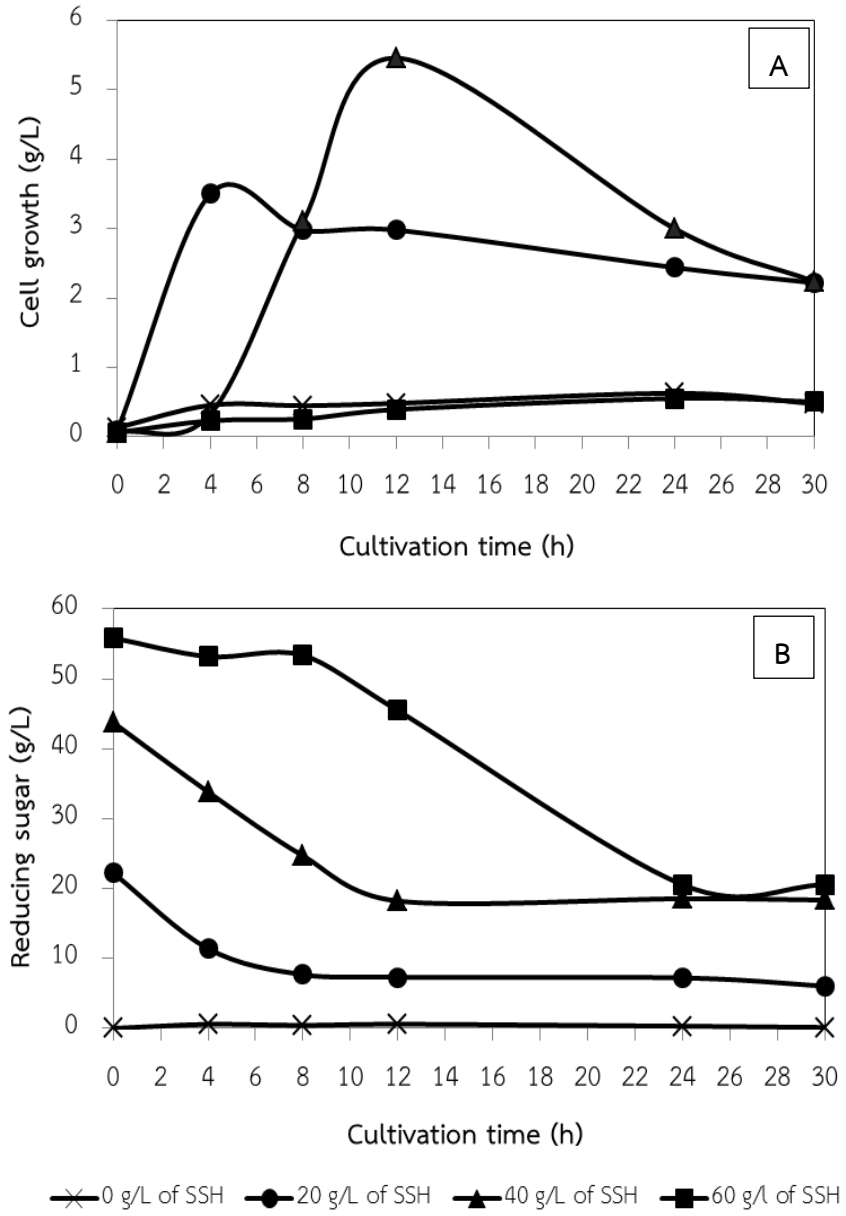

Figure 4.3 Time course of cell growth (A) and residual sugars (B) in succinic acid fermentation by *A. succinogenes* DSMZ 22257 from SSH as a carbon source.

The time course of cell growth and residual reducing sugars was shown in Figure 4.3A and 4.3B, respectively. The cell growth of *A. succinogenes* increased with the increasing of the initial SSH concentration from 0 to 40 g/L. The lag of cell growth was observed for 4 h. Noticeably, at the initial SSH concentration 60 g/L, the residual sugars were slowly consumed, resulting in a small amount of cell growth and succinic acid concentration.

The fermentation result using glucose as a carbon source indicated that cell growth of *A. succinogenes* and succinic acid concentration increased with increasing glucose concentration. Lee et al. (1999a) reported that cell growth and metabolites production from *A. succiniciproducens* were affected by the initial glucose concentration. A longer lag phase was accompanied by lower biomass and succinic acid concentration (at a high initial glucose concentration of 80 g/L). Liu et al. (2008) also reported that succinic acid production by *A. succinogenes* CGMCC1593 was inhibited by high initial glucose concentration. When the initial glucose concentration increased from 50 g/L to 75 g/L, the maximum specific growth rate dropped from 0.77 h^{-1} to 0.58 h^{-1}, which corresponded to a decline in the rate of succinic acid production from 1.3 g/L•h to 1.0 g/L•h. However, Lin, Du, Koutinas et al. (2008) reported that *A. succinogenes* tolerated up to 143 g/L of glucose with the maximum specific growth rate of 0.50 h^{-1} and cell growth was completely inhibited with glucose concentration over 158 g/L. A significant decrease in succinic acid yield and a prolonged lag phase were observed with glucose concentration above 100 g/L. Among the end-products investigated, formate was found to have the greatest inhibitory effect on succinic acid fermentation (Lin et al., 2008). Moreover, Guettler and coworker (1996) reported that *A. succinogenes* could grow in aqueous media containing over 150 g/L of glucose. Indeed, Liu et al. (2008) also reported that *A. succinogenes* could tolerate up to 160 g/L initial glucose concentration but with a reduction of yield and productivity of succinic acid. The tolerance of microorganism to glucose concentration might be due to the differences in growth media, such as the presence of a nitrogen source, $MgCO_3$ and a metal solution. These factors will be investigated in the future.

When using sorghum straw hydrolysate as a carbon source, a lag phase of 4 h was observed, while no distinct lag phase was seen with glucose as a carbon source. These results indicated that the inhibitory effect of higher concentration of sugars from SSH on *A. succinogenes* was also observed in this study. It might be explained that there were inhibitors such as 5-hydroxy methyl fufural and fufural which may have effect on cell growth and subsequently succinic acid production.

From this research, the yield of succinic acid concentration from SSH was 0.777 g/g substrate, close to the 0.870 g/g substrate from 60 g/L glucose. It indicated that the sugars liberated from sorghum straw could be used as an alternative carbon source. Moreover, sorghum straw was cheap, abundant and renewable. Therefore using agriculture biomass is a more cost efficient process and increasing the value to the agriculture waste.

4.5.3 Optimization of medium composition for succinic acid production using Plackett-Burman Design (PBD)

The methodology of Plackett-Burman Design (PBD) was a powerful and useful tool in rapidly searching key factors from a multivariable system. PBD did not determine the exact quantity, but it could provide some important information about each factor with relatively few experiments (Plackett, 1946). As shown in Table 4.3, PBD for 12 trials with two levels of concentrations was undertaken to evaluate the significance of six medium components. In order to determine the influence of the most important variables, a standardized Pareto chart (Figure 4.4) was employed. Analysis of the measured response variables enabled one to obtain standardized Pareto charts and predict versus an actual plot (Figure 4.5). A standardized Pareto chart consisted of bars with a length proportional to the absolute value of the estimated effects, divided by the standard error. The bar was displayed in order of the size of the effects, with the largest effects on top. The chart included a vertical line at the critical *t*-value for an alpha. The result showed that the confidence levels greater than 95% ($p < 0.05$) were acceptable.

Table 4.3 The experiment design using PBD and the result for the optimization of succinic acid production by *A. succinogenes* DSMZ 22257

Run Order	A	B	C	D	E	F	G[a]	H[a]	CDW[b] (g/L)	Succinic acid (g/L)	
										Actual	Predicted
1	1	1	1	-1	1	1	-1	1	0.8574	15.7461	15.7579
2	1	-1	1	-1	-1	-1	1	1	1.4615	11.2597	11.6458
3	-1	1	-1	-1	-1	1	1	1	0.4024	13.5968	12.9540
4	1	1	-1	1	-1	-1	-1	1	0.1126	12.1710	12.4159
5	1	-1	-1	-1	1	1	1	-1	0.1399	13.8538	14.0987
6	-1	1	1	1	-1	1	1	-1	1.6761	10.7548	11.3976
7	-1	-1	1	1	1	-1	1	1	1.3250	7.6586	7.2725
8	-1	-1	-1	1	1	1	-1	1	0.9068	10.2540	10.6401
9	-1	-1	-1	-1	-1	-1	-1	-1	1.2910	7.3643	7.3761
10	1	1	-1	1	1	-1	1	-1	1.2943	11.7459	11.5010
11	1	-1	1	1	-1	1	-1	-1	1.2057	14.0999	13.4571
12	-1	1	1	-1	1	-1	-1	-1	0.9226	8.1323	8.1205

[a] Dummy variable

[b] CDW means cell dry weight

From the present study, the chart included a vertical line at the critical *t*-value for α of 0.05. Effects for which the bars were smaller than the critical *t*-value were considered not significant and did not affect the response variables. The effect may be positive or negative. From Figure 4.4, yeast extract and $MgCO_3$ had a confidence level above 95%. Hence, these were considered to be significant for the succinic acid biosynthesis. From the PBD analysis using Minitab Program (version 17), a first-order regression equation was shown in Equation 4.3:

$$Y = 11.386 + 1.760A + 0.638B - 0.111C - 0.272D - 0.155E + 1.664F + 0.092G + 0.395H \quad \text{(Eq.4.3)}$$

In this equation, *Y* was the succinic acid production. A, B, C, D, E and F were the values of yeast extract, urea, $CaCl_2$, $MnCl_2$, Na_2CO_3 and $MgCO_3$, respectively, while G and H were dummy variables.

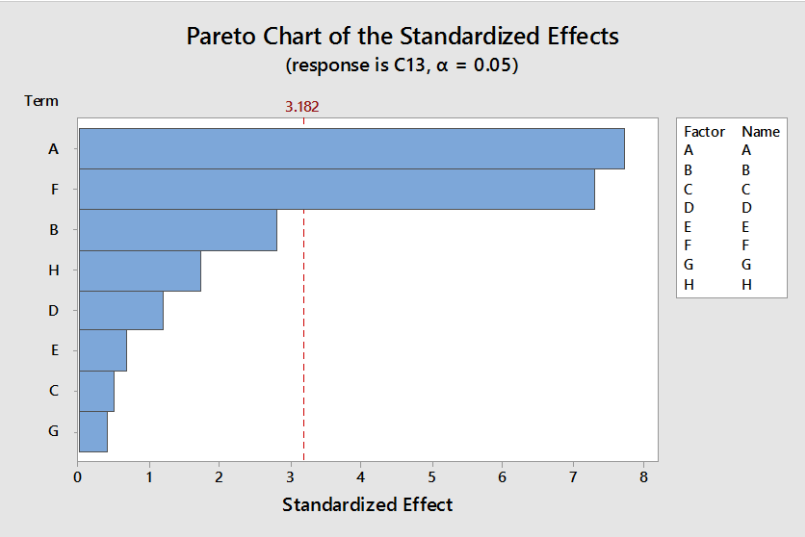

Figure 4.4 Pareto chart of standardized effects on the succinic acid production.

The chart included a vertical line (i.e., standardized effect = 3.182) at the critical t-value for **α** of 0.05. The bars were displayed in order of the size of the effects and the standardized effect of each term was shown on the top of its corresponding bar.

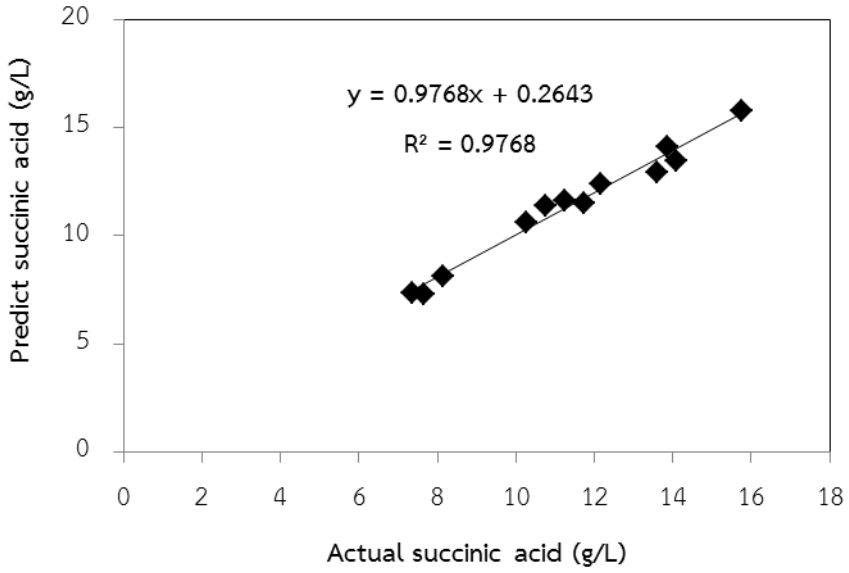

Figure 4.5 Parity plot between the actual and predicted values of succinic acid production by *A. succinogenes* DSMZ 22257.

The goodness of the regression was checked by the coefficient of determination R^2, whose value (R^2 = 97.68%) indicated that only 2.32% of the total variation could not be explained by the model. It was reasonable to use the regression model to analyze the trend in this response. The succinic acid production

was affected by the coefficients of yeast extract (1.760) and $MgCO_3$ (1.664). The positive coefficient indicated that the high level of yeast extract concentration was helpful for the succinic acid product.

Similarly, the positive result of $MgCO_3$ indicated that the high level of $MgCO_3$ concentration was helpful for the succinic acid production. The optimal medium composition was 50.0 g/L of yeast extract, 5.0 g/L of urea, 5.0 g/L of $CaCl_2$, 0.25 g/L of $MnCl_2$, 2.50 g/L of Na_2CO_3 and 50 g/L of $MgCO_3$. The maximum succinic acid concentration of 15.746 g/L was achieved. The productivity, specific productivity and CDW were 0.656 g/L•h, 15.746 g/L and 0.8574 g/L, respectively.

Statistical method: Plackett-Burman Design (PBD) was applied for a preliminary optimization of succinic acid fermentation medium by *A. succinogenes* DSMZ 22257. Yeast extract and $MgCO_3$ were screened to be the key factors for the succinic acid production. Yeast extract effected cell growth directly as a nutrient. It contained many trace substances such as folic acid, pantothenic acid, biotin, vitamin B1, B2, B6 and B12. This may be the reason why many kinds of vitamins could be omitted while the succinic acid could efficiently be produced.

However, the culture pH value was one of the key factors in the succinic acid production. $MgCO_3$ was used as a neutralizing agent and was added to the fermentation medium to adjust pH of the culture medium. Wang, Zhu, Li et al. (2012) reported the pattern of succinic acid production at a culture pH of 7.0 was similar to that obtained at 7.5. When the initial culture pH was adjusted to 6.5 or 8.0, a significant decrease of succinic acid production was observed. The highest succinic acid production of 48.2 g/L was obtained at a culture pH of 7.5. Consequently, the effect of medium optimization $MgCO_3$ may be partly from the influence of the pH. On the other hand, sufficient CO_2 supplement in the fermentation broth could strongly influence the metabolic flux of carbon and the activities of phosphoenolpyruvate (PEP) carboxykinase, which were the important committed steps for the biosynthesis of succinic acid (McKinlay and Vieille, 2008). As an important CO_2 donor in the *A. succinogenes* fermentation, $MgCO_3$ could react with organic acids in fermentation broth and caused an increase in the dissolved concentrations of HCO_3^-, CO_3^{2-} and CO_2.

Zhu, Wang, Liu et al. (2012) reported that a higher amount of $MgCO_3$ was more effective on promoting the succinic acid production by *A. succinogenes*. Moreover, the maximum succinic acid production of 61.92 g/L was obtained at 159.22 mM dissolved CO_2 concentration, which was supplied by 40 g/L $MgCO_3$ with 100% CO_2 gas. This explained that the dissolved CO_2 concentration was another factor affecting succinic acid synthesis. Indeed, during the fermentation process, insoluble $MgCO_3$ caused turbid broth, which made the cells spread uniformly in the broth. This effectively avoids cell flocculation. These entire properties make $MgCO_3$ to be one of the key factors significantly improved for succinic acid production.

4.5.4 Optimization fermentative medium for succinic acid production using CCD and RSM

A three-variable-five-level matrix of CCD was employed to identify the optimized conditions and the interactive effects. SSH, yeast extract, and $MgCO_3$ were selected as the variables for CCD. The concentrations of succinic acid for each individual run along with the predicted responses were summarized in Table 4.4.

Table 4.4 Design matrix of centered central composite design (CCD) for succinic acid production

Run No.	SSH	YE	$MgCO_3$	CDW	Succinic acid (g/L)	
	A	B	C	(g/L)	Actual	Predicted
1	-1	1	1	3.6501	15.664	15.198
2	0	0	0	4.1818	15.540	15.739
3	0	0	1.68	2.5723	12.963	13.716
4	1	-1	1	8.9385	15.280	15.239
5	-1	1	-1	3.5926	10.665	9.758
6	-1.68	0	0	3.0034	9.873	10.709
7	0	0	0	4.6130	15.834	15.739
8	1	1	1	3.8369	17.547	16.709
9	0	0	0	3.5783	15.281	15.739
10	0	1.68	0	4.6848	15.446	16.460
11	-1	-1	1	3.3915	12.758	12.070
12	0	0	0	3.7076	15.576	15.739
13	1	1	-1	4.4980	18.112	17.852
14	0	0	-1.68	4.2393	7.435	8.024
15	1.68	0	0	3.7651	19.663	20.170
16	0	-1.68	0	4.6561	10.187	10.516
17	0	0	0	3.8657	16.714	15.739
18	0	0	0	3.8226	15.726	15.739
19	-1	-1	-1	3.3483	4.261	4.151
20	1	-1	-1	6.8835	14.385	13.904

SSH, sorghum straw hydrolysated; YE, yeast extract; CDW, cell dry weight

The highest succinic acid concentration of 19.6631 g/L and CDW 3.765 g/L were achieved at 24 h when the concentrations of SSH, yeast extract, and $MgCO_3$ were 48.40, 30.00, and 30.00 g/L, respectively (Run 15). The lowest succinic acid concentration at 4.2606 g/L and CDW 3.3483 g/L were obtained when SSH, yeast extract, and $MgCO_3$ concentrations were 35, 25, and 25 g/L, respectively (Run 19).

Based on the response data were analyzed in the Design-Expert software, the optimized concentrations of SSH, yeast extract, and $MgCO_3$ were 45 (1), 34.55 (0.91), and 29.25 (-0.15) g/L and the predicted concentration of succinic acid was 19.059 g/L.

The succinic acid was a 17.85% improvement over that achieved with the one-factor-at-a-time method (16.172 g/L) (Phuengjayaem and Teeradakorn, 2016).

The response data were analyzed in the Design-Expert software. The application of multiple regression analysis to the experimental data, the following quadratic equation (Eq. 4.4), which using an empirical relationship between succinic acid and the test variables in coded units:

$$Y = 15.74 + 2.82A + 1.77B + 1.69C - 0.11A^2 - 0.80B^2 - 1.72C^2 - 0.41AB - 1.65AC - 0.62BC \quad \text{(Eq.4.4)}$$

Where Y was the succinic acid produced of SSH (A), yeast extract (B), and $MgCO_3$ (C). The statistical significance of the above equation was verified by the *F* test, and the ANOVA for the response surface quadratic model is shown in Table 4.5.

Table 4.5 Analysis of variance (ANOVA) for the quadratic model[a]

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	
Model	264.838	9	29.42644	43.46636	< 0.0001	significant
A	108.1898	1	108.1898	159.8093	< 0.0001	
B	42.70366	1	42.70366	63.0784	< 0.0001	
C	39.15152	1	39.15152	57.83146	< 0.0001	
A^2	0.161373	1	0.161373	0.238368	0.6359	
B^2	9.137898	1	9.137898	13.49777	0.0043	
C^2	42.74366	1	42.74366	63.13749	< 0.0001	
AB	1.374426	1	1.374426	2.030191	0.1847	
AC	21.67095	1	21.67095	32.01058	0.0002	
BC	3.073836	1	3.073836	4.540423	0.0589	
Residual	6.769934	10	0.676993			
Lack of Fit	5.54262	5	1.108524	4.516057	0.0618	not significant
Pure Error	1.227314	5	0.245463			
Total	271.6079	19				

[a] Coefficient of determination (R^2)= 0.9751; Adjusted R^2=0.9526; Coefficient of variation (CV)=5.90; Adeq precision=27.533

The model *F* value of 43.466 and values of probability (*P*) >*F* (< 0.0001) indicated that the model terms were significant. The regression equation presented that the R^2 was 0.9751 (Table 4.5), which indicated reasonable of the model. This result suggests

that approximately 97.51% of the variance in the response could be explained by this model (Figure 4.6). The adjusted R^2, which corrected the R^2 value for the sample size and for the number of terms, was 0.9526. The model was goodness and the predicted response was better as the R^2 value becomes closer to 1.0 however, values of R^2 > 0.75 indicated the reasonable of the model (Ferreira, Duarte, Ribeiro et al., 2009).

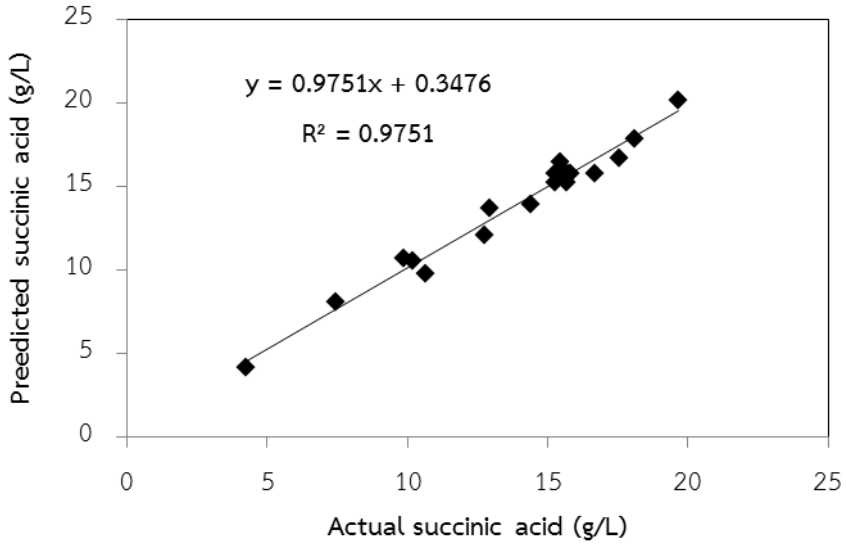

Figure 4.6 The relationship between actual and predicted value of succinic acid from the model of CCD by *A. succinogenes* DSMZ 22257.

The value of lack of fit indicates the probability of unfitness between the model predicted and actual values. According to the result, the lack of fit *F* value was 4.52 implied there was a 6.18% chance that the large of lack of fit. The *P* value of lack of fit was 0.0618, i.e., greater than 0.05, indicating that the model was appropriate. It could occur because noise, the lack of fit value was not significant in relation to the pure error. Non-significant lack of fit indicated that the model was a good fit. The adequate precision value, which measured the signal to noise ratio, was 27.533. The ratio greater than 4 was desirable (Song, Zhang, Kuang et al., 2007c). Therefore this model could be appropriate to predict succinic acid production.

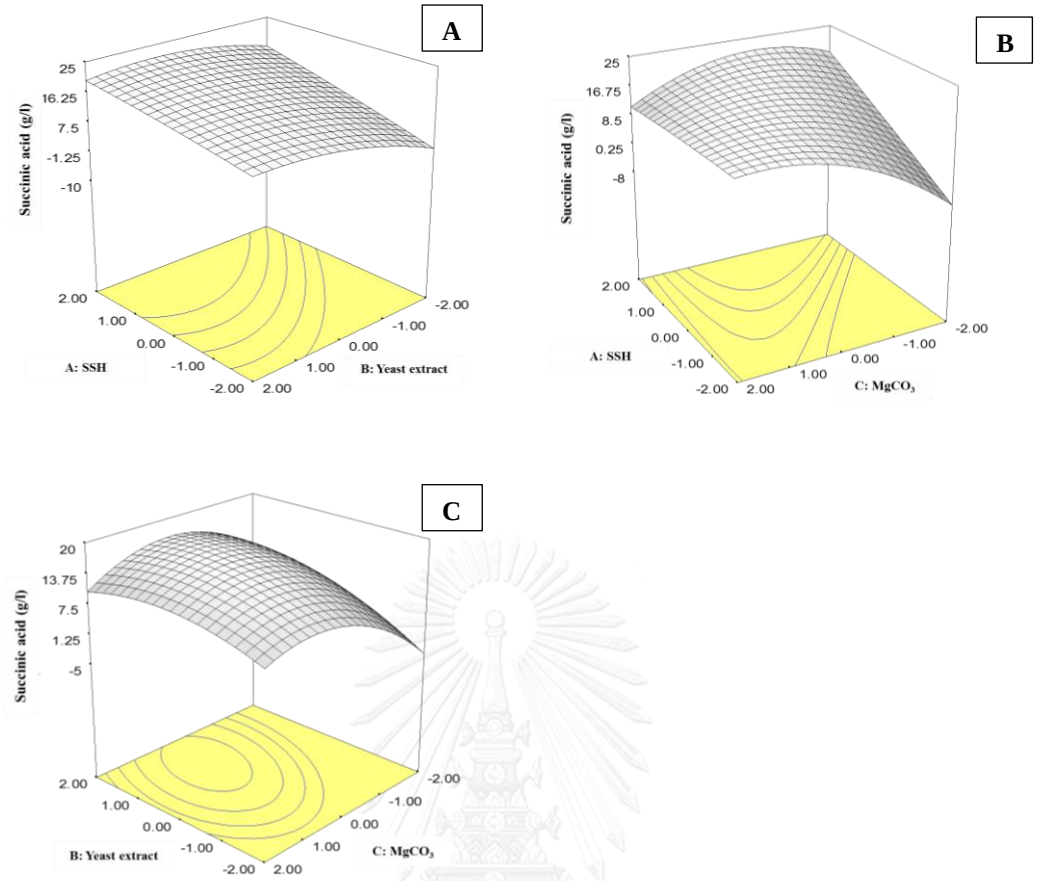

Figure 4.7 Response surface plots and contour plots

(A) Combined effects of SSH and yeast extract with constant magnesium carbonate (30 g/L);

(B) Combined effects of SSH and magnesium carbonate with constant yeast extract (30 g/L);

(C) Combined effects of yeast extract and magnesium carbonate with constant SSH (40 g/L)

The response surface curves were then plotted to study the interaction between the key variables and to determine the optimal concentration of each variable for maximized succinic acid production. As shown in Figure 4.7A, the effects of SSH and yeast extract on succinic acid production were determined when the other variable was at its center point. When yeast extract was at a low level resulting in the low yield of succinic acid. Significant improvement in succinic acid production could be obtained by increasing yeast extract concentration in the range 21.6-35.0 g/L. Figure 4.7B had shown the effects of SSH and $MgCO_3$ when yeast extract was at its center point. When the concentration of $MgCO_3$ was 30 g/L, the succinic acid concentration increased with adding the concentration of SSH in the range 21.6-38.4 g/L; while with adding $MgCO_3$ at a higher concentration (>35.00 g/L) resulting in

succinic acid was slightly decreased. Adding suitable amount of $MgCO_3$ could induce to a higher of succinic acid. From Figure 4.7C illustrated the interaction of yeast extract and $MgCO_3$ on the concentration of succinic acid production, when SSH concentration was at its center point, succinic acid production could be improved by increasing the concentration of $MgCO_3$ to 35 g/L with adding the concentration of SSH in the range 25.0-35.0 g/L while 38.4 g/L of $MgCO_3$ slightly decreased to succinic acid.

$MgCO_3$ was considered as the main factor to control the pH of the fermentation broth. It provide magnesium ions for phosphoenolpyruvate carboxykinase which was a key enzyme in succinic acid production (Lee et al., 1999a). When the SSH was at a low level, an increase in $MgCO_3$ did not improve the succinic acid production. However, when the SSH and $MgCO_3$ were at high levels, succinic acid concentration could be improved. Similar, results have been reported by Yu et al. (2010), who reported that corncob hydrolysate was used for succinic acid production by *A. succinogenes.* The medium optimization, yeast extract (11 g/L) and $MgCO_3$ (38 g/L) were selected as the nitrogen source and control pH, respectively. The maximum succinic acid of 23.64 g/L was produced with a yield of 0.58 g/g substrate (Yu et al., 2010). Shen, Wang, Qin et al. (2014) reported that cane molasses was used for succinic acid production by *A. succinogenes*, the important factors were cane molasses (85 g/L), yeast extract (8.84 g/L), and $MgCO_3$ (63.10 g/L). In batch culture gave the maximal succinic acid of 57.960 g/L with a yield of 0.679 g/g substrate were obtained. Although, the maximum succinic acid of 19.6631 g/L obtained in our research was lower the above researches however the yield of succinic acid at 0.687 g/g substrate (base on sugar utilization) was higher than these.

4.5.5 Validation of the model

To validate the fermentation variables, the results from the run of the combination levels of the 3 key variables (initial total sugars of SSH, yeast extract, and $MgCO_3$) were predicted based on the CCD quadatic model (Eq 4.3). The applicability of the model and the accuracy of the prediction were checked based on verification experiments performed in three replicate using the optimized conditions representing the maximum point of the concentration of succinic acid to verify the modelling results. The predicted concentration of succinic acid was 19.059

g/L and the concentration determined by experiment was 19.527±0.624 g/L. The result shows that there was no significant difference between actual and predicted value (2.45%). This indicated that the actual values obtained was in good agreement with the predictions of of the quadratic regression model. Thus, the quadatic model from this study was suitable for predicting succinic acid production from SSH and also optimization of the fermentative medium.

4.6 Conclusions

In this study, the optimization succinic acid production by *A. succinogenes* DSMZ 22257 using Plackett-Burman design was developed. This method was demonstrated to be effective in selecting the significant factors and enhancing succinic acid production in *A. succinogenes* DSMZ 22257 fermentation. Yeast extract, and $MgCO_3$ were screened to be key factors for the succinic acid production. Then key medium were optimized by Central Composite Design (CCD) using a Response Surface Methodology (RSM). The optimized concentrations of SSH, yeast extract and $MgCO_3$ were 45 (1), 34.55 (0.91) and 29.25 (-0.15) g/L, respectively. The predicted model of succinic acid production was developed in terms of fermentation variables by RSM and an ANOVA test was performed. From statistical analysis, the concentration of succinic acid 19.059 g/L was obtained. Succinic acid was increased by 17.85% compared with 16.172 g/L obtained from the one-factor-at-a-time method. Therefore, the RSM approach could be quite efficient and useful for the optimization of succinic acid production.

Chapter V

Development and optimization of succinic acid production by *Actinobacillus succinogenes* NP9-aA7

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Manuscript of this chapter is under preparation.

5.1 Abstract

In this research, the potential succinic acid bacterium, isolate NP9-aA7, was isolated from bovine rumen. It is a non-motile, non-spore-forming and gram-negative rod. The phenotypic characterization and phylogenetic analysis based on the 16S rRNA gene sequence indicates that the isolate NP9-aA7 is similar to *Actinobacillus succinogenes*. The statistical method combining a Plackett-Burman Design (PBD) and a Box-Behnken Design (BBD) using a Response Surface Methodology (RSM) were developed to optimize succinic acid production by *A. succinogenes* NP9-aA7. The key medium consisted 74 g/L of glucose, 30 g/L of yeast extract and 60 g/L of $MgCO_3$ which gave a maximum succinic acid production of 60.087 g/L with a yield of 0.816 g/g glucose after 36 h of cultivation times. The predicted concentration of succinic acid was 59.886 g/L. The result from the quadratic model showed the percentage errors between the actual and predicted values for succinic acid production varied from 0.335% to 4.515% and thus were not significantly different. Consequently, the RSM method was useful to optimize the concentration of medium and to provide key factors for scaled-up succinic acid fermentation with *A. succinogenes* NP9-aA7. In addition, a mixed alkaline neutralizer at 3:1 of $MgCO_3$ to $Mg(OH)_2$ ratio resulting in increasing their solubility with improved succinic acid production. In case, using 40 g/L of SSH as an alternative carbon source, maximum succinic acids of 19.139 g/L with a yield of 0.632 g/g substrate were obtained. This result suggested that SSH could be used as an alternative carbon source for succinic acid production by *A. succinogenes* NP9-aA7.

Keywords: *Actinobacillus succinogenes* NP9-aA7, Succinic acid, Plackett-Burman Design (PBD), Box Behnken-Design (BBD) and Response Surface Methodology (RSM)

5.2 Introduction

Succinic acid is an intermediate product of the tricarboxylic acid (TCA) cycle and a fermentative end-product of anaerobic metabolism. Thus, it is synthesized in almost all microbial, plant and animal cells. The organisms suitable for effective production of succinic acid can be divided into fungi and bacteria. Various microorganisms have been screened and studied to produce succinic acid using different sources of carbon. The production of succinic acid by bacteria - only Gram-positive bacteria such as *Corynebacterium glutamicum* (Inui et al., 2004) and *Enterococcus faecalis* (Wee et al., 2002) have been reported. Gram-negative bacteria have been isolated from various anaerobic environments such as domestic sludge, cattle waste, rice paddy, marine shipworm, oral cavity of dog, rumen and gastro-intestinal tract (Song and Lee, 2006). Succinic acid is generated by a number of anaerobic microorganisms including *Anaerobiospirillum succiniciproducens*, *Propionibacterium* sp., *Escherichia coli*, *Pectinatus* sp., *Bacteroides* sp., *Ruminococcus flavefaciens*, *Actinobacillus succinogenes*, *Bacteroides fragilis* (Beauprez et al., 2010). Most bacteria which are isolated from the rumen of ruminants, including *A. succinogenes, A. succiniciproducens* and *M. succiniciproducens* (Song and Lee, 2006), are the best candidates for succinic acid production as they produce succinic acid as a major fermentation product. The rumen is a unique microbial ecosystem present in many species of herbivorous mammals known as ruminants which is characterized by carbon dioxide, methane and traces of hydrogen production (Lee et al., 1999b).

There are many factors involved in the development of succinic acid production, therefore a number of experiments should be run concurrently, with the interactions between these factors investigated. For fermentation medium optimization, Plackett-Burman Design (PBD) is a good method for rapid screening of many key medium components and to identify the most significant independent factors (Liu and Tang, 2010). The concentration of the key medium is optimized by a Box-Behnken Design (BBD) using a Response Surface Methodology (RSM) for estimation of the relationships between the response and the key factors. Compared with the one-factor-at-a-time method, a statistical experimental design has the advantages of

reduction of the number of experiments and improving statistical interaction analysis (Ren et al., 2008).

The aim of this study is to screen succinic acid producing bacteria from various sources. Then the potential isolate is characterized and identified. Consequently, it will be shown that the succinic acid production process can be optimized using statistical methods.

5.3 Materials and Methods

5.3.1 Screening and characterization of isolates

5.3.1.1 Microorganism screening and isolation of succinic acid production

Bacterial strains were isolated from bovine rumen fluid from Kasetsart University Kamphaeng Saen Campus, Nakorn prathom province in Thailand. The samples were immediately enriched in 5 mL of enrichment broth consisting of 20 g/L glucose, 5 g/L polypeptone, 5 g/L yeast extract, 3 g/L K_2HPO_4, 2 g/L NaCl, 2 g/L $(NH_4)_2SO_4$, 0.2 g/L $CaCl_2{\cdot}2H_2O$, 0.4 g/L $MgCl_2{\cdot}6H_2O$, and 15 g/L $MgCO_3$. They were then incubated at 37 ºC for 48-72 h under anaerobic conditions using an anaerobic pack (MGC, Japan). Positive tubes were subcultured for the enrichment agar plate diluted to 10^{-6} in Phosphate Buffered Saline (PBS) buffer. Then 0.1 mL of the diluted cultures was spread onto enrichment agar plates and incubated under anaerobic conditions at 37 °C. After 24-48 h, visible colonies were picked and re-streaked on fresh enrichment agar plates and incubated overnight at 37 ºC for 24 h under anaerobic conditions.

A single colony extracted from the enrichment agar plates was streaked on screening agar plates consisting of 20 g/L glucose, 1 g/L NaCl, 5 g/L yeast extract, 3 g/L K_2HPO_4, 1 g/L $(NH_4)_2SO_4$, 0.2 g/L $CaCl_2.2H_2O$, 0.2 g/L $MgCl_2.6H_2O$, 15 g/L $MgCO_3$ and 15 g/L agar. The pH of the media was adjusted to 6.5 and they were incubated overnight at 37 ºC under anaerobic conditions. Acid-producing isolates exhibited a clear zone around the colonies that were selected and purified. They were maintained on a TSA agar plate or slant which consisted of 17 g/L pancreatic digest of casein, 3 g/L soy peptone, 2 g/L glucose, 5 g/L NaCl, and 2.5 g/L KH_2PO_4. Then, positive isolates were stored at -70 °C or lyophilized for further study.

5.3.1.2 Morphological characteristics

Isolates were observed including gram stain (Buck, 1982), endospore stain (Schaeffer and Fulton, 1933), cell morphology and colony appearance (color, shape, margin, optical property and elevation) after grown on GAM agar plate at 37°C under anaerobe for 3 days (Tanasupawat et al., 1998).

5.3.1.3 Phenotypic characterization

5.3.1.3 Phenotypic characterization

The physiological characteristics included different pH values (3.5-9), temperatures (20-50°C) and NaCl concentrations (6% w/v NaCl) were tested using MRS broth (MRS; de Man, Rogosa and Sharpe) (Appendix A).

The biological characteristics including, gas formation (Barrow and Feltham, 1993), catalase activity (Gagnon et al., 1959), nitrate reduction (Conn and Breed, 1919), arginine hydrolysis (Niven et al., 1942), starch hydrolysis (Iverson and Millis, 1974) Slime formation (Barrow and Feltham, 1993) and acid formation from various carbohydrates was tested as described by Tanasupawat et al. (Tanasupawat et al., 2010). Incubation condition was at 37°C for up to 5 days.

5.3.1.4 16S rRNA gene sequence and phylogenetic analysis

The 16S rRNA gene of isolates was PCR amplified using primers, 20F (5'-AGTTTGATCCTGGCTC-3') and 1530R (5'-AAGGAGGTGATCCAGCC-3'). The amplified 16S ribosomal RNA gene sequences were analyzed with Macrogen®, from Korea using primers, 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 518F (5'-CCAGCAGCCGCGGTAATACG-3'), 800R (5'TACCAGGGTATCTAATCC'3) and 1492R (5'TACGGYTACCTTGT-TACGACTT'3) for DNA sequencing. Sequence alignment was determined by the EzTaxon database (http://www.ezbiocloud.net/eztaxon). Multiple alignments of sequences were performed by the program BioEdit version 7.0.2 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). A phylogenetic tree was constructed by the neighbor-joining method using the program MEGA, version 6 (Tamura et al., 2013). A bootstrap analysis of Felsenstein (Felsenstein, 1985) was performed to determine confidence values of individual branches in the phylogenetic tree with 1000 replications.

5.3.1.5 Accession number

The sequence of the 16S rRNA gene of isolate NP9-aA7 has been deposited in the DNA Data Bank of Japan *(DDBJ)* database under the following accession number: LC192793.

5.3.2 Optimization succinic acid production

5.3.2.1 Inoculum medium

Inoculum consisted of 50 ml of 3% tryptic soy broth (TSB) medium (pancreatic digest of casein 17.00 g/L, soy peptone 3.00 g/L, glucose 3.00 g/L, NaCl 5.00 g/L, and K_2HPO_4 2.50 g/L). The inoculate cultures were grown in a rotary shaker at 37 °C and agitated at 200 rpm under anaerobic conditions using an anaerobic pack (MGC, Japan) for 24 h.

5.3.2.2 Fermentation Medium

The fermentation was conducted in a 250 ml flask with 50 ml of the medium containing: 30.0 g/L of yeast extract, 2.0 g/L urea, 2 g/L $MgCl_2.6H_2O$, 1.5 g/L $CaCl_2$, 0.07 g/L $MnCl_2$, 4.4 g/L Na_2HPO_4, 3.3 g/L NaH_2PO_4, 30 g/L $MgCO_3$ and the pH was adjusted to 7 (Liang et al., 2011b). 0.3 µg/L of biotin and 0.2 µg/L of thiamin were prepared by sterile membrane filtration (0.22 µm nylon, Millipore Express, Ireland) and added. Glucose was separately sterilized at 115 °C for 20 minutes and added to the medium to maintain the initial concentration of 60.00 g/L. It was incubated at 37 $^{\circ}C$ with agitation at 200 rpm for 48 h with 10% seed inoculum (TSB medium). All chemicals were purchased from Merck (Merck KGaA, Darmstadt, Germany) unless otherwise described.

5.3.2.3 Key medium components screened for succinic acid production using Plackett-Burman Design (PBD)

The experiment using the Plackett & Burman Design (PBD) with 12 trials and 8 variables (Glucose, Yeast extract, $CaCl_2$, $MgCl_2$, $MnCl_2$, Na_2HPO_4, NaH_2PO_4 and $MgCO_3$) was carried out on two levels namely, minimum and maximum, coded as “-1” and “+1,” respectively, with triplicates of the center point (Table 5.1). The statistical software package “Minitab 17”, was used for analyze the experimental data.

Table 5.1 Experimental designs using the Plackett–Burman Design for succinic acid production by *A. succinogenes* NP9-aA7 under anaerobic cultivation at 37°C, with agitation at 200 rpm for 36 h.

Factor	A	B	C	D	E	F	G	H
	Glucose	Yeast extract	$CaCl_2$	$MnCl_2$	$MnCl_2$	Na_2HPO_4	NaH_2PO_4	$MgCO_3$
Low(-1)	20	5	1	1	0.1	1.5	1	15
High(+1)	60	15	3	3	0.3	4.5	3	45

5.3.2.4 Effect of various concentrations of biotin on cell growth and succinic acid production by A. succinogenes NP9-aA7 using a one-factor-at-a-time method

Biotin was added to the production medium in the range of 0 to 200 µg/L. Biotin was prepared by sterile membrane filtration (0.22 µm nylon, Millipore Express, Ireland) and added.

5.3.2.5 Effects of different nitrogen sources on cell growth and succinic acid production by A. succinogenes NP9-aA7 using the one-factor-at-a-time method

To optimize the nitrogen sources, different inorganic and organic nitrogen sources (in same percent equivalent) such as, $(NH_4)_2SO_4$ (15 g/L), NH_4Cl (10.3 g/L), KNO_3 (19.2 g/L), peptone (24 g/L), urea (6.8 g/L) corn steep liquor (CSL) (4.8%, v/v) and beef extract (30 g/L) were compared with yeast extract (30 g/L) in anaerobic conditions (Liu et al., 2008).

5.3.2.6 Effects of different alkaline neutralizer on cell growth and succinic acid production by A. succinogenes NP9-aA7 using the one-factor-at-a-time method

The pH of the medium was maintained with the addition of different alkaline neutralizers such as, $CaCO_3$, $MgCO_3$, $Ca(OH)_2$, NaOH, Na_2CO_3, $NaHCO_3$ and $Mg(OH)_2$ in order to optimize succinic acid production.

5.3.2.7 Optimization medium composition for succinic acid production by A. succinogenes NP9-aA7 using Response Surface Methodology (RSM)

A Box-Behnken design (BBD) was performed in order to evaluate the effect of key medium components on the response surface in the region of examination. The design matrix was composed of three variables (glucose, yeast extract, and

magnesium carbonate) with three levels coded as 1, 0, and +1. Experiments were conducted using the conventional 'one-factor-at-a-time' method to select the suitable factors for maximum succinic acid production. The ranges and levels of three variables i.e. glucose (*A*), yeast extract (*B*), and magnesium carbonate (*C*) are listed in Table 5.2. The test variables were coded according to the following equation (Kilic et al., 2002):

$$X_i=(X_i-X_{i*})/\Delta X_i, \qquad \text{(Eq. 5.1)}$$

where X_i is the coded value of the i^{th} independent variable, X_i is the uncoded value of the i^{th} independent variable, X_{i*} is the uncoded i^{th} independent variable at the center point, and ΔXi is the step change value.

Table 5.2 Experimental ranges and levels of the three independent variables used in RSM in terms of actual and coded factors

	A	B	C
	Glucose	**Yeast extract**	**$MgCO_3$**
Low(-1)	40	10	20
Med (0)	60	20	40
High(+1)	80	30	60

The response surface analysis was based on multiple linear regressions that take into account the main, quadratic, and interaction effects in accordance with the following equation:

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_1 X_i + \sum_{i=1}^{3} \beta_2 X_i X_j + \sum_{i=1}^{3} \beta_3 X_i^2 \qquad \text{(Eq. 5.2)}$$

where Y is the predicted response, and X_1, X_2 and X_3 are the parameter values for the independent variables. The constants β_0, β_1, β_2 and β_3 are coefficient estimates for succinic acid production. The constant β_0 was the intercept term, β_1 was the liner effect, β_2 was the interaction, and β_3 was the quadratic effect. Process performance was estimated by analyzing the succinic acid concentration produced after 36 h of cultivation time. In optimization, the response can be related to chosen factors by quadratic models. The result from statistical analysis, including analysis of variance (ANOVA) to obtain the interactive effects between the process variables and the response, and the quality of fit of the polynomial model, was represented by the

coefficient of determination R^2, and the *F*-test used to check the statistical significance in the same program.

5.3.2.8 Determination of the optimum ratio of alkaline neutralizers on cell growth and succinic acid production by A. succinogenes NP9-aA7 using the one-factor-at-a-time method

The effect of the different alkaline neutralizer ratios at 3:1, 2:1, 1:1, 1:2 and 1:3 of $MgCO_3$:$Mg(OH)_2$ used to control pH were investigated. The culture conditions were the same as in the above experiments.

5.3.2.9 Effect of various concentration of SSH on cell growth and succinic acid production by A. succinogenes NP9-aA7 using one-factor-at-a-time method

The fermentation process was performed using various concentrations of SSH in the range 0-60 g/L under anaerobic conditions at 37 ^{o}C with agitation at 200 rpm for 36 h. The sorghum straw used in this study was obtained from the Suphanburi Field Crop Research Center, Suphanburi, Thailand. Pretreatment methods for the sorghum straw using 3% of H_2SO_4 at 120°C for 10 minutes were described by Poonsrisawat et al. (Poonsrisawat et al., 2013).

5.4 Analytical methods

5.4.1 Cell concentration

The insoluble $MgCO_3$ in the sample was removed by adding 0.2 M HCl. Then the cell concentration was measured as optical density at wavelength 660 nm using a spectrophotometer (UV160, Shimadzu Corporation, Japan) (Zheng et al., 2009).

5.4.2 Reducing sugars

The reducing sugars in the sorghum straw hydrolysate (SSH) was measured by DNS (3, 5-dinitrosalicylic acid colorimetric) method applied from Miller (1959), with D-glucose as the standard. The mixture contained; 50 µl of sample and 150 µl of DNS reagent were heated in a boiling water bath for 10 min. Then cooled immediately on ice bath and added 1 mL of distilled water. The absorbance was measured by spectrophotometer at 540 nm.

5.4.3 High-performance liquid chromatography (HPLC)

Fermentation products (succinic, acetic and formic acid) were analyzed with HPLC. Twenty microliters of sample were filtered (0.45 Am, 13 mm membrane disc filters) and loaded on HPLC using a system equipped with a cation-exclusion column (Aminex HPX-87H; 300 mm 7.8 mm; Bio-Rad Chemical) and a refractive index detector (Shimadzu Model RID-6A). The mobile phase was 5 mM of H_2SO_4 solution at a flow rate of 0.6 mL/min with the column operated at 55 °C.

5.4.4 Statistical analysis

All experiments were tested in triplicate and the related data were expressed as average values. The statistical software package “Minitab 17” was used to analyze the results from the PBD experimental data. Statistical Package for the Social Sciences (SPSS) for Windows program version 15 was used to analyze the data from the optimization condition for succinic acid production by *A. sucinogenes* NP9-aA7. The effect of various biotin concentrations, different nitrogen sources, different alkaline neutralizers, the ratio of alkaline neutralizer, and various SSH concentrations used as a carbon source were examined. The statistical software package ‘Design-Expert 6.0 (trial version)’ was used to analyze the results from BBD and carried out a regression analysis for the equations and for an estimation of the statistical significance of the different quadratic equation models.

5.5 Results and discussion

5.5.1 Screening and characterization of isolate NP9-aA7

The following is a summary of 49 isolates in total obtained from bovine rumen and bovine rumen fluid. Among these isolates, isolate NP9-aA7 was selected because it showed a distinctive ability to produce a relatively large amount of succinic acid. Moreover, it was able to utilize a variety of carbon sources such as, D-Amygdalin, L-Arabinose, Cellobiose, Gluconate, Glucose, Lactose, D-Mannitol, Ribose, Salicin, Sorbitol and D-Xylose under anaerobic conditions (Table 5.3). Colonies of isolate NP9-aA7 appearing on the screening agar plate after 24 h of incubation were gray, punctiform, convex, whole, translucent and 1–1.5 mm in diameter. The NP9-aA7 was a non-spore forming and Gram negative rod.

Table 5.3 Comparison of phenotypic characteristics of isolates *A. succinogenes* NP9-aA7 and type strain

Characteristic	Accession no.	
	NP9-aA7	Type strain (Guettler et al., 1999)
Arginine hydrolysis	-	-
Gas from glucose	-	-
Catalase	+	+
Nitrate reduction	+	+
Growth in 6% NaCl	+	-
Growth at pH 3.5	+	-
pH 5.0	+	6-7.4
pH 9.0	-	-
20 °C	-	-
40 °C	+	37
50 °C	-	-
Acid from:		
D-Amygdalin	+	+
L-Arabinose	+	+
Cellobiose	+	+
D-Fructose	-	+
D-Galactose	-	+
Gluconate	+	+
Glucose	+	+
Lactose	+	+
Maltose	-	+
D-Mannitol	+	+
D-Mannose	-	+
Melibiose	-	-
∞-Methyl-D-glucoside	-	-
Raffinose	-	+
Rhamnose	-	-
Ribose	+	+
Salicin	+	+
Sorbitol	+	+
Sucrose	-	+
Trehalose	-	-
D-Xylose	+	+

+, Positive reaction; -, negative reaction.

The 16S rRNA sequence (1,440 bases) was determined for Isolate NP9-aA7. Similarity analysis between the strain *Actinobacillus succinogenes* 130z^{T} and other representatives of the family Pasteurellaceae was determined to be 99.93% similar (Table 5.4). Subsequently, a phylogenetic tree was constructed as seen in Figure 5.1.

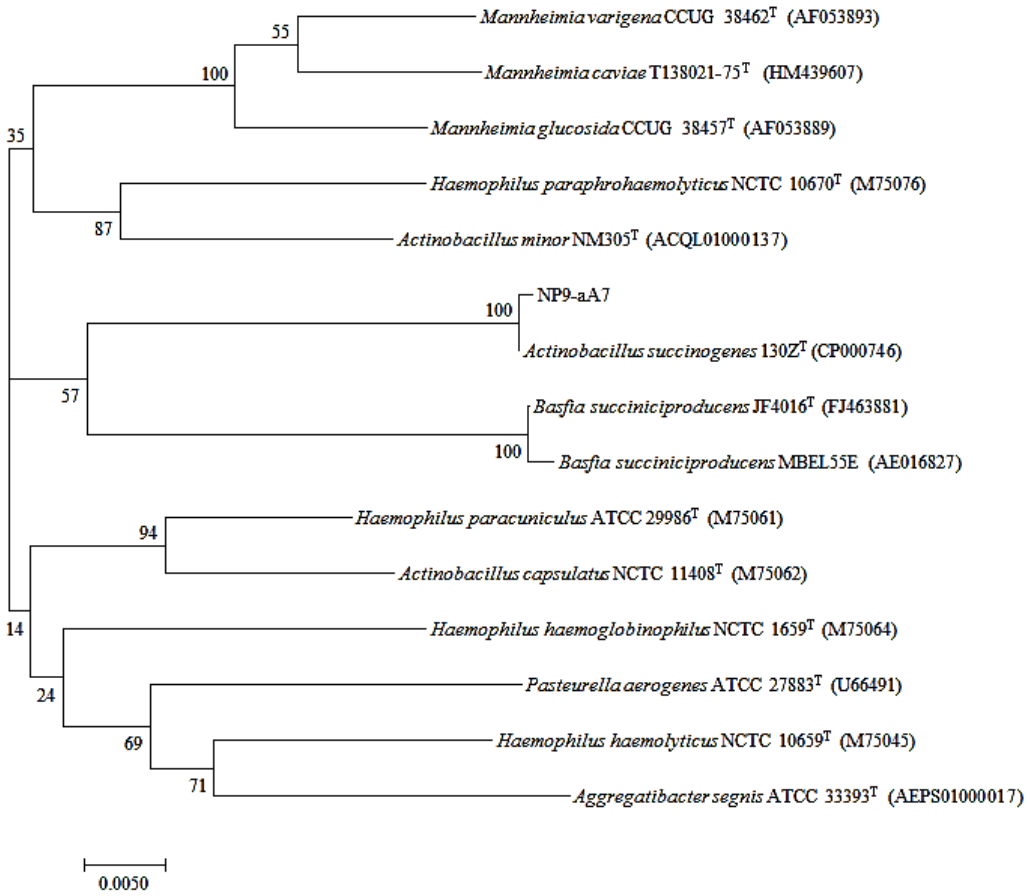

Figure 5.1 Neighbor-joining phylogenetic tree of isolates NP9-aA7 based on 16S rRNA gene sequences. Bootstrap values were expressed as percentages of 1000 replications.

Table 5.4 The level of sequence homology between NP9-aA7 and other related strains

Accession no.	Species	Similarity (%)
CP000746	*Actinobacillus succinogenes*	99.93
M75070	*Avibacterium volantium*	94.28
AE016827	*Basfia succiniciproducens*	94.26
M75059	*Avibacterium gallinarum*	94.21
M75064	*Haemophilus haemoglobinophilus*	94.21
M75076	*Haemophilus paraphrohaemolyticus*	94.11
M75045	*Haemophilus haemophilus*	94.07

5.5.2 Growth profile and succinic acid production by *A. succinogenes* NP9-aA7

The isolate NP9-aA7 was cultivated under the anaerobic condition in the incubator shaker at 37 $^{\circ}$C, 200 rpm. The sample was investigated every 3 h until 60 h. Then cell growth and succinic acid were determined. These results were presented in Table 5.5 and Figure 5.3.

Table 5.5 The result of growth profile and succinic acid production of *A. succinogenes* NP9-aA7

Cultivation Time (h)	Residual glucose (g/L)	Glucose utilization[b] (%)	OD660	Succinic (g/L)	Formic (g/L)	Acetic (g/L)	Succinic acid yield (g/g glucose)	Succinic acid productivity (g/L*h)
0	60.575±[c]4.156	4.911±6.524	0.286±0.003	0.311±0.311	1.468±1.469	0.080±0.081	0.105±0.105	0.000±0.000
3	39.079±3.253	35.486±5.371	0.427±0.056	3.069±2.370	3.238±0.412	0.228±0.012	0.144±0.111	1.023±0.790
6	34.505±0.000	43.037±0.000	2.800±0.149	2.052±0.051	6.985±0.061	1.159±0.094	0.079±0.002	0.342±0.008
9	0.273±0.095	99.549±0.156	4.550±0.226	4.094±0.110	9.704±0.318	2.104±0.079	0.068±0.002	0.455±0.012
12	0.211±0.039	99.652±0.064	4.705±0.524	4.585±0.426	11.072±0.318	2.376±0.059	0.077±0.007	0.382±0.036
15	0.129±0.052	99.786±0.086	5.225±0.795	6.343±0.993	10.536±0.416	2.876±0.261	0.106±0.017	0.423±0.066
18	0.134±0.039	99.778±0.065	5.288±0.053	6.778±0.738	11.039±0.899	3.499±0.784	0.113±0.012	0.377±0.041
21	0.092±0.031	99.848±0.050	5.545±0.498	7.245±0.783	9.117±0.818	3.214±0.269	0.121±0.013	0.345±0.037
24	0.045±0.025	99.925±0.041	6.265±0.598	10.106±0.317	7.765±0.436	4.091±0.142	0.168±0.005	0.421±0.013
30	0.063±0.071	99.897±0.117	8.045±0.425	12.808±0.159	6.305±0.475	4.973±0.107	0.213±0.003	0.427±0.005
36	0.000±0.000	100.000±0.000	7.185±0.262	16.983±1.192	4.637±0.056	6.060±0.383	0.283±0.020	0.472±0.033
42	0.000±0.000	100.000±0.000	6.525±0.819	16.888±0.420	4.631±0.448	6.272±0.228	0.281±0.007	0.402±0.010
48	0.000±0.000	100.000±0.000	6.845±0.252	15.624±1.063	3.461±0.405	5.276±0.418	0.260±0.018	0.326±0.022
60	0.000±0.000	100.000±0.000	7.440±0.286	18.352±2.126	3.617±0.074	6.054±0.520	0.306±0.035	0.306±0.035

[a] Glucose utilization was defined as the percentage of glucose concentration utilized by the bacteria in initial glucose.

[b] Succinic acid was obtained from cells grown in anaerobic conditions for 36 h.

[c] ±, Standard deviation was calculated from triplicate samples.

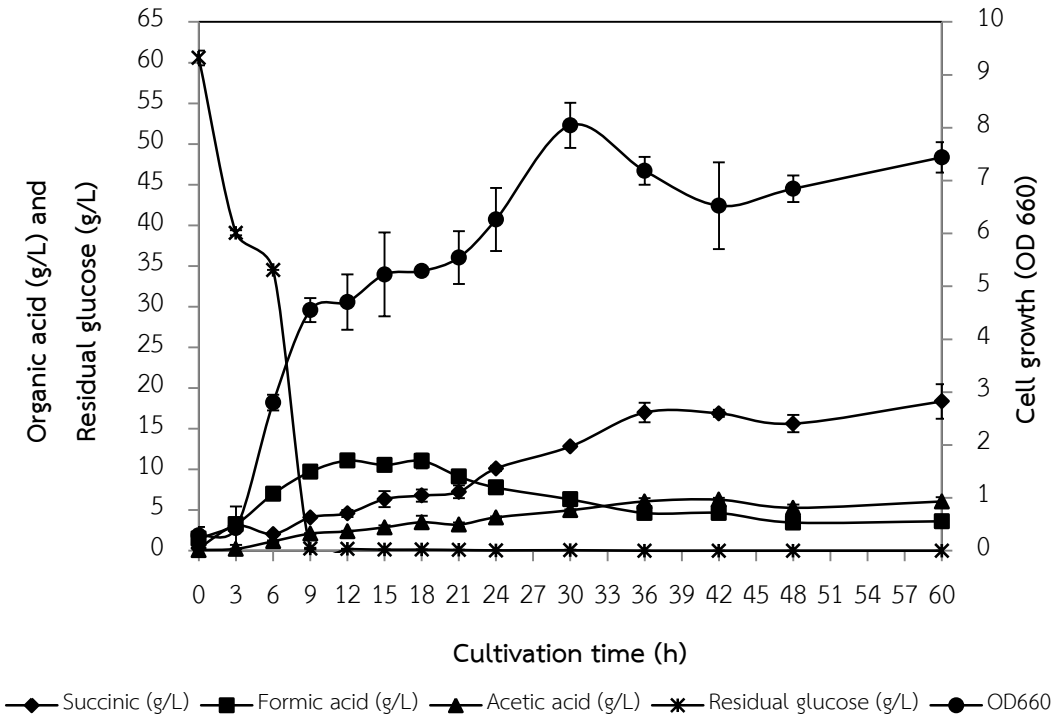

Figure 5.2 Profiles of cell growth, residual glucose and organic acid production by *A. succinogenes* NP9-aA7

From Figure 5.2, *A. succinogenes* NP9-aA7 was cultivated under anaerobic conditions in an incubator shaker at 37 °C, and agitated at 200 rpm. The sample was collected every 3 h until 60 h. Then, cell growth and succinic acid levels were determined. The lag phase was found at 3 h then fast cell growth from 3 to 30 h. After that, there was little change in growth profile while there was a slight decrease in residual glucose in the first 9 h and it was completely consumed after 9 h. In this case, the succinic acid was slightly increased to 12.808 g/L after 30 h. The highest succinic acid production of 16.983 g/L was obtained at the level of maximum cell growth (36 h). The phenomenon noted in the growth profile illustrate that succinic acid was produced with a growth associate. It might be that the succinic acid was a primary metabolite but the maximum succinic acid was produced in the stationary phase of cell growth. The optimum succinic acid production was found to be after 36 h.

This result were analyzed the significance of statistical by multiple comparison using Duncan method (Appendix D) resulting in separate the result into 8 groups, optimum cell growth at 27 h however optimum succinic acid was found to be after 36 h.

Jiang, Xu, Xi et al. (2013) reported the effect of time course of *A. succinogenes* NJ113 on cell growth, the residual sugar concentration and the organic acid production during the anaerobic batch fermentation using in a 3 L fermenter. A sugar mixture (35 g/L) for batch fermentation was composed of 18 g/L of cellobiose, 8 g/L of glucose, 5 g/L of arabinose and 4 g/L of xylose, which was consistent with the sugar ratio of sugarcane bagasse cellulose hydrolysate. The cells grew rapidly and reached a maximum concentration of 5.38 g/L at 9 h, and 31.7 g/L sugars were consumed by 33 h, with 3.3 g/L of residual cellobiose. The production of succinic acid and acetic acid continuously increased with the sugar consumption. A total of 21.7 g/L succinic acid was obtained with a productivity of 0.66 g/L•h and a yield of 68.4%. Succinic acid and acetic acid production significantly increased from 4 h to 15 h, accompanied by an increase in the ratio of succinic acid to acetic acid of 1.8 by the end of the batch fermentation.

Corresponding to Jiang et al. (2013) determined the ability of *A. succinogenes* NJ113 to use cellobiose, batch fermentation was carried out with cellobiose concentrations ranging from 30 to 70 g/L, which resulted in corresponding succinic acid concentrations increasing from 20.7 to 38.9 g/L. A final succinic acid concentration of 30.3 g/L with a yield of 67.8% was achieved from an initial cellobiose concentration of 50 g/L via batch fermentation in anaerobic bottles in anaerobic bottles for 36 h.

Barros, Freitasb, Padilhaa et al. (2013) studied the biological production of succinic acid from glycerol is an attractive process by *A. succinogenes.* The fermentative process was conducted at temperature 37°C, agitation 150 rpm in different time periods (24, 48, 72, 96 h) using free cells. The best result was observed in glycerol from biodiesel as substrate 1.62 g/L in 48 h of fermentation.

5.5.3 Key medium components screened for succinic acid production by *A. succinogenes* NP9-aA7 using Plackett-Burman Design (PBD)

The methodology of Plackett-Burman Design (PBD) was described the above section (5.3.2.3). It was used for screening the key factors from a complex medium of succinic acid production by *A. succinogenes* NP9-aA7 under anaerobic conditions at 37 ℃ for 36 h.

Table 5.6 Experimental design using PBD and the results for the optimization of succinic acid production by *A. succinogenes* NP9-aA7

Run Order	A	B	C	D	E	F	G	H[a]	Cell growth (OD660)	Succinic acid (g/L)	
										Actual	Predicted
1	1	1	1	-1	1	1	-1	1	9.660	24.4620	24.3771
2	-1	1	-1	-1	-1	1	1	1	12.980	14.4720	15.7054
3	-1	-1	-1	-1	-1	-1	-1	-1	6.555	5.5270	5.4421
4	1	-1	1	1	-1	1	-1	-1	4.880	11.2260	12.4594
5	1	1	-1	1	1	-1	1	-1	5.285	11.2080	13.3649
6	-1	-1	1	1	1	-1	1	1	6.500	8.9130	7.9046
7	-1	-1	-1	1	1	1	-1	1	7.590	12.5790	13.5874
8	1	-1	1	-1	-1	-1	1	1	3.450	13.4440	14.4524
9	-1	1	1	-1	1	-1	-1	-1	5.440	9.5992	9.6840
10	1	1	-1	1	-1	-1	-1	1	5.630	27.3800	25.2231
11	-1	1	1	1	-1	1	1	-1	4.245	9.2630	8.0296
12	1	-1	-1	-1	1	1	1	-1	3.945	10.4340	8.2771

[a], 3 Dummy variables data were not shown and [b], CDW mean cell dry weight

As shown in Table 5.7, PBD for 12 trials with two levels of concentrations was undertaken to evaluate the significance of eight medium components and 3 numbers of dummy variable data (not shown). Analysis of the measured response variables enabled to obtain standardized Pareto charts (Figure 5.3) and predicted versus actual plot (Figure 5.4).

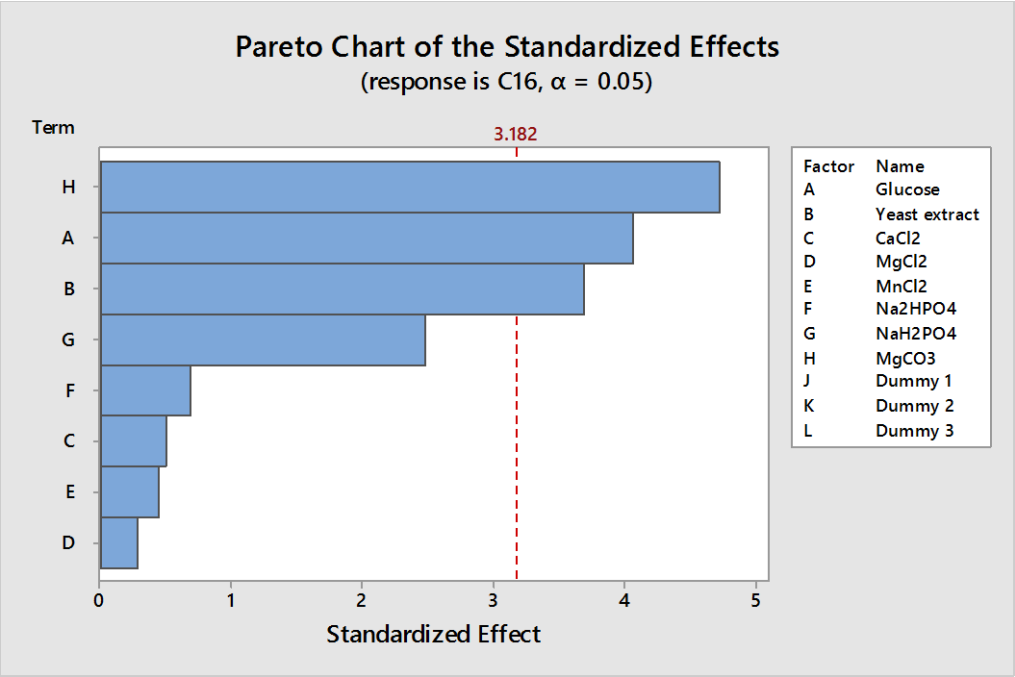

Figure 5.3 Pareto chart of standardized effects on succinic acid production.

From Figure 5.3 is a standardized Pareto chart consisting of bars with a length proportional to the absolute value of the estimated effects, divided by the standard error. The bar displays the order of the size of the effects, with the largest effects on top. The chart includes a vertical line at the critical *t*-value for alpha. Confidence levels greater than 95% ($p < 0.05$) were acceptable. Effects for which the bars were smaller than the critical *t*-value are considered not significant and did not affect the response variables. The effect may be positive or negative. From Figure 2, it can be seen that yeast extract and $MgCO_3$ had a confidence level above 95%. Hence, these were considered to be significant for succinic acid biosynthesis. A first-order regression equation is shown in Equation 5.3 from the PDB analysis using Minitab Program (version 17):

$$Y = 13.209 + 3.150\ A + 2.855\ B - 0.391\ C + 0.219\ D - 0.343\ E + 0.530\ F - 1.920\ G + 3.666\ H \quad \text{(Eq.5.3)}$$

where *Y* is the succinic acid concentration, A, B, C, D, E, F, G and H are the value of glucose, yeast extract, urea, $CaCl_2$, $MnCl_2$, Na_2CO_3 and $MgCO_3$, respectively, while J, K and L are dummy variables. The results of analysis of variance from PBD are shown in Table 5.7.

Table 5.7 Analysis of Variance of Linear model from PBD

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	8	429.609	53.701	7.46	0.063
Linear	8	429.609	53.701	7.46	0.063
Glucose	1	119.075	119.075	16.54	0.027
Yeast extract	1	97.819	97.819	13.59	0.035
$CaCl_2$	1	1.835	1.835	0.25	0.648
$MgCl_2$	1	0.577	0.577	0.08	0.796
$MnCl_2$	1	1.412	1.412	0.20	0.688
Na_2HPO_4	1	3.376	3.376	0.47	0.543
NaH_2PO_4	1	44.234	44.234	6.15	0.089
$MgCO_3$	1	161.281	161.281	22.41	0.018
Error	3	21.593	7.198		
Total	11	451.202			

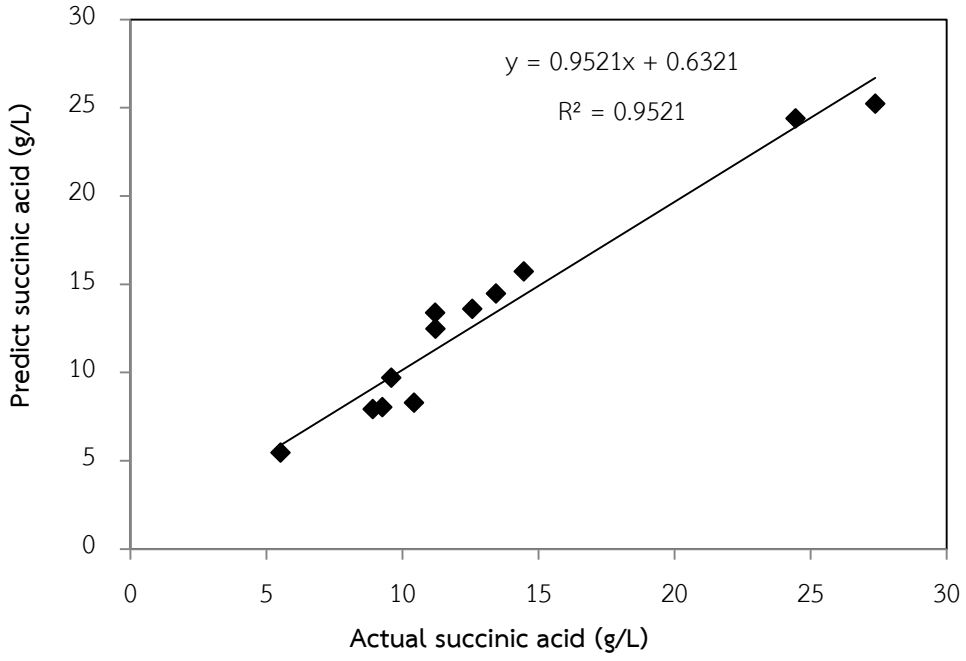

Figure 5.4 The relationship between actual and predicted value of succinic acid from the model of PBD by *A. succinogenes* NP9-aA7.

The goodness of the regression was checked by the coefficient of determination R^2 whose value of 95.21% indicates that only 4.79% of the total

variation could not be explained by the model. It was therefore considered reasonable to use the regression model to analyze the trend in the response. Consequently, yeast extract and $MgCO_3$ were selected for further study to obtain the optimal production. Succinic acid production was affected by the coefficients of glucose (3.150), yeast extract (2.855) and $MgCO_3$ (3.666). The positive coefficient indicated that the high level of yeast extract concentration aided succinic acid production. Similarly, the positive result of $MgCO_3$ indicates that the high level of $MgCO_3$ concentration helped succinic acid production. The optimal medium composition was 60.0 g/L of glucose, 15 g/L of yeast extract, 1.0 g/L of $Cacl_2$, 3.0 g/L of $MgCl_2$, 0.10 g/L of $MnCl_2$, 1.50 g/L of Na_2HPO_4, 1.0 g/L of Na_2HPO_4 and 45 g/L of $MgCO_3$. A maximum succinic acid concentration of 27.380 g/L was achieved while productivity and cell growth were 0.912 g/L·h and OD_{660} 5.630, respectively, whereas the control produced a maximum succinic acid of 19.054 g/L. The optimal concentrations of three key components including glucose, yeast extract and $MgCO_3$ were further studied using response surface methodology (RSM).

This result accordance to previous research in part of PBD results by *A. succinogenes* (Chapter IV) Glucose, Yeast extract and $MgCO_3$ were also screened to be key factors had a confidence level above 95% and hence were considered to significantly influence the succinic acid production. The optimal concentrations of three key components including glucose, yeast extract and $MgCO_3$ were further studied using response surface methodology (RSM). The optimal concentrations of three key components including glucose, yeast extract and $MgCO_3$ were further studied using response surface methodology (RSM).

Similar to the previous research, a preliminary optimization of succinic acid fermentation medium by *Actinobacillus succinogenes*, yeast extract, and $MgCO_3$ were identified to be the key medium components by Plackett-Burman Design.

The research of Yu et al. (2010) studied the optimization of fermentation media for succinic acid production using corncob by *A. succinogenes* cultivated under 12 different conditions of PBD. They report that initial sugar concentration, yeast extract and $MgCO_3$ are important for succinic acid production.

Zhu et al. (2012) reported on the optimization of fermentation media for succinic acid production by *Actinobacillus succinogenes* ATCC 55618 using PBD. They identified glucose, yeast extract, and $MgCO_3$ to be key medium components. A maximum succinic acid production of 51.9 g/L was obtained with 80.0 g/L of glucose, 60.0 g/L of $MgCO_3$, and 10.0 g/L of yeast extract.

Shen et al. (2014) used a PBD to screen the key variables for succinic acid production from cane molasses by *Actinobacillus succinogenes* GXAS137. The important parameters were found to be 85 g/L of total sugars of cane molasses, 8.84 g/L of yeast extract, and 63.1 g/L of $MgCO_3$. The maximal succinic acid production reached 57.43±0.86 g/L.

The culture pH value was also found to be one of the key factors in succinic acid production in a study by Samuelov et al. (Samuelov, Lamed, Lowe et al., 1991). $MgCO_3$ was used as an alkaline neutralizer and added to the fermentation medium to adjust the pH. Furthermore, the addition of CO_2 in the fermentation medium strongly affected the metabolic flux of carbon and the activities of phosphoenolpyruvate (PEP) carboxykinase, which were important steps for the biosynthesis of succinic acid (McKinlay et al., 2007). Therefore, it is concluded that sufficient CO_2 is another key factor affecting succinic acid accumulation. As an important CO_2 donor in the *A. succinogenes* fermentation, $MgCO_3$ interacts with organic acids in the fermentation medium resulting in an increase in the dissolved concentrations of HCO_3^-, CO_3^{2-}, and CO_2. When gaseous CO_2 was used with $MgCO_3$, a larger quantity of $MgCO_3$ was more effective in promoting succinic acid synthesis. However, insoluble $MgCO_3$ caused a turbid culture medium, which caused the cells to be evenly distributed in the broth. This effectively avoids cell flocculation. All these features make $MgCO_3$ one the key factors that significantly improves succinic acid production.

Yeast extract was also screened and found to be a key factor because it affected cell growth directly as the nutrient. Yeast contains many trace elements such as folic acid, pantothenic acid, biotin, and vitamin B1, B2, B6, and B12. This may explain why many kinds of vitamins could be omitted while succinic acid could

nonetheless accumulate efficiently. To conclude, the variables of glucose, $MgCO_3$ and yeast extract had a confidence level above 80% and hence were considered to significantly influence succinic acid production (Zhu et al., 2012). Further study is required to determine optimal concentrations of these three key components.

5.5.4 Effect of various concentrations of biotin on cell growth and succinic acid production by *A. succinogenes* NP9-aA7 using the one-factor-at-a-time method

A. succinogenes NP9-aA7 was cultivated under the anaerobic condition with different biotin concentration in the range 0-200 µg/L in the incubator shaker at 37 oC, 200 rpm for 36 h. Result was shown in Table 5.8 and Figure 5.5

Table 5.8 Effects of various concentrations of biotin on cell growth and succinic acid production by *A. succinogenes* NP9-aA7

Biotin conc. (µg/L)	Residual glucose (g/L)	Glucose utilization (%)[a]	OD 660	Succinic (g/L)	Formic (g/L)	Acetic (g/L)	Succinic acid yield (g/g glucose)	Succinic acid productivity (g/L•h)[b]
0	0.027±[c]0.010	99.953±0.017	5.491±0.396	14.528±1.398	4.980±0.143	5.190±0.916	0.247±0.019	0.403±0.039
50	0.035±0.008	99.941±0.014	14.565±0.948	44.191±5.073	5.276±0.313	3.098±0.217	0.716±0.069	1.227±0.141
100	0.043±0.008	99.926±0.014	14.730±0.715	45.575±3.602	5.252±0.671	13.109±0.440	0.745±0.049	1.265±0.100
150	0.035±0.004	99.941±0.007	13.450±0.433	47.415±2.889	3.257±3.846	2.557±1.137	0.802±0.039	1.317±0.080
200	0.044±0.012	99.926±0.020	14.545±0.436	48.361±1.463	2.921±3.033	1.858±1.726	0.812±0.019	1.343±0.041

[a] Glucose utilization was defined as the percentage of glucose concentration utilized by the bacteria in initial glucose.

[b] Succinic acid was obtained from cells grown in anaerobic conditions for 36 h.

[c] ±, Standard deviation was calculated from triplicate samples.

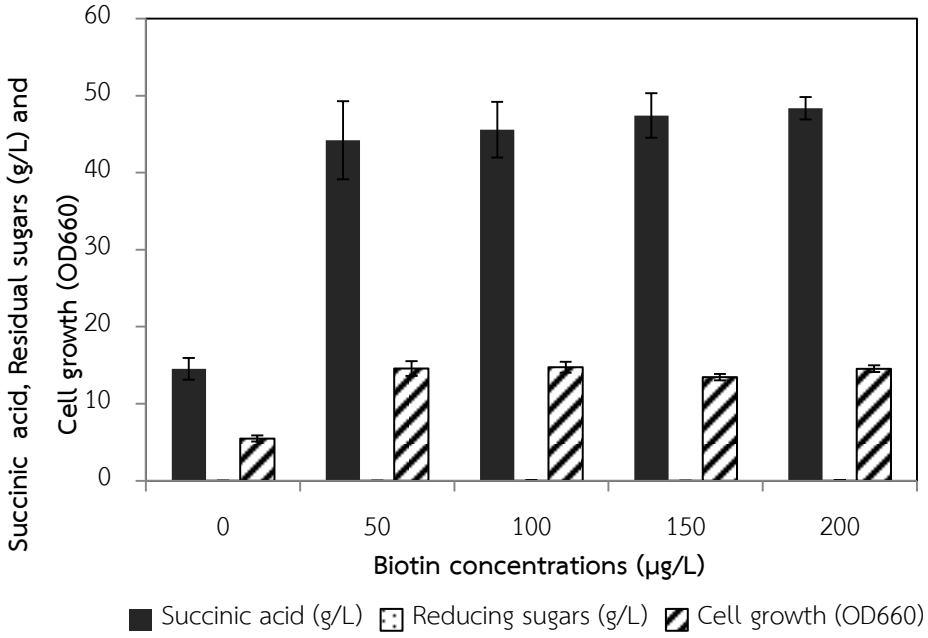

Figure 5.5 Effects of various concentrations of biotin on cell growth and succinic acid production by *A. succinogenes* NP9-aA7

These results illustrate that biotin had an effect on succinic acid production by *A. succinogenes* by NP9-aA7. Succinic acid was increased from 14.528 g/L (without added biotin) to 44.191 g/L (50 µg/L of biotin) which moreover improved to 48.361 g/L with the addition of 200 µg/L of biotin.

Result from statistical analysis by SPSS showed the p-value was shown of 0.000 from ANOVA table (Appendix D) could be explain that using different biotin concentration had effect on succinic acid production. Then these results were analyzed by multiple comparisons using Tukey, Duncan and Scheffe method for determine the optimum biotin concentration for succinic acid production. Result were investigated that when using biotin 50-200 µg/L gave succinic in the range 44.191-48.361 g/L and no significant in group. *P*-value was shown of 0.235, 0.061and 0.343 by Tukey, Duncan and Scheffe method, respectively (Appendix D). Moreover, the biotin concentration could be reduced demand yeast extract concentration in a more for promote succinic acid production. Also biotin was a considered factor. It

could be concluded that 50 μg/L of biotin was optimal for succinic acid production by *A. succinogenes* NP9-aA7.

Nghiem, Davison, Thompson et al. (1996) studied the effects of biotin concentrations on succinic acid production were also investigated in anaerobic bottle cultivation supplemented with 0–200 μg/L of biotin, with 90 g/L of glucose resulting in cell growth and succinic acid concentration increased with increasing biotin from 0 to 100 μg/L, and glucose utilization had no distinct change at the biotin concentration over 100 μg/L. therefore 100 μg/L of biotin sutable for promote the cell growth and succinic acid production. Addition of 50 mg/L of biotin during succinic acid fermentation by *A. succiniciproducens* is reported to cause a 16% increase in glucose utilization, with the maximum concentration of succinic acid increasing by 17%.

Chen, Li, Ma et al. (2011) studied the effect of biotin concentration on cell growth and succinic acid production with yeast cell hydrolysate (YCH) by *A. succinogenes*. Result showed that supplementation with a vitamin mixture (The supplemented vitamins and their concentrations were according to the reference (Du, Lin, Koutinas et al., 2007) and as following (per liter): cyanocobalamin, 10 μg/L; biotin, 200 μg/L; folic acid, 200 μg/L; thiamine, 500 μg/L; riboflavin, 500 μg/L; nicotinic acid, 500 μg/L; pantothenic acid, 500 μg/L; p-aminobenzoic acid, 500 μg/L; thioctic acid, 500 μg/L; and pyridoxine, 1 μg/L) improves the ferment ability of YCH. When biotin was added into the YCH-based medium, cell growth and succinic acid production both improved, and this improvement was comparable to the addition of all ten of the tested vitamins (Jiang, Chen, Liu et al., 2010).

According to the research of Xi et al. (2012) studied the effect of biotin on succinic acid production by *A. succinogenes* NJ 113. The succinic acid production was increased with addition of biotin in the minimal chemically defined medium (CDM) but they reported that in the initial CDM, the biotin concentration was 10 mg/L, and vitamins acted as cofactors, coenzymes, or prosthetic groups for enzymes and were required in very small amounts (Lin, Hanson and Cronan, 2010). Thus, the biotin

concentration was reduced to investigate its effect on succinic acid production. The optimal biotin concentration range was 50 mg/L to 150 µg/L.

In contrast, a research has been reported that the ratio of succinic acid to acetic acid of 9.0 and succinic acid yield of 79.3% was obtained by *A. succinogenes* CGMCC1593 at the glucose concentration of 50 g/L supplemented with 15 g/L of yeast extract in anaerobic bottle fermentation (Liu et al., 2008). It seemed that lower succinic acid and higher acetic acid were obtained with biotin-supplemented YCH than with yeast extract. Biotin is a water-soluble vitamin required by all organisms because of its essential role as a carboxyl carrier in carboxylation reactions in metabolic pathways such as gluconeogenesis, fatty acid synthesis, and amino acid catabolism (McMahon, 2002). No biotin-dependent pyruvate carboxylase gene has been found in any Pasteurellaceae genome sequence (McKinlay and Vieille, 2008), so the role of biotin in succinic acid fermentation by *A. succinogenes* with YCH-based medium was not related to pyruvate carboxylation. This conclusion was confirmed by the lack of change in the succinic acid to acetic acid ratio with increasing biotin concentration.

The metabolic pathway of *A. succinogenes* converts glucose into phosphoenolpyruvate (PEP) then splits into the two branches: the acetate-producing C3 pathway and the succinate-producing C4 pathway. Biotin plays an important role in electron transfer in the anaerobic respiratory chain of *A. succinogenes*. PEP was converted into fermentation products via the C3 pathway (formate, acetate, and ethanol) and the C4 pathway (succinate), with malic enzyme and oxaloacetate (OAA) decarboxylase forming reversible shunts between these pathways. The increase in the biotin concentration was conducive to PEP flow to the C4 pathway, while the flux of the C3 pathway was reduced (Xi et al., 2012).

5.5.5 Effects of different nitrogen sources on cell growth and succinic acid production by *A. succinogenes* NP9-aA7 using the one-factor-at-a-time method

The effect of different inorganic and organic nitrogen sources (in same percent equivalent) are shown in Table 5.9 and Figure 5.6.

Table 5.9 Effects of different in nitrogen source on cell growth and succinic acid production by *A. succinogenes* NP9-aA7

N. source	Residual glucose (g/L)	Glucose utilization (%)[a]	OD 660	Succinic (g/L)	Formic (g/L)[c]	Acetic (g/L)[c]	Succinic acid yield (g/g glucose)	Succinic acid productivity (g/L*h)[b]
$(NH_4)_2SO_4$	0.122±[c]0.011	99.797±0.018	0.805±0.043	15.282±2.125	0.056±0.022	0.752±0.026	0.255±0.036	0.424±0.083
KNO_3	0.259±0.134	99.568±0.223	0.598±0.120	3.722±1.457	0.000±0.000	0.334±0.057	0.062±0.024	0.103±0.057
NH_4Cl	0.130±0.037	99.783±0.061	2.855±0.117	35.164±0.995	0.381±0.381	1.122±0.238	0.587±0.017	0.977±0.039
Beef extract	0.145±0.018	99.758±0.029	13.425±0.365	51.027±1.278	3.876±0.313	4.789±1.183	0.853±0.021	1.417±0.050
CSL	0.077±0.027	99.872±0.044	4.780±1.295	27.939±0.333	0.121±0.121	5.934±1.585	0.466±0.005	0.776±0.013
Peptone	0.120±0.024	99.799±0.040	2.350±0.057	36.175±2.070	0.641±0.112	2.533±0.498	0.604±0.035	1.005±0.081
Urea	0.020±0.264	99.673±0.188	0.733±0.017	3.955±1.592	0.012±0.012	4.983±0.936	0.066±0.027	0.110±0.063
Yeast extract	0.103±0.002	99.828±0.003	14.900±1.746	49.756±6.763	6.463±0.151	4.501±0.354	0.831±0.113	1.382±0.266

[a] Glucose utilization was defined as the percentage of glucose concentration utilized by the bacteria in initial glucose.

[b] Succinic acid was obtained from cells grown in anaerobic conditions for 36 h.

[c] ±, Standard deviation was calculated from triplicate samples.

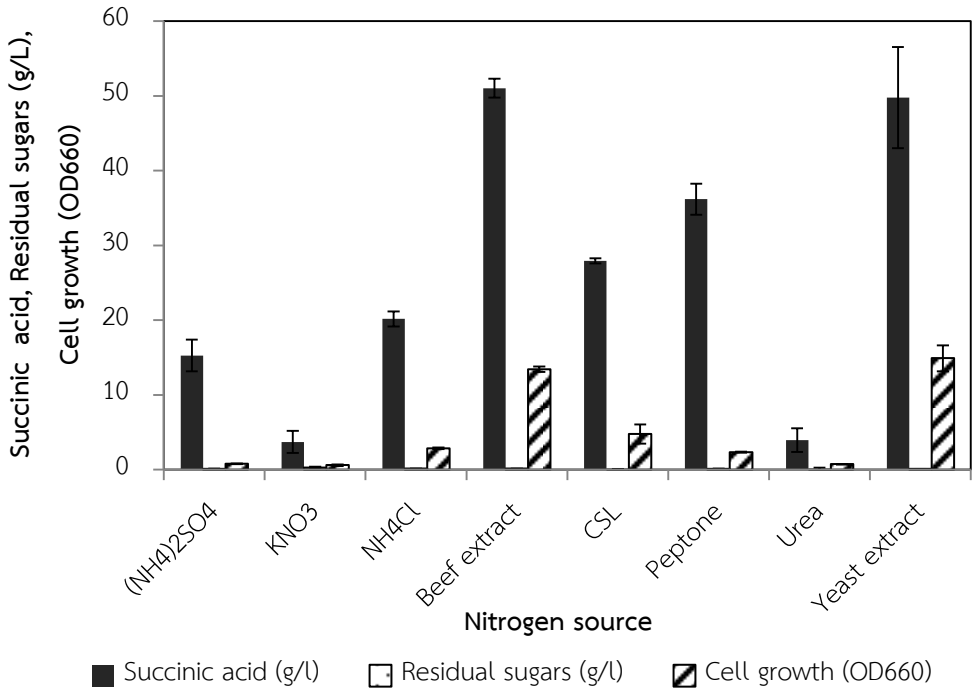

Figure 5.6 Effects of different nitrogen sources on cell growth and the production of succinic acid by *A. succinogenes* NP9-aA7

From Figure 5.6, it can be seen that poor cell growth was observed with all the inorganic nitrogen sources tested, and yeast extract was found to be the best nitrogen source for succinic acid production by *A. succinogenes* NP9-aA7. Maximum succinic acid production of 51.027 g/L and cell growth of 13.425 (OD_{660}) were obtained when using 30 g/L beef extract as a nitrogen source followed by succinic acid of 49.756 g/L and cell growth of 14.900 (OD_{660}) when using yeast extract as a nitrogen source.

From statistical analysis by SPSS, the *p*-value was shown of 0.000 from ANOVA table (Appendix D) could be explain that using different nitrogen source had effect on succinic acid production. Then these results were analyzed by multiple comparisons using Tukey, Duncan and Scheffe method were divide into 5 groups. The best groups, including beef extract and yeast extract, were found not to be significantly different with a p-value of 0.999, 0.584 and 1.000 respectively following the Tukey, Duncan and Scheffe method. Maximum succinic acid production of 49.756 g/L was obtained when using 30 g/L of yeast extract as a nitrogen source with no significant difference between yeast extract and beef extract. However, the cost of beef extract is higher than yeast extract. From these results it can be concluded that the use of yeast extract of 30 g/L was the best nitrogen source for succinic acid fermentation by *A. succinogenes* NP9-aA7. The cost of yeast Extract was 2900.00 ฿ for 500 g while the cost of beef Extract was 5150.00 ฿ for 500 g.

Similarly, Liu et al. (Liu et al., 2008) studied the effect of different inorganic and organic nitrogen sources compared with yeast extract (15 g/L in same percent equivalent) on cell growth and succinic acid production by *A. succinogenes* CGMCC1593. Poor cell growth was observed with all the inorganic nitrogen sources tested, while yeast extract was found to be the best nitrogen source for succinic acid production.

It has been reported that inorganic nitrogen sources are not effective to promote cell growth of *A. succinogenes*. A chemically defined medium needs amino acids and vitamins for cell growth (Yang, Le, Hu et al., 2008). Therefore, the effects of inorganic nitrogen sources were not considered. Among the complex nitrogen

sources evaluated, yeast extract produced the best results for both cell growth and succinic acid production.

5.5.6 Effects of different alkaline neutralizers on cell growth and succinic acid production by *A. succinogenes* NP9-aA7 using the one-factor-at-a-time method

The effects of different alkaline neutralizers were compared such as $Ca(OH)_2$, $CaCO_3$, $NaHCO_3$, $NaCO_3$, NaOH, $Mg(OH)_2$ and $MgCO_3$ with 10g/L compare to the control (no adding alkaline neutralizer). Results were showed in Table 5.10 and Figure 5.7.

Table 5.10 Effect of different alkaline neutralizers on cell growth and succinic acid production by *A. succinog*enes NP9-aA7

Neutralizing agent	Residual glucose (g/L)	Glucose utilization (%)[a]	Cell growth (OD 660)	Succinic (g/L)	Formic (g/L)	Acetic (g/L)[c]	Succinic acid yield (g/g glucose)	Succinic acid productivity (g/L*h)[b]
$Ca(OH)_2$	0.071±[c]0.009	99.881±0.015	2.400±0.321	10.628±0.546	1.488±0.059	0.822±0.073	0.177±0.009	0.295±0.021
$CaCO_3$	0.003±0.008	99.991±0.010	11.810±1.381	23.536±0.219	3.796±0.210	3.010±0.297	0.392±0.004	0.654±0.009
$NaHCO_3$	0.066±0.028	99.890±0.047	8.995±2.462	11.883±0.454	3.562±0.485	2.255±0.165	0.198±0.008	0.330±0.018
$Na2CO_3$	0.077±0.024	99.872±0.040	9.875±0.268	20.443±3.516	2.836±0.980	1.877±0.954	0.341±0.059	0.522±0.079
NaOH	0.198±0.098	99.670±0.163	3.215±2.170	0.829±0.322	2.475±0.470	1.532±1.371	0.014±0.005	0.023±0.013
$Mg(OH)_2$	0.001±0.043	99.945±0.025	8.245±1.178	30.427±0.313	2.708±0.663	1.538±0.293	0.507±0.005	0.845±0.012
$MgCO_3$	0.112±0.025	99.814±0.041	5.760±0.301	35.755±1.776	3.834±0.363	0.297±0.059	0.597±0.030	0.993±0.070
Control[d]	0.008±0.095	99.874±0.037	3.185±0.023	9.826±0.003	0.984±0.985	0.495±0.495	0.164±0.000	0.273±0.000

[a] Glucose utilization was defined as the percentage of glucose concentration utilized by the bacteria in initial glucose.

[b] Succinic acid was obtained from cells grown in anaerobic conditions for 36 h.

[c] ±, Standard deviation was calculated from triplicate samples.

[d] **Control, No Alkaline**

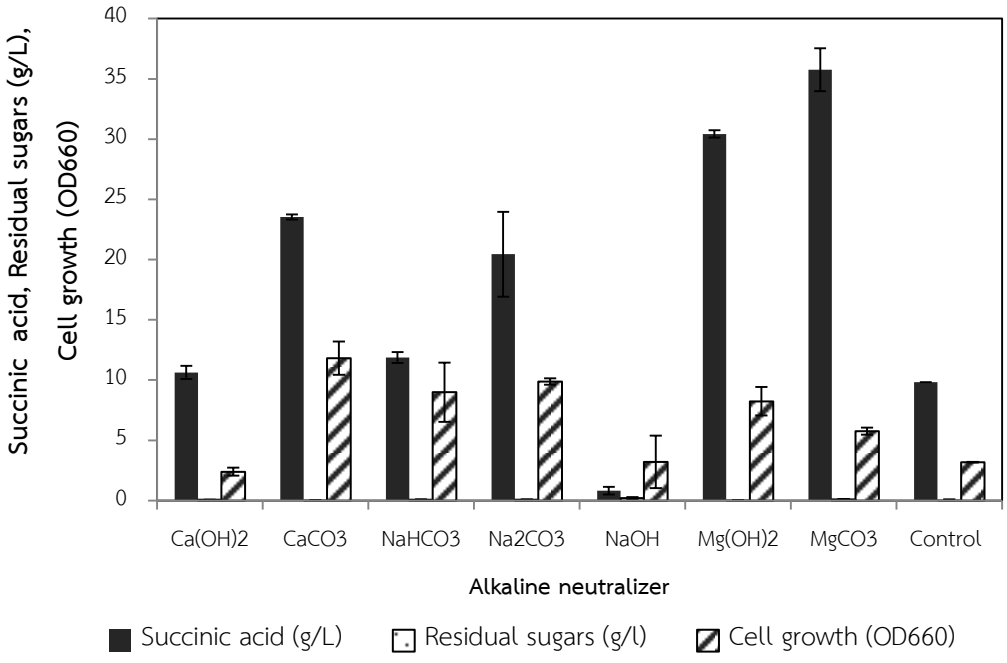

Figure 5.7 Effect of different alkaline neutralizer on cell growth and succinic acid production by *A. succinogenes* NP9-aA7.

The statistical analysis by SPSS, the p-value was shown of 0.000 from ANOVA table (Appendix D) could be explain that using different alkaline neutralizing agent had effect on succinic acid production. Then these results were analyzed by multiple comparisons using Tukey, Duncan and Scheffe method were divide into 4 groups. The best group including, including $MgCO_3$ and $Mg(OH)_2$, were not significantly different with a p-value of 0.453, 0.052 and 0.724 respectively, by the Tukey Duncan and Scheffe method. Maximum succinic acid production of 35.755g/L was obtained when using 10 g/L of $MgCO_3$ as an alkaline neutralizing agent. $MgCO_3$ not only controls pH and provides CO_2, but also supplements the cofactor. Magnesium ion was reported as the cofactor for many key enzymes in the succinic acid synthesis pathway such as phosphoenolpyruvate carboxylase (PEPC) (Bazaes, Toncio, Laivenieks et al., 2007), phosphoenolpyruvate carboxykinase (PEPCK), and pyruvate carboxylase (PC) (Adina-Zada, Zeczycki and Attwood, 2012). Magnesium ions also play a key role in the affinity of CO_2 with PEPC, which catalyzed the carboxylation of phosphoenolpyruvate to the first C4 metabolite oxaloacetate (Kai, Matsumura and Izui, 2003). Malate dehydrogenase, another key enzyme in the synthesis of succinic acid, was a NADH dependent enzyme that catalyzes the conversion of oxaloacetate to malate, and Mg^{2+} plays a key role in this catalytic reaction (Liang, Ma, Liu et al., 2011a).

Similarly, (Liang et al., 2011b) reported on the effects of different alkaline neutralizers on cell growth and succinic acid production by *A. succinogenes*. The glucose consumption in fermentation with Na_2CO_3, $NaHCO_3$, $Mg(OH)_2$, and $MgCO_3$ as alkaline neutralizers was much higher compared with $Ca(OH)_2$, $CaCO_3$, NaOH, and NH_3H_2O. The use of $MgCO_3$ as an alkaline neutralizer resulted in the best cell growth, glucose utilization, and succinic acid production.

When NaOH was used as an alkaline neutralizing agent, it resulted in the lowest succinic acid production perhaps because a high Na^+ level could result in a hypertonic environment, which has a negative effect on metabolism and cell growth (Liu et al., 2008). Moreover, OH^- is a strong base. Na^+ also plays a very important role in maintaining the transmembrane pH gradient, cell osmotic pressure, and

regulation of intracellular pH. Lee et al. (Lee, Lee and Chang, 2010) reported that the OD600 of *A. succiniciproducens* significantly decreased when the concentration of NaCl exceeded 4 g/L.

Some reports have shown that Ca^{2+} is toxic to *M. succiniciproducens* during succinic acid fermentation (Song, Huh, Lee et al., 2007a). Ca^{2+} can alter the normal fluidity and permeability of cell membranes, which can affect cell growth and cell metabolism resulting in low succinic acid production when $Ca(OH)_2$ or $CaCO_3$ are used as alkaline neutralizers.

From the results of this experiment it can be concluded that $MgCO_3$ is effective for succinic acid production. The cost of $MgCO_3$ was 5923.13 Thai ฿ for 1 kg and the cost of $Mg(OH)_2$ was 6219.29฿ for 1 kg (Sigma-aldrich, WGK Germany). Therefore, to supplement the strong alkalinity of OH^- and high solubility of $Mg(OH)_2$ and to provide the CO_3^{2-} of $MgCO_3$, $MgCO_3$ was selected as an affordable mixed alkaline neutralizer to control pH for succinic acid production by *A. succinogenes* NP9-aA7.

5.5.7 Optimization medium composition for succinic acid production by *A. succinogenes* NP9-aA7 using Response Surface Methodology (RSM)

A three-variable-three-level (Table 5.11) matrix of BBD was employed to determine the optimized conditions and the interactive effects. Glucose, yeast extract, and $MgCO_3$ were selected as the factors for BBD. The succinic acid concentrations for each individual run along with the predicted responses are summarized in Table 5.12

Table 5. 11 Experimental ranges and levels of the three independent variables used in RSM in terms of actual and coded factors.

	A	B	C
	glucose	**Yeast extract**	**$MgCO_3$**
Low(-1)	40	10	20
Med (0)	60	20	40
High(+1)	80	30	60

Table 5.12 Experimental design using Box-Behnken Design (BBD) and the results for the optimization of succinic acid production by *A. succinogenes* NP9-aA7

Run No.	Glucose	YE	$MgCO_3$	Cell growth	Succinic acid (g/L)	
	A	B	C	(OD 660)	Actual	Predicted
1	1	1	0	18.035	51.528	49.637
2	0	0	0	14.025	35.768	39.889
3	0	1	1	21.200	57.630	56.198
4	-1	0	-1	12.865	32.483	29.161
5	0	0	0	13.965	39.898	39.889
6	-1	1	0	1.690	17.827	21.913
7	1	0	1	11.470	31.033	34.355
8	0	-1	1	8.210	21.959	22.722
9	-1	-1	0	10.815	29.530	31.421
10	0	0	0	15.995	38.898	39.889
11	1	0	-1	12.140	30.599	33.254
12	0	-1	-1	9.410	26.130	27.561
13	0	0	0	16.705	42.718	39.889
14	0	1	-1	15.420	36.508	35.744
15	1	-1	0	10.550	2.556	-1.530
16	-1	0	1	17.720	46.330	43.675
17	0	0	0	14.597	42.163	39.889

YE, yeast extract

The highest succinic acid concentration of 57.630 g/L was attained at 24 h when the concentrations of glucose, yeast extract, and $MgCO_3$ were 60.00, 30.00, and 60.00 g/L, respectively, with cell growth of (OD_{660}) 21.200 (Run 3). The lowest succinic acid concentration was 2.556 g/L, which was obtained when glucose, yeast extract, and $MgCO_3$ concentrations were 80, 10, and 20 g/L, respectively (Run 15).

Based on the software analysis, the optimized concentrations of glucose, yeast extract, and $MgCO_3$ were 74 (0.74), 30 (1.00), and 60 (1.00) g/L and the predicted concentration of succinic acid was 59.886 g/L. This was a 15.83% improvement over that attained with the one-at-a-time method (49.856 g/L of

succinic acid was obtained from 30 g/L of yeast extract as a nitrogen source with 30 g/L of $MgCO_3$ as an alkaline neutralizer). The response data were analyzed using Design-Expert software. The application of RSM yielded the following regression equation, which was an empirical relationship between succinic acid and the test variables in coded units:

$$Y = 39.889 - 1.307A + 10.415B + 3.904C + 15.169AB - 3.353AC + 6.323BC - 7.487A^2 - 7.0418B^2 + 2.709C^2 \quad \text{(Eq5.4)}$$

where *Y* is the succinic acid produced as a function of glucose (A), yeast extract (B), and $MgCO_3$ (C). The statistical significance of the above equation was checked with the *F* test, and an ANOVA for the response surface quadratic model shown in Table 5.13.

Table 5.13 Analysis of variance (ANOVA) for the Response Surface Quadratic Model[a]

Source	Sum of Squares	DF	Mean Square	F-Value	Prob > F	
Model	2613.407	9	290.379	17.972	0.0005	significant
A	13.661	1	13.661	0.845	0.3884	
B	867.735	1	867.735	53.704	0.0002	
C	121.934	1	121.934	7.547	0.0286	
A^2	236.023	1	236.023	14.608	0.0065	
B^2	208.789	1	208.789	12.922	0.0088	
C^2	30.898	1	30.898	1.912	0.2092	
AB	920.371	1	920.371	56.962	0.0001	
AC	44.975	1	44.975	2.784	0.1392	
BC	159.934	1	159.934	9.898	0.0162	
Residual	113.103	7	16.158			
Lack of Fit	81.964	3	27.321	3.510	0.1283	not significant
Pure Error	31.139	4	7.785			
Total	2726.510	16				

[a] Coefficient of determination (R^2)= 0.9585; Adjusted R^2=0.9052; Coefficient of variation; SS: sum of squares; DF: degrees of freedom; MS: mean square; R^2: determination coefficient; adj R^2: adjusted R^2; pred R^2: predicted R^2. Std. Dev. 4.02 R^2-Squared 0.9585, Mean 34.3269, Adj R-Squared 0.9052, C.V. 11.71 Pred R-Squared 0.5012, PRESS1360.08 and Adeq Precision 18.725.

The Model F-value of 17.972 implies the model was significant. There was only a 0.05% chance that a "Model F-Value" this large could occur due to noise.

Values of probability (*P*) "Prob > F" of 0.0005 indicate that the model terms were significant. In this case B, C, A^2, B^2, AB, BC were significant model terms. Values of probability greater than 0.05 indicate the model terms were not significant. The "Lack of Fit F-value" was 3.510 implying the Lack of Fit was not significant relative to the pure error. There was a 12.83% chance that a "Lack of Fit F-value" this large could occur due to noise. A non-significant lack of fit indicates that the model was a good fit (Song et al., 2007c). The adequate precision value, which measured the signal to noise ratio, was 18.725. A ratio greater than 4 is satisfactory, so this model could be used to design the space. The quadratic equation shows that the R^2 value was 0.9585 (Table 5.13), which indicates opportuneness of the model. This result indicates that approximately 95.85% of the variability in the dependent variable can

be explained by this model. The model was stronger and the predicted response was better as the R^2 value approached 1.0, however, values of R^2 > 0.75 indicate aptness of the model (Ferreira et al., 2009). Therefore, the model was considered suitable to predict succinic acid production.

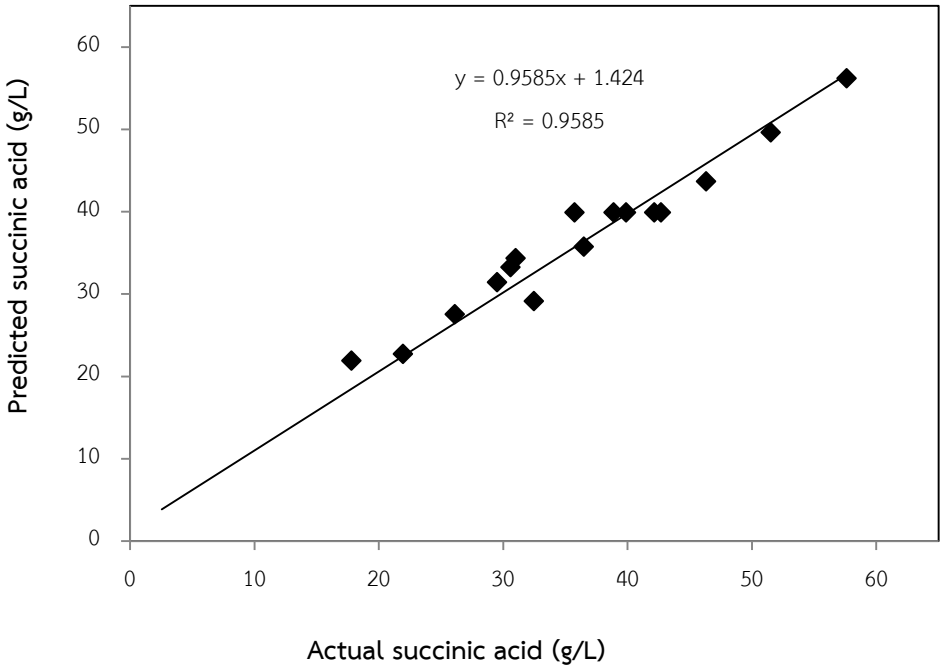

Figure 5.8 The relationship between the actual and predicted values of succinic acid production by *A. succinogenes* NP9-aA7.

The response surface curves were plotted to investigate the interactions between the factors and to determine the optimal concentration of each factor for maximum succinic acid production (Figure 5.9).

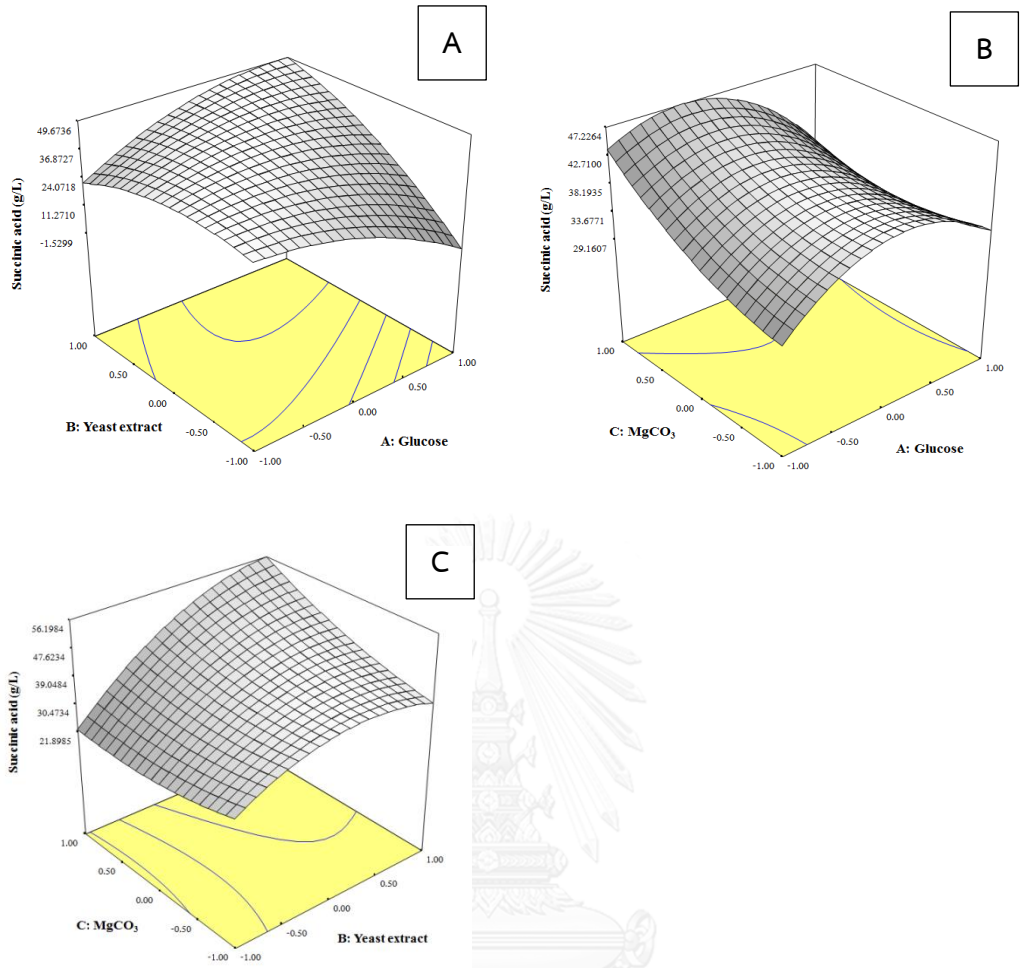

Figure 5.9 Response surface plots and contour plots

(A) Combined effects of glucose and yeast extract with constant magnesium carbonate (40 g/L);

(B) Combined effects of glucose and magnesium carbonate with constant yeast extract (20 g/L);

(C) Combined effects of yeast extract and magnesium carbonate with constant glucose (60 g/L)

As shown in Figure 5.9A, the effects of glucose and yeast extract on the production of succinic acid were determined when the other factor was at its center point although the increasing glucose concentration does not increase succinic acid production when yeast extract is at a low level. Similar to cases of low levels of glucose (40 g/L), succinic acid did not increase with increasing yeast extract concentration.

Figure 5.9B shows that the succinic acid production was improved significantly by increasing the amount of yeast extract to 30 g/L while increasing glucose from 40 to 80 g/L. With yeast extract at a center point (20 g/L), adding enough $MgCO_3$ led to a higher concentration of succinic acid. When glucose was in the range of 50-70 g/L, succinic acid production was not encouraged. Magnesium carbonate is considered as one of the main factors to control the pH in fermentation, and provides magnesium ions for PEPCK which is a key enzyme in succinic acid production (Lee et al., 1999b). When the glucose was at a low level, an increase in $MgCO_3$ did not improve the succinic acid concentration. However, if the glucose and $MgCO_3$ were at high levels, more succinic acid was attained.

Figure 5.9C illustrates the interaction of yeast extract and $MgCO_3$ on the concentration of succinic acid when glucose concentration was at its center point (60 g/L). It is clearly shown that succinic acid concentration was improved by increasing the amount of $MgCO_3$ with the amount of yeast extract added.

Zhu et al. (2012) reported that a method combining the Plackett-Burman Design (PBD), the steepest ascent method (SA), and the Box-Behnken Design (BBD) was developed to optimize succinic acid production by *Actinobacillus succinogenes* ATCC 55618. First, glucose, yeast extract, and $MgCO_3$ were identified to be the key medium components by PBD. Then, preliminary optimization was performed using the SA method to reach an optimal region of the key medium components. After that, the production of succinic acid was optimized concurrently by using BBD from which the optimal concentration was found to be 84.6 g/L of glucose, 14.5 g/L of yeast extract, and 64.7 g/L of $MgCO_3$. Confirmation experiments demonstrate that the maximal succinic acid production of 52.7 ± 0.8 g/L was obtained from the identified optimal conditions.

(Zhang et al., 2012) used the model in terms of fermentation factors from RSM to predict the succinic acid production by *A. succinogenes,* strain BE-1. The glucose, yeast extract, and interactive effect of yeast extract and $MgCO_3$ were found to be the most significant factors in succinic acid production in this case. The optimum values for glucose, yeast extract, and $MgCO_3$ concentrations were found to

be 27.43, 9.56, and 23.32 g/L, respectively. This resulted in a predicted value of 19.08 g/L, which was increased by 28.9% compared with the 14.80 g/L obtained from the one-at-a-time method.

5.5.8 Model Validation

To validate the fermentation parameters, results from the run of the combination levels of the 3 key factors (glucose, yeast extract, and $MgCO_3$) were predicted based on the BBD quadratic model. The applicability of the model and the accuracy of the prediction were checked based on verification experiments performed in triplicate using the optimized conditions representing the maximum point of the concentration of succinic acid to verify the modelled results (Table 5.14).

Table 5.14 The arrangement and results of confirmatory trials

No.	Residual glucose (g/L)	Glucose utilization (%)[a]	OD 660	Succinic (g/L)	Formic (g/L)[c]	Acetic (g/L)[c]	Succinic acid yield (g/g glucose)	Succinic acid productivity (g/L•h)[b]
1	0.421±[c]0.117	99.431±0.158	14.749±1.453	60.087±1.866	2.888±0.148	4.072±0.151	0.816±0.025	1.669±0.073
2	0.221±0.169	99.695±0.234	17.404±3.320	58.595±1.518	2.985±0.412	4.422±0.861	0.812±0.021	1.628±0.060
3	0.105±0.137	99.857±0.186	13.916±0.583	57.079±0.180	3.720±0.995	5.152±1.459	0.776±0.002	1.586±0.007
4	0.251±0.267	99.615±0.409	15.525±3.118	55.857±0.661	3.302±0.344	5.083±0.837	0.860±0.011	1.552±0.026
5	0.144±0.037	99.786±0.055	16.346±1.432	54.420±1.369	3.131±0.226	4.780±0.056	0.809±0.021	1.512±0.054

[a] Glucose utilization was defined as the percentage of glucose concentration utilized by the bacteria in initial glucose.

[b] Succinic acid was obtained from cells grown in anaerobic conditions for 36 h.

[c] ±, Standard deviation was calculated from triplicate samples.

Table 5.15 Comparison between the actual and predicted succinic acid using a quadratic equation from BBD

No.	Glucose	YE	$MgCO_3$	Succinic acid			
				Actual	Predicted	Residual (g/L)	Error (%)
1	74.0	30.0	60.0	60.087	59.886	0.201	0.335
2	72.4	30.0	60.0	58.595	59.838	-1.243	2.122
3	73.6	29.9	60.0	57.079	59.791	-2.712	4.752
4	65.2	30.0	60.0	55.857	58.443	-2.586	4.630
5	67.4	29.0	60.0	54.420	58.207	-2.514	4.515

YE, Yeast extract

Validation of the model was also conducted and the percentage of errors between the actual and predicted values for succinic acid production varied from 0.335 % to 4.515 %. The predicted average concentration of succinic acid was 57.207±2.227 g/L and the average concentration determined by experiment was 59.233±0.834 g/L. These results show that there was no significant difference between actual and predicted values (3.54%). This indicates that the actual values obtained were in good agreement with the predictions of the quadratic equation model. Therefore, the model was considered suitable for predicting succinic acid production. Correspondingly, 74 g/L of glucose, 30 g/L of yeast extract, and 60 g/L of $MgCO_3$ were obtained as the optimal points of the model. The optimal medium producing the actual maximum succinic acid was 60.087 g/L and the predicted maximum of succinic acid was 59.886 g/L. Results from this study suggest that the RSM approach could be quite efficient and useful for the optimization of succinic acid fermentation conditions and the optimal conditions provide key medium components for scaled-up succinic acid fermentation with *A. succinogenes* NP9-aA7.

5.5.9 Effect of different ratio of $Mg(OH)_2$ and $MgCO_3$ on cell growth and succinic acid production by *A. succinogenes* NP9-a7 using the one-factor-at-a-time method

A. succinogenes NP9-aA7 was cultivated at different ratios of 3:1, 2:1, 1:1, 1:2 and 1:3 of $MgCO_3$ and $Mg(OH)_2$ under anaerobic conditions at 37 ^{O}C, with agitation at 200 rpm for 36 h of cultivation time. Results were showed in Table 5.16 and Figure 5.10.

Table 5.16 Comparative analysis of succinic acid production with mixed alkaline neutralizers, $Mg(OH)_2$, or $MgCO_3$ as pH regulators.

$MgCO_3$:$Mg(OH)_2$	Residual glucose (g/L)	Glucose utilization (%)[a]	OD 660	Succinic (g/L)	Formic (g/L)[c]	Acetic (g/L)[c]	Succinic acid yield (g/g glucose)	Succinic acid productivity (g/L•h)[b]
(3:1)	0.196±[c]0.180	99.674±0.300	15.140±2.321	48.644±0.073	17.611±10.635	4.481±0.101	0.813±0.003	1.351±0.003
(2:1)	0.349±0.273	99.418±0.454	13.930±2.893	37.605±3.070	6.694±0.533	4.832±0.401	0.630±0.053	1.045±0.121
(1:1)	0.295±0.212	99.509±0.353	11.640±2.234	32.995±1.339	7.601±0.221	4.163±0.233	0.552±0.022	0.917±0.053
(1:2)	0.428±0.019	99.286±0.032	10.720±3.568	28.470±0.035	8.083±1.867	3.288±0.448	0.478±0.001	0.791±0.001
(1:3)	0.295±0.180	99.509±0.300	10.850±0.125	27.823±0.547	7.396±0.746	2.071±1.800	0.466±0.010	0.773±0.021

[a] Glucose utilization was defined as the percentage of glucose concentration utilized by the bacteria in initial glucose.

[b] Succinic acid was obtained from cells grown in anaerobic conditions for 36 h.

[c] ±, Standard deviation was calculated from triplicate samples.

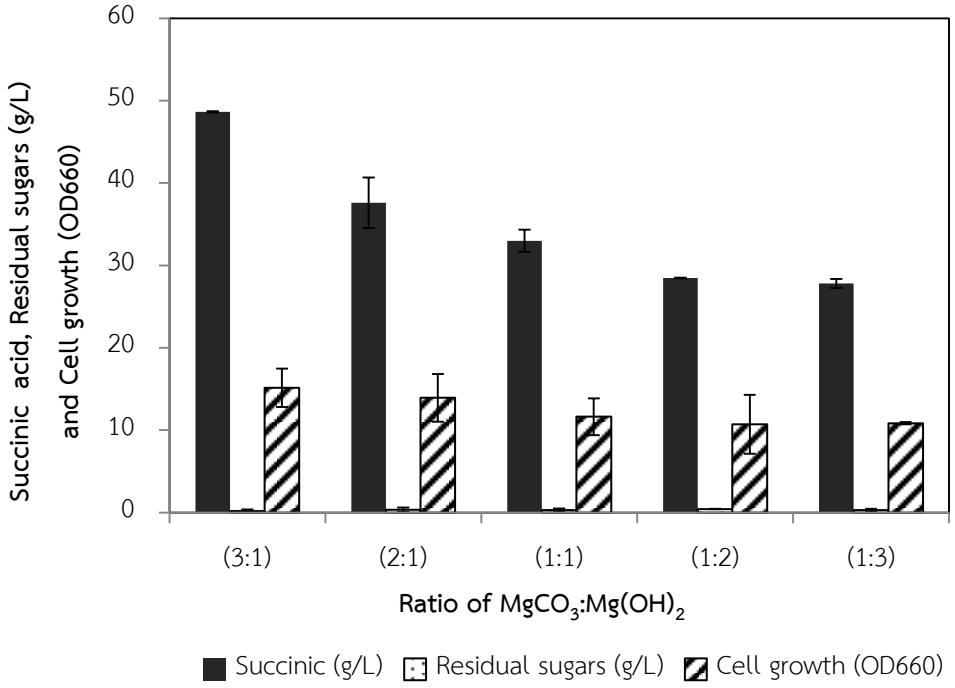

Figure 5.10 Comparison of different mass ratios of $MgCO_3$ and $Mg(OH)_2$ in the production of succinic acid in batch fermentation.

Although the cost of succinic acid production could be reduced by substituting $MgCO_3$ by $Mg(OH)_2$ as the alkaline neutralizer, the price of $Mg(OH)_2$ was still high (the prices of $MgCO_3$ and $Mg(OH)_2$ is 940 and 380 dollars per ton, respectively). Moreover, $Mg(OH)_2$ could be increased solubility of alkaline neutralizer.

As shown in Table 5.17, with the mass ratio of $MgCO_3$ and $Mg(OH)_2$ at 3:1, the succinic acid production was higher than the other maximum of succinic acid production of 48.644 g/L with a yield of 0.813 g/g and cell growth of (OD_{660}) 17.611. Succinic acid gradually decreased with a decreasing proportion of $MgCO_3$ in the mixture. Only $MgCO_3$ (60 g/L) was used as a control medium when it produced a maximum of succinic acid of 41.525 g/L, lower than 3:1 of $MgCO_3$ to $Mg(OH)_2$ (data not shown), while cell growth (OD_{660}) was 16.590 which is very close to a mixed alkaline neutralizer. Also, the Mg^{2+} concentration in the mixed alkaline neutralizer (of which the ratio of $MgCO_3$and $Mg(OH)_2$ was 3:1) was enough for the growth of cells and production of succinic acid. Moreover, the multiple comparisons using Tukey,

Duncan and Scheffe method were divided into 5 groups. The best group including the ratio of $MgCO_3$ and $Mg(OH)_2$ was 3:1 follow by the ratio of $MgCO_3$ and $Mg(OH)_2$ was 2:1 and control (only $MgCO_3$) no significant in group with a p-value of 0.167 by Tukey and 0.061 by Scheffe method (Appendix D). Therefore, 3:1 was the optimal mass ratio of $MgCO_3$ and $Mg(OH)_2$ for succinic acid production.

5.5.10 Utilization of sweet sorghum straw hydrolysate as carbon source for succinic acid fermentation by *A. succinogenes* NP9-aA7

A. succinogenes NP9-aA7 was cultivated with various concentrations of SSH in the range of 20-80 g/L under anaerobic conditions in the incubator shaker at 37 $^{\circ}C$, 200 rpm for 48 h of cultivation times. Results were showed in Table 5.17 and Figure 5.11

Table 5.17 Effect of various concentrations of SSH on cell growth and succinic acid production by *A. succinogenes* NP9-aA7

SSH (g/L)	Residual glucose (g/L)	Glucose utilization (%)[a]	OD 660	Succinic (g/L)	Formic (g/L)	Acetic (g/L)	Succinic acid yield (g/g glucose)	Succinic acid productivity (g/L•h)[b]
20	0.253±[c]0.038	98.360±0.244	5.180±0.009	5.151±4.329	8.298±4.259	1.908±2.459	0.339±0.285	0.102±0.137
40	6.132±0.435	83.225±1.191	4.615±0.102	19.139±0.685	2.797±3.280	0.610±0.056	0.632±0.027	0.542±0.011
60	55.409±2.009	9.131±3.295	0.790±0.087	0.000±0.000	3.848±3.776	3.202±4.128	0.000±0.000	0.000±0.000
80	72.387±1.058	1.747±1.437	0.908±0.008	0.000±0.000	6.493±4.590	3.142±4.537	0.000±0.000	0.000±0.000
Control [c]	0.022±0.017	99.966±0.027	6.235±0.061	40.847±1.993	1.983±3.112	3.965±3.435	0.648±0.032	1.125±0.075

[a] Glucose utilization was defined as the percentage of glucose concentration utilized by the bacteria in initial glucose.

[b] Succinic acid was obtained from cells grown in anaerobic conditions for 36 h.

[c] ±, Standard deviation was calculated from triplicate samples.

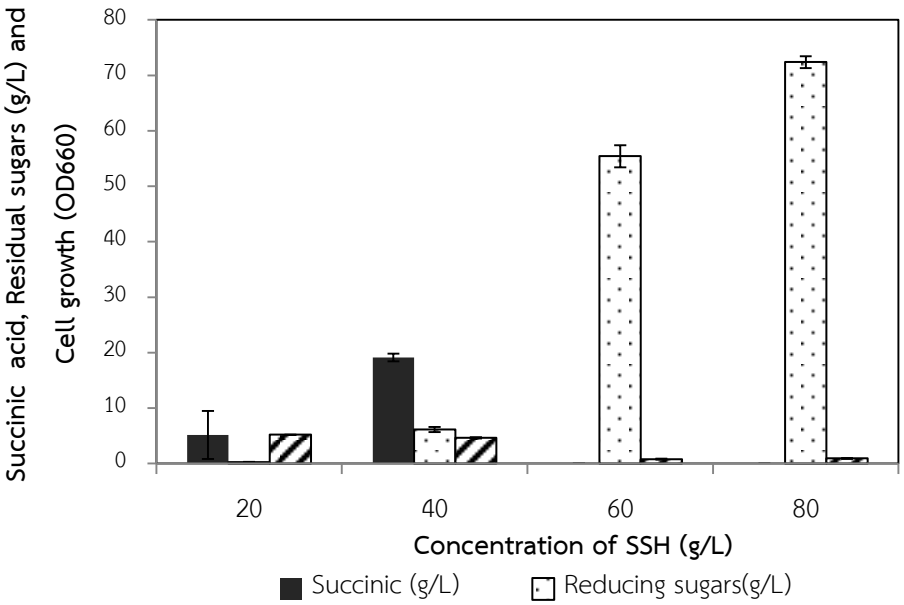

Figure 5.11 Effect of initial SSH concentration on organic acid production

It this result, can be seen that the cell growth of *A. succinogenes* NP9-aA7 and succinic acid production were examined when different concentrations of SSH were used as a carbon source. It was found to be able to grow in SSH in the range of 20-40 g/L. The maximum cell growth of (OD_{660}) was 4.615 and succinic acid was 19.139 g/L with a yield of 0.632 g/g substrate at initial 40 g/L of SSH concentration.

While a small level of growth was obtained when SSH was increased above 40 g/L, no succinic acid was detected.

Recently, many researchers have reported the effects of the inhibitory compounds in lignocellulosic hydrolysate on cell growth and succinic acid production. For instance, Wang et al.(Wang, Wang, Wang et al., 2013) studied inhibition effects of furfurals and 5-hydroxymethylfurfural (5-HMF) on cell growth and succinic acid production by engineered *Escherichia coli*. Cell growth and succinic acid production were severely inhibited by furfural and HMF with both concentrations higher than 0.8 g/L. while cell growth was completely inhibited when the concentration of furfural was over 6.4 g/L, or the concentration of HMF was over 12.8 g/L. At the concentration of maximum toleration, which was 3.2 g/L, furfural decreased cell growth by 77.8% and the succinic acid value by 36.1% while HMF decreased the cell growth by 13.6% and the succinic acid value by 18.3%. Activity measurements of key enzymes demonstrate that phosphoenolpyruvate carboxylase, malate dehydrogenase, fumarate reductase were all inhibited by furfural and HMF. In 2014 Wang et al. (Wang, Zhang, Zhang et al., 2014) studied the effects of inhibitory compounds in lignocellulosic hydrolysate on cell growth and succinic acid production which had been partially mitigated by the overexpression of the mgtA gene. Mg^{2+} might be transported more easily into the cells when the mgtA gene is overexpressed.

Lower succinic acid production from SSH as a carbon source may be due to the inhibiting components from the pretreated SSH, such as HMF and furfural. However, it was still able to produce succinic acid. Therefore, further study should examine the inhibition and detoxification effects of inhibitors in the pretreated SSH to optimize conditions and improve the production process.

Result of optimization succinic acid production by *A. succinogenes* from this section were summarized in the Table 5.18

Table 5.18 Summary of optimization condition for succinic acid production

Factors	Condition	Result	Succinic acid (g/L)	Control	Remark
Cell growth	Control medium (glucose 60g/L, yeast extract 30 g/L and $MgCO_3$ 30 g/L) was cultivated for 0-60 h	36 h was suitable cultivation time.	16.983	15.624[a]	-
PBD design	Glucose, Yeast extract, $CaCl_2$, $MgCl_2$, $MnCl_2$, Na_2HPO_4, NaH2PO4, $MgCO_3$ with 3 dummy variables	Glucose, Yeast extract, $MgCO_3$ were key factors.	27.380	19.054	Biotin was not added
Effect of biotin	Vary biotin 0-200 µg/L	The result was the same with adding biotin in the range 50-200 µg/L.	44.191	14.528 (no biotin)	-
Effect of nitrogen source	$(NH_4)_2SO_4$, KNO_3, NH_4Cl, Beef extract, CSL, Peptone, Urea and Yeast extract	30 g/L of yeast extract was the best nitrogen source.	49.756	49.756	-
Effect of alkaline neutralizer	Vary 10 g/L of NaOH, $NaHCO_3$, Na_2CO_3 $CaCO_3$, $Ca(OH)_2$, $Mg(OH)_2$ and $MgCO_3$	$MgCO_3$ was the best alkaline neutralizer.	35.755	9.826 (no alkaline)	-
RSM	Glucose 40-80 g/L Yeast extract 10-30 g/L $MgCO_3$ 20-60 g/L	Maximum succinic acid was obtained from glucose 74 g/L, yeast extract 30 g/L and $MgCO_3$ 60 g/L.	57.630	39.889	Optimum medium consisted of glucose 74g/L, yeast extract 30 g/L and $MgCO_3$ 60 g/L.
Vary ratio of alkaline neutralizer	RSM medium	Optimum ratio was 3:1 of $MgCO_3$:Mg_2OH	48.644	41.525	-
Validate Model	Six experiments represented for check the model.	F value = 2.948 sig. = 0.112 (>0.05 no significant)	60.087	-	This model suitable for predict succinic acid
Vary sugar from SSH	RSM medium	Optimum SSH concentration was 40 g/L	19.139	41.525	-

[a] Succinic acid was obtained from cells grown in anaerobic conditions for 60 h.

5.6 Conclusions

The growth performance of *A. succinogenes* NP9-aA7 producing the highest amount of succinic acid was 16.983 g/L at 36 h of cultivation time. Results from the one-factor-at-a-time method demonstrate that yeast extract and $MgCO_3$ were the best nitrogen source and alkaline neutralizer, respectively. The statistical method combining a Plackett-Burman Design (PBD) and a Box-Behnken Design (BBD) using Response Surface Methodology (RSM) were developed to optimize succinic acid production by *A. succinogenes* NP9-aA7. Key factors consisted of quantities of glucose of 74 g/L, yeast extract at 30 g/L and $MgCO_3$ of 60 g/L which produced a maximum of succinic acid to 60.087 g/L with a yield of 0.816 g/g of glucose after 36 h cultivation time under anaerobic conditions. The predicted concentration of succinic acid was 59.886 g/L. The result from the quadatic model showed the percentage errors between the actual and predicted values for succinic acid production varied from 0.335% to 4.515% were not significantly different. This result suggests that the combined method is a powerful way to optimize conditions for succinic acid production by *A. succinogenes* NP9-aA7. The use of a mixed alkaline neutralizer at a ratio of 3:1 of $MgCO_3$ to $Mg(OH)_2$ increases solubility with improved succinic acid production. In this case, using SSH as a carbon source for succinic acid production, a maximum cell growth (OD_{660}) of 4.615 and succinic acid production of 19.139 g/L with a yield of 0.632 g/g substrate at 40 g/L of SSH concentration were obtained. Further study should examine the inhibition and detoxification of inhibitors in pretreated SSH to optimize conditions and improve the efficiency of the production process to make it more advantageous when scaled up for succinic acid fermentation with *A. succinogenes* NP9-aA7.

Chapter VI

Scale up to two liter fermenter

Sukanya Phuengjayaem, Siriluk Teeradakorn

Manuscript of this chapter is under preparation.

6.1 Abstract

In the fermentation of succinic acid under the anaerobic condition, the increasing gas CO_2 has a significant effect on acid production because CO_2 was included into the backbone of three carbon compound to generate four carbon oxaloacetate through PEP carboxylase to increase succinic acid production. Result from this study indicated that CO_2 partial pressure with $MgCO_3$ has significant effect of on the production of succinic acid in batch fermentations in a 2-L fermenter. The optimum medium composition from previous study consist of 74 g/L of glucose, 30 g/L of yeast extract and 60 g/L of alkaline neutralizer (including 45 g/L of $MgCO_3$ and 15 g/L of $Mg(OH)_2$) could be improved the succinic acid production to 72.930 g/L with a yield of 1.393 g/g glucose at the CO_2 partial pressure of 50.66 kPa after 24 h of cultivation time. Similar to using SSH as a carbon source for succinic acid production under optimum condition in a 2-L fermenter, it could be improve the succinic acid production. The results obtained in this study may be useful for reducing the cost of succinic acid fermentation process.

Further study should be examined for the inhibition and detoxification of inhibitor in pretreated SSH for optimization condition and improvement to the process efficiency more advantageously then scaled-up succinic acid fermentation with *A. succinogenes* NP9-aA7.

Keyword: Carbon dioxide (CO_2), Succinic acid, Two L fermenter, Sorghum straw hydrolysate (SSH)

6.2 Introduction

Theoretically, succinic acid can be formed fermentative from glucose with following stoichiometry (Lee et al., 1999a):

$$Glucose + 2CO_2 + 4H \rightarrow 2Succinic\ acid + 2H_2O$$

Therefore, the supplies of CO_2 and electron donors are necessary to achieve good succinic acid production. The research explained that CO_2 could significantly affect the cell growth of anaerobic bacteria (Dehority, 1971; Ohta et al., 1989). Also, the level of CO_2 in culture medium could affect the metabolic fluxes and the distribution of fermentation products. In the fermentation of succinic acid under the anaerobic condition, the increasing gas CO_2 has a significant effect on acid production because CO_2 was included into the backbone of three carbon compound to generate four carbon oxaloacetate through PEP carboxylase to increase the production of succinic acid (Stols and Donnelly, 1997).

The pH of culture medium is important as it affects the solubility of CO_2 in the medium, and thus affects the availability of CO_2 for microorganisms. Because of the poor solubility of gaseous CO_2 at 1 atm, many kinds of carbonate and bicarbonate salts were employed as indirect CO_2 donor to enhance the dissolved CO_2 concentration in fermentation broth. $MgCO_3$ is a preferable carbonate because the addition of $MgCO_3$ would not lead to a radical change of culture pH, and an increase of Mg^{2+} concentration in fermentation broth showed little negative effect on the metabolism profile and morphology of succinic acid production strain (Liu et al., 2008). Some investigators tried to demonstrate the relationship between extra CO_2 donors and succinic acid production (Lee et al., 1999a; Song, Lee, Choi et al., 2007b; Van der Werf et al., 1997). However there were a few different features in physiological and biochemical characteristics among various kinds of succinic acid producing strains, and the current results were weak in promoting succinic acid production (Lee et al., 1999a; Song et al., 2007b).

These reason showed that CO_2 availability has a great impact on biomass and product formations. The pH is also a key factor because it affects the solubility of CO_2 in the medium, and as a consequence influences the availability of CO_2 for

micro-organisms. Because different micro-organisms can tolerate different CO_2 levels during the fermentation, the best CO_2 concentration should be obtained on an individual basis for each micro-organism and medium used (Cheng, Zhao, Zeng et al., 2012). Therefore, it was necessary to investigate the optimum of CO_2 partial pressure on the accumulation of succinic acid by *A. succinogenes* NP9-aA7 in a 2-L fermenter.

6.3 Material and Method

6.3.1 Anaerobic fermentation in a 2-L fermenter

The optimal of medium composition as above were verified in a 2-L fermenter (B.E. Marubishi Co., Inc., Thailand) containing 1.2 L of fermentation medium, as well as the initial carbon sources was 74 g/L of glucose or 40 g/L of SSH. The pH was controlled at 7.0 with 5 N NaOH and 5 N H_3PO_4 and temperature was maintained at 37 °C, 200 rpm with 10% (v/v) inoculated of preculture broth (***A. succinogenes*** DSMZ 22257 or NP9-aA7) and external CO_2 gas sparging rate was 1.0 vvm. CO_2 and N_2 were from linde industrial gases, Thailand (Linde, Thailand). Foam was controlled by adding Antifoam 289 (Sigma Chemical Co., St. Louis, MO). Samples were taken every 3 h for 48 h for analysis of the cell growth was monitored by measuring the absorbance at 660 nm (OD660), residual glucose by DNS method and the products by HPLC.

6.3.2 Effect of CO_2 partial pressure with $MgCO_3$ on cell growth and succinic acid production by *A. succinogenes* NP9-aA7 in a 2-L fermeter

The significance of gaseous CO_2 partial pressure on succinic acid production was studied by various CO_2 partial pressures at 25.33, 50.66, 75.99 and 101.33 kPa (100% CO_2 gas) and the corresponding dissolved CO_2 concentration in the fermentation broth was 5.05, 10.11, 15.16, and 20.22 mM. $MgCO_3$ was added to the broth after a separate sterilization before the inoculation.

6.3.3 Utilization of sweet sorghum straw hydrolysate as carbon source for succinic acid fermentation by NP9-aA7

The optimum of medium composition as above and 40 g/L of SSH (optimum concentration for succinic acid production from flask scale) were added. The

external CO_2 partial pressure at 50.33 kPa gas sparging rate was 1.0 vvm. The system was operated according above experiment. Samples were taken every 3 h for 60 h for analysis of the cell growth by spectrophotometer (OD660), residual glucose by DNSA method and the products by HPLC.

6.4 Analytical

6.4.1 Cell concentration

The insoluble $MgCO_3$ in the sample was removed by add 0.2 M HCl. Then the cell concentration was measured as optical density at wavelength 660 nm using a spectrophotometer (UV160, Shimadzu Corporation, Japan) (Zheng et al., 2009).

6.4.2 Reducing sugars

The reducing sugars in the sorghum straw hydrolysate (SSH) was measured by DNS (3, 5-dinitrosalicylic acid colorimetric) method applied from Miller (1959), with D-glucose as the standard. The mixture contained; 50 µl of sample and 150 µl of DNS reagent were heated in a boiling water bath for 10 min. Then cooled immediately on ice bath and added 1 mL of distilled water. The absorbance was measured by spectrophotometer at 540 nm.

6.4.3 High-performance liquid chromatography (HPLC)

Fermentation products (succinic, acetic and formic acid) were analyzed with HPLC. Twenty microliter of sample were filtered (0.45 Am, 13 mm membrane disc filters) and loaded on HPLC using a system equipped with a cation-exclusion column (Aminex HPX-87H; 300 mm 7.8 mm; Bio-Rad Chemical) and a refractive index detector (Shimadzu Model RID-6A). The mobile phase was 5 mM of H_2SO_4 solution at a flow rate of 0.6 mL/min with the column operated at 55 °C.

6.4.4 Statistical analysis

All experiments were tested in triplicate and the related data were expressed as averages values. Statistical Package for the Social Sciences (SPSS) for Windows program version 15 was used to analysis the data from optimization condition for succinic acid production by *A. sucinogenes* NP9-aA7 in a 2-L fermenter. Effect of CO_2 partial pressure and cultivation times were examined.

6.5 Results and discussion

6.5.1 Succinic acid fermentation by *A. succinogenes* strain DSMZ 22257 and NP9-aA7 using control medium in a 2-L fermenter

Based on the above results obtained in the anaerobic flask, a batch fermentation of glucose as a carbon source was also carried out in a 2-L fermenter. The result in Table 6.1 indicated that organic acid production and sugar utilization in fermenter were consistent with those in the anaerobic flask.

Table 6.1 Fermentation results of *A. succinogenes* DSMZ 22257 in a 2-L fermenter

Cultivation time (h)	Succinic acid (g/L)	Formic acid (g/L)	Acetic acid (g/L)	Residual total sugar (g/L)	Sugar utilization (%)	Succinic acid yield (%)
0	-	-	-	60.002	0.000	0.000
6	30.228	2.345	4.434	31.551	47.417	50.381
12	46.001	2.016	6.493	20.168	66.388	76.668
18	45.553	3.508	7.593	8.427	74.462	75.922
24	45.573	4.634	7.764	15.324	74.462	75.954
36	44.088	5.558	7.625	11.943	80.095	73.480
48	43.988	6.033	7.348	9.952	83.413	73.313

As shown in Table 6.1, succinic acid concentration increased with extent cultivation time from 0 to 12 h. After 12 h of cultivation time, it had no distinct change succinic acid yield. While sugar utilization was slightly increased from 47.417% to 83.413% from 6 h to 48 h of cultivation time, respectively. In addition, formic and acetic acid concentrations were continuously increased with extent cultivation time from 0-48 h.

Table 6.2 Fermentation results of *A. succinogenes* NP9-aA7 using control medium in a 2-L fermenter

Time (h)	Residual glucose (g/L)	Glucose utilization (%)[a]	OD 660	Succinic (g/L)[b, c]	Formic (g/L)[c]	Acetic (g/L)[c]	Succinic acid yield (g/g glucose)	Succinic acid productivity (g/L*h)
0	61.462±1.062	4.709±8.156	1.213±0.049	0.804±0.020	2.103±0.195	0.294±0.026	0.025±0.043	0.000±0.000
3	54.345±4.674	19.248±17.046	2.352±0.062	3.420±0.083	3.624±0.336	0.944±0.085	0.108±0.096	1.187±0.028
6	54.381±2.609	26.762±5.455	4.597±0.028	8.618±0.210	5.263±0.488	2.517±0.226	0.462±0.091	1.496±0.035
9	44.857±3.946	39.497±6.639	11.525±0.205	18.530±0.451	7.894±0.732	6.234±0.559	0.666±0.096	2.144±0.050
12	39.178±2.591	47.022±4.635	12.030±0.015	22.472±0.547	8.380±0.777	7.096±0.636	0.674±0.101	1.950±0.046
15	33.355±6.144	54.747±8.421	12.260±0.122	32.311±0.787	8.380±0.777	9.075±0.813	0.845±0.203	2.243±0.052
18	28.646±1.019	61.113±0.492	11.767±0.031	32.547±0.793	9.296±0.862	9.727±0.872	0.744±0.057	1.883±0.044
21	28.323±3.050	61.516±5.532	9.380±0.072	36.279±0.883	9.375±0.869	10.449±0.937	0.827±0.070	1.799±0.042
24	20.523±2.200	71.896±3.876	8.773±0.479	37.394±0.911	9.058±0.840	10.582±0.949	0.729±0.074	1.622±0.038
27	0.200±0.025	99.027±1.230	7.213±0.255	40.384±0.983	9.667±0.896	11.159±1.000	0.570±0.036	1.557±0.036
30	0.229±0.052	98.988±1.156	5.540±0.046	40.365±0.983	9.448±0.876	11.374±1.020	0.570±0.036	1.401±0.033
33	0.170±0.008	99.066±1.221	5.500±0.062	37.413±0.911	8.349±0.774	10.122±0.907	0.528±0.033	1.180±0.028
36	0.173±0.006	99.064±1.229	5.570±0.580	39.134±0.953	8.598±0.797	10.562±0.947	0.552±0.034	1.132±0.026
39	0.164±0.002	99.075±1.226	5.420±0.477	39.134±0.953	8.598±0.797	10.562±0.947	0.552±0.034	1.045±0.024
42	0.160±0.006	99.080±1.228	5.780±0.165	37.822±0.921	7.722±0.716	9.210±0.826	0.533±0.033	0.938±0.022
45	0.162±0.003	99.077±1.228	5.720±0.062	37.822±0.921	7.722±0.716	9.210±0.826	0.533±0.033	0.875±0.020
48	0.165±0.008	99.074±1.233	5.980±0.096	38.207±0.930	7.747±0.718	9.337±0.837	0.539±0.034	0.829±0.019

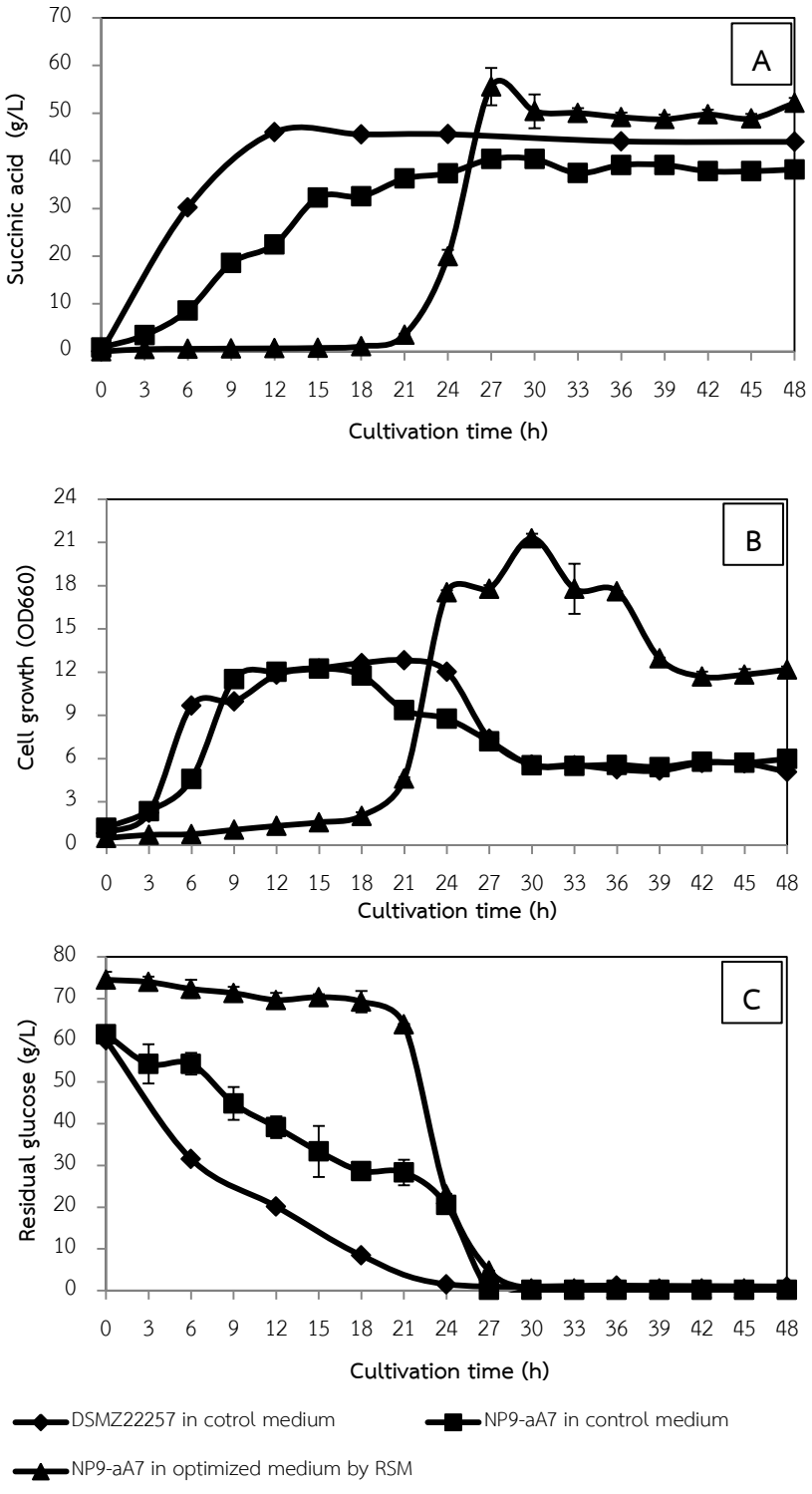

Figure 6.1 The comparison succinic acid fermentation by *A. succinogenes* DSMZ 22257 and NP9-aA7 in a 2-L fermenter. (A) the succinic acid production, (B) cell growth and (C) the sugar consumption.

The result of fermentation using glucose as a carbon source in a 2-L fermenter by *A. succinogenes* strain DSMZ 22257 and NP9-aA7 were showed in Figure 6.1. The results were shown that cell growth of DSMZ 22257 reached a maximum OD660 of 9.690 at 12 h, and then entered a stationary phase, in which cell density increased slowly to 12.826 at 21 h after that OD660 was declined slowly from 24h to 48 h of cultivation time. Corresponding to the maximum succinic acid of 46.001 g/L was obtained at 12 h of cultivation times while glucose rapidly decreased from 0-12 h. After 12 h, there was no significant change of succinic acid concentration. However, the concentrations of acetic acid and formic acid were appreciably higher compared with long cultivation time, which led to a slight decrease in the succinic acid yield from 76.668% to 73.313%. These results indicated that at 12 h of cultivation time was optimum for succinic acid fermentation by *A. succinogenes* with a high succinic acid and low concentration of byproducts.

According the research of Zheng et al. (2009), optimization condition of succinic acid by *A. succinogenes* CGMCC1593 was studied. The results were shown that cell growth reached a maximum OD660 of 7.9 at 12 h, and then entered a steady phase, in which cell density declined slowly. Succinic acid concentration continuously increased until the sugar was depleted at 12 h. At the end fermentation (48 h), the concentration and yield of succinic acid reached 45.5 g/1 and 80.7%, respectively.

Compared with *A. succinogenes* NP9-aA7 that produced 40.384 g/L of succinic acid at 27 h with a yield of 0.802 g/L (Table 6.2) closely to succinic acid was produced from strain DSMZ 22257. Strain NP9-aA7 could be performed to efficiently produce succinic.

The phenomenon of *A. succinogenes* NP9-aA7 in control medium, the cell growth was maximum OD660 of 11.525 at 9 h. Then cell growth entered a stationary phase to 18 h, in which cell density increased slowly to 12.260 at 15 h after 21 h OD660 was declined slowly until 30 h of cultivation times cell entered a steady. Similarly to the maximum succinic acid of 40.384 g/L was obtained at 27 h of cultivation times while glucose was decreased rapidly within 18 h then was

completely utilized at 27 h of cultivation times. However, at the same time for maximum succinic acid NP9-aA7 gave a high concentration of acetic acid and formic acid were 11.159 and 9.667, respectively. It was a compelling reason to do that we have to add the concentration of succinic acid with low concentration of by product.

6.5.2 Succinic acid fermentation by *A. succinogenes* NP9-aA7 using optimized medium in a 2-L fermenter

Succinate production was scaled up to the working volume of 1.2 L in a 2-L fermenter, with 10 % of at OD660 of 0.7. The result in Table 6.3 indicated that organic acid production and sugar utilization in fermenter were consistent medium with those in optimum from the anaerobic flask.

Table 6.3 Fermentation results of *A. succinogenes* NP9-aA7 using optimized medium in a 2-L fermenter

Time (h)	Residual glucose (g/L)	Glucose utilization (%)[a]	OD 660	Succinic (g/L)[b, c]	Formic (g/L)[c]	Acetic (g/L)[c]	Succinic acid yield (g/g glucose)	Succinic acid productivity (g/L•h)
0	74.509±1.880	0.000±0.000	0.488±0.036	0.000±0.000	1.515±0.140	0.000±0.000	0.000±0.000	0.000±0.000
3	73.970±1.307	0.752±0.854	0.713±0.051	0.488±0.034	1.577±0.146	0.181±0.016	0.485±0.544	0.163±0.004
6	72.317±2.160	2.927±2.543	0.763±0.025	0.553±0.039	2.278±0.211	0.187±0.017	1.748±2.714	0.092±0.002
9	71.310±1.526	4.255±2.567	1.063±0.015	0.618±0.044	4.109±0.381	0.204±0.018	0.301±0.263	0.069±0.002
12	69.657±1.735	6.462±0.268	1.333±0.068	0.662±0.047	4.139±0.384	0.249±0.022	0.138±0.016	0.055±0.001
15	70.340±0.693	5.517±3.289	1.583±0.025	0.741±0.052	4.179±0.387	0.373±0.033	0.221±0.102	0.049±0.001
18	69.262±2.555	6.972±2.616	2.033±0.245	1.094±0.077	6.146±0.570	0.507±0.045	0.242±0.126	0.061±0.001
21	63.798±0.165	14.249±2.234	4.623±0.081	3.503±0.247	7.973±0.739	1.783±0.160	0.335±0.053	0.167±0.004
24	23.075±0.108	68.534±1.297	17.547±0.142	19.956±1.406	1.005±0.093	4.806±0.431	0.391±0.026	0.832±0.019
27	4.759±0.025	92.950±1.232	17.790±0.246	58.080±3.277	15.392±1.427	6.807±0.610	0.839±0.055	2.151±0.149
30	0.479±0.018	98.655±1.205	21.290±0.303	50.398±3.552	13.974±1.296	6.252±0.560	0.685±0.043	1.680±0.039
33	0.472±0.016	98.665±1.208	17.780±1.733	50.043±1.001	14.548±1.349	6.382±0.572	0.681±0.012	1.516±0.037
36	0.473±0.015	98.663±1.207	17.600±0.046	49.170±0.983	14.309±1.327	6.331±0.567	0.669±0.012	1.366±0.033
39	0.528±0.054	98.590±1.217	12.950±0.062	48.724±0.974	13.966±1.295	6.320±0.567	0.663±0.012	1.249±0.031
42	0.518±0.052	98.605±1.189	11.690±0.346	49.723±0.994	14.033±1.301	6.150±0.551	0.677±0.012	1.184±0.029
45	0.488±0.020	98.643±1.207	11.830±0.381	48.887±0.978	13.941±1.292	6.210±0.557	0.665±0.012	1.086±0.027
48	0.494±0.022	98.636±1.203	12.180±0.208	52.135±1.043	14.903±1.382	6.682±0.599	0.709±0.012	1.086±0.027

Based on the above results, batch fermentation was implemented in a 2-L. At 27 h, concentrations of succinic, formic and acetic acids were 55.576 g/L, 15.392 g/L, and 6.807 g/L, respectively. This result could be validated the model of optimum medium composition for succinic acid production from base flask scale (Table 6.3).

Correspondingly, the optimal point of the model at 74 g/L of glucose, 30 g/L of yeast extract and 60 g/L of $MgCO_3$ were obtained. The optimal medium gave the actual maximum succinic acid was 60.087 g/L and predicted maximum of succinic acid was 59.886 g/L. the model was validated in the fermentation resulting in the highest succinic acid concentration and yield at 27 h reached 58.080 g/L with a yield of 0.839 g/g glucose. The result shows that there was no significant difference between actual and predicted value (3.02%), indicate that the actual values obtained was in good agreement with the predictions of of the quadratic regression model. Therefore, the model was suitable for predicting succinic acid production.

However, the succinic acid production can occur with cultivation time less than flask scale, while the cell growth higher than flask scale. This result might be due to in the fermentation has been operated the system throughout fermentation such as aeration rate and pH controller which the culture pH value was one of the key factors on the cell growth and the production of succinic acid.

6.5.3 Effect of CO_2 partial pressure with $MgCO_3$ on cell growth and succinic acid production by *A. succinogenes* NP9-aA7 in a 2-L fermenter

The effect of the supply of gaseous CO_2 on the fermentation process was studied by adding various the CO_2 partial pressures of 25.33, 50.66, 75.99, and 101.33 kPa, respectively. The other culture conditions were the same as the above experiments. The result of glucose utilization, cell growth and organic acid production were showed in Table 6.4-6.7.

Table 6.4 The CO_2 partial pressure of 25.33 kPa in anaerobic fermentation of succinic acid

Time (h)	Residual glucose (g/L)	Glucose utilization (%)[a]	OD 660	Succinic (g/L)[b, c]	Formic (g/L)[c]	Acetic (g/L)[c]	Succinic acid yield (g/g glucose)	Succinic acid productivity (g/L*h)
0	73.719±1.374	1.446±1.837	1.772±0.015	0.273±0.578	0.642±1.113	0.088±0.153	1.492±2.584	0.000±0.000
3	74.258±3.022	0.725±4.040	2.146±0.182	1.748±0.315	0.590±0.502	0.586±0.143	1.573±2.969	0.561±0.105
6	68.148±2.810	8.894±3.756	11.304±0.165	5.278±0.396	1.809±0.150	1.884±0.375	0.918±0.474	0.870±0.066
9	59.917±0.795	19.898±1.063	16.476±0.055	5.703±8.065	2.669±2.334	2.551±2.218	0.528±0.458	0.853±0.896
12	47.480±1.464	36.524±1.957	18.876±0.422	18.529±2.428	6.754±1.657	7.387±2.370	0.708±0.109	1.603±0.202
15	40.975±0.323	45.221±0.432	18.348±0.327	23.452±1.030	2.564±3.277	6.994±0.545	0.691±0.016	1.558±0.069
18	43.059±0.623	42.434±0.832	18.132±0.397	26.160±1.035	5.716±0.465	5.165±1.188	0.822±0.026	1.449±0.058
21	34.218±0.224	54.255±0.300	17.964±0.389	31.030±2.205	6.008±0.508	7.018±0.681	0.757±0.041	1.462±0.105
24	0.637±0.008	99.149±0.010	12.072±1.300	53.478±3.927	12.498±3.151	14.935±2.997	0.739±0.048	2.283±0.164
27	0.644±0.009	99.139±0.012	13.224±0.198	49.655±5.267	3.106±1.501	2.050±3.551	0.691±0.063	1.899±0.195
30	0.645±0.004	99.138±0.006	13.872±0.055	47.821±2.823	8.985±0.904	7.155±5.302	0.658±0.036	1.628±0.094
33	0.701±0.030	99.063±0.041	12.876±0.385	43.633±0.741	7.813±0.625	9.551±0.600	0.595±0.013	1.337±0.022
36	0.629±0.013	99.159±0.018	10.740±0.150	45.000±1.380	8.384±0.674	11.019±0.685	0.606±0.013	1.249±0.038
39	0.625±0.006	99.165±0.008	10.380±0.075	45.561±2.073	8.032±0.752	10.231±0.822	0.612±0.020	1.163±0.053
42	0.623±0.007	99.167±0.010	9.612±0.343	44.755±0.863	8.963±1.133	10.851±0.732	0.610±0.015	1.078±0.021
45	0.617±0.003	99.174±0.004	7.704±0.072	47.771±0.565	10.255±1.820	13.378±2.373	0.650±0.012	1.072±0.013
48	0.623±0.002	99.167±0.003	7.560±0.095	57.205±4.350	12.958±2.988	15.731±2.881	0.791±0.053	1.222±0.091

Table 6.5 The CO_2 partial pressure of 50.66 kPa in anaerobic fermentation of succinic acid

Time (h)	Residual glucose (g/L)	Glucose utilization (%)[a]	OD 660	Succinic (g/L)[b, c]	Formic (g/L)[c]	Acetic (g/L)[c]	Succinic acid yield (g/g glucose)	Succinic acid productivity (g/L*h)
0	72.209±1.085	2.420±1.467	0.413±0.008	0.000±0.000	0.000±0.000	0.000±0.000	0.000±0.000	0.000±0.000
3	63.834±6.149	13.737±8.310	0.450±0.035	1.665±0.367	1.382±0.862	0.117±0.203	0.177±0.125	0.555±0.104
6	61.858±4.478	16.409±6.051	0.507±0.010	0.974±0.626	1.257±0.954	0.115±0.200	0.034±0.010	0.162±0.087
9	61.822±2.886	16.457±3.900	1.382±0.006	1.454±0.111	1.621±1.304	0.487±0.170	0.115±0.030	0.162±0.011
12	61.822±2.886	16.457±3.900	8.292±0.309	4.781±0.082	2.324±0.554	1.945±0.673	0.416±0.107	0.398±0.005
15	44.677±6.942	39.626±9.382	32.496±0.670	21.485±0.029	7.264±0.663	8.692±3.623	0.763±0.200	1.432±0.018
18	27.424±1.163	62.940±1.572	43.212±0.021	45.374±0.710	13.006±1.618	16.069±6.916	0.989±0.024	2.521±0.031
21	24.261±2.990	67.214±4.041	43.524±0.072	65.787±3.314	15.283±1.799	19.824±8.702	1.389±0.086	3.133±0.115
24	21.781±3.642	70.566±4.922	37.572±0.525	72.930±0.380	5.790±7.349	20.661±8.712	1.393±0.093	3.039±0.044
27	0.377±0.007	99.491±0.009	27.387±0.180	72.888±0.452	14.812±2.375	19.101±8.185	0.914±0.000	2.477±0.025
30	0.398±0.003	99.463±0.004	28.092±0.583	72.317±1.032	16.538±3.036	6.992±7.944	0.995±0.000	2.411±0.028
33	0.387±0.011	99.476±0.015	27.996±0.162	67.112±2.470	14.972±2.806	19.706±8.865	0.917±0.000	1.976±0.054
36	0.385±0.013	99.480±0.018	27.180±0.225	67.714±3.273	15.623±3.015	29.397±17.642	0.962±0.000	1.881±0.066
48	0.376±0.018	99.492±0.024	28.958±0.895	54.995±4.171	12.353±2.324	6.077±5.412	0.800±0.000	1.146±0.065

Table 6.6 The CO_2 partial pressure of 75.99 kPa in anaerobic fermentation of succinic acid

Time (h)	Residual glucose (g/L)	Glucose utilization (%)[a]	OD 660	Succinic (g/L)[b, c]	Formic (g/L)[c]	Acetic (g/L)[c]	Succinic acid yield (g/g glucose)	Succinic acid productivity (g/L*h)
0	72.461±2.241	2.331±3.021	0.470±0.013	0.000±0.000	0.000±0.000	0.000±0.000	0.000±0.000	0.000±0.000
3	72.173±2.552	2.719±3.440	0.820±0.073	0.000±0.000	0.000±0.000	0.000±0.000	0.000±0.000	0.000±0.000
6	70.196±2.366	9.065±3.189	0.820±0.073	0.758±0.013	1.355±1.075	0.281±0.059	0.159±0.070	0.126±0.054
9	71.167±6.350	4.075±8.559	0.473±0.010	0.731±0.012	1.212±0.965	0.304±0.068	-0.927±0.880	0.081±0.035
12	63.080±5.791	14.976±7.805	0.482±0.014	0.584±0.009	1.009±0.792	0.178±0.062	0.074±0.030	0.049±0.017
15	64.194±2.412	13.474±3.251	0.588±0.075	0.581±0.009	1.089±0.794	0.228±0.081	0.073±0.017	0.039±0.013
18	64.661±2.288	12.844±3.084	0.652±0.018	0.718±0.010	1.261±0.909	0.351±0.198	0.088±0.024	0.040±0.007
21	61.606±1.436	16.962±1.935	3.757±0.010	2.722±0.037	1.762±0.646	0.871±0.067	0.223±0.024	0.130±0.003
24	48.271±0.486	34.936±0.655	16.383±0.982	10.420±0.135	3.673±0.343	3.213±0.340	0.397±0.007	0.434±0.021
27	0.157±0.010	99.789±0.013	35.680±0.121	39.922±0.526	11.826±1.262	11.149±0.862	0.542±0.000	1.479±0.026
30	0.144±0.022	99.805±0.030	36.050±0.288	55.403±0.728	12.594±1.020	11.487±1.262	0.751±0.000	1.847±0.039
33	0.214±0.054	99.711±0.072	36.800±0.418	59.125±0.809	13.248±2.123	12.663±1.973	0.835±0.001	1.792±0.093
36	0.168±0.004	99.773±0.005	35.387±0.061	58.529±0.781	13.052±1.710	12.418±1.526	0.805±0.000	1.626±0.019
48	0.155±0.007	99.791±0.010	27.680±0.040	57.141±0.777	12.250±1.358	12.225±2.146	0.801±0.000	1.190±0.049

Table 6.7 The CO_2 partial pressure of 101.33 kPa in anaerobic fermentation of succinic acid

Time (h)	Residual glucose (g/L)	Glucose utilization (%)[a]	OD 660	Succinic (g/L)[b, c]	Formic (g/L)[c]	Acetic (g/L)[c]	Succinic acid yield (g/g glucose)	Succinic acid productivity (g/L*h)
0	70.951±0.187	4.120±0.252	0.462±0.019	0.000±0.000	0.000±0.000	0.000±0.000	0.000±0.000	0.000±0.000
3	68.615±0.553	7.277±0.748	0.902±0.400	0.636±0.547	0.997±1.451	2.388±3.797	0.121±0.101	0.212±0.182
6	65.632±2.117	11.309±2.860	0.945±0.160	0.415±0.327	1.811±0.695	1.503±2.362	0.056±0.051	0.069±0.054
9	63.179±0.062	1.109±0.084	1.108±0.150	0.219±0.053	0.907±0.726	0.098±0.057	0.275±0.044	0.024±0.006
12	61.275±1.979	3.683±2.675	1.115±0.148	0.419±0.116	0.868±0.705	0.138±0.051	0.237±0.164	0.035±0.010
15	62.684±3.052	15.292±4.125	1.480±0.090	2.118±0.106	1.952±1.640	0.728±0.156	0.207±0.072	0.141±0.007
18	55.639±3.742	24.812±5.056	13.410±0.090	19.562±0.372	5.992±0.487	5.747±0.316	1.133±0.200	1.087±0.021
21	21.673±0.389	70.711±0.525	31.060±0.105	42.738±1.754	11.137±1.946	11.213±0.819	0.848±0.036	2.035±0.084
24	16.030±0.249	78.337±0.337	40.947±3.511	53.462±1.168	13.571±0.755	12.393±0.924	0.957±0.021	2.228±0.049
27	0.302±0.009	99.592±0.013	34.720±0.280	63.583±2.846	14.895±2.738	13.891±1.326	0.895±0.042	2.355±0.105
30	0.323±0.013	99.563±0.018	31.653±0.566	65.847±0.391	12.402±1.083	3.519±6.095	0.787±0.003	1.862±0.013
33	0.313±0.001	99.576±0.002	32.733±0.600	62.638±2.752	11.734±1.810	11.520±1.288	0.741±0.040	1.595±0.083
36	0.317±0.014	99.572±0.019	32.533±0.046	59.235±3.796	13.289±2.180	12.876±1.550	0.834±0.055	1.645±0.105
48	0.321±0.015	99.567±0.020	27.720±0.080	61.113±1.739	14.046±1.327	4.738±8.207	0.861±0.026	1.273±0.036

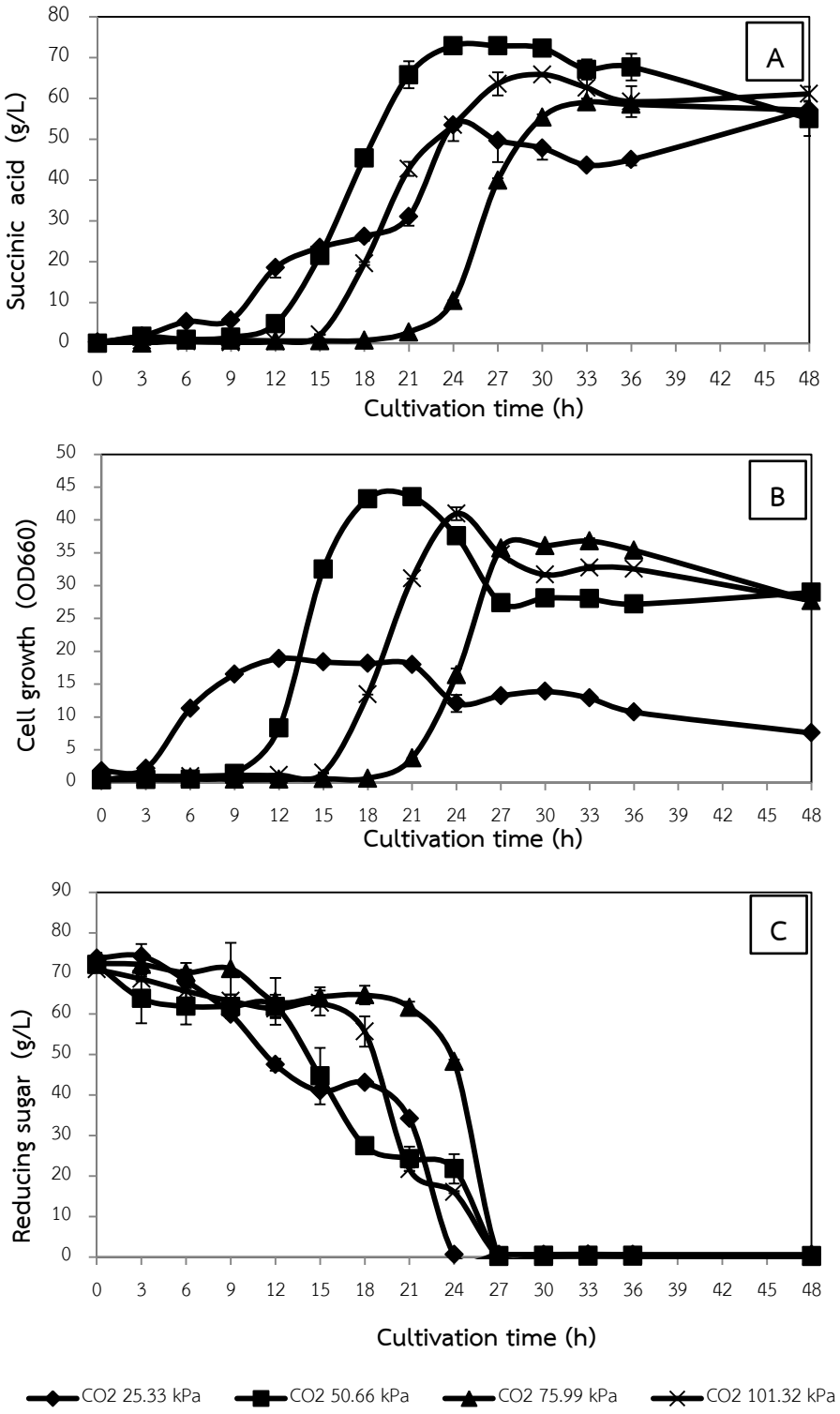

Figure 6.2 Effect of the supply of gaseous CO_2 on succinic acid production by *A. succinogenes* NP9-aA7. (A) the succinic acid production, (B) cell growth and (C) the sugar consumption.

Figure 6.2 illustrated that when CO_2 adding in the fermentation result of succinic acid was increased. A maximum succinic acid was 53.462 g/L from CO_2 at 25 kPa after 24 h of cultivation times. The lag phase of cell growth was observed for 3 h of cultivation time. The log phase of cell growth was in the range of 3-12 h, a maximum of cell growth at OD660 of 18.876 then into stationary phase within 12-24 h. After that the cell growth was slightly decreased to 7.560 (OD_{660}) at 48 h of cultivation times while the production of succinic acid was appeared on the end of the stationary phase (24 h of cultivation times).

When the pressure of CO_2 was increased to 50.66 kPa investigated that the succinic acid was accumulated. The maximum succinic acid of 72.930 g/L with a yield of 1.393 g/g glucose was obtained after 27 h of cultivation times. The succinic acid was increased by 36.44%. According to the maximum of cell growth (OD_{660}) was increased to 43.212 at 18 h of cultivation times. Clearly demonstrated that the increasing of CO_2 has effect on cell growth and succinic acid production however, the lag phase of cell growth was longer than low pressure of CO_2. The longer lag period was found in 75.99 kPa and 101.32 kPa and succinic acid production was 65.847 and 59.235 g/L from 101.32 and 75.99 kPa of CO_2, respectively because different micro-organisms can tolerate different CO_2 levels during the fermentation, the best CO_2 concentration should be obtained on an individual basis for each micro-organism and medium used (Cheng et al., 2012). Result from this study investigated that optimal CO_2 at 50.66 kPa adding in the medium stimulated to produce succinic acid.

There were reported about CO_2 could strongly effect the metabolic flux of carbon and the activities of phosphoenolpyruvate (PEP) carboxykinase, which were the important committed steps for the biosynthesis of succinic acid (McKinlay and Vieille, 2008). The quantitative determination of the dissolved CO_2 concentration in the fermentation broth was beneficial to study the impact of CO_2 partial pressure on the production of succinic acid. Song et al. (2007b) and (Lee et al., 1999a) reported that succinic acid production could be enhanced by increasing CO_2 partial pressure in the fermentation of *M. succiniciproducens* and *A. succiniciproducens.*

Zou et al. (2011) studied the effect of CO_2 on production of succinic acid by *A. succinogenes* ATCC 55618. When the CO_2 partial pressures were 25.33, 50.66, 75.99, and 101.33 kPa, the dissolved CO_2 concentrations in the fermentation broth was 5.05, 10.11, 15.16, and 20.22 mM, respectively. The succinic acid productions were 8.84, 10.21, 10.44, and 10.97 g/L as obtained on 48 h at the CO_2 partial pressure of 25.33, 50.66, 75.99, and 101.33 kPa, respectively, and its corresponding productivities were 0.18, 0.21, 0.22, and 0.23 g/L·h. These indicated that when gaseous CO_2 was used as the sole CO_2 donor, the available dissolved CO_2 concentration was not high enough to increase the production of succinic acid in the fermentation of *A. succinogenes*.

On the contrary, as reported by Lu, Eiteman and Altman (2009) and Samuelov et al. (1991), a higher available CO_2 concentration could cause higher succinic acid production by increasing the activity of PEP carboxykinase.

In addition, Zou et al. (2011) reported that the production of succinic acid reached 56.14 and 60.38 g/L after 72 h when 40 g/L $MgCO_3$ was used as the only CO_2 donor and 40 g/L $MgCO_3$ was supplied at the CO_2 partial pressure of 101.33 kPa, and the corresponding productivity was 0.80 and 0.84 g/L·h. The succinic acid production was just decreased by 7.03% without the supply of gaseous CO_2. Similar, the patterns of acetic acid production was increase with increasing CO_2 partial pressures. The concentrations of other by-products such as formic acid, lactic acid and ethanol were relatively constant. And there was no significant effect on the cell growth whether gaseous CO_2 was used. They were explained that $MgCO_3$ may be used as indirect CO_2 molecule donor to promote the production of succinic acid in the fermentation process of *A. succinogenes* and the dissolved concentrations of HCO_3^-, CO_3^{2-} and CO_2 could be enhanced with the addition of $MgCO_3$ in the fermentation broth. However, $MgCO_3$ may not be used as $CO3^{2-}$ donor because there were few reports that CO_3^{2-} could be used directly as substrate by succinic acid producing microorganisms. HCO_3^- and CO_2 could be used as the co-substrate of PEP carboxylase and improve the production of succinic acid (Samuelov et al., 1991), HCO_3^- was much less permeable to lipid cell membrane than the uncharged CO_2 molecule because was a kind of

polar molecular and there was no HCO_3^- transporter on the membrane of *A. succinogenes* which could deliver HCO_3^- from the broth into the cell (Badger and Price, 2003). So the higher concentration of HCO_3^- could not promote the production of succinic acid. On the other hand, when the levels of dissolved CO_2 reached 159.22 mM, there would be insoluble $MgCO_3$ that could cause turbid broth. The cells were spread uniformly in the broth, which was helpful to eliminate the cell flocculation and indirectly promoting the succinic acid biosynthesis.

Lee et al. (1999a) studied the effect of CO_2 aeration at different pH on cell growth and succinate formation using *A. succiniciproducens* were investigated. Succinate yield increased from 0.84 g/g substrate without CO_2 supply to 0.88 g/g substrate with CO_2 supply at pH 6.5. At pH 7.2, both dry cell weight and succinate yield decreased sharply. They concluded that different succinate yields were due to different CO_2 solubility at different pH. It was reasonable to explain succinate yield increase with CO_2 aeration at pH 6.5. Different to succinate yield, biomass was adversely affected by CO_2 aeration. These phenomena suggest that CO_2 has selective inhibition on metabolism by altering intracellular enzymatic activities.

Song et al. (2007b) studied the effect of the CO_2 availability on biomass formation and succinate fermentation under various CO_2 partial pressures were investigated in batch culture using *M. succiniciproducens* MBEL55E. Biomass formation was strongly inhibited at low CO_2 availability. Biomass formation and succinate production enhanced in proportion as CO_2 availability improved. The increasing of CO_2 availability in the medium, batch cultures were fulfilled with varied concentrations of $NaCO_3$, $MgCO_3$, or $CaCO_3$ implement as an additional CO_2 source. When 119 mM of $MgCO_3$ corresponding 141 mM dissolved CO_2 concentration was added, biomass formation and succinate fermentation were further increased. Compared with the yields of biomass and succinate at dissolved CO_2 concentration of 8.74 mM, the yields of succinic acid increased at dissolved CO_2 concentration of 141 mM by 49% and 52%, respectively. However, biomass formation and succinic acid production were inhibited to some degree in the media with 238 mM $MgCO_3$ corresponding to dissolved CO_2 concentration of 163 mM.

It could be concluded that the higher dissolved CO_2 concentration was beneficial for the succinic acid biosynthesis. But the dissolved CO_2 concentration was not the only factor affecting succinic acid synthesis. The fermentations were conducted by adding $MgCO_3$ to enhance the dissolved CO_2 concentration. However, it has necessary to define optimum condition of CO_2 and $MgCO_3$ for succinic acid production. In this research, gaseous CO_2 was used with $MgCO_3$ necessary to use for control the pH resulting in more effective on promoting the succinic acid production. Optimal conditions for succinic acid production were CO_2 of 50.66 kPa with 60 g/L as an alkaline neutralizer.

6.5.4 Utilization of sorghum straw hydrolysate as carbon source for succinic acid fermentation by *A. succinogenes* NP9-aA7

Succinic acid production was scaled up to the working volume of 1.2 L in a 2-L fermenter, with the optimal SSH concentration of 40 g/L and an initial cell concentration at OD_{660} of 0.7. The other culture conditions were the same as the above experiments. The result of glucose utilization, cell growth and organic acid production were showed in table 6.7.

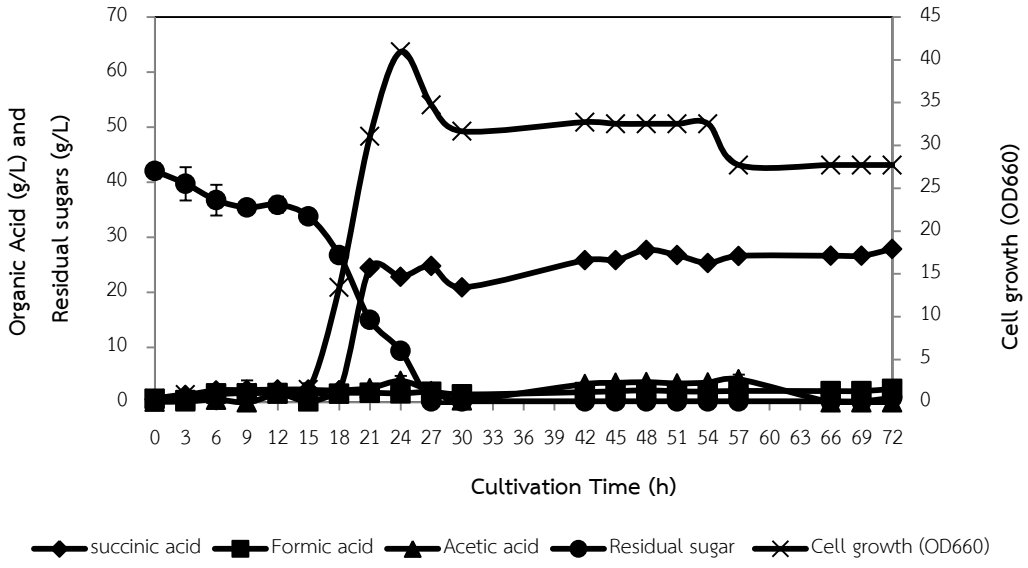

Figure 6.3 Utilization of SSH as carbon source for succinic acid fermentation by *A. succinogenes* NP9-aA7.

At 21 h, concentrations of succinic acid, formic acid and acetic acid were 24.454 g/L, 1.718 g/L, and 2.567 g/L, respectively, under optimum condition from the above in batch fermentation (Figure 6.3). After 21 h, the succinic acid was relatively stable until 72 h (27.873 g/L of succinic acid) and maximum succinic acid yield and succinic acid productivity of 0.338 g/g substrate and 0.776 g/L•h, respectively were obtained. The succinic acid from this experiment could be improve from flask scale (the maximum cell growth (OD660) was 4.615 and succinic acid was 19.139 g/L with a yield of 0.632 g/g substrate at 40 g/L of SSH concentration). The lag phase was observed at 15 h of cultivation time longer than glucose as a carbon source at same condition but shorter than flask scale however, the lag phase in this case shorter than SSH as a carbon source at same condition in flask scale.

In comparison, *A. succinogenes* CGMCC1593 produced succinate at a concentration of 45.3 g/L from pretreated sugarcane molasses in 5 L fermenter with batch fermentation. The strain also excreted high concentrations of acetic acid and formic acid, more than 5 g/L and 1.5 g/L, respectively. In addition, the succinate concentration of 55.2 g/L from sugarcane molasses was obtained when it was cultivated by fed-batch fermentation (Liu et al., 2008).

Further study should be examined the inhibition and detoxification of inhibitor in pretreated SSH for optimization condition and improvement the process efficiency more advantageous then scaled-up succinic acid fermentation with *A. succinogenes* NP9-aA7.

6.6 Conclusions

There was significant effect of CO_2 partial pressure with $MgCO_3$ on the production of succinic acid in batch fermentations in a 2-L fermenter. The optimum medium composition from previous study consist of 74 g/L of glucose, 30 g/L of yeast extract and 60 g/L of alkaline neutralizer (including 45 g/L of $MgCO_3$ and 15 g/L of $Mg(OH)_2$) could be improved the highest succinic acid production to 72.930 g/L with a yield of 1.393 g/g glucose at the CO_2 partial pressure of 50.66 kPa after 24 h of cultivation time. Similar to using SSH as a carbon source for succinic acid production under optimum condition in a 2-L fermenter, it could be improve the succinic acid

production. The results obtained in this study may be useful for reducing the cost of succinic acid fermentation process.

Further study should be examined the inhibition and detoxification of inhibitor in pretreated SSH for optimization condition and improvement the process efficiency more advantageous then scaled-up succinic acid fermentation with *A. succinogenes* NP9-aA7.

Chapter VII

Summary, Conclusion and Future work

The chart below described the steps used for isolation, screening, characterization and optimization of succinic acid.

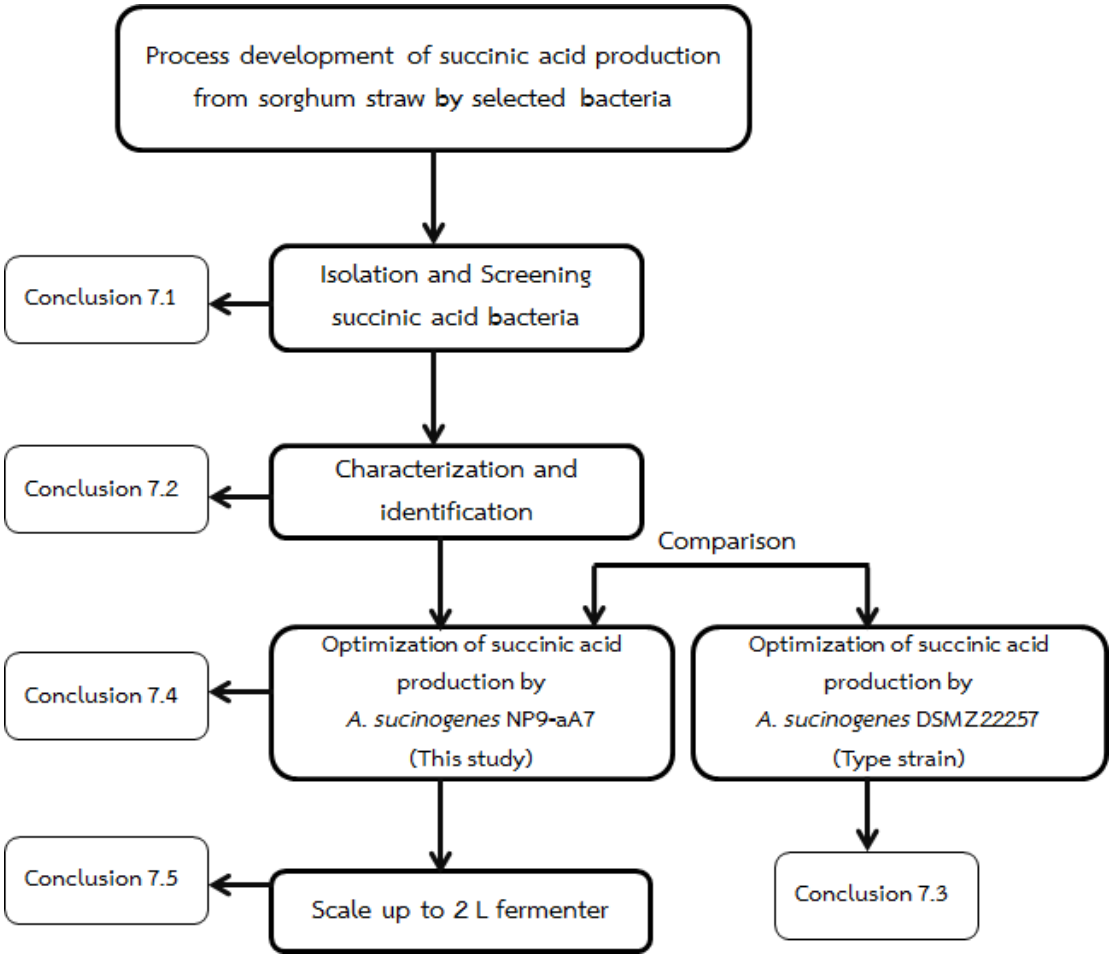

Figure 7.1 Summary of the steps for isolation, screening, characterization and optimization of succinic acid production

7.1 Isolation and screening succinic acid bacteria

The succinic acid producing bacteria were screened from 7 sources and 6 provinces in Thailand. The result from primary screening, 171 isolates exhibited a clear zone on the screening medium. Secondary screening, 165 isolates with succinic acid ability were obtained by TLC. From the quantitative analysis, concentration of succinic acid was in the range of 0.186-45.554 g/L. Subsequently, 58 isolate were further characterized including morphological, physiological and biochemical characteristics.

7.2 Characterization and identification of succinic acid producing strain

Fifty-eight isolates were divided into 11 groups. Representative isolate from each group have been identified based on its 16S rRNA sequence analysis. Isolates from group I, II, III and IV were closely related to *Enterococcus* sp. except isolate NP8-aB2 was closely related to *Streptococcus* sp.. Isolates from group V were closely related to *Lactobacillus* sp. Isolates from group VI were closely related to *Enterococcus* sp.*, Clostridium* sp. and *Lactococcus* sp.. Isolate from group VII were closely related to *Enterococcus* sp. and *Lactococcus* sp.. Isolate from group VIII were closely related to *Enterococcus* sp. and *Clostridium* sp. Isolate from group IX was closely related to *Clostridium* sp. Isolate from group X belongs to *Pasteurellaceae* family and were closely related to *Proteus* sp. and *Actinobacillus* sp.. Isolate from group XI were closely related to *Enterococcus* sp.

Among 58 isolates, the strain *Actinobacillus succinogenes* NP9-aA7 from group X was selected to further study because it produced high succinic acid of 42.539 g/L with a yield of 0.709 g/g glucose. It was facultative anaerobe and resistant to low pH and non-pathogenic. The potential isolate NP9-aA7 needs to be integrated with the fermentation process by optimizing the medium composition for cell growth and promote the succinic acid production.

7.3 Optimization succinic acid production by *A.succinogenes* DSMZ 22257 (Type strain)

From one factor-at-a-time method, the maximum succinic acid concentration of 52.180 g/L, corresponding to a yield of 0.870 g/g glucose was obtained from 60 g/L of glucose. When using 40 g/L of SSH as a carbon source, succinic acid of 16.671 g/L, corresponding to yield of 0.777 g/g substrate was achieved after 24 h of cultivation times.

Statistical method: Plackett-Burman Design (PBD) was applied for a preliminary optimization of succinic acid fermentation medium by *A. succinogenes* DSMZ 22257. The results from PBD, yeast extract and $MgCO_3$ were identified as the key medium components. Then key medium were optimized by Central Composite Design (CCD) using a Response Surface Methodology (RSM). The optimized concentrations of SSH, yeast extract and $MgCO_3$ were 45 (1), 34.55 (0.91) and 29.25 (-0.15) g/L, respectively. From statistical analysis, the concentration of succinic acid 19.059 g/L was obtained. This was a 17.85% improvement over that attained with the one-factor-at-a-time method.

7.4 Optimization of succinic acid production by *A. succinogenes* NP9-aA7 (This study)

Results from the one-factor-at-a-time method demonstrated that *A. succinogenes* NP9-aA7 gave a succinic acid of 49.756 g/L when using yeast extract and $MgCO_3$ as a nitrogen source and alkaline neutralizer, respectively. The statistical method combining a Plackett-Burman Design (PBD) and a Box-Behnken Design (BBD) using Response Surface Methodology (RSM) showed that the key factors consisted 74 of glucose, 30 g/L of yeast extract and 60 g/L of $MgCO_3$, resulting in a maximum of succinic acid of 60.087 g/L with a yield of 0.816 g/g glucose after 36 h cultivation times. Effect of a mixed alkaline neutralizer resulting in at 3:1 of $MgCO_3$ to $Mg(OH)_2$ ration increases solubility with improved succinic acid production.

In case, using 40 g/L of SSH as an alternative carbon source, maximum succinic acids of 19.139 g/L with a yield of 0.632 g/g substrate were obtained.

7.5 Scale up to 2-L fermenter

From the optimum condition for succinic acid production by *A. succinogenes* NP9-aA7 was cultivated in a 2-L fermenter. A maximum succinic acid of 58.080 g/L after 27 h of cultivation times was obtained.

The effects of CO_2 partial pressure with alkaline neutralizers were also investigated. The CO_2 partial pressure of 50.66 kPa and alkaline neutralizers (45 g/L of $MgCO_3$ and 15 g/L of $Mg(OH)_2$) enhance succinic acid production to 72.930 g/L with a yield of 1.393 g/g glucose after 24 h of cultivation times. In case using SSH 40 g/L as a carbon source, succinic acid of 27.703 g/L was obtained after 48 h of cultivation times.

7.6 Suggestion for future work

Based on the experimental results in this thesis, the following work should be investigated for further study in order to improve the yield of succinic acid production.

1. To use agricultural material as an alternative carbon source for economical succinic acid production.
2. To reduce the inhibitor (detoxification) in the agricultural material using physical treatment such as membrane separation, chemical treatment such as activated carbon treatment and biological treatment such as microbial detoxification.
3. It is possible to find the new spiecies from NS18-A1 and SP17-B1. Because they were belonged to *C. amygdalinum* BR-10(T) with similarity of 97.82% and 97.84% similarity, respectively.
4. Using CO_2 as a medium buffering is a great advantage of helping to reduce greenhouse gases.

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APPENDIX

APPENDIX A

Culture media and Chemical preparation

1. Arginine broth

Yeast extract	0.03	g
Peptone	0.05	g
NaCl	0.05	g
K_2HPO_4	0.003	g
L (+) arginine HCL	0.055	g
Phenol red	0.0001	g
Tween 80	0.001	g
Agar	0.0055	g
Distilled water	1000	mL

Dissolved and adjusted pH to 7.2 with NaOH before added agar. Then the medium was melted by microwave and added phenol red. The medium was sterilized by autoclave at 121°C for 15 min.

2. Nitrate broth

KNO_3	0.01	g
Yeast extract	0.03	g
Peptone	0.05	g
NaCl	0.05	g
Tween 80	0.001	mL
Agar	0.0055	g
Distilled water	1000	mL

Dissolved and adjusted pH to 6.8 with NaOH before added agar. Then the medium was melted by microwave and sterilized by autoclave at 121°C for 15 min.

3. Sulfanilic acid solution

Sulphanilic acid	0.8	g
5 N Acetic acid	100	mL

Dissolved and gentle heating in a fume hood.

4. *N*,*N*-dimethyl-l-naphthylamine solution

N,*N*-dimethyl-l-naphthylamine	0.5	g
5 N Acetic acid	100	mL

Dissolved and gentle heating in a fume hood.

5. Starch agar plate

Starch	2	g
Yeast extract	0.5	g
Peptone	0.5	g
Agar	2	g
Distilled water	1000	mL

Dissolved and adjusted pH to 7.0 with NaOH. Then the medium was melted by microwave and sterilized by autoclave at 121°C for 15 min and poured into plate.

6. Iodine reagent

Iodine solution	10	mL

Dissolved and adjusted volume to 20 mL with distilled water.

7. Slime agar plate

Sucrose	0.2	g
Yeast extract	0.05	g
Peptone	0.05	g
Agar	0.2	g
Distilled water	1000	mL

Dissolved and adjusted pH to 6.8-7.0 with NaOH. Then the medium was sterilized by autoclave at 121°C for 15 min and poured into plate.

8. Medium for acid from carbohydrates test

Carbohydrates	0.05	g
Yeast extract	0.05	g
Peptone	0.05	g
Salt solution	0.05	mL
Distilled water	1000	mL

Dissolved and adjusted pH to 6.8 with NaOH. Then bromocresol purple was added the medium and sterilized by autoclave at 121℃ for 15 min.

9. Salt solution

$MgSO_4.7H_2O$	4	g
$MnSO_4.4H_2O$	0.2	g
$FeSO_4.7H_2O$	0.2	g
NaCl	0.2	g
Distilled water	100	mL

Dissolved and added 0.5 mL into medium test.

10. DNSA reagent (Miller, 1959)

3,5 dinitrosalicylic acid	2.65	g
Sodium Hydroxide	4.95	g
Sodium potassium tartrate	76.50	g
Sodium metabisulfite	2.05	g
Phenol	1.90	mL

The 3, 5 dinitrosalicylic acid, NaOH, sodium potassium tartrate and sodium metabisulfite were dissolved in distilled water then phenol was added in the mixer. This reagent was stirred until homogeneously then adjusted the final volume to 1 L and stored in amber bottle.

APPENDIX B

Result of 16S rRNA gene sequence

1. Isolate AY2-bA2

Organism; *E. casseliflavus* (99.73%)

Nucleotide; 1383 bp

Source; Bark of *Ficus religiosa* L.

Accession No.; LC122272

AGAGTTTGATCCTGGCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAACGCTTTTTCTTTCA
CCGGAGCTTGCTCCACCGAAAGAAAAAGAGTGGCGAACGGGTGAGTAACACGTGGGTAACCTGCCCATCAGAA
GGGGATAACACTTGGAAACAGGTGCTAATACCGTATAACACTATTTTCCGCATGGAAGAAAGTTGAAAGGCGC
TTTTGCGTCACTGATGGATGGACCCGCGGTGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGAT
GCATAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGC
AGTAGGGAATCTTCGGCAATGGACGAAAGTCTGACCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCG
TAAAACTCTGTTGTTAGAGAAGAACAAGGATGAGAGTAAAACGTTCATCCCTTGACGGTATCTAACCAGAAAG
CCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAA
AGCGAGCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGG
GAGACTTGAGTGCAGAAGAGGAGAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAGGAACAC
CAGTGGCGAAGGCGGCTCTCTGGTCTGTAACTGACGCTGAGGCTCGAAAGCGTGGGGAGCGAACAGGATTAG
ATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTGGAGGGTTTCCGCCCTTCAGTGCTGCAGCA
AACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCAC
AAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTTTGACCACT
CTAGAGATAGAGCTTCCCCTTCGGGGGCAAAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGA
TGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATTGTTAGTTGCCATCATTTAGTTGGGCACTCTAGCGAG
ACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACA
CGTGCTACAATGGGAAGTACAACGAGTTGCGAAGTCGCGAGGCTAAGCTAATCTCTTAAAGCTTCTCTCAGTT
CGGATTGTAGGCTGCAACTCGCCTACATGAAGCCGGAATCGCTAGTAATCGCGGATCAGCACGCCGCGGTG

2. Isolate AY2-bB2

Organism; *E. casseliflavas* (99.69%)

Nucleotide; 1295bp

Source; Bark of *Ficus religiosa* L.

Accession No.; LC120365

TGAGTAACACGTGGGTAACCTGCCCATCAGAAGGGGATAACACTTGGAAACAGGTGCTAATACCGTATAACAC
TATTTTCCGCATGGAAGAAAGTTGAAAGGCGCTTTTGCGTCACTGATGGATGGACCCGCGGTGCATTAGCTAG
TTGGTGAGGTAACGGCTCACCAAGGCCACGATGCATAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACT
GAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGACGAAAGTCTGACCGAGC
AACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTTGTTAGAGAAGAACAAGGATGAGAGTAGA
ACGTTCATCCCTTGACGGTATCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAG
GTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCC
CCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAGAGTGGAATTCCATGTG
TAGCGGTGAAATGCGTAGATATATGGAGGAACACCAGTGGCGAAGGCGGCTCTCTGGTCTGTAACTGACGCTG
AGGCTCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGT
GTTGGAGGGTTTCCGCCCTTCAGTGCTGCAGCAAACGCATTAAGCACTCCGCCGGGGGAGTACGACCGCAAGG
TTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAG
AACCTTACCAGGTCTTGACATCCTTTGACCACTCTAGAGATAGAGCTTCCCCTTCGGGGGCAAAGTGACAGGT
GGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATTGTT
AGTTGCCATCATTTAGTTGGGCACTCTAGCGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCA
AATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGGAAGTACAACGAGTTGCGAAGTCGCGA
GGCTAAGCTAATCTCTTAAAGCTTCTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGCCGGAATC
GCTAGTAATCGCGGATCAGCACGCCGCGGTGAATACGTTCCCGGGCCTTGTACACAC

3. Isolate NP2-A3

Organism; *E. duran* (99.68%)

Nucleotide; 1285 bp

Source; Soil

Accession No.; LC122273

TCGTACGCTTCTTTTTCCACCGGAGGCTTGCTCCACCGGAAAAAGAAGAGTGGCGAACGGGTGAGTAACACGT
GGGTAACCTGCCCATCAGAAGGGGATAACACTTGGAAACAGGTGCTAATACCGTATAACAATCGAAACCGCAT
GGTTTTGATTTGAAAGGCGCTTTCGGGTGTCGCTGATGGATGGACCCGCGGTGCATTAGCTAGTTGGTGAGGT
AACGGCTCACCAAGGCCACGATGCATAGCCGACCTGAGAGGGTGATCGGCCACATTGGGACTGAGACACGGC
CCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGACGAAAGTCTGACCGAGCAACGCCGCGT
GAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTTGTTAGAGAAGAACAAGGATGAGAGTAACTGTTCATCCC
TTGACGGTATCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGT
TGTCCGGATTTATTGGGCGTAAAGCGAACGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACC
GGGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAGAGTGGAATTCCATGTGTAGCGGTGAAA
TGCGTAGATATATGGAGGAACACCAGTGGCGAAGGCGGCTCTCTGGTCTGTAACTGACGCTGAGGCTCGAAAG
CGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTGGAGGGTT
TCCGCCCTTCAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAA
AGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAG
GTCTTGACATCCTTTGACCACTCTAGAGATAGAGCTTCCCCTTCGGGGGCAAAGTGACAGGTGGTGCATGGTT
GTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATTGTTAGTTGCCATCA
TTTAGTTGGGCACTCTAGCAAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGC
CCCTTATGACCTGGGCTACACACGTGCTACAATGGGAAGTACAACGAGTCGCGAAGTCGCGAGGCTAAGCTAA
TCTCTTAAAGCTTCTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTA

4. Isolate NS13-dB1

Organism; *E. faecium* (99.93%)

Nucleotide; 1376bp

Source; Bovine rumen

Accession No.; LC122274

AGCTTGCTCCACCGGAAAAAGAAGAGTGGCGAACGGGTGAGTAACACGTGGGTAACCTGCCCATCAGAAGGG
GATAACACTTGGAAACAGGTGCTAATACCGTATAACAATCGAAACCGCATGGTTTTGATTTGAAAGGCGCTTTC
GGGTGTCGCTGATGGATGGACCCGCGGTGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCCACGATGC
ATAGCCGACCTGAGAGGGTGATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAG
TAGGGAATCTTCGGCAATGGACGAAAGTCTGACCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTA
AAACTCTGTTGTTAGAGAAGAACAAGGATGAGAGTAACTGTTCATCCCTTGACGGTATCTAACCAGAAAGCCA
CGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGC
GAGCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGA
GACTTGAGTGCAGAAGAGGAGAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAGGAACACCA
GTGGCGAAGGCGGCTCTCTGGTCTGTAACTGACGCTGAGGCTCGAAAGCGTGGGGAGCAAACAGGATTAGATA
CCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTGGAGGGTTTCCGCCCTTCAGTGCTGCAGCTAAC
GCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAA
GCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTTTGACCACTCT
AGAGATAGAGCTTCCCCTTCGGGGGCAAAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATG
TTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATTGTTAGTTGCCATCATTCAGTTGGGCACTCTAGCAAGAC
TGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACG
TGCTACAATGGGAAGTACAACGAGTTGCGAAGTCGCGAGGCTAAGCTAATCTCTTAAAGCTTCTCTCAGTTCG
GATTGCAGGCTGCAACTCGCCTGCATGAAGCCGGAATCGCTAGTAATCGCGGATCAGCACGCCGCGGTGAATA
CGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGA

5. Isolate SP8-B4

Organism; *E. faecium* (99.30%)

Nucleotide; 1376 bp

Source; Soil

Accession No.; LC122275

GAGTGGCGAACGGGTGAGTAACACGTGGGTAACCTGCCCATCAGAAGGGGATAACACTTGGAAACAGGTGCTA
ATACCGTATAACAATCGAAACCGCATGGTTTTGATTTGAAAGGCGCTTTCGGGTGTCGCTGATGGATGGACCC
GCGGTGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCCACGATGCATAGCCGACCTGAGAGGGTGATC
GGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGACG
AAAGTCTGACCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTTGTTAGAGAAGAAC
AAGGATGAGAGTAACTGTTCATCCCTTGACGGTATCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCC
GCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTCTTAAGTC
TGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAGAG
TGGAATTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAGGAACACCAGTGGCGAAGGCGGCTCTCTGGTC
TGTAACTGACGCTGAGGCTCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAAC
GATGAGTGCTAAGTGTTGGAGGGTTTCCGCCCTTCAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGA
GTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTC
GAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTTTGACCACTCTAGAGATAGAGCTTCCCCTTCGGGG
GCAAAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCG
CAACCCTTATTGTTAGTTGCCATCATTCAGTTGGGCACTCTAGCAAGACTGCCGGTGACAAACCGGAGGAAGG
TGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGGAAGTACAACGAG
TTGCGAAGTCGCGAGGCTAAGCTAATCTCTTAAAGCTTCTCTCAGTTCGGATTGCAGGCTGCAACTCGCCTGC
ATGAAGCCGGAATCGCTAGTAATCGCGGATCAGCACGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCG
CCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGAGGTAACCTTTTTGGAGCCAGCCG

6. Isolate NS13-aB1

Organism; *E. hirae* (100%)

Nucleotide; 1349bp

Source; Bovine rumen

Accession No.; LC122276

GCGAACGGGTGAGTAACACGTGGGTAACCTGCCCATCAGAAGGGGATAACACTTGGAAACAGGTGCTAATACC
GTATAACAATCGAAACCGCATGGTTTTGATTTGAAAGGCGCTTTCGGGTGTCGCTGATGGATGGACCCGCGGT
GCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCATAGCCGACCTGAGAGGGTGATCGGCCA
CATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGACGAAAGTC
TGACCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTTGTTAGAGAAGAACAAGGAT
GAGAGTAACTGTTCATCCCTTGACGGTATCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTA
ATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTCTTAAGTCTGATGT
GAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAGAGTGGAAT
TCCATGTGTAGCGGTGAAATGCGTAGATATATGGAGGAACACCAGTGGCGAAGGCGGCTCTCTGGTCTGTAAC
TGACGCTGAGGCTCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAG
TGCTAAGTGTTGGAGGGTTTCCGCCCTTCAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGA
CCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCA
ACGCGAAGAACCTTACCAGGTCTTGACATCCTTTGACCACTCTAGAGATAGAGCTTCCCCTTCGGGGGCAAAG
TGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCC
TTATTGTTAGTTGCCATCATTTAGTTGGGCACTCTAGCAAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGA
TGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGGAAGTACAACGAGTCGCAA
AGTCGCGAGGCTAAGCTAATCTCTTAAAGCTTCTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAG
CCGGAATCGCTAGTAATCGCGGATCAGCACGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTC
ACACCACGAGAGTTTGTAACACCCGAAGTCGGTGAG

7. Isolate NS13-dA1

Organism; *E. hirae* (100.00%)

Nucleotide; 1419bp

Source; Bovine rumen

Accession No.; LC122277

GCTTCTTTTTCCACCGGAGCTTGCTCCACCGGAAAAAGAGGAGTGGCGAACGGGTGAGTAACACGTGGGTAAC
CTGCCCATCAGAAGGGGATAACACTTGGAAACAGGTGCTAATACCGTATAACAATCGAAACCGCATGGTTTTG
ATTTGAAAGGCGCTTTCGGGTGTCGCTGATGGATGGACCCGCGGTGCATTAGCTAGTTGGTGAGGTAACGGCT
CACCAAGGCGACGATGCATAGCCGACCTGAGAGGGTGATCGGCCACATTGGGACTGAGACACGGCCCAAACT
CCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGACGAAAGTCTGACCGAGCAACGCCGCGTGAGTGAA
GAAGGTTTTCGGATCGTAAAACTCTGTTGTTAGAGAAGAACAAGGATGAGAGTAACTGTTCATCCCTTGACGG
TATCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGG
ATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGG
GTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAGAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGA
TATATGGAGGAACACCAGTGGCGAAGGCGGCTCTCTGGTCTGTAACTGACGCTGAGGCTCGAAAGCGTGGGG
AGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTGGAGGGTTTCCGCCC
TTCAGTGCTGCAGCTAACGCATTTAAGCACTCTCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAA
TTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTT
GACATCCTTTGACCACTCTAGAGATAGAGCTTCCCCTTCGGGGGCAAAGTGACAGGTGGTGCATGGTTGTCGT
CAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATTGTTAGTTGCCATCATTTAG
TTGGGCACTCTAGCAAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTT
ATGACCTGGGCTACACACGTGCTACAATGGGAAGTACAACGAGTCGCAAAGTCGCGAGGCTAAGCTAATCTCT
TAAAGCTTCTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGCCGGAATCGCTAGTAATCGCGGAT
CAGCACGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAACACCC
GAAGTCGGTGAGGTAACCTTTTGGAGCCAGCCGCC

8. Isolate NS15-aA1

Organism; *E. faecium* (100.00%)

Nucleotide; 1333 bp

Source; Bovine rumen

Accession No.; LC122278

GGAGTGGCGAACGGGTGAGTAACACGTGGGTAACCTGCCCATCAGAAGGGGATAACACTTGGAAACAGGTGC
TAATACCGTATAACAATCGAAACCGCATGGTTTTGATTTGAAAGGCGCTTTCGGGTGTCGCTGATGGATGGAC
CCGCGGTGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCATAGCCGACCTGAGAGGGTG
ATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGA
CGAAAGTCTGACCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTTGTTAGAGAAGA
ACAAGGATGAGAGTAACTGTTCATCCCTTGACGGTATCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAG
CCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTCTTAAG
TCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAG
AGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAGGAACACCAGTGGCGAAGGCGGCTCTCTGG
TCTGTAACGACGCTGAGGCTCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAA
CGATGAGTGCTAAGTGTTGGAGGGTTTCCGCCCTTCAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGG
AGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATT
CGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTTTGACCACTCTAGAGATAGAGCTTCCCCTTCGGG
GGCAAAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGC
GCAACCCTTATTGTTAGTTGCCATCATTTAGTTGGGCACTCTAGCAAGACTGCCGGTGACAAACCGGAGGAAG
GTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGGAAGTACAACGA
GTCGCAAAGTCGCGAGGCTAAGCTAATCTCTTAAAGCTTCTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTA
CATGAAGCCGGAATCGCTAGTAATCGCGGATCAGCACGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACC
GCCCGTCACACCACGAGAGTTT

9. Isolate NS15-aA2

Organism; *E. hirae* (99.50%)

Nucleotide; 1387 bp

Source; Bovine rumen

Accession No.; LC122279

GGGAAGGGCGGCGTGCTATACATGCAGTCGAACGCTTCTTTTTCACCGGAGCTTGCTCCACCGGAAAAAGAGG
AGTGGCGAACGGGTGAGTAACACGTGGGTAACCTGCCCATCAGAAGGGGATAACACTTGGAAACAGGTGCTAA
TACCGTATAACAATCGAAACCGCATGGTTTTGATTTGAAAGGCGCTTTCGGGTGTCGCTGATGGATGGACCCG
CGGTGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCATAGCCGACCTGAGAGGGTGATCG
GCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGACGA
AAGTCTGACCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTTGTTAGAGAAGAACA
AGGATGAGAGTAACTGTTCATCCCTTGACGGTATCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCG
CGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTCTTAAGTCT
GATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAGAGT
GGAATTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAGGAACACCAGTGGCGAAGGCGGCTCTCTGGTCT
GTAACTGACGCTGAGGCTCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACG
ATGAGTGCTAAGTGTTGGAGGGTTTCCGCCCTTCAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAG
TACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCG
AAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTTTGACCACTCTAGAGATAGAGCTTCCCCTTCGGGGG
CAAAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGC
AACCCTTATTGTTAGTTGCCATCATTTAGTTGGGCACTCTAGCAAGACTGCCGGTGACAAACCGGAGGAAGGT
GGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGGAAGTACAACGAGT
CGCAAAGTCGCGAGGCTAAGCTAATCTCTTAAAGCTTCTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACA
TGAAGCCGGAATCGCTAGTAATCGCGGATCAGCACGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGC
CC

10. Isolate SP5-A5

Organism; *E. hirae* (100.00%)

Nucleotide; 1283 bp

Source; Soil

Accession No.; LC122280

TAACCTGCCCATCAGAAGGGGATAACACTTGGAAACAGGTGCTAATACCGTATAACAATCGAAACCGCATGGT
TTTGATTTGAAAGGCGCTTTCGGGTGTCGCTGATGGATGGACCCGCGGTGCATTAGCTAGTTGGTGAGGTAAC
GGCTCACCAAGGCGACGATGCATAGCCGACCTGAGAGGGTGATCGGCCACATTGGGACTGAGACACGGCCCA
AACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGACGAAAGTCTGACCGAGCAACGCCGCGTGAG
TGAAGAAGGTTTTCGGATCGTAAAACTCTGTTGTTAGAGAAGAACAAGGATGAGAGTAACTGTTCATCCCTTG
ACGGTATCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGT
CCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGG
GAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAGAGTGGAATTCCATGTGTAGCGGTGAAATGCG
TAGATATATGGAGGAACACCAGTGGCGAAGGCGGCTCTCTGGTCTGTAACTGACGCTGAGGCTCGAAAGCGTG
GGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTGGAGGGTTTCCG
CCCTTCAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGA
ATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCT
TGACATCCTTTGACCACTCTAGAGATAGAGCTTCCCCTTCGGGGGCAAAGTGACAGGTGGTGCATGGTTGTCG
TCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATTGTTAGTTGCCATCATTTA
GTTGGGCACTCTAGCAAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCT
TATGACCTGGGCTACACACGTGCTACAATGGGAAGTACAACGAGTCGCAAAGTCGCGAGGCTAAGCTAATCTC
TTAAAGCTTCTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGCCGGAATCGCTAGTAATCGCGGA
TCAGCACGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCG

11. Isolate SP6-A5

Organism; *E. hirae* (100.00%)

Nucleotide; 1284 bp

Source; Soil

Accession No.; LC122281

AACACGTGGGTAACCTGCCCATCAGAAGGGGATAACACTTGGAAACAGGTGCTAATACCGTATAACAATCGAA
ACCGCATGGTTTTGATTTGAAAGGCGCTTTCGGGTGTCGCTGATGGATGGACCCGCGGTGCATTAGCTAGTTG
GTGAGGTAACGGCTCACCAAGGCGACGATGCATAGCCGACCTGAGAGGGTGATCGGCCACATTGGGACTGAG
ACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGACGAAAGTCTGACCGAGCAAC
GCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTTGTTAGAGAAGAACAAGGATGAGAGTAACTGT
TCATCCCTTGACGGTATCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGG
CAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGG
CTCAACCGGGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAGAGTGGAATTCCATGTGTAGC
GGTGAAATGCGTAGATATATGGAGGAACACCAGTGGCGAAGGCGGCTCTCTGGTCTGTAACTGACGCTGAGGC
TCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTG
GAGGGTTTCCGCCCTTCAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGA
AACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACC
TTACCAGGTCTTGACATCCTTTGACCACTCTAGAGATAGAGCTTCCCCTTCGGGGGCAAAGTGACAGGTGGTG
CATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATTGTTAGTT
GCCATCATTTAGTTGGGCACTCTAGCAAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATC
ATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGGAAGTACAACGAGTCGCAAAGTCGCGAGGCT
AAGCTAATCTCTTAAAGCTTCTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGCCGGAATCGCTA
GTAATCGCGGATCAGCACGCCGCGGTGAATACGTTCCCGGGCCTTG

12. Isolate SP9-A3

Organism; *E. hirae* (100.00%)

Nucleotide; 1284 bp

Source; Soil

Accession No.; LC122282

AACACGTGGGTAACCTGCCCATCAGAAGGGGATAACACTTGGAAACAGGTGCTAATACCGTATAACAATCGAA
ACCGCATGGTTTTGATTTGAAAGGCGCTTTCGGGTGTCGCTGATGGATGGACCCGCGGTGCATTAGCTAGTTG
GTGAGGTAACGGCTCACCAAGGCGACGATGCATAGCCGACCTGAGAGGGTGATCGGCCACATTGGGACTGAG
ACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGACGAAAGTCTGACCGAGCAAC
GCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTTGTTAGAGAAGAACAAGGATGAGAGTAACTGT
TCATCCCTTGACGGTATCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGG
CAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGG
CTCAACCGGGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAGAGTGGAATTCCATGTGTAGC
GGTGAAATGCGTAGATATATGGAGGAACACCAGTGGCGAAGGCGGCTCTCTGGTCTGTAACTGACGCTGAGGC
TCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTG
GAGGGTTTCCGCCCTTCAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGA
AACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACC
TTACCAGGTCTTGACATCCTTTGACCACTCTAGAGATAGAGCTTCCCCTTCGGGGGCAAAGTGACAGGTGGTG
CATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATTGTTAGTT
GCCATCATTTAGTTGGGCACTCTAGCAAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATC
ATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGGAAGTACAACGAGTCGCAAAGTCGCGAGGCT
AAGCTAATCTCTTAAAGCTTCTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGCCGGAATCGCTA
GTAATCGCGGATCAGCACGCCGCGGTGAATACGTTCCCGGGCCTTG

13. Isolate BK1-A1

Organism; *E.saccharolyticus* sub sp. (100.00%)

Nucleotide; 1364bp

Source; Bark of *Samanae saman*

Accession No.; LC122283

TTCGGTTCATTGGAAAAGAGGAGTGGCGAACGGGTGAGTAACACGTGGGTAACCTGCCCATCAGAGGGGGATA
ACACTTGGAAACAGGTGCTAATACCGCATAACGCTTTTTCTCGCATGAGAGAAAGCTGAAAGGCGCTTTTGCG
TCACTGATGGATGGACCCGCGGTGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCATAGC
CGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGG
AATCTTCGGCAATGGACGAAAGTCTGACCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACT
CTGTTGTTAGAGAAGAACAAGGATGAGAAGAGAATGTTCATCCCTTGACGGTATCTAACCAGAAAGCCACGGC
TAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGC
GCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGAGACTT
GAGTGCAGAAGAGGAGAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAGGAACACCAGTGGC
GAAGGCGGCTCTCTGGTCTGTAACTGACGCTGAGGCTCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTG
GTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTGGAGGGTTTCCGCCCTTCAGTGCTGCAGCAAACGCATT
AAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGT
GGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTTTGACCACTCTAGAGA
TAGAGCTTTCCCTTCGGGGACAAAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGG
TTAAGTCCCGCAACGAGCGCAACCCCTATTGTTAGTTGCCATCATTTAGTTGGGCACTCTAGCGAGACTGCCG
GTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTA
CAATGGGAAGTACAACGAGTTGCGAAGTCGCGAGGCTAAGCTAATCTCTTAAAGCTTCTCTCAGTTCGGATTG
TAGGCTGCAACTCGCCTACATGAAGCCGGAATCGCTAGTAATCGCGGATCAGCACGCCGCGGTGAATACGTTC
CCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAA

14. Isolate NS15-bB2

Organism; *L. fermentum* (99.78%)

Nucleotide; 1391 bp

Source; Bovine rumen

Accession No.; LC122284

CTGGCTCAGGATGAACGCCGGCGGTGTGCCTAATACATGCAAGTCGAACGCGTTGGCCCAATTGATTGATGGT
GCTTGCACCTGATTGATTTTGGTCGCCAACGAGTGGCGGACGGGTGAGTAACACGTAGGTAACCTGCCCAGAA
GCGGGGGACAACATTTGGAAACAGATGCTAATACCGCATAACAACGTTGTTCGCATGAACAACGCTTAAAAGA
TGGCTTCTCGCTATCACTTCTGGATGGACCTGCGGTGCATTAGCTTGTTGGTGGGGTAACGGCCTACCAAGGC
GATGATGCATAGCCGAATTGAGAGACTGATCGGCCACAATGGGACTGAGACACGGCCCATACTCCTACGGGAG
GCAGCAGTAGGGAATCTTCCACAATGGGCGCAAGCCTGATGGAGCAACACCGCGTGAGTGAAGAAGGGTTTC
GGCTCGTAAAGCTCTGTTGTTAAAGAAGAACACGTATGAGAGTAACTGTTCATACGTTGACGGTATTTAACCA
GAAAGTCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGATTTATTGGG
CGTAAAGAGAGTGCAGGCGGTTTTCTAAGTCTGATGTGAAAGCCTTCGGCTTAACCGGAGAAGTGCATCGGAA
ACTGGATAACTTGAGTGCAGAAGAGGGTAGTGGAACTCCATGTGTAGCGGTGGAATGCGTAGATATATGGAAG
AACACCAGTGGCGAAGGCGGCTACCTGGTCTGCAACTGACGCTGAGACTCGAAAGCATGGGTAGCGAACAGG
ATTAGATACCCTGGTAGTCCATGCCGTAAACGATGAGTGCTAGGTGTTGGAGGGTTTCCGCCCTTCAGTGCCG
GAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGCC
CGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCTACGCGAAGAACCTTACCAGGTCTTGACATCTTGCGC
CAACCCTAGAGATAGGGCGTTTCCTTCGGGAACGCAATGACAGGTGGTGCATGGTCGTCGTCAGCTCGTGTCG
TGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTTACTAGTTGCCAGCATTAAGTTGGGCACTCTA
GTGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGACGACGTCAGATCATCATGCCCCTTATGACCTGGGCT
ACACACGTGCTACAATGGACGGTACAACGAGTCGCGAACTCGCGAGGGCAAGCAAATCTCTTAAAACCGTTCT
CAGTTCGGACTGCAGGCTGCAACTCGCCTGCACGAAGTCGGAATCGCTAGTAATCGCGGATCAGCATGCCGCG
GTGAAT

15. Isolate NS13-bA1

Organism; *L. oris* (99.85%)

Nucleotide; 1359 bp

Source; Bovine rumen

Accession No.; LC122285

GCTTGCACTGATTTGACGTTGGATTCCCAGTGAGCGGCGGACGGGTGAGTAACACGTGGGCAACCTGCCCCAA
AGCGGGGGATAACATTTGGAAACAGGTGCTAATACCGCATAACTTGGAAAACCACATGGTTTTCCAATAAAAG
ATGGTTTCGGCTATCACTTTGGGATGGGCCCGCGGTGCATTAGCTAGTTGGTAAGGTAACGGCTTACCAAGGC
GATGATGCATAGCCGAGTTGAGAGACTGATCGGCCACAATGGAACTGAGACACGGTCCATACTCCTACGGGAG
GCAGCAGTAGGGAATCTTCCACAATGGGCGCAAGCCTGATGGAGCAACACCGCGTGAGTGAAGAAGGGTTTC
GGCTCGTAAAACTCTGTTGTTGGAGAAGAACGTGCGTAAGAGTAACTGTTTACGCAGTGACGGTATCCAACCA
GAAAGTCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGATTTATTGGG
CGTAAAGCGAGCGCAGGCGGTTGCTTAGGTCTGATGTGAAAGCCTTCGGCTTAACCGAAGAAGTGCATCGGAA
ACCGGGCGACTTGAGTGCAGAAGAGGACAGTGGAACTCCATGTGTAGCGGTGGAATGCGTAGATATATGGAAG
AACACCAGTGGCGAAGGCGGCTGTCTGGTCTGCAACTGACGCTGAGGCTCGAAAGCATGGGTAGCGAACAGG
ATTAGATACCCTGGTAGTCCATGCCGTAAACGATGAGTGCTAGGTGTTGGAGGGTTTCCGCCCTTCAGTGCCG
AAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGCC
CGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCTACGCGAAGAACCTTACCAGGTCTTGACATCTTGCGC
CAACCTCAGAGATGAGGCGTTCCCTTCGGGGACGCAAAGACAGGTGGTGCATGGTCGTCGTCAGCTCGTGTCG
TGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTTACTAGTTGCCAGCATTCAGTTGGGCACTCTA
GTGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGACGACGTCAGATCATCATGCCCCTTATGACCTGGGCT
ACACACGTGCTACAATGGCCGGTACAACGAGCAGCTAACCCGCGAGGGTGTGCAAATCTCTTAAAGCCGGTCT
CAGTTCGGACTGCAGTCTGCAACTCGACTGCACGAAGTCGGAATCGCTAGTAATCGCGGATCAGCATGCCGCG
GTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGA

16. Isolate SP14-B2

Organism; *Lc. Fermosensis* (99.83%)

Nucleotide; 1398 bp

Source; Dog mouth

Accession No.; LC122286

CTATTTTCATGAAGAGCGGCGAACGGGTGAGTAACGCGTGGGAAATCTGCCGAGTAGCGGGGGACAACGTTTG
GAAACGAACGCTAATACCGCATAACAATGAGAATCGCATGATTCTTATTTGAAAGAAGCAATTGCTTCACTACT
TGATGATCCCGCGTTGTATTAGCTAGTTGGTAGTGTAAAGGACTACCAAGGCGATGATACATAGCCGACCTGA
GAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCG
GCAATGGGGGCAACCCTGACCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTTGTT
AGAGAAGAACGTTAAGTAGAGTGGAAAATTACTTAAGTGACGGTATCTAACCAGAAAGGGACGGCTAACTACG
TGCCAGCAGCCGCGGTAATACGTAGGTCCCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGTGG
TTTCTTAAGTCTGATGTAAAAGGCAGTGGCTCAACCATTGTGTGCATTGGAAACTGGGAGACTTGAGTGCAGG
AGAGGAGAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAGGAACACCGGAGGCGAAAGCGG
CTCTCTGGCCTGTAACTGACACTGAGGCTCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCA
CGCCGTAAACGATGAGTGCTAGCTGTAGGGAGCTATAAGTTCTCTGTAGCGCAGCTAACGCATTAAGCACTCC
GCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGT
GGTTTAATTGGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCCGATGCTATCCTTAGAGATAAGGAGT
TACTTCGGTACATCGGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCC
GCAACGAGCGCAACCCTTATTACTAGTTGCCATCATTAAGTTGGGCACTCTAGTGAGACTGCCGGTGATAAAC
CGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGATG
GTACAACGAGTCGCCAACCCGCGAGGGTGCGCTAATCTCTTAAAACCATTCTCAGTTCGGATTGCAGGCTGCA
ACTCGCCTGCATGAAGTCGGAATCGCTAGTAATCGCGGATCAGCACGCCGCGGTGAATACGTTCCCGGGCCTT
GTACACACCGCCCGTCACACCACGGAAGTTGGGAGTACCCAAAGTAGGTTGCCTAACCGCAAGGAGGGCGCTT
CCTAAGGTAAGAC

17. Isolate SP14-A3

Organism; *Lc. garviae* (99.85%)

Nucleotide; 1368 bp

Source; Dog mouth

Accession No.; LC122287

AAGATAGCTTGCTATTTTCATGAAGAGCGGCGAACGGGTGAGTAACGCGTGGGAAATCTGCCGAGTAGCGGGG
GACAACGTTTGGAAACGAACGCTAATACCGCATAACAATGAGAATCGCATGATTCTTATTTGAAAGAAGCAATT
GCTTCACTACTTGATGATCCCGCGTTGTATTAGCTAGTTGGTAGTGTAAAGGACTACCAAGGCGATGATACAT
AGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTA
GGGAATCTTCGGCAATGGGGGCAACCCTGACCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAA
CTCTGTTGTTAGAGAAGAACGTTAAGTAGAGTGGAAAATTACTTAAGTGACGGTATCTAACCAGAAAGGGACG
GCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTCCCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGA
GCGCAGGTGGTTTCTTAAGTCTGATGTAAAAGGCAGTGGCTCAACCATTGTGTGCATTGGAAACTGGGAGACT
TGAGTGCAGGAGAGGAGAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAGGAACACCGGAG
GCGAAAGCGGCTCTCTGGCCTGTAACTGACACTGAGGCTCGAAAGCGTGGGGAGCAAACAGGATTAGATACCC
TGGTAGTCCACGCCGTAAACGATGAGTGCTAGCTGTAGGGAGCTATAAGTTCTCTGTAGCGCAGCTAACGCAT
TAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGG
TGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATACTCGTGCTATCCTTAGA
GATAAGGAGTTCCTTCGGGACACGGGATACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGG
GTTAAGTCCCGCAACGAGCGCAACCCTTATTACTAGTTGCCATCATTAAGTTGGGCACTCTAGTGAGACTGCC
GGTGATAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCT
ACAATGGATGGTACAACGAGTCGCCAACCCGCGAGGGTGCGCTAATCTCTTAAAACCATTCTCAGTTCGGATT
GCAGGCTGCAACTCGCCTGCATGAAGTCGGAATCGCTAGTAATCGCGGATCAGCACGCCGCGGTGAATACGTT
CCCGGGCCTTGTACACACCGCCCGTCACACCACGGAAGTTGGGAGTACCCAAAGTA

18. Isolate SP15-A2

Organism; *Lc garviae* (99.93%)

Nucleotide; 1343 bp

Source; Dog mouth

Accession No.; LC122288

TGAAGAGCGGCGAACGGGTGAGTAACGCGTGGGAAATCTGCCGAGTAGCGGGGGACAACGTTTGGAAACGAA
CGCTAATACCGCATAACAATGAGAATCGCATGATTCTTATTTGAAAGAAGCAATTGCTTCACTACTTGATGATC
CCGCGTTGTATTAGCTAGTTGGTAGTGTAAAGGACTACCAAGGCGATGATACATAGCCGACCTGAGAGGGTGA
TCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGG
GGCAACCCTGACCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTTGTTAGAGAAGA
ACGTTAAGTAGAGTGGAAAATTACTTAAGTGACGGTATCTAACCAGAAAGGGACGGCTAACTACGTGCCAGCA
GCCGCGGTAATACGTAGGTCCCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGTGGTTTCTTAA
GTCTGATGTAAAAGGCAGTGGCTCAACCATTGTGTGCATTGGAAACTGGGAGACTTGAGTGCAGGAGAGGAGA
GTGGAATTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAGGAACACCGGAGGCGAAAGCGGCTCTCTGGC
CTGTAACTGACACTGAGGCTCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAA
CGATGAGTGCTAGCTGTAGGGAGCTATAAGTTCTCTGTAGCGCAGCTAACGCATTAAGCACTCCGCCTGGGGA
GTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTC
GAAGCAACGCGAAGAACCTTACCAGGTCTTGACATACTCGTGCTATCCTTAGAGATAAGGAGTTCCTTCGGGA
CACGGGATACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCG
CAACCCTTATTACTAGTTGCCATCATTAAGTTGGGCACTCTAGTGAGACTGCCGGTGATAAACCGGAGGAAGG
TGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGATGGTACAACGAG
TCGCCAACCCGCGAGGGTGCGCTAATCTCTTAAAACCATTCTCAGTTCGGATTGCAGGCTGCAACTCGCCTGC
ATGAAGTCGGAATCGCTAGTAATCGCGGATCAGCACGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCG
CCCGTCACACCACGGAAGTTGGGAGTACCC

19. Isolate SP15-B2

Organism; *Lc. garviae* (99.78%)

Nucleotide; 1360 bp

Source; Dog mouth

Accession No.; LC122289

GAAGATAGCTTGCTATTTTCATGAAGAGCGGCGAACGGGTGAGTAACGCGTGGGAAATCTGCCGAGTAGCGGG
GGACAACGTTTGGAAACGAACGCTAATACCGCATAACAATGAGAATCGCATGATTCTTATTTGAAAGAAGCAA
TTGCTTCACTACTTGATGATCCCGCGTTGTATTAGCTAGTTGGTAGTGTAAAGGACTACCAAGGCGATGATACA
TAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGT
AGGGAATCTTCGGCAATGGGGGCAACCCTGACCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAA
AACTCTGTTGTTAGAGAAGAACGTTAAGTAGAGTGGAAAATTACTTAAGTGACGGTATCTAACCAGAAAGGGA
CGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTCCCAAGCGTTGTCCGGATTTATTGGGCGTAAAGC
GAGCGCAGGTGGTTTCTTAAGTCTGATGTAAAAGGCAGTGGCTCAACCATTGTGTGCATTGGAAACTGGGAGA
CTTGAGTGCAGGAGAGGAGAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAGGAACACCGGA
GGCGAAAGCGGCTCTCTGGCCTGTAACTGACACTGAGGCTCGAAAGCGTGGGGAGCAAACAGGATTAGATACC
CTGGTAGTCCACGCCGTAAACGATGAGTGCTAGCTGTAGGGAGCTATAAGTTCTCTGTAGCGCAGCTAACGCA
TTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCG
GTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATACTCGTGCTATCCTTAG
AGATAAGGAGTTCCTTCGGGACACGGGATACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTG
GGTTAAGTCCCGCAACGAGCGCAACCCTTATTACTAGTTGCCATCATTAAGTTGGGCACTCTAGTGAGACTGC
CGGTGATAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGC
TACAATGGATGGTACAACGAGTCGCCAACCCGCGAGGGTGCGCTAATCTCTTAAAACCATTCTCAGTTCGGAT
TGCAGGCTGCAACTCGCCTGCATGAAGTCGGAATCGCTAGTAATCGCGGATCAGCACGCCGCGGTGAATACGT
TCCCGGGCCTTGTACACACCGCCCGTCACACCACGGAAGTTGGGAGT

20. Isolate AY1-bA1

Organism; *C. bifermentans* ATCC 638 (T) (99.85%)

Nucleotide; 1293bp

Source; Bark of *Ficus religiosa* L.

Accession No.; LC192840

GTGAGTAACGCGTGGGTAACCTGCCCTGTACACACGGATAACATACCGAAAGGTATACTAATACGGGATAACA
TACGAAAGTCGCATGGCTTTTGTATCAAAGCTCCGGCGGTACAGGATGGACCCGCGTCTGATTAGCTAGTTGG
TAAGGTAATGGCTTACCAAGGCAACGATCAGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGAACTGAGAC
ACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCAACGC
CGCGTGAGCGATGAAGGCCTTCGGGTCGTAAAGCTCTGTCCTCAAGGAAGATAATGACGGTACTTGAGGAGGA
AGCCCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGGGCTAGCGTTATCCGGAATTACTGGGCGT
AAAGGGTGCGTAGGTGGTTTTTTAAGTCAGAAGTGAAAGGCTACGGCTCAACCGTAGTAAGCTTTTGAAACTA
GAGAACTTGAGTGCAGGAGAGGAGAGTAGAATTCCTAGTGTAGCGGTGAAATGCGTAGATATTAGGAGGAATA
CCAGTAGCGAAGGCGGCTCTCTGGACTGTAACTGACACTGAGGCACGAAAGCGTGGGGAGCAAACAGGATTA
GATACCCTGGTAGTCCACGCCGTAAACGATGAGTACTAGGTGTCGGGGGTTACCCCCCTCGGTGCCGCAGCTA
ACGCATTAAGTACTCCGCCTGGGAAGTACGCTCGCAAGAGTGAAACTCAAAGGAATTGACGGGGACCCGCACA
AGTAGCGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCTAAGCTTGACATCCCACTGACCTCT
CCCTAATCGGAGATTTCCCTTCGGGGACAGTGGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAG
ATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGCCTTTAGTTGCCAGCATTAAGTTGGGCACTCTAGAGG
GACTGCCGAGGATAACTCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGCTTAGGGCTACAC
ACGTGCTACAATGGGTGGTACAGAGGGTTGCCAAGCCGCGAGGTGGAGCTAATCCCTTAAAGCCATTCTCAGT
TCGGATTGTAGGCTGAAACTCGCCTACATGAAGCTGGAGTTACTAGTAATCGCAGATCAGAATGCTGCGGTGA
ATGCGTTCCCGGGTCTTGTACACACCGCCCGTCACACCATGGAAGTTGGGGGCG

21. Isolate AY5-bA1

Organism; *C. tertium* DSM 2485 (T) (100.00 %)

Nucleotide; 1225bp

Source; Bark of *Ficus religiosa* L.

Accession No.; LC192789

GAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACATTGGGACTGAGAC
ACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGGGAAACCCTGATGCAGCAACGC
CGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTCTTCAGGGACGATAATGACGGTACCTGAGGAGGA
AGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCGAGCGTTGTCCGGATTTACTGGGCGT
AAAGGGAGCGTAGGCGGATTTTTAAGTGAGATGTGAAATACCCGGGCTCAACTTGGGTGCTGCATTTCAAACT
GGAAGTCTAGAGTGCAGGAGAGGAGAGTGGAATTCCTAGTGTAGCGGTGAAATGCGTAGAGATTAGGAAGAAC
ACCAGTGGCGAAGGCGACTCTCTGGACTGTAACTGACGCTGAGGCTCGAAAGCGTGGGGAGCAAACAGGATT
AGATACCCTGGTAGTCCACGCCGTAAACGATGAATACTAGGTGTAGGGGTTGTCATGACCTCTGTGCCGCGCA
AACGCATTAAGTATTCCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGAATTGACGGGGGCCCGCAC
AAGCAGCGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCTAGACTTGACATCTCCTGCATTAC
TCTTAATCGAGGAAGTCCCTTCGGGGACAGGATGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGA
TGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATTGTTAGTTGCTACCATTTAGTTGAGCACTCTAGCGAG
ACTGCCCGGGTTAACCGGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGTCTAGGGCTACACA
CGTGCTACAATGGCAAGTACAAAGAGATGCGATACCGCGAGGTGGAGCTAAACTATAAAACTTGTCTCAGTTC
GGATTGTAGGCTGAAACTCGCCTACATGAAGCTGGAGTTGCTAGTAATCGCGAATCAGAATGTCGCGGTGAAT
ACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGAGAGTTGGCAATACCCAAAGTTCGTGAGCTAACC
CGTAAGGGAGGCAGCGACCTAAGGTAGGGTCAGCGATTGGGGTGAAGTCGTAACAAGGTA

22. Isolate NP7-cB3

Organism; *Lactobacillus ruminis* NBRC 102161(T) (100.00 %)

Nucleotide; 1321bp

Source; Bovine rumen fluid

Accession No.; LC192790

GGCGAACGGGTGAGTAACACGTAGGCAACCTGCCCAAAAGAGGGGGATAACACTTGGAAACAGGTGCTAATAC
CGCATAACCATGAACACCGCATGATGTTCATGTAAAAGACGGCTTTTGCTGTCACTTTTGGATGGGCCTGCGG
CGTATTAACTTGTTGGTGGGGTAATGGCCTACCAAGGTGATGATACGTAGCCGAACTGAGAGGTTGATCGGCC
ACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGAAAGT
CTGATGGAGCAACGCCGCGTGAATGAAGAAGGCCTTCGGGTCGTAAAATTCTGTTGTCAGAGAAGAACGTGCG
TGAGAGTAACTGTTCACGTATTGACGGTATCTGACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGT
AATACGTAGGTGGCGAGCGTTGTCCGGATTTATTGGGCGTAAAGGGAACGCAGGCGGTCTTTTAAGTCTGATG
TGAAAGCCTTCGGCTTAACCGAAGTAGTGCATTGGAAACTGGAAGACTTGAGTGCAGAAGAGGAGAGTGGAAC
TCCATGTGTAGCGGTGAAATGCGTAGATATATGGAAGAACACCAGTGGCGAAAGCGGCTCTCTGGTCTGTAAC
TGACGCTGAGGTTCGAAAGCGTGGGTAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAG
TGCTAAGTGTTGGAGGGTTTCCGCCCTTCAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGG
TCGCAAGACTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCA
ACGCGAAGAACCTTACCAGGTCTTGACATCTTCTGACAATTCCAGAGATGGAACGTTCCCTTCGGGGACAGAA
TGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCC
TTATTGTCAGTTGCCATCATTAAGTTGGGCACTCTGGCGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGA
TGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGACGGTACAACGAGTCGCTA
ACTCGCGAGGGCAAGCTAATCTCTTAAAGCCGTTCTCAGTTCGGATTGCAGGCTGCAACTCGCCTGCATGAAG
TCGGAATCGCTAGTAATCGCGAATCAGCATGTCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTC
ACACCAT

23. Isolate NP8-aB2

Organism; *Streptococcus lutetiensis* CIP 106849 (T) (99.84%)

Nucleotide; 1262bp

Source; Bovine rumen fluid

Accession No.; LC192791

TACTAGCGGGGGATAACTATTGGAAACGATAGCTAATACCGCATAACAGCATTTAACACATGTTAGATGCTTG
AAAGGAGCAATTGCTTCACTAGTAGATGGACCTGCGTTGTATTAGCTAGTTGGTGAGGTAACGGCTCACCAAG
GCGACGATACATAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGG
GAGGCAGCAGTAGGGAATCTTCGGCAATGGGGGCAACCCTGACCGAGCAACGCCGCGTGAGTGAAGAAGGTT
TTCGGATCGTAAAGCTCTGTTGTAAGAGAAGAACGTGTGTGAGAGTGGAAAGTTCACACAGTGACGGTAACTT
ACCAGAAAGGGACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTCCCGAGCGTTGTCCGGATTTAT
TGGGCGTAAAGCGAGCGCAGGCGGTTTAATAAGTCTGAAGTTAAAGGCAGTGGCTTAACCATTGTTCGCTTTG
GAAACTGTTAGACTTGAGTGCAGAAGGGGAGAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGATATATGG
AGGAACACCGGTGGCGAAAGCGGCTCTCTGGTCTGTAACTGACGCTGAGGCTCGAAAGCGTGGGGAGCAAAC
AGGATTACATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAGGTGTTAGGCCCTTTCCGGGGCTTAGTG
CCGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGG
GCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCCG
ATGCTATTCCTAGAGATAGGAAGTTTCTTCGGAACATCGGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTG
TCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCCTATTGTTAGTTGCCATCATTAAGTTGGGCACT
CTAGCGAGACTGCCGGTAATAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGG
GCTACACACGTGCTACAATGGTTGGTACAACGAGTCGCGAGTCGGTGACGGCAAGCAAATCTCTTAAAGCCAA
TCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGTCGGAATCGCTAGTAATCGCGGATCAGCACGCC
GCGGTGAATACGTTCCCGGGCCTT

24. Isolate NP9-aA3

Organism; *Proteus mirabilis* ATCC 29906 (T) (99.92 %)

Nucleotide; 1257bp

Source; Bovine rumen

Accession No.; LC192792

TAGAGGGGGATAACTACTGGAAACGGTGGCTAATACCGCATAATGTCTACGGACCAAAGCAGGGGCTCTTCGG
ACCTTGCACTATCGGATGAACCCATATGGGATTAGCTAGTAGGTGGGGTAAAGGCTCACCTAGGCGACGATCT
CTAGCTGGTCTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAG
TGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTAGGGTTGTA
AAGTACTTTCAGCGGGGAGGAAGGTGATAAGGTTAATACCCTTATCAATTGACGTTACCCGCAGAAGAAGCAC
CGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGC
GCACGCAGGCGGTCAATTAAGTCAGATGTGAAAGCCCCGAGCTTAACTTGGGAATTGCATCTGAAACTGGTTG
GCTAGAGTCTTGTAGAGGGGGGTAGAATTCCATGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAATACCGG
TGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATA
CCCTGGTAGTCCACGCTGTAAACGATGTCGATTTAGAGGTTGTGGTCTTGAACCGTGGCTTCTGGAGCTAACG
CGTTAAATCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGC
GGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTACTCTTGACATCCAGCGAATCCTTTAG
AGATAGAGGAGTGCCTTCGGGAACGCTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTT
GGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCACGTAATGGTGGGAACTCAAAGGAGAC
TGCCGGTGATAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGAGTAGGGCTACACACG
TGCTACAATGGCAGATACAAAGAGAAGCGACCTCGCGAGAGCAAGCGGAACTCATAAAGTCTGTCGTAGTCCG
GATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGTAGATCAGAATGCTACGGTGAATA
CGTTCCCGGGCCTTGTAC

25. Isolate NP9-aA7

Organism; *A. succinogenes* 130Z (T) (99.86 %)

Nucleotide; 1440bp

Source; Buffalo rumen fluid

Accession No.; LC192793

CCTGGCTCAGATTGAACGCTGGCGGCAGGCTTAACACATGCAAGTCGAACGGTAACGGGTGGAAAGCTTGCTT
TCCATGCTGACGAGTGGCGGACGGGTGAGTAATGCTTGGGGATCTGGCTTATGGAGGGGGATAACGACGGGA
AACTGTCGCTAATACCGCGTAATGTCTAAGGACTAAAGGGTGGGATTTTCGGACCGCCCGCCATAAGATGAGC
CCAAGTGGGATTAGGTAGTTGGTGGGGTAAAGGCCTACCAAGCCGACGATCTCTAGCTGGTCTGAGAGGATGA
CCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCGCAATGGG
GGCAACCCTGACGCAGCCATGCCGCGTGAATGAAGAAGGCCTTCGGGTTGTAAAGTTCTTTCGGTGGTGAGGA
AGGCGAATAAGTTAACAGCTTATTCGATTGACGTTAGCCACAGAAGAAGCACCGGCTAACTCCGTGCCAGCAG
CCGCGGTAATACGGAGGGTGCGAGCGTTAATCGGAATAACTGGGCGTAAAGGGCACGCAGGCGGCTATTTAA
GTGAGATGTGAAATCCCCGGGCTTAACCTGGGAACTGCATTTCAGACTGGGTAGCTAGAGTACTTTAGGGAGG
GGTAGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAATACCGAAGGCGAAGGCAGCCCCTTG
GGAACGTACTGACGCTCATGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCTGTA
AACGCTGTCGATTTGGGGATTGGGCGATAAGCCTGGTGCCCGAAGCTAACGTGATAAATCGACCGCCTGGGGA
GTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTC
GATGCAACGCGAAGAACCTTACCTACTCTTGACATCCTCAGAATCCGGTAGAGATATCGGAGTGCCTTCGGGA
ACTGAAAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGGTTAAGTCCCGCAACGAGC
GCAACCCTTATCCTTTGTTGCCAGCATGTAGAGATGGGAACTCAAAGGAGACTGCCGGTGATAAACCGGAGGA
AGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGAGTAGGGCTACACACGTGCTACAATGGCGTATACAGA
GGGAAACGAGCCTGCGAGGGGGAGTGAATCTCAGAAAGTGCGTCGTAGTCCGGATTGGAGTCTGCAACTCGA
CTCCATGAAGTCGGAATCGCTAGTAATCGCGAATCAGCATGTCGCGGTGAATACGTTCCCGGGCCTTGTACAC
ACCGCCCGTCACACCATGGGGAGTGGGTTGTACCAGAAGTAGATAGCTTAACCGAAAG

26. Isolate NP9-cA4

Organism; *L. reuteri* JCM 1112 (T) (99.70 %)

Nucleotide; 1319bp

Source; Buffalo rumen fluid

Accession No.; LC192794

GGCGGACGGGTGAGTAACACGTAGGTAACCTGCCCCGGAGCGGGGGATAACATTTGGAAACAGATGCTAATAC
CGCATAACAACAAAAGCCACATGGCTTTTGTTTGAAAGATGGCTTTGGCTATCACTCTGGGATGGACCTGCGG
TGCATTAGCTAGTTGGTAAGGTAACGGCTTACCAAGGCGATGATGCATAGCCGAGTTGAGAGACTGATCGGCC
ACAATGGAACTGAGACACGGTCCATACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGGCGCAAGC
CTGATGGAGCAACACCGCGTGAGTGAAGAAGGGTTTCGGCTCGTAAAGCTCTGTTGTTGGAGAAGAACGTGCG
TGAGAGTAACTGTTCACGCAGTGACGGTATCCAACCAGAAAGTCACGGCTAACTACGTGCCAGCAGCCGCGGT
AATACGTAGGTGGCAAGCGTTATCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTGCTTAGGTCTGATG
TGAAAGCCTTCGGCTTAACCGAAGAAGTGCATCGGAAACCGGGCGACTTGAGTGCAGAAGAGGACAGTGGAAC
TCCATGTGTAGCGGTGGAATGCGTAGATATATGGAAGAACACCAGTGGCGAAGGCGGCTGTCTGGTCTGCAAC
TGACGCTGAGGCTCGAAAGCATGGGTAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGATGAG
TGCTAGGTGTTGGAGGGTTTCCGCCCTTCAGTGCCGGAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGA
CCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCT
ACGCGAAGAACCTTACCAGGTCTTGACATCTTGCGCTAACCTTAGAGATAAGGCGTTCCCTTCGGGGACGCAA
TGACAGGTGGTGCATGGTCGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCC
TTGTTACTAGTTGCCAGCATTAAGTTGGGCACTCTAGTGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGA
CGACGTCAGATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGACGGTACAACGAGTCGCAA
ACTCGCGAGAGCAAGCTAATCTCTTAAAGCCGTTCTCAGTTCGGACTGTAGGCTGCAACTCGCCTACACGAAG
TCGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTC
ACACC

27. Isolate NS14-dA2

Organism; *E. lactis* BT159 (T) (99.70 %)

Nucleotide; 1346bp

Source; Bovine rumen tissue

Accession No.; LC192795

GTGGCGAACGGGTGAGTAACACGTGGGTAACCTGCCCATCAGAAGGGGATAACACTTGGAAACAGGTGCTAAT
ACCGTATAACAATCGAAACCGCATGGTTTTGATTTGAAAGGCGCTTTCGGGTGTCGCTGATGGATGGACCCGC
GGTGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCCACGATGCATAGCCGACCTGAGAGGGTGATCGG
CCACATTGGGACTGAGACACGGCCCAAACTCATACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGACGAAA
GTCTGACCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTTGTTAGAGAAGAACAAG
GATGAGAGTAACTGTTCATCCCTTGACGGTATCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCG
GTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTCTTAAGTCTGA
TGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAGAGTGG
AATTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAGGAACACCAGTGGCGAAGGCGGCTCTCTGGTCTGT
AACTGACGCTGAGGCTCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGAT
GAGTGCTAAGTGTTGGAGGGTTTCCGCCCTTCAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTA
CGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAA
GCAACGCGAAGAACCTTACCAGGTCTTGACATCCTTTGACCACTCTAGAGATAGAGCTTCCCCTTCGGGGGCA
AAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAA
CCCTTATTGTTAGTTGCCATCATTCAGTTGGGCACTCTAGCAAGACTGCCGGTGACAAACCGGAGGAAGGTGG
GGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGGAAGTACAACGAGTTG
CGAAGTCGCGAGGCTAAGCTAATCTCTTAAAGCTTCTCTCAGTTCGGATTGCAGGCTGCAACTCGCCTGCATG
AAGCCGGAATCGCTAGTAATCGCGGATCAGCACGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCC
GTCACACCACGAGAGTTTGTAACACCCGAAGTC

28. Isolate NS18-A1

Organism; *C. amygdalinum* BR-10 (T) (97.82%)

Nucleotide; 1491bp

Source; Fruit fermented

Accession No.; LC192796

GAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAGCGAAGCAGTTTGAATGAAGCTTTCGGATGGATTTCAA
ATTGACTGAGCGGCGGACGGGTGAGTAACGCGTGGGTAACCTGCCTCATACAGGGGGATAACAGTTGGAAAC
GACTGCTAATACCGCATAAGCGCACAGTGCCGCATGGCACGGTGCGAAAAACTCCGGTGGTATGAGATGGACC
CGCGTCTGATTAGGTAGTTGGTGAGGTAACGGCCCACCAAGCCGACGATCAGTAGCCGACCTGAGAGGGTGAC
CGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGG
GAAACCCTGATCCAGCGACGCCGCGTGACTGAAGAAGTATTTCGGTATGTAAAGGTCTATCAGCAGGGAAGAA
AATGACGGTACCTGACTAAGAAGCCCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGGGCAAGC
GTTATCCGGATTTACTGGGTGTAAAGGGAGCGTAGACGGCGCTGCAAGTCTGGAGTGAAAGCCCGGGGCTCAA
CCCCGGGACTGCTTTGGAAACTGTGGGGCTGGAGTGCAGGAGAGGTAAGTGGAATTCCTAGTGTAGCGGTGAA
ATGCGTAGATATTAGGAGGAACACCAGTGGCGAAGGCGGCTTACTGGACTGTAACTGACGTTGAGGCTCGAAA
GCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAATACTAGGTGTTGGGGAGC
AAAGCTCTTCGGTGCCGCCGCAAACGCAATAAGTATTCCACCTGGGGAGTACGTTCGCAAGAATGAAACTCAA
AGGAATTGACGGGGACCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAA
GTCTTGACATCGGAATGACCGTCTCGTAACGGAGACTTCCCTTCGGGGCATTCCAGACAGGTGGTGCATGGTT
GTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTAGTAGCCAGCA
GGTAAAGCTGGGCACTCTGAGGAGACTGCCAGGGATAACCTGGAGGAAGGTGGGGATGACGTCAAATCATCAT
GCCCCTTATGATTTGGGCTACACACGTGCTACAATGGCGTAAACAAAGGGAGGCAAAGCCGCGAGGTGGAGCA
AATCCCAAAAATAACGTCTCAGTTCGGATTGTAGTCTGCAACTCGACTACATGAAGCTGGAATCGCTAGTAATC
GCGGATCAGAATGCCGCGGTGAATACGTTCCCGGGTCTTGTACACACCGCCCGTCACACCATGGGGAGTTGGT
AACGCCCGAAGTCAGTGACCCAACCGCAAGGGATGGAGCTGCCGAAGGCGGGACTGATAACTGGGGGTGAAG
TCGTAACAAGGTAGCCGTATCGGAAGGTGCGCTG

29. Isolate SP10-A5

Organism; *Enterococcus hirae* ATCC 9790 (T) (100.00%)

Nucleotide; 1356bp

Source; Soil

Accession No.; LC192797

GAGGAGTGGCGAACGGGTGAGTAACACGTGGGTAACCTGCCCATCAGAAGGGGATAACACTTGGAAACAGGT
GCTAATACCGTATAACAATCGAAACCGCATGGTTTTGATTTGAAAGGCGCTTTCGGGTGTCGCTGATGGATGG
ACCCGCGGTGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCATAGCCGACCTGAGAGGGT
GATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATG
GACGAAAGTCTGACCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTTGTTAGAGAA
GAACAAGGATGAGAGTAACTGTTCATCCCTTGACGGTATCTAACCAGAAAGCCACGGCTAACTACGTGCCAGC
AGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTCTTA
AGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGG
AGAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAGGAACACCAGTGGCGAAGGCGGCTCTCT
GGTCTGTAACTGACGCTGAGGCTCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGT
AAACGATGAGTGCTAAGTGTTGGAGGGTTTCCGCCCTTCAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTG
GGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTT
AATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTTTGACCACTCTAGAGATAGAGCTTCCCCTT
CGGGGGCAAAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAAC
GAGCGCAACCCTTATTGTTAGTTGCCATCATTTAGTTGGGCACTCTAGCAAGACTGCCGGTGACAAACCGGAG
GAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGGAAGTACA
ACGAGTCGCAAAGTCGCGAGGCTAAGCTAATCTCTTAAAGCTTCTCTCAGTTCGGATTGTAGGCTGCAACTCG
CCTACATGAAGCCGGAATCGCTAGTAATCGCGGATCAGCACGCCGCGGTGAATACGTTCCCGGGCCTTGTACA
CACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGA

30. Isolate SP13-B2

Organism; *Clostridium butyricum* DSM 10702 (T) (98.57%)

Nucleotide; 1190 bp

Source; Mount of dog

Accession No.; LC192798

CGCTGGCGGCGTGCTTAACACATGCAAGTCGAGCGATGAAGCTCCTTCGGGAGTGGATTAGCGGCGGACGGG
TGAGTAACACGTGGGTAACCTGCCTCATAGAGGGGAATAGCCTTTCGAAAGGAAGATTAATACCGCATAAGAT
AGTAGTATCGCATGGTACAGCTATTAAAGGAGTAATCCGCTATGAGATGGACCCGCGTCGCATTAGCTAGTTG
GTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACATTGGGACTGAG
ACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGGGAAACCCTGATGCAGCAAC
GCCGCGTGAGTGATGACGGCCTTCGGATTGTAAAACTCTGTCTTTGGGGACGATAATGACGGTACCCAAGGAG
GAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTACTGGGC
GTAAAGGGAGCGTAGGTGGATATTTAAGTGGGATGTGAAATACTCGGGCTTAACCTGGGTGCTGCATTCCAAA
CTGGATATCTAGAGTGCAGGAGAGGAAAGGAGAATTCCTAGTGTAGCGGTGAAATGCGTAGAGATTAGGAAGA
ATACCAGTGGCGAAGGCGCCTTTCTGGACTGTAACTGACACTGAGGCTCGAAAGCGTGGGGAGCAAACAGGAT
TAGATACCCTGGTAGTCCACGCCGTAAACGATGAATACTAGGTGTAGGGGTTGTCATGACCTCTGTGCCGCCG
CTAACGCATTAAGTATTCCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGAATTGACGGGGGCCCGC
ACAAGCAGCGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCTAGACTTGACATCTCCTGAATT
ACTCTGTAATGGAGGAAGCCACTTCGGTGGCAGGAAGACACGTGGTGCATGGTTGTCGTCAGATCGTGTCGTG
AGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATTGTTAGTTGCTACCACTTAGTTGAGCACTCTAGC
GAGACTGCCCGGGTTAACCGGCAAGAAGGTGGAGATGACGTCGAATCATCATGCCCCTTATGTCTAGGGCTAC
ACACATGCTACAATGGTCGGTACAA

31. Isolate SP17-B1

Organism; *Clostridium amygdalinum* BR-10 (T) (97.84%)

Nucleotide; 1398bp

Source; Bovine rumen

Accession No.; LC192799

TTCTGGCTCAGGATGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAGCGAAGCAGTTTGAATGAAGCTTT
CGGATGGATTTCAAATTGACTGAGCGGCGGACGGGTGAGTAACGCGTGGGTAACCTGCCTCATACAGGGGGAT
AACAGTTGGAAACGACTGCTAATACCGCATAAGCGCACAGTGCCGCATGGCACGGTGCGAAAAACTCCGGTGG
TATGAGATGGACCCGCGTCTGATTAGGTAGTTGGTGAGGTAACGGCCCACCAAGCCGACGATCAGTAGCCGAC
CTGAGAGGGTGACCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTGGGGAATA
TTGGACAATGGGGGAAACCCTGATCCAGCGACGCCGCGTGACTGAAGAAGTATTTCGGTATGTAAAGGTCTAT
CAGCAGGGAAGAAAATGACGGTACCTGACTAAGAAGCCCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACG
TAGGGGGCAAGCGTTATCCGGATTTACTGGGTGTAAAGGGAGCGTAGACGGCGCTGCAAGTCTGGAGTGAAA
GCCCGGGGCTCAACCCCGGGACTGCTTTGGAAACTGTGGGGCTGGAGTGCAGGAGAGGTAAGTGGAATTCCT
AGTGTAGCGGTGAAATGCGTAGATATTAGGAGGAACACCAGTGGCGAAGGCGGCTTACTGGACTGTAACTGAC
GTTGAGGCTCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAATACT
AGGTGTTGGGGAGCAAAGCTCTTCGGTGCCGCCGCAAACGCAATAAGTATTCCACCTGGGGAGTACGTTCGCA
AGAATGAAACTCAAAGGAATTGACGGGGACCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCG
AAGAACCTTACCAAGTCTTGACATCGGAATGACCGTCTCGTAACGGAGACTTCCCTTCGGGGCATTCCAGACA
GGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATC
CTTAGTAGCCAGCAGGTAAAGCTGGGCACTCTGAGGAGACTGCCAGGGATAACCTGGAGGAAGGTGGGGATG
ACGTCAAATCATCATGCCCCTTATGATTTGGGCTACACACGTGCTACAATGGCGTAAACAAAGGGAGGCAAAG
CCGCGAGGTGGAGCAAATCCCAAAAATAACGTCTCAGTTCGGATTGTAGTCTGCAACTCGACTACATGAAGCT
GGAATCGCTAGTAATCGCGGATCAGAATGCCGCGGTGAATACGTTCCCGGGTCTTGTACACACCGCCCGTCAC
ACCATGGGAGTTGGT

32. Isolate NS17-B1

Organism; *Clostridium indolis* DSM 755 (T) (99.84%)

Nucleotide; 1238bp

Source; Fruit fermented

Accession No.; LC192800

TAACGCGTGGGTAACCTGCCTCATACAGGGGGATAACAGTTGGAAACGACTGCTAATACCGCATAAGCACACA
GTGCCGCATGGTACGGTGTGAAAAACTCCGGTGGTATGAGATGGACCCGCGTCTGATTAGGTAGTAGGTGAGG
TAACGGCCCACCAAGCCGACGATCAGTAGCCGACCTGAGAGGGTGACCGGCCACATTGGGACTGAGACACGG
CCCAAACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGGGAAACCCTGATCCAGCGACGCCGCG
TGAGTGAAGAAGTGTTTCGGCATGTAAAGCTCTATCAGCAGGGAAGAAAATGACGGTACCTGACTAAGAAGCC
CCGGCTAACTACGTGCCAGCAGCCGCGGGTAATACGTAGGGGGGCAAGCGTTATCCGGATTTACTGGGTGTAA
AGGGAGCGTAGACGGCGATGCAAGTCTGGAGTGAAAGCCCGGGGCTCAACCCCGGGGACTGCTTTGGAAACT
GTGTTGCTAGAGTGCAGGAGAGGTAAGTGGAATTCCTAGTGTAGCGGTGAAATGCGTAGATATTAGGAGGAAC
ACCAGTGGCGAAGGCGGCTTACTGGACTGTAACTGACGTTGAGGCTCGAAAGCGTGGGGAGCAAACAGGATTA
GATACCCCTGGTAGTCCACGCCGTAAACGATGAATACTAAGGTGTTGGGGAGCAAAGCTCTTCCGGTGCCGCC
GCTAACGCAATAAGTATTCCACCTGGGGGAGTACGTTCGCAAGAATGAAACTCAAAGGAATTGACGGGGACCC
GCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAAGTCTTGACATCGGAATGA
CCGGTCCGTAACGGGGCCTTCCCTTCGGGGCATTCCAGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGT
GAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTAGTAGCCAGCAAGTCAAGTTGGGCACTCT
GGGGAGACTGCCAGGGATAACCTGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGATTTGGGC
TACACACGTGCTACAATGGCGTAAACAAAGGGAAGCAAAGGAGTGATCCGGAGCAAACCCCAAAAATAACGTC
TCAGTTCGGATTGTAGTCTGCAACTCGACTACATGAAGCTGGAATCGCTAGTAATCGCGGATCAGAATGCCGC

APPENDIX C

Calibration curve and Standard graph

C1. Calibration curve for various concentration of glucose by DNS method

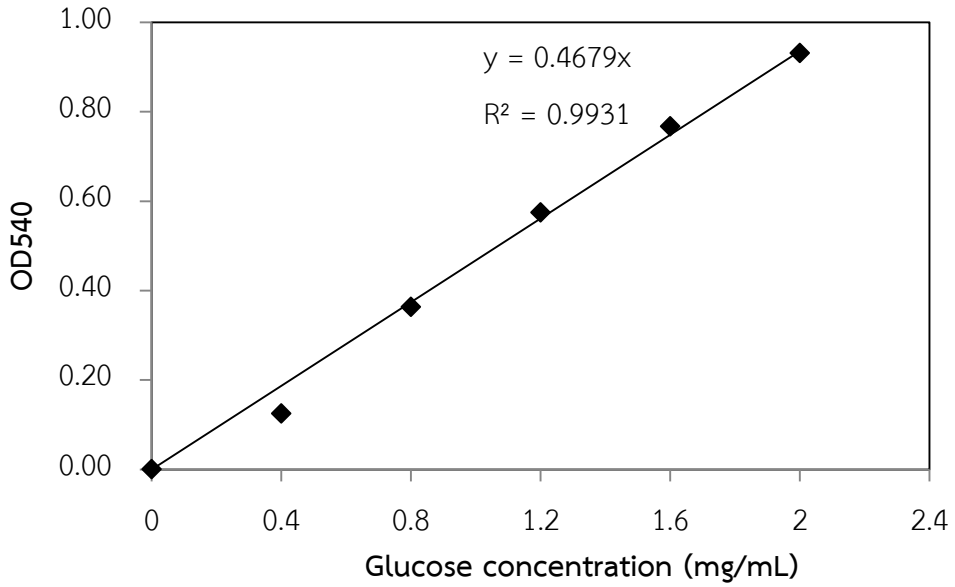


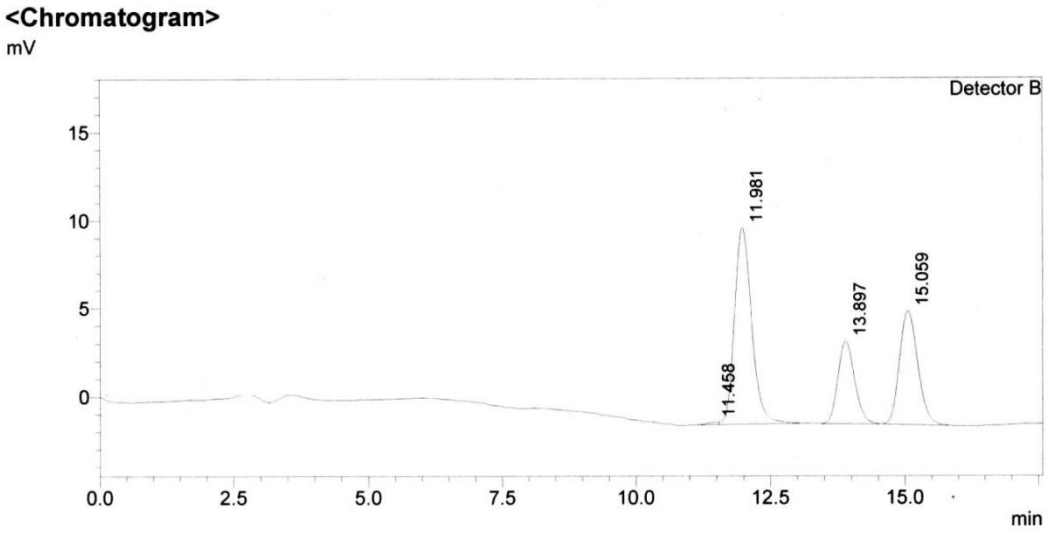
Figure B Calibration curve for various concentration of glucose by DNS method

Equation; Y = 0.4679 X

$$\text{Glucose concentration (g/L)} = \frac{\mathbf{OD540}}{\mathbf{0.4679}}$$

C2. Standard peaks of organic acid by HPLC

(Aminex HPX-87H Column)

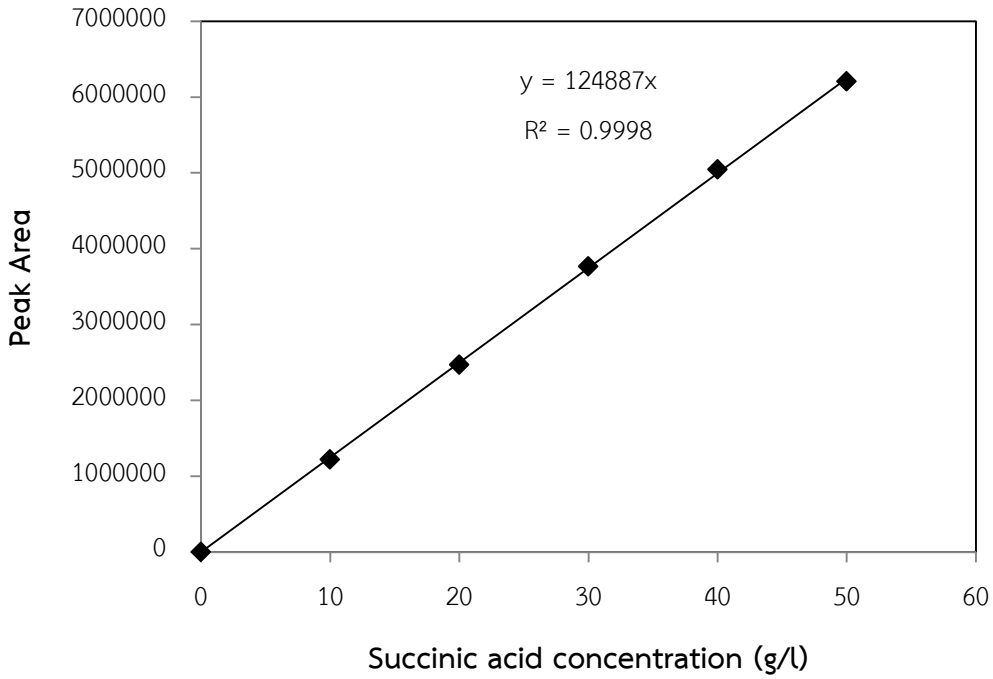


FigureC.2 A Standard peaks of organic acid on the Aminex HPX-87H Column

(Succinic acid, Formic acid and Acetic acid)

Peak	Name	Retention time	Area	Height
1	Succinic acid	11.981	247084	11118
2	Formic acid	13.897	101006	4652
3	Acetic acid	15.059	152550	6398

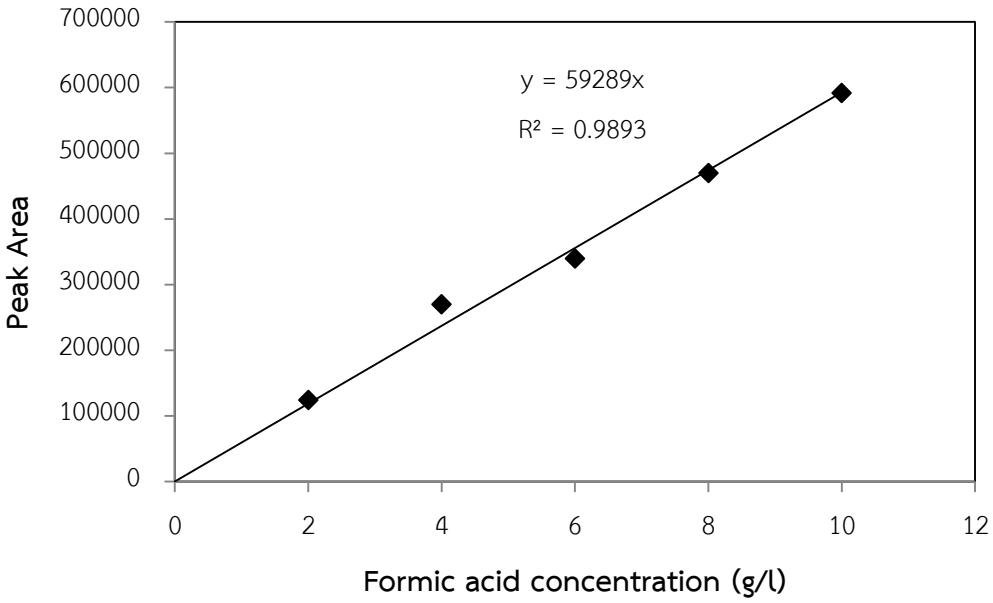
C3. Calibration curve for succinic acid by HPLC



Equation; $Y = 124887 \cdot X$

$$\text{Succinic acid (g/L)} = \frac{\text{Peak area}}{124887}$$

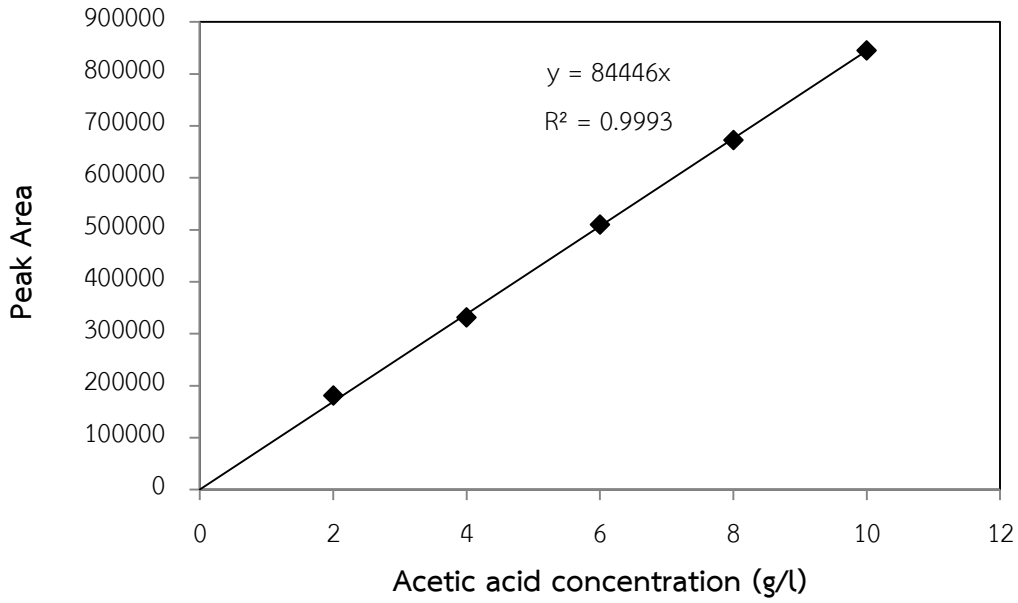
C4. Calibration curve for formic acid by HPLC



Equation; $Y = 59289 \cdot X$

$$\text{Formic acid (g/L)} = \frac{\text{Peak area}}{59289}$$

C5. Calibration curve for acetic acid by HPLC



Equation; $Y = 84446 \cdot X$

$$\text{Succinic acid (g/L)} = \frac{\text{Peak area}}{84446}$$

APPENDIX D

Statistical Analysis

Table D1 ANOVA result for cell growth of *A. succinogenes* NP9-aA7

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1471.717	13	113.209	102.773	.000
Within Groups	30.843	28	1.102		
Total	1502.560	41			

Table D2 Multiple comparison for cell growth of *A. succinogenes* NP9-aA7 by Tukey's Method

		N	Subset for alpha = .05							
	time		1	2	3	4	5	6	7	8
Tukey HSD[(a)]	0 h	3	.31100							
	6 h	3	2.05167	2.05167						
	3 h	3	3.06900	3.06900						
	9 h	3		4.09400	4.09400					
	12 h	3		4.58500	4.58500	4.58500				
	15 h	3			6.34300	6.34300				
	18 h	3			6.77800	6.77800				
	21 h	3				7.24500	7.24500			
	24 h	3					10.10600	10.10600		
	30 h	3						12.80800	12.80800	
	48 h	3							15.62433	15.62433
	42 h	3								16.88833
	36 h	3								16.98267
	60 h	3								18.35233
	Sig.		.129	.213	.153	.161	.101	.147	.112	.138

Means for groups in homogeneous subsets are displayed.; [a] Uses Harmonic Mean Sample Size = 3.000.

Table D3 Multiple comparison for cell growth of *A. succinogenes* NP9-aA7 by Duncan's Method

	time	N	Subset for alpha = .05							
			1	2	3	4	5	6	7	8
Duncan[(a)]	0 h	3	.31100							
	6 h	3	2.05167	2.05167						
	3 h	3		3.06900	3.06900					
	9 h	3			4.09400					
	12 h	3			4.58500					
	15 h	3				6.34300				
	18 h	3				6.77800				
	21 h	3				7.24500				
	24 h	3					10.10600			
	30 h	3						12.80800		
	48 h	3							15.62433	
	42 h	3							16.88833	16.88833
	36 h	3							16.98267	16.98267
	60 h	3								18.35233
	Sig.		.052	.245	.105	.330	1.000	1.000	.145	.117

Table D4 Multiple comparison for cell growth of *A. succinogenes* NP9-aA7 by Scheffe's Method

	time	N	Subset for alpha = .05							
			1	2	3	4	5	6	7	8
Scheffe[(a)]	0 h	3	.31100							
	6 h	3	2.05167	2.05167						
	3 h	3	3.06900	3.06900	3.06900					
	9 h	3	4.09400	4.09400	4.09400					
	12 h	3	4.58500	4.58500	4.58500					
	15 h	3		6.34300	6.34300	6.34300				
	18 h	3			6.77800	6.77800				
	21 h	3			7.24500	7.24500				
	24 h	3				10.10600	10.10600			
	30 h	3					12.80800	12.80800		
	48 h	3						15.62433	15.62433	
	42 h	3						16.88833	16.88833	
	36 h	3						16.98267	16.98267	
	60 h	3							18.35233	
	Sig.		.073	.071	.089	.185	.688	.089	.674	

Means for groups in homogeneous subsets are displayed.; [a] Uses Harmonic Mean Sample Size = 3.000.

Table D5 ANOVA for result of various concentration of biotin on cell growth and succinic acid production by *A. succinogenes* NP9-aA7

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2467.101	4	616.775	120.582	.000
Within Groups	51.150	10	5.115		
Total	2518.251	14			

Table D6 Multiple comparison for result of various concentrations of biotin on cell growth and succinic acid production by *A. succinogenes* NP9-aA7

	Biotin_conc	N	Subset for alpha = .05	
			1	2
Tukey HSD[(a)]	0 µg/L	3	14.52767	
	50 µg/L	3		44.19100
	100 µg/L	3		45.57500
	150 µg/L	3		47.41500
	200 µg/L	3		48.36067
	Sig.		1.000	.235
Duncan[(a)]	0 µg/L	3	14.52767	
	50 µg/L	3		44.19100
	100 µg/L	3		45.57500
	150 µg/L	3		47.41500
	200 µg/L	3		48.36067
	Sig.		1.000	.061
Scheffe[(a)]	0 µg/L	3	14.52767	
	50 µg/L	3		44.19100
	100 µg/L	3		45.57500
	150 µg/L	3		47.41500
	200 µg/L	3		48.36067
	Sig.		1.000	.343

Means for groups in homogeneous subsets are displayed.; [a] Uses Harmonic Mean Sample Size = 3.000.

Table D7 ANOVA for result of different nitrogen sources on cell growth and succinic acid production by *A. succinogenes* NP9-aA7

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	7352.907	7	1050.415	135.696	.000
Within Groups	123.855	16	7.741		
Total	7476.763	23			

Table D8 Multiple comparison for result of different nitrogen sources on cell growth and succinic acid production by *A. succinogenes* NP9-aA7

	Nitrogen_source	N	Subset for alpha = .05				
			1	2	3	4	5
Tukey HSD[a]	KNO_3	3	3.72167				
	Urea	3	3.95467				
	$(NH_4)_2SO_4$	3		15.28167			
	CSL	3			27.93867		
	NH4Cl	3			35.16400	35.16400	
	Peptone	3				36.17467	
	Yeast extract	3					49.75600
	Beef extract	3					51.02700
	Sig.		1.000	1.000	.084	1.000	.999
Duncan[(a)]	KNO_3	3	3.72167				
	Urea	3	3.95467				
	$(NH_4)_2SO_4$	3		15.28167			
	CSL	3			27.93867		
	NH4Cl	3				35.16400	
	Peptone	3				36.17467	
	Yeast extract	3					49.75600
	Beef extract	3					51.02700
	Sig.		.920	1.000	1.000	.662	.584
Scheffe[(a)]	KNO_3	3	3.72167				
	Urea	3	3.95467				
	$(NH_4)_2SO_4$	3		15.28167			
	CSL	3			27.93867		
	NH4Cl	3			35.16400		
	Peptone	3			36.17467		
	Yeast extract	3				49.75600	
	Beef extract	3				51.02700	
	Sig.		1.000	1.000	.140	1.000	

Means for groups in homogeneous subsets are displayed.; [a] Uses Harmonic Mean Sample Size = 3.000.

Table D9 ANOVA for result of different alkaline neutralizer on cell growth and succinic acid production by *A. succinogenes* NP9-aA7

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	3272.713	7	467.530	48.478	.000
Within Groups	154.306	16	9.644		
Total	3427.019	23			

Table D10 Multiple comparison for result of different alkaline neutralizer on cell growth and succinic acid production by *A. succinogenes* NP9-aA7

		N	Subset for alpha = .05			
	Alkaline		1	2	3	4
Tukey HSD(a)	NaOH	3	.82900			
	No alkaline	3	4.91400	4.91400		
	$CaCO_3$	3		10.62800		
	$NaHCO_3$	3		11.88267		
	$Ca(OH)_2$	3			23.53633	
	Na_2CO_3	3			23.77600	
	$Mg(OH)_2$	3			30.42700	30.42700
	$MgCO_3$	3				35.75500
	Sig.		.738	.177	.186	.453
Duncan(a)	NaOH	3	.82900			
	No alkaline	3	4.91400			
	$CaCO_3$	3		10.62800		
	$NaHCO_3$	3		11.88267		
	$Ca(OH)_2$	3			23.53633	
	Na_2CO_3	3			23.77600	
	$Mg(OH)_2$	3				30.42700
	$MgCO_3$	3				35.75500
	Sig.		.127	.627	.926	.052
Scheffe(a)	NaOH	3	.82900			
	No alkaline	3	4.91400	4.91400		
	$CaCO_3$	3	10.62800	10.62800		
	$NaHCO_3$	3		11.88267		
	$Ca(OH)_2$	3			23.53633	
	Na_2CO_3	3			23.77600	
	$Mg(OH)_2$	3			30.42700	30.42700
	$MgCO_3$	3				35.75500
	Sig.		.099	.420	.434	.724

Means for groups in homogeneous subsets are displayed.; [a] Uses Harmonic Mean Sample Size = 3.000.

Table D11 ANOVA for result of various ratio of $Mg(OH)_2$ and $MgCO_3$ on cell growth and succinic acid production by *A. succinogenes* NP9-aA7

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	873.157	4	218.289	94.719	.000
Within Groups	23.046	10	2.305		
Total	896.204	14			

Table D12 Multiple comparison for result of various ratio of $Mg(OH)_2$ and $MgCO_3$ on cell growth and succinic acid production by *A. succinogenes* NP9-aA7

	Ratio	N	Subset for alpha = .05			
			1	2	3	4
Tukey HSD(a)	1:3	3	27.82333			
	1:2	3	28.47000			
	1:1	3		32.99500		
	2:1	3			37.60500	
	3:1	3				48.64400
	Sig.		.983	1.000	1.000	1.000
Duncan(a)	1:3	3	27.82333			
	1:2	3	28.47000			
	1:1	3		32.99500		
	2:1	3			37.60500	
	3:1	3				48.64400
	Sig.		.613	1.000	1.000	1.000
Scheffe(a)	1:3	3	27.82333			
	1:2	3	28.47000	28.47000		
	1:1	3		32.99500	32.99500	
	2:1	3			37.60500	
	3:1	3				48.64400
	Sig.		.990	.056	.051	1.000

Means for groups in homogeneous subsets are displayed.; [a] Uses Harmonic Mean Sample Size = 3.000.

Table D13 ANOVA for result of model validation

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.730	1	.730	.055	.816
Within Groups	289.778	22	13.172		
Total	290.508	23			

Table D14 ANOVA for result of gaseous CO_2 and the addition of $MgCO_3$ on cell growth and succinic acid production by *A. succinogenes* NP9-aA7

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	125643.846[(a)]	55	2284.434	650.302	.000
Intercept	152351.966	1	152351.966	43369.546	.000
CO_2	7349.501	3	2449.834	697.386	.000
Cultivation_time	103212.163	13	7939.397	2260.083	.000
CO_2 * Cultivation_time	15082.182	39	386.723	110.087	.000
Error	393.442	112	3.513		
Total	278389.254	168			
Corrected Total	126037.288	167			

[a] R Squared = .997 (Adjusted R Squared = .995)

Table D15 Multiple comparison for result of gaseous CO_2 and the addition of $MgCO_3$ on cell growth and succinic acid production by *A. succinogenes* NP9-aA7

	CO_2	N	Subset			
			1	2	3	4
Tukey HSD[a,b]	CO_2 75 kPa	42	20.47381			
	CO_2 25.33 kPa	42		29.77540		
	CO_2 101.33 kPa	42			31.09419	
	CO_2 50.66 kPa	42				39.11286
	Sig.		1.000	1.000	1.000	1.000
Duncan[a,b]	CO_2 75 kPa	42	20.47381			
	CO_2 25.33 kPa	42		29.77540		
	CO_2 101.33 kPa	42			31.09419	
	CO_2 50.66 kPa	42				39.11286
	Sig.		1.000	1.000	1.000	1.000
Scheffe[a,b]	CO_2 75 kPa	42	20.47381			
	CO_2 25.33 kPa	42		29.77540		
	$CO_2$101.33 kPa	42			31.09419	
	CO_2 50.66 kPa	42				39.11286
	Sig.		1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.; Based on Type III Sum of Squares; The error term is Mean Square(Error) = 3.513.; [a] Uses Harmonic Mean Sample Size = 42.000.; [b] Alpha = .05.

Table D16 ANOVA for result of various cultivation time of the supply of gaseous 50.66 kPa of CO_2 on cell growth and succinic acid production by *A. succinogenes* NP9-aA7

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	39550.856	13	3042.374	1573.298	.000
Within Groups	54.145	28	1.934		
Total	39605.001	41			

Table D17 Multiple comparison for result of various cultivation time of the supply of gaseous 50.66 kPa of CO_2 on cell growth and succinic acid production by *A. succinogenes* NP9-aA7

	Cultivation_time	N	Subset for alpha = .05						
			1	2	3	4	5	6	7
Tukey HSD[(a)]	0 h	3	.00000						
	6 h	3	.97400	.97400					
	9 h	3	1.45367	1.45367					
	3 h	3	1.66467	1.66467					
	12 h	3		4.78100					
	15 h	3			21.48533				
	18 h	3				45.37433			
	48 h	3					54.99467		
	33 h	3						65.21600	
	21 h	3						65.78733	
	36 h	3						67.71367	
	30 h	3							72.31700
	27 h	3							72.88800
	24 h	3							72.93033
	Sig.		.963	.098	1.000	1.000	1.000	.632	1.000

Means for groups in homogeneous subsets are displayed.; [a] Uses Harmonic Mean Sample Size = 3.000.

Table D17 Multiple comparison for result of various cultivation time of the supply of gaseous 50.66 kPa of CO_2 on cell growth and succinic acid production by *A. succinogenes* NP9-aA7 (continues)

	Cultivation_time	N	Subset for alpha = .05							
			1	2	3	4	5	6	7	8
Duncan[(a)]	0 h	3	.00000							
	6 h	3	.97400							
	9 h	3	1.4536							
	3 h	3	1.6646							
	12 h	3		4.7810						
	15 h	3			21.4853					
	18 h	3				45.3743				
	48 h	3					54.9946			
	33 h	3						65.2160		
	21 h	3						65.7873	65.7873	
	36 h	3							67.7136	
	30 h	3								72.3170
	27 h	3								72.8880
	24 h	3								72.9303
	Sig.		.191	1.000	1.000	1.000	1.000	.619	.101	.616
Scheffe[(a)]	0 h	3	.0000							
	6 h	3	.9740							
	9 h	3	1.4536							
	3 h	3	1.6646							
	12 h	3	4.7810							
	15 h	3		21.4853						
	18 h	3			45.3743					
	48 h	3				54.9946				
	33 h	3					65.2160			
	21 h	3					65.7873			
	36 h	3					67.7136	67.7136		
	30 h	3						72.3170		
	27 h	3						72.8880		
	24 h	3						72.9303		
	Sig.		.237	1.000	1.000	1.000	.968	.137		

Means for groups in homogeneous subsets are displayed.; [a] Uses Harmonic Mean Sample Size = 3.000.

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Academic publications

1. Phuengjayaem, S., and Teeradakorn, S. 2016. Producing Succinic Acid with Actinobacillus succinogenes: Optimizing the Composition of the Medium Using Plackett-Burman Design. Chiang Mai University Journal of Natural Sciences, 15(3): 253-264.

2. Phuengjayaem, S., Phinkian, N., Tanasupawat S. and Teeradakorn, S. 2017. Diversity and Succinic Acid Production of Lactic Acid Bacteria Isolated from Animals, Soils and Tree Barks. Research Journal of Microbiology. (submitted)

3. Phuengjayaem, S., Tanasupawat, S. and Teeradakorn, S. Process development of succinic acid production by Actinobacillus succinogenes NP9-aA7 using statistical methods. (in preparation)