การคัดกรองและลักษณะสมบัติของแบคทีเรียที่ผลิตกรดซักซินิกและการหาค่าเหมาะที่สุดต่อการผลิต

นางสาวณัชชา ปิ่นเขียน

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SCREENING AND CHARACTERIZATION OF SUCCINIC ACID PRODUCING BACTERIA AND ITS
PRODUCTION OPTIMIZATION

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A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science Program in Biotechnology

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Thesis Title	SCREENING AND CHARACTERIZATION OF SUCCINIC ACID PRODUCING BACTERIA AND ITS PRODUCTION OPTIMIZATION
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ณัชชา ปิ่นเขียน : การคัดกรองและลักษณะสมบัติของแบคทีเรียที่ผลิตกรดซักซินิกและการหาค่าเหมาะ ที่สุดต่อการผลิต (SCREENING AND CHARACTERIZATION OF SUCCINIC ACID PRODUCING BACTERIA AND ITS PRODUCTION OPTIMIZATION) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: อ. ดร.ศิริลักษณ์ ธีระดากร, หน้า.

แบคทีเรียที่สามารถผลิตกรดซักซินิกได้ถูกคัดแยกจาก 6 แหล่งในประเทศไทย โดยเบื้องต้นสามารถคัด แยกได้แบคทีเรียทั้งหมด 310 ไอโซเลต มีเพียง 51 ไอโซเลตซึ่งถูกคัดเลือกโดยสามารถผลิตกรดซักซินิกได้จาก อาหารเฉพาะ ซึ่งแสดงผลบวกในขั้นตอนโครมาโทกราฟีแบบแผ่นบาง และยืนยันผลของการผลิตกรดซักซินิกด้วยโคร มาโทกราฟีของเหลวสมรรถนะสูง พบว่ากรดซักซินิกที่ได้มีปริมาณอยู่ในช่วง 0.553-52.028 กรัมต่อลิตร นำมา แยกแยะและแบ่งกลุ่มได้ 9 กลุ่ม ด้วยลักษณะทางสัณฐานวิทยา สรีระวิทยา และชีวเคมี ซึ่งไอโซเลตที่ผลิตกรดซัก ซินิกได้สูงที่สุดในแต่ละกลุ่มถูกนำไปศึกษาด้านยีน 16S rRNA โดยไอโซเลต CN1-OB13 (กลุ่มที่ 1) มีความคล้ายคลึง กับ *Escherichia fergusonii* ATCC 35469[T] (99.87%) ไอโซเลต PCH6-3 (กลุ่มที่ 2) มีความคล้ายคลึง กับ *Lactobacillus reuteri* JCM 1112[T] (99.71%) ไอโซเลต AY5-bA2 (กลุ่มที่ 3) มีความคล้ายคลึง กับ *Lactobacillus ruminis* NBRC 102161[T] (99.71%) ไอโซเลต AY5-bB4 (กลุ่มที่ 4) มีความคล้ายคลึง กับ *Clostridium sporogenes* DSM 795[T] (99.78%) ไอโซเลต PCH2-1 (กลุ่มที่ 5) มีความคล้ายคลึง กับ *Enterococcus faecium* CGMCC 1.2136[T] (99.86%) ไอโซเลต NP1-A2 (กลุ่มที่ 6) มีความคล้ายคลึง กับ *Enterococcus faecalis* ATCC 19433[T] (99.86%) ไอโซเลต CN2-OA2 (กลุ่มที่ 7) มีความคล้ายคลึง กับ *Enterococcus avium* ATCC 14025[T] (100%) ไอโซเลต NS15-bA2 (กลุ่มที่ 8) มีความคล้ายคลึง กับ *Enterococcus hirae* ATCC 9790[T] (100%) สุดท้ายคือ ไอโซเลต NS15-dA1 (กลุ่มที่ 9) มีความคล้ายคลึง กับ *Enterococcus durans* CECT411[T] (99.89%) โดยไอโซเลต NS15-bA2 และ NS15-dA1 สามารถผลิตกรดซัก ซินิกได้สูงสุด คือ 52.082 และ 50.862 กรัมต่อลิตร จาก 60 กรัมต่อลิตรของกลูโคสตามลำดับและไม่เคยมีรายงาน ว่า มีการนำไปศึกษาหาภาวะที่เหมาะต่อการผลิตกรดซักซินิก ดังนั้นทั้งสองไอโซเลตจึงถูกเลือกไปทำการศึกษาหา ภาวะที่เหมาะสมต่อการผลิตกรดซักซินิก จากนั้นศึกษาองค์ประกอบของอาหารที่มีผลต่อการผลิตกรดซักซินิก ได้แก่ แหล่งคาร์บอน (ความเข้มข้นของกลูโคส) แหล่งไนโตรเจน (อินทรีย์และอนินทรีย์) ค่าความเป็นกรด-ด่าง และ อุณหภูมิ ซึ่งภาวะที่เหมาะสมต่อการผลิตกรดซักซินิกโดยไอโซเลต NS15-dA1 และ NS15-bA2 คือ 60 กรัมต่อลิตร ของกลูโคสใช้เป็นแหล่งคาร์บอน 30 กรัมต่อลิตรของสารสกัดยีสต์ (สำหรับไอโซเลต NS15-dA1) และ 30 กรัมต่อ ลิตรของทริปโตน (สำหรับไอโซเลต NS15-bA2) ใช้เป็นแหล่งไนโตรเจน 0.2 กรัมต่อลิตรของแคลเซียมคลอไรด์ไดไฮ เดรต 0.2 กรัมต่อลิตรของแมกนีเซียมคลอไรด์เฮกซะไฮเดรต 0.07 กรัมต่อลิตรของแมงกานีสคลอไรด์ 4.4 กรัมต่อ ลิตรของไดโซเดียมไฮโดรเจนฟอสเฟต 3.3 กรัมต่อลิตรของโซเดียมไดไฮโดรเจนฟอสเฟต 30 กรัมต่อลิตรของ แมกนีเซียมคาร์บอเนต ที่ค่าความเป็นกรด-ด่าง 7.0 อุณหภูมิ และอุณหภูมิ 37 องศาเซลเซียส เป็นเวลา 24 ชั่วโมง กรดซักซินิกสูงสุดที่ผลิตได้คือ 51.692±0.1707 กรัมต่อลิตร และ 53.051±0.3538 กรัมต่อลิตร ตามลำดับ

สาขาวิชา   เทคโนโลยีชีวภาพ   ลายมือชื่อนิสิต ....

ปีการศึกษา 2558   ลายมือชื่อ อ.ที่ปรึกษาหลัก ....

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KEYWORDS: SUCCINIC ACID, PHENOTYPIC CHARACTERIZATION

NATCHA PINKIAN: SCREENING AND CHARACTERIZATION OF SUCCINIC ACID PRODUCING BACTERIA AND ITS PRODUCTION OPTIMIZATION. ADVISOR: SIRILUK TEERADAKORN, Ph.D., pp.

Succinic acid producing bacteria were isolated from 6 sources in Thailand. Firstly, a total 310 isolates, only 51 isolates were screened for their succinic acid production by selective medium plate. They showed positive on TLC method and they were confirmed to have succinic acid producing ability using HPLC. Succinic acid was obtained in the range of 0.553-52.028 g/l. They were divided into 9 groups based on morphological, physiological and biochemical characteristics. Isolates produced high succinic acid from each group were selected to study 16S rRNA gene sequence. Isolate CN1-OB13 (Group I) was closely related to *Escherichia fergusonii* ATCC 35469^T (99.87%). Isolate PCH6-3 (Group II) was closely related to *Lactobacillus reuteri* JCM 1112^T (99.71%). Isolate AY5-bA2 (Group III) was closely related to *Lactobacillus ruminis* NBRC 102161^T (99.71%). Isolate AY5-bB4 (Group IV) was closely related to *Clostridium sporogenes* DSM 795^T (99.78%). Isolate PCH2-1 (Group V) was closely related to *Enterococcus faecium* CGMCC 1.2136^T (99.86%). Isolate NP1-A2 (Group VI) was closely related to *Enterococcus faecalis* ATCC 19433^T (99.86%). Isolate CN2-OA2 (Group VII) was closely related to *Enterococcus avium* ATCC 14025^T (100%). Isolate NS15-bA2 (Group VIII) was closely related to *Enterococcus hirae* ATCC 9790^T (100%). Lastly, isolate NS15-dA1 (Group IX) was closely related to *Enterococcus durans* CECT411^T (99.89%). Isolate NS15-bA2 and NS15-dA1 could produce the highest succinic acid of 52.028 and 50.862 g/l from 60g/l of glucose, respectively and they were no reported of succinic acid production from other research. Thus these two isolates were selected to study optimization of succinic acid production. Next, the medium composition that affected to the succinic acid production; carbon sources (glucose concentration), nitrogen sources (organic and inorganic), pH and temperature were investigated. The optimum conditions on succinic acid production by isolate NS15-dA1 and NS15-bA2 was 60 g/l of glucose as a carbon source, 30 g/l of yeast extract (for isolate NS15-dA1) and 30 g/l of tryptone (for isolate NS15-bA2) as a nitrogen source, 0.2 g/l of $CaCl_2.2H_2O$, 0.2 g/l of $MgCl_2.6H_2O$, 0.07 g/l of $MnCl_2$, 4.4 g/l of Na_2HPO_4, 3.3 g/l of NaH_2PO_4, 30 g/l of $MgCO_3$ at pH 7.0 and 37°C for 24 h. The highest succinic acid of 51.692±0.1707 g/l and 53.051±0.3538 g/l were obtained, respectively.

Field of Study: Biotechnology	Student's Signature	....
Academic Year: 2015	Advisor's Signature	....

ACKNOWLEDGEMENTS

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CHAPTER I

INTRODUCTION

Succinic acid, also known as butanedioic acid or amber acid, is a dicarboxylic acid having the molecular formula of $C_4H_6O_4$. Succinic acid is a common natural organic acid present in humans, animals, plants, and microorganisms (Zeikus et al., 1999). The applications of succinic acid in agricultural, it is a known growth regulator (Dougall and Weyrauch, 1980) which can be used for seed treatment and plant rooting. In the food industry, succinic acid is used as a bread softening agent, a flavoring enhancer for beverages, and a catalyst for food seasoning preparation (Chimirri et al., 2010). In the pharmaceutical industry, succinic acid is also used in the manufacture of medicines for sedatives, antispasmers, antiplegm, antiphogistic, anrhoers, contraceptives, and cancer-curing (Sener et al., 1997). In the chemical industry, succinic acid can be used as a precursor for the production of many high value chemicals including adipic acid, 1,4-butanediol, tetrahydrofuran, 2-pyrrolidinone, N-methyl pyrrolidinone and gramma-butyrolactone (Yu et al., 2011). Finally, succinic acid is a precursor to many specialized polyesters such as polybutylene succinate (PBS), polyamide (Nylon^R x,4) and various green solvents (Xu and Guo, 2010). Due to its versatile applications, succinic acid is demanded worldwide. The global succinic acid production is estimated between 30,000 and 50,000 tons per year. According to a survey report from Markets and Markets, the market of succinic acid is expected to grow at a rate of 18.7% from 2011 to 2016. The global market for succinic acid in terms of revenue was estimated to be worth $182.8 million in 2010 and is expected to reach $496.0 million by 2016 (Nattrass et al., 2013).

Processes of succinic acid production have two processes. Firstly, succinic acid is produced by chemical process. Succinic acid is produced by catalytic hydrogenation of maleic acid or anhydride and maleic anhydride. Secondary, biological process, succinic acid can be produced anaerobically through fermentation by bacteria as it is an intermediate of the reductive TCA cycle. However, biological

process has been attracting interests of researchers because it affects to environmental less than chemical process. It is also notable that a greenhouse gas CO_2 is fixed into succinic acid during the fermentation (Zeikus, 1980) and chemical process has high conversion cost of maleic anhydride to succinic acid (Chimirri et al., 2010).

Many microorganisms can produce succinic acid. Bacteria strains such as *Ruminococcus flavefaciens, Bacteroides amylophilus, Succcinimonas amylolytica, Cytophaga succinicans and Enterococcus faecalis.* Fungal strains such as *Paecilomyces varioti, Aspergillus niger*, and *Penicillium simplicissimum*. But the productivity of fungi strains is much lower when compared with the bacterial strains (Coustou et al., 2005). Succinic acid producing bacteria can screen various sources such as *Succinatimonas hippie* from human faeces (Morotomi et al., 2010) and *Phascolarctobacterium succinatutens* from human feces (Watanabe et al., 2012). However, most succinic acid producing bacteria are mainly belonging to rumen of ruminants such as the cattle, sheep, antelopes, deer and giraffes. Due to the rumen microbial ecosystem is an anaerobic environment and they have a high level of nutrient supply (10-18 percent dry matter), temperature regulation (38-41℃), pH control (6-7) by buffer in saliva. That is optimum condition for microorganisms. *Actinobacillus succinogenes* 130Z (Guettler et al., 1999), *Mannheimia succiniproducens* MBEL55E (Lee et al., 2002), *Klebsiella pneumoniae* (Thakker et al., 2006), *Basfia succiniciproducens* (Kuhnert et al., 2010) *Anaerobiospirillum succiniciproducens* (Davis et al., 1976) and *Succinivibrio dextrinosolvens* (Bryant and Doetsch, 1955) were screened from rumen of ruminants.

The ability of succinic acid production in bacteria is different. The environment showed significant effect on succinic acid production of bacteria including media components and fermentation parameters such as temperature and pH also showed significant effect on succinate production. Thus that should be optimized according to the strain.

The objective of this study, succinic acid producing bacteria is screened from various sources such as soil, cattle dung and rumen of ruminants. The optimum conditions for succinic acid production from potential isolates are also studied.

CHAPTER II
LITERATURE REVIEWS

2.1 Succinic acid

Succinic acid, also known as butanedioic acid or amber acid, is a member of the C4-dicarboxylic acid family. It has the molecular formula of $C_4H_6O_4$ and the chemical structure is shown in Figure 1 (Fieser et al., 1932).
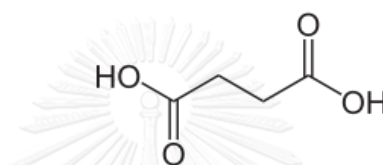
Figure 1. Chemical structure of succinic acid (Fieser et al., 1932)

Succinic acid is a colorless crystalline solid at room temperature, has negligible vapor pressure and a melting point of 185-187°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.

Presently, succinic acid can be used for many benefits. Various important chemicals such as 1,4-butanediol which is an important industrial solvent and raw material for polybutylene terephthalate resins, tetrahydrofuran which is a solvent and key ingredient of adhesives, printing inks, and magnetic tapes, succinate salt which is a flavor enhancer that can replace monosodium glutamate and gamma-butyrolactone which is a chemical intermediate, ingredient of paint removers and textile products. Furthermore succinic acid can be used as precursors of chemical industry such as biopolymer, detergent, surfactant, corrosion inhibitors and painting compound. In addition, succinic acid is as a additives to food ingredients, biophosphors, solvent additives, flavor additives, stimulants for plant growth and pharmaceuticals intermediates (Figure 2) (Song and Lee, 2006).
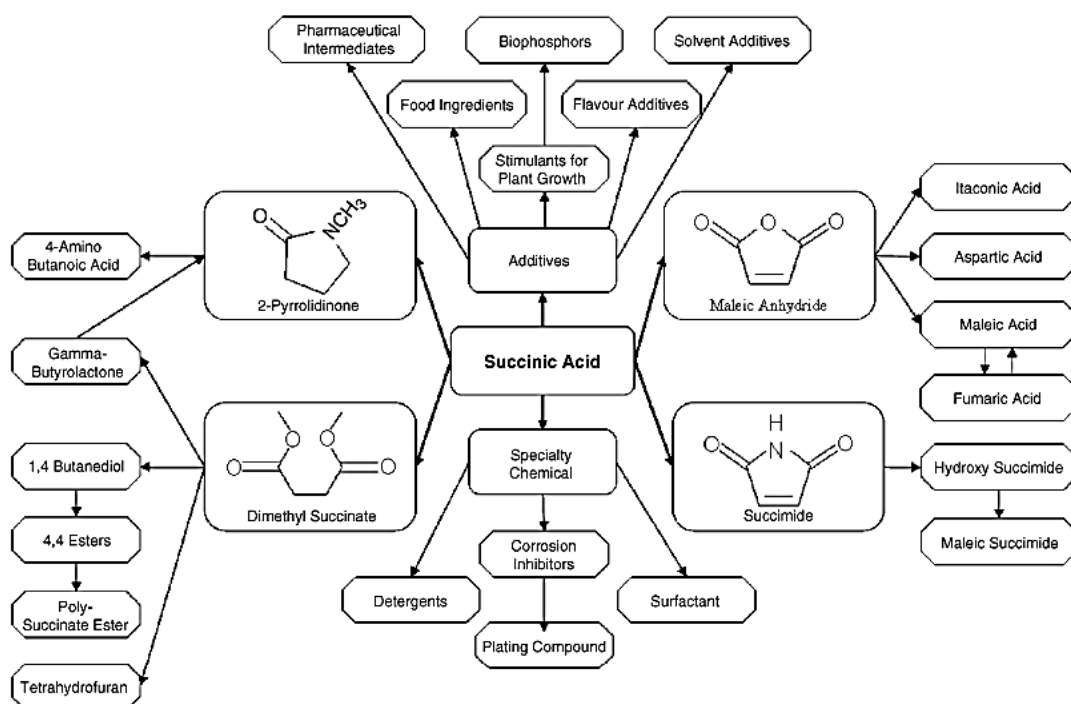
Figure 2. Various important chemicals and products that can be synthesized from succinic acid (Song and Lee, 2006)

2.2 Processes of succinic acid production

Currently, succinic acid is mostly produced by the chemical process, in which petroleum oil is used as a starting material. It is produced through reaction of oxidation, hydrogenation and hydration of n-butane. The first step in the reaction, petroleum oil is oxidation to n-butane. Next step, maleic anhydride is hydrogenated to succinic anhydride. 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 is used a Ni/Zr/Al/Si alloy. Finally step, succinic anhydride formed can be reacted with water to form succinic acid (Figure 3) (Sutton et al., 2002).
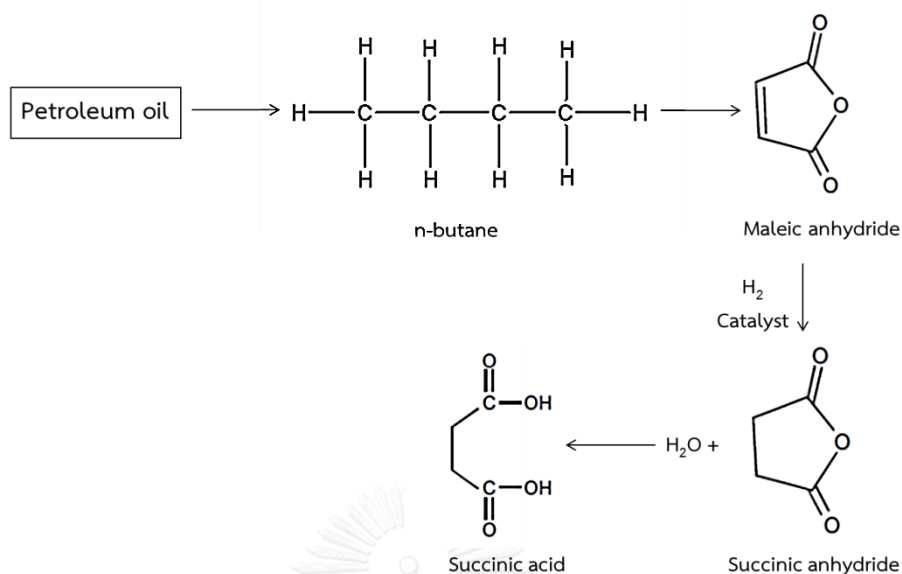
Figure 3. Chemical process of succinic acid production (Sutton et al., 2002)

In addition to chemical process, that has biological process. Firstly, renewable sources such as straw, corn stover or bagasse are digestion to glucose as a substrate. Then succinic acid is produced through anaerobic fermentation of microorganism due to succinic acid is an intermediate of the tricarboxylic acid (TCA) cycle and one of the fermentation end products of anaerobic metabolism (Figure 4). Phosphoenolpyruvate (PEP) carboxylation is strongly regulated by CO_2 levels. In bacteria, PEP carboxykinase functions catabolically to fix CO_2 and synthesize oxaloacetate from PEP. At low CO_2 levels, bacteria produce lactic acid or ethanol as a major end product. Under high CO_2 levels, succinic acid is the major product and only traces of lactic acid or ethanol are produced by bacteria (Samuelov et al., 1991).
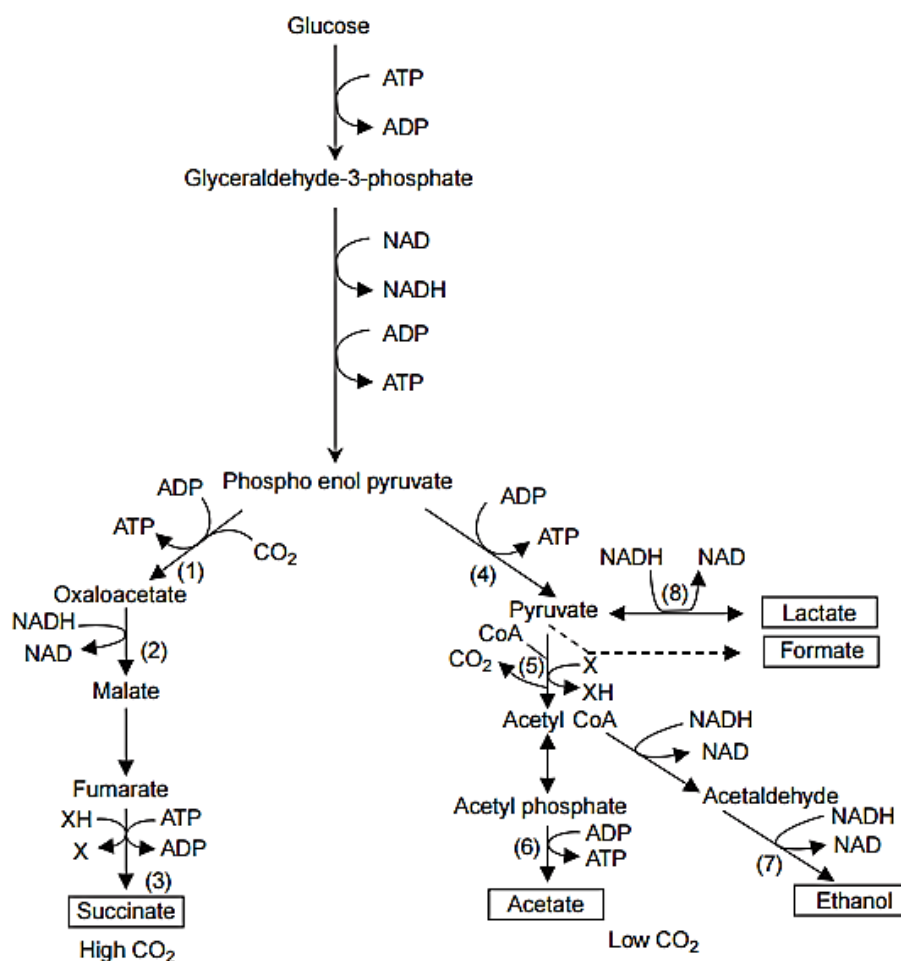
Figure 4. Pathway of succinic acid production from glucose by bacteria in anaerobic condition (Samuelov et al., 1991)

However chemical process for succinic acid production is continuously increasing because it uses oil as a substrate. As biological process uses renewable resources and has environment friendly approach (Kurzrock and Botz, 2010). Worldwide bio succinic acid production has grown from 15,000 metric ton a year in 1999 to the 180,000 metric ton in 2015. The reason of this increase is the growing bio succinic acid since petrochemical production has remained stable for years (Bechthold et al., 2008), it shows in Figure 5. Therefore, the biological process is interesting more than chemical process.
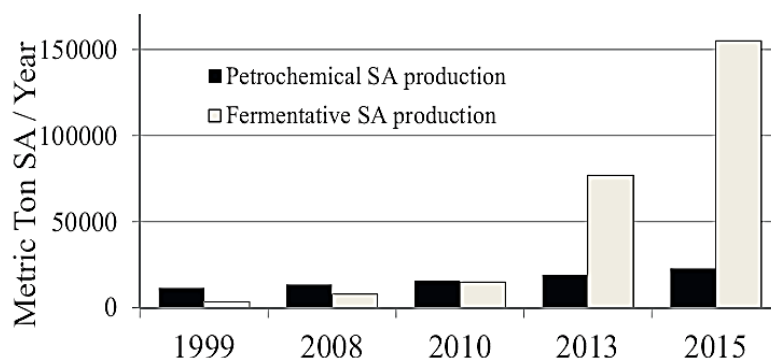
Figure 5. Evolution of worldwide succinic acid production in metric ton per year (Bechthold et al., 2008)

2.3 Succinic acid producing bacteria

Succinic acid is produced by various microorganisms. For example bacteria including *Actinobacillus succinogenes*, *Enterococcus flavescens*, *Enterococcus faecalis*, *Mannheimia succiniciproducens*, and *Lactobacillus* strains and fungi such as *Aspergillus niger* and *Penicillium simplicissimum*. The most ability of succinic acid production by fungi (Table 1), that can produce succinic acid lower than bacteria. Moreover, the use of fungi has been mostly limited to the manufacture of food and beverages due to the difficulties in fermentation, separation and purification (McIntyre and McNeil, 1997). Therefore bacteria are mostly used to produce succinic acid.

Table 1. Performances of succinic acid production by various microorganisms

Microorganisms	Substrate	Succinic acid (g/l)	Yield (g/g)	Productivity (g/l/h)	References
A. succinogenes	Glucose	45.8	0.83	1.55	(Guettler et al., 1998)
A. succiniciproducens	Glucose	34.4	0.86	1.8	(Lee et al., 1999b)
M. succiniciproducens	Glucose	10.5	0.59	1.75	(Song et al., 2007)
E. flavescens	Sucrose	2.82	0.14	0.47	(Agarwal et al., 2007)
E. faecalis	Glycerol	153	7.65	4.25	(Kang et al., 2000)
A. niger	Glucose	55.4	0.31	0.92	(David et al., 2003)
P. simplicissimum	Glucose	0.06	0.30	0.005	(Gallmetzer et al., 2002)

Succinic acid bacteria can discoved in various sources (Table 2), such as bovine rumen, human faeces and fecal of beagles dog. For example, *Anaerobiospirillum succiniciproducens* isolated from the mouth of the beagle dog is a gram-negative obligately anaerobic bacterium that produces succinate, acetate, formate, ethanol, and lactate, from glucose and lactose (Davis et al., 1976) and *Actinobacillus succinogenes* 130Z is a ruminal, facultative anaerobic bacteria that has the ability to utilize a wide range of substrates including L-arabinose, cellobiose, fructose, galactose, glucose, lactose, maltose, mannitol, mannose, sucrose, D-xylose, and salicin (Guettler et al., 1999). This strain can produces succinate in very high concentrations, along with acetate, pyruvate, formate, or ethanol. Succinate is also produced by microorganisms isolated from the digestive system of other animals. Sources of succinic acid producing bacteria are showed in Table 2 and most of the succinic acid producing bacteria is isolated from the rumen of ruminant.

Table 2. Sources of succinic acid producing bacteria

Microorganisms	Sources	References
Actinobacillus succinogenes	Bovine rumen	(Guettler et al., 1999)
Anaerobiospirillum succiniciproducens	Fecal of Beagles dog	(Davis et al., 1976)
Bacteroides ruminicola	Reticulo-rumen of a cow	(Bryant et al., 1958)
Bacteriodes succinogenes	Bovine rumen	(Stewart and Flint, 1989)
Basfia succiniciproducens	Bovine rumen	(Kuhnert et al., 2010)
Klebsiella pneumoniae	Rumen fluid of buffalo	(Thakker et al., 2006)
Mannheimia succiniciproducens MBEL55E	Bovine rumen	(Lee et al., 2002)
Phascolarctobacterium succinatutens	Human faeces	(Watanabe et al., 2012)
Succinatimonas hippie	Human faeces	(Morotomi et al., 2010)
Succinimonas amylolytica	Reticulo-rumen of cattle	(Bryant et al., 1958)
Succinivibrio dextrinosolvens	Rumen of cattle	(Bryant and Small, 1956)

Ruminants have stomachs with four chambers consisted of rumen, reticulum, omasum and abomasum, as shown in Figure 6. Food is first mixed with saliva and passed to the rumen, where it is mechanically broken down to smaller pieces and next passed to the reticulum. Here the food is further broken down and separate from indigestible non-food items before it is formed into cuds. These cuds or clumps of partially degraded food are then regurgitated into the animal's mouth (re-chewed) and then re-swallowed back into the rumen. The major of the anaerobic microorganisms that aid in cellulose breakdown inhabit the rumen, during this step of digestion and fermentation begins. The partially digested food then moves to the omasum, where water, vitamins and short chain fatty-acids from fermentation are absorbed into the animal's body. Before it is passed to the abomasum, pH is decreased and enzymes are released to further break down the material. The material is further broken down and then passed to the small and large intestines where nutrients are absorbed before the waste is excreted (Leschine, 1995). The process takes about 9-12 hours.
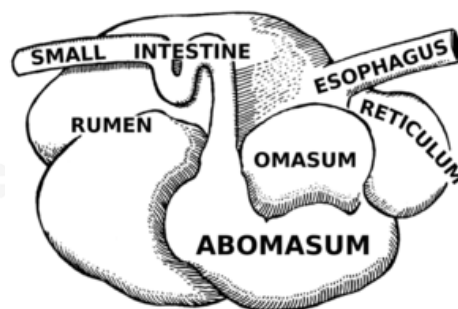
Figure 6. The four chambered stomach of ruminants. Microorganisms live primarily in the rumen, but all four chambers are essential for digestion.

(Mendez and Foresman, 2010)

In the rumen, succinic acid is an important precursor for propionate, which is absorbed through the rumen wall for subsequent oxidation to provide energy, biosynthetic precursors for the animal and it is well adapted for the maintenance of a large and diverse microbial population. There is a relatively constant supply of food and water. The temperature is relatively constant at about 39°C. The pH of the

ingesta is slightly acid by the influx of food, water and heavily buffered saliva and an equilibrium between the ruminal ingesta and the blood stream with regard to H ions (Masson and Phillipson, 1951). There is a constant removal of the products of microbial growth via secondary fermentations, passage to the lower digestive tract, and absorption through the rumen wall into the blood stream. The rumen has a low oxygen tension of the gaseous phase. Moreover microorganisms in the rumen include bacteria, fungi, protozoa, archaea and viruses. Bacteria, along with protozoa, are the predominant microbes and by mass account for 40-60% of total microbial matter in the rumen. Thus most succinic acid producing bacteria is screened from rumen of ruminants.

2.4 Screening succinic acid producing bacteria

2.4.1 Selective medium

Selective medium is used for screening succinic acid producing bacteria. Selective medium allows the growth of certain type of microorganisms, while inhibiting the growth of other microorganisms.

In 1999, Guettler et al. (Guettler et al., 1999) found *Actinobacillus succinogenes*, a novel succinic acid producing strain from bovine rumen. It could produce high succinic acid. The selective medium consists of glucose 20 g/l, poly peptone 10 g/l, yeast extract 5 g/l, K_2HPO_4 3 g/l, NaCl 1 g/l, $(NH_4)_2SO_4$ 1 g/l, $CaCl_2.2H_2O$ 0.2 g/l, $MgCl_2.6H_2O$ 0.2 g/l, $MgCO_3$ 15 g/l, agar 15 g/l and pH 6.5. Bacteria with succinic acid ability exhibits a clear zone around colony because of the selective medium with $MgCO_3$, magnesium (Mg^{2+}) reacts with succinic acid ($C_4H_6O_4$) to succinate salt ($MgC_4H_4O_4$), so a clear zone is observed.

Furthermore, in 2010, Morotomi et al. (Morotomi et al., 2010) found *Succinatimonas hippie* which is a novel succinic acid producing strain from human faeces. GAM or Gifu anaerobic medium is used to screen succinic acid producing bacteria. Sodium thioglycollate and L-Cystine hydrochloride are the reducing agents added in this medium to provide adequate anaerobiosis. In 2012, Watanabe et al. (Watanabe et al., 2012) found *Phascolarctobacterium succinatutens* which is a novel

succinic acid producing strain from human faeces. GAM or Gifu anaerobic medium is used to screen succinic acid producing bacteria.

Therefore, both selective mediums described above are used to screen succinic acid producing bacteria in this study.

2.5 Characterization of succinic acid producing bacteria

Bacterial characterization can be described and compared with descriptions of other organisms. It was divided into four steps as follows:

2.5.1 Morphological characteristics

Gram stain is a powerful, easy test that allows to different between the two major classes of bacteria. Comparison of the gram positive and gram negative bacterial cell walls are shown in Figure 7A: a gram positive bacterium has a thick peptidoglycan layer that contains teichoic and lipoteichoic acid. Figure 7B: a gram negative bacterium has a thin peptidoglycan layer and an outer membrane that contains lipopolysaccharide, phospholipids, and proteins. The periplasmic space between the cytoplasmic and outer membranes contains transport, degradative, and cell wall synthetic proteins. The outer membrane is joined to the cytoplasmic membrane at adhesion points and is attached to the peptidoglycan by lipoprotein links (Young, 2007).
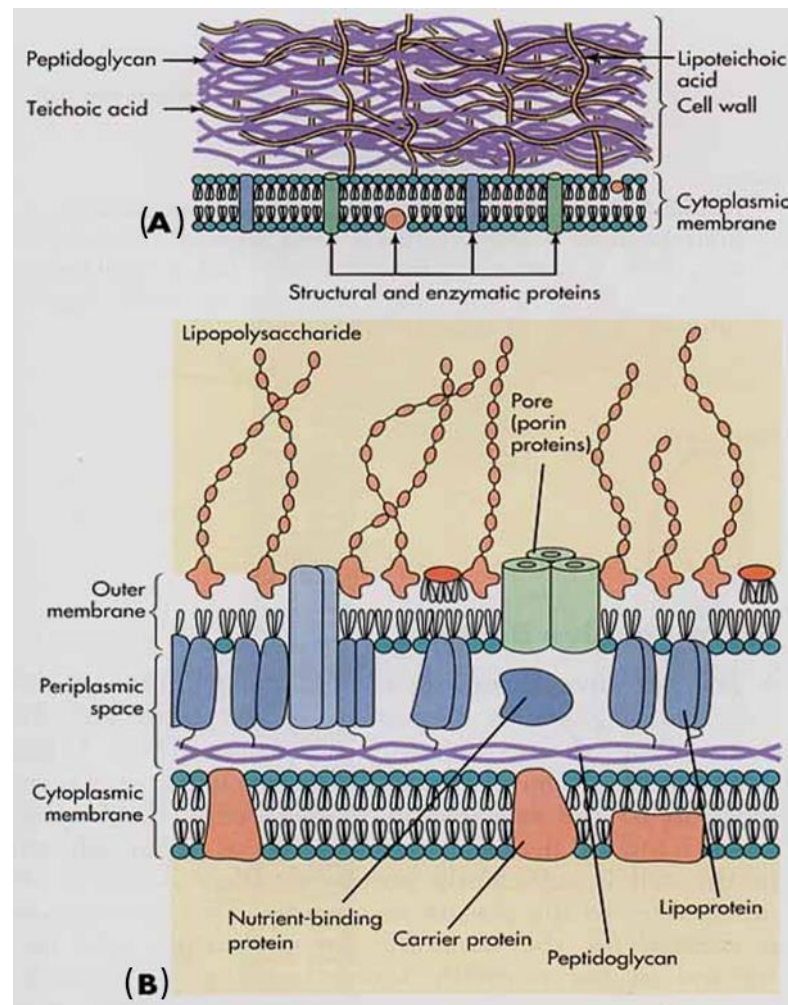
Figure 7. Comparison of the gram positive and gram negative bacterial cell walls. (A) is gram positive and (B) is gram negative bacteria. (Young, 2007)

Cell morphology includes shapes and arrangements are shown in Figure 8. The three most common bacterial cell shapes are cocci, bacilli or rod (spore-forming rods: mycobacteria, corynebacteria and streptomycetes) and spirilla (vibrios, spirillum and spirochete). Arrangements of bacteria consist of diplococci (cocci in pairs), neisseriae (coffee-bean shape in pairs), tetrads (cocci in packets of 4), sarcinae (cocci in packets of 8, 16, 32 cells), streptococci (cocci in chains), micrococci and staphylococci (large cocci in irregular clusters).
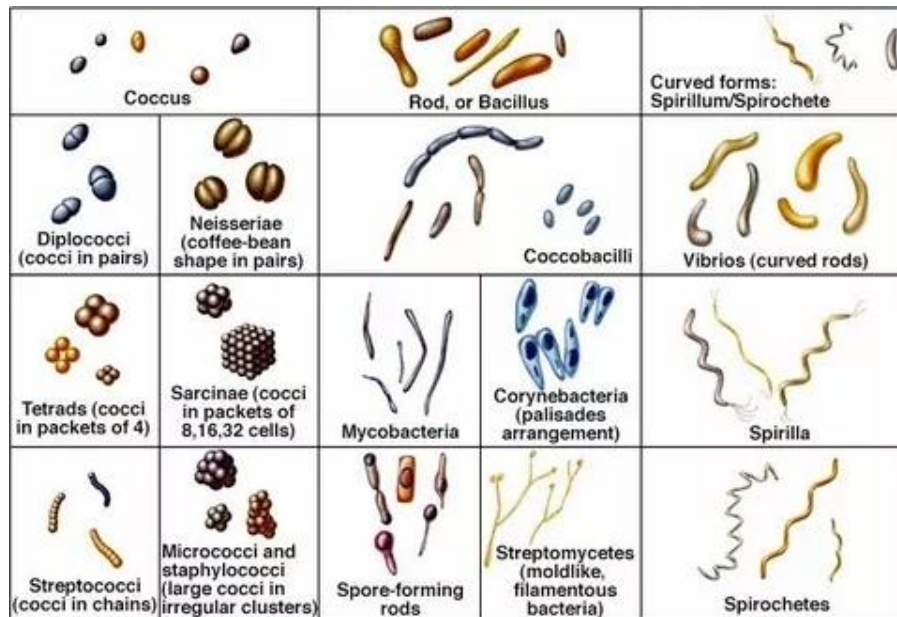
Figure 8. Cell morphology consists of shapes and arrangements (Hendrix, 1998)

Colonial appearance is used to identify species of bacteria. Features of the colonies may help to pinpoint the identity of the bacterium. Different species of bacteria can produce very different colonies. Figure 9 shows colonial appearance including general surface form (circular, filamentous, irregular rhizoid and spindle), margin (entire, undulate lobate, erose, filamentous and curled), elevation (flat, raised, convex, pulvinate and umbonate), color (orange pigment, yellow, white, tan etc.) and optical property (transparent (clear), opaque (not transparent or clear), translucent (almost clear) and iridescent (changing colors in reflected light)).
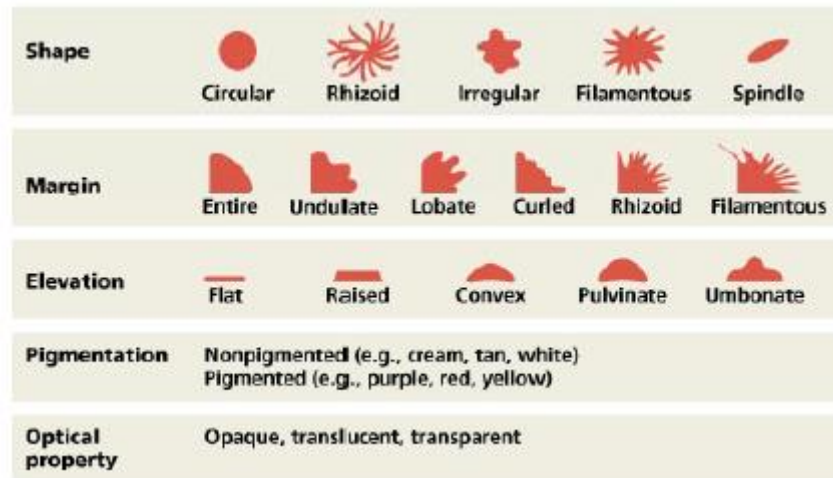
Figure 9. Colony morphology consists of shape, margin, elevation, pigmentation and optical property (Acharya, 2013)

2.5.2 Physiological characteristics

Growth in various salt concentrations can divide species of bacteria. Salt concentrations affect osmosis in cell. Osmophilic microorganisms can grow in high osmosis. Osmoduric microorganisms can survive in high osmosis but do not cell division. Halophilic microorganisms can grow in high salt concentrations. Haloduric microorganisms can survive to high concentrations of salt but cannot grow (Kim et al., 2014).

Optimum cells growth in the range of pH. Each species of microbe has its own characteristic range of pH values in which it grows and reproduces best. Bacteria are sensitive to the hydrogen ion concentration they find in their environment. The pH affects for large proteins, such as enzymes. Usually, the catalytic properties of the enzymes are lost and metabolism is paused (Blamire, 2000).

Optimum cells growth in different temperature. Psychrophiles can grow in cold temperatures, ranging from −20℃ to 10℃. Mesophiles is an organism that grows best in moderate temperature, neither too hot nor too cold, typically between 25℃ and 40℃ (Ingraham, 1958). Thermophiles are heat-loving that grows at

relatively high temperatures, between 41°C and 122°C (Pettipher et al., 1997). Temperature is another important factor and it also affects the growth and enzyme activity of the microorganism.

Effect of oxygen on growth. Obligate aerobes need oxygen because they cannot ferment or respire anaerobically. Obligate anaerobes (strict anaerobes) are killed by oxygen. Facultative anaerobes can grow with or without oxygen because they can metabolise energy aerobically or anaerobically. They use aerobic respiration generates more ATP than either fermentation or anaerobic respiration. Microaerophiles can grow only where a low concentration of oxygen has diffused into medium. Microaerophiles need oxygen because they cannot ferment or respire anaerobically. However, they are poisoned by high concentrations of oxygen (Todar, 2002).

2.5.3 Biochemical characteristics

Catalase test is produced by bacteria that respires using oxygen and protects them from the toxic by-products of oxygen metabolism. Catalase-positive bacteria include strict aerobes as well as facultative anaerobes if they able to respire using oxygen as a terminal electron acceptor. Catalase-negative bacteria may be anaerobes or facultative anaerobes that only ferment and do not respire using oxygen as a terminal electron acceptor (Clarke and Cowan, 1952).

Oxidase test is used to determine if a bacterium produces certain cytochrome C oxidases. It uses a reagent such as *N,N,N',N'*-tetramethyl-p-phenylenediamine (TMPD) or *N,N*-dimethyl-p-phenylenediamine (DMPD), which is also a redox indicator. The reagent is a dark-blue to red-brown color when oxidized and colorless when reduced. Oxidase-positive bacteria possess cytochrome oxidase or indophenol oxidase. These both catalyze the transport of electrons from donor compounds (NADH) to electron acceptors (oxygen). The reagent, TMPD dihydrochloride acts as an artificial electron donor for the enzyme oxidase. The oxidized reagent forms the colored compound indophenol blue. The cytochrome system is usually only present in aerobic organisms that are capable of using oxygen

as the terminal electron acceptor. The end-product of this metabolism is either water or hydrogen peroxide (broken down by catalase) (MacFaddin, 2000).

Urease test is used to determine the ability of an organism to split urea through the production of the enzyme urease. Many organisms have a urease enzyme which is able to split urea in the presence of water to release ammonia and carbon dioxide (Canteros et al., 1996). The ammonia combines with carbon dioxide and water to form ammonium carbonate that shows in Figure 10.

$$(NH_2)_2CO + 2H_2O \longrightarrow CO_2 + H_2O + 2NH_3$$

Figure 10. Reaction of urea produces ammonia and CO_2 (Cooper and Spencer, 1998)

Gelatin liquefaction is used to determine the ability of an organism to produce a gelatinase that hydrolyzes gelatin. Gelatinase allows the organisms that produce it to break down gelatin into smaller polypeptides, peptides, and amino acids that can cross the cell membrane and be utilized by the organism (Clarke and Cowan, 1952).

Lipase production is used to determine the ability of an organism to produce a lipase that hydrolyzes triglyceride. Lipases break down lipids. Organisms produce lipase that break down lipids into smaller fragments. Triglycerides are composed of glycerol and three fatty acids. The end products that can be used by the cell in energy production or other processes (Clarke and Cowan, 1952).

Gas production is produced by bacteria from carbohydrate such as glucose as a carbon source. When microbes ferment glucose, they produce gases mainly carbon dioxide and hydrogen. These gases bubble up through the medium and escape into the atmosphere. Tubes of broth media can be made with inverted tubes called Durham tubes. Gas producing microbes generate enough gas to force the medium from the tube, filling it with bubbles (Hayward, 1957).

Lysine decarboxylase is used to determine microbe that can use the amino acid lysine as a source of carbon and energy for growth (Falkow, 1958).

Ornithine decarboxylase is used to break ornithine down into putrescine and CO_2. Ornithine is not found in proteins but it is a raw material that bacteria convert into other essential molecules (Fay and Barry, 1972).

Casein hydrolysis is used to determine an organism that can produce the exoenzyme casesase. Casease is secreted out of the cells into the surrounding media, catalyzing the breakdown of milk protein, called casein, into small peptides and individual amino acids which are then taken up by the organism for energy use or as building material. The hydrolysis reaction causes the milk agar, normally the opacity of real milk, to clear around the growth area as the casein protein is converted into soluble and transparent end products-small chains of amino acids, dipeptides and polypeptides (Medina and Baresi, 2007).

Indole production is used to determine the ability of the organism to convert tryptophan into the indole. This division is performed by a chain of a number of different intracellular enzymes, a system generally referred to as tryptophanase. Indole is generated by reductive deamination from tryptophan via the intermediate molecule indolepyruvic acid. Tryptophanase catalyzes the deamination reaction, during which the amine (NH_2) group of the tryptophan molecule is removed. Final products of the reaction are indole, pyruvic acid, ammonium (NH^{4+}) and energy. Pyridoxal phosphate is required as a coenzyme (MacFaddin, 2000).

Methyl Red (MR) is used to determine different bacteria that convert dextrose and glucose to pyruvate using different metabolic pathways. Some of these pathways produce unstable acidic products which quickly convert to neutral compounds. Some bacteria use the butylene glycol pathway which produces neutral end products including acetoin and 2,3-butanediol. Other bacteria use the mixed acid pathway which produces acidic end products such as lactic, acetic, and formic acid. These acidic end products are stable and will remain acidic (Tille, 2014).

Voges-Proskauer test (VP) is used to detect organisms that utilize the butylene glycol pathway and produce acetoin. When the VP reagents are added to VP broth that has been inoculated with an organism that uses the butylene glycol pathway, the acetoin end product is oxidized in the presence of potassium hydroxide (KOH) to diacetyl. Creatine is also present in the reagent as a catalyst (Tille, 2014).

Citrate utlilization is used to determine the ability of bacteria to utilize sodium citrate as its only carbon source and inorganic ($NH_4H_2PO_4$) is the sole fixed nitrogen source. When an organic acid such as citrate is used as a carbon and energy source, alkaline carbonates and bicarbonates are produced ultimately. In addition, ammonium hydroxide is produced when the ammonium salts in the medium are used as the sole nitrogen source (Betty et al., 2007).

Arginine hydrolysis is used to determine bacteria that can use amino acid or arginine as a source of carbon and energy for growth. Use of arginine is accomplished by the enzyme arginine dihydrolase. A medium consists of arginine and a pH indicator. When arginine is used, the pH of the medium rises and the indicator changes color (Chen et al., 1982).

Phenylalanine deaminase is used to determine the ability of an organism to produce the enzyme deaminase. This enzyme removes the amine group from the amino acid phenylalanine and releases the amine group as free ammonia. As a result of this reaction, phenylpyruvic acid is also produced (Ederer et al., 1971).

Nitrate reduction is used for differentiation of bacteria on the basis of their ability to produce nitrate reductase enzyme that hydrolyze nitrate (NO_3^-) to nitrite (NO_2^-) which may then again be degraded to various nitrogen products like nitrogen oxide, nitrous oxide and ammonia (NH_3) depending on the enzyme system of the organisms and the atmosphere in which it is growing. In uninoculated nitrate broth and with cultures of organisms that do not reduce nitrate, the test for nitrite is negative until zinc dust or other reducing agent is added to the culture medium to reduce the nitrate contained in it. To detect small amounts residual nitrate the

amount of zinc added may be critical (Zobell, 1932). The tests are very sensitive and it is important to check the uninoculated medium for nitrite, which should not be present.

Starch hydrolysis is used to determine bacteria with ability to use starch as a carbon source for growth with an enzyme alpha-amylase. A medium consist of starch is used. After inoculation and overnight incubation, iodine reagent is added to detect the presence of starch. Iodine reagent complexes with starch to form a blue-black color in the culture medium. Clear zone a round colony is indicative of their ability to digest the starch in the medium due to the presence of alpha-amylase (Evans et al., 2004).

Slime formation is produced by some species. Slime is a polysaccharide layer that lies outside the cell envelope of bacteria. Slime formation can be found in both gram negative bacteria and gram positive bacteria. They should not be confused with bacterial outer membrane, which contains lipopolysaccharides and lipoproteins and is found only in gram negative bacteria (Yoshida et al., 2000).

Acid from carbohydrates is a metabolic process performed by almost all types of bacteria. This will result in the production of ATP that is the ultimate energy source of the organism and happen either in the presence or absence of atmospheric oxygen. The enzyme systems in bacteria allow them to oxidize environmental nutrient sources. Bacteria will use different energy sources in the medium depends on the specific enzymes of each bacteria. Many bacteria possess the enzymes system required for the oxidation and utilization of the simple sugar such as glucose. Some bacteria have the ability to degrade complex carbohydrates like lactose, sucrose or even polysaccharides. Such bacterium should possess the enzymes that should cleave the glycosidic bonds between the sugar units and the resulting simple carbohydrate can be transported into the cell. Lactose is a disaccharide consisting of the glucose and galactose connected by glycosidic bond. The bacteria which produce the enzyme lactase will break this bond and thus release free glucose that can be easily utilized by the organism. The characteristics

feature of the enzyme production in the bacteria enables them to use diverse carbohydrates and this will aid in the identification of unknown bacteria (Morello et al., 1985).

2.5.4 16S rRNA gene sequence and phylogenetic tree analysis

They are used to identified bacteria due to the rRNA gene is the most conserved (least variable) DNA in all cells. Portions of the rDNA sequence from distantly related organisms are remarkably similar. This means that sequences from distantly related organisms can be precisely aligned, making the true differences easy to measure. For this reason, genes that encode the rRNA (rDNA) have been used extensively to determine taxonomy, phylogeny (evolutionary relationships) and to estimate rates of species divergence among bacteria (Janda and Abbott, 2007). Thus the comparison of 16S rRNA gene sequence can show evolutionary relatedness among microorganisms.

Characterization of some succinic acid producing bacteria is shown in Table 3.

Table 3. Characterization of some succinic acid producing bacteria

Species	Morphological characteristics	Physiological characteristics	Biochemical characteristics	16S rRNA gene sequence and phylogenetic analysis	References
Actinobacillus succinogenes strain 130Z[T]	- Gram negative - Rod or coccobacillus. - Non-motile - Non-spore-forming - Facultatively anaerobic	- Mesophilic - Growth at 37-39°C - No growth at 20°C or 45°C - No growth in NaCl 4.5% (w/v)	- Positive for catalase, oxidase, β-galactosidase (ONPG) and nitrate reduction - Negative for arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, citrate utilization, H_2S, urease, indole, tryptophan deaminase, the Voges-Proskauer test, gelatin hydrolysis and gas production - Produce acid from amygdalin, L-arabinose, cellobiose, D-fructose, galactose, gluconate, D-glucose, lactose, maltose, D-mannose, D-mannitol, raffinose, D-ribose, D-sorbitol, salicin, sucrose and D-xylose - Not produce acid from D-arabinose, L-xylose, L-rhamnose, D-melibiose, trehalose, D-lyxose and L-arabitol	- The most closely related members of the family *Pasteurellaceae* have 16S rRNA gene similarities of 95.5% - Based upon morphological and biochemical properties, strain 130ZT is most similar to members of the genus *Actinobacillus* - Strain 130ZT be classified as a new species, *Actinobacillus succinogenes.*	(Guettler et al., 1999)

Table 3. Characterization of some succinic acid producing bacteria (continued)

Species	Morphological characteristics	Physiological characteristics	Biochemical characteristics	16S rRNA gene sequence and phylogenetic analysis	References
Klebsiella pneumoniae strain SAP	- Gram negative - Rod-shaped. - Non-motile - Facultatively anaerobic	- Growth at 10 and 37-39°C - No growth at 44.5°C	- Positive for Voges-Proskauer test, lysine decarboxylase and urease - Negative for gas production, arginine dihydrolase, and gelatin hydrolysis - Produce acid from D-glucose, galactose, L-rhamnose, sucrose, maltose, mannose, cellobiose, xylose, melibiose, lactose, D-mannitol, raffinose and D-sorbitol - Not produce acid from L-arabinose, fructose and ribose	The most closely of *Klebsiella* sp. have 16s rRNA gene similarities of 96.9%. The taxonomic position of isolate SAP was concluded to be new strain of *Klebsiella pneumoniae*	(Thakker et al., 2006)
Succinatimonas hippei	- Gram negative - Rod-shaped. - Non-motile - Non-spore-forming - Strictly anaerobic	- Growth at 35-40°C - No growth at 30 and 45°C	- Positive for acid phosphatase, alanine arylamidase and arginine arylamidase - Negative for catalase, oxidase, urease, hydrolysis of aesculin and gelatin and nitrate reduction - Produce acid from D-glucose, maltose, and xylose - Not produce acid from lactose, D-mannitol L-rhamnose, D-sorbitol and sucrose	- The most closely associated with the members of the family *Succinivibrionaceae* have 16s rRNA gene similarities of 87.9-92.3%. -Phenotypic and chemotaxonomic data suggest that strain YIT 12066^{T} represents a novel species of a new genus	(Morotomi et al., 2010)

2.6 Optimization of succinic acid production

Production medium

In 2010, Li et al. (Li et al., 2010) studied succinic acid production by *Actinobacillus succinogenes*. Production medium consists of glucose 60 g/l, yeast extract 30 g/l, urea 2 g/l, $CaCl_2.2H_2O$ 0.2 g/l, $MgCl_2.6H_2O$ 0.2 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 pH to 7.0. The result showed 45.8 g/l of succinic acid and a high yield of 1.23 g/g glucose. Optimum conditions of succinic acid production were studied by various researches as shown in Table 4.

In this study, four variables consist of initial glucose concentration, different nitrogen sources, initial pH and temperature that affects succinic acid production are investigated.
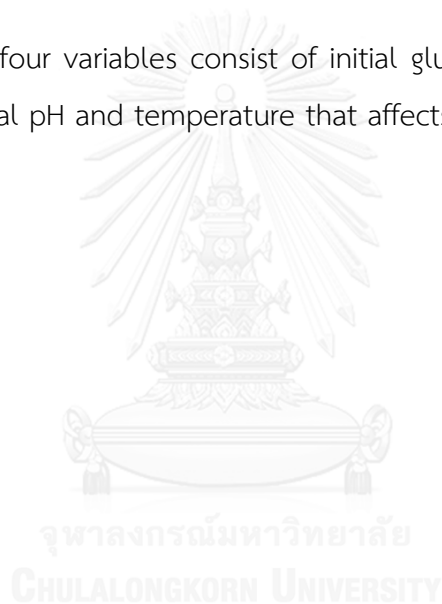
Table 4. Optimization of succinic acid production

Species	Carbon sources	Nitrogen sources	Initial pH	Temperature (°C)	Succinic acid (g/l)	References
Anaerobiospirillum succiniciproducens	Glucose 0.5, 1.0, 2.0, 5, 10, 20 and 40 g/l	Yeast extract, Polypeptone, Peptone, Soytone, Tryptone, Beef extract and Casamino acid	6.5	39°C	3.0 g/l of succinic acid from - 10 g/l of glucose - 4 g/l of polypeptone - pH 7.0 and 39°C	(Lee et al., 1999b)
Bacteroides fragilis	Glucose 5, 10, 15, 20, 25, 30, 35 and 40 g/l	Yeast extract, Beef extract, Peptone, Tryptone, Malt extract, Corn steep liquor, $(NH_4)_2HPO_4$, NH_4Cl and $NaNO_3$	4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0	20, 25, 30, 35, 37, 39, 45 and 50°C	2.8 g/l succinic acid from - 15 g/l of glucose - 25 g/l of peptone - pH 6.5 - 37°C	(Isar et al., 2006)
Enterococcus flavescens	Glucose, Fructose, Maltose, Xylose, Sucrose, Lactose, Galactose, Cane molasses, Starch, Glycerol, Sorbitol, Mannitol, Rhamnose and Arabinose	Yeast extract, Tryptone, Corn steep liquor, Beef extract, Urea, NH_4Cl, $(NH_4)_3PO_4$, $(NH_4)NO_3$, $(NH_4)_2CO_3$, $(NH_4)_2SO_4$, KNO_3 and $NaNO_3$	4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 and 9.0	25, 30, 35, 37, 39, 45 and 50°C	2.82 g/l of succinic acid from - 20 g/l of sucrose - 2 g/l of yeast extract - pH 6.5 - 39°C	(Agarwal et al., 2007)
Actinobacillus succinogenes CGMCC1593	Molasses 0, 5, 10, 15 and 20 g/l	Yeast extract, Peptone, Corn steep liquor, Beef extract, Dry yeast cell, NH_4Cl and KNO_3	7.0	37°C	46.4 g/l of succinic acid from - 65 g/l of molasses - 10 g/l of yeast extract - pH 7.0 and 37°C	(Liu et al., 2008)

Table 4. Optimization of succinic acid production (continued)

Species	Carbon sources	Nitrogen sources	Initial pH	Temperature (°C)	Succinic acid (g/l)	References
Mannheimia succiniciproducens LPK7	Glucose 5 g/l	Yeast extract, Peptone, Tryptone, Malt extract, Beef extract and Urea	6.0, 6.5, 7.0 and 7.5	36, 39, 42 and 45°C	3.67 g/l of succinic acid from - 5 g/l of glucose - 3 g/l of yeast extract - pH 7.5 - 39°C	(Oh et al., 2009)
Mannheimia succiniciproducens MBEL55E	Glucose 5, 10, 15, 20 and 25 g/l	Yeast extract 0, 1 and 2 g/l Peptone 0, 2 and 5 g/l	5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0 and 8.5	30, 32, 35 and 36°C	15 g/l of succinic acid from - 20 g/l of glucose - 2 g/l yeast extract - pH 6.5 - 37°C	(Raja and Dhanasekar, 2011)
Actinobacillus succinogenes	Glucose 10, 30, 50, 70 and 90 g/l	- Yeast extract - Yeast cell hydrolysate (YCH)	7.0	37°C	35.5 g/l of succinic acid from - 50 g/l of sucrose - 15 g/l of Yeast cell hydrolysate (YCH) - pH 7.0 - 37°C	(Chen et al., 2011)

CHAPTER III

MATERIALS AND METHODS

3.1 Sample sources

Sample sources including soils in Suphanburi, Surin and Nakhonpathom provinces, chicken manure in Suphanburi province, cattle dung in Nakhonsawan province, bovine rumen from a slaughter house in Nakhonsawan province, tree bark in Ayutthaya and pig manure in Chainat province, Thailand.

3.2 Chemicals and reagents

Chemicals	Company, country
Agar	Fluka, Germany
Agarose	Ajax Chemicals, Australia
Ammonium sulfate ($(NH_4)_2SO_4$)	Merck, Germany
D-Amygdalin ($C_{20}H_{27}NO_{11}$)	Wako, Japan
L-Arabinose ($C_5H_{10}O_5$)	Wako, Japan
L-Arginine hydrochloride	Alfa Aesar, UK
Biotin	Fluka, Germany
Calcium chloride dihydrate ($CaCl_2.2H_2O$)	Merck, Germany
Cellobiose ($C_{12}H_{22}O_{11}$)	Sigma, U.S.A
D-Fructose ($C_6H_{12}O_6$)	Ajax Chemicals, Australia
D-Galactose ($C_6H_{12}O_6$)	Ajax Chemicals, Australia
D-Glucose ($C_6H_{12}O_6$)	Ajax Chemicals, Australia
Lactose ($C_{12}H_{22}O_{11}$)	Wako, Japan
Magnesium carbonate ($MgCO_3$)	Sigma-Aldrich, U.S.A
Maltose ($C_{12}H_{22}O_{11}$)	Wako, Japan
D-Mannitol ($C_6H_{14}O_6$)	Wako, Japan
D-Mannose ($C_6H_{12}O_6$)	Wako, Japan

Chemicals	Company, country
Melibiose ($C_{12}H_{22}O_{11}$)	Wako, Japan
∞-Methyl-D-glucoside ($C_7H_{14}O_6$)	Alfa Aesar, UK
Poly peptone	Wako, Japan
di-Potassium hydrogen phosphate (K_2HPO_4)	Merck, Germany
Potassium dihydrogen phosphate (KH_2PO_4)	Merck, Germany
Potassium nitrate (KNO_3)	Merck, Germany
D-Raffinose pentahydrate ($C_{18}H_{32}O_{16}.5H_2O$)	Wako, Japan
L-Rhamnose monohydrate ($C_6H_{12}O_5.H_2O$)	Wako, Japan
D-Ribose ($C_5H_{10}O_5$)	Wako, Japan
D-Salicin ($C_{13}H_{18}O_7$)	Sigma-Aldrich, U.S.A
Sodium chloride (NaCl)	Ajax Chemicals, Australia
Sodium dihydrogen phosphate (NaH_2PO_4)	Merck, Germany
Sodium hydrogen phosphate (Na_2HPO_4)	Merck, Germany
Sodium gluconate ($C_6H_{12}O_7$)	Wako, Japan
D-Sorbitol ($C_6H_{14}O_6$)	Wako, Japan
Sucrose ($C_{12}H_{22}O_{11}$)	Merck, Germany
Sulfuric acid (H_2SO_4)	Merck, Germany
Thiamine	Fluka, Germany
Trehalose dihydrate ($C_{12}H_{22}O_{11}.2H_2O$)	Wako, Japan
Urea (CH_4N_2O)	Sigma-Aldrich, U.S.A
D-Xylose ($C_5H_{10}O_5$)	Wako, Japan
Yeast extract	Bio Springer, France
Zinc dust	Sigma-Aldrich, U.S.A

3.3 Equipments and supplies

Equipments and Supplies	Company, country
Aminex HPX-87H, 300 x 7.8 mm	Bio-Rad Laboratories.Inc., U.S.A
Autoclave (HV-50)	Hirayama manufacturing Corp., Japan
Bench-top centrifuge, WiseSpin® (CF10)	Dihan scientific Co., Ltd., South Korea
Cellulose membrane acetate filter (pore size 0.45 μm, 13 mmØ)	Sartorius Stedim Biotech GmbH, Germany
Freezer (-80℃) (SF-C697)	Sanyo Commercial Solution, Ltd., Thailand.
Gel/PCR DNA Fragments Extraction Kit DF100/DF300	Geneaid Biotech, Taiwan
High Performance Liquid Chromatography (HPLC)	LC-10AD, Shimadzu Corporation, Japan
Incubator (MIR 152)	Sanyo Electric Co., Ltd. Japan
Mupid®-EXU Submarine electrophoresis system	Advance Co, Ltd., Japan
pH meter (Accumet® AB15)	Fisher Scientific, Singapore
Refrigerated incubator shaker (Innova™ 4330)	New Brunswick Scientific Co., Inc., U.S.A
T100™ Thermal Cycler	Bio-Rad, Singapore
TLC plate (Silica gel 60G F254)	Merck, Germany
UV-Visible recording spectrophotometer (U-5100 Ratio-Beam)	Hitachi, U.S.A

3.4 Primers

Primers	Company, country
20 forward primer (Weisburg et al., 1991) (5'-AGTTTGATCCTGGCTC-3', Tm=48.3)	Thermo Scientific, Korea
1530 reverse primer (Weisburg et al., 1991) (5'-AAGGAGGTGATCCAGCC-3', Tm=54.1)	Thermo Scientific, Korea

3.5 Methods

3.5.1 Preparation of samples

All samples including soils, chicken manure, cattle dung, bovine rumen, tree bark and pig manure were packed in bags and stored at 4°C until use to screen for succinic acid producing bacteria. In case of bovine rumen, it was blended with sterile water (five-fold dilution) to create a suspension before use.

3.5.2 Screening of succinic acid producing bacteria

3.5.2.1 Enrichment medium

Three grams of cattle dung, chicken manure, soil, tree bark, pig manure and 50 µl of bovine rumen suspension were added in 5 ml of enrichment medium. The enrichment medium containing per liter: 20 g glucose, 5 g poly peptone , 3 g yeast extract, 5 g K_2HPO_4, 2 g NaCl, 2 g $(NH_4)_2SO_4$, 0.2 g $CaCl_2.2H_2O$, 0.4 g $MgCl_2.6H_2O$, 15 g $MgCO_3$, of NaN_3 and adjusted pH to 6.5 (Lee et al., 2002). The enrichment medium was steriled by autoclaving at 121°C for 15 min. Incubation condition was at 37°C under anaerobe for 72 h.

3.5.2.2 Solid medium (selective medium)

Fifty µl of sample were spread onto the modified Gifu anaerobic medium (GAM; Nissui Pharmaceutical) (Appendix A-1) with additional 15 g/l of agar (Morotomi et al., 2010). Plates were incubated at 37°C for 72 h under anaerobic conditions. Single colonies were selected, streaked on GAM agar and incubated in

the same as previous condition. Then the single colonies were picked and transferred to selective medium for succinic acid producing ability (Agarwal et al., 2005). Incubation condition was at 37°C under anaerobe for 24 h. The selective medium containing per liter: 20 g glucose, 10 g poly peptone, 5 g yeast extract, 3 g K_2HPO_4, 1 g NaCl, 1 g $(NH_4)_2SO_4$, 0.2 g $CaCl_2.2H_2O$, 0.2 g $MgCl_2.6H_2O$, 15 g $MgCO_3$, 15 g of agar and adjusted pH to 6.5 (Guettler et al., 1999). The selective medium was steriled by autoclaving at 121°C for 15 min. Isolates with succinic acid producing ability which exhibited a clear zone around colony were selected.

3.5.2.3 Liquid medium (production medium)

The Isolates with succinic acid producing ability were picked up and transferred to a production medium. The production medium containing, per liter: 60 g glucose, 30 g yeast extract, 2 g urea, 0.2 g $CaCl_2.2H_2O$, 0.2 g $MgCl_2.6H_2O$, 0.07 g $MnCl_2$, 4.4 g Na_2HPO_4, 3.3 g NaH_2PO_4, 30 g of $MgCO_3$ and adjusted pH to 7.0. The production medium was steriled by autoclaving at 121°C for 15 min. Glucose was separately sterilized at 115°C for 20 min and added to the production medium. Biotin (0.3 µg/l) and thiamin (0.2 µg/l) were prepared by sterile membrane filtration (0.22 µm nylon, Millipore Express, Ireland) and added to the production medium after that were steriled. Incubation condition was at 37°C for 72 h under anaerobe (Li et al., 2010). Then isolates produced succinic acid, the culture broth was centrifuged at 10,000 rpm for 5 min. The supernatants were analyzed for the presence of succinic acid using thin-layer chromatography (TLC) for qualitative test and high-performance liquid chromatography (HPLC) for quantitative test.

3.5.3 Succinic acid determination

3.5.3.1 Thin-layer chromatography (TLC)

The test samples (10 µl) and standard (succinic acid) 2 g/l were spotted onto a silica gel TLC plates and resolved using a solvent system comprised of ethanol, NH_4OH and water (20:5:3 v/v). A standard solution (1 mg/ml) of succinic acid was used as a reference. After 30 min, the air dried plates were sprayed with

bromocresol green (0.04% w/v in ethanol) and heated at 160°C for 5 min to reveal the organic acid spots (Agarwal et al., 2005).

3.5.3.2 High-performance liquid chromatography (HPLC)

Fermentation products (succinic acid) were analyzed by HPLC (high-performance liquid chromatography) system equipped with a cation-exclusion column and a refractive index detector. The mobile phase is 5 mM H_2SO_4 solution at a flow rate of 0.6 ml/min and the column was operated at 55°C (Agarwal et al., 2005).

3.5.4 Characterization of succinic acid producing bacteria

3.5.4.1 Morphological characteristics

Isolates were observed including gram stain (Appendix A-3) (Hucker and Conn, 1923), endospore stain (Appendix A-4) (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 and Okada, 1998).

3.5.4.2 Physiological characteristics

Isolates were examined after grown in GAM broth at 37°C under anaerobe for 72 h. Then they were dropped into medium for test. Growth in different NaCl concentrations (2% and 6% w/v NaCl), at pH values (3-9) and different temperatures (20-50°C) was tested using MRS broth (MRS; de Man, Rogosa and Sharpe) (Appendix A-2). The pH was adjusted with steriled 6M HCl and 6M NaOH (Barrow and Feltham, 1993).

3.5.4.3 Biochemical characteristics

Isolates were examined after grown in GAM broth at 37°C under anaerobe for 72 h. Then they were diluted using 0.85% NaCl solution (Tanasupawat and Okada, 1998) and dropped into medium for the following tests:

Catalase test, isolates were grown overnight on GAM agar plate and transferred to microscope slide. Then 3% H_2O_2 (Appendix A-5) was dropped onto colony on the microscope slide. After 5 min, any sign of bubbling was interpreted as a positive test. The absence of bubbling was interpreted as negative (Gagnon et al., 1959).

Gas production, isolates was determined by 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 to a sterile tube of arginine broth (Appendix A-6.1). Incubation condition was at 37°C for up to 5 days. After that a pink color showed the presence of positive reaction (Niven et al., 1942).

Nitrate reduction, isolates were dropped into nitrate broth (Appendix A-7.1) and inoculated for up to 5 days. Then sulfanilic acid solution (Appendix A-7.2) was added 3 drops and followed by 2 drops of *N,N*-dimethyl-l-naphthylamine solution (Appendix A-7.3). After 3 min a deep red color showed the presence of nitrite and thus the nitrate was reduced, indicated a positive reaction. But the result showed no color, added a small amount of zinc. After 5 min a color change to red indicated a negative reaction because nitrate must have been present and reduced to form nitrite (Conn and Breed, 1919).

Starch hydrolysis, isolates were streaked on starch agar plate (Appendix A-8.1). After incubation condition was at 37°C under anaerobe for 24 h, iodine reagent (Appendix A-8.2) was added to flood the plate. Clear zone around colonies was positive test. 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-9.1). 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-10.1). 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 (Barrow and Feltham, 1993).

The results from phenotypic characteristics were grouped using a hierarchical cluster in statistical package for the social sciences for windows (SPSS) program (version 15.0).

3.5.4.4 16S rRNA gene sequence and phylogenetic analysis

Isolates produced the highest succinic acid from each group were analyzed by colony polymerase chain reaction (PCR). Colonies were picked up and transferred to microtube contained 30 µl sterile distilled water. Then this microtube was boiled 95°C for 3-5 min. Amplification of the 16S rRNA gene was carried out in 50 µl of PCR reaction mixture. PCR reaction mixtures were shown in Table 5 (Tanasupawat et al., 2004).

Table 5. PCR reaction mixtures

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') (Weisburg et al., 1991)	2
1530R (5'-AAGGAGGTGATCCAGCC-3') (Weisburg et al., 1991)	2
dNTP (2 mM)	1
Taq DNA polymerase (5 U/µl)	0.25
Total	50

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-11.2) and after that 16S rRNA fragments were purified by Gel/PCR DNA Fragments Extraction Kit DF100/DF300 (Geneaid Biotech, Taiwan). The amplified 16S rRNA gene sequence was analyzed by Macrogen®, Korea (Tanasupawat et al., 2004).

Sequence alignment was corrected manually by using the program BioEdit (version 7.0.2). The sequence databases contained over 1000 sequences were saved by the program Notepad (version 6.2). The sequence similarity were compared the database from EzTaxon (www.ezbiocloud.net/eztaxon). Multiple alignments of the sequences determined were performed with a program CLUSTAL_X (version 1.83) (Tamura et al., 2011). A phylogenetic tree was constructed by the neighbor-joining method with (Saitou and Nei, 1987) the program MEGA (version 6.0) (Tamura et al., 2011). The confidence values of individual branches in the phylogenetic tree were determined by using the bootstrap analysis of Felsenstein based on 1000 replications (Felsenstein, 1985).

3.5.5 Optimization of succinic acid production

3.5.5.1 Inoculum preparation

Succinic acid producing bacteria, maintained in 10% skim milk and stored at -80℃, was propagated on modified GAM agar plate and incubated at 37℃ under anaerobe. The inoculum was prepared by adding a loop full of pure culture from modified GAM agar plate into 30 ml of GAM broth in 50 ml flask and incubated at 37℃ under anaerobe for 24 h in the incubator shaker at 200 rpm.

3.5.5.2 Production medium

Five percentage of inoculum (OD600 of 3.2) was transferred to a production medium and incubated at 37℃ under anaerobe for 24 h. The production medium was prepared as described in Section 3.5.2.3. Cell growth was monitored by measuring the absorbance at 660 nm (OD660) using a spectrophotometer. Cell dry weight (CDW) was calculated from a standard curve relating the OD660 to CDW. After that the culture broth was centrifuged at 10,000 rpm for 5 min. The supernatants were filtered with 0.45 μm cellulose membrane and analyzed for the presence of succinic acid using high-performance liquid chromatography (HPLC) and residual reducing sugar with DNS method (Li et al., 2010).

3.5.5.3 Effect of glucose concentration

Different concentration of glucose (30, 60, 90 g/l) was investigated. The culture broth was incubated at 37℃ under anaerobe condition for 72 h in the incubator shaker at 200 rpm.

3.5.5.4 Effect of nitrogen sources

Different type of nitrogen sources (yeast extract, peptone, tryptone, urea, KNO_3, NH_4Cl, $(NH_4)_2HPO_4$, $(NH_4)_2SO_4$ and NH_4NO_3) was investigated. The culture broth was incubated at 37℃ under anaerobe condition for 24 h in the incubator shaker at 200 rpm.

3.5.5.5 Effect of initial pH

Different initial pH 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0 was investigated. The production medium was adjusted with 5M HCl and 5M NaOH. The culture broth was incubated at 37°C under anaerobe condition for 24 h in the incubator shaker at 200 rpm.

3.5.5.6 Effect of temperature

Different temperature at 35, 37 and 39°C was investigated. The culture broth was incubated at 35, 37 and 39°C under anaerobe condition for 24 h in the refrigerated incubator shaker at 200 rpm.

3.5.6 Analysis methods

3.5.6.1 Cell growth

After the desired incubation period, cell growth was monitored by measuring the absorbance at 660 nm (OD660) using a spectrophotometer. 0.5 M HCl was added to samples (2:1 v/v) in order to dissolve $MgCO_3$ to form soluble magnesium chloride and carbon dioxide (Lin et al., 2008). Cell dry weight (CDW) was calculated from a standard curve relating the OD660 to CDW (Appendix B-2).

3.5.6.2 Residual sugars

The culture broth was centrifuged at 10,000 rpm for 5 min. The supernatants of 50 μl transferred to microtube and 150 μl of DNS reagent was added (Appendix A-12). After that the mixture was boiled using water bath for 5 min to red-brown color. Then cooling to room temperature and 1 ml of distilled water was added to the mixture. Record the absorbance with a spectrophotometer at 540 nm against the blank (Miller, 1959).

3.5.6.3 Succinic acid

The culture broth was centrifuged at 10,000 rpm for 5 min and the supernatants were filtered with 0.45 μm cellulose membrane. Succinic acid was

determined using HPLC with a RI detector. The analysis was performed using a Bio-Rad Aminex-87H column. The analysis conditions were as follows: sample volume 20 μl, 0.005 N H_2SO_4 as a mobile phase, flow rate 0.6 ml/min and column temperature at 45°C (Agarwal et al. 2005).

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Collected samples

Samples were collected 6 times from various sources in Thailand (Table 6) as follows:

The first times, eighteen isolates were obtained from soils which were collected from upper layer of soils in Suphanburi and Suratthani provinces.

The second times, sixty-five isolates were obtained from chicken manure (Hybrid Chickens) which collected from Suphanburi and 60 isolates were obtained from cattle dung (American Brahman Cattle) which was collected from Anantapong farm in Nakhonsawan province.

The third times, sixteen isolates were obtained from soils which were collected from upper layer of soils in Nakhonpathom province.

The fourth times, twenty-seven isolates were obtained from bovine rumen which was collected from Anantapong farm in Nakhonsawan province.

The fifth times, twenty-nine isolates were obtained from tree bark which was collected the second layer of bark (Bodhi Tree) from Ayutthaya province.

Lastly, ninety-five isolates were obtained from pig manure (Large White) which was collected from Chainat province.

Total 310 isolates were obtained from six sources in five provinces in Thailand.

Table 6. Collected samples from various sources in Thailand

Times	Samples	Province	Number of isolates
1	Soil (SPI/B)	Suphanburi	12
	Soil (SRI/A,B)	Suratthani	6
		Total	18
2	Chicken manure (SP4-A,B)	Suphanburi	16
	Chicken manure (SP5-A,B)	Suphanburi	10
	Chicken manure (SP6-A,B)	Suphanburi	7
	Chicken manure (SP7-A,B)	Suphanburi	8
	Chicken manure (SP8-A ,B)	Suphanburi	15
	Chicken manure (SP9-A,B)	Suphanburi	5
	Chicken manure (SP10-A,B)	Suphanburi	4
		Total	65
1	Cattle dung (NS1-A,B)	Nakhonsawan	6
	Cattle dung (NS2-A,B)	Nakhonsawan	6
	Cattle dung (NS3-A,B)	Nakhonsawan	7
	Cattle dung (NS4-A,B)	Nakhonsawan	3
	Cattle dung (NS5-A,B)	Nakhonsawan	6
	Cattle dung (NS6-A,B)	Nakhonsawan	6
	Cattle dung (NS7-A,B)	Nakhonsawan	4
	Cattle dung (NS8-A,B)	Nakhonsawan	4
	Cattle dung (NS9-A,B)	Nakhonsawan	4
	Cattle dung (NS10-A,B)	Nakhonsawan	4
	Cattle dung (NS11-A,B)	Nakhonsawan	4
	Cattle dung (NS12-A,B)	Nakhonsawan	6
		Total	60
		Total	125

Table 6. Samples were collected from various sources in Thailand (continued)

Times	Samples	Province	Number of isolates
3	Soil (NP1-A)	Nakhonpathom	4
	Soil (NP3-A,B)	Nakhonpathom	5
	Soil (NP5-A/B)	Nakhonpathom	4
	Soil (NP6-B)	Nakhonpathom	3
		Total	16
4	Bovine rumen (NS13-a,b,c)	Nakhonsawan	7
	Bovine rumen (NS14-a,b,c,d)	Nakhonsawan	9
	Bovine rumen (NS15-a,b,c,d)	Nakhonsawan	11
		Total	27
5	Tree bark (AY1-a,b)	Ayutthaya	6
	Tree bark (AY2-a,b)	Ayutthaya	4
	Tree bark (AY3-a,b)	Ayutthaya	10
	Tree bark (AY4-a,b)	Ayutthaya	3
	Tree bark (AY5-a,b)	Ayutthaya	6
		Total	29
6	Pig manure (CN1-O)	Chainat	10
	Pig manure (CN2-O)	Chainat	9
	Pig manure (CN3-O)	Chainat	6
	Pig manure (CN4-O)	Chainat	9
	Pig manure (CN5-O)	Chainat	2
	Pig manure (CN6-O)	Chainat	1
	Pig manure (CN1-6, PCH1-6)	Chainat	12
		Total	95
Total			310

4.2 Screening of succinic acid producing bacteria

4.2.1 Screening succinic acid producing bacteria using solid medium (selective medium)

Selective medium screened 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 a clear zone around colonies on selective medium. Due to the screening medium with $MgCO_3$, magnesium (Mg^{2+}) reacted with succinic acid ($C_4H_6O_4$) to succinate salt ($MgC_4H_4O_4$), so a clear zone was observed.

Out of a total of 310 isolates, only 51 isolates could produce succinic acid as they exhibited a clear zone around colonies on selective medium plate (Figure 11). Fifty-one isolates included 3 isolates from soil, 6 isolates from chicken manure, 4 isolates from cattle dung, 5 isolates from bovine rumen, 8 isolates from tree bark and 25 isolates from pig manure (Table 7).
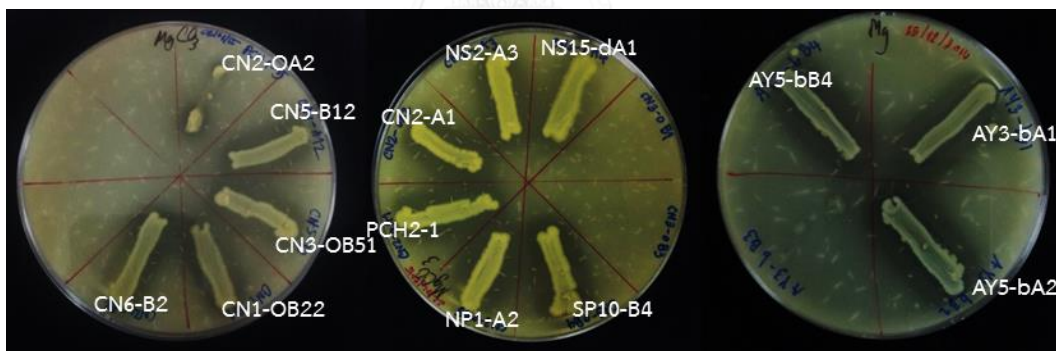
Figure 11. Isolates showing a clear zone around colonies on a selective medium

Table 7. Isolates exhibited a clear zone in selective medium

Times/Sources		Isolates	Total
1	Soil	SPI-B2, SRI-B1	2
2	Chicken manure	SP4-B5, SP5-A6, SP5-B4, SP8-A4, SP8-B1 ,SP10-B4	10
	Cattle dung	NS2-A1, NS2-A3, NS2-B3, NS3-B1	
3	Soil	NP1-A2	1
4	Bovine rumen	NS14-aA2, NS15-aB2, NS15-bA2, NS14-dB1, NS15-dA1	5
5	Tree bark	AY2-aA1, AY3-bA1, AY4-aA1, AY5-aB1, AY5-bA2, AY5-bB3, AY5-bB4, AY5-bB6	8
6	Pig manure	CN1-OB13, CN1-OB22, CN2-OA2, CN2-OB4, CN3-OB51, CN4-OA1, CN4-OB21, CN6-OB1, CN1-A1, CN1-B21, CN1-B3, CN2-A1, CN2-B5, CN3-B1, CN4-B1, CN5-A21, CN5-B11,CN5-B12, CN5-B2, CN6-B2, PCH1-2, PCH2-1 ,PCH4-3, PCH6-2, PCH6-3	25
		Total	**51**

4.2.2 Screening succinic acid producing bacteria using liquid medium (production medium)

Fifty-one isolates exhibited a clear zone around colonies were further analyzed with TLC. A standard succinic acid showed a clear yellow spot as shown in Figure 12. The positive isolates such as NS15-dA1, NS2-A3, AY5-bB4 and CN2-OA2 showed an R_f of 0.56. The R_f was calculated from distance of the spot on the TLC-plate/distance of the solvent front. The R_f values of other isolates were showed in Table 8.
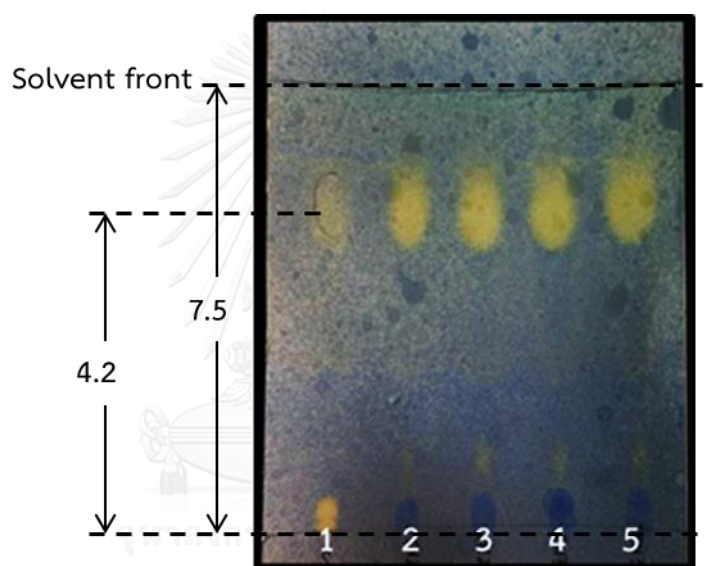
Figure 12. Analysis of some isolates for succinic acid production on TLC plate.
1: Standard succinic acid; 2: isolate NS15-dA1; 3: isolate NS2-A3; 4: isolate AY5-bB4;
5: isolate CN2-OA2.

All 51 isolates were confirmed to have succinic acid producing ability using HPLC. Succinic acid concentrations in the range of 0.5529-52.028 g/l and yield of succinic acid in the range of 0.009-0.867 g/g glucose were obtained from 60 g/l of glucose as a carbon source. Isolate NS15-bA2 from bovine rumen was able to produce greater amounts of succinic acid than other isolates (Table 8).

Therefore, all 51 isolates could produce succinic acid. They showed positive result in TLC and HPLC method.

Table 8. Determination of succinic acid ability of 51 isolates

Isolates	TLC method	HPLC method	
	R_f value	Succinic acid (g/l)	Yield (g/g glucose)
SPI-B2	0.55	1.938	0.032
SRI-B1	0.55	1.808	0.030
SP4-B5	0.57	41.291	0.688
SP5-A6	0.57	44.377	0.740
SP5-B4	0.57	42.589	0.710
SP8-A4	0.57	40.082	0.668
SP8-B1	0.55	39.931	0.666
SP10-B4	0.55	42.910	0.715
NS2-A1	0.55	38.846	0.647
NS2-A3	0.56	37.119	0.619
NS2-B3	0.56	40.861	0.681
NS3-B1	0.56	38.482	0.641
NP1-A2	0.56	49.415	0.824
NS14-aA2	0.57	48.355	0.806
NS15-aB2	0.57	46.478	0.775
NS15-bA2	0.57	52.028	0.867
NS14-dB1	0.57	47.861	0.798
NS15-dA1	0.56	50.862	0.848
AY2-aA1	0.55	3.831	0.064
AY3-bA1	0.55	12.914	0.215
AY4-aA1	0.55	29.143	0.486
AY5-aB1	0.55	5.170	0.086
AY5-bA2	0.55	8.096	0.135
AY5-bB3	0.55	0.553	0.009
AY5-bB4	0.56	3.157	0.053
AY5-bB6	0.55	7.056	0.118

* Standard succinic acid showed an R_f of 0.55, 0.56 and 0.57

Table 8. Determination of succinic acid ability of 51 isolates (continued)

Isolates	TLC method	HPLC method	
	R_f value	Succinic acid (g/l)	Yield (g/g glucose)
CN1-OB13	0.56	49.036	0.817
CN1-OB22	0.56	41.103	0.685
CN2-OA2	0.56	48.892	0.815
CN2-OB4	0.55	45.591	0.760
CN3-OB51	0.55	45.086	0.751
CN4-OA1	0.55	42.834	0.714
CN4-OB21	0.55	43.255	0.721
CN6-OB1	0.56	47.651	0.794
CN1-A1	0.56	46.294	0.772
CN1-B21	0.58	44.312	0.739
CN1-B3	0.58	45.832	0.764
CN2-A1	0.56	4.128	0.069
CN2-B5	0.58	1.447	0.024
CN3-B1	0.58	3.430	0.057
CN4-B1	0.56	2.103	0.035
CN5-A21	0.55	45.160	0.753
CN5-B11	0.55	39.620	0.660
CN5-B12	0.56	44.102	0.735
CN5-B2	0.55	42.747	0.712
CN6-B2	0.56	34.614	0.577
PCH1-2	0.56	46.454	0.774
PCH2-1	0.55	50.411	0.840
PCH4-3	0.55	6.362	0.106
PCH6-2	0.55	43.709	0.728
PCH6-3	0.55	5.686	0.095
51 isolates		0.553-52.028	0.009-0.867

* Standard succinic acid showed an R_f of 0.55, 0.56 and 0.58

4.3 Characterization of succinic acid producing bacteria

4.3.1 Morphological characteristics

All isolates with succinic acid production ability were studied for morphological characteristics. The result of morphological characteristics of 51 isolates was shown in Table 9. Out of a total of 51 isolates, only 21 isolates were rods consisted of 10 isolates were gram negative (Figure 13A), 7 isolates were gram positive (Figure 13B) and 4 isolates were gram positive and spore forming (Figure 13C). The other isolates were gram positive and cocci (coccus, cocci in pair and cocci in chain) (Figure 13D).
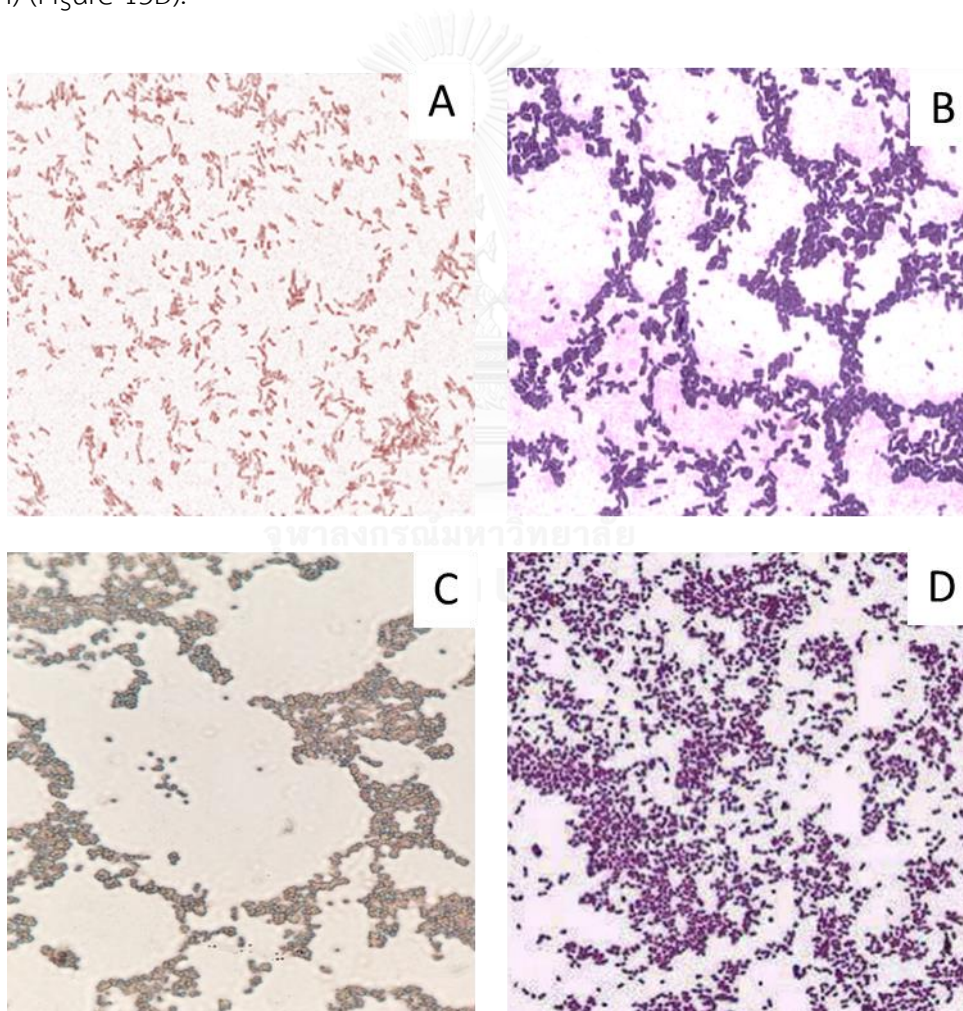
Figure 13. Cell morphological based on gram stain and endospore stain

(A, Gram negative rods; B, Gram positive rods; C, Gram positive rods and

spore forming; D, Gram positive cocci/pair/chain)

Table 9. Morphological characteristics of 51 isolates

Isolates	Gram stain	Cell morphology		Colony appearance				
		Shape	Spore	Color	Shape	Margin	Optical property	Elevation
CN1-OB13	Negative	Rods	Non-spore forming	Yellow	Circular	Entire	Translucent	Convex
CN1-OB22	Negative	Rods	Non-spore forming	Yellow	Circular	Entire	Translucent	Translucent
CN2-OB4	Negative	Rods	Non-spore forming	Yellow	Circular	Entire	Translucent	Translucent
CN4-OA1	Negative	Rods	Non-spore forming	Yellow	Circular	Entire	Translucent	Translucent
CN1-B3	Negative	Rods	Non-spore forming	Yellow	Circular	Entire	Translucent	Translucent
CN1-A1	Negative	Rods	Non-spore forming	Yellow	Circular	Entire	Translucent	Translucent
CN5-A21	Negative	Rods	Non-spore forming	Yellow	Circular	Entire	Translucent	Translucent
CN5-B11	Negative	Rods	Non-spore forming	Yellow	Circular	Entire	Translucent	Translucent
CN5-B12	Negative	Rods	Non-spore forming	Yellow	Circular	Entire	Translucent	Translucent
CN6-B2	Negative	Rods	Non-spore forming	Yellow	Circular	Entire	Translucent	Translucent
Total								10 isolates
AY5-bA2	Positive	Rods	Non-spore forming	White	Circular	Entire	Opaque	Flat
AY5-bB6	Positive	Rods	Non-spore forming	White	Circular	Entire	Opaque	Flat
CN2-A1	Positive	Rods	Non-spore forming	White	Circular	Entire	Opaque	Flat
CN3-B1	Positive	Rods	Non-spore forming	White	Circular	Entire	Opaque	Flat
CN4-B1	Positive	Rods	Non-spore forming	White	Circular	Entire	Opaque	Flat
PCH4-3	Positive	Rods	Non-spore forming	White	Circular	Entire	Opaque	Flat
PCH6-3	Positive	Rods	Non-spore forming	White	Circular	Entire	Opaque	Flat
Total								7 isolates

Table 9. Morphological characteristics of 51 isolates (continued)

| Isolates | Gram stain | Cell morphology | | Color | Colony appearance | | | |
		Shape	Spore		Shape	Margin	Optical property	Elevation
SRI-B1	Positive	Rods	Spore forming	Yellow-gray	Circular	Irregular	Opaque	Flat
AY5-bB3	Positive	Rods	Spore forming	Yellow-gray	Circular	Irregular	Opaque	Flat
AY5-bB4	Positive	Rods	Spore forming	Yellow-gray	Circular	Irregular	Opaque	Flat
CN2-B5	Positive	Rods	Spore forming	Yellow-gray	Circular	Irregular	Opaque	Flat
Total								4 isolates
SPI-B2	Positive	Cocci	Non-spore forming	White	Circular	Entire	Opaque	Convex
SP4-B5	Positive	Cocci	Non-spore forming	White	Circular	Entire	Opaque	Convex
SP5-A6	Positive	Cocci	Non-spore forming	White	Circular	Entire	Opaque	Convex
SP5-B4	Positive	Cocci	Non-spore forming	White	Circular	Entire	Opaque	Convex
SP8-A4	Positive	Cocci	Non-spore forming	White	Circular	Entire	Opaque	Convex
SP8-B1	Positive	Cocci	Non-spore forming	White	Circular	Entire	Opaque	Convex
SP10-B4	Positive	Cocci	Non-spore forming	White	Circular	Entire	Opaque	Convex
NS2-A1	Positive	Cocci	Non-spore forming	White	Circular	Entire	Opaque	Convex
NS2-A3	Positive	Cocci	Non-spore forming	White	Circular	Entire	Opaque	Convex
NS2-B3	Positive	Cocci	Non-spore forming	White	Circular	Entire	Opaque	Convex
NS3-B1	Positive	Cocci	Non-spore forming	White	Circular	Entire	Opaque	Convex
NP1-A2	Positive	Cocci	Non-spore forming	White	Circular	Entire	Opaque	Convex
NS14-aA2	Positive	Cocci	Non-spore forming	White	Circular	Entire	Opaque	Convex
NS15-aB2	Positive	Cocci	Non-spore forming	White	Circular	Entire	Opaque	Convex

Table 9. Morphological characteristics of 51 isolates (continued)

Isolates	Gram stain	Cell morphology		Colony appearance				
		Shape	Spore	Color	Shape	Margin	Optical property	Elevation
NS15-bA2	Positive	Cocci	Non-spore forming	White	Circular	Entire	Opaque	Convex
NS14-dB1	Positive	Cocci	Non-spore forming	White	Circular	Entire	Opaque	Convex
NS15-dA1	Positive	Cocci	Non-spore forming	White	Circular	Entire	Opaque	Convex
AY2-aA1	Positive	Cocci	Non-spore forming	White	Circular	Entire	Opaque	Convex
AY3-bA1	Positive	Cocci	Non-spore forming	White	Circular	Entire	Opaque	Convex
AY4-aA1	Positive	Cocci	Non-spore forming	White	Circular	Entire	Opaque	Convex
AY5-aB1	Positive	Cocci	Non-spore forming	White	Circular	Entire	Opaque	Convex
CN2-OA2	Positive	Cocci	Non-spore forming	White	Circular	Entire	Opaque	Convex
CN3-OB51	Positive	Cocci	Non-spore forming	White	Circular	Entire	Opaque	Convex
CN4-OB21	Positive	Cocci	Non-spore forming	White	Circular	Entire	Opaque	Convex
CN6-OB1	Positive	Cocci	Non-spore forming	White	Circular	Entire	Opaque	Convex
CN1-B21	Positive	Cocci	Non-spore forming	White	Circular	Entire	Opaque	Convex
CN5-B2	Positive	Cocci	Non-spore forming	White	Circular	Entire	Opaque	Convex
PCH1-2	Positive	Cocci	Non-spore forming	White	Circular	Entire	Opaque	Convex
PCH2-1	Positive	Cocci	Non-spore forming	White	Circular	Entire	Opaque	Convex
PCH6-2	Positive	Cocci	Non-spore forming	White	Circular	Entire	Opaque	Convex
Total								30 isolates

4.3.2 Physiological characteristics

The results of physiological characteristics were shown in Table 10. All 51 isolates could grow in 2% and 6% NaCl, at pH 5-7 and 20-45°C. No growth was observed at pH 3. Most isolates were facultative anaerobes but only 4 isolates, SRI-B1, AY5-bB3, AY5-bB4 and CN2-B2, were microaerophiles. The bacterial physiology at pH 9.0 and growth at 50°C showed different reactions.

Table 10. Physiological characteristics of 51 isolates

Isolates	Physiological characteristics											
	2% NaCl	6% NaCl	pH 3.0	pH 5.0	pH 7.0	pH 9.0	20°C	30°C	37°C	45°C	50°C	O_2
CN1-OB13	+	+	-	+	+	+	+	+	+	+	-	Facultative anaerobe
CN1-OB22	+	+	-	+	+	+	+	+	+	+	-	Facultative anaerobe
CN2-OB4	+	+	-	+	+	+	+	+	+	+	-	Facultative anaerobe
CN4-OA1	+	+	-	+	+	+	+	+	+	+	-	Facultative anaerobe
CN1-B3	+	-	-	+	+	+	+	+	+	+	-	Facultative anaerobe
CN1-A1	+	-	-	+	+	+	+	+	+	+	-	Facultative anaerobe
CN5-A21	+	+	-	+	+	+	+	+	+	+	-	Facultative anaerobe
CN5-B11	+	+	-	+	+	+	+	+	+	+	-	Facultative anaerobe
CN5-B12	+	+	-	+	+	+	+	+	+	+	-	Facultative anaerobe
CN6-B2	+	+	-	+	+	+	+	+	+	+	-	Facultative anaerobe
AY5-bA2	+	+	-	+	+	+	+	+	+	+	-	Facultative anaerobe
AY5-bB6	+	+	-	+	+	+	+	+	+	+	-	Facultative anaerobe
CN2-A1	+	+	-	+	+	+	+	+	+	+	-	Facultative anaerobe
CN3-B1	+	+	-	+	+	+	+	+	+	+	-	Facultative anaerobe
CN4-B1	+	+	-	+	+	+	+	+	+	+	-	Facultative anaerobe
PCH4-3	+	+	-	+	+	-	+	+	+	+	-	Facultative anaerobe
PCH6-3	+	+	-	+	+	-	+	+	+	+	-	Facultative anaerobe

Table 10. Physiological characteristics of 51 isolates (continued)

Isolates	Physiological characteristics											
	2% NaCl	6% NaCl	pH 3.0	pH 5.0	pH 7.0	pH 9.0	20°C	30°C	37°C	45°C	50°C	O₂
SRI-B1	+	-	-	+	+	-	-	+	+	+	+	Microaerophile
AY5-bB3	+	+	-	+	+	-	-	+	+	+	-	Microaerophile
AY5-bB4	+	+	-	+	+	-	-	+	+	+	-	Microaerophile
CN2-B5	+	+	-	+	+	-	-	+	+	+	-	Microaerophile
SPI-B2	+	+	-	+	+	+	+	+	+	+	+	Facultative anaerobe
SP4-B5	+	+	-	+	+	+	+	+	+	+	+	Facultative anaerobe
SP5-A6	+	+	-	+	+	+	+	+	+	+	+	Facultative anaerobe
SP5-B4	+	+	-	+	+	+	+	+	+	+	+	Facultative anaerobe
SP8-A4	+	+	-	+	+	+	+	+	+	+	-	Facultative anaerobe
SP8-B1	+	+	-	+	+	+	+	+	+	+	+	Facultative anaerobe
SP10-B4	+	+	-	+	+	+	+	+	+	+	+	Facultative anaerobe
NS2-A1	+	+	-	+	+	+	+	+	+	+	-	Facultative anaerobe
NS2-A3	+	+	-	+	+	+	+	+	+	+	+	Facultative anaerobe
NS2-B3	+	+	-	+	+	+	+	+	+	+	-	Facultative anaerobe
NS3-B1	+	+	-	+	+	+	+	+	+	+	-	Facultative anaerobe
NP1-A2	+	+	-	+	+	+	+	+	+	+	+	Facultative anaerobe
NS14-aA2	+	+	-	+	+	+	+	+	+	+	+	Facultative anaerobe
NS15-aB2	+	+	-	+	+	+	+	+	+	+	+	Facultative anaerobe

Table 10. Physiological characteristics of 51 isolates (continued)

Isolates	Physiological characteristics											
	2% NaCl	6% NaCl	pH 3.0	pH 5.0	pH 7.0	pH 9.0	20°C	30°C	37°C	45°C	50°C	O₂
NS15-bA2	+	+	-	+	+	+	+	+	+	+	-	Facultative anaerobe
NS14-dB1	+	+	-	+	+	+	+	+	+	+	+	Facultative anaerobe
NS15-dA1	+	+	-	+	+	+	+	+	+	+	-	Facultative anaerobe
AY2-aA1	+	+	-	+	+	+	+	+	+	+	+	Facultative anaerobe
AY3-bA1	+	+	-	+	+	+	+	+	+	+	+	Facultative anaerobe
AY4-aA1	+	+	-	+	+	+	+	+	+	+	-	Facultative anaerobe
AY5-aB1	+	+	-	+	+	+	+	+	+	+	-	Facultative anaerobe
CN2-OA2	+	+	-	+	+	+	+	+	+	+	-	Facultative anaerobe
CN3-OB51	+	+	-	+	+	+	+	+	+	+	+	Facultative anaerobe
CN4-OB21	+	+	-	+	+	+	+	+	+	+	-	Facultative anaerobe
CN6-OB1	+	+	-	+	+	+	+	+	+	+	+	Facultative anaerobe
CN1-B21	+	+	-	+	+	+	+	+	+	+	-	Facultative anaerobe
CN5-B2	+	+	-	+	+	+	+	+	+	+	+	Facultative anaerobe
PCH1-2	+	+	-	+	+	+	+	+	+	+	+	Facultative anaerobe
PCH2-1	+	+	-	+	+	+	+	+	+	+	+	Facultative anaerobe
PCH6-2	+	+	-	+	+	+	+	+	+	+	+	Facultative anaerobe

4.3.3 Biochemical characteristics

The results of biochemical characteristics were shown in Table 11. All 51 isolates showed negative reactions to starch hydrolysis and slime formation. But catalase, gas production, arginine hydrolysis, nitrate reduction and acid from carbohydrates showed different reaction in Table 11.

Table 11. Biochemical characteristics of 51 isolates

Characteristics	1	2	3	4	5	6	7	8	9	10
Catalase	+	+	+	+	+	+	+	+	+	+
Gas production	+	+	+	+	+	+	+	+	+	+
Arginine hydrolysis	-	-	-	-	-	-	-	-	-	-
Nitrate reduction	+	+	+	+	+	+	+	+	+	+
Starch hydrolysis	-	-	-	-	-	-	-	-	-	-
Slime formation	-	-	-	-	-	-	-	-	-	-
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*	+	+	+	+	+	+	+	+	+	-

+, Test was positive or acid was produced; -, test was negative or no acid produced

*, Different reactions

1, CN1-OB13; 2, CN1-OB22; 3, CN2-OB4; 4, CN4-OA1; 5, CN1-B3; 6, CN1-A1; 7, CN5-A21; 8, CN5-B11;

9, CN5-B12; 10, CN6-B2

Table 11. Biochemical characteristics of 51 isolates (continued)

Characteristics	11	12	13	14	15	16	17	18	19	20
Catalase*	-	-	-	-	-	-	-	+	+	+
Gas production*	-	-	-	-	-	-	-	+	+	+
Arginine hydrolysis	-	-	-	-	-	-	-	-	-	-
Nitrate reduction	-	-	-	-	-	-	-	-	-	-
Starch hydrolysis	-	-	-	-	-	-	-	-	-	-
Slime formation	-	-	-	-	-	-	-	-	-	-
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*	-	-	+	-	-	+	-	-	-	-

+, Test was positive or acid was produced; -, test was negative or no acid produced

*, Different reactions

11, AY5-bA2; 12, AY5-bB6; 13, CN2-A1; 14, CN3-B1; 15, CN4-B1; 16, PCH4-3; 17, PCH6-3; 18, SRI-B1;

19, AY5-bB3; 20, AY5-bB4

Table 11. Biochemical characteristics of 51 isolates (continued)

Characteristics	21	22	23	24	25	26	27	28	29	30
Catalase*	+	-	-	-	-	-	-	-	-	-
Gas production*	+	-	-	-	-	-	-	-	-	-
Arginine hydrolysis*	-	+	+	+	+	+	+	+	+	+
Nitrate reduction*	-	+	-	-	-	-	-	-	-	-
Starch hydrolysis	-	-	-	-	-	-	-	-	-	-
Slime formation	-	-	-	-	-	-	-	-	-	-
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*	-	+	+	+	+	+	+	+	+	+

+, Test was positive or acid was produced; -, test was negative or no acid produced

*, Different reactions

21, CN2-B5; 22, SPI-B2; 23, SP5-A6; 24, SP5-B4; 25, SP8-B1; 26; SP10-B4; 27, NS2-A3; 28, AY2-aA1;

29, NS14-aA2; 30, NS15-aB2

Table 11. Biochemical characteristics of 51 isolates (continued)

Characteristics	31	32	33	34	35	36	37	38	39	40
Catalase	-	-	-	-	-	-	-	-	-	-
Gas production	-	-	-	-	-	-	-	-	-	-
Arginine hydrolysis*	+	+	+	+	+	+	+	-	-	-
Nitrate reduction	-	-	-	-	-	-	-	-	-	-
Starch hydrolysis	-	-	-	-	-	-	-	-	-	-
Slime formation	-	-	-	-	-	-	-	-	-	-
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*	+	+	+	+	+	-	-	+	-	-

+, Test was positive or acid was produced; -, test was negative or no acid produced

*, Different reactions

31, NS14-dB1; 32, CN3-OB51; 33, CN5-B2; 34, PCH1-2; 35, PCH2-1; 36, NP1-A2; 37, CN6-OB1; 38, SP8-A4; 39, AY5-aB1; 40, CN2-OA2

Table 11. Biochemical characteristics of 51 isolates (continued)

Characteristics	41	42	43	44	45	46	47	48	49	50	51
Catalase	-	-	-	-	-	-	-	-	-	-	-
Gas production	-	-	-	-	-	-	-	-	-	-	-
Arginine hydrolysis*	-	-	+	+	+	+	+	+	+	+	+
Nitrate reduction	-	-	-	-	-	-	-	-	-	-	-
Starch hydrolysis	-	-	-	-	-	-	-	-	-	-	-
Slime formation	-	-	-	-	-	-	-	-	-	-	-
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	-	-	-	-	-	-	-	-	-	-	-

+, Test was positive or acid was produced; -, test was negative or no acid produced

*, Different reaction

41, CN4-OB21; 42, CN1-B21; 43, SP4-B5; 44, NS2-A1; 45, NS2-B3; 46, NS3-B1; 47, NS15-bA2; 48, NS15-dA1; 49, AY3-bA1; 50, AY4-aA1; 51, PCH6-2

       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. Fifty-one isolates were divided 9 groups (Figure 14 and 15). Group I, II, III and IV were rods as Group V, VI, VII, VIII and IX were cocci and the results from characteristics of each group were shown in Table 12 to 20.
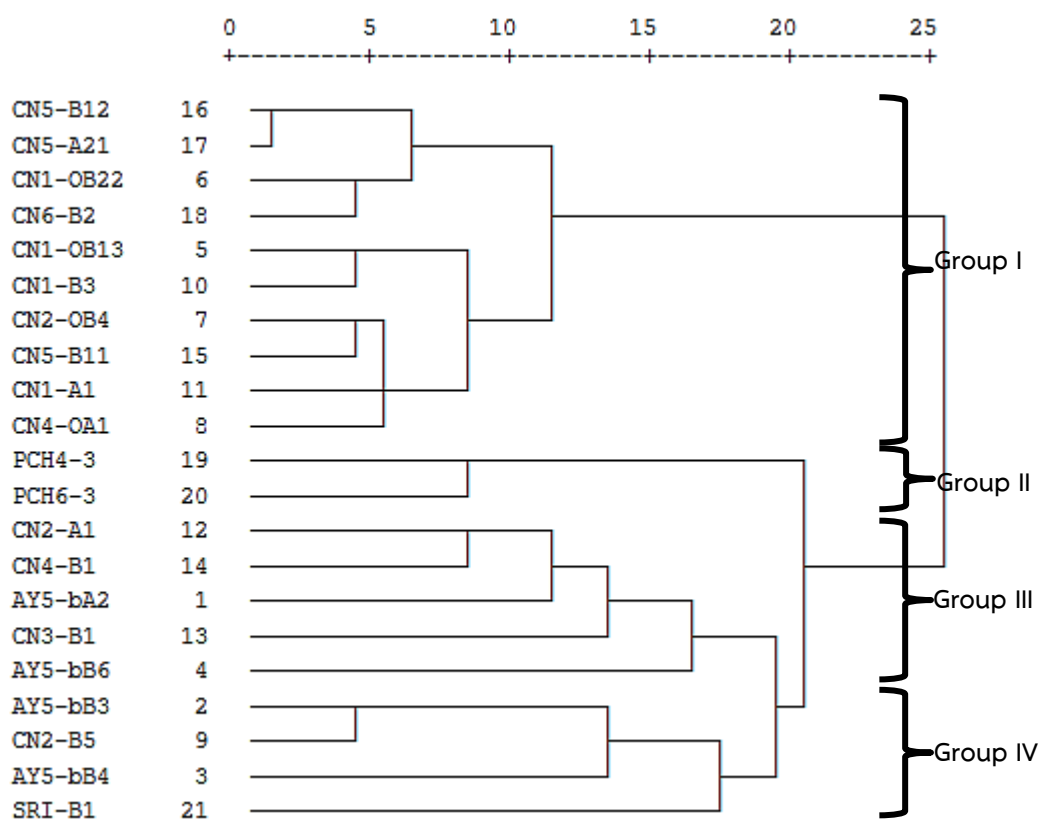
Figure 14. Dendrogram of the hierarchical cluster in SPSS program of rods based on morphological, physiological and biochemical characteristics
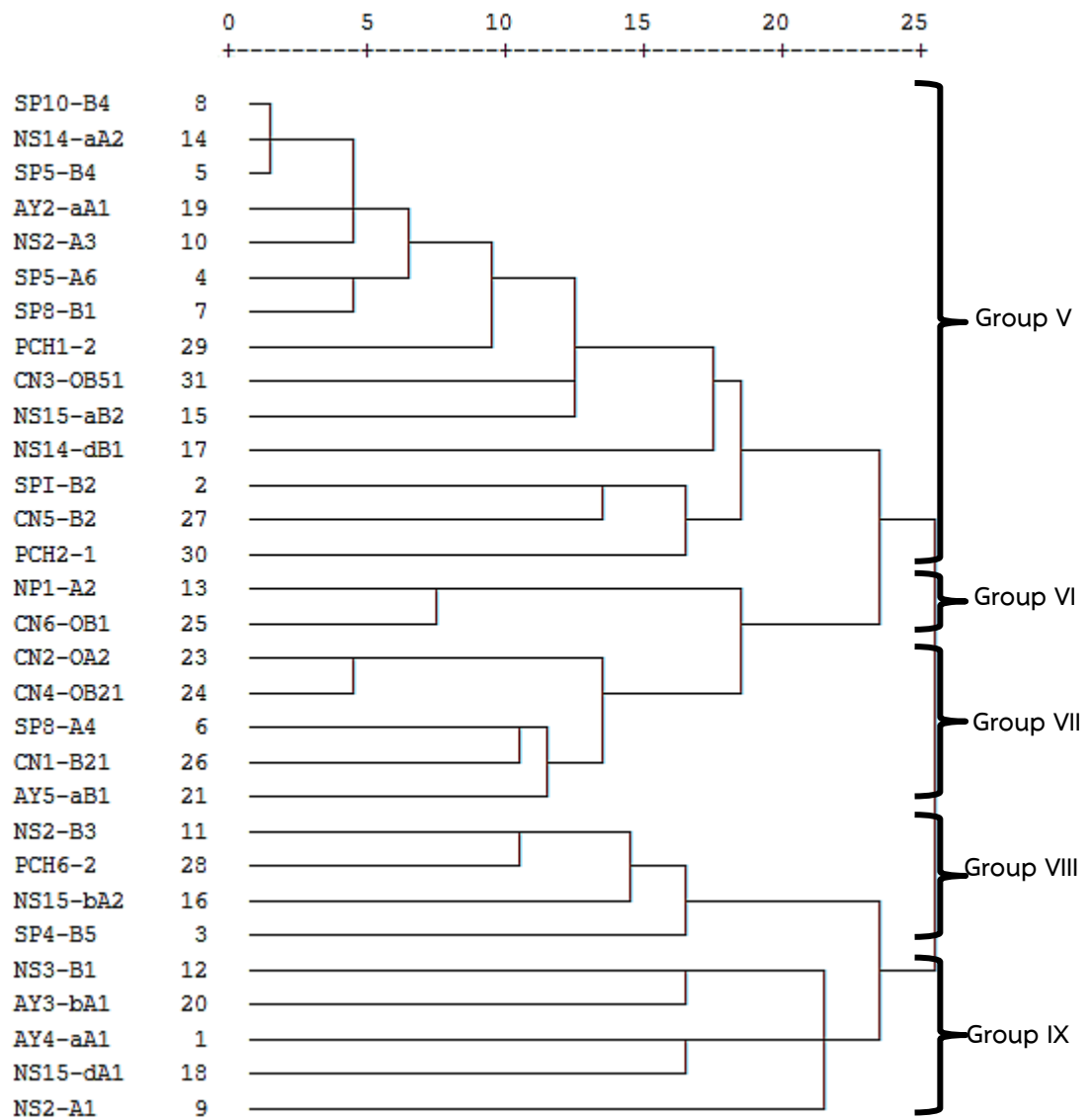
Figure 15. Dendrogram of the hierarchical cluster in SPSS program of cocci based on morphological, physiological and biochemical characteristics

Group I of isolates

Group I comprised of 10 isolates, namely, CN5-B12, CN5-A21, CN1-OB22, CN6-B2, CN1-OB13, CN1-B3, CN2-OB4, CN5-B11, CN1-A1 and CN4-OA1. Group I was screened from pig manure. All isolates were gram negative, rods and facultative anaerobes. Colonies were white, circular, entire, raised and translucent. Group I grew in the presence of 2% NaCl, at 20-45°C and pH 5-9. No growth was observed at 50°C and pH 3. They showed positive reactions to catalase, gas production and nitrate reduction. They showed negative reactions to arginine hydrolysis, starch hydrolysis and slime formation. Acid from L-arabinose, cellobiose, D-fructose, D-galactose, gluconate, glucose, lactose, maltose, D-mannitol, D-mannose, rhamnose, ribose, sorbitol and trehalose were positive reaction, while melibiose, ∞-methyl-D-glucoside, raffinose, sucrose were negative reaction. But growth in 6% NaCl and acid from D-amygdalin, rhamnose, salicin and D-xylose showed different reaction in Table 12.

Furthermore, the isolates in Group I could produce succinic acid in the range 34.614-49.036 g/l. Isolate CN1-OB13 produced the highest succinic acid of 49.036 g/l so isolate CN1-OB13 was representative for further study using 16S rRNA gene sequence analysis.

Table 12. Characteristics of Group I

Characteristics	1	2	3	4	5	6	7	8	9	10
Growth in 6% NaCl*	+	+	+	+	+	-	+	+	-	+
Growth at 45°C	+	+	+	+	+	+	+	+	+	+
Growth at 50°C	-	-	-	-	-	-	-	-	-	-
Catalase	+	+	+	+	+	+	+	+	+	+
Gas production	+	+	+	+	+	+	+	+	+	+
Arginine hydrolysis	-	-	-	-	-	-	-	-	-	-
Nitrate reduction	+	+	+	+	+	+	+	+	+	+
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*	+	+	+	-	+	+	+	+	+	+

+, Test was positive or acid was produced; -, test was negative or no acid produced

*, Different reactions

1, CN5-B12; 2, CN5-A21; 3, CN1-OB22; 4, CN6-B2; 5, CN1-OB13; 6, CN1-B3; 7, CN2-OB4; 8, CN5-B11;

9, CN1-A1; 10, CN4-OA1

Group II of isolates

Group II comprised of 2 isolates, namely, PCH4-3 and PCH6-3. Group II was screened from pig manure. All isolates were gram positive, rods and facultative anaerobes. Colonies were white, circular, entire, opaque and flat. Group II showed positive reactions to gas production and arginine hydrolysis. They negative reactions to catalase, nitrate reduction, starch hydrolysis and slime formation. They were grown in the presence of 2%, 6% NaCl, at 20-45°C and pH 5-7. No growth was observed at 50°C, pH 3 and pH 9. Acid from L-arabinose, D-fructose, D-galactose, glucose, lactose, maltose, D-mannose, melibiose, ribose and sucrose were positive reaction, while cellobiose, D-mannitol, ∞-methyl-D-glucoside, rhamnose, salicin, sorbitol, and trehalose were negative reaction. But acid from D-amygdalin, gluconate, raffinose and D-xylose showed different reaction in Table 13.

Furthermore, the isolates in Group II could produce succinic acid of 5.686 and 6.362 g/l. Isolate PCH6-3 produced the highest succinic acid of 6.362 g/l so isolate PCH6-3 was representative for further study using 16S rRNA gene sequence analysis.

Table 13. Characteristics of Group II

Characteristics	1	2
Growth in 6% NaCl	+	+
Growth at 45°C	+	+
Growth at 50°C	-	-
Catalase	-	-
Gas production	+	+
Arginine hydrolysis	+	+
Nitrate reduction	-	-
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*	+	-

+, Test was positive or acid was produced; -, test was negative or no acid produced

*, Different reactions

1, PCH4-3; 2, PCH6-3

Group III of isolates

Group III comprised of 5 isolates, namely, CN2-A1, CN4-B1, AY5-bA2, CN3-B1 and AY5-bB6. Group III was screened from tree bark and pig manure. All isolates were gram positive, short rods and facultative anaerobes. Colonies were white, circular, entire, opaque and convex. Group III showed positive reactions to arginine hydrolysis. They negative reactions to catalase, gas production, nitrate reduction, starch hydrolysis and slime formation. They were grown in the presence of 2%, 6% NaCl, at 20-37°C and pH 5-9. No growth was observed at 50°C and pH 3. Acid from cellobiose, D-fructose, D-galactose, gluconate, glucose, maltose, D-mannose, raffinose and salicin were positive reaction, while L-arabinose, D-mannitol, sorbitol, trehalose and D-xylose were negative reaction. But acid from D-amygdalin, lactose, ∞-Methyl-D-glucoside, rhamnose, ribose, sucrose and D-xylose showed different reactions in Table 14.

Furthermore, the isolates in Group III could produce succinic acid in the range 2.103-8.098 g/l. Isolate AY5-bA2 produced the highest succinic acid of 8.098 g/l so isolate AY5-bA2 was representative for further study using 16S rRNA gene sequence analysis.

Table 14. Characteristics of Group III

Characteristics	1	2	3	4	5
Growth in 6% NaCl	+	+	+	+	+
Growth at 45°C	+	+	+	+	+
Growth at 50°C	-	-	-	-	-
Catalase	-	-	-	-	-
Gas production	-	-	-	-	-
Arginine hydrolysis	-	-	-	-	-
Nitrate reduction	-	-	-	-	-
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*	+	-	-	-	-

+, Test was positive or acid was produced; -, test was negative or no acid produced

*, Different reactions

1, CN2-A1; 2, CN4-B; 3, AY5-bA2; 4, CN3-B1; 5, AY5-bB6

Group IV of isolates

Group IV comprised of 4 isolates, namely, AY5-bB3, AY5-bB4, CN2-B5 and SRI-B1. Group IV was screened from soil, tree bark and pig manure. All isolates were gram positive, rods, spore forming and microaerophiles. Colonies were yellow-gray, circular, irregular, opaque and flat. Group IV showed positive reactions to catalase and gas production. They negative reactions to arginine hydrolysis, nitrate reduction, starch hydrolysis and slime formation. They were grown in the presence of 2% NaCl, at 30-45°C and pH 5-7. No growth was observed at 20°C, pH 3 and 9. Acid from D-fructose, gluconate, glucose, maltose, were positive reaction, while D-amygdalin, L-arabinose, D-mannitol, D-mannose, melibiose, rhamnose, sorbitol, trehalose and D-xylose were negative reaction. But growth in 6% NaCl, at 50°C and acid from cellobiose, D-galactose, lactose, ∞-methyl-D-glucoside, ribose, salicin and sucrose showed different reaction in Table 15.

Furthermore, the isolates in Group IV could produce succinic acid in the range 1.447-3.157 g/l. Isolate AY5-bB4 produced the highest succinic acid of 3.157 g/l so isolate AY5-bB4 was representative for further study using 16S rRNA gene sequence analysis.

Table 15. Characteristics of Group IV

Characteristics	1	2	3	4
Growth in 6% NaCl*	+	+	+	-
Growth at 45°C	+	+	+	+
Growth at 50°C*	-	-	-	+
Catalase	+	+	+	+
Gas production	+	+	+	+
Arginine hydrolysis	-	-	-	-
Nitrate reduction	-	-	-	-
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	-	-	-	-

+, Test was positive or acid was produced; -, test was negative or no acid produced

*, Different reactions

1, AY5-bB3; 2, AY5-bB4; 3, CN2-B5; 4, SRI-B1

Group V of isolates

Group V comprised of 14 isolates, namely, SP10-B4, NS14-aA2, SP5-B4, AY2-aA1, NS2-A3, SP5-A6, SP8-B1, PCH1-2, CN3-OB51, NS15-aB2, NS14-dB1, SPI-B2, CN5-B2 and PCH2-1. Group V was screened from soil, chicken manure, cattle dung, bovine rumen and pig manure. All isolates were gram positive, cocci and facultative anaerobes. Colonies were white, circular, entire, opaque and convex. Group V showed positive reactions to arginine hydrolysis. They negative reactions to catalase, gas production, nitrate reduction, starch hydrolysis and slime formation. They were grown in the presence of 2%, 6% NaCl, at 20-50°C and pH 5-9. No growth was observed at pH 3. Acid from D-amygdalin, L-arabinose, cellobiose, D-fructose, D-galactose, gluconate, glucose, lactose, maltose, D-mannitol, D-mannose, melibiose, ribose, salicin, sucrose, trehalose and D-xylose were positive reaction, while ∞-Methyl-D-glucoside was negative reaction. But acid from sorbitol and rhamnose showed different reaction in Table 16.

Furthermore, the isolates in Group V could produce succinic acid in the range 1.938-50.411 g/l. Isolate PCH2-1 produced the highest succinic acid of 50.411 g/l so isolate PCH2-1 was representative for further study using 16S rRNA gene sequence analysis.

Table 16. Characteristics of Group V

Characteristics	1	2	3	4	5	6	7	8	9	10	11
Growth in 6% NaCl	+	+	+	+	+	+	+	+	+	+	+
Growth at 45°C	+	+	+	+	+	+	+	+	+	+	+
Growth at 50°C	+	+	+	+	+	+	+	+	+	+	+
Catalase	-	-	-	-	-	-	-	-	-	-	-
Gas production	-	-	-	-	-	-	-	-	-	-	-
Arginine hydrolysis	+	+	+	+	+	+	+	+	+	+	+
Nitrate reduction	-	-	-	-	-	-	-	-	-	-	-
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	+	+	+	+	+	+	+	+	+	+	+

+, Test was positive or acid was produced; -, test was negative or no acid produced

*, Different reactions

1, SP10-B4; 2, NS14-aA2; 3, SP5-B4; 4, AY2-aA1; 5, NS2-A3; 6, SP5-A6; 7, SP8-B1; 8, PCH1-2; 9, CN3-OB51;
10, NS15-aB2; 11, NS14-dB1

Table 16. Characteristics of Group V (continued)

Characteristics	12	13	14
Growth at 6% NaCl	+	+	+
Growth at 45°C	+	+	+
Growth at 50°C	+	+	+
Catalase	-	-	-
Gas production	-	-	-
Arginine hydrolysis	+	+	+
Nitrate reduction*	+	-	-
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	+	+	+

+, Test was positive or acid was produced; -, test was negative or no acid produced

*, Different reactions

12, SPI-B2; 13, CN5-B2; 14, PCH2-1

Group VI of isolates

Group VI comprised of 2 isolates, namely, NP1-A2 and CN6-OB1. They were screened from soil and pig manure. All isolates were gram positive, cocci and facultative anaerobes. Colonies were white, circular, entire, opaque and convex. GroupVI showed positive reactions to arginine hydrolysis. They negative reactions to catalase, gas production, nitrate reduction, starch hydrolysis and slime formation. They were grown in the presence of 2%, 6% NaCl, at 20-45°C and pH 5-9. No growth was observed at 50°C and pH 3. Acid from D-amygdalin, cellobiose, D-fructose, D-galactose, gluconate, glucose, maltose, D-mannitol, D-mannose, ribose, salicin, sorbitol and were positive reaction, while L-arabinose, melibiose, ∞-methyl-D-glucoside, raffinose, rhamnose and D-xylose were negative reaction. But acid from lactose, rhamnose, sucrose and trehalose showed different reaction in Table 17.

Furthermore, the isolates in Group VI could produce succinic acid in the range 47.651-49.415 g/l. Isolate NP1-A2 produced the highest succinic acid of 49.415 g/l so isolate NP1-A2 was representative for further study using 16S rRNA gene sequence analysis.

Table 17. Characteristics of Group VI

Characteristics	1	2
Growth at 6% NaCl	+	+
Growth at 45°C	+	+
Growth at 50°C	+	+
Catalase	-	-
Gas production	-	-
Arginine hydrolysis	+	+
Nitrate reduction	-	-
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	-	-

+, Test was positive or acid was produced; -, test was negative or no acid produced

*, Different reactions

1, NP1-A2; 2, CN6-OB1

Group VII of isolates

Group VII comprised of 5 isolates, namely, CN2-OA2, CN4-OB21, SP8-A4, CN1-B21 and AY5-aB1. They were screened from chicken manure, tree bark and pig manure. All isolates were gram positive, cocci and facultative anaerobes. Colonies were white, circular, entire, convex and opaque. They negative reactions to catalase, gas production, arginine hydrolysis, nitrate reduction, starch hydrolysis and slime formation. They were grown in the presence of 2%, 6% NaCl, at 20-45°C and pH 5-9. No growth was observed at 50°C and pH 3. Acid from D-amygdalin, cellobiose, D-fructose, D-galactose, gluconate, glucose, maltose, D-mannitol, D-mannose, melibiose, ribose, salicin and sorbitol were positive reaction, while raffinose was negative reaction. But acid from L-arabinose, lactose, melibiose, ∞-methyl-D-glucoside, rhamnose, sucrose and D-xylose showed different reaction in Table 18.

Furthermore, the isolates in Group VII could produce succinic acid in the range 5.170-48.892 g/l. Isolate CN2-OA2 produced the highest succinic acid of 48.892 g/l so isolate CN2-OA2 was representative for further study using 16S rRNA gene sequence analysis.

Table 18. Characteristics of Group VII

Characteristics	1	2	3	4	5
Growth at 6% NaCl	+	+	+	+	+
Growth at 45°C	+	+	+	+	+
Growth at 50°C	-	-	-	-	-
Catalase	-	-	-	-	-
Gas production	-	-	-	-	-
Arginine hydrolysis	-	-	-	-	-
Nitrate reduction	-	-	-	-	-
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*	-	-	+	-	-

+, Test was positive or acid was produced; -, test was negative or no acid produced

*, Different reactions

1, CN2-OA2; 2, CN4-OB21; 3, SP8-A4; 4, CN1-B21; 5, AY5-aB1

Group VIII of isolates

Group VIII comprised of 4 isolates, namely, NS2-B3, PCH6-2, NS15-bA2 and SP4-B5. They were screened from chicken manure, cattle dung, bovine rumen and pig manure. All isolates were gram positive, cocci and facultative anaerobes. Colonies were white, circular, entire, convex and opaque. They showed positive reactions to arginine hydrolysis. They negative reactions to catalase, gas production, nitrate reduction, starch hydrolysis and slime formation. They were grown in the presence of 2% and 6% NaCl, at 20-45°C and pH 5-9. No growth was observed at pH 3. Acid from D-amygdalin, cellobiose, D-fructose, D-galactose, glucose, lactose, maltose, D-mannose, melibiose, ribose, salicin, sucrose and trehalose were positive reaction, while L-arabinose, gluconate, D-mannitol, sorbitol and D-xylose were negative reaction. But growth at 50°C and acid from ∞-methyl-D-glucoside, raffinose and rhamnose showed difference reaction in Table 19.

Furthermore, the isolates in Group VIII could produce succinic acid in the range 40.861-52.028 g/l. Isolate NS15-bA2 produced the highest succinic acid of 52.028 g/l so isolate NS15-bA2 was representative for further study using 16S rRNA gene sequence analysis.

Table 19. Characteristics of Group VIII

Characteristics	1	2	3	4
Growth at 6% NaCl	+	+	+	+
Growth at 45°C	+	+	+	+
Growth at 50°C*	-	+	-	+
Catalase	-	-	-	-
Gas production	-	-	-	-
Arginine hydrolysis	+	+	+	+
Nitrate reduction	-	-	-	-
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	-	-	-	-

+, Test was positive or acid was produced; -, test was negative or no acid produced

*, Different reaction

1, NS2-B3; 2, PCH6-2; 3, NS15-bA2; 4, SP4-B5

Group IX of isolates

Group IX comprised of 5 isolates, namely, NS3-B1, AY3-bA1, AY4-aA1, NS15-dA1 and NS2-A1. They were screened from cattle dung, tree bark and bovine rumen. All isolates were gram positive, cocci and facultative anaerobes. They showed positive reactions to arginine hydrolysis. They negative reactions to catalase, gas production, nitrate reduction, starch hydrolysis and slime formation. They were grown in the presence of 2% and 6% NaCl, at 20-45°C and pH 5-9. No growth was observed at 50°C and pH 3. Acid from cellobiose, D-fructose, D-galactose, glucose, lactose, D-mannose, ribose, sucrose and D-xylose were positive reaction, while L-arabinose, ∞-methyl-D-glucoside, raffinose and D-xylose were negative reaction. But acid from D-amygdalin, gluconate, maltose, D-mannitol, melibiose, rhamnose, salicin and sorbitol showed different reaction in Table 20.

Furthermore, the isolates in Group IX could produce succinic acid in the range 29.143-50.862 g/l. Isolate NS15-dA1 produced the highest succinic acid of 50.862 g/l so isolate NS15-dA1 was representative for further study using 16S rRNA gene sequence analysis.

Table 20. Characteristics of Group IX

Characteristics	1	2	3	4	5
Growth at 6% NaCl	+	+	+	+	+
Growth at 45°C	+	+	+	+	+
Growth at 50°C	-	-	-	-	-
Catalase	-	-	-	-	-
Gas production	-	-	-	-	-
Arginine hydrolysis	+	+	+	+	+
Nitrate reduction	-	-	-	-	-
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	-	-	-	-	-

+, Test was positive or acid was produced; -, test was negative or no acid produced

*, Different reaction

1, NS3-B1; 2, AY3-bA1; 3, AY4-aA1; 4, NS15-dA1; 5, NS2-A1

4.3.4 16S rRNA gene sequence and phylogenetic analysis

Isolate CN1-OB13, PCH6-3, AY5-bA2, AY5-bB4, PCH2-1, NP1-A2, CN2-OA2, NS15-bA2 and NS15-dA1 which produced the highest succinic acid from each group, 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 shown in Figure 16.
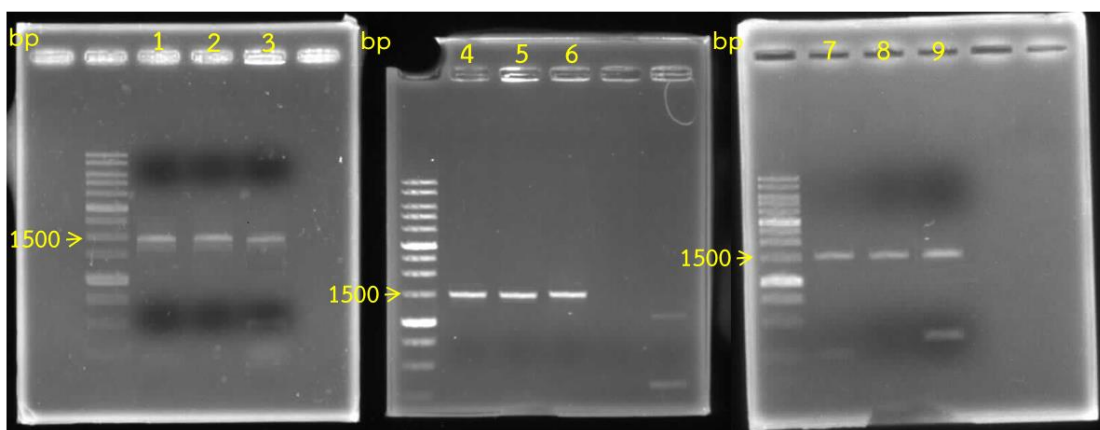

Figure 16. PCR products on 1% agarose gel based on 16S rRNA gene sequence
1, Isolate NP1-A2; 2, isolate PCH6-3; 3, isolate AY5-bA2; 4, isolate AY5-bB4;
5, isolate PCH2-1; 6, isolate CN1-OB13; 7, isolate CN2-OA2; 8, isolate NS15-dA1;
9, isolate NS15-bA2

Isolate NP1-A2, NS15-dA1, NS15-bA2, PCH2-1 and CN2-OA2 were closely related to *Enterococcus* sp., Isolate AY5-bA2 and PCH6-3 were closely related to *Lactobacillus* sp., Isolate AY5-bB4 was closely related to *Clostridium* sp. and isolate CN1-OB13 was closely related to *Escherichia* sp. (Figure 17). The results of percentages similarities were shown in Table 21.
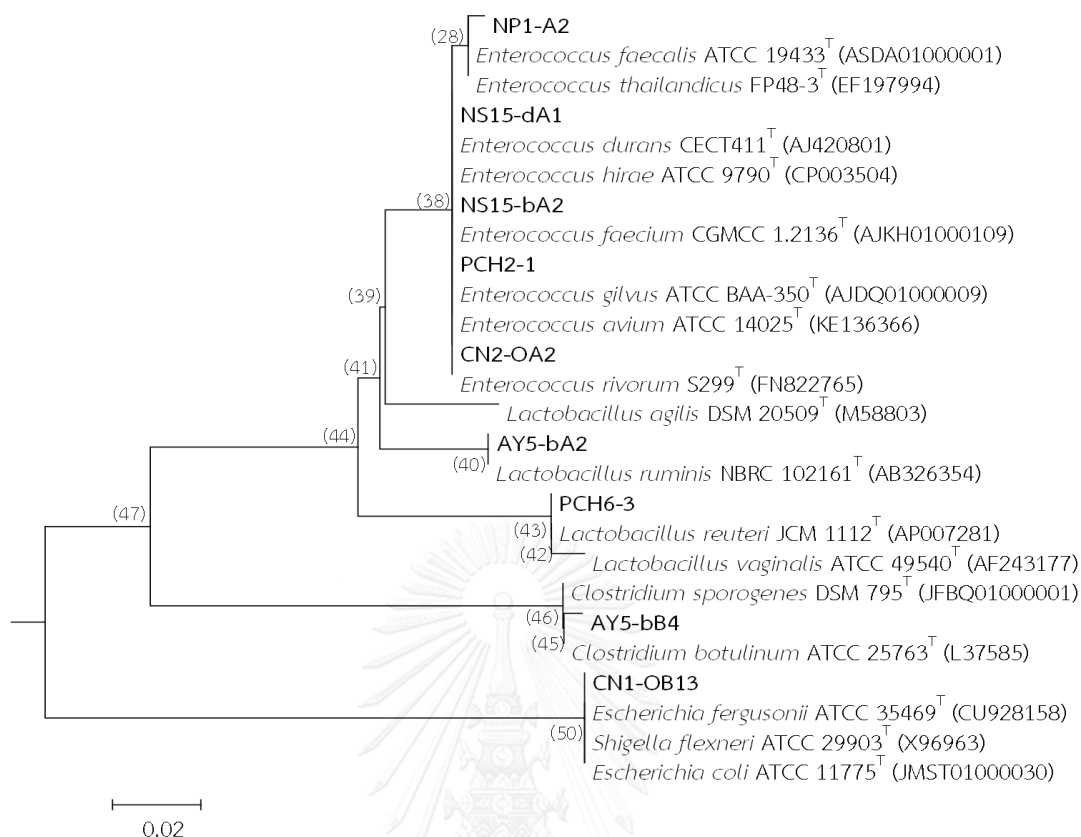
Figure 17. Neighbour-joining-tree showing the phylogenetic position of 9 isolates based on 16S rRNA gene sequence. Bar = 0.02 nucleotide substitution per site.

Table 21. Percentages similarities of isolate CN1-OB13, PCH6-3, AY5-bA2, AY5-bB4, PCH2-1, NP1-A2, CN2-OA2, NS15-bA2 and NS15-dA1

Group	Isolates	Species	% Similarity
I	CN1-OB13	*Escherichia fergusonii* ATCC 35469[T]	99.87
II	PCH6-3	*Lactobacillus reuteri* JCM 1112[T]	99.71
III	AY5-bA2	*Lactobacillus ruminis* NBRC 102161[T]	99.71
IV	AY5-bB4	*Clostridium sporogenes* DSM 795[T]	99.78
V	PCH2-1	*Enterococcus faecium* CGMCC 1.2136[T]	99.86
VI	NP1-A2	*Enterococcus faecalis* ATCC 19433[T]	99.86
VII	CN2-OA2	*Enterococcus avium* ATCC 14025[T]	100
VIII	NS15-bA2	*Enterococcus hirae* ATCC 9790[T]	100
IX	NS15-dA1	*Enterococcus durans* CECT 441[T]	99.89

The results of morphological, physiological and biochemical characteristics of isolates (CN1-OB13, PCH6-3, AY5-bA2, AY5-bB4, PCH2-1, NP1-A2, CN2-OA2, NS15-bA2 and NS15-dA1) were compared type strains (Table 22). Their results showed similar to type strains. Thus they according to the results of 16S rRNA gene sequence.

Table 22. Characteristics of 9 representative isolates from each group and type strains

Characteristics	1	T[1]	2	T[2]	3	T[3]	4	T[4]	5	T[5]	6	T[6]	7	T[7]	8	T[8]	9	T[9]
Growth in 2% NaCl	+	ND	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth in 6% NaCl	+	ND	+	+	+	+	+	ND	+	+	+	+	+	+	+	+	+	+
Growth at 20°C	+	+	+	+	+	+	+	ND	+	+	+	+	+	+	+	+	+	+
Growth at 30°C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth at 37°C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth at 45°C	+	ND	+	+	+	+	+	ND	+	+	+	+	+	+	+	+	+	+
Growth at 50°C	-	ND	-	-	-	-	+	ND	+	+	+	+	-	-	-	-	-	-
Catalase	+	+	+	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-
Gas production	+	+	+	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-
Starch hydrolysis	-	ND	-	-	-	-	-	ND	-	-	-	-	-	-	-	-	-	-
Slime formation	-	ND	-	-	-	-	-	ND	-	-	-	-	-	-	-	-	-	-
Arginine hydrolysis	-	-	-	-	-	-	-	-	+	+	+	+	-	-	+	+	+	+
Nitrate reduction	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-

+, Test was positive or acid was produced; -, test was negative or no acid produced; ND, No data

1, CN1-OB13; T[1], *Escherichia fergusonii* ATCC 35469[T] (Farmer et al., 1985); 2, PCH6-3; T[2], *Lactobacillus reuteri* JCM 1112[T] (Kandler et al., 1980); 3, AY5-bA2; T[3], *Lactobacillus ruminis* NBRC 102161[T] (Sharpe et al., 1973); 4, AY5-bB4; T[4], *Clostridium sporogenes* DSM 795[T] (Poehlein et al., 2015); 5, PCH2-1; T[5], *Enterococcus faecium* CGMCC 1.2136[T] (Manero and Blanch, 1999; Schleifer and Baltz, 1984); 6, NP1-A2; T[6], *Enterococcus faecalis* ATCC 19433[T] (Manero and Blanch, 1999; Schleifer and Baltz, 1984); 7, CN2-OA2; T[7], *Enterococcus avium* ATCC 14025[T] (Collins et al., 1984; Manero and Blanch, 1999); 8, NS15-bA2; *Enterococcus hirae* ATCC 9790[T] (Farrow and Collins, 1985); 9, NS15-dA1; T[9], *Enterococcus durans* CECT411[T] (Collins et al., 1984)

Table 22. Characteristics of 9 representative isolates from each group and type strains (continued)

Characteristics	1	T[1]	2	T[2]	3	T[3]	4	T[4]	5	T[5]	6	T[6]	7	T[7]	8	T[8]	9	T[9]
Acid from:																		
D-Amygdalin	-	ND	-	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+
L-Arabinose	+	+	+	+	-	-	-	-	+	+	-	-	+	+	-	-	-	-
Cellobiose	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+
D-Fructose	+	ND	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Galactose	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+
Gluconate	+	ND	-	+	+	+	+	ND	+	+	+	+	+	+	-	+	+	-
Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Lactose	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Mannitol	+	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-
D-Mannose	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+
Melibiose	-	-	-	-	+	-	-	-	+	+	+	-	+	+	+	-	-	+
∞-Methyl-D-glucoside	-	-	+	+	-	+	+	+	-	+	-	-	-	-	+	-	-	-
Raffinose	-	-	+	+	-	-	-	-	-	+	-	-	+	+	+	+	-	-
Rhamnose	+	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
Ribose	+	ND	+	+	+	+	+	ND	+	+	+	+	+	+	+	+	+	+
Salicin	+	+	-	-	+	+	-	-	+	+	+	+	+	+	+	+	+	+
Sorbitol	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
Sucrose	-	-	-	-	-	-	-	-	+	+	-	-	+	+	+	+	+	+
Trehalose	+	+	-	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+
D-Xylose	+	+	-	-	-	-	-	-	+	+	-	-	+	+	-	-	-	-

+, Test positive or acid was produced; -, test was negative or no acid produced; ND, No data

1, CN1-OB13; T[1], *Escherichia fergusonii* ATCC 35469[T] (Farmer et al., 1985); 2, PCH6-3; T[2], *Lactobacillus reuteri* JCM 1112[T] (Kandler et al., 1980); 3, AY5-bA2; T[3], *Lactobacillus ruminis* NBRC 102161[T] (Sharpe et al., 1973); 4, AY5-bB4; T[4], *Clostridium sporogenes* DSM 795[T] (Poehlein et al., 2015); 5, PCH2-1; T[5], *Enterococcus faecium* CGMCC 1.2136[T] (Manero and Blanch, 1999; Schleifer and Baltz, 1984); 6, NP1-A2; T[6], *Enterococcus faecalis* ATCC 19433[T] (Manero and Blanch, 1999; Schleifer and Baltz, 1984); 7, CN2-OA2; T[7], *Enterococcus avium* ATCC 14025[T] (Collins et al., 1984; Manero and Blanch, 1999); 8, NS15-bA2; *Enterococcus hirae* ATCC 9790[T] (Farrow and Collins, 1985); 9, NS15-dA1; T[9], *Enterococcus durans* CECT411[T] (Collins et al., 1984)

Isolates NS15-bA2 (*Enterococcus hirae* ATCC 9790[T]) was screened bovine rumen which produced the highest succinic acid of 0.87 g/g glucose. Similary, *Actinobacillus succinogenes* was screened from bovine rumen which produced yield of succinic acid of 0.83 g/g glucose (Guettler et al. 1999) and *Anaerobiospirilum succiniciproducens* was screened from bovine rumen which produced yield of succinic acid of 0.86 g/g glucose (Lee et al., 1999b). Moreover, chicken manure, tree bark and pig manure were new sources of succinic acid producing bacteria. These sources were no reported for screening of succinic acid bacteria from other research.

From the results of morphological, physiological and biochemical characteristics, most isolates were facultative anaerobic bacteria since the collected samples were exposed to air and strict anaerobe died in the presence of oxygen. Moreover, isolate CN1-OB13 (Group I) was *Escherichia fergusonii* ATCC 35469[T], isolate PCH6-3 (Group II) and AY5-bA2 (Group III) were *Lactobacillus* spp. and isolate PCH2-1, NP1-A2, CN2-OA2, NS15-bA2 and NS15-dA1 (Group V to IX) were *Enterococcus* spp. Most species were facultative anaerobe and tolerant to a wide range of environmental conditions, including extreme temperature (20-45°C), pH (5-9) and high sodium chloride concentrations. Isolate AY5-bB4 (Group IV) was *Clostridium* spp. comprised of a few isolate since this species was strictly anaerobic bacteria.

Escherichia fergusonii ATCC 35469[T] (isolate CN1-OB13; Group I) produced high succinic acid but it infected open wounds in humans and resisted the ampicillin, gentamicin and chloramphenicol (Mahapatra et al., 2005; Savini et al., 2008) (Table 23). Thus these species was not selected for further study optimization of succinic acid production.

Lactobacillus reuteri JCM 1112[T] (isolate PCH6-3; Group II), *Lactobacillus ruminis* NBRC 102161[T] (isolate AY5-bA2; Group III) and *Clostridium sporogenes* DSM 795[T] (isolate AY5-bB4; Group VI) produced a small amount of succinic acid thus they were not selected for further study optimization of succinic acid production (Table 23).

Enterococcus faecium CGMCC 1.2136[T] (isolate PCH2-1; Group V), *Enterococcus faecalis* ATCC 19433[T] (NP1-A2; Group VI) produced high succinic acid but these two species were reported of succinic acid production from other research (Table 23).

Kang et al. (Ryu et al., 1999) reported that *Enterococcus faecium* and *Enterococcus faecalis* gave the yield of succinic acid of 0.33 g/g glycerol and 0.82 g/g glycerol, respectively. Thus these species was not selected for further study optimization of succinic acid production.

    Enterococcus avium ATCC 14025^T (isolate CN2-OA2; Group VII) produced high succinic acid but it was intraabdominal infections and endocarditis in humans and resisted the vancomycin (Chao et al., 2013) (Table 23). Thus these species was not selected for further study optimization of succinic acid production.

    Enterococcus hirae ATCC 9790^T (isolate NS15-bA2; Group VIII) and *Enterococcus durans* CECT411^T (isolate NS15-dA1; Group IX) produced greater amounts of succinic acid than other groups (Table 23) and these two species were no reported of succinic acid production from other research.

    Therefore we selected isolate NS15-bA2 (*Enterococcus hirae* ATCC 9790^T) and NS15-dA1 (*Enterococcus durans* CECT411^T) for further study about succinic acid production.

Table 23. Summary of advantages and disadvantages of 9 groups

Isolates	Species	Succinic acid (g/l)	Advantages	Disadvantages	References
CN1-OB13 (Group I)	*Escherichia fergusonii* ATCC 35469[T]	49.036	- Produced high succinic acid	- Infected open wounds in humans - Resistant to the antibiotic ampicillin, gentamicin and chloramphenicol	(Mahapatra et al., 2005; Savini et al., 2008)
PCH6-3 (Group II)	*Lactobacillus reuteri* JCM 1112[T]	6.362	- Inhibit the growth of *E. coli* from affecting their hosts. - Prevention of gut infections - Probiotics	- Produced low succinic acid	(Kaneuchi et al., 1988)
AY5-bA2 (Group III)	*Lactobacillus ruminis* NBRC 102161[T]	8.098	- Suppressing antibiotic-resistant pathogens - Probiotics	- Produced low succinic acid	(O' Donnell et al., 2015; Yun et al., 2005)
AY5-bB4 (Group IV)	*Clostridium sporogenes* DSM 795[T]	3.157	- Having potential to be beneficial in cancer treatments aiming to reduce damage to non-cancerous cells within the host - Using tryptophan to synthesize 3-indolepropionic acid (IPA) as a potent antioxidant within the human body and brain	- Produced low succinic acid	(Chyan et al., 1999; Wikoff et al., 2009)

Table 22. Summary of advantages and disadvantages of 9 groups

Isolates	Species	Succinic acid (g/l)	Advantages	Disadvantages	References
PCH2-1 (Group V)	*Enterococcus faecium* CGMCC 1.2136[T]	50.411	- Used in fermenting foods such as cheese and vegetables - Probiotics - Produced high succinic acid	- Causing diseases such as neonatal meningitis and endocarditis. - Resistant to the antibiotic vancomycin	(Kang and Lee, 2005; Mascini et al., 2006)
NP1-A2 (Group VI)	*Enterococcus faecalis* ATCC 19433[T]	49.415	- Probiotics in chicken, pig and cattle feed to lower diarrhea in the animals - Produced high succinic acid	- Resistant to beta-lactam antibiotics because they contain penicillin-binding proteins (PBPs)	(Franz et al., 2011; Rocas et al., 2004)
CN2-OA2 (Group VII)	*Enterococcus avium* ATCC 14025[T]	48.892	- Produced high succinic acid	- Resistant to the antibiotic vancomycin - Intraabdominal infections and endocarditis in human	(Chao et al., 2013)
NS15-bA2 (Group VIII)	*Enterococcus hirae* ATCC 9790[T]	52.028	- Probiotics for animal nutrition - Produced high succinic acid	- Resistant to the antibiotic vancomycin	(Bourafa et al., 2015; Franz et al., 2011)
NS15-dA1 (Group IX)	*Enterococcus durans* CECT411[T]	50.862	- Antimicrobial activity - Antioxidant ability - Probiotics - Produced high succinic acid	- Resistant to the antibiotic vancomycin	(Cercenado et al., 1995; Franz et al., 2011)

4.4 Optimization of succinic acid production by the potential isolates

To know the optimization condition for the succinic acid production by the potential succinic acid producing bacteria (isolate NS15-dA1 and NS25-bA2), the following factors were studied; carbon sources, nitrogen sources, pH and temperature. Control was performed using a medium consisted of 60 g/l of glucose, 30 g/l of yeast extract, 2 g/l of urea and cultivated at pH 7.0, 37°C for 24 h.

4.4.1 Effect of glucose concentration

From Figure 18, when initial 30 g/l of glucose was used, glucose was consumed completely within 12 h. Similarly, 60 g/l of glucose was used, glucose was consumed completely within 24 h. Contrary, increasing glucose to 90 g/l, results in excessive carbon sources. At 24 h, the highest succinic acid concentrations of 50.014±0.5104 and 52.472±0.1129 g/l were obtained from isolate NS15-dA1 and isolate NS15-bA2, respectively (Table 23).

Table 24. Effect of glucose concentration on succinic acid production by isolate NS15-dA1 and NS15-bA2.

Isolates	Glucose (g/l)	Succinic acid (g/l)			
		12 h	24 h	36 h	48 h
NS15-dA1	30	26.502±0.1201[a]	27.130±0.0223	26.652±0.3412	26.592±0.6016
	60	40.105±0.6821	50.014±0.5104	49.933±0.0188	49.237±0.4530
	90	37.881±0.5111	46.961±0.0992	46.178±0.0469	46.329±0.7301
NS15-bA2	30	15.240±0.4602	25.934±0.1089	24.855±0.2201	24.974±0.0651
	60	21.938±0.3241	52.472±0.1129	51.370±0.0329	49.391±0.2465
	90	12.376±0.2099	47.231±0.0907	46.568±0.0421	46.324±0.7382

[a] Each value was an average of three parallel replicates and was presented as mean ± standard deviation
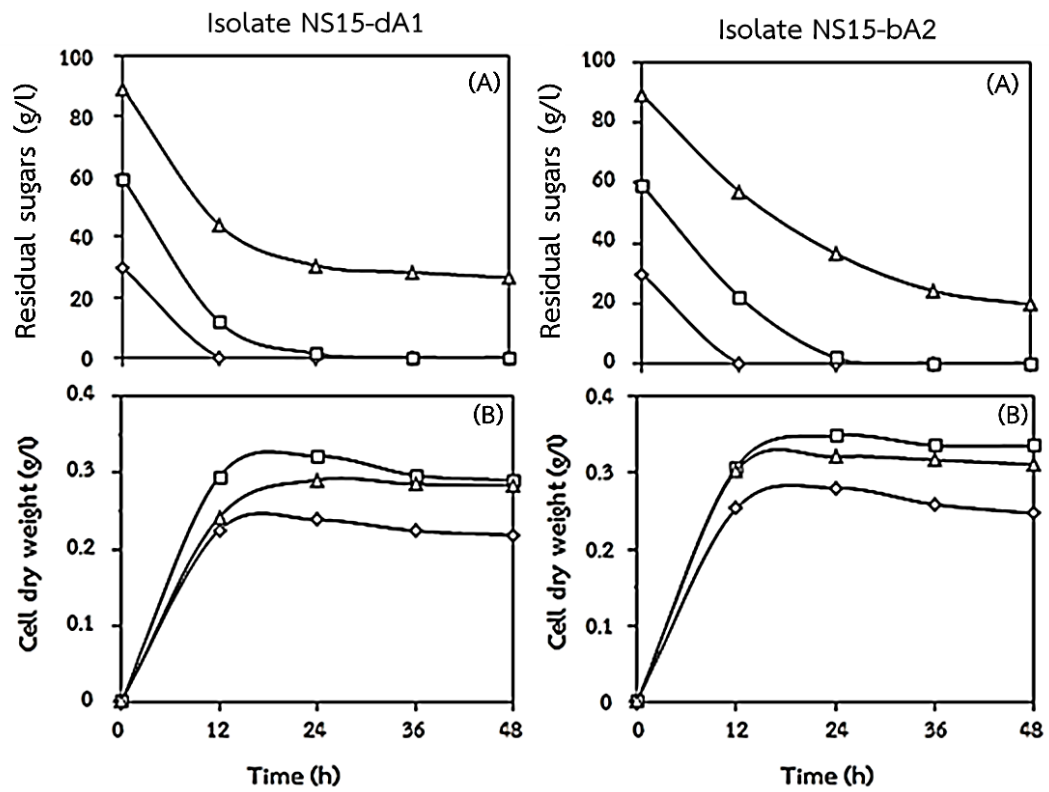
Figure 18. Time courses of residual sugars (A) and cell dry weight (B) when cultivated isolate NS15-dA1 and NS15-bA2 using glucose in the range 30-90 g/l as a carbon source. (◇ : 30 g/l; ▢ : 60 g/l; △ : 90 g/l)
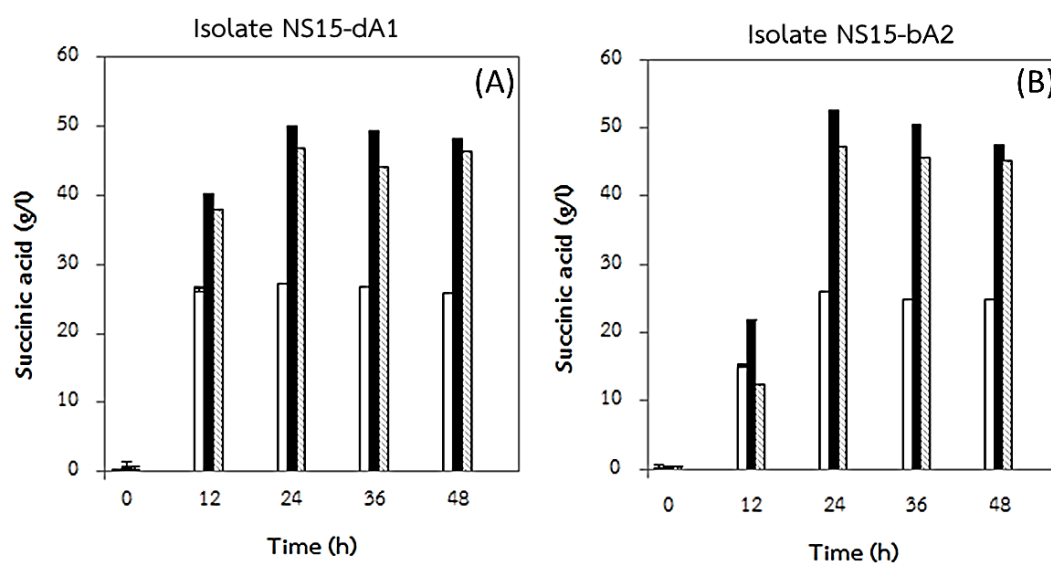
Figure 19. Succinic acid production by isolate NS15-dA1 (A) and NS15-bA2 (B) using glucose in the range 30-90 g/l as a carbon source. (□: 30 g/l; ■:60 g/l; ▨: 90 g/l) (The error bars in the figure indicated the standard deviations among three parallel replicates.)

Gonzáleza et al. (Gonzáleza et al., 2008) studied the effect of various glucose concentrations (10-100 g/l) on succinic acid production by *Actinobacillus succinogenes* ZT-130. They reported that 54.7 g/l of glucose was the optimum carbon source resulting in high succinic acid of 33.8 g/l. Lee et al. (Lee et al., 1999b) studied the effect of various glucose concentrations (0.5-40 g/l) on succinic acid production by *Anaerobiospirillum succiniciproducens*. They reported that 20g/l of glucose was the optimum carbon source resulting in high succinic acid of 33.8 g/l and a maximum specific growth rate of 0.350 h^{-1}.

Chen et al. (Chen et al., 2011) reported that high glucose concentration was due to the osmotic effects in the succinic acid fermentation. The cells growth and succinic acid concentration were also inhibited by high glucose concentration, which probably was due to substrate inhibition, a common issue in fermentation (Kotzamanidis et al., 2002).

Therefore, initial 60 g/l of glucose and 24 h of cultivation time were chosen for the further study.

4.4.2 Effect of different nitrogen sources

Among the nitrogen sources; yeast extract, peptone, tryptone, urea, KNO_3, $NHCl_4$, $(NH_4)_2SO_4$, $(NH_4)_2HPO_4$ and NH_4NO_3, yeast extract yielded the highest succinic acid of 49.962±0.0206 g/l and cell growth of 0.2945±0.0027 g/l by isolate NS15-dA1 (Table 24). That is optimum for both cell growth as well as succinic acid production. Yeast extract, apart from acting as nitrogen source also supplies vitamins and trace metals. Therefore, it affected the growth of the organism and thus increased succinic acid production. Furthermore, Kang et al. (Kang et al., 2000) reported that *Enterococcus faecalis* RKY1 used yeast extract as a nitrogen source and produced the highest succinic acid of 27 g/l. Lee et al. (Lee et al., 2002) observed that *M. succiniciproducens* MBEL55E could produce high succinic acid of 14 g/l when using yeast extract as a nitrogen source.

In case of isolate NS15-bA2 found to be the best nitrogen source Moreover trypetone is optimum for both cell growth of 0.3734±0.0096 g/l as well as succinic acid production of 53.892±0.0502 g/l (Table 24). Tryptone provides nitrogen, amino acids, and vitamins for the growing bacteria. Furthermore, Isar et al. (Isar et al., 2006) reported tryptone was the best nitrogen sources resulting in the production of 2.0 g/l of succinic acid by *Bacteroides fragilis*. Similarly from Agarwal et al. (Agarwal et al., 2007) among the various organic nitogen sources tested, tryptone maximally enhanced the production of both succinic acid (3.8 g/l) as well enzyme activity (PPCK, Phosphoenolpyruvate carboxykinase) by *Enterococcus flavescens*.

Therefore, yeast extract was chosen for the further study by isolate NS15-dA1 and tryptone was chosen for the further study by isolate NS15-bA2.

Table 25. Effect of nitrogen sources on succinic acid production and cell dry weight by isolate NS15-dA1 and NS15-bA2.

Nitrogen sources	Isolate NS15-dA1		Isolate NS15-bA2	
	Succinic acid (g/l)	Cell dry weight (g/l)	Succinic acid (g/l)	Cell dry weight (g/l)
Control	50.133±0.6773[a]	0.2882±0.0152	52.044±0.4178	0.2841±0.0133
Yeast extract	49.962±0.4025	0.2946±0.0279	52.432±0.9788	0.3067±0.9788
Peptone	40.811±0.2650	0.2248±0.0180	39.201±1.0186	0.2345±0.0235
Tryptone	42.239±0.2096	0.2307±0.0240	53.892±0.0502	0.3734±0.0098
Urea	3.555±0.1559	0.0098±0.0006	1.070±0.0594	0.1024±0.0002
KNO_3	7.262±0.1191	0.0492±0.0037	17.461±0.0645	0.0495±0.0012
$NHCl_4$	0.829±0.0716	0.0101±0.0006	0.774±0.0155	0.0105±0.0006
$(NH_4)_2SO_4$	1.632±0.0269	0.0103±0.0006	1.332±0.2014	0.0107±0.0003
$(NH_4)_2HPO_4$	6.765±0.1894	0.0407±0.0055	14.693±1.0586	0.0423±0.0043
NH_4NO_3	2.087±0.0373	0.0158±0.0081	0.751±0.4179	0.0118±0.0013

[a] Each value was an average of three parallel replicates and was presented as mean ± standard deviation
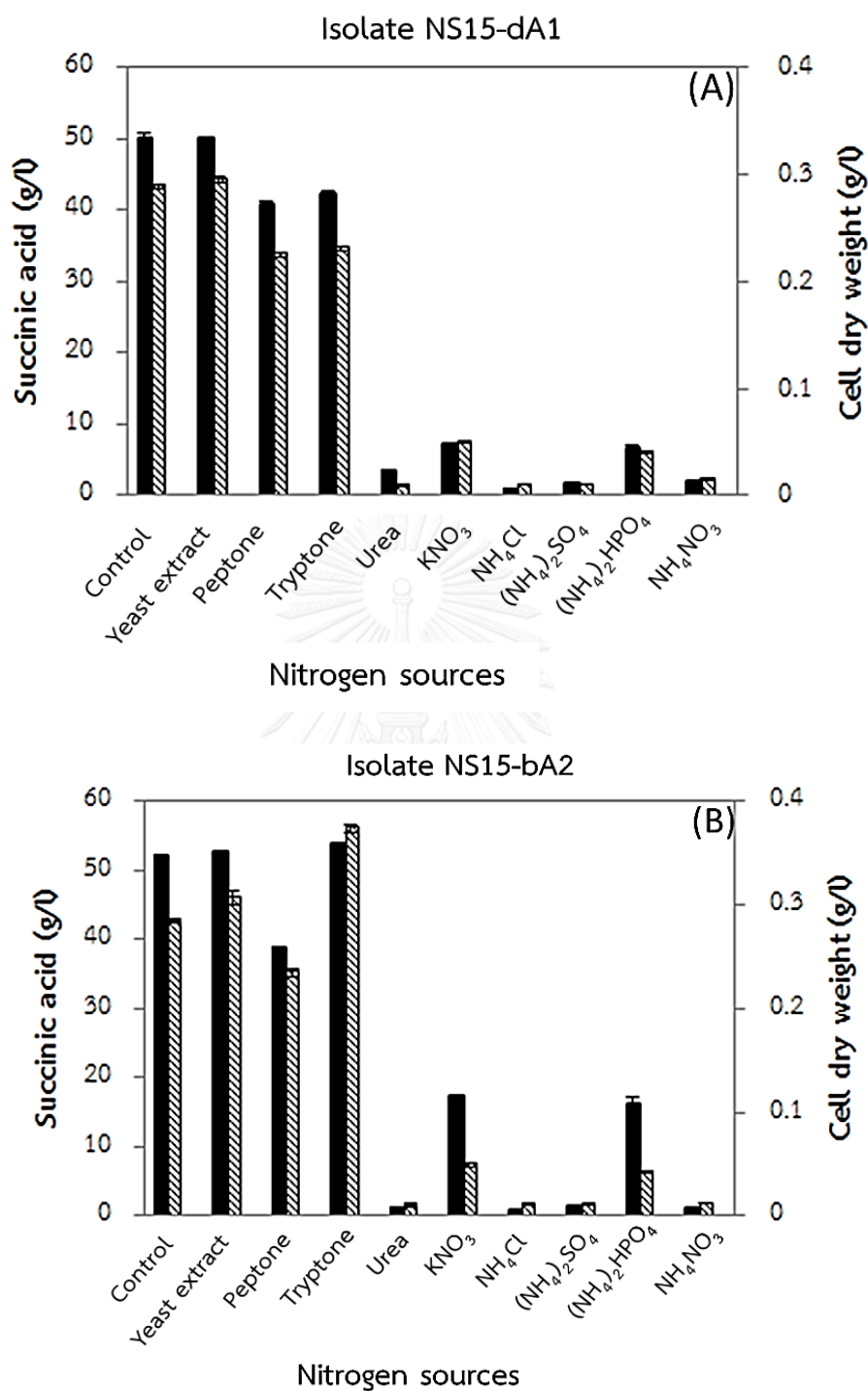
Figure 20. Effect of different nitrogen sources on succinic acid production and cell dry weight by isolate NS15-dA1 and NS15-bA2. (■: Succinic acid; ▨: CDW)

(The error bars in the figure indicated the standard deviations among three parallel replicates.)

4.4.3 Effect of initial pH

The effect of different level of initial pH 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0 on succinic acid production was shown in Figure 21. At initial pH 7.0 was found to be an optimum initial pH which result the highest of succinic acid of 50.703±0.4734 g/l and cell growth of 0.3116±0.0020 g/l by NS15-dA1 (Table 25). Similarly, initial pH 7.0 was found to be an optimum initial pH which result the highest of succinic acid of 53.212±0.2029 g/l and cell growth of 0.3419±0.0109 g/l by NS15-bA2 (Table 25).

Wee et al. (Wee et al., 2002) reported that pH 7-8 is the optimum pH for succinic acid production by *Enterococcus faecalis*. Similar, Ryu et al. (Ryu et al., 1999) reported at pH 7, *Enterococcus faecalis* could produce the maximum succinic acid of 65.9 g/l. Moreover Lee et al. (Lee et al., 2002) observed that *M. succiniciproducens* MBEL55E, a succinic acid producer grew well in the pH range of 6.0-7.5. Most probable reason could be that the activity of the enzyme responsible for succinic acid production was maximally induced within a given pH range or value. Similar Samuelov et al. (Samuelov et al., 1991) reported the influence of pH on the level of fermentative enzyme responsible for end-product formation in the cells grown at pH 6.2, both the PPCK (Phosphoenolpyruvate carboxykinase) activity and succinic acid production reached high value.

Therefore, pH 7.0 was chosen for the further study by isolate NS15-dA1 and NS15-bA2.

Table 26. Effect of initial pH on succinic acid production and cell dry weight by isolate NS15-dA1 and NS15-bA2

Initial pH	Isolate NS15-dA1		Isolate NS15-bA2	
	Succinic acid (g/l)	Cell dry weight (g/l)	Succinic acid (g/l)	Cell dry weight (g/l)
Control	51.016±0.2658[a]	0.2958±0.0001	52.633±1.0178	0.3264±0.0024
5.0	12.822±0.0798	0.1997±0.0286	21.948±0.2640	0.0771±0.0035
5.5	31.167±0.3424	0.2111±0.0134	37.136±0.4864	0.2246±0.0060
6.0	40.286±0.2932	0.1844±0.0162	43.860±0.5238	0.1962±0.0010
6.5	46.171±0.2081	0.2590±0.0051	48.170±0.2616	0.2756±0.0011
7.0	50.703±0.4734	0.3116±0.0129	53.212±0.2029	0.3419±0.0108
7.5	48.484±0.2918	0.2632±0.0025	47.761±0.4023	0.2408±0.0015
8.0	48.917±0.1367	0.1891±0.0295	43.557±0.4836	0.2012±0.0070

[a] Each value was an average of three parallel replicates and was presented as mean ± standard deviation
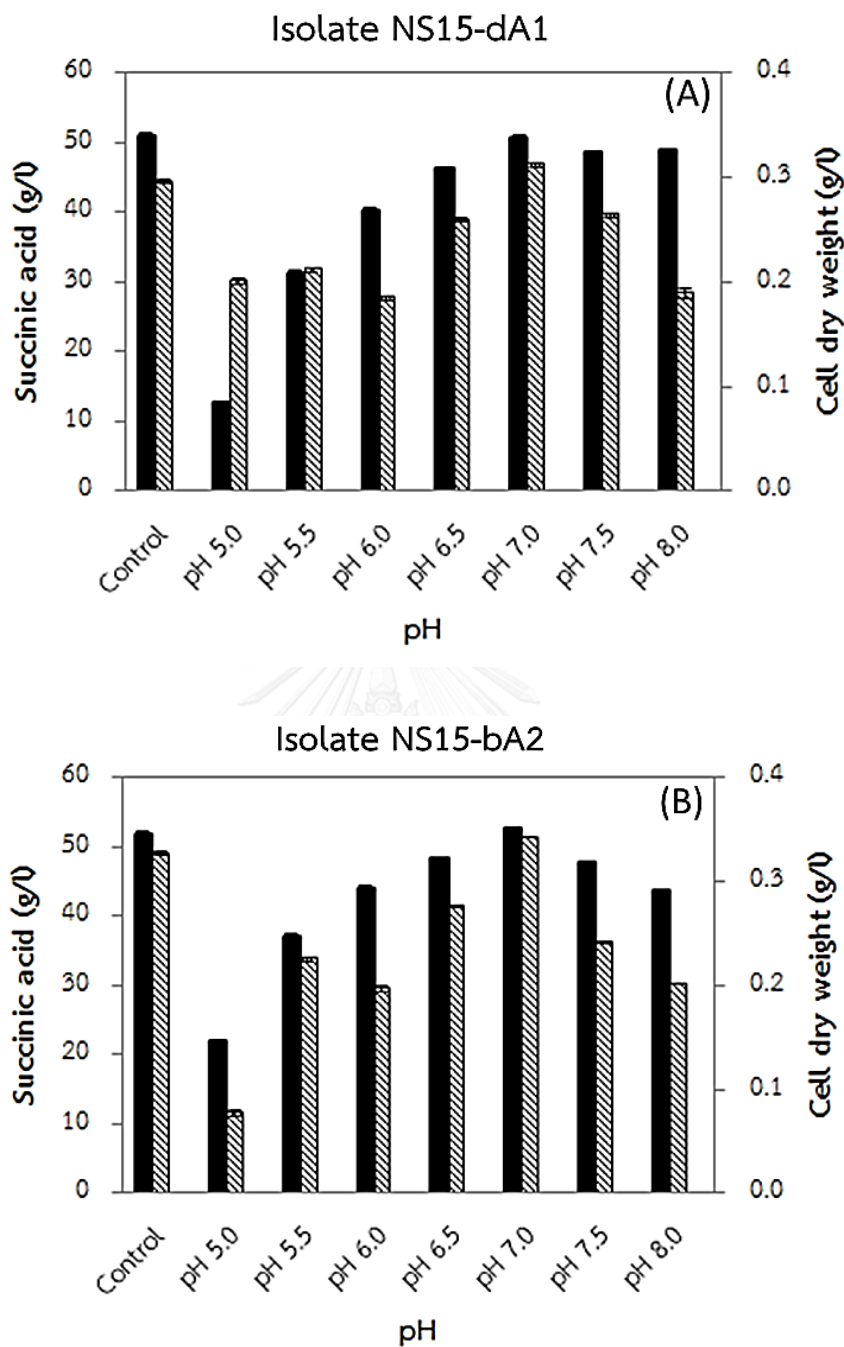
Figure 21. Effect of initial pH on succinic acid production and cell dry weight by

isolate NS15-dA1 and NS15-bA2. (■: Succinic acid; ▨: CDW)

(The error bars in the figure indicated the standard deviations among three parallel replicates.)

4.4.4 Effect of temperature

The effect of different temperature of 35, 37 and 39℃ on succinic acid production was shown in Figure 22. At 37℃ was found to be the optimum temperature and highest succinic acid of 51.692±0.1707 g/l and cell growth of 0.3099±0.0003 g/l were obtained by isolate NS15-dA1. Similarly, at 37℃ was found to be the optimum temperature and highest succinic acid of 53.051±0.3538 g/l and cell growth of 0.3463±0.0165 g/l were obtained by isolate NS15-bA2 (Table 26).

The results of the effect of temperature was similar as phenotypic characterization, isolate NS15-dA1 and NS15-bA2 showed well cell growth in the range 20-45℃. Isar et al. (Isar et al., 2006) reported at 37±2℃ was the optimal temperature for succinic acid production from *Bacteroides fragilis*. Macy et al. (Macy et al., 1978) observed that 37±1℃ was the most suitable temperature of succinic acid production form *Bacteroides fragilis*. Lee et al. (Lee et al., 1999a) reported that at 37±1℃ was the optimal temperature for growth and succinic acid production by *Anaerobiospirillum succiniciproducens*. Huh et al. (Huh et al., 2004) reported also *Mannheimia succiniciproducens* produced maximum succinic acid at 37℃. The probable reason, at 37℃ may be optimal for enzyme activity.

Therefore, 37℃ was chosen for the further study by isolate NS15-dA1 and NS15-bA2.

Table 27. Effect of temperature on succinic acid production and cell dry weight by isolate NS15-dA1 and NS15-bA2

| Temperature | Isolate NS15-dA1 | | Isolate NS15-bA2 | |
	Succinic acid (g/l)	Cell dry weight (g/l)	Succinic acid (g/l)	Cell dry weight (g/l)
Control	51.526±0.2512[a]	0.3077±0.0013	51.061±0.2658	0.3374±0.0019
35	50.730±0.2452	0.2887±0.0077	42.693±0.3539	0.2988±0.0034
37	51.692±0.1707	0.3099±0.0003	53.051±0.3538	0.3463±0.0165
39	49.085±0.6682	0.2692±0.0078	45.294±0.3180	0.3027±0.0013

[a] Each value was an average of three parallel replicates and was presented as mean ± standard deviation
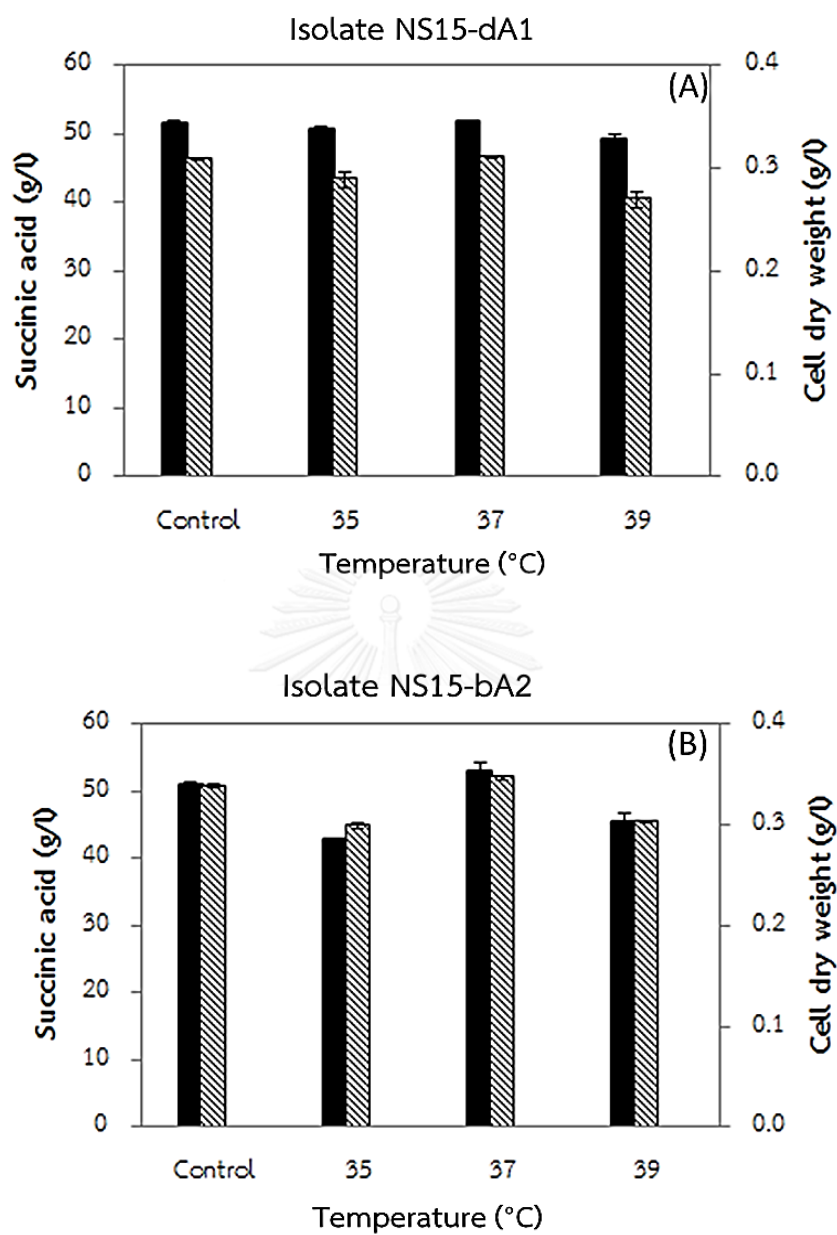
Figure 22. Effect of temperature on succinic acid production and cell dry weight by

isolate NS15-dA1 and NS15-bA2. (■: Succinic acid; ▨: CDW)

(The error bars in the figure indicated the standard deviations among three parallel replicates.)

Therefore, the optimum condition for succinic acid production by isolate NS15-dA1 and NS15-bA2 were 60 g/l of glucose, 30 g/l of yeast extract (for isolate NS15-dA1), 30 g/l of tryptone (for isolate NS15-bA2) at initial pH 7.0 and 37°C. The results of cell dry weight (g/l), succinic acid concentration (g/l), yield (g/g glucose) and productivity (g/l/h) were shown in Table 27.

Table 28. Summary of high succinic acid production under optimum condition

Isolates	Glucose (g/l)	Cell dry weight (g/l)	Succinic acid (g/l)	Yield (g/g glucose)	Productivity (g/l/h)
NS15-dA1	60	0.310±0.0003[a]	51.692±0.1707	0.861±0.0028	2.154±0.0196
NS15-bA2	60	0.346±0.0165	53.051±0.3538	0.884±0.0188	2.210±0.0305

[a] Each value was an average of three parallel replicates and was presented as mean ± standard deviation

Yield of succinic acid production by isolate NS15-dA1 (*Enterococcus hirae* ATCC 9790[T]) and NS15-bA2 (*Enterococcus durans* CECT411[T]) compared with other species (Table 28). These two isolates were potential for succinic acid production.

Table 29. Performances of succinic acid production by bacteria

Bacteria	Succinic acid (g/l)	Yield (g/g glucose)	Productivity (g/l/h)	References
L. casei	14.8	0.74	0.31	(Kaneuchi et al., 1988)
L. reuteri	13.4	0.67	0.28	(Kaneuchi et al., 1988)
E. faecalis	24.6	1.23	1.02	(Ryu et al., 1999)
E. flavescens	2.8	0.14	0.47	(Kang et al., 2000)
E. faecium	16.7	0.84	0.69	(Ryu et al., 1999)
E. hirae (isolate NS15-bA2)	53.1	0.89	2.21	This study
E. durans (isolate NS15-dA1)	51.7	0.86	2.15	This study

CHAPTER V

CONCLUSION

In this study, the screening, characterization and optimization of succinic acid producing bacteria isolated in Thailand were studied. Succinic acid producing bacteria were screened from 6 sources and 6 provinces in Thailand. A total of 310 isolates, only 51 isolates could produce succinic acid as they exhibited clear zone around colonies on selective medium plate. They were analyzed by TLC method and they showed positive result by this method. Then they were confirmed to have succinic acid producing ability using HPLC. Succinic acid concentrations from these isolates were in the range of 0.553-52.028 g/l. Then 51 isolates were characterized including morphological, physiological and biochemical characteristics. Twenty-one isolates were rods and 30 isolates were cocci. They were divided into 9 groups based on morphological, physiological and biochemical characteristics. Nine isolates were selected for study 16S rRNA gene sequence and phylogenetic analysis because they produced the highest succinic acid from each group. Isolate CN1-OB13 (Group I) was closely related to *Escherichia fergusonii* ATCC 35469[T] (similarity percentage values of 99.87). Isolate PCH6-3 (Group II) was closely related to *Lactobacillus reuteri* JCM 1112[T] (similarity percentage values of 99.71). Isolate AY5-bA2 (Group III) was closely related to *Lactobacillus ruminis* NBRC 102161[T] (similarity percentage values of 99.71). Isolate AY5-bB4 (Group IV) was closely related to *Clostridium sporogenes* DSM 795[T] (similarity percentage values of 99.78). Isolate PCH2-1 (Group V) was closely related to *Enterococcus faecium* CGMCC 1.2136[T] (similarity percentage values of 99.86). Isolate NP1-A2 (Group VI) was closely related to *Enterococcus faecalis* ATCC 19433[T] (similarity percentage values of 99.86). Isolate CN2-OA2 (Group VII) was closely related to *Enterococcus avium* ATCC 14025[T] (similarity percentage values of 100). Isolate NS15-bA2 (Group VIII) was closely related to *Enterococcus hirae* ATCC 9790[T] (similarity percentage values of 100). Lastly, isolate NS15-dA1 (Group IX) was closely related to *Enterococcus durans* CECT411[T] (similarity percentage values of 99.89).

Isolate NS15-bA2 and isolate NS15-dA1 were selected to study optimization of succinic acid production because these isolates could produce the highest succinic acid of 52.028 and 50.862 g/l from 60 g/l of glucose, respectively.

The optimum conditions on succinic acid production by isolate NS15-dA1 and NS15-bA2 was 60 g/l of glucose as a carbon source, 30 g/l of yeast extract (for isolate NS15-dA1) and 30 g/l of tryptone (for isolate NS15-bA2) as a nitrogen source, at pH 7.0 and 37°C. The highest succinic acid of 51.692±0.1707 g/l and 53.051±0.3538 g/l were obtained, respectively.

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APPENDIX

APPENDIX A

Culture media, Reagent and Buffer preparation

1. Modified Gifu anaerobic medium (GAM; Nissui Pharmaceutical)

Peptic digest of animal tissue	10	g
Papaic digest of soyabean meal	3	g
Proteose peptone	10	g
Digested serum	13.5	g
Yeast extract	5	g
Beef extract	2.2	g
Liver extract	1.2	g
Dextrose	3	g
KH_2PO_4	2.5	g
NaCl	3	g
Starch, Soluble	5	g
L-cysteine HCl	0.3	g
$C_2H_3O_2SNa$	0.3	g
Distilled water	1000	ml
pH	7.3	

Dissolved and adjusted pH to 7.3 with HCl. Medium was sterilized by autoclave at 121°C for 15 min.

2. MRS broth (MRS; de Man, Rogosa and Sharpe)

Enzymatic digest of animal tissue	10	g
Beef extract	10	g
Yeast extract	5	g
Dextrose	20	g
Sodium acetate	5	g
Polysorbate 80	1	g
Potassium Phosphate	2	g
Ammonium citrate	2	g
Magnesium sulfate	0.1	g
Manganese sulfate	0.05	g
Distilled water	1000	ml
pH	6.5	

Dissolved and sterilized by autoclave at 121°C for 15 min.

3. Gram stain

Colony was smear on a clean slide. Slide was fixed by passing through flame. The smear was covered with crystal violet solution for 30 s, next washed with water and drained. Then the smear was covered with iodine solution for 30 s and washed with water and drained. 95% ethanol was used decolorized and wash with water. After that safranin solution was flooded and allowed to counter stain about 30 s. Blot slide was dried and examined under oil immersion (100X).

4. Endospore stain

Colony was smear on a clean slide. Slide was fixed by passing through flame. The smear was covered with malachite green solution and heated for 5 minutes till it starts to evaporate. Then the slide was cooled to room temperature for 2 minutes. Next washed with water and drained. After that safranin solution was flooded and allowed to counter stain about 2 min. Blot slide was dried and examined under oil immersion (100X).

5. Catalase test

5.1 H_2O_2 solution

H_2O_2	3	ml

Dissolved and adjusted volume to 100 ml with distilled water.

6. Arginine hydrolysis

6.1 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.

7. Nitrate reduction test

7.1 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℃ for 15 min.

7.2 Sulfanilic acid solution

Sulphanilic acid	0.8	g
5 N Acetic acid	100	ml

Dissolved and gentle heating in a fume hood.

7.3 *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.

8. Starch hydrolysis

8.1 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℃ for 15 min and poured into plate.

8.2 Iodine reagent

Iodine solution	10	ml

Dissolved and adjusted volume to 20 ml with distilled water.

9. Slime formation

9.1 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℃ for 15 min and poured into plate.

10. Acid from carbohydrates

10.1 Medium 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.

10.2 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.

11. Polymerase chain reaction (PCR)

11.1 6X DNA loading dye

Tris (hydroxymethyl) aminomethane	1.21	g
Bromophenol blue	0.03	g
Xylene cyanol FF	0.03	g
Glycerol	60	ml
Sodium laureth sulfate (SLES)	1	g
Ethylenediaminetetraacetic acid	37.22	g

Dissolve Tris (hydroxymethyl) aminomethane with distilled water and adjust pH to 8.0 with HCl. After dissolve EDTA and glycerol, pH was adjust to 7.6 with HCl and bromophenol blue and xylene cyanol FF were added. Then the volume was brought up to 100 ml with distilled water. 6X DNA loading dye was stored at 4°C.

11.2 1% (w/v) Agarose gel

Agarose	1	g
10X TAE buffer	100	ml

Dissolved and heated by microwave until agarose gels were dissolved well. After agarose solution cool down to about 45°C, poured the agarose solution into the case and leave to solidify at room temperature.

11.3 10X Tris acetate- ethylenediaminetetraacetic acid (TAE) buffer

Tris (hydroxymethyl) aminomethane	48.4	g
Acetic acid (glacial)	11.4	g
Ethylenediaminetetraacetic acid	3.7	g
NaOH	1	g

Dissolved Tris (hydroxymethyl) aminomethane and NaOH with distilled water. EDTA was added and mixed thoroughly, and then acetic acid was added brought up to volume to 1000 ml with distilled water. Diluted 10 times before used.

11.4 Ethidium bromide solution

Ethidium bromide 50 mg

Dissolved and adjusted volume to 100 ml with distilled water. The solution was stored in the amble bottle.

12. DNS reagent

3,5-dinitrosalicylic acid 1 g

Sodium potassium tartrate 30 g

2M NaOH 20 ml

Distilled water 80 ml

APPENDIX B

Standard curve

1. Standard curve of glucose by DNS assay

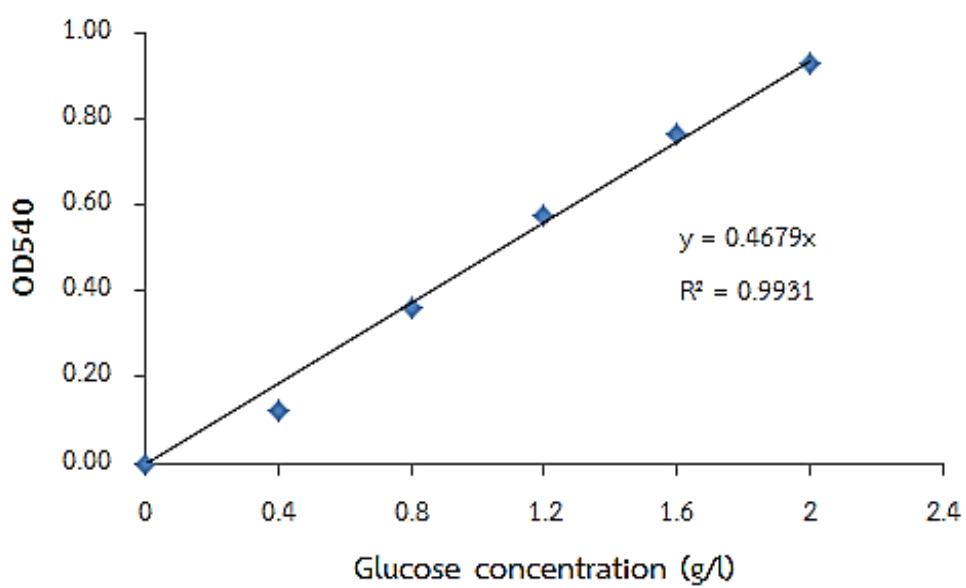**Figure B1.** Standard curve of glucose concentration

Equation; Y=0.4679X

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

2. **Standard curve of cell dry weight (CDW)**

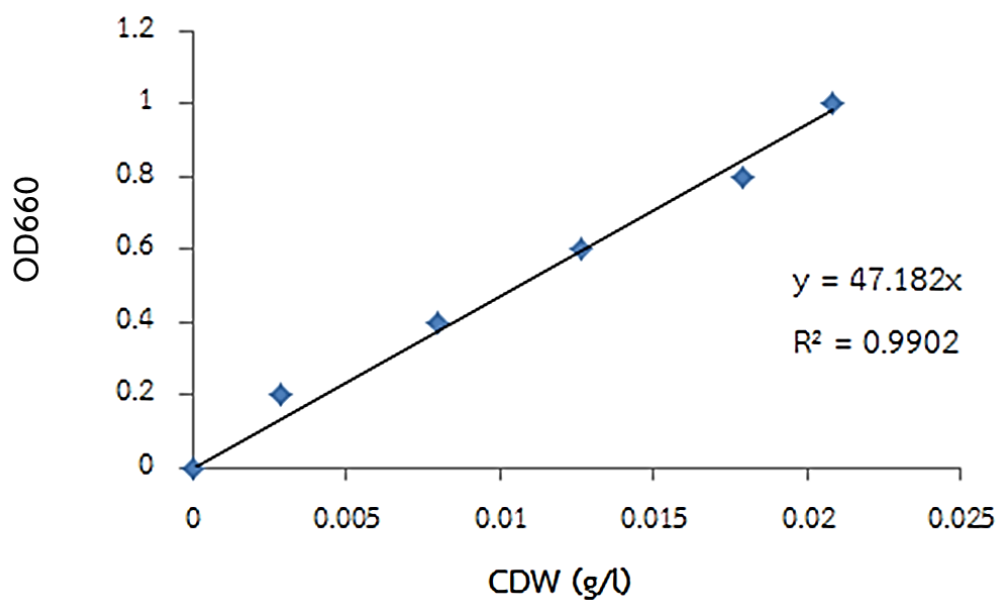

Figure B2-1. Standard curve of cell dry weight (CDW) of isolate NS15-dA1

Equation; Y= 47.182X

$$\text{Cell dry weight (g/l)} = \frac{OD660}{47.182}$$

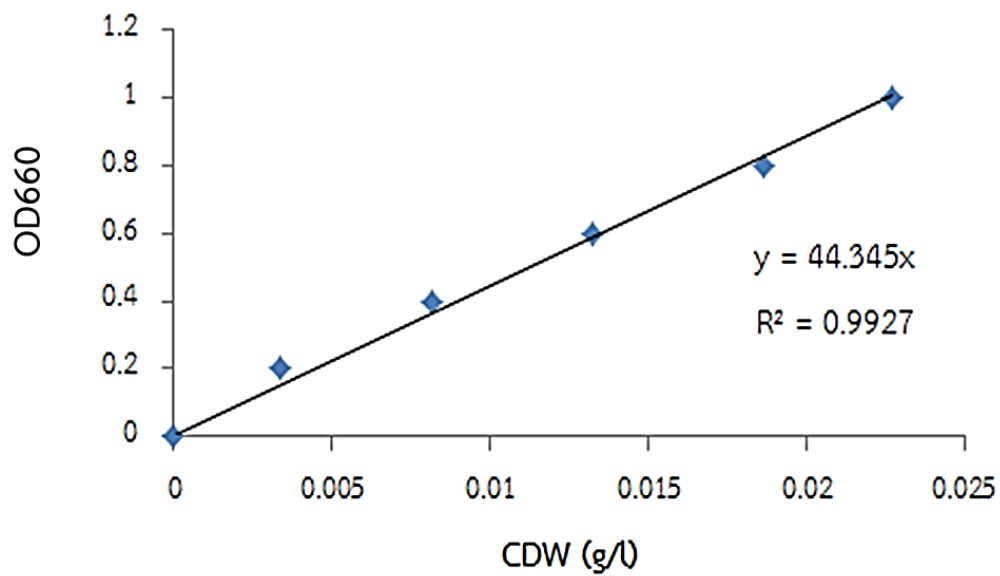**Figure B2-2.** Standard curve of cell dry weight (CDW) of isolate NS15-bA2

Equation; Y= 44.345X

$$\text{Cell dry weight (g/l)} = \frac{\text{OD660}}{44.345}$$

3. Standard curve of succinic acid by HPLC (High Performance Liquid
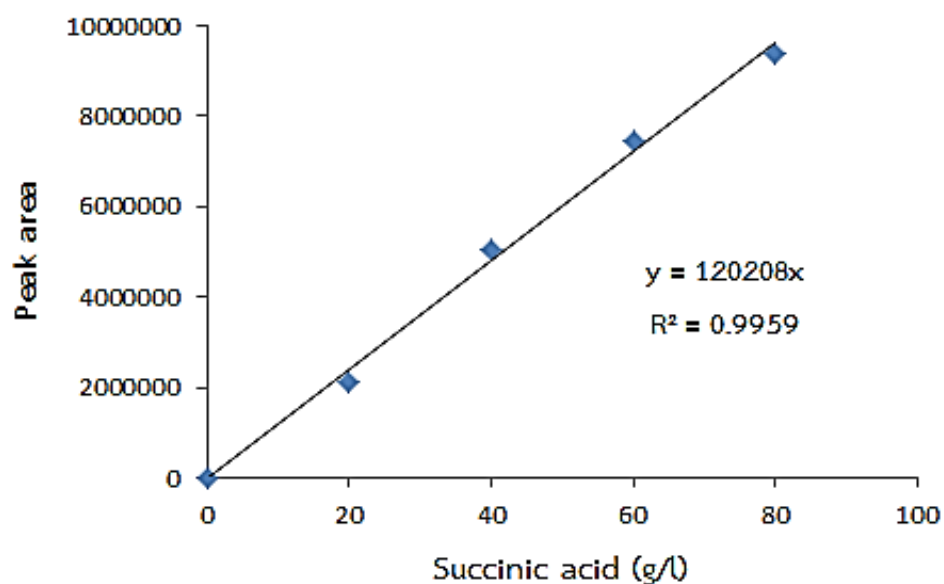
    Chromatography)

Figure B3. Standard curve of succinic acid concentration

Equation; Y= 120208X

$$\text{Succinic acid concentration (g/l)} = \frac{\text{Peak Area}}{120208}$$

APPENDIX C

The results of optimization on succinic acid production

1. Effect of glucose concentration

Table C1.1 Residual sugars and cell dry weight and succinic acid yield by isolate
NS15-dA1 and NS15-bA2 for 48 h

Isolates	Residual sugars (g/l)				
	0 h	12 h	24 h	36 h	48 h
NS15-	29.748±0.0201[a]	0.000±0.0021	0.000±0.0002	0.000±0.0006	0.000±0.0002
dA1	59.247±0.1419	11.695±0.1081	1.250±0.0208	0.000±0.0002	0.000±0.0003
	89.097±0.2067	43.869±0.3125	30.484±0.1990	28.202±0.0484	26.425±0.1380
NS15-	29.659±0.0620	0.000±0.0001	0.000±0.0009	0.000±0.0008	0.000±0.0014
bA2	59.473±0.3029	22.168±0.4011	1.789±0.02149	0.000±0.0003	0.000±0.0002
	89.505±0.3024	57.265±0.3207	36.418±0.3166	24.064±0.1353	19.568±0.0371

Isolates	Cell dry weight (g/l)				
	0 h	12 h	24 h	36 h	48 h
NS15-	0.0017±0.0001[a]	0.2251±0.0004	0.2384±0.0009	0.2238±0.0001	0.1876±0.0006
dA1	0.0015±0.0001	0.2950±0.0011	0.3217±0.0008	0.2963±0.0007	0.2887±0.0009
	0.0013±0.0001	0.2419±0.0005	0.2906±0.0090	0.2849±0.0004	0.2842±0.0080
NS15-	0.0018±0.0001	0.2530±0.0011	0.2801±0.0001	0.2584±0.0002	0.2469±0.0012
bA2	0.0016±0.0002	0.3071±0.0011	0.3484±0.0049	0.3356±0.0020	0.3252±0.0008
	0.0014±0.0001	0.3024±0.0007	0.3207±0.0006	0.3166±0.0053	0.3105±0.0037

[a] Each value was an average of three parallel replicates and was presented as mean ± standard
deviation.

Table C1.2 Yield of succinic acid by isolate NS15-dA1 and NS15-bA2 for 48 h

Isolates	Glucose (g/l)	Yield of succinic acid (g/g glucose)			
		12 h	24 h	36 h	48 h
NS15-dA1	30	0.441±0.0020[a]	0.452±0.0037	0.444±0.0057	0.443±0.0101
	60	0.668±0.0111	0.834±0.0085	0.832±0.0003	0.821±0.0075
	90	0.631 ±0.0085	0.723±0.0017	0.769±0.0007	0.772±0.0122
NS15-bA2	30	0.254±0.0077	0.432±0.0018	0.414±0.0037	0.416±0.0011
	60	0.366±0.0054	0.875±0.0019	0.856±0.0005	0.823±0.0041
	90	0.206±0.0034	0.787±0.0015	0.776±0.0007	0.772±0.0123

[a] Each value was an average of three parallel replicates and was presented as mean ± standard deviation.

2. Effect of different nitrogen sources

Table C2.1 Residual sugars by isolate NS15-dA1 and NS15-bA2

Nitrogen sources	Residual sugars (g/l) of Isolate NS15-dA1		Residual sugars (g/l) of Isolate NS15-bA2	
	0 h	24 h	0 h	24 h
Control	59.791±0.0012[a]	0.000±0.0012	59.655±0.6041	0.000±0.0006
Yeast extract	59.519±0.4921	0.000±0.0011	59.723±0.0075	0.000±0.0008
Peptone	59.723±1.2029	9.852±0.0260	59.927±0.6015	9.802±0.0165
Tryptone	59.724±0.8202	9.734±0.0159	59.179±0.0165	0.000±0.0020
Urea	59.451±0.0241	46.293±0.0075	59.926±0.2601	52.475±0.0038
KNO_3	59.519±0.0165	51.071±0.0142	59.519±1.5617	49.146±0.0085
$NHCl_4$	59.519±0.0075	47.244±0.0254	59.451±0.9241	47.176±0.0214
$(NH_4)_2SO_4$	59.247±0.3511	45.319±0.0026	59.315±0.2210	46.496±0.0164
$(NH_4)_2HPO_4$	59.383±0.1923	46.043±0.0142	59.383±1.3086	36.531±0.0203
NH_4NO_3	59.383±0.5421	49.146±0.0081	59.362±0.6165	50.664±0.0492

[a] Each value was an average of three parallel replicates and was presented as mean ± standard deviation.

Table C2.2 Yield of succinic acid by isolate NS15-dA1 and NS15-bA2

Nitrogen sources	Yield of succinic acid (g/g glucose)	
	Isolate NS15-dA1	Isolate NS15-bA2
Control	0.836±0.0013[a]	0.867±0.0069
Yeast extract	0.832±0.0067	0.874±0.0146
Peptone	0.681±0.0044	0.653±0.0170
Tryptone	0.704±0.0035	0.898±0.0008
Urea	0.119±0.0026	0.018±0.0010
KNO_3	0.121±0.0019	0.291±0.0011
$NHCl_4$	0.014±0.0012	0.013±0.0003
$(NH_4)_2SO_4$	0.027±0.0004	0.022±0.0034
$(NH_4)_2HPO_4$	0.113±0.0032	0.245±0.0176
NH_4NO_3	0.035±0.0006	0.013±0.0007

[a] Each value was an average of three parallel replicates and was presented as mean ± standard deviation.

3. Effect of initial pH

Table C3.1 Residual sugars by isolate NS15-dA1 and NS15-bA2

Initial pH	Residual sugars (g/l) of Isolate NS15-dA1		Residual sugars (g/l) of Isolate NS15-bA2	
	0 h	24 h	0 h	24 h
Control	59.791±0.3065[a]	0.000±0.0002	59.605±0.0076	0.000±0.0003
5.0	59.519±0.0577	47.054±0.0644	59.449±0.0214	38.622±0.6621
5.5	59.753±0.0032	22.165±0.0542	59.723±0.0034	20.892±0.1659
6.0	59.723±0.8700	18.735±0.0125	59.882±0.6807	15.083±0.0206
6.5	59.451±0.0097	4.095±0.0038	59.304±0.0067	0.000±0.0001
7.0	59.519±0.0164	0.000±0.0012	59.199±0.0250	0.000±0.0002
7.5	59.519±0.2008	0.000±0.0002	59.519±0.1509	0.000±0.0003
8.0	59.247±0.0792	0.000±0.0004	59.674±0.0084	15.431±0.8192

[a] Each value was an average of three parallel replicates and was presented as mean ± standard deviation

Table C3.2 Yield of succinic acid by isolate NS15-dA1 and NS15-bA2

| Initial pH | Yield of succinic acid (g/g glucose) | |
	Isolate NS15-dA1	Isolate NS15-bA2
Control	0.850±0.0044[a]	0.877±0.0169
5.0	0.214±0.0013	0.366±0.0044
5.5	0.519±0.0057	0.619±0.0081
6.0	0.671±0.0049	0.731±0.0087
6.5	0.770±0.0035	0.803±0.0044
7.0	0.845±0.0079	0.887±0.0034
7.5	0.808±0.0049	0.796±0.0067
8.0	0.815±0.0023	0.726±0.0081

[a] Each value was an average of three parallel replicates and was presented as mean ± standard deviation

4. Effect of temperature

Table C4.1 Residual sugars by isolate NS15-dA1 and NS15-bA2

| Temperature (°C) | Residual sugars (g/l) of Isolate NS15-dA1 | | Residual sugars (g/l) of Isolate NS15-bA2 | |
	0 h	24 h	0 h	24 h
Control	59.330±0.0954[a]	0.000±0.0001	59.605±0.0090	0.000±0.0001
35	59.682±0.0064	0.000±0.0001	59.023±0.0087	5.960±0.1056
37	59.291±0.1318	0.000±0.0002	59.190±0.1030	0.000±0.0003
39	59.873±0.0943	8.615±0.1807	59.245±0.0098	10.229±0.0796

| Temperature (°C) | Yield of succinic acid (g/g glucose) | |
	Isolate NS15-dA1	Isolate NS15-bA2
Control	0.859±0.0042[a]	0.851±0.0044
35	0.846±0.0041	0.712±0.0059
37	0.862±0.0028	0.884±0.0059
39	0.818±0.0111	0.755±0.0053

[a] Each value was an average of three parallel replicates and was presented as mean ± standard deviation

APPENDIX D

16S rRNA gene sequence

Primer

20 F : AGTTTGATCCTGGCTC

1530 R : AAGGAGGTGATCCAGCC

1.   Isolate CN1-OB13

GGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAACGGTAACAGGAAGCAGC

TTGCTTCTTTGCTGACGAGTGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGG

GATAACTACTGGAAACGGTAGCTAATACCGCATAACGTCGCAAGACCAAAGAGGGGGACCTTCG

GGCCTCTTGCCATCGGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAACGGCTCACCTAG

GCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAGA

CTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCC

GCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGGAGTAAAGTTA

ATACCTTTGCTCATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCG

GTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTTGT

TAAGTCAGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCTGATACTGGCAAGCTTGAGT

CTCGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACC

GGTGGCGAAGGCGGCCCCCTGGACGAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAAC

AGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTTGGAGGTTGTGCCCTTGAGG

CGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAAC

TCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGA

AGAACCTTACCTGGTCTTGACATCCACGGAAGTTTTCAGAGATGAGAATGTGCCTTCGGGAACC

GTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAA

CGAGCGCAACCCTTATCCTTTGTTGCCAGCGGTCCGGCCGGGAACTCAAAGGAGACTGCCAGTG

ATAAACTGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGACCAGGGCTACACAC

GTGCTACAATGGCGCATACAAAGAGAAGCGACCTCGCGAGAGCAAGCGGACCTCATAAAGTGC

GTCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGTGGA

TCAGAATGCCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTG

GGTTGCAAAAGAAGTAGGTAGCTTAACCTTCGGGAGGGCGCTTACCACTTTGTGATTCATGACT
GGGGTGAAGTCGTAACAAGGTAACCGTAGGGGAACCT

2. Isolate PCH6-3

CTGATTGATGGTGCTTGCACCTGATTGACGATGGATCACCAGTGAGTGGCGGACGGGTGAGTAA
CACGTAGGTAACCTGCCCCGGAGCGGGGGATAACATTTGGAAACAGATGCTAATACCGCATAAC
AACAAAAGCCACATGGCTTTTGTTTGAAAGATGGCTTTGGCTATCACTCTGGGATGGACCTGCG
GTGCATTAGCTAGTTGGTAAGGTAACGGCTTACCAAGGCGATGATGCATAGCCGAGTTGAGAGA
CTGATCGGCCACAATGGAACTGAGACACGGTCCATACTCCTACGGGAGGCAGCAGTAGGGAAT
CTTCCACAATGGGCGCAAGCCTGATGGAGCAACACCGCGTGAGTGAAGAAGGGTTTCGGCTCG
TAAAGCTCTGTTGTTGGAGAAGAACGTGCGTGAGAGTAACTGTTCACGCAGTGACGGTATCCAA
CCAGAAAGTCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCC
GGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTGCTTAGGTCTGATGTGAAAGCCTTCGGCTT
AACCGAAGAAGTGCATCGGAAACCGGGCGACTTGAGTGCAGAAGAGGACAGTGGAACTCCATG
TGTAGCGGTGGAATGCGTAGATATATGGAAGAACACCAGTGGCGAAGGCGGCTGTCTGGTCTG
CAACTGACGCTGAGGCTCGAAAGCATGGGTAGCGAACAGGATTAGATACCCTGGTAGTCCATGC
CGTAAACGATGAGTGCTAGGTGTTGGAGGGTTTCCGCCCTTCAGTGCCGGAGCTAACGCATTAA
GCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCAC
AAGCGGTGGAGCATGTGGTTTAATTCGAAGCTACGCGAAGAACCTTACCAGGTCTTGACATCTT
GCGCTAACCTTAGAGATAAGGCGTTCCCTTCGGGGACGCAATGACAGGTGGTGCATGGTCGTCG
TCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTTACTAGTTGC
CAGCATTAAGTTGGGCACTCTAGTGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGACGAC
GTCAGATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGACGGTACAACGAGT
CGCAAGCTCGCGAGAGTAAGCTAATCTCTTAAAGCCGTTCTCAGTTCGGACTGTAGGCTGCAAC
TCGCCTACACGAAGTCGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCC
GGGCCTTGTACACACCGCCCGTCACACCATGGGAGTTT

3. Isolate AY5-bA2

CGAATGCTTGCATTCACCGAAAGAAGCTTAGTGGCGAACGGGTGAGTAACACGTAGGCAACCTG
CCCAAAAGAGGGGGATAACACTTGGAAACAGGTGCTAATACCGCATAACCATGGACATCGCATG
ATGTTCATGTAAAAGACGGCTTTTGCTGTCACTTTTGGATGGGCCTGCGGCGTATTAACTTGTT
GGTGGGGTAACGGCCTACCAAGGTGATGATACGTAGCCGAACTGAGAGGTTGATCGGCCACATT
GGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACG
AAAGTCTGATGGAGCAACGCCGCGTGAATGAAGAAGGCCTTCGGGTCGTAAAATTCTGTTGTCA
GAGAAGAACGTGCGTGAGAGTAACTGTTCACGTATTGACGGTATCTGACCAGAAAGCCACGGCT
AACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTA
AAGGGAACGCAGGCGGTCTTTTAAGTCTGATGTGAAAGCCTTCGGCTTAACCGAAGTAGTGCAT
TGGAAACTGGAAGACTTGAGTGCAGAAGAGGAGAGTGGAACTCCATGTGTAGCGGTGAAATGC
GTAGATATATGGAAGAACACCAGTGGCGAAAGCGGCTCTCTGGTCTGTAACTGACGCTGAGGTT
CGAAAGCGTGGGTAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCT
AAGTGTTGGAGGGTTTCCGCCCTTCAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAG
TACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTG
GTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCTTCTGACAATTCCAGAGAT
GGAACGTTCCCTTCGGGGACAGAATGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAG
ATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATTGTCAGTTGCCATCATTAAGTTGGGCA
CTCTGGCGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCC
CTTATGACCTGGGCTACACACGTGCTACAATGGACGGTACAACGAGTCGCTAACTCGCGAGGGC
AAGCTAATCTCTTAAAGCCGTTCTCAGTTCGGATTGCAGGCTGCAACTCGCCTGCATGAAGTCG
GAATCGCTAGTAATCGCGAATCAGCATGTCGCGGTGAATACGTTCCCGGGCCTTGTACACACCG
CCCGTCACACCATGAGAGTTTGTAACACCCA

4. Isolate AY5-bB4

GCGATGAAGCTTCCTTCGGGAAGTGGATTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCT
GCCTCAAAGTGGGGGATAGCCTTCCGAAAGGAAGATTAATACCGCATAACATAAGAGAATCGCA
TGATTTTCTTATCAAAGATTTATTGCTTTGAGATGGACCCGCGGCGCATTAGCTAGTTGGTAAG
GTAACGGCTTACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACATTGGAAC
TGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCGCAATGGGGGAAACC
CTGACGCAGCAACGCCGCGTGGGTGATGAAGGTCTTCGGATTGTAAAGCCCTGTTTTCTGGGAC
GATAATGACGGTACCAGAGGAGGAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACG
TAGGTGGCGAGCGTTGTCCGGATTTACTGGGCGTAAAGGGTGCGTAGGCGGATGTTTAAGTGG
GATGTGAAATCCCCGGGCTTAACCTGGGGGCTGCATTCCAAACTGGATATCTAGAGTGCAGGAG
AGGAAAGCGGAATTCCTAGTGTAGCGGTGAAATGCGTAGAGATTAGGAAGAACACCAGTGGCG
AAGGCGGCTTTCTGGACTGTAACTGACGCTGAGGCACGAAAGCGTGGGTAGCAAACAGGATTA
GATACCCTGGTAGTCCACGCCGTAAACGATGGATACTAGGTGTAGGGGGTATCAACTCCCCCTG
TGCCGCAGTTAACACAATAAGTATCCCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGG
AATTGACGGGGGCCCGCACAAGCAGCGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACC
TTACCTGGACTTGACATCCCTTGCATAGCCTAGAGATAGGTGAAGCCCTTCGGGGCAAGGAGAC
AGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTAGGTTAAGTCCTGCAACGAGCGC
AACCCTTGTTATTAGTTGCTACCATTAAGTTGAGCACTCTAATGAGACTGCCTGGGTAACCAGG
AGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGTCCAGGGCTACACACGTGCTACAA
TGGTAGGTACAATAAGACGCAAGACCGTGAGGTGGAGCAAAACTTATAAAACCTATCTCAGTTC
GGATTGTAGGCTGCAACTCGCCTACATGAAGCTGGAGTTGCTAGTAATCGCGAATCAGAATGTC
GCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGAGAGCTGGTAACACCC
GAAGTCCGTGAGGT

5. PCH2-1

AGTGGCGAACGGGTGAGTAACACGTGGGTAACCTGCCCATCAGAAGGGGATAACACTTGGAAA
CAGGTGCTAATACCGTATAACAATCAAAACCGCATGGTTTTGATTTGAAAGGCGCTTTCGGGTG
TCGCTGATGGATGGACCCGCGGTGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCCAC
GATGCATAGCCGACCTGAGAGGGTGATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCT
ACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGACGAAAGTCTGACCGAGCAACGCCGCGTG
AGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTTGTTAGAGAAGAACAAGGATGAGAGTAACTG
TTCATCCCTTGACGGTATCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAAT
ACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTCTTAAGT
CTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAG
AAGAGGAGAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAGGAACACCAGTGG
CGAAGGCGGCTCTCTGGTCTGTAACTGACGCTGAGGCTCGAAAGCGTGGGGAGCAAACAGGAT
TAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTGGAGGGTTTCCGCCCTTC
AGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAA
GGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAA
CCTTACCAGGTCTTGACATCCTTTGACCACTCTAGAGATAGAGCTTCCCCTTCGGGGGCAAAGT
GACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAG
CGCAACCCTTATTGTTAGTTGCCATCATTCAGTTGGGCACTCTAGCAAGACTGCCGGTGACAAA
CCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCT
ACAATGGGAAGTACAACGAGTCGCGAAGTCGCGAGGCTAAGCTAATCTCTTAAAGCTTCTCTCA
GTTCGGATTGCAGGCTGCAACTCGCCTGCATGAAGCCGGAATCGCTAGTAATCGCGGATCAGCA
CGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCAC

6. Isolate NP1-A2

GACGGGTGAGTAACACGTGGGTAACCTACCCATCAGAGGGGGATAACACTTGGAAACAGGTGC
TAATACCGCATAACAGTTTATGCCGCATGGCATAAGAGTGAAAGGCGCTTTCGGGTGTCGCTGA
TGGATGGACCCGCGGTGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCCACGATGCATA
GCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGG
CAGCAGTAGGGAATCTTCGGCAATGGACGAAAGTCTGACCGAGCAACGCCGCGTGAGTGAAGA
AGGTTTTCGGATCGTAAAACTCTGTTGTTAGAGAAGAACAAGGACGTTAGTAACTGAACGTCCC
CTGACGGTATCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGT
GGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTCTTAAGTCTGATGT
GAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGA
GAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAGGAACACCAGTGGCGAAGGC
GGCTCTCTGGTCTGTAACTGACGCTGAGGCTCGAAAGCGTGGGGAGCAAACAGGATTAGATACC
CTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTGGGAGGGTTTCCGCCCTTCAGTGCTG
CAGCAAACGCATTAAGCACTCCGCCTGGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATT
GACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTTCGAAGCAACGCGAAGAACCTTA
CCAGGTCTTGACATCCTTTGACCACTCTAGAGATAGAGCTTTCCCTTCGGGGACAAAGTGACAG
GTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAA
CCCTTATTGTTAGTTGCCATCATTTAGTTGGGCACTCTAGCGAGACTGCCGGTGACAAACCGGA
GGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAAT
GGGAAGTACAACGAGTCGCTAGACCGCGAGGTCATGCAAATCTCTTAAAGCTTCTCTCAGTTCG
GATTGCAGGCTGCAACTCGCCTGCATGAAGCCGGAATCGCTAGTAATCGCGGATCAGCACGCCG
CGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGT

7. Isolate CN2-OA2

GAGCTTGCTCCACCGAAAGAAAAGGAGTGGCGAACGGGTGAGTAACACGTGGGTAACCTGCCC
ATCAGAAGGGGATAACACTTGGAAACAGGTGCTAATACCGTATAACAATCGAAACCGCATGGTT
TCGGTTTGAAAGGCGCTTTTGCGTCACTGATGGATGGACCCGCGGTGCATTAGCTAGTTGGTGA
GGTAACGGCTCACCAAGGCAACGATGCATAGCCGACCTGAGAGGGTGATCGGCCACATTGGGA
CTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGACGCAAG
TCTGACCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTTGTTAGAGA
AGAACAAGGATGAGAGTAGAATGTTCATCCCTTGACGGTATCTAACCAGAAAGCCACGGCTAAC
TACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAG
CGAGCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTG
GAAACTGGGAAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCATGTGTAGCGGTGAAATGCGTA
GATATATGGAGGAACACCAGTGGCGAAGGCGGCTCTCTGGTCTGTAACTGACGCTGAGGCTCG
AAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAA
GTGTTGGAGGGTTTCCGCCCTTCAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTA
CGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGGACATGTGGT
TTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTTTGACCACTCTAGAGATAG
AGCTTCCCCTTCGGGGGCAAAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGAT
GTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATTGTTAGTTGCCATCATTTAGTTGGGCACT
CTAGCGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCT
TATGACCTGGGCTACACACGTGCTACAATGGGAAGTACAACGAGTCGCGAAGTCGCGAGGCTAA
GCTAATCCTTAAAGCTTCTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGCCGGAA
TCGCGTAGTAATCGCGGATCAGCACGCCGCGGTGAATACGTTGCCCGGGCCTTGTACACACCGC
CCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGAGGTAA

8. Isolate NS15-bA2

GCTTCTTTTTCCACCGGAGCTTGCTCCACCGGAAAAAGAGGAGTGGCGAACGGGTGAGTAACAC
GTGGGTAACCTGCCCATCAGAAGGGGATAACACTTGGAAACAGGTGCTAATACCGTATAACAAT
CGAAACCGCATGGTTTTGATTTGAAAGGCGCTTTCGGGTGTCGCTGATGGATGGACCCGCGGTG
CATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCATAGCCGACCTGAGAGGGT
GATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCT
TCGGCAATGGACGAAAGTCTGACCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAA
AACTCTGTTGTTAGAGAAGAACAAGGATGAGAGTAACTGTTCATCCCTTGACGGTATCTAACCA
GAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGG
ATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAA
CCGGGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAGAGTGGAATTCCATGTG
TAGCGGTGAAATGCGTAGATATATGGAGGAACACCAGTGGCGAAGGCGGCTCTCTGGTCTGTAA
CTGACGCTGAGGCTCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCG
TAAACGATGAGTGCTAAGTGTTGGAGGGTTTCCGCCCTTCAGTGCTGCAGCTAACGCATTTAAG
CACTCTCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCAC
AAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCT
TTGACCACTCTAGAGATAGAGCTTCCCCTTCGGGGGCAAAGTGACAGGTGGTGCATGGTTGTCG
TCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATTGTTAGTTGC
CATCATTTAGTTGGGCACTCTAGCAAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGAC
GTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGGAAGTACAACGAGT
CGCAAAGTCGCGAGGCTAAGCTAATCTCTTAAAGCTTCTCTCAGTTCGGATTGTAGGCTGCAAC
TCGCCTACATGAAGCCGGAATCGCTAGTAATCGCGGATCAGCACGCCGCGGTGAATACGTTCCC
GGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGAGGTAAC
CTTTTGGAGCCAGCCGCC

9. Isolate NS15- dA1

TCGTACGCTTCTTTTTCCACCGGAGGCTTGCTCCACCGGAAAAAGAAGAGTGGCGAACGGGTGA
GTAACACGTGGGTAACCTGCCCATCAGAAGGGGATAACACTTGGAAACAGGTGCTAATACCGTA
TAACAATCGAAACCGCATGGTTTTGATTTGAAAGGCGCTTTCGGGTGTCGCTGATGGATGGACC
CGCGGTGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCCACGATGCATAGCCGACCTGA
GAGGGTGATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGG
GAATCTTCGGCAATGGACGAAAGTCTGACCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGA
TCGTAAAACTCTGTTGTTAGAGAAGAACAAGGATGAGAGTAACTGTTCATCCCTTGACGGTATC
TAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTT
GTCCGGATTTATTGGGCGTAAAGCGAACGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCG
GCTCAACCGGGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAGAGTGGAATTC
CATGTGTAGCGGTGAAATGCGTAGATATATGGAGGAACACCAGTGGCGAAGGCGGCTCTCTGGT
CTGTAACTGACGCTGAGGCTCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCA
CGCCGTAAACGATGAGTGCTAAGTGTTGGAGGGTTTCCGCCCTTCAGTGCTGCAGCTAACGCAT
TAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCG
CACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACAT
CCTTTGACCACTCTAGAGATAGAGCTTCCCCTTCGGGGGGCAAAGTGACAGGTGGTGCATGGTTG
TCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATTGTTAGT
TGCCATCATTTAGTTGGGCACTCTAGCAAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATG
ACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGGAAGTACAACGA
GTCGCGAAGTCGCGAGGCTAAGCTAATCTCTTAAAGCTTCTCTCAGTTCGGATTGTAGGCTGCA
ACTCGCCTA

VITA

Miss Natcha Pinkian was born on November 4, 1990 in Nakhonsawan, Thailand. She received her Bachelor's degree of Science in Bioechnology from the Faculty of Engineering and Industrial Technology, Silpakorn University in 2012. She has been studying for a Master Degree of Science in Biotechnology at Faculty of Sciences, Chulalongkorn University since 2013.

Academic presentations

1. Poster presentation:

Pinkian, N., Phuengjayaem, S., Tanasupawat, S and Teeradakorn, S. "Screening and characterization of succinic acid bacteria from Thailand." International Conference on Food and Applied Bioscience. 4-5 February 2016. The Empress Hotel, Chiang Mai, Thailand. (Best Poster Award)

2. Publication:

Pinkian, N., Phuengjayaem, S., Tanasupawat, S and Teeradakorn, S. "Screening and characterization of succinic acid bacteria from Thailand." Chiang Mai University Journal of Natural Sciences (submitted).