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กระบวนการการผลิตกรดซัคซินิกจากวัสดุจำพวกลิกโนเซลลูโลสโดย *Actinobacillus* sp.

Process development of succinic acid production from lignocellulosic materials by *Actinobacillus* sp.

โดย

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ชื่อโครงการวิจัย กระบวนการการผลิตกรดซัคซินิกจากวัสดุจำพวกลิกโนเซลลูโลสโดย *Actinobacillus* sp.

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เดือนและปีที่ทำวิจัยสำเร็จ มีนาคม 2558

บทคัดย่อ

ทำการเก็บตัวอย่างแหล่งดินและมูลสัตว์จากจังหวัดสุรินทร์(SR1-2) สุพรรณบุรี(SP1-10) นครสวรรค์(NS1-12) และนครปฐม(NP1-6) รวมทั้งหมด 30 ตัวอย่าง เพื่อคัดเลือกแบคทีเรียที่สามารถผลิตกรดซัคซินิกได้ พบว่ามี 230 สายพันธุ์ที่สามารถเจริญเติบโตในอาหารแข็งสำหรับคุณสมบัติการผลิตกรดซัคซินิก โดยพบว่าเป็นแบคทีเรียแกรมบวก 216 สายพันธุ์และแบคทีเรียแกรมลบ 14 สายพันธ์ โดยแบคทีเรียแกรมลบที่มีศักยภาพในการผลิตกรดซัคซินิก ได้แก่ isolate SP8/A4 และ NS2/A2 โดยสามารถผลิตกรดซัคซินิกได้ 42.08 และ 43.26 กรัม/ลิตรตามลำดับ ทำการศึกษานำร่องในการผลิตกรดซัคซินิกจากวัสดุประเภทลิกโนเซลลูโลส โดย *Actinobacillus succinogenes* DSMZ 22257 พบว่าเมื่อใช้สารละลายย่อยสลายของชานข้าวฟ่าง(เทียบเท่าน้ำตาลรีดิวส์) 60 กรัม/ลิตรเป็นแหล่งคาร์บอน การเจริญของจุลินทรีย์จะมีระยะการเจริญเติบโตในช่วงระยะปรับตัวนานขึ้นและมีการเจริญสูงสุด 2.665 กรัม/ลิตรและสามารถผลิตกรดซัคซินิกได้ 13.820 กรัม/ลิตร เมื่อใช้สารแหล่งคาร์บอนคือกลูโคส 60 กรัม/ลิตร จุลินทรีย์มีการเจริญสูงสุด 3.185 กรัม/ลิตรและสามารถผลิตกรดซัคซินิกได้ 44.799 กรัม/ลิตร

Project Title Process development of succinic acid production from lignocellulosic materials by *Actinobacillus sp*.

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ABSTRACT

Total 30 samples were collected from soil and bovine dung in Surin (SR1-2), Suphanburi (SP1-10), Nakornsawan(NS1-12) and Nakhonprathom (NP1-6) province, Thailand. Two hundreds thirty bacterial isolates with succinic acid ability were obtained. Two hundreds and sixteen isolates were gram-positive and fourteen isolates were gram negative bacteria. Two of these negative isolates, isolate SP8/A4 and NS2/A2, have a potential for succinic acid production with 42.08 and 43.26 g/l, respectively. *Actinobacillus succinogenes* DSMZ 22257 was used as a representative for succinic acid production from lignocellulosic material. The use of 60 g/l (equivalent reducing sugar) sorghum straw hydrolyzate as a carbon source, longer lag phase was found and the highest amount cell growth of 2.665 g/l and succinic acid of 13.820 g/l were obtained. When using glucose 60g/l as a carbon source, a maximum cell growth of 3.185 g/l and succinic acid 44.799 g/l were obtained.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter I

Introduction

1.1 Succinic acid and application

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

1.2 Succinic acid production

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

1.3 Screening of succinic acid bacteria

Succinic acid is synthesized in almost microbials, plants and animal cells. Those efficient microbials can be divided into fungi and bacteria (Song and Lee, 2006).

Fungal succinic acid producers; *Fusarium* sp., *Aspergillus* sp. and *Penicillium* sp.; are rarely described in literatures. *P. simplicissimum* produces succinate and citrate under anaerobic and aerobic conditions. A strong increase in succinate excretion was observed when the respiratory chain was inhibited, either by sodium azide or anaerobic condition. However, the use of succinic acid derived from fungi has been mostly limited to the

manufacture of food and beverages due to the difficulties in fermentation, separation and purification (Magnuson and Lasure, 2004).

Yeast; *Saccharomyces cerevisiae* is an organism producing succinic acid as a metabolic byproduct under aerobic and/or anaerobic conditions. *S. cerevisiae* has been much studied to achieve high concentration of succinic acid in wine manufacturing (Song and Lee, 2006).

A little gram-positive bacteria; *Corynebacterium glutamicum*, *Enterococcus faecalis* and *Ruminococcus flavefaciens* have been studied for succinic acid production. Several engineered *C. glutamicum* strains were created by disruption and replacement of genes and their optimal culture conditions were developed. Succinic acids can be produced by gram-negative bacteria; *Anaerobiospirillum succiniciproducens, Actinobacillus succinogenes, Bacteroides amylophilus, Escherichia coli, Mannheimia succiniciproducens, Prevotella ruminicola, Succcinimonas amylolytica, Succinivibrio dextrinisolvens, Wolinella succinogenes* and *Cytophaga succinicans* (Guettler et al., 1999). There have been isolated in various anaerobic environments such as domestic sludge, cattle waste, rice paddy, marine shipworm, mouth of dog, rumen and gastro-intestines.

At present, bacteria isolated from the rumen such as *A. succinogenes, A. succiniciproducens* and *M. succiniciproducens* are the best candidates for succinic acid production. This is most likely due to that the rumen is a highly efficient organ providing an environment to produce succinic acid. The rumen is a unique microbial ecosystem found in many species of herbivorous mammals known as ruminants, caused by carbon dioxide, methane, traces of hydrogen production. Moreover, a lot of vitamins and amino acids are abundant in the rumen resulting in minimal medium necessary.

The primary role of the rumen is to provide pre-gastric digestion of various polysaccharide materials, which is mediated by a variety of microorganisms in the rumen containing of 10^9-10^{10} bacterial, 10^5–10^6 protozoan and 10^3–10^4 fungal cells/ml of rumen fluid. The production of C4 dicarboxylic acids in the rumen reduces energy loss associated with methanogenesis (30–40 mol% of CH_4 is present in the ruminal gas) by increasing the amount of metabolizable energy available to the animal in the form of propionic acid. Although the C4 dicarboxylic compounds such as oxaloacetic, malic, fumaric and succinic acids are not detected in the ruminal fluid. The large amounts of these acids are produced by CO_2 fixation reactions using 60–70 mol% of CO_2 present in the ruminal gas. The major C3

compounds in the cell used for carboxylation reaction are phosphoenolpyruvate (PEP) and pyruvate. In particular, succinic acid is converted to propionic acid, which can account for 20% (w/w) of total volatile fatty acids (VFAs) in the rumen by succinic acid utilizing bacteria such as *Veillonella parvula*, *Selenomonas ruminantium* and *Succiniclasticumruminis* sp. Propionic acid produced by this way is absorbed through the rumen wall for subsequent oxidation to provide energy and biosynthetic precursors for the animals. Therefore, it is reasonable to think that some microorganisms present in the rumen will be a good succinic acid producer (Song and Lee, 2006).

Guettler and co-worker (1999) reported that the rumen bacteria, *A. succinogenes* 130Z, generates high concentrations of succinic acid from a variety of carbon substrates, and the fluoroacetate-resistant mutant of *A. succinogenes* can generate 110 g/l of succinic acid with an apparent yield of 1.2 mole succinic acid/mole glucose.

1.4 *Actinobacillus succinogenes*

A. succinogenes was originally isolated from bovine ruminal contents and belongs to the family *Pasteurellaceae basedon* its 16S rRNA sequence analysis. The phenotypic analysis showed that this organism is a facultative anaerobic, non-motile,pleomorphic and gram-negative rod or occasionally filamentous bacterium. It shows a distinctive ability to produce a relatively large amount of succinic acid from a broad range of carbon sources such as arabinose, cellobiose, fructose, galactose, glucose, lactose, maltose, mannitol, mannose, sorbitol,sucrose, xylose or salicin under anaerobic condition (Song & Lee, 2006). These properties allow fermentation of cane molasses, whey and wheat hydrolysates that are much cheaper carbon sources than refined sugar and glucose (Beauprez, Mey & Soetaert, 2010).

Naturally it produces high concentrations of succinate as a fermentation end product in addition to formate, acetate, and ethanol. *A. succinogenes* converts glucose to phosphoenolpyruvate (PEP), at which point metabolism splits into the following two branches: the formate, acetate and ethanol producing C3 pathway, and the succinate producing C4 pathway Contrast *E. coli* or *A. succiniciproducens*, *A. succinogenes* is a moderate osmophile and has good tolerance to a high concentration of glucose, which is beneficial for fermentation .Comprehensive physiological and genetic studies relating to succinic acid production in *A. succinogenes* have been performed (McKinlay, Zeikus & Vieille, 2007).

PEP carboxylation, which is the important committed step for succinic acid production in rumen bacteria, is strongly regulated by CO_2 levels. Theoretically, 1 mol of CO_2 is required toform 1 mol of succinic acid. The higher CO_2 level resulted in an increased succinic acid production at the expense of ethanol and formic acid. This is most likely due to the increased carboxylationof PEP to oxaloacetate rather than PEP conversion to pyruvate. Also, the addition of extra electron donors including hydrogen and electrically reduced neutral red resulted in thesignificant increase of succinic acid production. These observations are consistent with that the use of more reduced sugars such as arabitol, mannitol and sorbitol resulted in significant increases in the succinic acid and ethanol production compared with glucose.

Zheng and co-worker used SSF technique for succinic acid production by *A.succinogenes* in a 5 L stirred bioreactor with corn stover as the raw material. The corresponding operation conditions were summarized as follows: SSF operation at 38 ^{o}C for 48 h, diluted alkaline pretreated corn stover as substrate with concentration of 70 g/l, enzyme loading of 20 FPU cellulase and 10 U cellobiase per gram substrate. The maximal succinic acid concentration and yield could reach 47.4 g/l and 0.72 g/g-substrate, respectively. This result suggested an industrial potential of succinic acid production by using SSF and corn stover.

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

1.5 Pathway of succinic acid production

Succinic acid has a positive influence on human metabolism and there is no risk of its accumulation in the human body, it has been used in food industries. Succinic acid is an intermediate of the tricarboxylic acid (TCA) cycle and one of the fermentation end-products of anaerobic metabolism (Song and Lee, 2006).

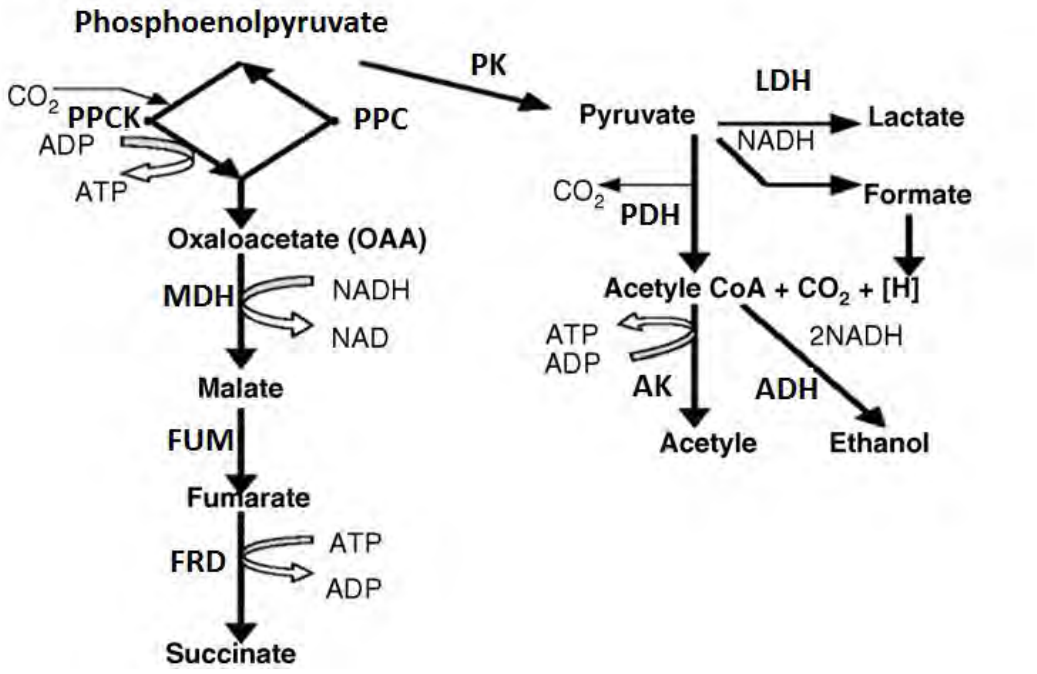

Figure 1 Pathway leading to production of succinic acid and pyruvate from phosphoenolpyruvate. PPCK = phosphoenolpyruvate carboxykinase; MDH, malate dehydrogenase; FUM=fumarase; FRD = fumarate reductase; PK = pyruvate kinase; LDH = lactate dehydrogenase; PDH = pyruvate dehydrogenase; AK, acetate kinase; ADH = alcohol dehydrogenase.

Some bacteria mainly utilize the PEP carboxylation reaction, while others use multiple pathways to form succinic acid. Phosphoenolpyruvate is one of the central intermediates either converted into pyruvate resulting in the formation of the fermentation products (acetate, formate, ethanol and lactate) or it is converted into oxaloacetate resulting in the formation of end products succinate and propionate through the reversible arm of tricarboxylic acid (TCA) cycle. Under anaerobic conditions, the flux of PEP towards either oxaloacetate or pyruvate is affected by environmental factors such as pH, temperature, hydrogen, carbon dioxide and nutritional factors such as carbon, nitrogen sources and metal ions of the growth medium. Five key enzymes responsible for succinic acid production were identified to be phosphoenolpyruvate carboxylase (PPC), phosphoenolpyruvate carboxykinase (PPCK), malate dehydrogenase (MDH), fumarase (FUM) and fumarate reductase (FRD). Therefore, it was importance to investigate the effects of different environmental and nutritional parameters on succinic acid production and on the activities of these TCA cycle enzymes involved in the production pathway (Agarwal et al., 2007).

1.6 Lignocellulosic material

Sorghum (*Sorghum bicolor* (L.) *Moench*) is a cane-like plant with high sugar content. Stem is rich in sugar and juice which Brix between 15% and 23%. It is a high photosynthetic efficiency with high biomass yield crop and it is an interesting annual plant because it can adapt to a wide range of climate from the tropics to cool temperate areas. It is also drought tolerant and has waterlogging resistant (resistant to flood land; soaked in the flood for one week, sorghum can quickly return to growth after flood), salinity resistant (between 0.5% and 0.9%, higher than maize, wheat and rice) and alkalinity resistance properties (Li and Halbrendt, 2009). This plant mainly composed of soluble (glucose and sucrose) and insoluble carbohydrates (cellulose and hemicellulose). Soluble carbohydrates are easily converted to succinic acid, while insoluble carbohydrates conversion to succinic acid by acid or enzymatic hydrolysis of the biopolymer to soluble oligosaccharides subsequently by fermentation to succinic acid. Sorghum is a potential renewable material for the production of bio-succinate since it is abundant in Thailand.

1.7 Problem of succinic acid production

The formation of byproducts such as acetic, formic and lactic acids is a major problem that has to be solved because it reduces the succinic acid yield and productivity, while increases the complexity and cost of succinic acid purification and recovery. Additionally, attempts to produce succinic acid by the cost-effective were presented by using renewable biomass which is much less expensive than refined carbohydrates. The levels of CO_2, culture pH and medium components have been known to be critical factors affecting both cell growth and succinic acid production. The increased CO_2 availability exerted a positive influence on succinic acid yield, while it depressed cell growth resulting in the decreased succinic acid productivity (Lee et al., 1999).

This work firstly concerned with the screening for bacteria with succinic acid ability. *The Actinobacillus succinogenes* DSMZ 22257 is used for comparison the degree of succinic acid production. The process variables such as the economical carbon source, medium components and the neutralizing agents are also investigated to maximize the succinic acid production but minimize the formation of by-products.

Chapter II
Materials and methods

2.1 Screening and isolation of succinic acid bacteria

2.1.1 Microorganism

The samples were collected from bovine dung from surin province (SR1-3), buffalo dung in Suphanburi province (SP1-2), soil sample in Suphanburi province (SP3-10), bovine dung in Nakhonsawan (NS1-10) and bark of tree from Nakhonprathom (NP1-6), Thailand.

One gram of sample was added into 3 ml of enrichment broth consisted of glucose 20 g/l, polypeptone 5 g/l, yeast extract 5 g/l, K_2HPO_4 3 g/l, NaCl 2 g/l, $(NH_4)_2SO_4$ 2 g/l, $CaCl_2 \cdot 2H_2O$ 0.2 g/l, $MgCl_2 \cdot 6H_2O$ 0.4 g/l, and $MgCO_3$ 15 g/l. General anaerobic cultivation techniques were used for the growth of this organism. Strict anaerobic conditions are ensured by using anaerobic pack (MGC, Japan). The samples were incubating at 37 ºC for overnight. The positive tubes were subculture to the enrichment agar plate and incubated at 37 ºC for overnight. The visibly colonies were picked and re-streaked on fresh enrichment agar plate and incubated overnight at 37 ºC under anaerobic condition (Bryant, 1972 and Hungate, 1966).

Screening medium consisted of glucose 20 g/l, NaCl 1 g/l, yeast extract 5 g/l, K_2HPO_4 3 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 and agar 15 g/l. pH an adjusted to 6.5. The single colony from enrichment medium was transferred to screening medium agar plates and incubated overnight at 37 ºC under anaerobic condition. This single colony was called “isolated bacterial strain” which used for study of characterization and identification (Agarwal, et al. 2005).

2.2 Production medium (Li et al., 2010)

The fermentation media was inoculated with 10% of the seeds prepared and grown in an anaerobic flask containing 50 ml of 3% Tryptic Soy Broth (TSB) medium (glucose 5 g/l, casein peptone tryptic digest 10 g/l, yeast extract, 10 g/l, NaCl 5 g/l and K_2HPO_4 2.5 g/l). They were grown in the rotary shaker at 37 °C and 200 rpm for 24 h. The ability of succinic acid producing strain was investigated by fermentation under anaerobic conditions. The fermentation medium consisted of yeast extract 30.0 g/l, urea 2.0 g/l, $MgCl_2.6H_2O$ 2 g/l, $CaCl_2$ 1.5 g/l, $MnCl_2$ 0.07 g/l, Na_2HPO_4 4.4 g/l, NaH_2PO_4 3.3 g/l, $MgCO_3$ 30 g/l and adjusted

pH to 7. The medium was autoclaved at 121ºC for 15 minutes. Glucose was separately sterilized at 115 °C for 20 min and added to the medium to maintain the initial concentration of 60.00 g/l. 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.

2.3 Analysis of succinic acid

2.3.1 Thin layer chromatography (TLC) (Agarwal, et al., 2005)

Thin layer chromatography was applied to develop inexpensive, efficient and fast methods for primary detection of succinic acid. The test samples (10 µl) and standard succinic acid 2 g/l were spotted onto a silica gel TLC plates (Silica gel 60 F254, E. Merck, Germany) and resolved using a solvent system comprising of ethanol, ammonium hydroxide and water (20:5:3 v/v) for 30 min. The air dry plates were sprayed with bromocresol green (0.04% w/v in ethanol) and heated at 160ºC for 5 min to reveal the organic acid spots.

2.3.2 High-performance liquid chromatography (HPLC)

Succinic acid produced during the fermentation process was analyzed by high-performance liquid chromatography (HPLC). Before injection into a column, all samples were centrifuged at 12,000 rpm for 15 min and then filtered through a cellulose membrane acetate filter (0.45 µm, 13 mm membrane disc filters). The condition for analysis process was shown below.

Column	Rezex ROA-Organic Acid H+(8%) HPLC Column (300 x 7.8 mm)
Guard column	Rezex ROA-Organic Acid H+(8%) HPLC Guard Column (50 x 7.8 mm)
Mobile Phase	0.005 N Sulfuric Acid
Flow Rate	0.5 mL/min
Detection	UV 210 nm
Temperature	55 °C
Injection volume	20 µl
Retention time	25 min

Standard succinic acid (20 µl) was used as control in the system. Peaks area of samples were identified and quantified by comparison with retention times (RT) of analytical standards (Oxalic acid, Tartaric, Malic acid, Citric acid, Succinic acid).

2.4 Succinic acid production by *Actinobacillus succinogenes* DSMZ 22257 in shake flask

To study the effect of different carbon sources on cell growth and succinic acid production, the economical carbon sources, glucose and sorghum straw (SS) hydrolyzate were compared. Sorghum straw was obtained from The Suphanburi Field Crops Research Center in Thailand. The SS consisted of 44.51% cellulose, 38.62% hemicellulose, 6.18% lignin and 10.69% ash. Chopped SS was dried in an oven at 70°C to a constant weight. Thirty grams of chopped sorghum straw was suspended in 300 ml of 3% aqueous H_2SO_4 solution and heated at 120°C for 10 minutes (Poonsrisawat et al., 2013). After pretreatment, the hydrolyzate was neutralized with 40% NaOH, centrifuged and filtered through 0.45 μm filters before analyzing total reducing sugars with the DNS method and monomeric sugars (glucose, xylose, galactose, arabinose and mannose) by HPLC.

Fermentation in anaerobic flask was carried out as described previously (section 2.2). *Actinobacillus succinogenes* DSMZ 22257 (the Institute Deutsche Sammlung von Mikroorganismen und Zellkulturen, Germany) was cultured in 150 ml anaerobic flasks containing 50 ml inoculum medium and incubated at 37 °C for 48 h. Cell concentration was measured as optical density at wavelength 660 nm using a spectrophotometer (UV160, Shimadzu Corporation, Japan) after removing the insoluble $MgCO_3$ with 0.2 M HCl (Zheng et al., 2009). The amount of succinic acid was quantified by HPLC.

2.5 Succinic acid production by *Actinobacillus succinogenes* DSMZ 22257 in 2-L bioreactor

Batch fermentation was carried out at 37^{o}C in 2-L bioreactor. The fermentation medium was the same as described in section 2.4. External CO_2 gas sparging rate was controlled at 0.05 vvm and agitation speed was 200 rpm for 48 h. The pH was controlled at 7.0 by automatic addition of 5 M H_3PO_4 or 5 M NaOH. Cell concentration was measured as optical density at wavelength 660 nm using a spectrophotometer (UV160, Shimadzu Corporation, Japan) after removing the insoluble $MgCO_3$ with 0.2 M HCl (Zheng et al., 2009) and the amount of succinic acid was quantified by HPLC.

Chapter III
Results and Discussion

3.1 Screening and isolation of succinic acid bacteria

Microorganisms were first screened based on colony characteristics on enrichment agar plate shown in Figure 2. Mostly isolated colony appearing on the agar plate after 24 h of incubation were smooth and white, 1–2 mm in diameter, non-spore-forming and gram-positive.

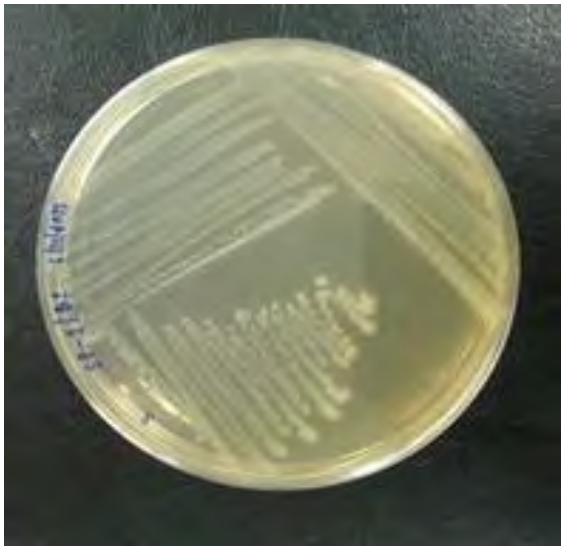

Figure 2 The colony characteristics on enrichment agar plate

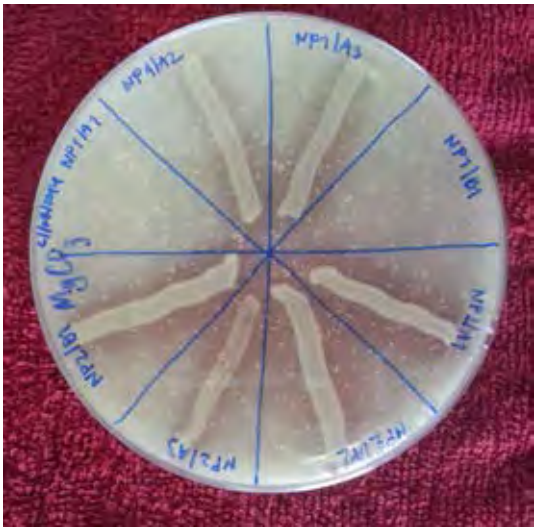

Figure 3 Characteristics of clear zone from isolated bacterial strain with succinic acid ability on screening agar plate

From Figure 3, the isolated bacteria with ability of succinic acid showed a narrow clear zone on screening agar plate. Then these bacteria were further cultivated in the production medium and analyzed for succinic acid by TLC and HPLC.

3.2 Analysis of succinic acid

The TLC method showed clear yellow spots (Figure 4) of different standard organic acids (succinic, lactic, fumaric, malic, citric and α-ketoglutaric acid) with distinct retention factor (Rf) values (Table 1).

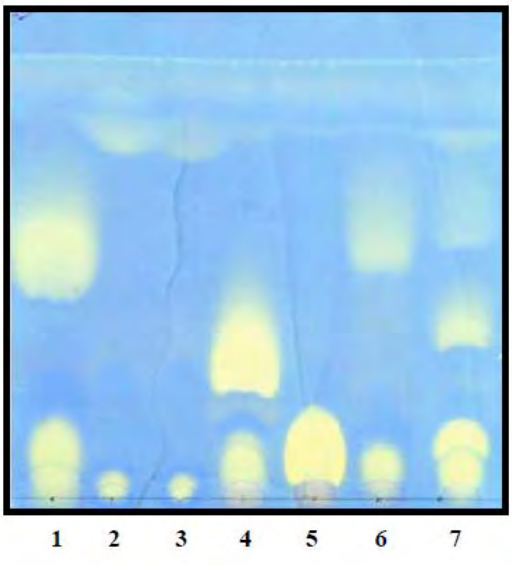

Figure 4 Resolution of different standard organic acids on TLC plate. (1) Succinic acid; (2) lactic acid; (3) fumaric acid; (4) malic acid; (5) citric acid; (6) α-ketoglutaric acid; (7) mixture of these organic acids.

Table 1 Analysis of different organic acids on TLC plate

Organic acids	Rf values
Succinic acid	0.56
Lactic acid	0.81
Fumaric acid	0.81
Malic acid	0.33
Citric acid	0.16
α-ketoglutaric acid	0.58

Succinic acid was resolved in 30 min and showed a prominent yellow spot with an Rf value of 0.56. Among 32 isolated bacterial strains tested from bovine dung in Surin province and buffalo dung in Suphanburi, 11 isolates were found to produce succinic acid. Total 166 isolates from soil in Suphanburi and bovine dung in Nakhonsawan province, 75 isolates were produced succinic acid. In addition, 32 isolates from bark of tree in Nakhonpathom province, 17 isolates were found to produce succinic acid.

Table 2 Summary results of total isolated bacterial strains with succinic acid ability in the present work

Isolates	**Sample source**	**Number of isolates**	**Number of bacteria gram**		**Number of succinic acid producing strains**	**Highest succinic acid (g/l)**
			Positive	**Negative**		
SR1-2	bovine dung from Surin province	14	11	3	3	2.03 g/l (SR2-A1)
SP1-2	buffalo dung in Suphanburi province	18	15	3	8	2.66 g/l (SP2-A1)
SP3-10	soil sample in Suphanburi province	105	99	6	59	51.95 g/l (SP8-B4)
NS1-12	bovine dung in Nakhonsawan	61	59	2	16	50.86 g/l (NS2-B3)
NP1-6	bark of tree from Nakhonprathom	32	32	0	17	56.48 g/l (NP2-A3)

A total number of 230 anaerobic bacterial isolates, 216 isolates were gram positive bacteria and 14 isolates were gram negative bacteria. Only 14 gram negative bacteria were further investigated for the succinic acid production and the results were shown in Table 3.

Table 3 Results of succinic acid production on TLC plates and HPLC analysis by isolated gram negative bacteria

Isolates	Sample sources	Number of gram negative bacteria	Number of positive test on TLC and HPLC	Highest succinic acid (g/l)
SR1-2	bovine dung from Surin province	3	2	1.866 g/l (SR2/A2)
SP1-2	buffalo dung in Suphanburi province	3	2	1.938 g/l (SP1/B2)
SP3-10	soil sample in Suphanburi province	6	1	42.08 g/l (SP8/A4)
NS1-12	bovine dung in Nakhonsawan	2	2	43.26 g/l (NS2/A2)
NP1-6	bark of tree from Nakhonprathom	0	0	-

The results from Table 3, only 7 isolates of gram negative bacteria could be produce succinic acid. Among these strains SP8/A4 and NS2/A2 gave a maximum succinic acid of 42.08 g/l and 43.26 g/l, respectively. Both isolates exhibit a potential succinic producing strains.

3.3 Succinic acid production by *A. succinogenes* DSMZ 22257 in shake flask

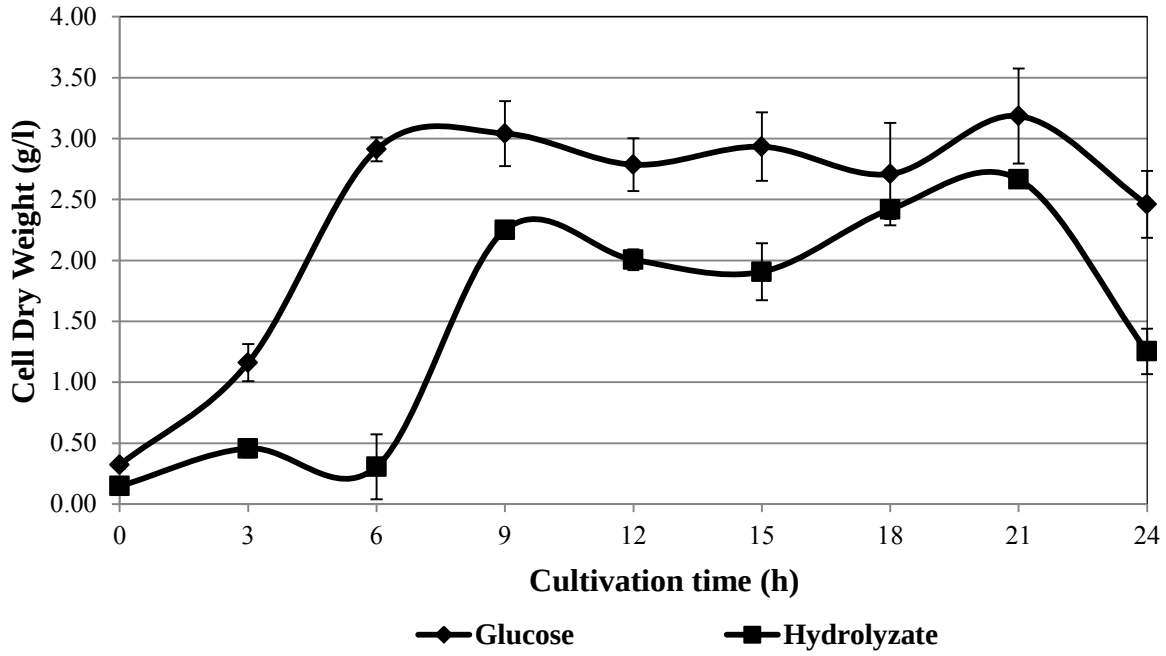

Figure 5 Effect of glucose and sorghum straw hydrolyzate on cell growth of *Actinobacillus succinogenes* DSMZ 22257

As shown in Figure 5, illustrated growth from sorghum straw hydrolysate was seen after a lag phase of 6 h, while no distinct lag phase with glucose as a carbon source. The maximum cell growth of 2.665 g/l was obtained at 21 h of cultivation.

Many studies have reported about inhibitors of microbial growth in diluted acid lignocellulosic hydrolysates, including furfural, hydroxymethylfurfural (HMF), acetic acid (Saha, 2003). The research of Chen and coworker (2011), they found 0.6 g/l of HMF, 0.3 g/l of furfural, 1.0 g/l of formic acid, and 3.4 g/l of acetic acid in corn fiber acid hydrolyzate at a total sugar concentration of 70 g/l. In addition, the other components were limiting in vitamins-supplemented yeast cell hydrolyzed, causing lower succinic acid productivity. Thus, the longer lag phase and lower sugar consumption rate could be a synergistic effect of inhibitors in the acid hydrolyzate.

Table 4 Fermeantation of *Actinobacillus succinogenes* DSMZ 22257 in shake flask using glucose and sorghum straw hydrolyzate as carbon sources

Carbon sources	Succinic acid (g/l)	Formic acid (g/l)	Acetic acid (g/l)	Residual total sugar (g/l)	Sugar utilization (%)	Succinic acid yield (%)
Glucose	44.799±0.394	1.457±1.621	1.120±0.672	0.920±1.273	98.467±2.121	75.828±0.667
SS hydrolyzate	13.820±1.384	2.561±0.130	0.832±0.871	6.500±0.424	89.167±0.070	25.831±2.587

Data are means ± standard deviations of results from triplicate cultures.

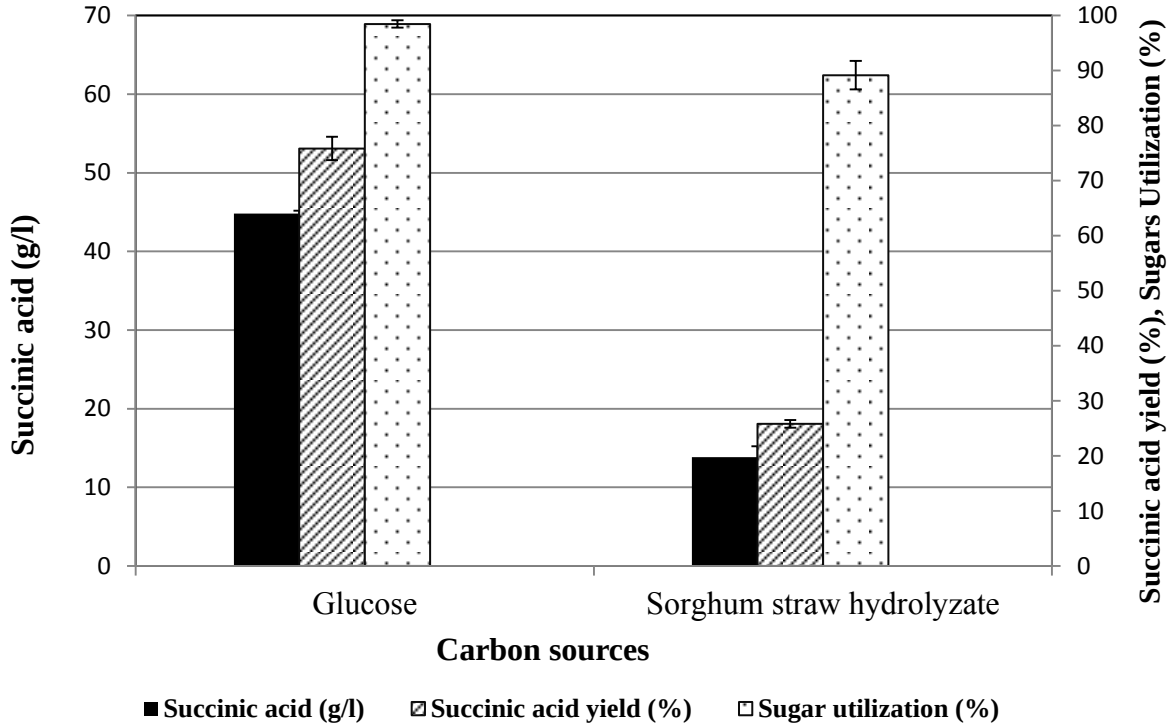

Figure 6 Effect of glucose and sorghum straw hydrolyzate on succinic acid production , succinic acid yield and sugar utilization

(■ Succinic acid (g/l), ▨ Succinic acid yield (%), □ Sugar utilization (%))

From Figure 6, *A.succinogenes* DSMZ 22257 was grown in an anaerobic flask for 48 h with an initial reducing sugar concentration of 60 g/l. Maximum succinic acid of 44.799 g/l and sugars utilization of 98.467% were obtained from glucose as a carbon source. Sorghum straw hydrolyzate gave the lower succinic acid of 13.820 g/l and sugars utilization of 89.167 % and succinic acid yield of 25.831%.

3.4 Succinic acid production by *A. succinogenes* DSMZ 22257 in a 2 L bioreactor

Based on the above results obtained in an anaerobic shake flask, batch fermentations of glucose as a carbon source was carried out in 2 L stirred bioreactor. The results from Table 5 indicated that succinic acid production and sugar utilization in stirred bioreactor were consistent with those in the anaerobic shake flask.

Table 5 Fermeantation of *Actinobacillus succinogenes* DSMZ 22257 in a 2 L bioreactor using glucose as a carbon source

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

As shown in Table 5, the amount of succinic acid increased with the extent cultivation time from 0 to 12 h. After 12 h of cultivation, it had no distinct change in succinic acid yield. Sugar utilization was slightly increased 47.417% to 83.413% from 6 to 48 h of cultivation time. In addition, by products, formic and acetic acid, were continuously increased with the extent cultivation time from 0-48 h.

From Figure 7, the maximum succinic acid of 46.001 g/l was obtained from 12 h of cultivation times while glucose concentration rapidly decreased from 0-12 h. After 12 h was no significant change of succinic acid concentration and slightly decreased glucose concentration. However, the concentrations of acetic acid and formic acid were appreciably higher compared with long cultivation time, which led to a slight decrease in the succinic acid yield from 76.668% to 73.313%. These results indicate that 12 h of cultivation time was optimum for succinic acid fermentation by *A. succinogenes* DSMZ 22257.

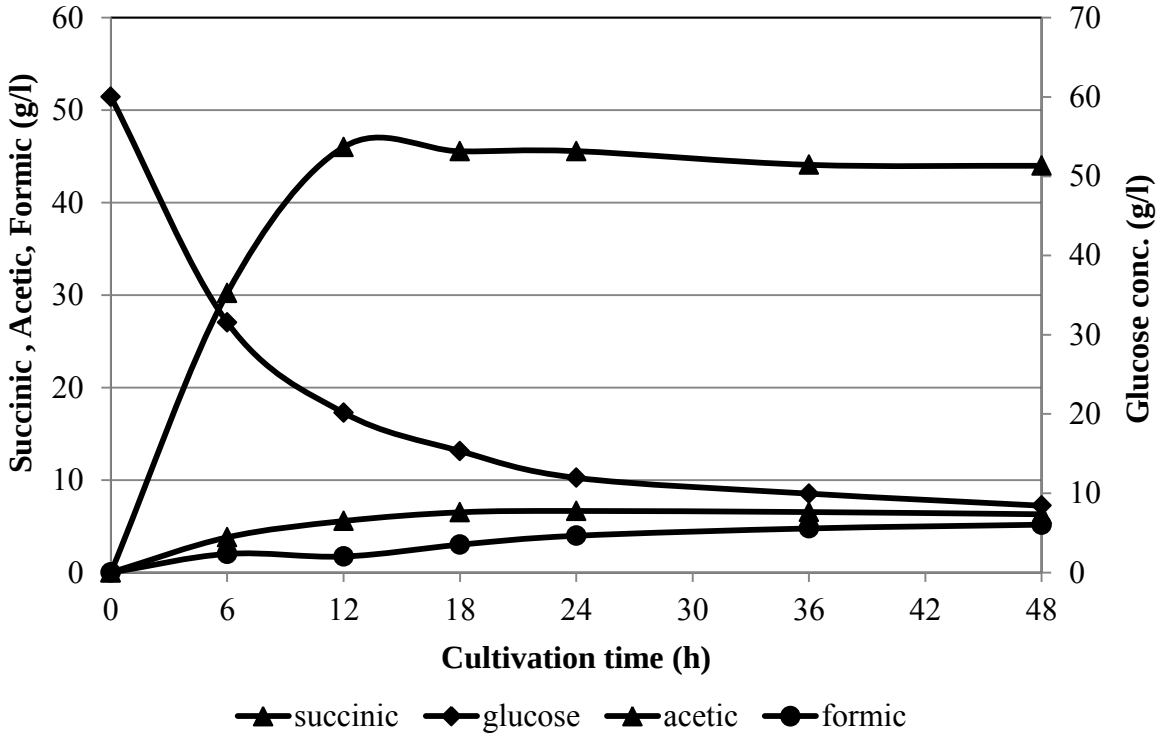

Figure 7 Fermeantation of *Actinobacillus succinogenes* DSMZ 22257 in a 2 L bioreactor using glucose as a carbon source

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

Chapter IV

Conclusion

The samples were collected from Surin (SR1-2), Suphanburi (SP1-10), Nakornsawan (NS1-12) and Nakhonprathom province (NP1-6), Thailand. Two hundreds thirty bacterial isolates with succinic acid ability were obtained. Two hundreds sixteen were gram-positive and fourteen isolates were gram-negative bacteria. Among these gram negative bacterial isolates, SP8/A4 and NS2/A2 were potential for succinic acid production gave a succinic acid concentration higher than 40 g/l.

Actinobacillus succinogenes DSMZ 22257 was used as a representative of gram negative bacteria for investigating growth and succinic acid production from agriculture biomass. Sorghum straw hydrolyzate was used replaced glucose as a carbon source. A growth lag phase was seen for 6 h when using sorghum straw hydrolyzate while no distinct lag phase was seen with glucose as a carbon source and gave a maximum cell growth of 2.665 g/l at 21 h.

Effects of carbon sources on the succinic acid production from glucose and sorghum straw hydrolyzate with an initial reducing sugar concentration of 60 g/l were compared. Maximum succinic acid of 44.799 g/l and sugars utilization of 98.467% were obtained from glucose as a carbon source. Sorghum straw hydrolyzate gave the lower succinic acid of 13.820 g/l and sugars utilization of 89.167 % and succinic acid yield of 25.831%.

The production of succinic acid in a 2 L bioreactor using glucose as a carbon source, a maximum succinic acid of 46.001 g/l was obtained at 12 h of cultivation time. However the attempt to improve the yield of succinic acid production from sorghum straw is undertaken. Furthermore, the potential gram negative bacterial isolates with succinic ability are also carried out.

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APPENDIX A

Standard peaks of organic acid by HPLC

(Rezex ROA-Organic Acid Column)

App ID 5515

Organic Acids

Column:	Rezex ROA-Organic Acid
Dimensions:	300 x 7.8 mm
Part No.:	00H-0138-K0
Mobile Phase:	0.005 N Sulfuric Acid
Flow Rate:	0.5 mL/min
Detection:	UV @ 210 nm
Temperature:	55 °C
Sample:	1. Oxalic 2. Citric 3. Tartaric 4. Succinic 5. Formic 6. Acetic

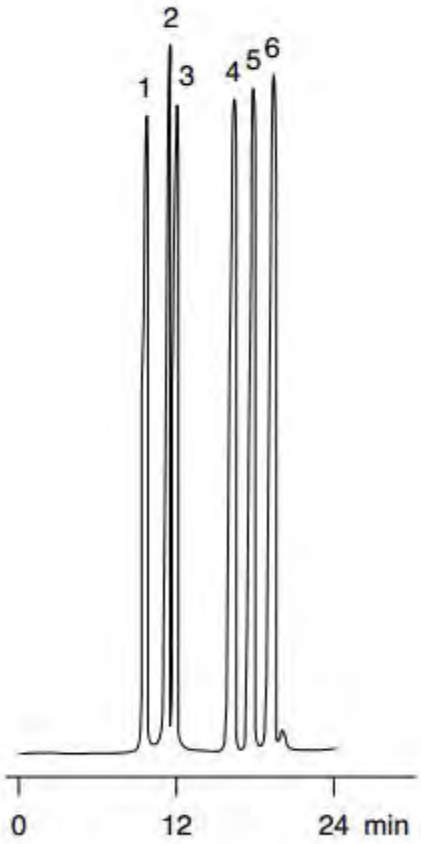

Figure A1 A Standard peaks of organic acid on the Rezex ROA-Organic Acid Column (Oxalic acid, Citric acid, Tartaric acid, Succinic acid, Formic acid and Acetic acid)

APPENDIX B

Calibration curve for various concentration of glucose by DNSA method

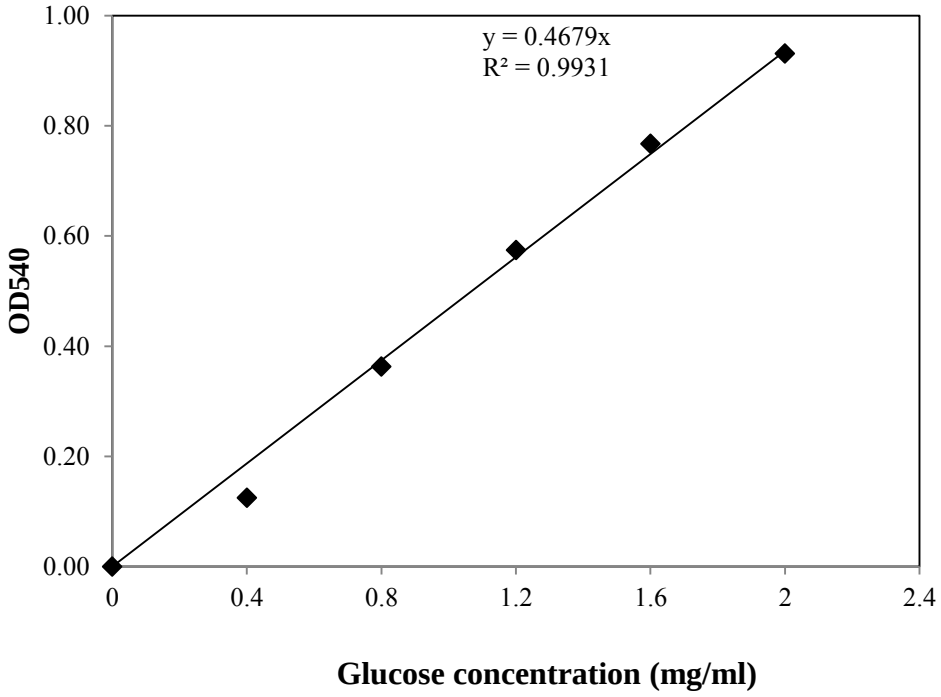

Figure B1 Calibration curve for various concentration of glucose by DNSA method

Equation; $Y = 0.4679\ X$

Glucose concentration (g/l) $= \dfrac{\text{OD } 540}{0.4679}$

ประวัติคณะผู้วิจัย

1. ชื่อหัวหน้าโครงการ

1. ชื่อ

(ไทย) นางสาว ศิริลักษณ์ ธีระดากร

(อังกฤษ) Miss Siriluk Teeradakorn

2. เลขหมายประจำตัวประชาชน 3-1012-02325-69-0

3. ตำแหน่งปัจจุบัน อาจารย์ A5

4. หน่วยงาน สถาบันเทคโนโลยีชีวภาพและวิศวกรรมพันธุศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

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5. ประวัติการศึกษา

ปริญญา	สาขาวิชา	มหาวิทยาลัย	ปีที่ได้รับ พ.ศ.
วิทยาศาสตร์บัณฑิต	จุลชีววิทยา	จุฬาลงกรณ์	2525
วิทยาศาสตร์มหาบัณฑิต	เทคโนโลยีชีวภาพ	จุฬาลงกรณ์	2529
วิทยาศาสตร์ดุษฎีบัณฑิต	เทคโนโลยีชีวภาพ	โอซาก้า (ประเทศญี่ปุ่น)	2541

6. ความชำนาญ/ความสนใจพิเศษ

6.1 เทคโนโลยีการหมัก

6.2 การแปรสภาพชีวมวล

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(ดร. ศิริลักษณ์ ธีระดากร)

(หัวหน้าโครงการวิจัย)

วันที่ 31 เดือนมีนาคม พ.ศ. 2558