การแยกเชื้อแบคทีเรีย *Corynebacterium glutamicum* ที่เจริญและผลิตกรดอะมิโนกลูตามิคได้ดีที่อุณหภูมิสูง จากดินที่ปนเปื้อนมูลนกในจังหวัดร้อยเอ็ด

นางสาวภวรรณตรี ไพศรีศาล

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาชีวเวชศาสตร์
มหาวิทยาลัยเทคโนโลยีสุรนารี
ปีการศึกษา 2556

ISOLATION OF THERMOTOLERANT GLUTAMIC ACID PRODUCING *CORYNEBACTERIUM GLUTAMICUM* FROM AVIAN FECES CONTAMINATED SOIL IN ROI-ET

Pawantree Paisrisan

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

Suranaree University of Technology

Academic Year 2013

ISOLATION OF THERMOTOLERANT GLUTAMIC ACID PRODUCING *CORYNEBACTERIUM GLUTAMICUM* FROM AVIAN FECES CONTAMINATED SOIL IN ROI-ET

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

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เชื้อแบคทีเรีย *Corynebacterium glutamicum* ที่มีความสามารถทนอุณหภูมิสูงและผลิตกรดอะมิโลกลูตามิคได้ดีถูกคัดแยกจากดินและดินที่ปนเปื้อนมูลนกที่เก็บจากจังหวัดร้อยเอ็ดของประเทศไทย จากผลการคัดแยก เราได้รับเชื้อ PP25 PP29 และ PP80 ซึ่งเชื้อเหล่านี้ถูกจำแนกว่าเป็นเชื้อ *C. glutamicum* โดยอาศัยการทดสอบทาง 16S rRNA gene ผลการทดสอบพบว่า ลำดับ 16S rDNA ของ เชื้อ PP25 PP29 และ PP80 มีความคล้ายคลึงกับเชื้อ *C. glutamicum* KY9002 (สายพันธุ์ดั้งเดิม) ด้วยความเหมือนร้อยละ 99

เชื้อ PP25 PP29 และ PP80 สามารถเจริญเติบโตได้ที่อุณหภูมิ 30 ถึง 40 องศาเซลเซียส อุณหภูมิที่เหมาะสมต่อการสร้างกรดอะมิโนกลูตามิคของเชื้อเหล่านี้ คือ 38 ถึง 38.5 องศาเซลเซียส ซึ่งเป็นอุณหภูมิที่ไม่เหมาะสมต่อการเจริญและการสร้างกรดอะมิโนกลูตามิคของเชื้อสายพันธุ์ดั้งเดิม ความสามารถในการเจริญและสร้างกรดอะมิโนกลูตามิคได้ที่อุณหภูมิสูงของเชื้อ PP25 PP29 และ PP80 นี้ชี้ให้เห็นว่า จีโนมโปรไฟล์สำหรับการทนอุณหภูมิสูงและการสร้างกรดอะมิโนกลูตามิคของเชื้อเหล่านี้อาจมีวิวัฒนาการเปลี่ยนแปลงแตกต่างกันไปตามธรรมชาติ

การเปลี่ยนแปลงของโปรไฟล์ของ 16S rRNA gene ของเชื้อ PP25 PP29 และ PP80 ถูกศึกษาโดยการวิเคราะห์ phylogenetic tree ผลการวิเคราะห์พบว่าเชื้อ PP25 PP29 และ PP80 มีสายวิวัฒนาการที่แตกต่างจาก *C. glutamicum* สายพันธุ์ดั้งเดิม โดย PP25 มีสายวิวัฒนาการที่แตกต่างและแยกออกไปจากสายวิวัฒนาการของ PP29 และ PP80

สาขาวิชาเภสัชวิทยา ลายมือชื่อนักศึกษา_____

ปีการศึกษา 2556 ลายมือชื่ออาจารย์ที่ปรึกษา_____

PAWANTREE PAISRISAN : ISOLATION OF THERMOTOLERANT GLUTAMIC ACID PRODUCING *CORYNEBACTERIUM GLUTAMICUM* FROM AVIAN FECES CONTAMINATED SOIL IN ROI-ET. THESIS ADVISOR : Dr. NAWARAT NANTAPONG, Ph.D. 62 PP.

CORYNEBACTERIUM GLUTAMICUM/ THERMOTOLERANT/ AVIAN FECES CONTAMINATED SOIL/ GLUTAMIC ACID/ COOLING COST

Corynebacterium glutamicum, a thermotolerant bacterium with an ability to produce high level of L-glutamic acid, was isolated from soil and soil contaminated with avian feces collected from Roi-Et, Thailand. As a result, we obtained PP25, PP29, and PP80 strains which were identified as *C. glutamicum* based on 16S rRNA gene analysis. The results revealed that 16S rDNA sequences of PP25, PP29, and PP80 were closely related to *C. glutamicum* KY9002 with 99% similarity.

The PP25, PP29, and PP80 were able to grow at a temperature range from 30-40°C. The optimum temperature range for L-glutamic acid production of these strains was at 38-38.5°C which was not an appropriate fermentation condition for the typical strain. The ability to ferment L-glutamic acid at an elevated temperature of PP25, PP29, and PP80 suggested that genomic profiles for thermal tolerance and glutamate productivity of these strains probably evolved spontaneously in nature.

The evolutionary of 16S rRNA gene profiles of PP25, PP29, and PP80 were determined by phylogenetic tree analysis. The results showed that PP25, PP29 and

PP80 were phylogenetically distinct from the *C. glutamicum* KY9002. The results suggested that PP25 diverged from a common ancestor of PP29 and PP80.

School of Pharmacology Student's Signature_____

Academic Year 2013 Advisor's Signature_____

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Pawantree Paisrisan

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LIST OF ABBREVIATIONS

μg/ml	=	Microgram per milliliter
μl	=	Microliter
AFLP	=	Amplified fragment length polymorphism analysis
C. glutamicum	=	*Corynebacterium glutamicum*
DNA	=	Deoxyribonucleic acid
EMP	=	Embden-Meyerhof-Parnas
EtBr	=	Ethidium bromide
g/l	=	Gram per liter
HEPE	=	High-energypulse-electron
LB	=	Luria-Burtani
mg/ml	=	Milligram per milliliter
MSG	=	Monosodium glutamate
NADH	=	Nicotinamide adenine dinucleotide
PCR	=	Polymerase chain reaction
RF	=	Retention factor
rRNA	=	Ribosomal ribonucleic acid
TBE	=	Tris-borate-EDTA
TCA	=	Tricarboxylic acid cycle
v/v	=	Volume by volume
w/v	=	Weight by volume

CHAPTER I

INTRODUCTION

1.1 Background/Problem

Corynebacterium glutamicum is an aerobic, Gram-positive, non-sporulating and biotin auxotroph bacterium. It was first isolated from soil by Kinoshita in 1956 (Kinoshita, Udaka and Shimono, 1957). *C. glutamicum* is an important industrial microorganism for its high production of amino acids such as glutamate and lysine (Hermann, 2003; Leuchtenberger, Huthmacher and Drauz, 2005; Toshihiro, Fukuda and Kondo, 2007). L-Glutamate in the form of monosodium L-glutamate (MSG) has been used as a flavor enhancer, a precursor of drugs, cosmetics, pharmaceutical compounds, fodder and as supplements (Bourke and Kohn, 2003; Department of Energy Cold Fusion Review, 2004). Various methods of culturing *C. glutamicum* have been employed in order to maximize the productivity of L-glutamic acid. For example, excretion of glutamic acid can be induced by biotin limitation, surfactants addition, antibiotics addition and temperature shift (Delaunay, Lapujade, Engasser and Goergen, 2002; Duperray, Jezequel, Ghazi, Letellier and Shechter, 1992; Nunheimer, Birnbaum, Ihnen and Demain, 1970; Shiio, Otsuka and Takahashi, 1962). However, biotin limitation strategy is the dominating process used for the commercial production of glutamate (Zheng et al., 2002).

In the process of L-glutamic acid fermentation, temperature is one of the most important factors directly related to the production efficiency. Most of microbe-based

industrial strains are usually high temperature sensitive. The productivity of L-glutamic acid is gradually decreased when the culture temperature is raised by heat released along the fermentation. Thus, a cooling system has to be employed to remove the heat, providing an appropriate environment for cell growth and amino acid production. Therefore, thermotolerant strains are preferred in the production processes due to their low cost in temperature control. For these reasons, the researchers have been try to isolate the novel glutamate producing bacteria that can grow at high temperature. This is particularly relevance to reduce costs and improve productivity of glutamate fermentation.

In this study, we attempted to isolate thermotolerant *C. glutamicum* strains that could efficiently produce and secrete high amount of L-glutamic acid from soils in Northeastern Thailand.

1.2 Research objectives

1.2.1 To isolate and identify *C. glutamicum* strains that can grow and produce glutamic acid at high temperatures.

1.2.2 To compare the growth and the ability of glutamic acid production between wild-type and isolated strains.

1.3 Research hypothesis

Soil and avian feces contaminated soil obtained from Roi-Et, Thailand. These soils have a high amount of ammonia, which was suitable for the growth of *C. glutamicum*. Therefore, the research hypothesis of this study was that

thermotolerant *C. glutamicum* could be isolated from soil and avian feces contaminated soil obtained from Roi-Et, Thailand.

1.4 Scope and limitation of the study

This work involves the isolation of *C. glutamicum* from soil collected from Roi-Et, Thailand. Identification of *C. glutamicum* was primarily based on the taxonomic comparison with wild-type strain. Finally, the growth and the ability of glutamic acid production between wild-type and isolated strains were compared.

1.5 Expected results

The expected result from this study was to obtain the strain of *C. glutamicum* that can grow and produce high level of glutamic acid at higher temperatures.

CHAPTER II

LITERATURE REVIEW

2.1 General information of glutamic acid

Glutamic acid is one of many amino acids that are the building blocks of proteins. The chemical structure of glutamate is show in Figure 2.1. Glutamic acid is a multifunctional amino acid involved in taste perception, excitatory neurotransmission and intermediary metabolism. It plays an important role in gastric phase digestion with multiplicity effects in the gastrointestinal tract when consumed with nutrients by enhancing gastric exocrine secretion (Zolotarev, Khropycheva, Uneyama and Torii, 2009). A number of studies have also shown the possible usefulness of glutamic acid in enhancing nourishment in the elderly and in patients with poor nutrition (Kondoh, Mallick and Torii, 2009).

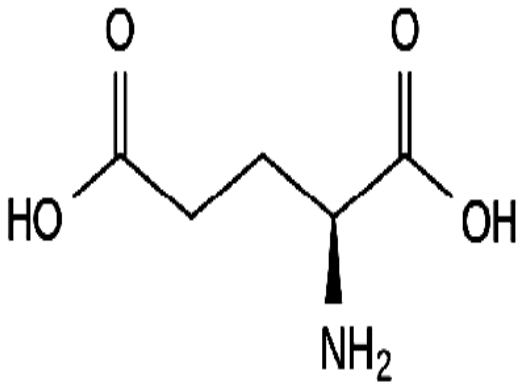

Figure 2.1 Chemical structure of glutamate (Jinap and Hajeb, 2010).

Glutamate is found in two forms, bound and free forms, in nature. Bound form of glutamate is linked to protein, while free glutamate is not bound together with other amino acids in protein. Only free form of glutamate affects in enhancing the flavour of food and gave the unique taste known as umami, delicious taste, to food (Kurihara, 2009). The umami taste was first described in 1908 by Kikunae Ikeda (Ikeda, 1908), the professor at the University of Tokyo. He began a research project to identify the substance in konbu, kelp like seaweed, which had been used for the preparation of soup stocks in Japan for many centuries. His research was base on the hypothesis that one or more taste substances may exist in kelp that could not be categorized as bitter, sour, salty, or sweet. In the same year, he had discovered the sodium salt of glutamic acid to be the taste component. Then, he filed a patent claim for a process to produce monosodium glutamate (MSG), as a new seasoning (Ikeda, 1908). In 1909, Saburousuke Suzuki began collaboration with Ikeda and then the first monosodium glutamate was produced commercially under the trade name Ajinomoto. L-Glutamate in the form of monosodium L-glutamate has been used as a flavor enhancer, a precursor of drugs, cosmetics, pharmaceutical compounds, fodder and as supplements. As reported by Kumagai (Kumagai, 2000), the estimated worldwide production of MSG was one million tons in 2001. Hermann (Hermann, 2003) reported that the annual production level of MSG is around 1.5 million tons and the market is growing by 6% each year. A recent survey shows that the annual production in 2009 is about 2 million tons (Sano, 2009). The production of monosodium L-glutamate is shown in Table 2.1.

Table 2.1 Production of monosodium glutamate (MSG).

Years	Production of MSG (ton/year)
1954	7,652
1960	22,177
1970	92,935
1988	340,000
1994	592,000
2001	1,000,000
2003	1,100,000
2006	1,500,000
2009	2,000,000

2.2 History of glutamic acid production

The manufacturing of glutamic acid was first produced by extraction method in which vegetable proteins like soy meal, wheat gluten, corn gluten, or yeast were hydrolyzed with hydrochloric acid (HCl) (Ikeda, 1908). The hydrolysate was then concentrated under reduced pressure, further acidified by the addition of concentrated HCl. Finally, hydrolysate was cooled to crystallize glutamic acid hydrochloride and then purified as MSG. However, production of glutamic acid by hydrolysis was limited because of the technical drawbacks of this method (Sano, 2009). For example, hydrogen chloride gas generated from hydrolysis method caused environmental problems including air pollution and toxic to human health.

During the Second World War American quartermasters realized that Japanese army rations tasted great. After the war, they introduced monosodium glutamate to the food industry and the world-wide use of monosodium glutamate began to extensive. The demand for glutamic acid was increased after World War II (Leuchtenberger, Huthmacher and Drauz, 2005). However, the amount of glutamic acid obtained from protein hydrolysis was very little and high production costs. Thus, it was not suitable

for large-scale production of glutamic acid. To meet growing demand for L-glutamic acid, the development of a more inexpensive and efficient process for producing L-glutamic acid is desired (Izui, Moriya, Hara and Ito, 1999).

Nowadays, glutamic acid is largely produced through microbial fermentation method. This method could reduce the production costs and environmental problems (Sano, 2009). The fermentation method is a production process of glutamic acid by culturing glutamic acid producing bacteria in a liquid medium contained carbohydrate and ammonium under aerobically condition (Figure 2.2). The glutamic acid producing bacteria synthesized glutamic acid and excreted it into the medium. The cultured medium is filtered and the glutamic acid precipitated as monosodium glutamate crystals, which are dried before packing for use in the food industry and as seasoning (Leung and Foster, 1996).

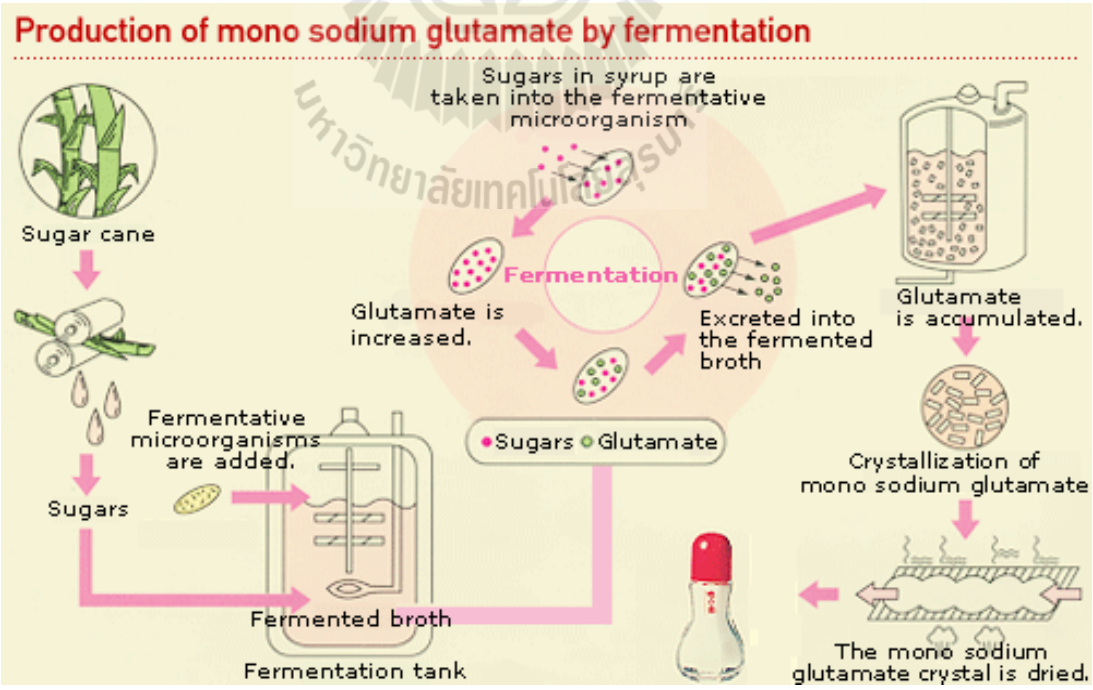

Figure 2.2 Production of monosodium glutamate by fermentation (George, 2004).

The fermentative production of glutamate is the process that utilized glucose as a sole carbon source (Kelle, Hermann and Bathe, 2005; Kimura, 2005). One molecule of glucose breaks down into two molecules of pyruvate through the Embden-Meyerhof-Parnas (EMP) pathway. Pyruvate is converted into acetyl-CoA and is channeled into the tricarboxylic acid (TCA) cycle. The acetyl-CoA condenses with oxaloacetate to form citrate, and then converted to isocitrate. Isocitrate converted to α-ketoglutarate, which is the key precursor of glutamic acid. α-Ketoglutarate converted into L-glutamic acid through reductive amination with free ammonium (NH_4^+) ions (Shiio, Otsuka and Tsunoda, 1959). The glutamate production pathway from glucose is shown in Figure 2.3.

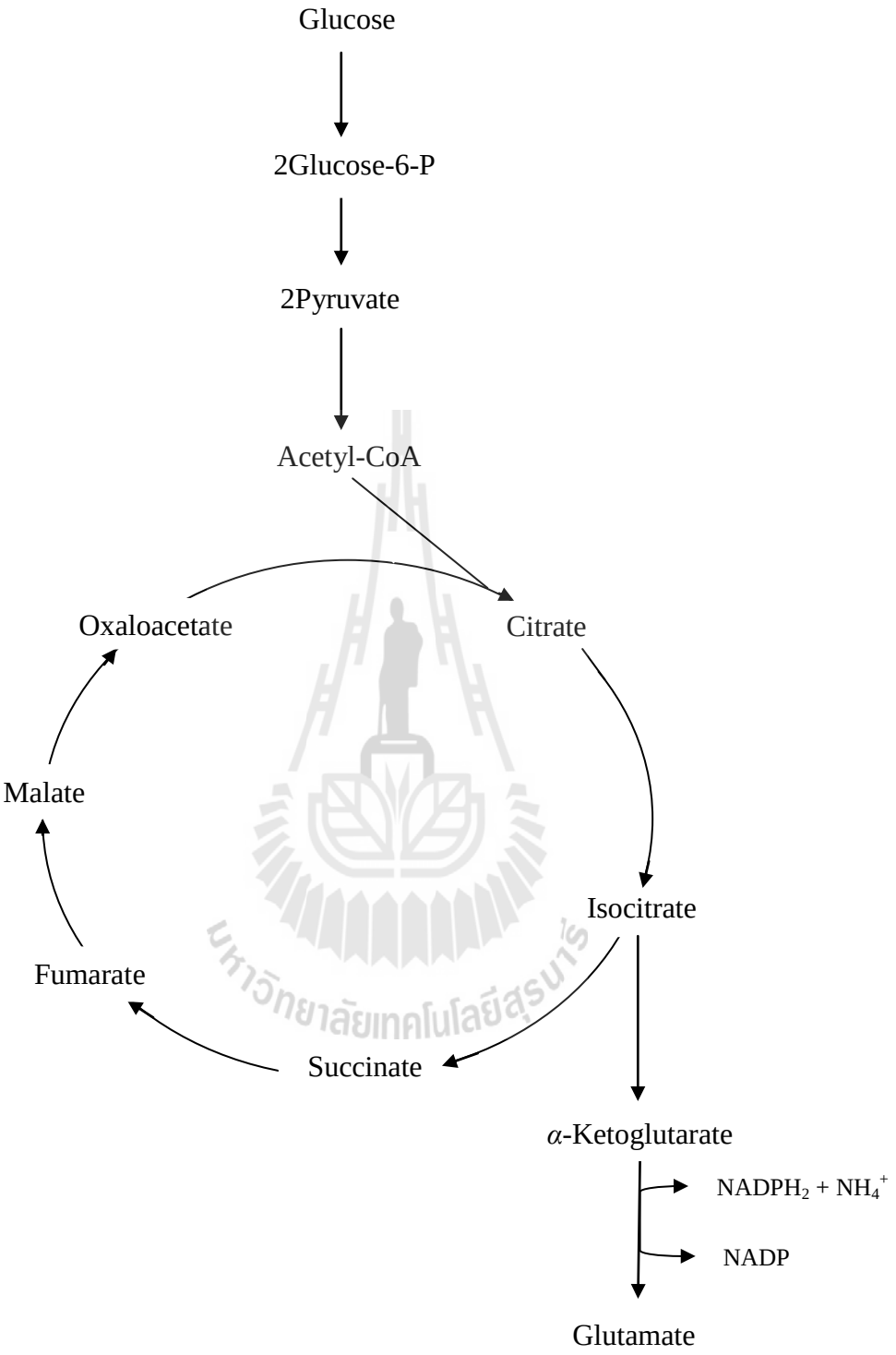

Figure 2.3 Biosynthesis of glutamic acid using glucose as the carbon source (Modified according from Kinoshita and Nakayama, 1978).

The fermentation process for industrial production of glutamic acid by microorganisms was first introduced by Kinoshita and co workers in 1957 (Kinoshita, Udaka and Shimono, 1957). They isolated the novel bacterium, *Corynebacterium glutamicum*. It was originally isolated from a soil sample contaminated with avian feces, collected from Tokyo, Japan. *C. glutamicum* was identified as a glutamic acid producing bacteria because its ability to produce and secrete high amount of glutamic acid into the culture medium.

C. glutamicum is a Gram positive, rod-shaped (Figure 2.4), non-sporulating, and non-motile bacterium. The cells were often arranged in V-formations (Figure 2.4) (Abe, Takayama and Kinoshita, 1967; Yamada and Komagata, 1972). Most strains of *C. glutamicum* formed pale yellow, cream-white or yellow colonies (Figure 2.5) (Helene, Joachim and Siegfried, 2002; Hideaki et al., 2008; Seiler and Hennlich, 1983; Wolfgang, 2006; Woodruff, 1981).

C. glutamicum occur commonly in nature in soil, soil contaminated with bird feces, sewage and manure, vegetables, and fruits. These natural habitats have high ammonia, nitrogen source, which is appropriate for growth of *C. glutamicum*.

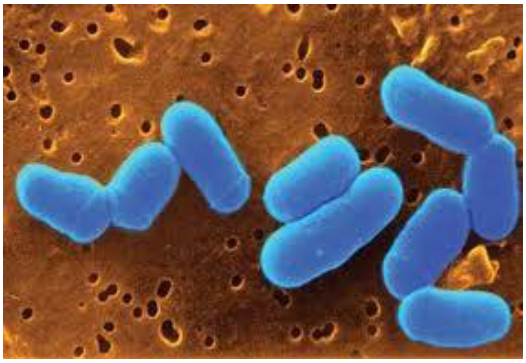

Figure 2.4 The cells of *C. glutamicum* were often arranged in V-formations (Abe, Takayama, and Kinoshita, 1967).

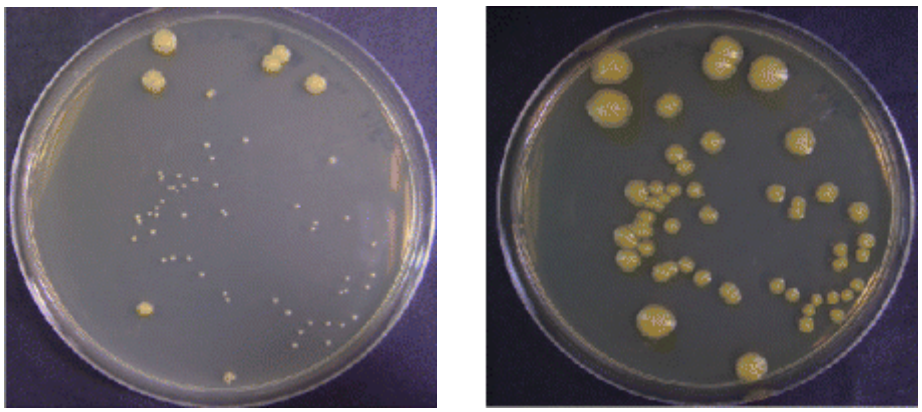

Figure 2.5 Colony morphology of *C. glutamicum* (Hideaki et al., 2008).

The cell wall of *C. glutamicum* has a special structure which is different from other gram positive bacteria. It has thick cell wall consisting of arabinogalactan and mycolic acids, which is limited to secretion process through the cell wall. Mycolic acids are the major components in the surface bilayer of the cell wall of mycolata bacteria, *Corynebacteria*, *Nocardia* and *Mycobacteria* genera (Eggeling, Krumbach and Sahm, 2001). A schematic model of the *C. glutamicum* cell wall is shown in Figure 2.6.

The related species of *C. glutamicum* such as *Corynebacterium lilium*, *Corynebacterium callunae*, *Corynebacterium herculis*, *Brevibacterium fravum*, *Brevibacterium lactofermentum*, *Brevibacterium divaricatum*, *Brevibacterium ammoniagenes*, *Brevibacterium thiogenetalis*, and *Microbacterium ammoniaphilum* were later isolated and identified as glutamic acid producing strains (Abe, Takayama and Kinoshita, 1967; Minnikin, Collins and Goodfellow, 1979; Suzuki, Kaneko and Komagata, 1981). They were able to produce L-glutamic acid from carbohydrate under aerobic condition. However, *C. glutamicum* is commonly used on an industry because of its highest excretion of glutamic acid.

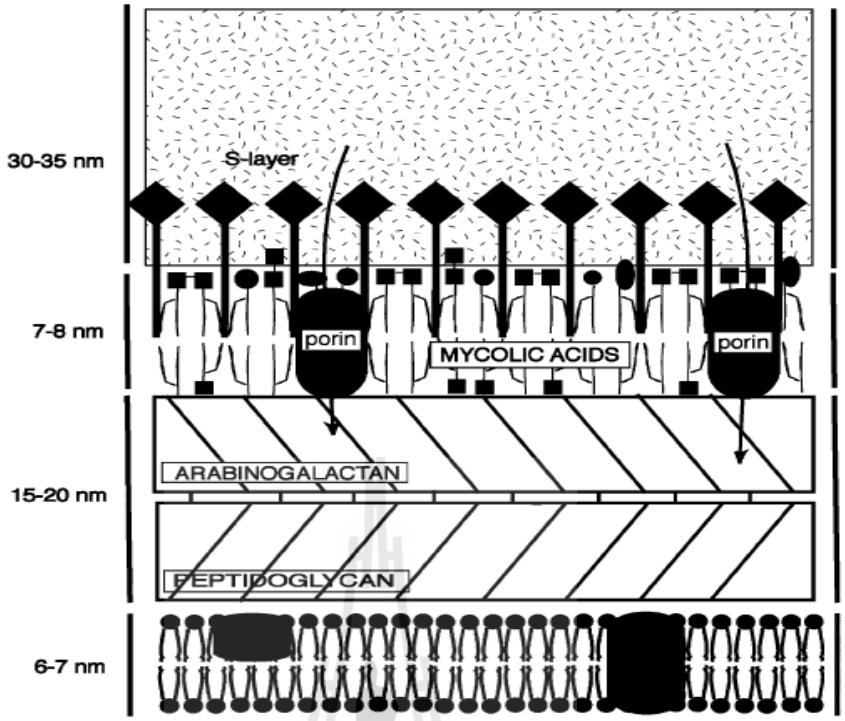

Figure 2.6 A schematic model of the *C. glutamicum* cell wall (Eggeling, Krumbach, and Sahm, 2001).

Glutamate excretion of *C. glutamicum* can be induced by biotin limitation (Hoischen and Krimer, 1989) or penicillin treatment (Eggeling, Krumbach and Sahm, 2001) or a fatty acid ester surfactant (Duperray, Jezequel, Ghazi, Letellier and Shechter, 1992). *C. glutamicum* is a biotin auxotroph which requires biotin for cell growth, but L-glutamic acid excretion is typically performed under biotin-limited conditions. Biotin limitation triggers glutamate excretion by inhibition of fatty acid synthesis which leads to a decrease availability of phospholipids and consequently membrane alterations. Due to a decreased fatty acid and phospholipid content under biotin limitation, the cell membrane permeability for glutamate is enhanced (Hoischen and Krimer, 1989). Under the condition of excess biotin, the glutamate secretion was induced by adding of penicillin or surfactants such as Tween 40 and Tween 60.

Addition of surfactants such as Tween 40 and Tween 60 inhibited fatty acid synthesis, while addition of penicillin inhibited peptidoglycan biosynthesis. It has been considered that surfactants and penicillin directly attack the cell membrane and cell wall, respectively, resulting in the alterations of cell surface structure. Since these treatments correlated with alterations in cell surface structure, it had been thought that glutamate leaks passively through a membrane. However, the mechanism of the glutamate production and excretion of *C. glutamicum* is still unclear (Hoischen and Krämer, 1990).

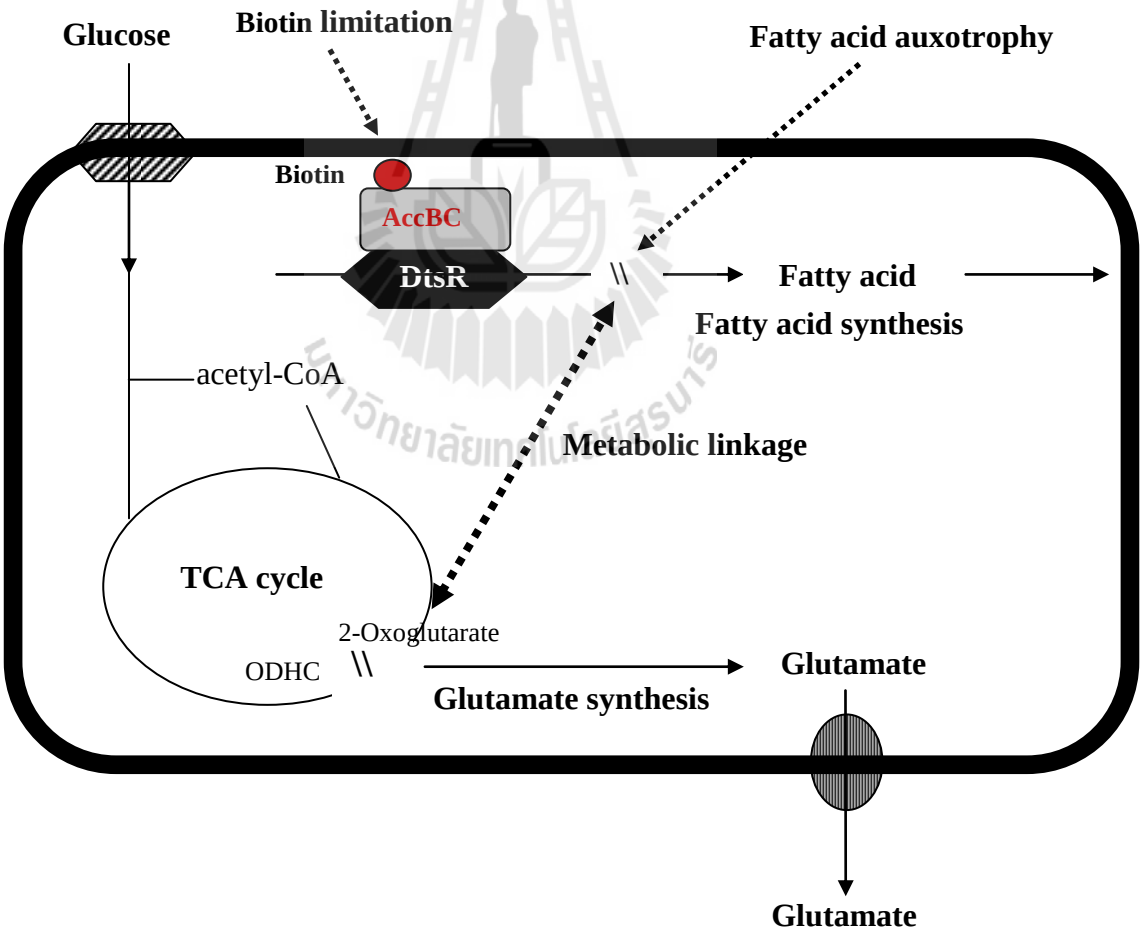

Figure 2.7 Model for the induction of glutamate excretion by *C. glutamicum* (Nakamura, Hirano, Ito and Wachi, 2007).

2.3 Application of thermotolerant microorganisms in the fermentation industry

In the fermentation process, temperature is one of the most important factors directly related to the production efficiency. The productivity is gradually decreased when the temperature of the medium rises due to fermentation heat generated by the microorganism themselves during the fermentation because of most of microorganisms used on an industrial scale are usually high temperature sensitive. In addition, global warming situation has brought indoor temperature increase beyond 30°C even in night time in many countries (Moghadami, Soudi, Sepehr and Rezvanianzade, 2013). That is the serious challenge to not only glutamic acid fermentation but also other fermentation industries, since they need a large amount of cooling water to maintain the optimum fermentation temperature. In submerged cultures, a large amount of heat is generated during fermentation and thus cooling costs become rather expensive. These problems could be alleviated by the use of thermotolerant strain. Use of thermotolerant microorganisms can be exemplified as one improvement of fermentative production using microorganism for industrial application. Therefore, thermotolerant microorganisms are preferred in the fermentation process due to their low cost in temperature control.

In the ethanol fermentation process, *Saccharomyces cerevisiae* has been used. Most of *S. cerevisiae* used for ethanol production on an industrial scale are usually mesophilic group (28-35°C). Thus, thermotolerant *S. cerevisiae* is preferred to reduce cooling cost of ethanol fermentation.

Balakumar et al. (2001) isolated thermotolerant yeast from distillery of the Jaffna peninsula, Sri Lanka. The selected strain was identified as *S. cerevisiae* S1.

This strain was adapted by heat treating underwent 15 cycles, 50°C each for 3 h. Therefore, the thermotolerance of yeast strain was developed. Ethanol production of *S. cerevisiae* S1 was 46 g l^{-1}, 38 g l^{-1}, and 26 g l^{-1} at 40, 43, and 45°C, respectively in nutrient medium consisted of 100 g glucose.

Fernández et al. (2003) isolated thermotolerant *S. cerevisiae* from the residual cream. The selected strain was evaluated in laboratory-scale batch fermentations at temperatures between 30 and 45°C. The high ethanol yield of this strain was 0.41 g g-1 at 40°C demonstrated the thermotolerance of the selected strain.

In 2008, Dong-jian and coworkers improved the thermotolerance and ethanol tolerance of an industrial yeast strain SM-3 by genome shuffling technique (Dong-jian, Chang-lu and Kui-ming, 2009). They obtained F34 strain, which could grow on plate cultures up to 55°C. It was found capable of completely utilizing 20% (w/v) glucose at 45-48°C, producing 9.95% (w/v) ethanol, and tolerating 25% (v/v) ethanol stress.

Ying et al. (2012) improved *S. cerevisiae* by using genome shuffling. They obtained improved strain, R32. The R32 strain produced 81.4 ± 2.7 g/l ethanol after fermentation at 42°C, which were higher than an original strain.

In 2012, Zhang et al. (2012) improved thermotolerance of *S. cerevisiae* by treated with high-energypulse-electron (HEPE) beam radiation. After HEPE beam radiation, they obtained the Y43 strain grew well at 45°C, which indicated that this strain could be advantageous for fermentation at higher temperatures. The ethanol production of Y43 was 83.1 g l^{-1} after fermentation at 43°C for 48 h. The ethanol yield of Y43 was 0.42 g g^{-1}, which was about 81.5% of the theoretical yield.

Fakruddin et al. (2013) isolated and characterized stress tolerant, high potential ethanol producing yeast strains from agro industrial waste of Bangladesh. The strains were coded as P, C, T and DB2. All the selected strains in this study were able to grow at temperature up to 46°C.

For industrial acetic acid fermentation, the major genera such as *Acetobacter*, *Gluconobacter*, and *Gluconoacetobacter* were used. Optimum temperature in the process of acetic acid fermentation is about 30°C and slight temperature increase by 2-3°C causes a serious failure in both fermentation rate and fermentation efficiency. Thus, thermotolerant strains of acetic acid bacteria that can work optimally at 37-40°C are also preferred to use in acetic acid fermentation. However, little has so far been reported about vinegar fermentation by thermotolerant acetic acid bacteria.

Saeki et al. (1997) screened some thermophilic acetic acid bacteria showing the same fermentation efficiency at 38-40°C as that of mesophilic strains at 30°C.

In 1980, Ohmori et al. (1980) isolated three *Acetobacter* strains with the ability to produce acetic acid at 37°C from vinegar mash. Isolated strain retained full activity to produce acetic acid in continuous submerged culture at 35°C and produced 45% of activity at 38°C, while the usual strain of *Acetobacter* completely lost its activity at 35°C. Thus the use of this strain may reduce the cooling costs of industrial vinegar production.

In 1999, Lu et al. (1999) isolated thermotolerant bacteria with high production of acetic acid from spoiled banana in Taiwan. The isolate, I14-2, was considered to be an *Acetobacter* sp. according to phenotypic and chemotaxonomic characteristics. This bacterium retained 97% and 68% of acetic acid-producing activity after 3 d incubation

at 35°C and 37°C, respectively, compared with that when incubated at 30°C. Thus, they suggested that this strain was thermotolerance.

Bassirou et al. (2007) isolated a thermotolerant acetic acid bacterium, designated strain CWBI-B418T, in Senegal from mango fruit. Optimal growth temperature on YGM medium of isolate is 35°C, but good growth is observed between 28 and 40°C.

2.4 Application of thermotolerant *C. glutamicum* in glutamic acid fermentation

For glutamate production, thermotolerant *C. glutamicum* strains are preferred in the production processes due to their low cost in temperature control. Many researchers tried to improve thermotolerance ability of *C. glutamicum* by temperature shift and genome shuffling method.

Delaunay et al. (2002) developed a fed-batch process for a temperature-sensitive strain *C. glutamicum* 2262, which produced glutamate titers up to 85 g/L in a biotin-rich medium by first growing at 33°C, and then subjected to a temperature shift to 39°C.

In 2012, Zheng et al. (2012) improved the thermotolerance of *C. glutamicum* by genome shuffling technique. They obtained the shuffled strain, F343, which could grow at 44°C, while even no growth was observed for parental strains at this temperature. The yield of L-glutamic acid of F343 was increased by 1.8-time comparing with that of the ancestor strains at 38°C. The genetic diversity between F343 and the ancestor strain was also evaluated by Amplified Fragment Length Polymorphism (AFLP) analysis. The result suggested that the phenotypes for both thermotolerance and L-glutamic acid production in F343 were evolved.

For this reason, we attempt to isolated novel thermotolerant *C. glutamicum*. We hope that, the thermotolerant *C. glutamicum* obtained from our study could be use on an industrial scale. In addition, it may be use as a model organism to develop glutamic acid producing ability.

CHAPTER III

MATERIALS

3.1 Bacterial strains

A type strain of *C. glutamicum*, KY9002 (ATCC13032), was kindly gifted from Kyowa Hakko Kogyo (Tokyo, Japan).

Escherichia coli DH5α (F-, φ80d*lacZ*ΔM15, Δ(*lacZYAargF*)U169, *deoR*, *recA*1, *endA*1, *hsdR*17(rk-, mk+), *phoA*, *supE*44, λ-, *thi*-1, *gyrA*96, *relA*1) (Woodcock et al., 1989) was used for transformation and plasmid construction.

3.2 Media

Luria-Burtani (LB) medium contained (per 1 liter) 5 g of yeast extract, 10 g of tryptone, 10 g of NaCl. The final pH was adjusted to 7.0.

Glucose minimum medium contained (per 1 liter) 10 g of glucose, 1 g of KH_2PO_4, 3 g of K_2HPO_4, 3 g of NH_4Cl, 2 g of urea, 0.5 g of $MgSO_4$-$7H_2O$, 0.01 g of $FeSO_4$-$7H_2O$, 0.001 g of $MnSO_4$-$7H_2O$, 0.5 g of casamino acid, 0.001 g of thiamin-HCl, 30 µg of biotin, and 1 ml of metal mixture. One liter of metal mixer was prepared by dissolving 0.990 g of $FeSO_4$-$7H_2O$, 0.880 g of $ZnSO_4$-$7H_2O$, 0.393 g of $CuSO_4$-$5H_2O$, 0.072 g of $MnCl_2$-$4H_2O$, 0.088 g of $Na_2B_4O_7$-$10H_2O$, and 0.037 g of $(NH_4)_6Mo_7O_2$-$4H_2O$ in distilled water. The pH of the medium was adjusted to 7.2.

Nitrate broth contained (per 1 liter) 3 g of beef extract, 5 g of peptone, 1 g of KNO_3. The final pH was adjusted to 7.2.

Christensen's urea medium was prepared by dissolving 1 g of peptone, 1 g of glucose, 2 g of KH_2PO_4, 5 g of NaCl in 1 liter of distilled water. The final pH was adjusted to 6.8-6.9.

Nutrient gelatin medium contained (per 1 liter) 3 g of beef extract, 5 g of peptone, and 120 g of gelatin. The final pH was adjusted to 7.0.

Sodium casein agar consisted of (per 1 liter) 2 g skimmed milk, 1 g glucose, 0.2 g K_2HPO_4, 0.2 g $MgSO_4$-$7H_2O$, 0.001 g $FeSO_4$-$7H_2O$ and 15 g agar. The pH of medium was adjusted to 7.0.

Carbohydrate fermentation broth (pH 7.0) was prepared by dissolving 3 g of beef extract, 5 g of peptone, 10 g of carbohydrate, and 4 ml of 1.6% phenol red in 1 liter of distilled water.

All solid media were prepared by adding 15 g of agar into 1 liter of medium.

The sterilization of the medium was performed by autoclaving at 120°C, 15 p.s.i. for 20 min.

Bile esculin agar slant was purchased from MedEx solutions limited partnership, Thailand. Bile esculin agar contained (per 1 liter) 40 g of bile salts, 5 g of meat peptone, 3 g of meat extract, 1 g of esculin, 0.5 g of $C_6H_{11}O_7FeNO_7$ and 1.5% of agar. The pH of bile esculin agar was 6.6-6.8.

3.3 Antibiotics

Narlidixic acid (Sigma-Aldrich, USA) stock solution was prepared by dissolving in sterile distilled water to give the final concentration of 50 mg/ml.

Ampicillin (Bio basic, Canada) stock solution was prepared by dissolving in sterile distilled water to give the final concentration of 100 mg/ml.

All the antibiotic stock solutions were filter sterilized through a 0.2 µm pore filter (Sartorius stedim biotech, USA) and stored at -20°C until use.

3.4 Buffers and solutions

3.4.1 Buffers for gel electrophoresis of DNA

1x TBE buffer consisted of 89 mM Tris-HCl (pH 8), 89 mM boric acid, and 2.5 mM EDTA.

6x Gel loading dye contained 10 mM Tris-HCl (pH 7.5), 50 mM EDTA, 30% glycerol, 0.06% bromphenol blue, and 0.12% orange G.

3.4.2 Solutions for determination of glutamate

The solvent system consisted of 60% (v/v) butanol, 15% (v/v) glacial acetic acid, and 25% (v/v) distilled water.

Ninhydrin solution consisted of 0.2% (w/v) ninhydrin in saturated butanol.

3.4.3 Solutions for gram staining

Primary stain solution contained (per 100 ml) 2 g of crystal violet, 20 ml of 95% ethanol, and 80 ml of 1% $(NH_4)_2C_2O_4$.

Counter stain solution composed of (per 100 ml) 10 ml safranin O (2.5% safranin O (w/v) in 95% ethanol), and 90 ml distilled water.

Mordant solution composed of 0.33 g of iodine, 0.66 g KI, and 100 ml distilled water.

All solutions were stored in amber bottle until use.

3.5 Primers

The names of the oligonucleotides and their sequences are listed in Table 3.1.

Table 3.1 List of primers used in this work.

Name	Sequences (5'-3')	Sources
C.ndh-N1	CTTGCCGTGCGCGTCGACCAGCAAACGCTA	Nantapong et al., 2004
C.ndh-C3/1	GCGGAATTCACCTGCGGTACCTCACACGTC	Nantapong et al., 2004
27F	AGAGTTTGATCCTGGCTCAG	Lane, 1991
1525R	AAAGGAGGTGATCCAGCC	Lane, 1991
M13/pUC Forward	CCCAGTCACGACGTTGTAAAACG	Helianti et al., 2010
M13/pUC Reverse	AGCGGATAACAATTTCACACAGG	Helianti et al., 2010

3.6 Plasmid

Plasmid pUCm-T used in this work was purchased from Bio Basic, Canada. Map of this plasmid is shown in Figure 3.1.

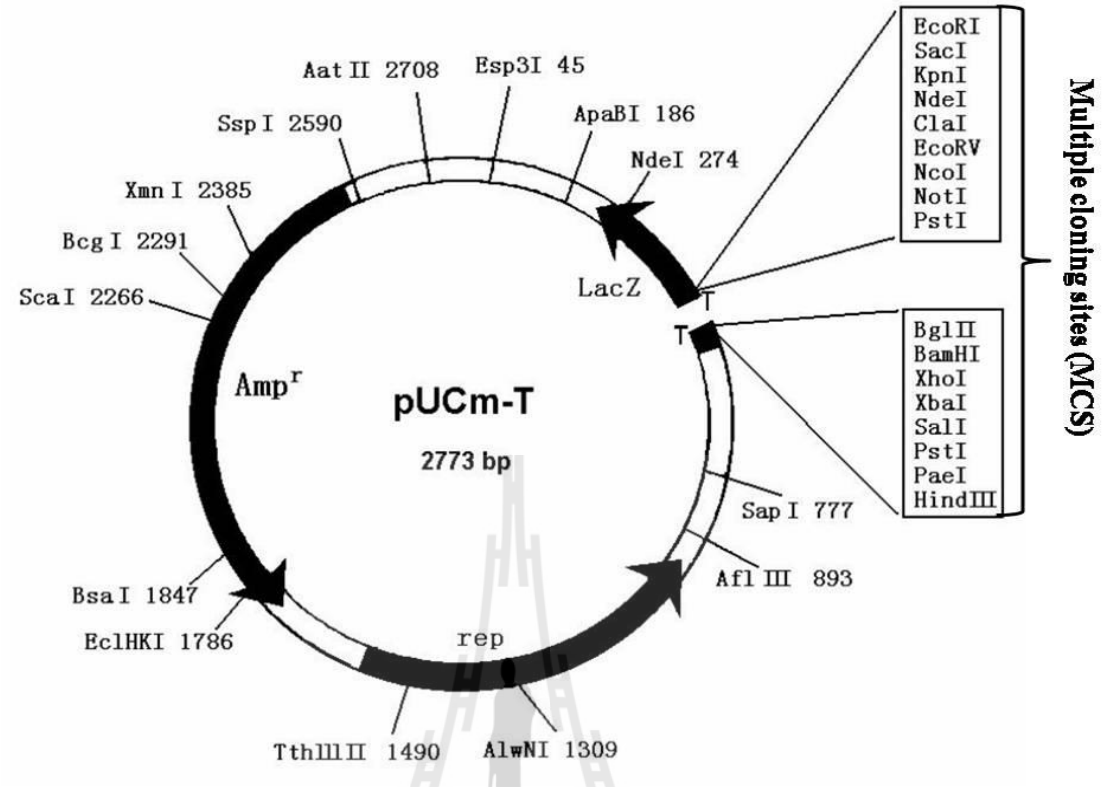

Figure 3.1 Map of plasmid pUCm-T.

The size of this plasmid is about 2.77 kb. It contains the multiple cloning sites (MCS), *β*-galactosidase (*lac*Z), ampicillin-resistance gene (amp^R), and an *Escherichia coli* origin of replication (*rep*).

3.7 Miscellaneous materials

Sodium azide was purchased from Sigma-Aldrich, USA. The NaN_3 stock solution was prepared by dissolving in sterile distilled water to give the final concentration of 20 mg/ml. It was filter sterilized through a 0.2 μm pore filter (Sartorius stedim biotech, USA) and stored at -20°C until use.

5-Bromo-4-chloro-3-indolyl-*β*-D-galactoside (X-gal) was purchased from Bio basic, Canada.

*Hin*d III digested λ-DNA marker was purchased from Promega, U.S.A.

TopTaq Master Mix Kit used for PCR amplification was purchased from Qiagen, Germany.

CHAPTER IV

METHODS

4.1 Isolation of *C. glutamicum* from soil

One gram of soil sample was transferred into 20 ml of sterile LB broth containing 50 µg/ml of nalidixic acid and 20 µg/ml of sodium azide. The culture broth was incubated aerobically at 37°C on an incubation shaker at 120 rpm, for 24 h. After incubation, the culture was serially diluted with sterile saline solution (0.9% NaCl) and plated on LB agar containing 50 µg/ml of nalidixic acid and 20 µg/ml of sodium azide. The plates were incubated at 37°C for 24 h or until colonies appeared.

4.2 Gram staining

Gram staining method was based on those modified by Hucker and Conn (1923) (Hucker and Conn, 1923). Bacterial cells were mixed with a drop of distilled water on a glass slide and allowed to air dry. Air dried smear was heat fixed by passing through a flame. The smear was flooded with crystal violet solution, stood for 1 min and rinsed with running water. Gram iodine was added on the slide and leaved for 1 min. The slide was washed with water and decolorized in absolute alcohol. Safranin solution was dropped on the slide, stood for 2 min and rinsed with running water. Gram straining is used to differentiate between Gram positive and Gram negative bacteria based on their chemical constituents and structural of differences of

their cell wall. The cell wall of Gram positive bacteria contains a dense layer of peptidoglycan, traping the crystal violet-iodine complex through many cross linkages (Roland and Larose, 1952). Therefore, Gram positive bacteria retained crystal violet and appeared dark blue or purple color. Gram negative bacteria have a thin layer of peptidoglycan surrounded by a layer of lipopolysaccharide, called outer membrane. The crystal violet-iodine complex will be lost from outer membrane during decolorization with absolute alcohol. Thus, Gram negative bacteria appear red or pink color after counterstaining with safranin.

4.3 Biochemical analysis

Gelatin digestion test (Clarke and Cowan, 1952) was done by stab-inoculating the tested bacteria into nutrient gelatin. The inoculated tubes were incubated at 37°C for 48 h. The gelatin-positive bacteria secrete gelatinases, an enzyme hydrolyzed gelatin to amino acids (Figure 4.2), resulting in the liquefaction of the medium after placing in refrigeration.

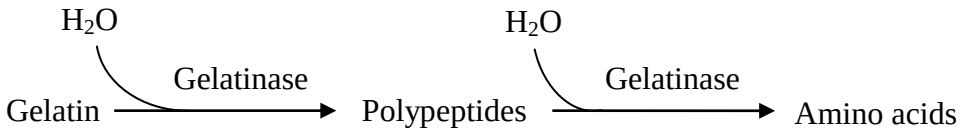

Figure 4.2 The reaction of gelatin digestion.

Urease test (Clarke and Cowan, 1952) was performed by transferring bacterial cells into Christiansen's urea broth. The inoculated tubes were incubated at 37°C for 48-72 h. The bacteria having an ability to produce urease hydrolyzed urea to ammonia

(Figure 4.3), creating an alkaline condition. Urease positive is indicated by changing of the phenol red indicator in the culture medium from yellow to pink.

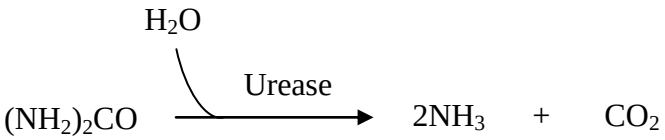

Figure 4.3 The reaction of urea hydrolysis.

Esculin hydrolysis test (Clarke and Cowan, 1952) was performed by streaking bacterial cells on an esculin agar slant. The inoculated tube was incubated at 37°C for 24 h. The esculin-positive bacteria hydrolyzed the esculin to esculetin (Figure 4.4). The esculetin reacted with ferric citrate in the medium to form a dark brown or black color.

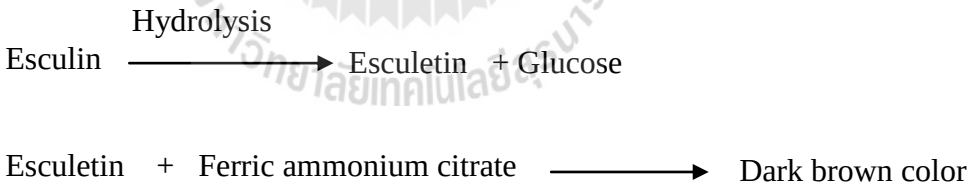

Figure 4.4 Chemical reaction of bile esculin hydrolysis.

Carbohydrate fermentation test (Clarke and Cowan, 1952) was done by inoculating bacterial cells into carbohydrate broth. The inoculated tubes were incubated at 37°C for 48 h. Positive bacteria fermented carbohydrate and produced

organic acids. Positive result is indicated by changing of phenol red indicator in the culture medium from red to yellow under acidic condition.

Casein digestion test (Clarke and Cowan, 1952) was performed by streaking bacterial cells on skimmed milk agar. The plate was incubated at 37°C for 48 h. The caseinase-positive bacteria degraded casein, a white protein in milk, into amino acids (Figure 4.5), generating clear zone around the bacterial colonies.

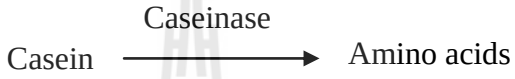

Figure 4.5 Chemical reaction of casein digestion.

Nitrate reduction test (Skerman, 1967) was done by inoculating bacterial cells into nitrate broth and incubated at 37°C for 3-5 days. The positive bacteria produce the enzymes nitrate reductase and nitrite reductaste. An enzyme nitrate reductase converts nitrate to nitrite. Then, nitrite reductaste enzyme reduces nitrite to nitrogen gas (Figure 4.6). The positive result is observed by appearing of nitrogen bubbles in an inverted Durham tube.

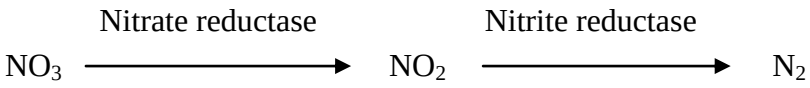

Figure 4.6 Chemical reaction of nitrate reduction.

4.4 Polymerase Chain Reaction (PCR)

4.4.1 Preparation of DNA template by freeze-thaw technique

The bacterial strains were streaked on LB agar plates and incubated at 37°C for 24 h. The single colony was transferred into microcentrifuge tube containing 10 µl of distilled water. The cell samples were incubated at -80°C for 5 min. Then, the suspensions were thawed at room temperature. The freezing and thawing of cells were repeated about 5-7 times. The suspension was centrifuged at 7,000 *g* for 5 min and the supernatant was used as DNA template for PCR amplification.

4.4.2 PCR amplification of *ndh* gene and 16s rRNA gene

The *ndh* gene was amplified with C.ndh-N1 and C.ndh-C3/1 primers (Table 3.1). The 16S rRNA gene was amplified by using primers 27F and 1525R (Table 3.1). The 25 µl of PCR mixture is shown in Table 4.1.

Table 4.1 PCR reaction mixture.

Reaction mixture	**Volume (µl)**
1. Genomic DNA template	5
2. Top Taq master mixed (2x)	12.5
3. Forward primer (10 µM)	1
4. Reverse primer (10 µM)	1
5. Distilled water	5.5
Final volume	**25**

The PCR amplification was performed by using DNA thermal cycler (BIO-RAD, USA). Amplification condition of *ndh* gene was performed by preheating at

95°C for 5 min followed by 30 cycles of 95°C for 1 min, 65°C for 1 min and 72°C for 1 min 30 sec. Final extension was performed at 72°C for 7 min.

Amplification condition of 16S rRNA gene was performed by preheating at 95°C for 5 min followed by 30 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min 30 sec. Final extension was performed at 72°C for 7 min.

4.5 Agarose gel electrophoresis

Agarose solution contained 0.8% (w/v) agarose in 1x TBE buffer. The agarose was dissolved by boiling in a microwave oven and poured into a gel casting tray. Gel was allowed to set at room temperature for 30-60 min. The DNA sample was mixed with 6x loading dye and loaded onto the gel. Electrophoresis was performed at a constant voltage (80-100 V) for 40-50 min or until the bromphenol blue presented in loading dye has migrated near the bottom of the gel. The gel was stained with 0.5 µg/ml ethidium bromide (EtBr) for 10-15 min and de-stained in distilled water to remove residual EtBr. The DNA sample was determined under UV transilluminator by comparing the size with *λ Hin*d III marker (Figure 4.7).

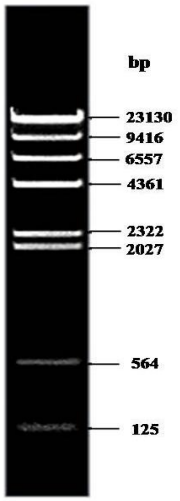

Figure 4.7 *λ Hin*d III marker.

4.6 Ligation of 16S rDNA into pUCm-T vector

The 16S rDNA was obtained by PCR amplification. The amplified 16S rDNA fragments were purified by using PCR clean-up gel extraction kit (Machery-nagel, Germany). The purified fragments were ligated to pUCm-T vector. The ligation mixture (Table 4.2) was incubated at 4°C overnight.

Table 4.2 Ligation mixture.

Reaction mixture	Volume (µl)
1. 10x Ligation buffer	1
2. pUCm-T vector (Figure 3.1)	1
3. Purified PCR product	X
4. Sterilized water	7-X
5. T4 DNA ligase	1
Total volume	**10**

4.7 Preparation of *E. coli* competent cells

A single colony of *E. coli* DH5α grown on LB agar was transferred to 2 ml of LB broth. The tube was incubated overnight at 37°C with 200 rpm shaking condition. One milliliter of overnight culture was transferred into 250 ml-Elenmayer flask containing 100 ml of sterilized LB broth. The culture was incubated at 37°C with 200 rpm shaking speed until OD_{600} reached 0.4-0.5. Then, the flask was placed on ice for 15-20 min and transferred to pre-chilled sterile 50 ml conical tubes. Bacterial cells were harvested by centrifugation at 7,000 *g*, 4°C for 10 min and washed twice with 10 ml of ice-cold $CaCl_2$ (0.1 M). The cell pellets were gently resuspended in 10 ml of ice-cold $CaCl_2$ (0.1 M) and kept on ice for 30 min followed by centrifugation at 7,000 *g*, 4°C for 10 min. The cell pellets were suspended in 2-3 ml of 0.1 M $CaCl_2$ containing 15% glycerol. The 100 µl cell suspension was aliquoted into pre-chilled

microcentrifuge tubes. The aliquots were dipped in liquid nitrogen and stored at -80°C until use.

4.8 Transformation of recombinant DNA into *E. coli* DH5α

Transformation steps were performed according to the manufacturer's instructions (Bio Basic, Canada). Competent cells were thawed on ice. The DNA was mixed with 100 µl competent cells and incubated on ice for 1 h. The mixture was heated at 42°C for 90 sec and immediately cooled on ice for 2 min. Transformation mixture was transferred into 1 ml of LB broth and standed at 37°C for 1 h. The 20 µl of transformants were spreaded on LB agar containing 100 µg/ml of ampicillin and 80 µg/ml of X-gal. The plates were incubated at 37°C overnight. The recombinant clones were selected by blue-white screening method. Thansformants containing pUCm-T exhibit a functional *lacZ* gene encoded *β*-galactosidase. *β*-Galactosidase is an enzyme that catalyzed the hydrolysis of X-gal to form blue pigments. Bacterial cells expressing *β*-galactosidase enzyme appear blue colony on the medium containing X-gal. The positive transforants harboring vector with an interrupted *lacZ* gene cannot utilize X-gal and thus appear white.

4.9 16S rDNA sequencing and construction of phylogenetic tree

Recombinant plasmid pUCm-T containing 16S rDNA was submitted for sequencing at Macrogen, Korea. The M13/pUC forward and M13/pUC reverse primers (Table 3.1) which bound specifically to pUCm-T were used for sequencing. The resulting sequences were analyzed using EditSeq and Clone Manager softwares. Each sequence was aligned with 16S rRNA gene available in the GenBank nucleotide

databases (NCBI, USA). Multiple sequences alignment was analyzed by using CLUSTAL X. A phylogenetic tree was constructed by using MEGA (Version 4.1) software.

4.10 Measurement of bacterial growth

The tested strains were pre-cultured on 5 ml LB broth at 37°C for 24 h. Then, 1 ml of an overnight cultured was transferred into 500-ml Erlenmeyer flask containing 99 ml of glucose minimum medium. The flask was cultivated at tested condition. The bacterial cells were collected every 3 h and measured turbidometrically at 600 nm (OD_{600}) (CE 1011 UV-VIS Spectrophotometer, Cecil Instruments, UK). The cell culture was diluted since the measured OD was above 0.9. The bacterial growth was calculated by multiplying with the dilution factor.

4.11 Determination of cell dry weight

The bacterial cultured was centrifuged at 7,000 *g* for 5 min to separate bacterial cells and cultured broth. The cells were washed twice with distilled water. The pellets were dried at 105°C for 4 h. The dry weight of the cells was measured by using 4 digit analytical balance (Gemini GR Series Analytical Balances, H&C Weighing Systems, USA).

4.12 Determination of glutamic acid production

The fermented broth was applied to TLC silica gel 60 (Merck AG, Germany) and developed with solvent system consisting of butanol, glacial acetic acid, and distilled water in the ratio of 60: 15: 25. The development was carried out in a

saturated tank for 2-2.30 h until the solvent front moved to the appropriate distance (Figure 4.8).

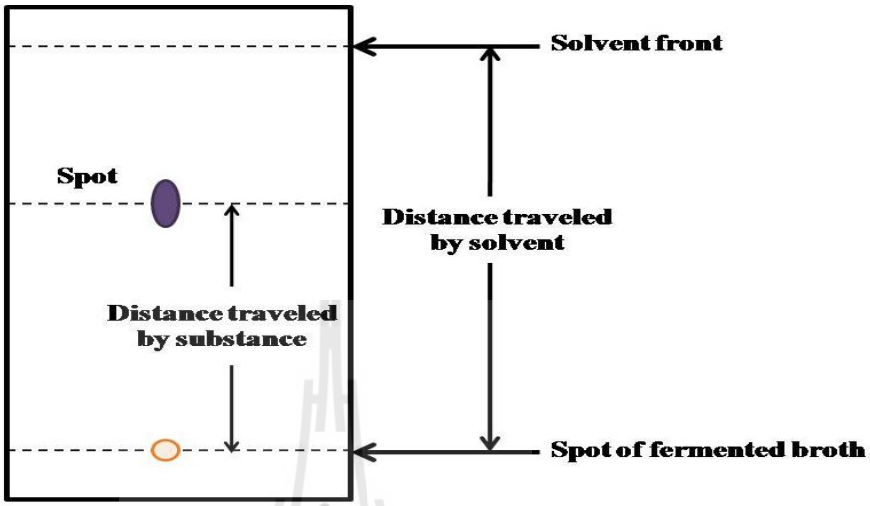

Figure 4.8 Thin layer chromatography analysis.

The plate was dried for 30-40 min and developed with 0.2% ninhydrin in saturated butanol.

The retention factor (RF) of sample was compared with the standard glutamic acid. The RF value was calculated from the following formula:

$$RF = \frac{\text{Distance traveled by substance}}{\text{Distance traveled by solvent front}}$$

The spots were scraped and extracted with 75% ethanol containing 0.005% $CuSO_4$-$5H_2O$ for 2 h at room temperature. The extracts were measured spectrophotometrically at 570 nm (UV-VIS Spectrophotometer, Cecil Instruments, UK). The glutamate concentration was obtained by comparing with calibration standard curve of known concentration of L-glutamic acid (0.1, 0.3, 0.5, 1.0, and 2.0 µmole).

CHAPTER V

RESULTS AND DISCUSSION

5.1 Optimization of culture conditions for isolation of thermotolerant *C. glutamicum*

Complex media such as brain heart infusion broth, LB5G (LB medium supplemented with 5 g glucose) broth or tryptone, peptone, yeast extract based medium have been used to isolate *C. glutamicum* (Vallino and Stephanopoulos, 1993; Marx, de Graaf, Wiechert, Eggeling and Sahm, 1996; Moritz, Striegel, de Graaf and Sahm, 2000; Petersen, 2000). However, no selective media or enrichment media were specifically used for isolation of these organisms from the environmental samples. In 2007, Kitchakarn and coworkers (Kithakarn and Trakulnaleamsai, 2007) used D2 broth supplemented with 10 µg/ml of nalidixic acid and 2 µg/ml of sodium azide to isolate thermotolerant *C. glutamicum* from soils and soil contaminated with avian feces. They suggested that nalidixic acid and sodium azide helped to inhibit the growth of unwanted bacteria that might be contaminated in the samples.

Nalidixic acid is effective against Gram positive bacteria which functions to inhibit a subunit of DNA gyrase, a topoisomerase that induces the negative supercoiling of DNA during DNA replication (Cook, Brown, Boyle and Goss, 1966; Boyle, Cook and Goss, 1969; Crumplin and Smith, 1975). Since, *C. glutamicum* posses a special cell wall structure differed from other Gram positive bacteria. Cell

wall of this bacterium contains a thick layer of peptidoglycan surrounded by mycolic acid. It has been shown that mycolic acid layer limits the influx of narlidixic acid into the bacterial cells and thus *C. glutamicum* is resistant to nalidixic acid (Park et al., 2008). On the other hand, sodium azide inhibits intracellular ATP synthesis by interrupting the function of cytochrome oxidase, an enzyme of the bacterial electron transport chain (Sousa et al., 2012). Since, electron transport chain of *C. glutamicum* contains an alternative cyanide-sensitive bypass oxidase which has been shown to resist to sodium azide (Das, Silaghi-Dumitrescu, Ljungdahl and Kurtz, 2005; Kabus, Niebisch and Bott, 2006). Thus, *C. glutamicum* is able to grow in the presence of sodium azide.

Our first attempted to isolate *C. glutamicum* from soil samples was performed by using LB medium containing the same condition of nalidixic acid and sodium azide described by Kitchakarn, 2007 (Kithakarn and Trakulnaleamsai, 2007). However, a lot of contaminations of others soil bacterium were observed and found to overgrow *Corynebacterium* spp. (Figure 5.1). In order to improve the isolation frequency of *C. glutamicum* from soil samples, we therefore tried to optimize a selective medium used for these microorganism. The efficacy of nalidixic acid and sodium azide were evaluated by determining their proper concentrations within the medium. The LB medium supplemented with nalidixic acid and sodium azide were varied from 5-65 µg/ml and 2-26 µg/ml, respectively. *C. glutamicum* KY9002, wild type strain, and five other bacteria (*Bacillus subtilis*, *Escherichia coli*, *Bacillus cereus*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*) that might occur as contaminants from soil were inoculated onto the tested media (Table 5.1). The growth of these bacterial strains were observed after two days of incubation at 30°C (Table

5.1). As the results, all tested contaminant strains including *C. glutamicum* could grow on the medium containing the same concentrations of nalidixic acid and sodium azide used by Kitchakarn et al. The growth of all tested contaminants, except *C. glutamicum* KY9002, were inhibited when the amount of nalidixic acid and sodium azide in the medium reached 45 and 18 µg/ml, respectively. However, 50 µg/ml nalidixic acid and 20 µg/ml sodium azide were found to be more suitable for the isolation of *C. glutamicum* from natural sources (Figure 5.2). In addition, the numbers of contaminants bacteria were significantly decreased when using glucose minimum medium instead of LB medium (Figure 5.3). From all of these results, we decided to use glucose minimum medium supplemented with 50 µg/ml nalidixic acid and 20 µg/ml sodium azide for the isolation of *C. glutamicum* from soil samples.

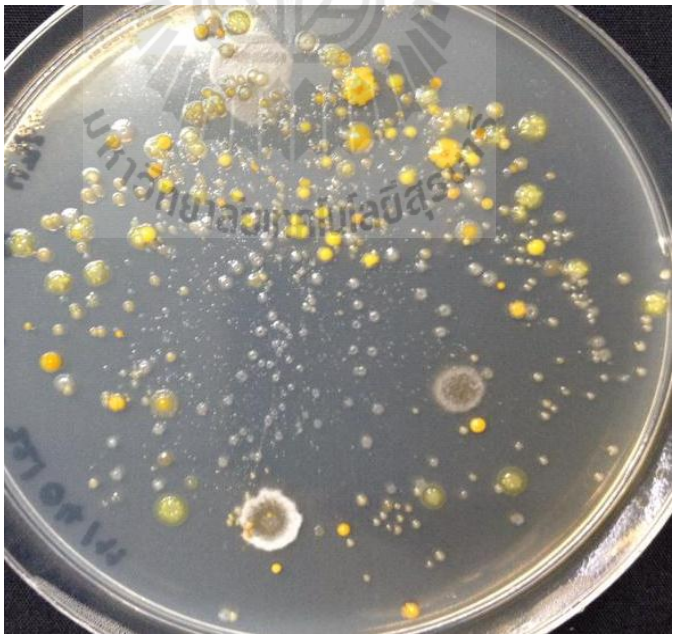

Figure 5.1 Colonies of several soil contaminants were overgrow *C. glutamicum* on LB agar plate supplemented whit 10 µg/ml nalidixic acid and 2 µg/ml sodium azide of sodium azide after incubation at 37°C for 24 h.

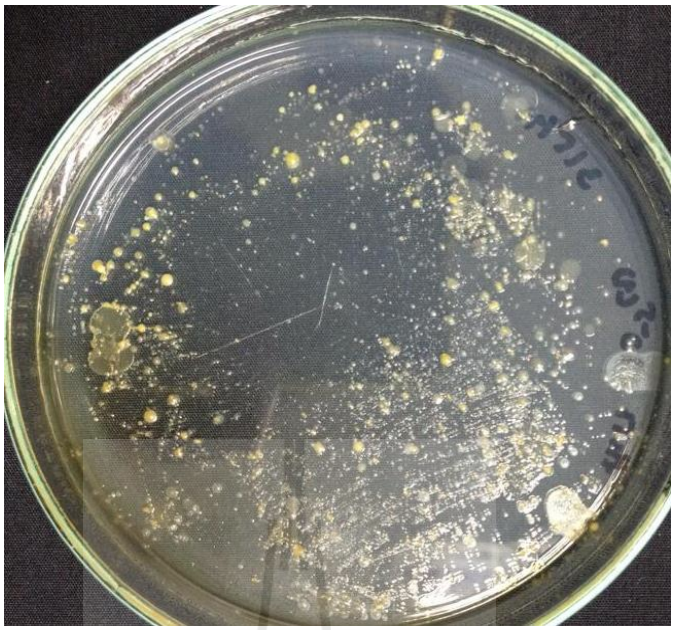

Figure 5.2 The reduced contaminants colonies on LB medium supplemented with 50 µg/ml nalidixic acid and 20 µg/ml sodium azide after incubated at 37°C for 24 h.

Figure 5.3 The reduced contaminants colonies on glucose minimum medium supplemented with 50 µg/ml nalidixic acid and 20 µg/ml sodium azide after incubated at 37°C for 24 h.

Table 5.1 Inhibition of contaminants bacteria by the different concentrations of nalidixic acid and sodium azide.

Concentrations (µg/ml)		Tested strains					
nalidixic acid	NaN_3	*C. glutamicum* (KY9002)	*Bacillus subtilis*	*Escherichia coli*	*Bacillus cereus*	*Staphylococcus aureus*	*Pseudomonas aeruginosa*
5	2	✓	✓	✓	✓	✓	✓
10	4	✓	✓	✓	✓	✓	✓
15	6	✓	✓	✓	✓	✓	✓
20	8	✓	✓	✓	✓	x	✓
25	10	✓	x	✓	x	x	✓
30	12	✓	x	✓	x	x	✓
35	14	✓	x	✓	x	x	✓
40	16	✓	x	✓	x	x	✓
45	18	✓	x	x	x	x	x
50	20	✓	x	x	x	x	x
55	22	x	x	x	x	x	x
60	24	x	x	x	x	x	x
65	26	x	x	x	x	x	x

✓ : growth, x : no growth

5.2 Isolation of thermotolerant *C. glutamicum*

To isolate the thermotolerant *C. glutamicum*, bird feces and soil samples were collected from Roi-Et, Thailand. Bird feces and soil samples were spread on glucose minimum agar containing 50 µg/ml of nalidixic acid and 20 µg/ml of sodium azide. The yellow, smooth, circular, and translucent colonies (Figure 5.4) similar to those of

C. glutamicum were selected. As a result, we obtained 120 isolates which were named as PP1 to PP120, respectively.

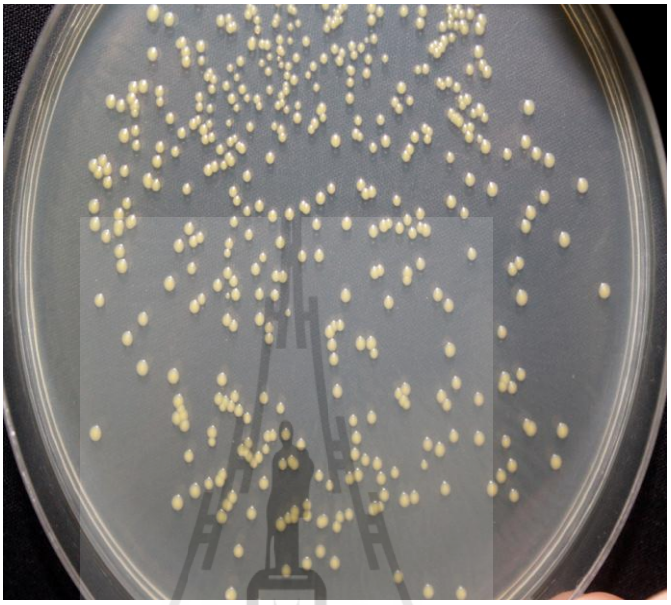

Figure 5.4 Colonies morphology of the PP25, PP29, and PP80 grew on LB agar plate after incubated at 37°C for 24 h.

5.3 Identification of isolated strains

The 120 isolates were roughly classified as *C. glutamicum* based on Gram reaction, cellular morphology, and PCR amplification of *C. glutamicum ndh* gene encoded NADH dehydrogenase II (NdhII). All isolates were Gram positive, rod-shaped, and non spore-forming (Figure 5.5).

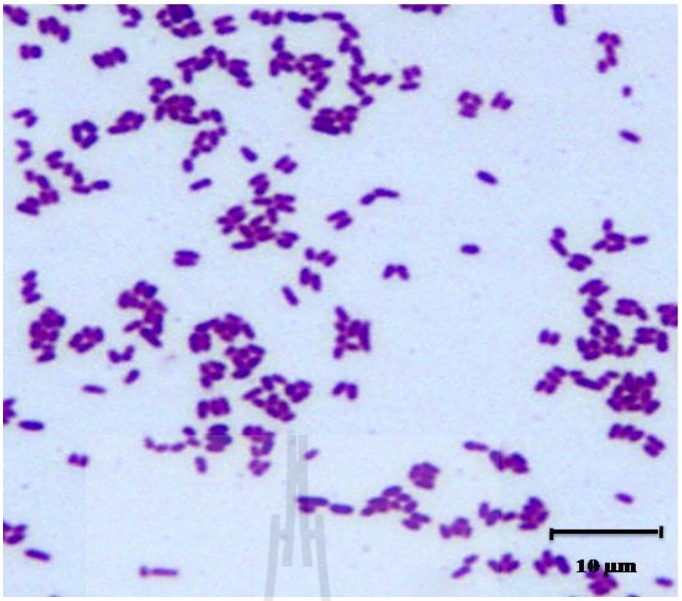

Figure 5.5 Gram stain of the PP25, PP29, and PP80 grew on LB agar and incubated at 37°C for 24 h (Bar = 1 µm, Magnification: x 1000).

The 120 isolates were amplified with C.ndh-N1 and C.ndh-C3/1 primers. This set of primers bound specifically to 5′- and 3′-ends of *ndh* gene and generated a 2 kb DNA fragment. Among 120 isolates, 44 isolates showed the 2 kb-bands of PCR products. These 44 strains were individually examined their glutamic acid production efficiency by culturing on 5 ml LB broth at 37°C. Among 44 strains, PP25, PP29, and PP80 produced highest amount of L-glutamic acid (data not shown). PP25, PP29, and PP80 were then identified as *C. glutamicum* by biochemical analysis. Their biochemical results were compared with wild type *C. glutamicum* (Table 5.2). The tested strains gave positive results for urease, glucose fermentation, fructose fermentation, sucrose fermentation, and nitrate reduction tests, while gelatin digestion, esculin hydrolysis, and casein digestion tests were negative (Table 5.2). These results were identical to those observed in wild type strain of *C. glutamicum*; however, these

were also indicated *C. ammoniagenes* strain (Figure 5.6). Thus, PP25, PP29, and PP80 were confirmed as *C. glutamicum* by 16S rRNA gene analysis. The approximately 1.25 kb 16S rDNA sequences obtained from these strains were aligned with 16S rDNA sequences available in the GenBank database. The results revealed that 16S rDNA sequences of PP25, PP29, and PP80 were closely related to *C. glutamicum* KY9002 with 99% similarity.

Table 5.2 Biochemical characteristics of PP25, PP29, and PP80.

Biochemical tests	Results			
	PP25	PP29	PP80	*C. glutamicum* (KY9002)
Gelatin digestion	-	-	-	-
Urease test	+	+	+	+
Esculin hydrolysis	-	-	-	-
Glucose fermentation	+	+	+	+
Fructose fermentation	+	+	+	+
Sucrose fermentation	+	+	+	+
Casein digestion	-	-	-	-
Nitrate reduction	+	+	+	+

-: Negative, +: Positive

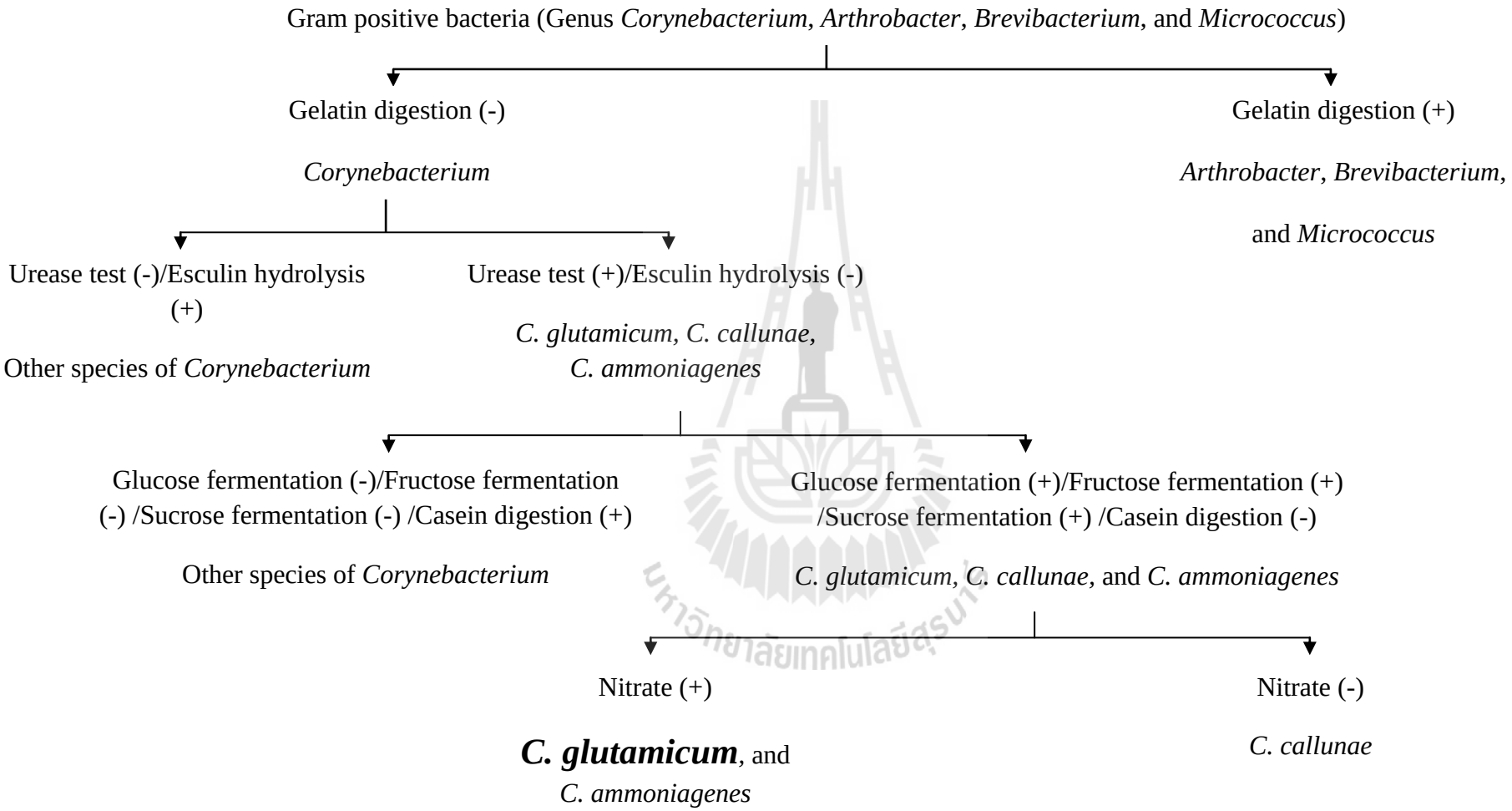

Figure 5.6 Biochemical analysis of *C. glutamicum*.

5.4 Comparison of growth of PP25, PP29, and PP80 with *C. glutamicum* wild type

To determine the growth of PP25, PP29, and PP80, bacterial cells were cultured on 100 ml of glucose minimum medium by varying incubation temperature from 30-41°C. The ability to grow of PP25, PP29, and PP80 were determined by comparing their growth curve with *C. glutamicum* wild type. Growth curve of PP25, PP29, PP80, and wild type were obtained from turbidity measurement at OD_{600}. The results showed that PP25, PP29, and PP80 could grow at 30-37°C which was similar to wild type (Figure 5.7A, B, and C). When the fermentation temperature had reached 38°C (Figure 5.7D) and above (Figure 5.8A, B, C, and D), the growth of wild type was ceased while PP25, PP29, and PP80 continued to grow.

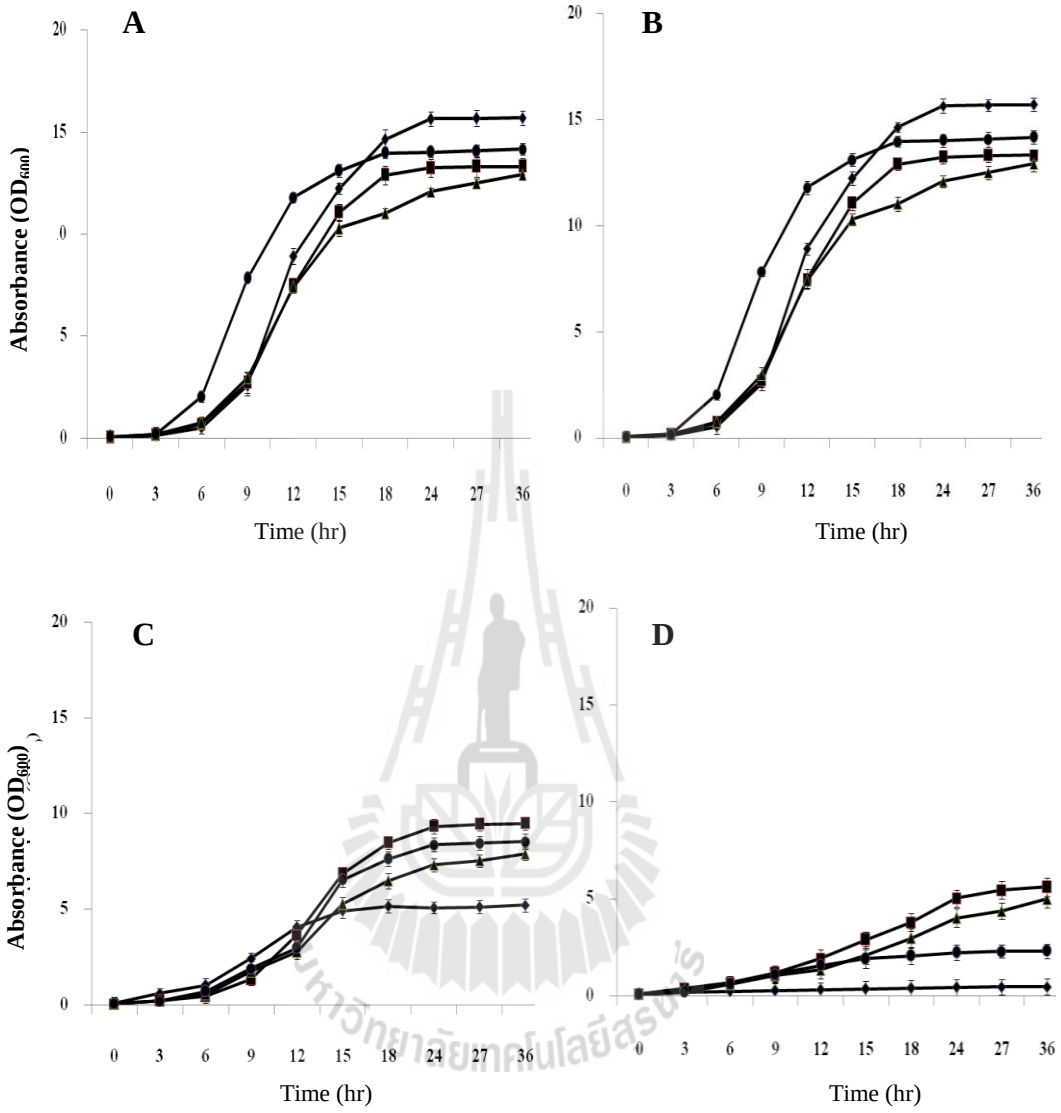

Figure 5.7 Comparison of growth between wild type *C. glutamicum* and PP25, PP29 and PP80. The tested strains were incubated at temperature ranging from 30°C (A), 35°C (B), 37°C (C), and 38°C (D). The ◆, ■, ▲, and ● bars represent the growth of wild type *C. glutamicum*, PP25, PP29 and PP80, respectively. Error bars represents SD. Each value is a mean of n=3 experiments.

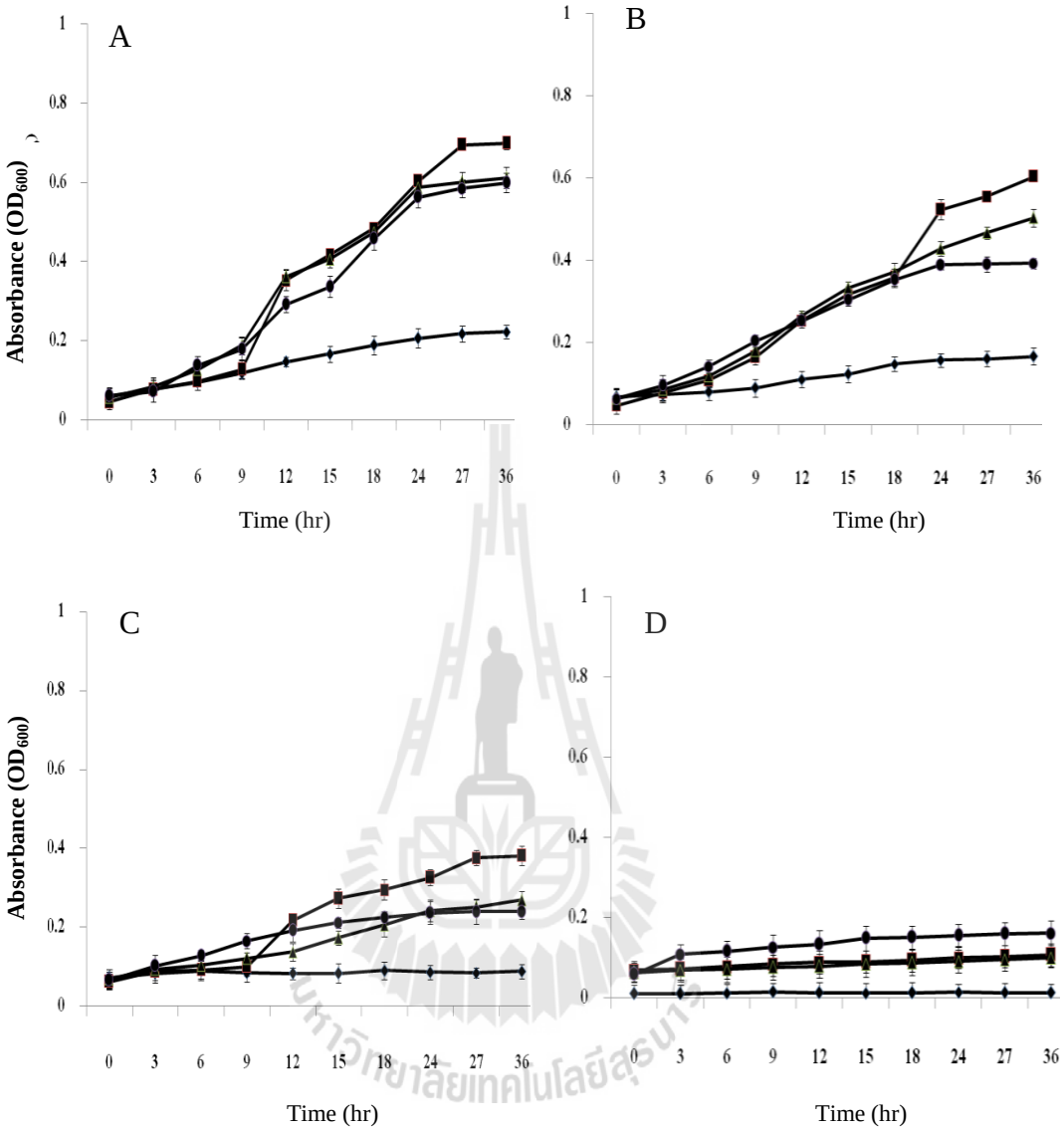

Figure 5.8 Comparison of growth between wild type *C. glutamicum* and PP25, PP29 and PP80. The tested strains were incubated at temperature ranging from 38.5°C (A), 39°C (B), 40°C (C), and 41°C (D). The ◆, ■, ▲, and ● bars represent the growth of wild type *C. glutamicum*, PP25, PP29 and PP80, respectively. Error bars represents SD. Each value is a mean of n=3 experiments.

5.5 Comparison of glutamic acid production of PP25, PP29, and PP80 with *C. glutamicum* wild type

Determination of glutamic acid production of PP25, PP29, and PP80 were performed on 100 ml of glucose minimum medium by varying incubation temperature from 30-41°C. Their glutamic acid yield was estimated by TLC method and then compared with wild type. The results showed that PP25, PP29, and PP80 excreted lower L-glutamic acid than wild type at 30°C, while their L-glutamic acid production increased with increasing temperature which was even higher than that of wild type (Figure 5.9). The glutamic acid production of PP25, PP29, and PP80 were significantly higher than wild type at 37-40°C. The highest yield of glutamic acid of PP25 was observed at 38°C, while PP29, and PP80 were observed at 38.5°C (Figure 5.9).

The highest L-glutamic acid production per cell of PP25, PP29, and PP80 were 1.99, 2.82, and 3.64 (g glutamic acid/ g dried cell) (Figure 5.10). The optimum temperature for glutamic acid production per cell of PP25 was observed at 38.5°C, while PP29, and PP80 were observed at 39°C.

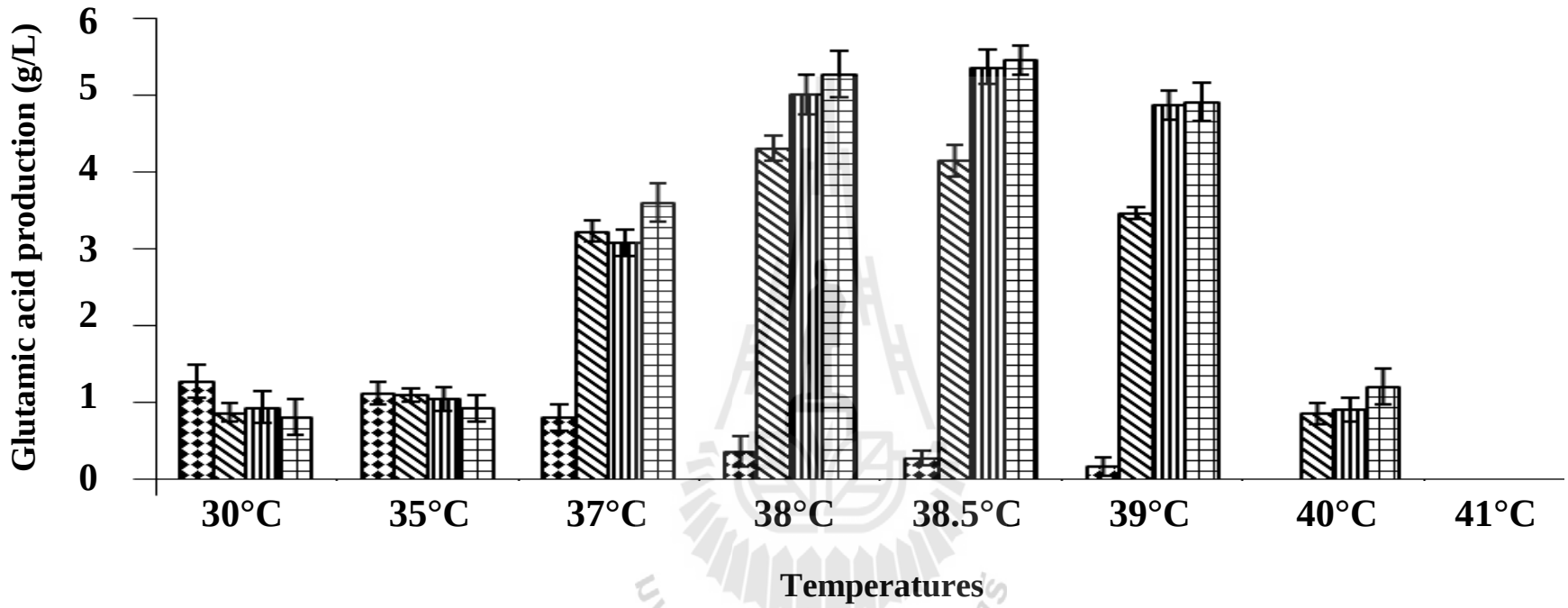

Figure 5.9 Comparison of glutamic acid production between wild type *C. glutamicum* and selected strains. The tested strains were grown in 500 ml-flask containing 100 ml of glucose minimum medium with shaking speed of 200 rpm and incubated at temperature ranging from 30°C-41°C. The ▩ , ▨, ▥, and ▦ bars represent the L-glutamic acid production of *C. glutamicum* wild type, PP25, PP29 and PP80, respectively. Error bars represents SD. Each value is a mean of n=3 experiments. Means followed by different letters differ significantly at P≤0.05 using Dunnett's one way ANNOVA.

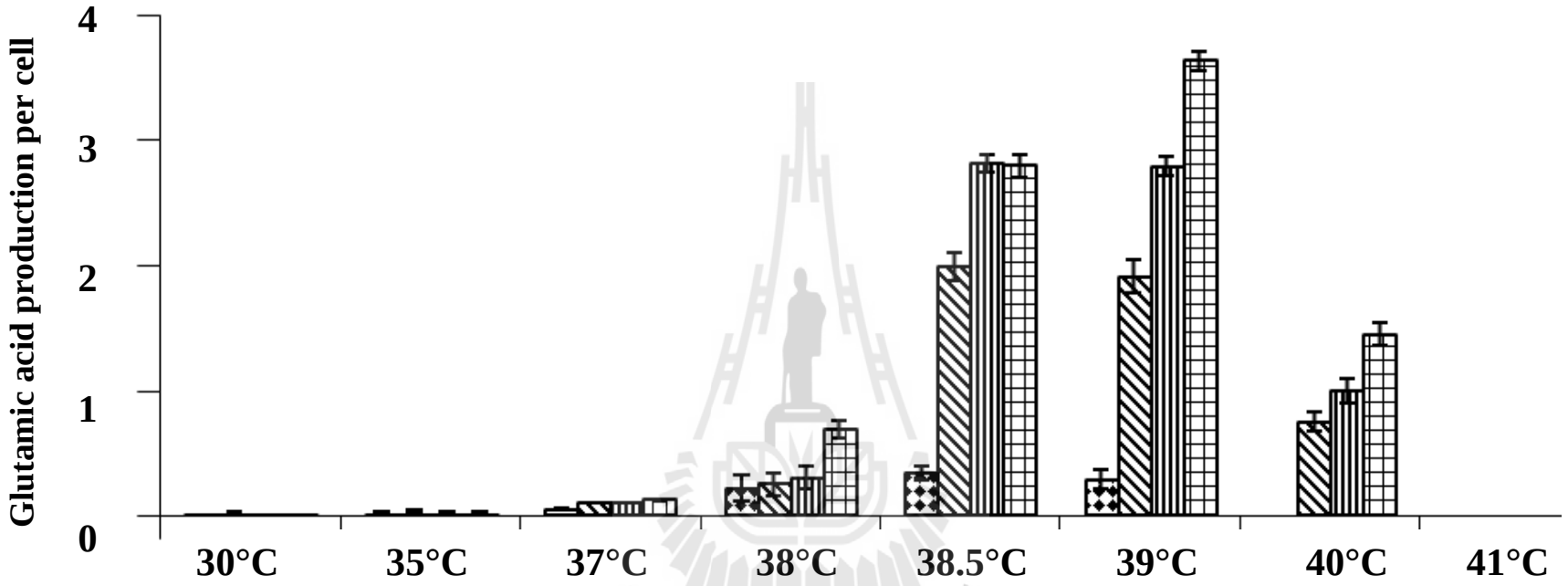

Figure 5.10 Comparison of glutamic acid production per cells between wild type *C. glutamicum* and selected strains. The tested strains were grown in 500 ml-flask containing 100 ml of glucose minimum medium with shaking speed of 200 rpm and incubated at temperature ranging from 30°C-41°C. The ⊠ , ⊘, ▥, and ▦ bars represent the L-glutamic acid production of *C. glutamicum* wild type, PP25, PP29 and PP80, respectively. Error bars represents SD. Each value is a mean of n=3 experiments. Means followed by different letters differ significantly at P≤0.05 using Dunnett's one way ANNOVA.

In 2012, Zheng and coworkers improved the thermotolerance of *C. glutamicum* by genome shuffling technique (Zheng et al., 2012). Ultraviolet (UV) irradiation and diethyl sulfate (DES) were used as the mutagenizing agents to generate the initial population diversity of mutants from two ancestors, *C. glutamicum* S9114 and *C. glutamicum* ATCC1376. Mutant strains of S9114 and ATCC1376 with subtle improvements in temperature tolerance were used for genome shuffling. The technique of genome shuffling was performed by 3 rounds protoplast fusions. The shuffled strain, F343, could grow at 44°C, while even no growth was observed for parental strains. The yield of L-glutamic acid of F343 was increased by 1.8-time comparing with that of the ancestor strains at 38°C. The genetic diversity between F343 and S9114 was also evaluated by Amplified Fragment Length Polymorphism (AFLP) analysis. The result suggested that the phenotypes for both thermotolerance and L-glutamic acid production in F343 were evolved. PP25, PP29 and PP80 obtained from this study shares some characters with F343 in which they can grow and produce L-glutamic acid at 38°C or above. However, the temperature limit for growth and optimum temperature for L-glutamic acid production of PP25, PP29 and PP80 are different from F343. The highest temperature for growth of PP25, PP29 and PP80 are 40°C, while F343 is 44°C. F343 retains the same level of L-glutamic acid production at temperature 32-38°C whereas the highest glutamate fermentation of PP25, PP29 and PP80 are around 38°C. Therefore, we suggested that some of the genomic features of PP25, PP29 and PP80 may be similar to F343, while others may not.

CHAPTER VI

CONCLUSIONS

We successfully isolate *C. glutamicum* named as PP25, PP29, and PP80 which were isolated from avian feces collected from Roi-Et, Thailand. PP25, PP29, and PP80 could grow and produce glutamic acid at 38°C and above while *C. glutamicum* wild type failed to grow at this temperature. These results indicated that PP25, PP29, and PP80 possess enhanced thermotolerance glutamate fermentation.

The ability of L-glutamic acid fermentation at an elevated temperature of PP25, PP29, and PP80 suggested that genomic profiles for temperature and glutamate productivity of these strains were probably be evolved in the nature spontaneously.

In order to ensure that genomic profiles of PP25, PP29 and PP80 were difference in wild type, their genomic profiles were determined by phylogenetic tree analysis. A phylogenetic tree was constructed using the neighbor-joining method. The phylogenetic analysis of PP25, PP29, and PP80 based on comparison of 16S rDNA sequences revealed that these strains were closely related to various strains of *C. glutamicum* (Figure 6.1). The results showed that PP25 was highly similar to *C. glutamicum* R. Thus, PP25 may be has the same ancestor with *C. glutamicum* R, less similar to *C. glutamicum* KY9002. In contrast, *C. glutamicum* PP29 and PP80 were closely related to *C. glutamicum* KY9002. Therefore, PP29 and PP80 may be shared a common ancestor with *C. glutamicum* KY9002. The results suggested that PP25 diverged from a common ancestor of PP29 and PP80.

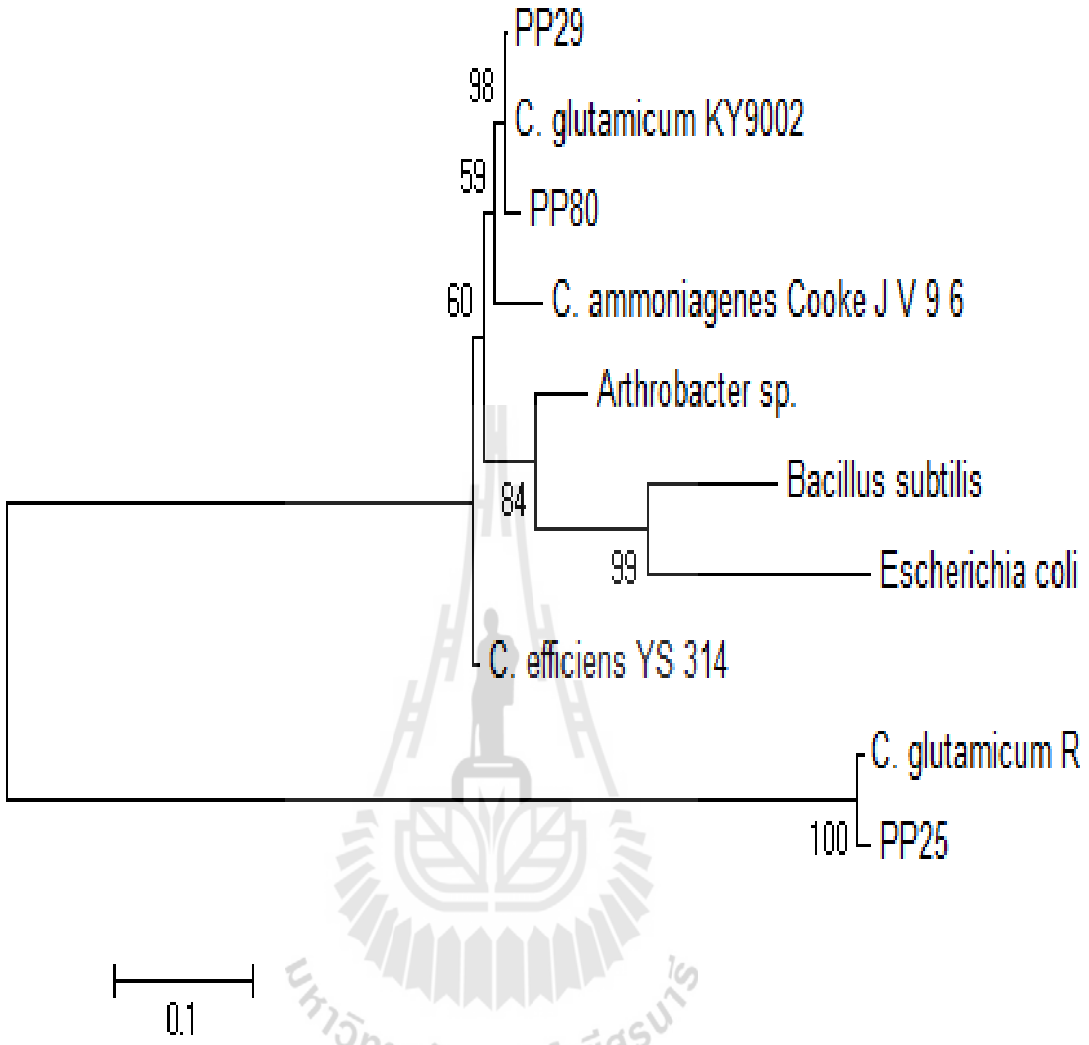

Figure 6.1 Phylogenetic tree representing the evolutionary relationship of PP25, PP29, PP80 and other related taxa. 16S rRNA gene sequences were aligned using ClustalW and a neighbour-joining phylogenetic tree was generated using the maximum composite likelihood method and MEGA4. Numbers at nodes are percentage bootstrap values based on 1,000 replications. GeneBank accession numbers of the sequences are indicated in the parentheses. Bar indicates 5 nucleotides substitution per 1,000 nucleotides.

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