THESIS APPROVAL

GRADUATE SCHOOL, KASETSART UNIVERSITY

Master of Science (Microbiology)

DEGREE

Microbiology	Microbiology
FIELD	**DEPARTMENT**

TITLE: Strain Improvement of *Corynebacterium glutamicum* DS50 by UV Mutagenesis for Enhancing L-Glutamate Production

NAME: Mr. Suphoj Noisakul

THIS THESIS HAS BEEN ACCEPTED BY

THESIS ADVISOR

(Assistant Professor Savitr Trakulnaleamsai, Ph.D.)

THESIS CO-ADVISOR

(Associate Professor Napavarn Nopparatnaraporn, Dr.Eng.)

ACTING DEPARTMENT HEAD

(Associate Professor Kooranee Tuitemwong, Ph.D.)

APPROVED BY THE GRADUATE SCHOOL ON _____

DEAN

(Associate Professor Gunjana Theeragool, D.Agr.)

THESIS

STRAIN IMPROVEMENT OF *Corynebacterium glutamicum* DS50 BY UV MUTAGENESIS FOR ENHANCING L-GLUTAMATE PRODUCTION

SUPHOJ NOISAKUL

A Thesis Submitted in Partial Fulfillment of
the Requirements for the Degree of
Master of Science (Microbiology)
Graduate School, Kasetsart University
2013

Suphoj Noisakul 2013: Strain Improvement of *Corynebacterium glutamicum* DS50 by UV Mutagenesis for Enhancing L-Glutamate Production. Master of Science (Microbiology), Major Field: Microbiology, Department of Microbiology. Thesis Advisor: Assistant Professor Savitr Trakulnaleamsai, Ph.D. 122 pages.

For strain improvement, *Corynebacterium glutamicum* DS50 isolated from soil contaminated with duck feces was subjected to UV-irradiation (6-8 minutes exposure) for increasing L-glutamate production. Out of 180 isolates screened, the highest L-glutamate producing strain namely UV-BB9 was selected. The UV-BB9 exhibited L-glutamate yield of 1.63 g/ g dried cell which was 11-folds higher than that of the wild-type strain in basal salt medium with 2.5 μg/ L biotin. In addition, the mutant seemed to be more sensitive to lysozyme and temperature at 37 ºC as compared to the wild-type. Furthermore, UV-BB9 exhibited high L-glutamate production although its growth was lower at 37 ºC. The results indicated that UV-irradiation was effective mutagenic agent for *C. glutamicum* strain improvement.

_____ _____ ___ / ___ / ___

Student's signature Thesis Advisor's signature

ACKNOWLEDGEMENTS

First of all, I would like to express my deepest gratitude to my thesis adnisor, Assistant Professor Savitr Trakulnaleamsai, whose deep scientific knowledge, continuous encouragement, advice and her teacher mind, made my thesis such an improving experience. I refer not only to her invaluable professional help but also to her admirable human care. She always inspre me in many acreative ideas and taught how to be a good student. I will never forget the crucial help and generous support that she gave me in many attempt to get a master degree and to improve myself in all scientific ideas. I would sincerely like to thank Associate Professor Napavarn Noparatnaraporn, my co-thesis advisor, in addition, Assistant Professor Wanna Malaphan, chairman in examination and Assistant Professor Patoomporn Chim-anage, for their valuable comments and thesis approving.

I would like thank to all my friends in the Department of Mirobiology, Faculty of Science, Kasetsart University who gave me a great support in many academic and personal life over many years in Kasetsart University. Thanks to Mrs. Srisuda Hanpakpoom and Ms. Wilawan Channarong, who advised me in some techniques in laboratory. Thanks to all colleagues that I did not mention here.

Finally, special appreciation expressed to my family for their support and understanding throughout my study.

Suphoj Noisakul

March 2013

TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF TABLES (Continued)

LIST OF FIGURES

LIST OF FIGURES (Continued)

LIST OF FIGURES (Continued)

LIST OF FIGURES (Continued)

LIST OF FIGURES (Continued)

LIST OF FIGURES (Continued)

LIST OF FIGURES (Continued)

STRAIN IMPROVEMENT OF *Corynebacterium glutamicum* DS50 BY UV MUTAGENESIS FOR ENHANCING L-GLUTAMATE PRODUCTION

INTRODUCTION

The market of amino acids is increasing with annual growth rate of 5-7% (Leuchtenberger *et al.*, 2005). With the exploitation of the wide spectrum of the uses of amino acids as food additives, pharmaceuticals, feed supplements, cosmetics, and polymer precursor, the demand for amino acids has increased (Shimizu and Hirasawa, 2006). Due to the biggest market among the amino acids is that of L-glutamate which the main use is as a flavor enhancer, the annual production was more than 1.5 million tons per year (Schultz *et al*., 2007).

Corynebacterium glutamicum is a facultative anaerobic, nonpathogenic, biotin-auxotrophic Gram-positive soil bacterium that was isolated by Kinoshita *et al* (1957). Since that time, different strains of this species are used industrially for the production of L-glutamate and several other amino acids. Coryneform bacteria have a thick cell wall, and this is probably plays as a permeability barrier for L-glutamate excretion. A variety of treatments were applied to triggering L-glutamate excretion such as biotin limitation (Shiio *et al.*, 1962a, b), addition of detergents such as Tween-40 or Tween-60 (Duperray *et al.*, 1992; Takinami *et al.*, 1965), addition of penicillin (Nunheimer *et al.*, 1970), use of temperature-sensitive strains cultured at higher temperature (Momose and Takagi, 1978; Delaunay *et al.*, 1999), or addition of ethambutol (Radmacher *et al.*, 2005). These all resulted change in membrane tension, leading to excretion of L-glutamate. However, these strategies could not be account for fully L-glutamate overproduce because of its capacity is under controlled by the putative genes. Therefore, mutagenesis and screening/ selection for higher producing microbial strains is the most necessary.

Today, strain improvement can be performed by two means: (i) mutation by classical genetic methods and (ii) genetic recombination techniques such as protoplast fusion, transformation, conjugation and recombinant DNA technology, including metabolic engineering (Demain and Adrio, 2008). The advantage of mutation/ selection is simplicity, since it requires little knowledge of the genetics, biochemistry and physiology of product biosynthetic pathway. Moreover, it does not need sophisticated equipment and requires minimal specialized technical manipulation (Gonzalez and Tomasini, 2003).

In a previous study, glutamic acid-producing bacterium *C. glutamicum* strain DS50 was isolated from soil contaminated with duck-feces in Thailand (Kitchakarn, 2007). The optimal temperature of this strain is about 35-37 ºC, and can grow at elevated temperature 42 ºC together with L-glutamate production. However, the amounts of L-glutamate produced is very low compared to that of industrial strains. Here, in order to establish a higher L-glutamate-producing mutant strains, we selected *C. glutamicum* DS50 as a starting strain for UV mutagenesis. Then, we screened for higher L-glutamate-producing and study some physiological characteristics of the selected mutant. In addition, we investigated the growth and L-glutamate production in jar fermenter using batch process. The experimental results obtained by this study could be further use as an alternative way for utilize indigenous microbial resources for amino acid production in future.

OBJECTIVES

1. To optimize the induced-mutation condition for strain improvement of *C. glutamicum* DS50

2. To screen the higher L-glutamate-producing mutants.

3. To characterize some physiological characteristics of the selected mutants.

4. To investigate growth and L-glutamate production of the selected mutants.

LITERATURE REVIEW

1. Glutamic Acid

1.1. Biological properties of glutamic acid

Glutamic acid is a non-essential amino acid having an acidic carboxyl group on its side chain (Figure 1) which can serve as both an acceptor and a donor of ammonia, toxic to the body. Glutamic acid is coupled with ammonia, to transport safely to the liver, where it is eventually converted to urea for excretion by the kidneys and to purines (necessary for the synthesis of genetic material). Glutamic acid coupled with ammonia is called glutamine, the monoamide form of glutamic acid. Only the L-stereoisomer occurs in mammalian proteins. It is abundant constituent of proteins. The glutamic acid-glutamine interconversion is of central importance to the regulation of the levels of toxic ammonia in the body. Once glutamine is incorporated into proteins, its relatively unreactive side-chain amide participates in very few reactions. In addition to the function of protein synthesis, it participates in important brain function, as an excitatory neurotransmitter. Glutamine is not essential to the human diet, since it can be synthesized in the body from glutamic acid. Free glutamic acid can be converted reversibly to ketoglutaric acid, an intermediate in the Krebs cycle, and can be degraded to carbondioxide and water, or transform into sugars. The monosodium salt of L-glutamate is widely used as a condiment.

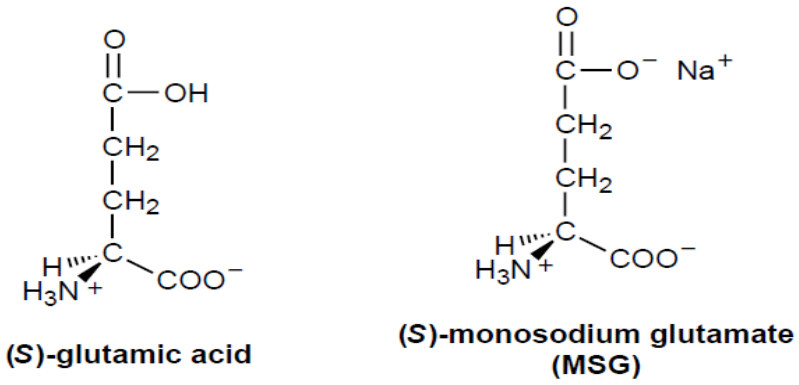


Figure 1 Structure of L-glutamate and L-monosodium glutamate (MSG).

Source: Ault (2004)

1.2. Physico-chemical properties of glutamic acid

Formula	$C_5H_9NO_4$
Molecular weight	147.13
Synonyms	H-Glu-OH; 2-Aminoglutaric acid; L-Glutaminic acid; (+)-Glutamic acid; (+)-L-glutamate; (S)-(+)-Glutamic acid
Physical state	White crystals or crystalline powder, slightly characteristic taste and acid taste, non-toxic. Freely soluble in formic acid and hot water, slightly soluble in cold water. Hardly dissolves in ether, acetone and cold acetic acid. Insoluble in ethanol and methanol.
Melting point	200 ºC (decomposes)

2. *Corynebacterium glutamicum*

2.1 General information

In 1957, Kinoshita and coworker isolated a bacterial strain which has the ability to over produce and excrete extracellularly L-glutamate in the minimal medium containing glucose as carbon source and the so called bacterium was named *C. glutamicum*. In taxonomic terms, it is belongs to the family of *Corynebacteriaceae* (Reershemius, 2008). A large number of different *Corynebacterium* species have been isolated from various human clinical and veterinary sources. In addition, non-medical corynebacteria are found in a broad variety of different habitats such as soil, plants, animal fodder, and dietary products (Liebl, 2005). During growth, bacteria obviously must be supplied with an energy source, a carbon source, a nitrogen source, and all other macronutrients and trace elements to meet the requirements defined by the cellular chemical composition. In addition, variably, other organic growth supplements like amino acids, nucleotide bases, or vitamins are needed for growth. Most strains of nonmedical *Corynebacterium* species grow well at 30 ºC in standard peptone-yeast extract media like the *Corynebacterium* medium, containing 1% casein peptone, 0.5% yeast extract, 0.5% glucose, and 0.5% NaCl, pH 7.2-7.4. Importantly, strains of glutamic acid producing corynebacteria, *C. glutamicum* and similar bacteria, are dependent upon the presence of biotin in the growth medium, some additionally require thiamine or p-amino benzoic acid (Abe, 1967). *Corynebacterium* cells are not motile and do not sporulate. The cells are Gram-positive and non-acid-fast. Under the light microscope, at about 400- to 1000-fold magnification, these species appeared as rod-shaped cells with a typical, somewhat irregular coryneform morphology. Cells are often arranged in V-formations, because of their snapping mode of cell division (Figure 2). Sometimes, packages of several cells in parallel arrangement, as palisades, are also found. Although some observed morphological differences can depend on the media used and the culture age, the typical morphological features of *Corynebacterium* are normally recognized easily when *C. glutamicum* is grown in various media or when inspected after different incubation periods (Leibl, 2005). In physiological aspects, Corynebacteria are catalase-positive, aerobic or facultative

anaerobic bacteria. The optimum growth temperature is between 25-37 ºC. It was reported by Onishi *et a.,l* (2003) that representative wild-type strain of *C. glutamicum* also grew rapidly at 40 ºC, but apparently this trait of thermotolerance is easily lost during the breeding of lysine production strains, presumably because of secondary mutations during repeated round of random mutagenesis and selection.

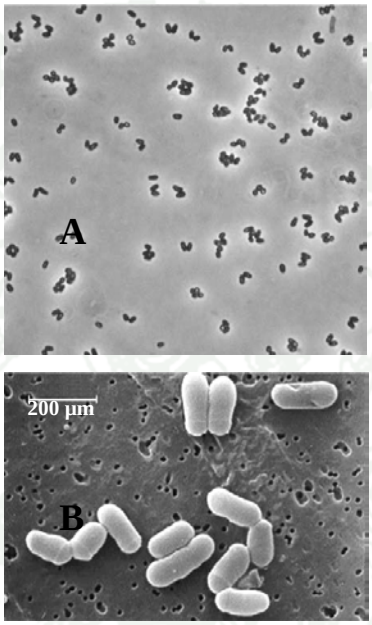


Figure 2 Phase-contrast micrograph of *C. glutamicum* cells grown on complex medium (A). Same cells placed on a nucleopore membrane and viewed by scanning electron microscopy (B). Note frequent V-type arrangement of cell pairs, due to snapping division is observed.

Source: Liebl (2005)

2.2 Biosynthesis of L-glutamate from glucose and acetate as a carbon source

After glucose enters cytosol in the form of glucose-6-phosphate and then transformed to phosphoenolpyruvate (PEP) through glycolysis reaction, it is subsequently converted to oxaloacetate catalyzed by PEP carboxylase (PEPCx). Likewise, PEP carboxykinase (PEPCk) catalyzed the conversion of oxaloacetate to PEP (Figure 3). Malate is then transformed into citrate and isocitrate via oxaloacetate

catalyzed by malate quinine oxidoreductase (MQO) and aconitase (CAN), which either serve as preliminary stage for L-glutamate production, catalyzed by isocitrate dehydrogenase (ICD) to form α-ketoglutarate (2-oxoglutarate) or channeled to the glyoxylate cycle. In case of acetate as a carbon source, the energy gained and intermediate formation are conducted chiefly via the glyoxylate cycle for *C. glutamicum*. Therefore, there is a competition between (i) the isocitrate lyase (ICL) reaction which forms succinate and glyoxylate, necessary for their growth and (ii) the ICD reaction which leads to the production of a key precursor, α-ketoglutarate as shown in Figure 8. (Crueger and Crueger, 1990; Eikmanns, 2005)

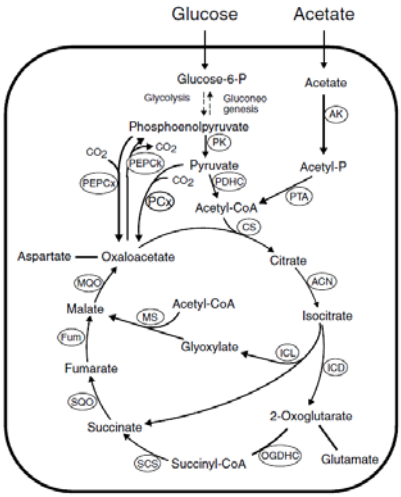


Figure 3 Biosynthetic pathway in *C. glutamicum* on glucose or acetate as a carbon source. The enzymes abbreviation are shown here as follows; ACN: aconitas; AK: acetate kinase; CS: citrate synthase; Fum: fumarase; ICD: isocitrate dehydrogenase; ICL: isocitrate lyase; MS: malate synthase; MQO: malate quinone oxidoreductase; OGDHC: 2-oxoglutarate dehydrogenase complex; PCx: pyruvate carboxylae; PDHC: pyruvate dehydrogenase complex; PEPCk: phosphoenol pyruvate (PEP) carboxykinase; PEPCx: phosphoenol pyruvate (PEP) carboxylase; PK: pyruvate kinase; PTA: phosphoenoltransacetylase; SCS: succinyl-CoA synthetase and SQO: succinate menaquinone oxidoreductase.

Source: Eikmanns (2005)

The key precursor of L-glutamate production is α-ketoglutarate or 2-oxoglutarate which is consequently converted into L-glutamate through reductive amination with free ammonium ion (NH_4^+), as shown in Figure 4. The last step is catalyzed by the NADP-dependent glutamate dehydrogenase or L-glutamate dehydrogenase (GDH) as shown in Figure 5. The $NADPH_2$ required at this stage of the reaction is furnished through the preceding oxidative decarboxylation of isocitrate to 2-oxoglutarate by isocitrate dehydrogenase. After that, $NADPH_2$ is then regenerated by reductive amination of 2-oxoglutarate. The strains used for commercially L-glutamate production has a block in α-ketoglutarate dehydrogenase (KDH). In the absence of NH_4^+ ion, α-ketoglutarate accumulated due to the malfunction in TCA cycle. Therefore, cells are use an alternative way to supplied sufficient TCA intermediate for their reaction (Crueger and Crueger, 1990).

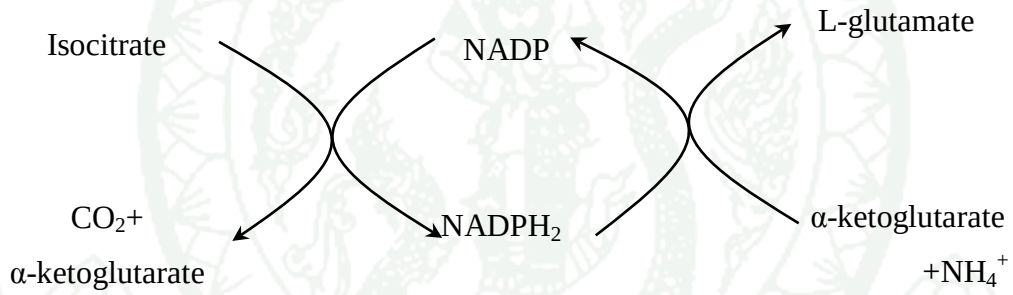


Figure 4 The reductive amination reaction of α-ketoglutarate in the presence of NH_4^+

Source: Crueger and Crueger (1990)

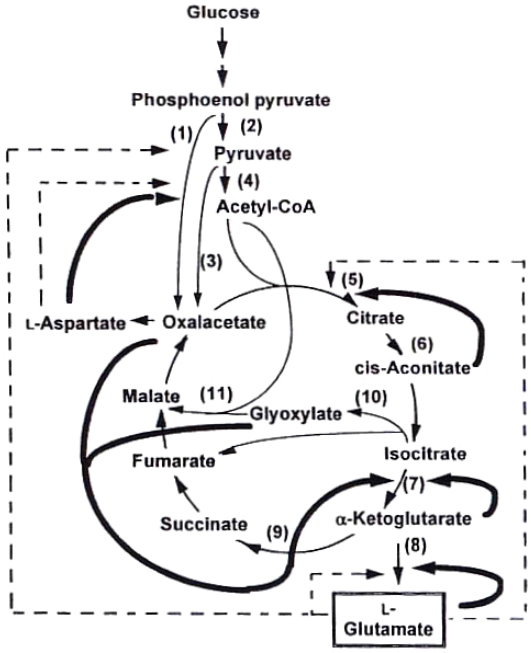


Figure 5 Biosynthesis of L-glutamate in *C. glutamicum* and their regulation. Straight lines represent feedback inhibition and dashed lines represent feedback repression. For the enzyme involves in the figure, it is indicated by a number as followed; PEPCx (1), PK (2), PCx (3), PDHC (4), CS (5), ACN (6), ICD (7), GDH (8), KDH (9), ICL (10) and MS (11).

Source: Reershemius (2008)

The most significant factor for L-glutamate overproduction is the activity of the enzymes GDH and KDH, in Figure 5. During over-producing process, the velocity of α-ketoglutarate to L-glutamate catalyzed by GDH is higher 150 folds than the side reaction of the substrate catalyzed by KDH, which leads back to TCA cycle (Shiio *et al.*, 1980). The Figure 5 illustrated the versatile regulation mechanisms in biosynthesis pathways for L-glutamate which feedback inhibition and feedback repression are indicated. Due to the complexity and various connection in these metabolic pathways, the great obstacles are to modifying these metabolic fluxes in needed direction (Reershemius, 2008).

The important factors for the cultivation process in order to achieve high volume of L-glutamate are the optimum concentration of biotin to influence, support cell growth and the excretion of product into the environment (Clement *et al.*, 1986). Another critical factor to prevent side reaction and undesirable product is the oxygen supply. Under partially anaerobic conditions , another and additional products such as lactic acid could be obtained (Kole *et al*., 1986).

3. L-glutamate production by *C. glutamicum*

3.1 L-glutamate production concerning with the leak model

C. glutamicum requires biotin for their growth. L-glutamate is not produced by wild-type of *C. glutamicum* in the presence of excess biotin in the culture medium. However, significant production of L-glutamate occurs when biotin is not sufficient (Shiio *et al*., 1962 a, b). Although in the presence of excess biotin, the addition of a detergent compound such as polyoxyethylene sorbitan monopalmitate (Tween 40) or polyoxyethylene sorbitan monostearate (Tween 60) causes *C. glutamicum* to produce large amounts of L-glutamate (Takinami *et al*., 1965). Addition of penicillin, one of the β-lactam antibiotics, also enhance the overproduction of L-glutamate by *C. glutamicum* (Nara *et al*., 1964). Similarly, the addition of ethambutol (Radmacher *et al*., 2005), a chemotherapeutic used to treat tuberculosis, or the addition of cerulenin (Hashimoto *et al.*, 2006) trigger L-glutamate production by wild-type of *C. glutamicum*. Temperature-sensitive mutants produce L-glutamate after a heat-shock (Momose and Tagaki, 1978; Delaunay *et al.*, 1999, 2002), although this process is not stable (Uy *et al.*, 2003). Since biotin is a cofactor of acetyl-Co A carboxylase, which is necessary for fatty acid synthesis, it was believed that the cell membrane permeability increased when biotin was not sufficient in the culture medium. In the same way, addition of detergent, penicillin, ethambutol, or cerulenin alters the composition of the cell membrane or cell wall of *C. glutamicum*. Later, it was believed that the permeability of the cell membrane and cell wall should be change by these processes. This explanation was called the leak model of L-glutamate production which implied the leakage of overproduced

L-glutamate through enjured cell membrane by these kinds of treatments (Kawahara *et al.*, 1997). However, Shiratsuchi *et al.*, (1995**)** proposed that large amounts of lysine and L-glutamate were simultaneous produced by cultivating a lysine-producing mutant of *Brevibacterium lactofermentum* with the addition of surface-active agent or penicillin. The results indicated that L-glutamate production of coryneform bacteria could be explained with difficultly only using the regulation of L-glutamate permeability. Moreover, the specific increase of L-glutamate production and reduction of lysine production in this simultaneous fermentation could not be accounted for by the leakage model.

3.2 L-glutamate production concerning with the compositions of cell surface structure

3.2.1 Cell surface structure of *C. glutamicum*

C. glutamicum belongs to the mycolic acid-containing actinomycetes. In terms of phylogenetic, it is highly related to *Mycobacterium* sp., *Nocardia* sp. and *Rhodococcus* sp. Mycobacteria have highly hydrophobic, lipid-bound cell walls. The cell wall lipids mainly consist of a long chain fatty acids called mycolic acid. Mycolic acid is an α-alkyl-β-hydroxylated fatty acid. It is known that *C. glutamicum* has two major types of mycolic acids that is $C_{32:0}$3OH and $C_{34:1}$3OH, and other types, $C_{34:0}$, $C_{36:0,}$ $C_{36:1}$, $C_{36:2}$. The chain length of mycolic acids found in *C. glutamicum* are shorter than those found in other related species, 50-56 carbons for *Mycobacterium* sp. and 34-48 for *Rhodococcus* sp (Shimizu and Hirasawa, 2006).

C. glutamicum has both of cell wall structure and compositions similar to mycobacteria (Figure 6). The cytoplasmic membrane is covered with a thick layer of peptidoglycan surrounding by a layer of arabinogalactan.
The composition of peptidoglycan in *C. glutamicum* is the same as in *E. coli* in which β-1, 4 linked N-acetylglucosamine and N-acetylmuramic acid-containing side chains of peptide, L-alanyl-D-glutamyl-*meso*-diaminopimelyl-D-alanine (Schleifer and Kandler, 1972). As in mycobacteria, mycolic acid is covalently bound to the terminal

hexa-arabinosyl motif of arabinogalactan and forming layer in the most exterior part. This is because of the outer layer consisting of arabinogalactan and mycolic acid, probably function as a permeability barrier (Shimizu and Hirasawa, 2006).

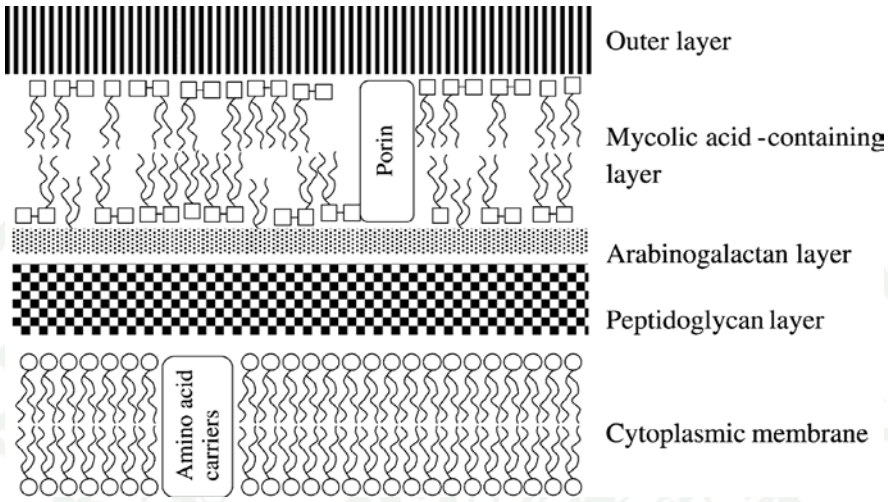


Figure 6 The organization of cell surface in *C. glutamicum*.

Source: Shimizu and Hirasawa (2006)

3.2.2 Lipid and fatty acid compositions concerning with L-glutamate production

Since the scientist discovered that *C. glutamicum* showed auxotrophic phenotype for biotin. That is leads them to interest in the relationship between membrane permeability and L-glutamate overproduction by biotin limitation (Shiio *et al*., 1963), and also another situation.

Hoischen and Kramer (1990) reported the relationship of membrane alteration during L-glutamate overproduction. They found that not only decreased in total fatty acids content, phospholipid, under biotin limitation but also changed in the ratio of saturated/ unsaturated fatty acid in which decreased in oleic acid and increased in palmitic acid. However, distribution of phospholipid species under this condition was unchanged. They also found that time consuming in the

change of fatty acid or lipid composition were not correlated to the change in overproduction process and specific fatty acid or other lipid compounds related to this process was also not found. As a result, they summarized that membrane alteration was required but not sufficient for L-glutamate overproduction.

Nampoothiri *et al* (2000) studied the effects of overexpression or deletion of the genes involved with lipid or fatty acid biosynthesis on L-glutamate overproduction by *C. glutamicum*. They found that changes in expression of that genes caused severe alteration of phospholipid composition and temperature-sensitive growth. These was obviously confirmed by overexpression of *fadD15* (encoding acyl-CoA ligase), *pgsA2* (encoding phosphatidyl glycerophosphate synthase) and *cdsA* (encoding CDP-diacylglycerol synthase) genes, respectively. The mutants of *cls* gene encoding caldiolipin synthase showed significantly temperature sensitive growth. Furthermore, when changing the expression of phospholipid or fatty acid biosynthesis genes, an alteration occurs not only in phospholipid composition and growth phenotype but also in L-glutamate efflux. Overexpression of *acp* gens encoding acyl carrier protein has a strongly effect to reduced L-glutamate accumulation triggered by Tween 60 addition. In contrast it was enhanced by overexpression of *cma* (cyclopropane mycolic acid synthase), *cls* or *plsC* (acylglycerol-3-phosphate acyltransferase) genes. The *cls* and *cma* mutants showed increased Tween 60-sensitivity and accumulated glutamate less than the wild-type. In addition, the strains posses overexpress of *acp*, *cls*, *pgsA2* and *cdsA* genes showed higher amount of L-glutamate than the wild-type with no Tween 60 addition. However, no correlation between phospholipid composition and L-glutamate efflux was found.

3.2.3 Mycolic acid-containing layer concerning with L-glutamate production

It is generally known that *C. glutamicum* and another members in the groups of coryneform bacteria have the outer layer of peptidoglycan. This layer consists of mycolic acid and plays an important role as a permecbility barrier and also resists to some kind of antibiotics (Mitani *et al*., 2005).

Hashimoto *et al* (2006) studied the relationship between mycolic acid formation and L-glutamate production. They reported that under a normal growth condition *C. glutamicum* have three major kinds of mycolic acid, C_{30}, C_{32} and C_{34}, respectively. Especially for C_{32}, it is the most abundant forms up about 70 % of the total mycolic acid. This type of mycolic acid was composed of two C_{16} carbon atom fatty acids, palmitate, which is one of the most abundant fatty acid found in *C. glutamicum*. Another plenty of fatty acid, oleic acid ($C_{18:1}$) was scarcely present in mycolic acid. The total mycolic acid content of *C. glutamicum* was reduced under all conditions, biotin limitation, Tween 40 addition, penicillin addition and cerulenin addition. The latter is the antibiotic that inhibiting biosynthesis of fatty acid and it also induce L-glutamate overproduction. Furthermore, the content of short-chain mycolic acid increased as by biotin limited and cerulenin addition. It is indicating that the treatment applied for triggering L-glutamate production causes defective in mycolic acid layer. Therefore, it is a great possibility to stated that mycolic acid functions as cell membrane permeability in L-glutamate production.

Radmacher *et al* (2005) investigated the biosynthesis of mycolic acid during L-glutamate overproduction. Their results showed that the use of ethambutol which inhibits the formation of mycolic acid, leads to the induction of L-glutamate overproduction. Due to the target of ethambutol is the arabinosyltransferase encoded by *embCAB* gene in *Mycobacterium tuberculosis* (Telenti *et al*., 1997; Escuyer *et al.*, 2001) and *C. glutamicum* also has the homolog of *embC* called *emb* gene which its expression involved in L-glutamate efflux. Therefore, the use of this drug to suppress the *emb* gene affects L-glutamate

overproduction. Moreover, ethambutol treatment caused (i) reduce the arabinan deposition in cell wall due to the activity of arabinosyltransferase, transfers arabinan from *β*-D-Ara*f*-1-monophosphorylpolyprenol to arabinogalactan (Belanger *et al*., 1996), encoded by *emb* gene was reduced, and (ii) the reduction of mycolic acid content in the cell wall. These data suggested that an alteration of the mycolic acid-containing layer trigger L-glutamate overproduction.

3.2.4 Peptidoglycan layer concerning with L-glutamate production

It is known that *C. glutamicum* has the ability to resist to lytic enzyme. It might be because of its mycolic acid-containing outer layer of the peptidoglycan layer. Therefore, the lysozyme-sensitive mutants of *C. glutamicum* are believed to have defects in cell surface structure.

Hirasawa *et al* (2000) studied the lysozyme-sensitive mutants of *C. glutamicum* and L-glutamate production by these mutants. *C. glutamicum* mutant strain KY9714 was originally isolated as a lysozyme-sensitive mutant (Katsumata *et el*., 1991) that cannot grow at 37 ºC. The wild-type strain KY9611 exhibited a normal rod shape at both 30 and 37 ºC but the mutant strain became a little fat rods or club-shaped rod even at the permissive temperature (30 ºC). They became swollen at the restrictive temperature at 37 ºC as shown in Figure 7. This morphology is typical of temperature-sensitive mutant of *C. glutamicum* (Kijima *et al*., 1998). The *ltsA* gene was cloned as the gene that complements both of temperature- and lysozyme-sensitivity of the KY9714 strain. The *ltsA* gene product showed highly homologous with PurF-type glutamine-dependent asparagines synthetase that belonging to the glutamine-dependent-aminotransferase of various organisms. However, the *ltsA* gene cannot complement the asparagine auxotrophy of an *E. coli asnA asnaB* double mutant. The *ltsA* mutants have the ability to produce L-glutamate at the elevated temperatures as shown in Table 1. Therefore, the relationship between a defect cell surface due to the *ltsA* mutation and L-glutamate production was found.

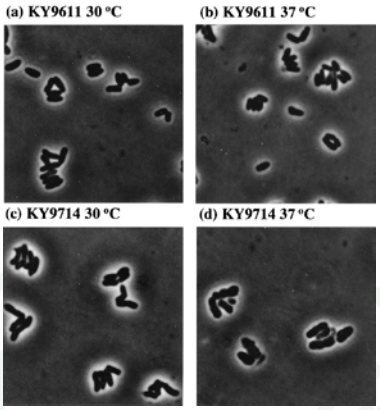


Figure 7 The cell morphology of *C. gluamicum* wild-type strain KY9611 (a and b) and the lysoxyme-sensitive mutant strain KY9714 (c and d). Cells were grown in L broth at 30 ºC (a and c) and 37 ºC (b and d). Phase-contrast microphotographs are illustrated.

Source: Hirasawa *et al* (2000)

Table 1 L-glutamate production by *C. glutamicum* KY9611 wild-type, KY9714 (*ltsA9714*) and KY9611 (*ltsA::kan*) strains. The cells were cultured in basal salt (BS) medium, per liter: 5 g of $(NH4)_2SO_4$, 5 g of urea, 2 g of KH_2PO_4, 2 g of K_2HPO_4, 0.25 g of $MgSO_4.7H_2O$, 0.01 g of $FeSO_4.7H_2O$,0.01 g of $MnSO_4.4–5H_2O$, 0.01 g of $CaCl_2.2H_2O$, 0.03 mg of $ZnSO_4.7H_2O$,0.1 mg of H_3BO_4, 0.07 mg of $CoCl_2.6H_2O$, 0.03 mg of $CuCl_2.2H_2O$, 0.01 mg of $NiCl_2$, 0.1 mg of $NaMo_2O_4.2H_2O$, 50 g of glucose, and 200 mg of biotin, pH7.0.

Strains	L-glutamate production (g/ L)		
	at 30 ºC	at 35 ºC	at 37 ºC
KY9611 (wild-type strain)	0.07±0.01	0.22±0.01	0.29±0.02
KY9714 (*ltsA9714*)	0.10±0.01	0.82±0.12	0.62±0.08
KY9611 (*ltsA::kan*)	0.47±0.02	1.10±0.13	2.45±0.18

Source: Hirasawa *et al.* (2000), Shimizu and Hirasawa (2006)

From the phenotypic and mutational analysis, It was shown that the gene product of *ltsA* gene is a glutamine-dependent amidotransferase, catalyses transferring of the amido residue of glutamine to substrate, concerned with the formation of cell surface structure. Thus, mutation at the *ltsA* gene leads to L-glutamate overproduction. Although the acceptor of amino residue of LtsA protein has not been identified but it is believed that it might be involved in the formation of mycolic-containing layer in *C. glutamicum*.

Mitani *et al* (2005) characterized the LtsA protein from *Rhodococcus reythropolis* which is related to *C. glutamicum*. They reported that the lysozym-sensitivty of *R. erythropolis ltsA* mutant was suppressed by introduction of *C. glutamicum ltsA* and *Bacillus subtilis asnB*, one of the homologs of *C. glutamicum ltsA* (Yoshida *et al.*, 1999). They also found that the lysozyme-sensitivity of *R. erythropolis ltsA* mutant was suppressed by the addition of NH_4Cl into culture medium. Furthermore, the glutaminase activity of the LtsA protein that was inactivated by site-directed mutagenesis can be restored by the addition of NH_4Cl as well. It is indicating that NH_3 as well as glutamine can be used as an amido donor for LtsA protein in *R. erythropolis*.

3.3 L-glutamate production concerning with metabolic flux

Asakura *et al* (2007) stated that even L-glutamate production is suppressed in the presence of excess biotin, the addition of certain fatty acid ester surfactant or penicillin to the medium also induces L-glutamate overproduction. For example, 220 mM L-glutamate is produced from 360 mM glucose by the wild-type *C. glutamicum* under the L-glutamate-producing condition of adding 1 g/ L Tween 40, whereas only 22 mM L-glutamate is produced without Tween 40 addition. It is indicating that at least 10-fold more L-glutamate is metabolically synthesized under producing conditions than under non-producing conditions. Therefore, metabolic flux change is expected to be important for L-glutamate overproduction in *C. glutamicum*.

The 2-oxoglutarate dehydrogenase complex (ODHC) catalyzes the oxidative decarboxylation of 2-oxoglutarate to succinyl coenzyme A (succinyl-Co A), and glutamate dehydrogenase (GDH) catalyzes ammonia assimilation of 2-oxoglutarate to form L-glutamate. These two enzymes compete for 2-oxoglutarate at the branch point of the TCA cycle and L-glutamate biosynthesis. Shingu *et al* (1971) and Kawahara *et al* (1997) reported that ODHC activities are decreased under L-glutamate-producing condition. Also, Asakura *et al* (2007) compared ODHC activities and found a reduction in ODHC activity relative to GDH activity during L-glutamate production, suggesting that the change in metabolic flux caused by the reduction in ODHC activity was important for L-glutamate production. However, the reduction in ODHC activity was not enough to account fully for the high L-glutamate productivity achieved by L-glutamate-producing conditions, as the reduction in ODHC activity ranged from 40 to 90% (Shingu *et al*., 1971 and Kawahara *et al*., 1997) and did not always strictly correspond to the level of L-glutamate production.

Since the ODHC comprises three enzymes: 2-oxoglutarate dehydrogenase (E1o), dihydrolipoamide S-succinyltransferase (E2o) and dihydrolipoamide dehydrogenase (E3). Asakura *et al* (2007) constructed an *odhA* deletion mutant, which completely lack of ODHC activity to encodes E1o subunit, and found that it produced L-glutamate as efficiently as the induced wild-type strain. Furthermore, this efficient L-glutamate production was achieved without any induction or any alteration of the fatty acid composition of the cells. This finding indicated that the change in metabolic flux is the direct cause of the L-glutamate production. Moreover, they also analyzed the effects of L-glutamate producing inductions on the L-glutamate productivity of the *odhA* deletion and found that each of such conditions and the loss of ODHC activity had additive effects on L-glutamate production. They also found that the cells lacking *odhA* accumulated 2-oxoglutarate due to the loss of ODHC activity as shown in the Figure 8 (Asakura *et al*., 2007).

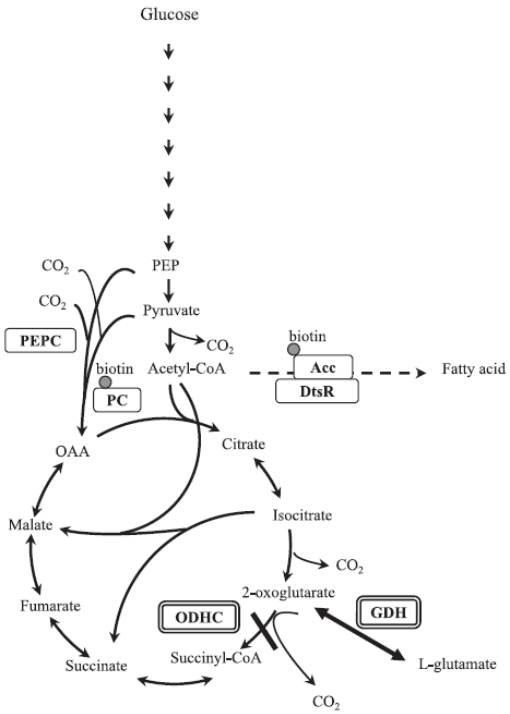


Figure 8 Summary of the speculated metabolic pathway form glucose to L-glutamate in *C. glutamicum.*

Source: Asakura *et al* (2007)

4. L-glutamate excretion in *C. glutamicum*

Although the discover of Asakura *et al* (2007) indicated that the disruption of *odhA* resulted in L-glutamate excretion without any induction. But the report of Nakamura *et al* (2007) shows a slightly conflict in which the additional mutations responsible for L-glutamate production were found in some *odhA* disruptants. After identification, they found the additional mutation on *NCgl1221* gene. They found that the N-terminal region of the NCgl1221 protein shows high homology to mechasensitive ion channels of *E. coli* and other microorganisms. Levina *et al* (1999) and Sukharev *et al* (2002) stated that these channels are activated by alteration of cell membrane tension, preventing the cell disruption by hypo-osmotic shock. Moreover, the C-terminal region of NCgl1221 protein does not resemble to that of any known

protein, this finding leads them to conclude that the NCgl1221 protein is the major exporter of L-glutamate in *C. glutamicum* and it was activated by alteration of cell membrane tension. After all, they proposed the model for L-glutamate production as followed:

1) The treatments used to trigger L-glutamate production caused an alteration of membrane tension

2) Changes in membrane tension affected the stricture of NCgl1221 protein to opened and

3) Activated NCgl1221 protein catalyzes L-glutamate excretion as demonstrated in Figure 9.

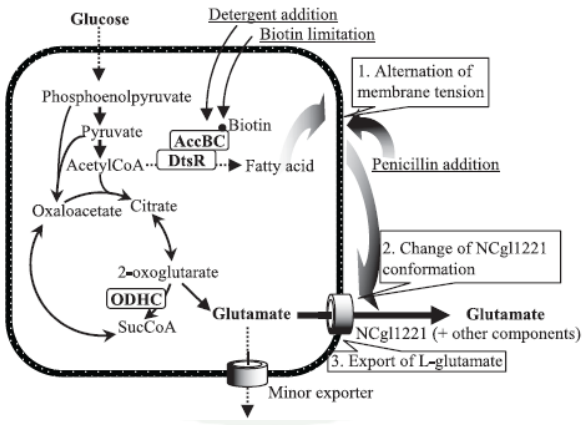


Figure 9 The proposed model of induction of L-glutamate production in *C. glutamicum*. The treatments with biotin limitation, penicillin addition, detergent addition, alter membrane tension by inhibiting fatty acid biosynthesis are leading to alteration of membrane tension. These change triggering the structural transformation of NCgl1221 protein, enable it to catalyze L-glutamate excretion.

Source: Nakamura *et al* (2007)

5. *C. glutamicum DS50*

C. glutamicum thermotolerant strain DS50 was isolated from duck-contaminated soil sample by Kitchakarn (2007). The optimal temperature for its growth was about 35-37 ºC, and has the ability to produce L-glutamate at 40-42 ºC. However, the production ability of this strain was lower in comparison with another production strains. Therefore, it is challenged to use *C. glutamicum* DS50 as a starting strain to generate the L-glutamate overproducing mutant strains by using the strain improvement methods.

6. Microbial strain improvement

The microbial production strain can be regarded as the heart of a fermentation industry. So, improvement of the production strains offers the greatest opportunities for cost reduction without significant capital outlay (Parekh *et al*., 2000). Improvement usually resides in increased yields of the desire metabolite. Today, strain improvement can be performed by two alternative strategies, classical genetic methods, and molecular genetic methods. Each has distinct advantages and in some cases all these approaches can be used in concert to increase production.

6.1 Classical genetic methods

Strain development by this strategy has typically relied on mutation, followed by random screening. After this careful fermentation tests are performed and new improved mutants are selected. Mutation can be carried out with physical mutagens like UV-light, chemical mutagens like N-methyl-N'-nitro-N-nitrosoguanidine (NTG), ethl methanesulfonate (EMS) or another potent mutagens (Baltz, 1999).

6.2 The advantages of mutation/ selection

The advantageous point of mutation and selection is simplicity, since it requires little knowledge of the genetics, biochemistry and physiology of the product biosynthetic pathway. Moreover, it does not need sophisticated equipment and requires minimal specialized technical manipulation. Another important advantage is effectiveness, since it leads to rapid titer increases (Gonzalez *et al*., 2003).

6.3 The selected research papers with classical genetic methods for microbial strain improvement

There are several previously reports with the successfully used of classical genetic methods, single dosage of physical chemical mutagens or in combination, to improve the microbial strains such as in filamentous fungi, yeast and bacteria.

6.3.1 In filamentous fungi

Rubinder *et al.* (2002) proposed a strain improvement program involving repeated mutagenesis and selection to generate amylase-hyperproducing, catabolite-repression-resistant and partially constitutive strains of *Thermomyces lanuginosus*. Spore suspension (1×10^7/ mL) of the wild-type strain was subjected to three cycle of mutagenesis (UV/ NTG) and a selection procedure to develop a mutant strains. One of the selected derepressed mutant strain III_{51} produced at about 7 and 3 fold higher specific activity of α-amylase (190 U/ mg protein) and glucoamylase (105 U/ mg protein), respectively, compared to those of the wild-type strain. Furthermore, the effect of production parameters on the selected strain was study using Box-Behnken design. The regression models computed showed significantly high R^2 values of 96 and 97 % for α-amylase and glucoamylase activities, indicating that they are appropriate for predicting relationships between corn flour, soybean meal and pH with α-amylase and glucoamylase production.

Bapiraju *et al.* (2004) proposed a stepwise mutation by using natural selection, UV-irradiation and NTG treatment to enhance lipase productivity of the wild-type strain *Rhizopus* sp. BTS-24. The best selected strains from the first step of mutation was subjected to the next step mutation following screened and picked-up for the last induced-mutation step. The results shown that the best natural selectant $BTNS_{12}$ exhibited 110% higher lipase activity than the strain BTS-24. The lipase yield of the best UV mutant $BTUV_3$ was 164% higher than the wild-type strain $BTNS_{12}$ and 185% time higher than the strain BTS-24. Furthermore, the lipase yield of the best NTG mutant $BTNT_2$ was 133% higher than the strain $BTUV_3$ and 232% higher than the strain BTS-24. The results indicated that UV and NTG were effective mutagenic agents for strain improvement of *Rhizopus* sp. BTS-24 for enhanced lipase productivity.

Khattab *et al.* (2005) initiated to use of mutagenesis in conjunction with protoplast fusion technique to generate extracellular glucose oxidase (GOD) overproducing strain of *Aspergillus niger*. Various strains of *A. niger* were screened for GOD activity. A suspension (2×10^9 spores/ mL) of the most effective producer, strain FS-3 (15.9 U/ mL) was mutagenized using UV-irradiation or EMS giving viability about 90%. Out of 400 mutants screened, 32 isolates were found to be resistance to 2-deoxy D-glucose, and 17 isolates of these exhibited higher GOD activities, from 114.5 to 332.1%, than the wild-type FS-3 strain. After determination of antifungal resistance of the highest producing mutants, four mutants were selected and used for protoplast fusion in three different intraspecific crosses. All of the fusants tested exhibited higher activities, from 285.5 to 394.2%, than the wild-type strain. Moreover, out of the 30 fusants isolated, 19 isolates were found to produce higher of GOD activities than their corresponding higher-producing parent strain.

Chand *et al.* (2005) proposed the isolation of cellulase producing fungi and a two novel mutation method for increase cellulase production. For the first, a germinating 5×10^8 spores/ mL of the potent strains was treated with 0.1 and 0.2 mg/ mL of 1-methyl-3-nitro-1-nitrosoguanidine (MNNG), ethidium bromide (EtBr) and UV-irradiation for 30 minutes and 1 hour duration and plated on selective

media with and without antifungal amphotericin B. The second method using the incorporation of mutagens (EtBr and MNNG) in the selective media in sublethal concentration (5 µg/ mL) with amphotericin B 2 µg/ mL. The results showed that the second method yielded maximum cellulose-producing mutants, which are also stable for cellulose production and also more potent than the mtants obtained by the first method indicating that this method could be applied to fungal strains for enzyme production.

Ahmed *et al.* (2007) reported that a mutant of *Penicillium roquefortii* were obtained after treated with UV-irradiation and EMS. The alkaloid-producing mutant strains derived by the mutation can be divided into four groups: 1) unstable to synthesized alkaloid, 2) with a non functional chain of clavin formation, 3) with roquefortine and 3, 12-dihydroroquefortine that were not found in the alkaloid fraction and 4) with a new compounds that were not found in the wild-type strain. Furthermore, the results also suggest the presence of not less than three different pathways of alkaloid formation in *P. roquefortii*. On the other hand, all of the selected mutants produced more lipase than the wild-type strain when using olive oil as a substrate. In contrast, many mutant strains produced less lipase than the wild-type strain when cotton seed oil was used as a substrate.

Adsul *et al.* (2007) proposed the mutation method for increased cellulose production of *Penicillium janthinellum* NCIM 1171. A suspension 107 spores/ mL of the wild-type were subjected to mutation with EMS for 24 hours followed by UV-irradiation for 3 minutes, giving 1% survival rate. Among the successive mutants screened, strain EMS-UV-8 showed enhanced cellulose production, clearance zone on Avicel containing plate (SM2 medium) and rapid growth on Walseth cellulose agar plate containing 0.2% 2-deoxy-D-glucose (SM3 medium). These mutants were transferred to Walseth cellulose plate containing higher concentration (1.5%) of 2-deoxy-D-glucose (SM4 medium). All these mutants showed approximately two-fold increase in activity of both Filter paper cellulase (FPase) and Endo-1, 4-β-D-glucanase (CMCase) in shake-flask culture when grown on basal medium containing 1% Cellulose-123 powder (CP-123) and 2.5% wheat

bran. The enzyme preparations from these mutants were used to hydrolyze Avicel. Higher hydrolysis yields of Avicel were obtained with enzyme preparation of the mutant strain EU1. The results indicating that this is the first report on the isolation, selection of the mutants based on hydrolysis of Avicel, which is the most crystalline substrate.

Jun *et al.* (2009) proposed the successive mutagenic steps to improve, and optimize cellulase production of *Trichoderma reesei* Rut-30. *T. reesei* Rut-30 10^7 spores/ mL were subjected to N-methyl-N'-nitro-N-ntrosoguanidine (NTG) for 6 hours followed by UV-irradiation for 15 minutes, resulting 1% survival rate. The successive mutants exhibited increased cellulase production, clear hydrolysis zone, and rapid growth on Avicel-containing plate. In particularly, the mutant strain NU-6 exhibited approximately two-fold increase in activity of both FPA and CMCase in shake-flask culture when grown on basal medium containing 1% peptone and 1% wheat bran. From the optimization for enzyme production using eight different media, a mixture of lactose and yeast cream was used as cellulase inducer, the mutant strain NU-6 exhibited highest enzyme and cell production with a 6.2 U/ mL FPase activity, 54.2 U/ mL CMCase activity, 0.39 U/ mL β-glucosidase activity and 12.6 mg/ mL fungal biomass, respectively. Also, this mutant strain excreted a large amounts of 291.3 U/ mL xylanase.

Chandra *et al.* (2009) developed a mutant strain of *Trichoderma citrinoviridae* by multiple exposured to EMS and EtBr. After each mutagenic treatment, enzyme production by the mutants that showed larger of clear zone formation was assessed in shake-flask culture, and the most promising strain was subjected to the next mutagenic treatment. The mutant EB-104 strain produced 0.63, 3.12, 8.22 and 1.94 IU/ mL FPase, endoglucanase, β-glucosidase abd cellobiase, respectively. These levels were 2.14, 2.10, 4.09 and 1.73-fold higher than that of the wild-type strain. Glucose up to 20 mM did not inhibit enzyme production of the mutant strain under submerged fermentation. From partially purified activity assay, the cellulase showed lack of inhibition by glucose. Furthermore, the partially purified

endoglucanase and β-glucosidase were activated by 2.0 and 2,6-fold, respectively, by 20 and 30 mM ethanol in the assay mixture.

Zambare (2010) reported that alkaline protease production of *Trichoderma reesei* MTCC-3929 can be improved by random mutagenesis with UV-irradiation and NTG treatment with 1% survival rate. The UV-mutants were screened for protease production based on clear zone formation on agar plate containing skimmed milk. The mutant UV-8 exhibited 9 mm clear zone diameter and 199.6 and 552.6 U/ mL protease activity for submerged (SMF) and solid state fermentation (SSF), respectively. The UV-8 was further mutated by NTG, resulting the mutant strain NTG-17 with 13 mm clear zone diameter. Furthermore, the mutant NTG-17 was found to produced 2.6 and 2.2-fold alkaline protease activity than the wild-type strain in SMF and SSF, respectively.

Huang *et al.* (2010) proposed a novel strategy for screening of high yield fumaric acid-producing *Rhizopus oryzae* ZD-35 by adding 50 mg/ L Nystatin into the production medium to restrict the spread of growing hyphae on agar plate containing bromocresol green as a pH indicator. Using this strategy, one high-yield mutant strain HJU11 was isolated from a large colony library of *R. oryzae* after treated with UV-irradiation giving 5% survival rate. Starting with an optimized glucose concentration of 85 g/ L, the mutant produced 57.4 g/ L fumaric acid in shake-flask culture and 41.1 g/ L in 5-L fermenter, which were 205 and 160% higher than that of the wild-type strain.

Xu *et al.* (2011) reported that cellulase production of *Trichoderma viridae* can be enhance after mutated by using successive mutation of 2 mL-10^6 spores/ mL with UV-irradiation, low-energy ion beam implantation, atmospheric pressure non-equilibrium discharge plasma (APNEDP), and NTG. After each mutagenic treatment, the cellulase activity of the mutants that exhibited the largest clear zone formation was assessed under solid state fermentation (SSF), and the most promising strain was subjected to the next mutagenic treatment. After four mutagenic steps, more than 3000 mutant colonies were produced, and about 1000 mutants were

assayed for cellulase activity. Among these mutants, *T. viridae* N879 mutant strain exhibited the greatest relevant activity, 2.38-fold FPase, 2.61-fold CMCase, 2.18-fold β-glucosidase and 2.27-fold cellobiohydrolase activities, compared with those of the wild-type strain under SSF using the inexpensive raw materials wheat straw as a substrate.

6.3.2 In yeasts

Sridhar *et al.* (2002) reported the effect of UV-irradiation on thermotolaerance, ethanol tolerance and osmotolerance of *Saccharomyces cerevisiae*. After using the screening and enrichment program for isolation of thermotolerance yeasts, the VS_1, VS_2, VS_3 and VS_4 strains, isolated from soil samples, appeared to be a thermotolerance, osmotolerance and ethanol tolerance than the other isolates. Among these, strain VS_1 and VS_3 were best performers, and the efforts were made to further improve their tolerance characters by treated with UV-irradiation to giving 50% survival rate. The mutant strains VS_1 40 and VS_3 100 produced 0.25 and 0.20 g/ L biomass more than the wild-type strains (VS_1 and VS_3) at 42 ºC using 2% glucose. At high glucose concentration, both mutants produced biomass which was 0.70 and 0.30 g/ L at 30 ºC, 0.10 and 0.20 g/ L at 40 ºC more than the wild-type strains. Ethanol 8.20 and 1.20 g/ L more than the wild-type strains at 42 ºC using 150 g/ L glucose were produced by these mutants. Further, more ethanol was produced by the mutants (VS_1 40 and VS_3 100) than the wild-type strains at high glucose concentrations of 5.0 and 6.0 g/ L at 30 ºC, 13.0 and 3.0 g/ L at 42 ºC, respectively. The results obtained indicate that UV-treated mutants (VS_1 40 and VS_3 100) acquired more osmotolerance, thermotolerance and ethanol tolerance than their wild-type strain.

Tan *et al.* (2003) reported that a mutant *Candida* sp. 99-125 with high lipase activity was achieved through a successive mutagenic treatment using UV-irradiation (5% survival rate), NTG (0.05% survival rate), and neutron mutation (with a density 10000 T). Lipase production of the mutant increased ten times (1108 U/ mL) compared with the wild-type strain (112 U/ mL). The fermentation medium

containing nitrogen sources, carbon sources and metal ions for lipase production by the mutant was optimized. The optimal medium composition consisted of 4% soybean meal, 2.5% (w/v) soybean oil, 0.1% (w/v) K_2HPO4, 0.1% (w/v) $(NH_4)_2SO_4$, 0.05% (w/v) $MgSO_4$, pH 7.0. The fermentation at 28 ºC with an agitation speed 500 rpm, aeration rate 1: 1 vvm in a 30-L fermenter revealed that high agitation speed increased the dissolved oxygen (DO) in the medium and enhanced lipase activity, and the excess of air did not stimulate the lipase production. The scale up was made from 30-L to 1500-L fermenter. The results indicated that production of lipase by this mutant strain is stable and reached to 8060 U/ mL.

Li *et al.* (2007) proposed the strain improvement to generate a SAM-accumulating mutant of *Candida* sp. Through successive mutagenic steps with NTG and UV-irradiation, the final SAM-accumulating mutant strain YQ-5 was achieved by UV-irradiate (for 30 seconds, resulting 11.13% survival rate) using ethionine and nystatin selection method. The YQ-5 mutant strain accumulated 112.1 mg/ g biomass, which was 3.14-fold higher than that of the wild-type strain S42-12. After cultivated in the optimal medium with a favorable fermentation condition, SAM content of the mutant strain reached at 1740 mg/ L.

Pasha *et al.* (2007) generated pentose-utilizing yeast by protoplast fusion followed by sequential mutation for ethanol fermentation using lignocellulosic substrate. After fused and selected based on growth at 42 ºC and ability to use xylose, the best selected fusants were mutated by UV and MNNG separately. After that, one mutant each was selected from two methods, and further mutated by a new mutation method of incorporating sub-lethal (5 mg/ L of EtBr and MNNG) concentration of mutagen in the media. Finally, the mutant fusant CP11 was found to be stable and used for lignocellulosic fermentation. The fusant yeast gave an ethanol yield of 0.459±0.012 g/ g substrate, productivity of 0.67±0.015 g/ L/ h and fermentation efficacy of 90%.

Shigematsu *et al.* (2010) generated barosensitive (viability loss-character under high pressure treatment) mutants of *Saccharomyces cerevisiae*. After subjected a suspension 10^7 cells/ mL to UV-irradiation for 3 minutes giving 10% survival rate, two barosensitive mutants were obtained. Both mutants exhibited significant loss of viability compared with the wild-type strains (KA31a and KA31α) in the pressure ranging from 175 to 250 MPa. Auxotrophic properties, ethanol fermentation and growth abilities were identical to those of the wild-type strain. The results indicated that these phenotypes are acceptable for use in fermentation control by high pressure treatment.

Cao *et al.* (2011) improved the production of S-adenosyl-L-methionine (SAM) of *Saccharomyces cerevisiae* CGMCC 1226 by using successive mutagenic with UV-irradiation coupled with ethionine-resistant screening procedure. After seven rounds of UV-irradiation followed with the selection procedure, the *S. cerevisiae* mutant strain CGMCC 2842 was selected as a higher SAM synthetase activity which was 2.7-fold increased as compared to the wild-type strain. The production in 15-L fermenter with fed-batch process reached 6.1 g/ L after cultivated for 36 hours, which was increased by 4.3-fold.

Taloria *et al.* (2012) investigated alcohol production of *Saccharomyces cerevisiae* by using twin approaches: 1) strain improvement and 2) increasing the activity of ADH by using metallic activator of enzyme. After applied random mutagenesis by UV exposure for 25 minutes, giving 2-3% survival rate, the mutant was found to produced 12% (v/v) alcohol in fermenter, whereas only 8% (v/v) was produced from the wild-type. Furthermore, addition of 20 mM Zinc sulphate resulted in maximum increased alcohol production. When the same concentration of Zinc sulphate was added to a 2-L fermenter, it resulted in 13.2% (v/v) alcohol production as compared to 8% (v/v) produced by the wild-type strain. Also, 3.2-fold increased in alcohol dehydrogenase activity in the presence of 20 mM Zinc sulphate as compared to the control.

6.3.3 In bacteria

Kadam *et al.* (2006) proposed the acclimatization technique followed by ultraviolet mutagenesis to develop strains of *Lactobacillus delbrueckii* for increase lactic acid production. Mutants obtained by acclimatization technique were isolated by transferring the wild-type strain to the liquid medium and lowering the pH of the medium at each transfer. The selection was based on the rapid growth of the culture in medium at initial pH 4.0 without calcium carbonate. Several repeated transfer were conducted until obtained substantial growth within 24 hours. The acclimatized cells were selected for further mutagenesis by exposing the cell suspension (10^6 cells/ mL) to UV-irradiation for 30 seconds where approximately 1% survival rate was obtained. Among the selected mutant strains, Uc-3 exhibited highest lactic acid production at 135 g/ L in batch fermentation with 150 g/ L of cane sugar, giving 90% lactic acid yield.

Haq *et al.* (2009) reported the strain improvement of *Bacillus licheniformis* strain GCB-30^{UCM} for enhance α-amylase production by using culture improvement through EMS treatment and selection. Five milliliter of EMS (50-300 µg/ mL) was transferred into bacterial suspension and placed at 30 ºC for 10-60 minutes. After that, the suspension was transferred to nutrient starch agar (NSA) medium and incubated at 40 ºC for 24 hours. The bacterial colonies showing bigger zone of starch hydrolysis were selected as a hyper-producer of α-amylase. Among these, only one isolate designated as *B. licheniformis* EMS-200^{40} gave 102.78±2.01 U/ mL/ min enzyme activity. The enzyme production was found to be maximal when use fermentation medium containing (g/ L) lactose 10.0, bactopeptone 14.0, yeast extract 6.0, KCl 1.0, $CaCl_2$ 0.25, $MgCl_2$ 0.2, $MnSO_4$ 0.001, $FeSO_4$ 0.0005, pH 6.5, incubated at 37 ºC for 72 hours. The volume of medium (50 mL) and inoculums size (4.0%) were also found t be optimize for enzyme production.

Khaliq *et al* (2009) proposed of the change in colony morphology and kinetics of tylosin production after mutagenesis of *Streptomyces fradiae* NRRL-20702. UV exposure for 100 and 120 seconds has resulted in 8 and 5% survival rate,

and caused development of six (three of each) morphologically altered colony types on agar plate. After screening using *Bacillus subtilis* bioassay, only morphological mutants indicated the production of tylosin. Among these, an increase of 2.7±0.22-fold in tylosin production (1500 mg/ L) in case of mutant UV-2 in complex medium was achieved as compared to the wild-type strain (550 mg/ L). In addition, gamma irradiation of the strain UV-2 using ^{60}Co (25 KGY, resulting 10% survival rate) gave one morphologically altered colony type, gamma-1, which gave 2500 mg/ L tylosin yield in complex medium. However, UV-irradiation associated changes were unstable with of tylosin activity, whereas mutant gamm-1 displayed high stability on subsequent culturing.

Zhao *et al* (2010) improved lipase production of *Serratia marcescens* ECU 1010 by subjecting the exponentially growing cells (1×10^8 cells/ mL) to UV-irradiation for 30 seconds, which resulted in 22% survival rate of cells. The treated suspension was cultured on tributyrin agar plates and incubated at 30 ºC for 2-3 days. After repeated UV mutagenesis for ten times, the mutant strain UV-01 was selected as a lipase-overproducing mutant based on the clear-halo formation by lypolysis on the tributyrin plate, but lost the ability of producing red pigment (prodigiosin). Furthermore, the mutant strain exhibited 2.3-fold higher lipase activity than the wild-type strain, and shows a good stability for enzyme production after ten serial transfers.

Joshi *et al* (2010) improved lactic acid production of *Lactobacillus lactis* by exposing cell suspension (10^8 cells/ mL) to UV-irradiation for 17 minutes, which gave approximately 1% survival rate. Lactic acid-overproducing mutants were selected based on rapid growth as well as clear zone of acid production. After that, the lactic acid-overproducing mutants were further mutated by exposing to UV-irradiation for 20 minutes. Subsequently, one mutant RM2-24 was found to produced 81 g/ L lactic acid, which was 3-fold higher than that of the wild-type strain. The highest lactic acid (110 g/ L) in batch fermentation was obtained with 150 g/ L sugar cane, with a 73% lactic acid yield.

Basavaraj *et al* (2010) improved the bacterial cellulose production of *Gluconacetobacter xylinus* NCIM 2526 by exposing the cell suspension (10^5 cells/ mL) to UV mutagenesis, and screening for their cellulose production abilities. The best UV-mutant was further subjected to EMS mutagenesis. The mutant $GHEM_4$ gave cellulose yield of 5.96 g/ L which was 50% more than that of the wild-type strain NCIM 2526.

Zhong *et al* (2011) improved the production of 1,3-Dihydroxyacetone (DHA) of *Gluconobacter oxydans* ZJB-605 by spreading the cells ont GYA plates, and placed under UV lamp and irradiated for 60 seconds. After that, screening for DHA-overproducing and DHA-resistant mutants were performed in shake-flask culture. The mutant *G. oxydans* ZJB 11001 was selected as it exhibited high DHA productivity (0.39±0.02 g/ L/ h), and also tolerate to high DHA concentrations. The maximum DHA concentration of 209.6±6.8 g/ L was obtained after 72 hours of fed-batch fermentation at30 ºC.

Xu *et al* (2011) reported the successful developed strain of *Bacillus subtilis* showing enhanced acetoin production. The mutants were induced by treating the wild-type strain N-12 (10^8 cells/ mL) with a UV light. The improved strains were mainly obtained with irradiation time of 40 and 60 seconds, which gave approximately 20-30% survival rate of the cells. The best UV-mutant was used as the starting strain for NTG mutation by treating cells with NTG at the interval time between 20 to 80 minutes. After that, highest acetoin-producing mutant was selected and used for the compound mutagens treatment. UV treatment time and NTG treatment time for the mutant was 40 seconds and 50 minutes, respectively. Among 208 survivors, the mutant strain TH-49 was found to produced highest 43.8 and 46.9 g/ L acetoin in flask and 10-L fermenter. It was almost 4-fold higher than that of the wild-type strain.

Agrawal *et al* (2012) reported the development of a β-galactosidase hyperproducing mutant of *Bacillus subtilis* strain PS by using the combination of chemical mutagen and UV mutagenesis. For EMS mutagenesis alone, the suspension

(10^6 cells/ mL) was treated with EMS (0.03 mL) at 37 ºC for 20 minutes. In case of UV mutagenesis, cell suspension was exposed to UV light for 25 minutes. In both case, approximately 50% survival rate of the cells were obtained. For the combination, the best of EMS-mutants were subsequently subjected to UV mutagenesis. The mutant PS-UM1, developed after UV exposure alone indicated a small increase in β-galactsidase production (718 U/ L) in comparison to the wild-type strain PS (675 U/ L). The mutant PS-CM5, developed after EMS exposure alone displayed a slightly better production (762 U/ L) than both of the previous strains. However, after the wild-type exposed to both the combination of UV and EMS, a better mutant PS-CM-UM3 was developed with 1.2-fold increase in production (806 U/ L). Moreover, optimization of culture conditions by classical one-variable-at-a-time approach was conducted to determine the optimum pH, temperature, and nitrogen sources. The mutant PS-CM-UM3 produced up to 1797 U/ L enzyme and was found to be stable for ten successive generations.

Gao *et al* (2012) developed a butanol-resistant mutant strain of *Clostridium acetobutylicum* ATCC 824 by exposing the cells to 0.8 mg/ mL NTG for 30 minutes, which gave approximately 28% survival rate of the cells. After that, the mutant cells were screened on agar plate containing various concentration of butanol. A selected butanol-resistant mutant strain 206 produced 50% higher than that of the wild-type strain when 60 g/ L glucose was used as a substrate. Furthermore, this strain was also able to produce of 23.47 g/ L in 80 g/ L glucose P2 medium after 70 hours fermentation, including 5.41 g/ L acetone, 15.05 g/ L butanol and 3.02 g/ L ethanol, resulting in an acetone-butanol-ethanol (ABE) yield and productivity of 0.32 g/ g and 0.34 g/ L/ h.

MATERIALS AND METHODS

1. Bacterial strains

Corynebacterium glutamicum strain DS50 isolated from duck feces contaminated-soil (Surawadee, 2007) was used throughout the study.

2. Culture media

2.1 For preservation and preparing inoculums or seed culture, A1 medium (see Appendix A, No 1) was used.

2.2 For L-glutamate production, cells were cultured in Basal Salt (BS) medium (see Appendix A, No 2) medium.

2.3 For lysozyme and temperature sensitivity tests, cells were grown in Lennox medium (see Appendix A, No 3).

3. Induced mutation by Ultraviolet (UV) light

3.1 Inoculum preparation

The inoculum was prepared by transfer 1 loop full of the active growing cell on A1 slant into 15×150 mm-test tube containing 5 mL A1 broth. The culture were grown at room temperature (32-33ºC) with aeration 350 rev min^{-1} (G10 Gyratory Shaker, New Brunswick Sciencetific, USA) for 18 hours and then re-culture in fresh medium at the same condition. Then, pipette 0.2 mL of culture into 250 mL-Erlenmeyer flask containing 50 mL A1 broth and cultivate at room temperature with shaking rate 350 rev min^{-1} until cell density at OD_{660} reaches 2.(corresponding to 10^7 cells/ mL, as cultivation for 4 hours (see Appendix B, No. 3).

3.2 The UV-induced mutation protocol

The UV-induced mutation was conducted by following method of Ekwealor and Obeta (2006) with a slight modification. The inoculums obtained from 3.1 were kept on ice for 30 minutes to stop further growth. Twenty mL of inoculum were transferred to a sterile centrifuge tube. After centrifugation at 10,000 rev min^{-1} (himac CR21F, HITACHI, Japan) for 5 minutes at 4ºC, the cells were washed twice with 0.85% NaCl and re-suspended with an equal volume of 0.85% w/v NaCl. After that, the cell suspension were poured onto a sterilized 9 cm-diameter petri-dish and allowed to exposed to UV light 254 nm at a distance of 30 cm in the laminar air flow that UV lamp (Sylvania, 15 W) was operated for 20 minutes to confirmed evenly emitted. Times for UV exposure were 4, 6, 8 minutes with a triplicate for each time interval. The UV-treated cells were pooled together and then plated out on A1 agar with and without serial dilution. The plates were subsequently incubated at 35ºC, 18-24 hours in dark condition to avoided photo-reactivation process. The five hundred colonies of UV-mutants from survival rate of 0.1-1 % (Karla, M. S. *et al*., 1973, Vaidya, R. J. *et al*., 2003) were randomly picked-up and kept as a master plate and used for further studies.

3.3 The screening of mutant for high L-glutamate production

The selection of mutant for high L-glutamate production were performed in test tube as a primary screening. One loop full of the culture from A1 agar slant was transferred to 15×150 mm-test tube containing 5 mL A1 broth. The culture was aerated at 350 rev min^{-1} at room temperature (32-33ºC) for 18 hours and then re-culture in fresh medium at the same condition. Transferring the inoculum 0.1 mL into 20×150 mm-test tube containing 5 mL Basal Salt medium. The culture were cultivated with shaking rate 220 rev min^{-1} (Bio-Shaker BR-300LF, TAITEC, Japan) at 37ºC for 24 hours. The qualitative analysis for L-glutamate in culture medium was performed using paper chromatographic method in which amino acid pattern was visualized by ninhydrin and compared with standard L-glutamate and lysine (1 µg/ µl

each). The potent mutants were kept in deep freezer under glycerol at - 80ºC for further studies.

4. Selection of L-glutamate-producing mutants

4.1 Seed culture preparation

The seed culture of selected mutants was prepared by inoculation 1 loop full of working stock into 15×150 mm-test tube containing 5 mL A1 broth. The culture were then aerated at 350 rev min^{-1} at room temperature for 18 hours. The culture broth 0.1 mL were transferred to a fresh medium and allowed to grow in the same condition. Then, 0.2 mL of seed culture were transferred to 250 mL-Erlenmeyer flask containing 50 mL A1 broth with aeration 350 rev min^{-1} at room temperature for 8-10 hours (OD_{660} reaches approximately 20).

4.2 Culture condition

The seed culture prepared as described in 4.1 were transferred to a triplicate of 250 mL-Erlenmeyer flask containing 50 mL Basal Salt medium with initial OD_{660} about 0.3. Samples were collected at time intervals until the cell density dropped. After that, the time-course of growth and L-glutamate as detected by paper chromatography were compared with *C. glutamicum* DS50 wild type.

5. Characterization of growth and L-glutamate production of the selected mutants

5.1 Temperature sensitivity test for the selected mutants

Temperature sensitivity test for the selected mutants were conducted by prepare seed culture as already explained in 4.1 using Lennox broth instead of A1 broth. The cell cultures with an initial OD_{660} equal to 0.3 were allowed to grow in 250 ml-Erlenmeyer flask containing 50 ml Lennox broth at 32 - 33 (room

temperature), 35, 37, 39, 41, 43 and 45ºC with triplicate for each culture conditions and then aerated by shaking at 220 rev min^{-1}. Growth profiles at each cultivation temperature that derived as absorbance value at OD_{660} were plotted against cultivation times from 0 to 24 hours.

5.2 Lysozyme sensitivity test for the selected mutants

Lysozyme sensitivity test for the selected mutants were took place by preparing seed culture as in the same condition as for temperature sensitivity test and then permitted to grow in a triplicate of 250 ml-Erlenmeyer flask containing 50 ml Lennox broth at room temperature with initial OD_{660} at about 0.06-0.07 and the aeration rate was set at 250 rev min^{-1}.(Innova 2100, New Brunswick Scientific, USA) The addition of enzyme at a concentration of 0 to 450 µg/ mL were suddenly started when OD_{660} reached 0.2 after cultivated for 3 hours (Hirasawa *et al*., 2000). To examine the inhibition effect of lysozyme, the culture broth that sampling every 1 hour of cultivation were placed on ice to avoid further lysis reaction and then immediately measured the optical density at 660 nm until it reaches the final point of cultivation at 10 hours. After that, the absorbance value that obtained as OD_{660} were plotted against cultivation time and then interpret as lysozyme inhibition profiles.

5.3 Optimization of temperature for L-glutamate production of the selected mutants

Optimization of temperature for L-glutamate production of the selected mutants were operated by transferring the seed culture that prepared as previously mentioned in 4.1 to 250 mL-Erlenmeyer flask containing 50 mL Basal Salt medium with an initial OD_{660} about 0.3. After that, the triplicate culture for each conditions were grown at 32, 35, 37, 39, 41 and 43ºC with shaking rate 220 rev min^{-1}. The culture broth were collected at only 0 and 24 hours of cultivation for further determined the reducing sugar and L-glutamate quantities by enzymatic method (Kasukabe *et al*., 1984).

5.4 Optimization of biotin contents for growth of the selected mutants

Optimization of biotin contents for growth of the selected mutants were done by transferring the seed culture as followed 4.1 and subsequently washed twice with 0.85% w/v NaCl. The washed cell were homogeneously re-suspended with Basal Salt medium without biotin and then finally adjust volume to 20 mL. After that, the cell suspension were transferred to 250 ml-Erlenmeyer flask containing 50 ml Basal Salt medium with various concentrations of biotin contents (200, 150, 100, 60, 30, 15, 10, 5, 2.5 and 0 µg/ L). The triplicate culture with an initial OD_{660} at about 0.3 were grown with the aeration rate of 220 rev min^{-1} at 37ºC. To observe growth characteristics, the culture broth were sampling in time intervals as cultivated from 0 to 24 hours for measuring the optical density at 660 nm and to quantify the amounts of L-glutamic acid using enzymatic method.

6. Study on growth and L-glutamate production of the selected mutant in jar fermenter

6.1 Inoculum preparation

6.1.1 Non-washed inoculums

Two flasks of inoculums obtained from 4.1 were pooled together giving a total volume of 100 mL. Then, transfer 50 mL into a jar fermenter which corresponding to 5 % of inoculums size (initial OD_{660} approximately 1).

6.1.2 Washed inoculums

After pooled 2 flasks of inoculums obtained from 4.1, a total volume of 100 mL were harvested and subsequently washed twice with 0.85 % NaCl. Then, re-suspend with 20 mL Basal Salt medium (OD_{660} approximately 100). After that, transfer the washed inoculum into a jar fermenter resulting initial OD660 approximately 1.

6.2 Culture conditions

Study on growth and L-glutamate production of the selected mutant in jar fermenter was conducted by using batch culture. The culture were grown in 3 L fermenter (BIONEER, BEM marubishi. CO., Ltd, Japan) with 1 L working volume of Basal Salt medium. In case of non-washed inoculums, the inoculums size was 5 % v/v of the culture medium which giving initial OD 660 approximately 1. The culture conditions were conducted with aeration rate of 0.5-1.0 vvm in Basal Salt medium with biotin 0, 200 and 400 μg/ L. In other case, the washed-inoculum was transferred into culture medium resulting initial OD 660 equally to 1. Culture conditions were operated with aeration rate of 1.0 vvm in Basal Salt medium with biotin 0 and 200 μg/ L. The pH was constantly controlled at 7.2 with 15% ammonia solution and 3 N HCl. A relatively high impeller agitation speed of 300 rev min^{-1} was kept unchanged at a fixed aeration rate as described previously to maintain sufficient oxygen level for the growing cells. The temperature for cultivation was adjusted to 37 ºC. The culture broth was collected at a time intervals for further assays of biomass, reducing sugar, L-glutamate both qualitative by chromatographic and quantitative by enzymatic method. Their growth profile and L-glutamate pattern were compared to those of the wild type strain.

7. Analysis procedures

7.1 Growth and maximum specific growth rate

To construct the growth curves, the optical density of cells that expressed as the absorbance value at 660 nm were measured using spectrophotometer (U-1800, HITACHI, Japan). To calculate the maximum specific growth rate (h^{-1}), the slope that observed from growth curve was calculated following the formula as shown in Appendix B, No 2.

7.2 Reducing sugar analysis protocol

Reducing sugar in the culture broth were determined by Nelson-Somogyi method (1944) as shown in Appendix C, No 3.

7.3 The analysis protocol for determining L-glutamate in culture media

7.3.1 Qualitative analysis of L-glutamate in culture media by paper chromatographic method (Apendix C, No 1)

The paper chromatogram that illustrated amino acid pattern throughout of thesis was performed as a fast and rapid method for detecting amino acid in the culture medium. Each spots appeared at each positions on paper chromatograph per one sheet were composed of:

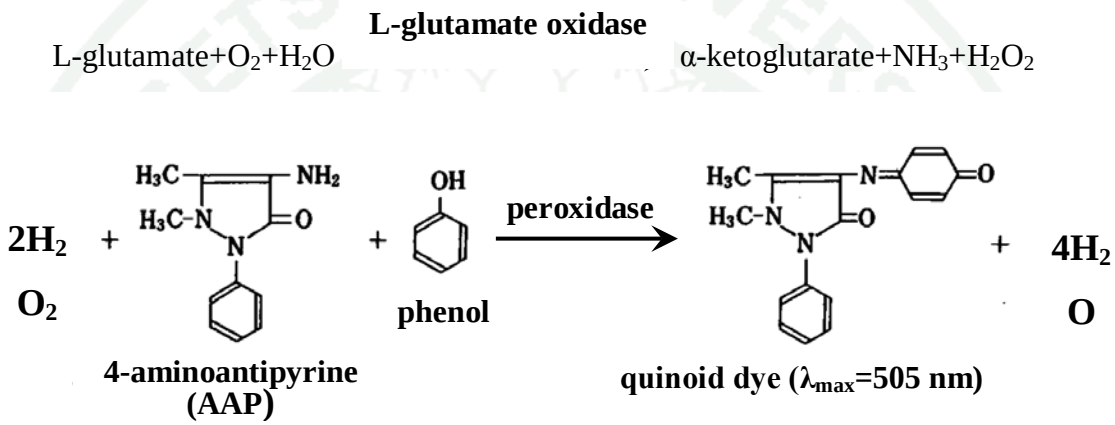
1) The spots appeared on the top-right and left side in each chromatography paper are standard amino acid L-lysine at a concentration of 1 µg/ µL.

2) The spots appeared on the bottom-right and left side in each chromatography paper are standard L-glutamate at a concentration of 1 µg/ µL and spots along the middle in each paper are sample produced by microorganism from any conditions that should be stated otherwise.

The cell-free supernate were spotted onto a chromatography paper (Whatman, No. 3). After saturated with solvent solution mixture of n-butanol: acetic acid: water (4: 1: 1 w/v) and completely dried at room temperature, amino acid pattern was appeared by spraying with a solution of 0.2% ninhydrin in acetone.

7.3.2 Quantitative analysis of L-glutamate in culture media by enzymatic method (Appendix C, No 2)

Quantitative analysis of L-glutamate in culture media by enzymatic method were carried-out based on the conversion of L-glutamate which catalyzed by L-glutamate oxidase giving three kinds of the end-product i.e. hydrogen peroxide, α-ketoglutaric acid and ammonia. In particular hydrogen peroxide, it was specifically reacted with phenol and 4-aminoantipyrine in the presence of horseradish peroxidase and the resulting quinoneimine chromogen which have maximum absorbance (λ_{max}) at 500 or 505 nm is produced as shown in the following equation.



RESULTS AND DISSCUSSION

Results

1. *Corynebacterium glutamicum* DS50 mutants derived from induced mutations by ultraviolet light

Coryebacterium glutamiucum DS50 mutants were obtained by induced mutations with ultraviolet light at 254 nm for 6 and 8 minutes. The effect of UV mutagenesis on the cell numbers and survival rates were summarized in Table 1. For the exposure time at 6 minutes, the number of mutagenized cells were decreased 5 to 6 folds as compared to control giving 16% survival rate.

Table 2 Effects of UV-irradiation at 254 nm on the cell number and survival rate of *C. glutamicum* DS50

Exposure time (minutes)	Cell number (CFU/ mL)	Survival rate (%)
0	$5\text{-}6.45\times10^{7}$	100
6	1.05×10^{7}	16
8	5.35×10^{3}	0.01

However, when the exposure time increased to 8 minutes the cell numbers and survival rate were dramatically decreased to 5.35×10^{3} CFU/ mL and 0.01 %, respectively. Consequently, single colony of the suspected mutants from both conditions were picked-up for 500 colonies by a sterilized toothpicks. After spot onto A1 agar plate and incubate at 35 ºC overnight or until growth was observed, the plates were kept at 4ºC as a master plate of mutant libraries for further experiments.

For primary screening, 100 single colonies from the batch of 0.01 % survival rate, representing 20% of the total 500 colonies of the master plate, were randomly selected for the best of L-glutamate excretion under shake-tube condition using Basalt Salt medium with biotin 200 µg/ L. It was found that all of the selected isolates have the ability to excrete of L-glutamate which detected by paper chromatography as the same level as that of the wild-type. Therefore, we could not find the improved strains from this condition (Figure 10). On the contrary, another selection strategy was performed using the mutant library of 16% survival rate. One hundred eighty colonies which corresponding to 36% of the total 500 colonies were randomly chosen. It was revealed that 5 mutant isolates namely, UV-BB9, UV-GG7, UV-JJ7, UV-MM8, UV-MM10 appeared to be good candidates in the point of L-glutamate excretion which detected by paper chromatography. The intensity of L-glutamate spot from these candidates showed the highest intensity among the other isolates (Figure 11).

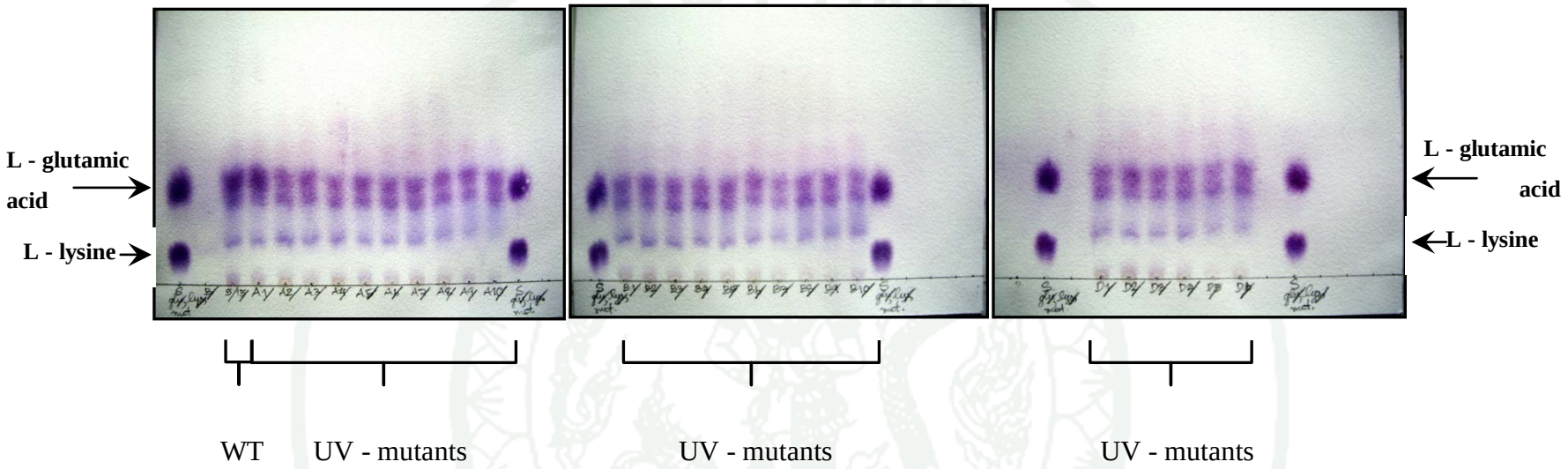


Figure 10 Example of primary screening of L-glutamate-producing mutants from 0.01 % survival rate. The mutants were randomly picked up and grown in 20×150 mm-test tube containing 5 mL Basal Salt medium (pH 7.2) with 200 µg/ L biotin under shaking at 220 rev min^{-1} at 37 ºC. Five microlitre of cell-free culture broth was spotted and visualized using ninhydrin by paper chromatography. For standard amino acid, L-glutamate and lysine (5 µg each) were used.

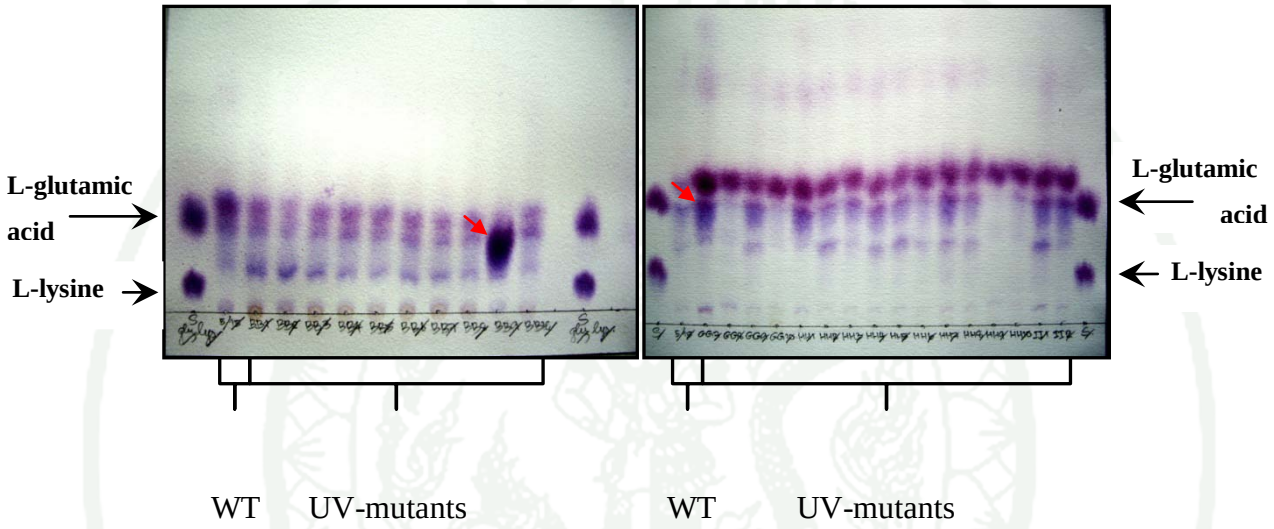


Figure 11 Example of primary screening of L-glutamate-producing mutants from 16 % survival rate. The mutants were randomly picked up and grown in 20×150 mm-test tube containing 5 mL Basal Salt medium (pH 7.2) with 200 µg/ L biotin under shaking at 220 rev min^{-1} at 37 ºC. Five microlitre of cell-free culture broth was spotted and visualized using ninhydrin by paper chromatography. For standard amino acid, L-glutamate and lysine (5 µg each) were used.

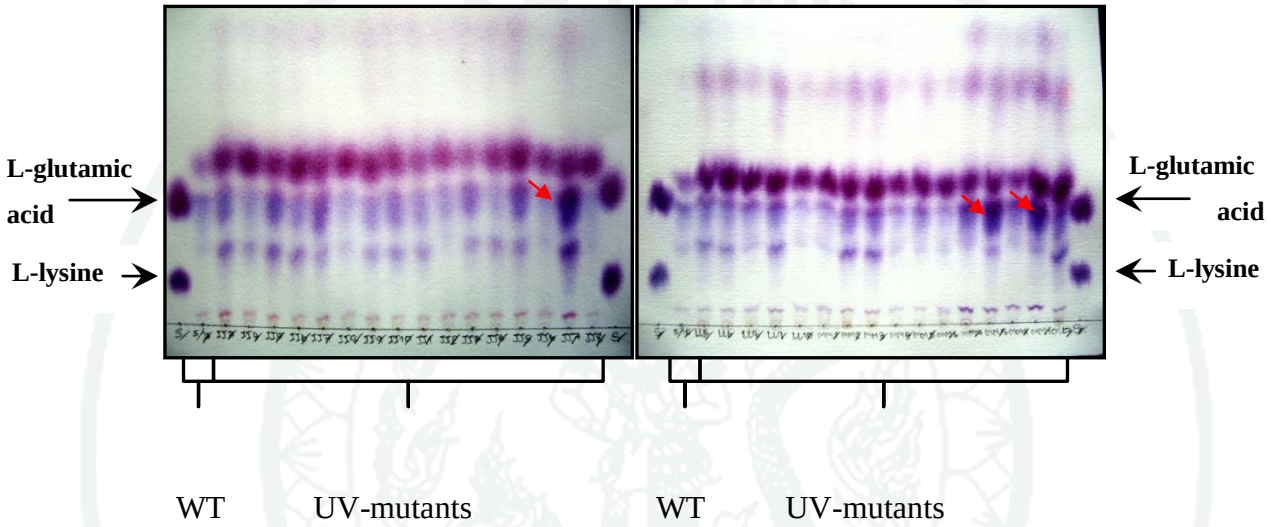


Figure 11 Continued

Five selected strains of UV mutants namely UV-BB9, UV-GG7, UV-JJ7, UV-MM8 and UV-MM10 obtained from primary screening were cultivated in 250 ml-Erlenmeyer flask containing 50 mL Basal Salt medium with biotin 200 µg/ L under aerated condition as a secondary screening. Their growth profile and L-glutamate excretion were determined and compared with those of the wild-type strain. It was found that only one mutant isolate namely UV-BB9 exhibited growth and L-glutamate pattern distinctly from those of the wild-type strain as shown in Figure 12. After cultivation for 15 hours, the maximum specific growth rate (h^{-1}), the maximum optical density at 660 nm of UV-BB9 were 0.59, 25.2, respectively. As considered from the L-glutamate pattern visualized by paper chromatography, the L-glutamate excreted by UV-BB9 was initially detected as a first point after cultivation for 4 hours.(Figure 12) After that, the spot intensity was gradually increased at the next point and remains constant until the last cultivation period. For the wild-type strain, the maximum specific growth rate (h^{-1}) and maximum optical density at 660 nm were 0.85 and 33.4 after cultivation for 10.5 hours. The L-glutamate produced by the wild-type strain could be detected at 10.5 hour onwards and the spot intensity was gradually increased until the cultivation time was over. (Figure 12) Comparison of growth characteristics and L-glutamate excretion pattern of both strains, it should be noticed that the UV-BB9 take a long a long lag phase time to enters log phase and maximum specific growth rate (h^{-1}), maximum turbidity, also, were lower than those of the wild-type strain. However, the ability to excrete L-glutamate of the mutant strain was obviously improved, which could be imply as constitutive production pattern, more than the wild-type strain. In case of another selected mutant strains, it was found that both of maximum turbidity and maximum specific growth rate (h^{-1}) were not significantly different from those of the wild-type strain. Based on growth characteristics and L-glutamate excretion pattern detected by paper chromatography as summarized in Table 3, we selected *C. glutamicum* strain UV-BB9 as a potent strain for further studies.

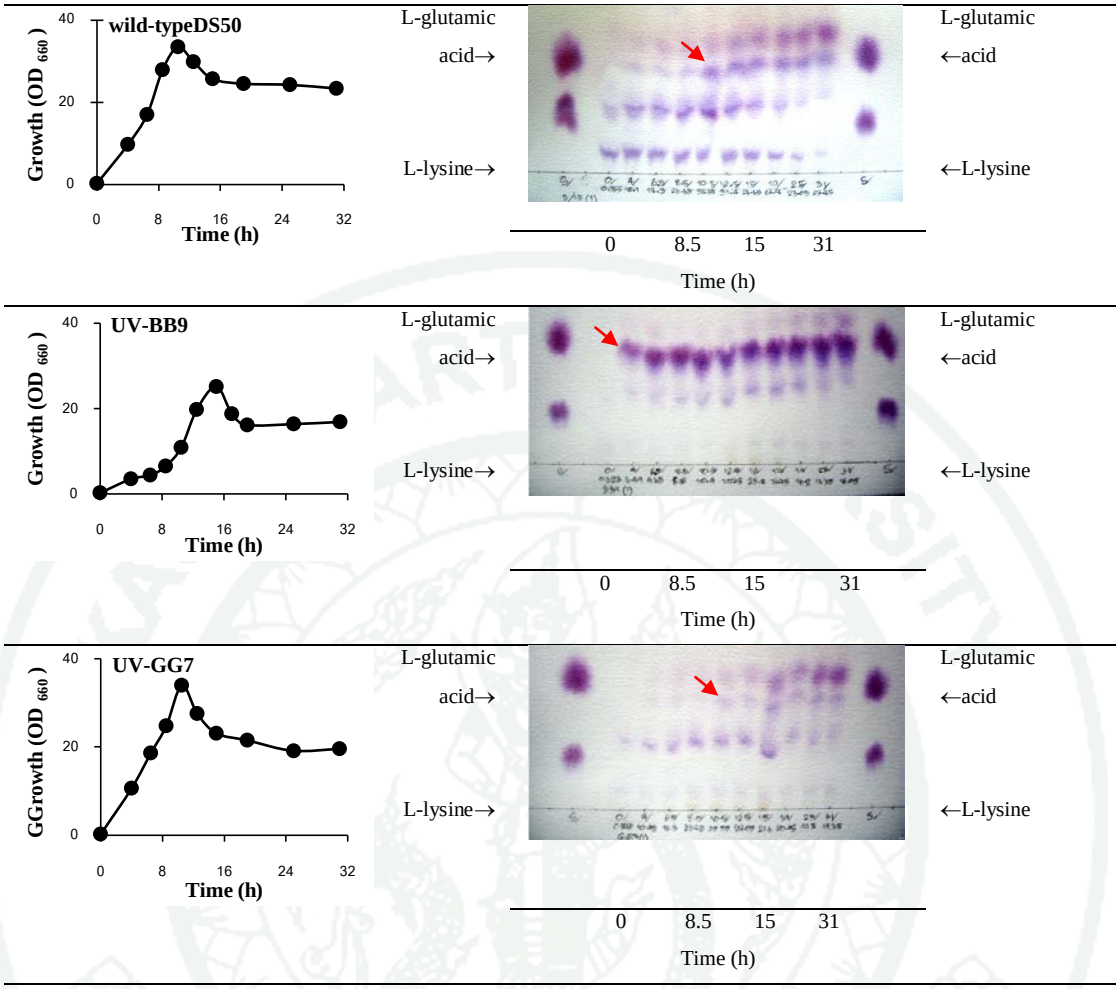


Figure 12 Growth profiles and chromatograms of L-glutamate obtained from *C. glutamicum* DS50 and five strains of the UV-mutant. Cells were cultured in 250-ml Erlenmeyer flask containing 50 mL Basal Salt medium with shaking rate 220 rev. min^{-1} at 37 ºC. Arrow indicated the times of initially detected of L-glutamate in the culture medium. Five microlitre of cell-free culture broth was spotted and visualized using ninhydrin. For standard amino acid, L-glutamate and lysine (5 µg each) were used.

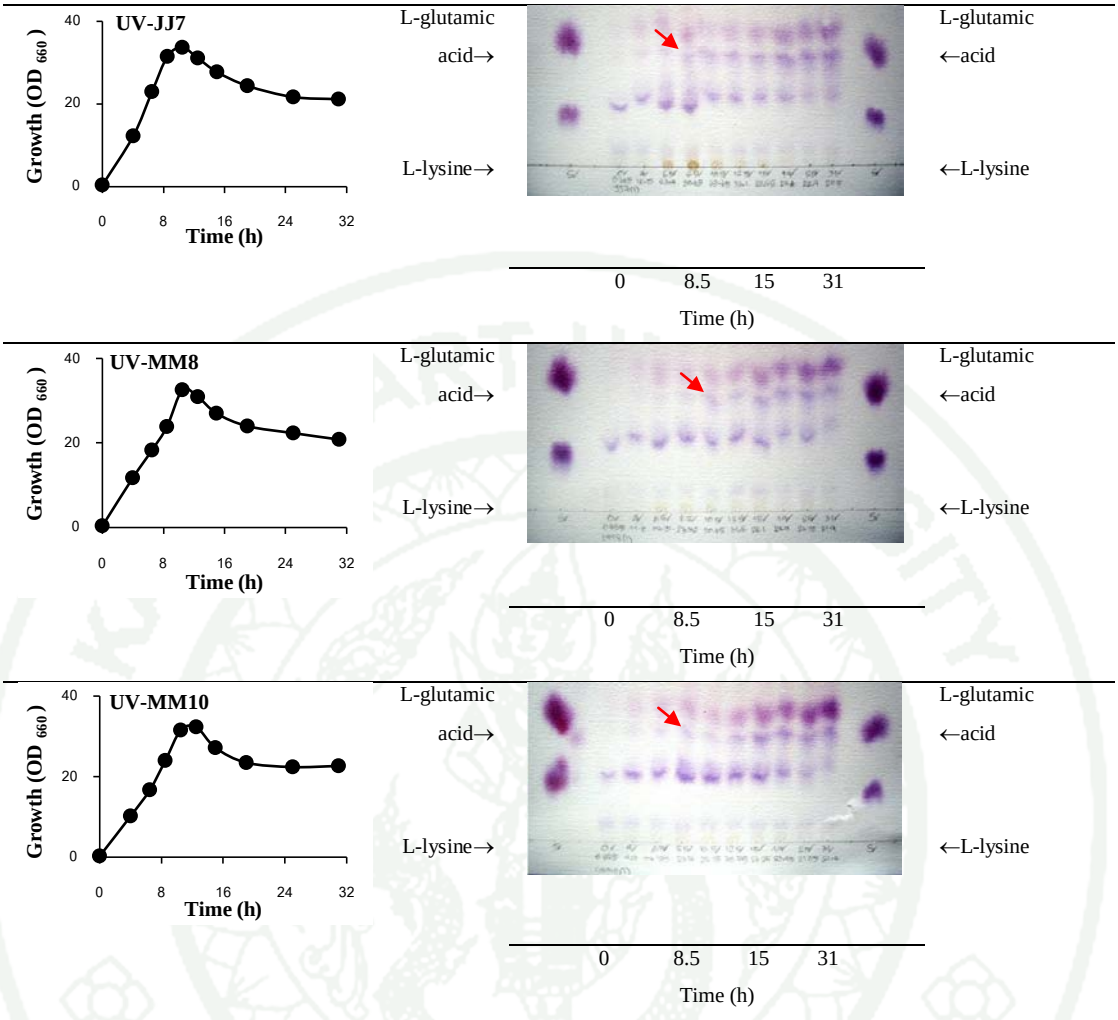


Figure 12 Continued

Table 3 Comparison of growth characteristics and L-glutamate excretion of *C. glutamicum* UV-mutants.

	C. glutamicum DS50	Selected mutant strains				
		UV-BB9	UV-GG7	UV-JJ7	UV-MM8	UV-MM10
1. Times required for OD_{660max} (h)	10.5	15	10.5	10.5	12.5	12.5
2. $OD_{660\ max}$	33.4	25.7	35.4	34.2	31.8	32.7
3. μ max (h^{-1})	0.85	0.59	0.89	0.87	0.90	0.89
4. Times required for initially detected of L-glutamate (h)	10.5	4	10.5	8.5	10.5	8.5

2. Physiological characteristics of *C. glutamicum* UV-BB9

The experimental results obtained from primary and secondary screening firmly indicated that growth characteristic of the UV-BB9 was decreased, but increased in L-glutamate excretion than that of the wild-type strain. We also strongly believed that the great of extracellulary L-glutamate excretion affected by an alteration of either cell wall or cell surface structure. Therefore, we selected two phenotypic characteristics which involved in cell surface structure, temperature and lysozyme sensitivity test, to examine physiological characteristics of the UV-BB9 mutant strain.

2.1 Temperature sensitive growth

Physiological characteristics of *C. glutamicum* UV-BB9 were performed with 2 set of the experiments. The first set of experiment is designated to examine the effect of temperature on growth characteristics of the wild-type, temperature sensitivity test, by investigating growth profiles under different cultivation temperatures. For the wild-type, the maximum specific growth rate (h^{-1}) at each temperatures were 0.63, 0.72, 0.72, 0.66, 0.39, 0.20, respectively, and the maximum OD_{660} at each temperatures were 18.77, 18.15, 13.88, 10.42, 4.21, 1.02, respectively, and no growth was observed when elevated temperature to 45 ºC as indicated in Figure 13 A. The optimum temperatures for growth of the wild-type were in the range of 32 to 39 ºC due to the highest values of growth rate and OD_{660}.

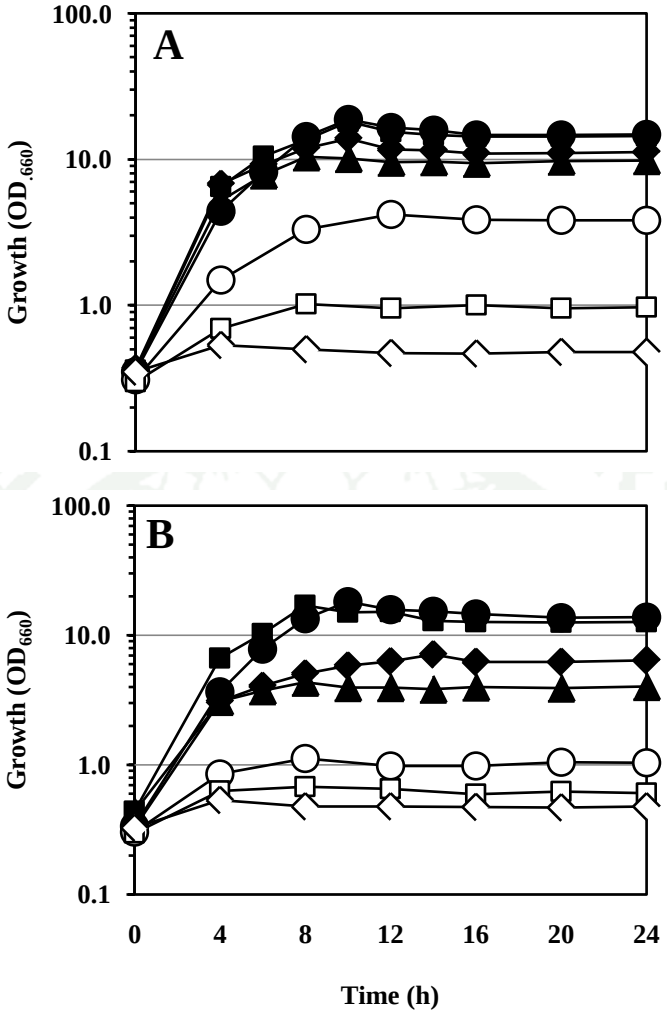


Figure 13 Effect of temperature on growth profile of *C. glutamicum* (A) DS50 wild type and (B) UV-BB9 mutant strains. Cells were grown in 250 mL-Erlenmeyer flask containing 50 mL Lennox medium pH 7.2 with shaking rate 220 rev min^{-1} and incubated at 32 ºC (●), 35 ºC (■), 37 ºC (◆), 39 ºC (▲), 41 ºC (○) and 43 ºC (□).

The results shown that the UV-BB9 was unable to grow at 45 ºC, the maximum specific growth rate (h^{-1}) at room temperature (32-33), 35, 37, 39, 41, 43ºC were 0.59, 0.67, 0.55, 0.49, 0.26, 0.18, respectively, and maximum OD_{660} were 18.17, 16.95, 7.08, 3.97, 1.12 and 0.68, respectively (Figure 13 B). The results also informed that the temperature of 32-35 ºC were optimum for growth of the mutant strain as can be seen from the maximum values of both specific growth rate h^{-1} and

OD_{660} which it was highest among other temperatures. In addition to the growth, the UV-BB9 have the highest growing temperature at 43 ºC as well as the wild-type strain. Therefore, it might be concluded that the mutant was slightly sensitive to temperature than the wild-type. The optimum temperature for growth of mutant was narrow, whereas wild-type was in broad range of temperature. However, the mutant still possess the thermotolerant characteristic.

2.2 Lysozyme sensitive growth

The second set of experiment was made on their sensitive growth of the wild-type strain and UV-BB9, lysozyme sensitivity test. The experiment undergoes based on the report of Hirasawa *et al.*, 2000 with a slight modification due to it is impossible to know whether higher or lower exactly concentrations of lysozyme for inhibiting growth of the tested strains. From the data on growth and L-glutamate excretion patterns visualized by paper chromatography, it is highly possible to speculate that the mutant strain UV-BB9 has less rigid cell wall than the wild-type strain. Based on this criteria, we started the experiment with lysozyme concentration in the range of 0-200 µg/ mL (for wild-type strain) and 0-45 µg/ mL (for UV-BB9 strain). Both of the tested strains with initial OD_{660} of 0.06-0.07 were allowed to grow aerobically at room temperature. The lysozyme was added to the culture medium after cultivated for 3 hours which corresponding to the turbidity about 0.2.

It was found that lysozyme at a concentration of 45 µg/ mL able to inhibited growth of the wild-type strain (Figure 14 A). For the UV-BB9, the result clearly indicated that 30 µg/ mL of lysozyme capable to inhibit growth of this strain after 2 hours of the enzyme addition (Figure 14 B). In final comparison, it can be concluded that UV-BB9 mutant strain was slightly sensitive to lysozyme than the wild-type strain. Moreover, the wild-type strain which was previously isolated as a thermotolerant strain by our laboratory also sensitive to lysozyme as well.

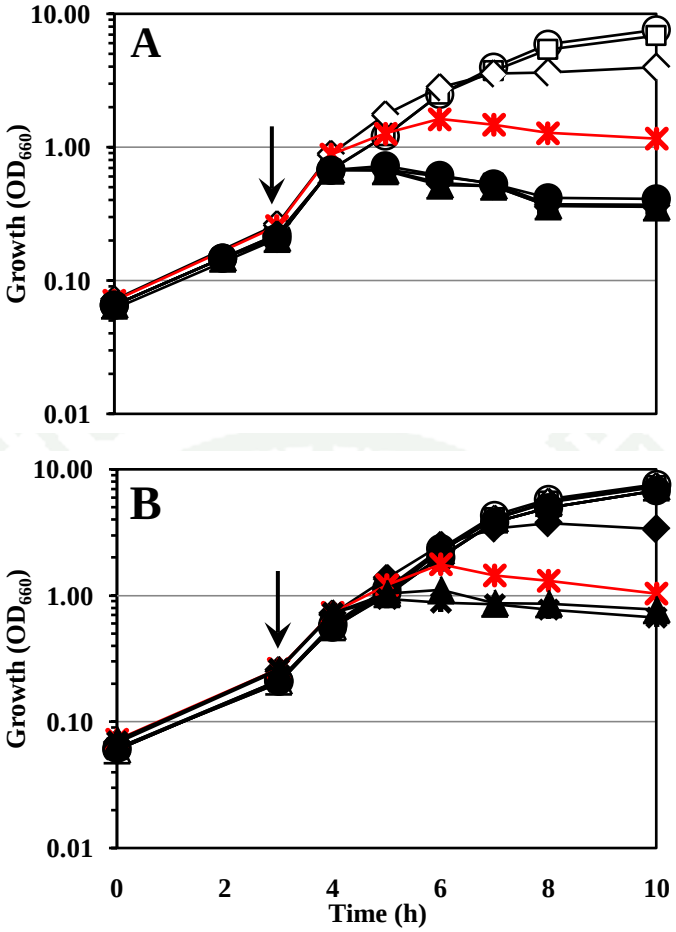


Figure 14 Effects of lysozyme in the range of 0 (○), 30 (□), 40 (◇), 45 (✱), 50 (△), 75 (✕), 100 (∗), 125 (●), 150 (■), 175 (◆) and 200 (▲) µg/ L on growth of *C. glutamicum* DS50 wild-type strain (A) and lysozyme in the rage of 0 (○), 7.5 (□), 10 (◇), 12.5 (△), 15 (✕), 17.5 (∗), 20 (●), 22.5 (■), 25 (◆), 30 (✱), 40 (▲) and 45 (✱) µg/ L on growth of the UV-BB9 mutant strain. Cells were cultured in 250 mL-Erlenmeyer flask containing 50 mL Lennox medium at room temperature with shaking rate 250 rev min^{-1}. Lysozyme was added to the culture at the time indicated by arrow. Growth was monitored by measuring the optical density at 660 nm.

3. Optimum temperatures and biotin contents for growth and L-glutamate production by *C. glutamicum* UV-BB9

From the previous results, it was found that the UV-BB9 has the ability to excrete a relatively large amounts of L-glutamate but not good in growth as compared with the wild-type stain. Therefore, higher product yield obtaining by the mutant strain might be caused by the defective on cell surface structure, not from high cell mass. It is noted that some extrinsic factors such as temperature, biotin contents have directly effect to L-glutamate production and which may cause the change of cell surface in *C. glutamicum* leading to the excretion of L-glutamate. This part of experiment attempts to find out the optimum temperatures, biotin contents, for growth and also for L-glutamate production by the wild-type and mutant strains.

3.1 Optimum temperature for L-glutamate production

For the optimum temperature for growth and L-glutamate production, the wild-type strain exhibited good growth at 32, 35 and 37 °C, yielding a dried cell weight by 6.7, 6.7 and 7.2 g/ L , and when elevated temperature to 39, 41 °C, the growth gradually decreased yielding dried cell weight by 3.7, 0.2 g/ L, respectively. No growth was observed at 43 °C (Figure 15 A). The wild-type consumed glucose by 25.0, 25.6, 25.4, 19.3 and 9.4 g/ L, respectively, as shown in Figure 15 B. Looking at the production at each temperature, the wild-type produced L-glutamate of 0.2 (32 °C), 0.3 (35 °C), 0.4 (37 °C), 0.4 (39 °C) and 0.2 (41 °C) g/ L (Figure 15 C), giving product yield (g glutamate/ g dried cell) of 0.03 (32 °C), 0.04 (35 °C), 0.06 (37 °C), 0.11 (39 °C) and 1.00 (41 °C) as shown in Figure 15 D. It was excepted for 42 °C, the product yield could not be calculate because it was obtained from cell lysis due to high temperature, not from cell growth. Comparing growth and L-glutamate production of the wild-type, it can be concluded that the optimum temperature for growth and L-glutamate production 37 °C because it is giving highest dried cell weight (7.2 g/ L), glucose consumption (25.4 g/ L), L-glutamate (0.4 g/L) and product yield (0.06 g/ g dried cell), respectively.

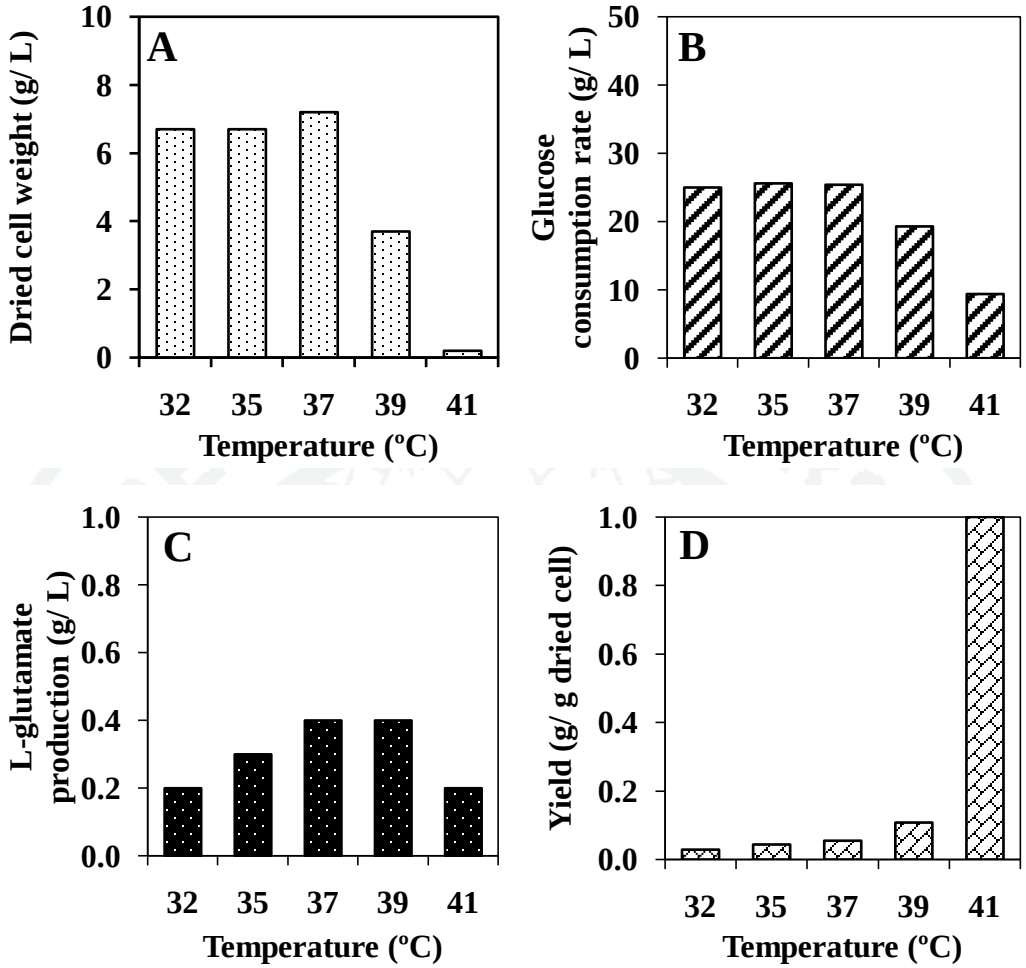


Figure 15 Growth as dried cell weight (A), glucose consumption rate (B), L-glutamate production (C) and yield (D) of *C. glutamicum* DS50 wild-type. The cells were grown for 24 hours in 250 mL-Erlenmeyer flask containing 50 mL Basal Salt medium pH 7.2 with biotin 200 µg/ mL at various temperatures with shaking rate 220 rev min^{-1}.

For the optimum temperature for growth and L-glutamate production of the UV-BB9, it was exhibited good growth at 32, 35, 37 °C, yielding 6.4, 6.4 and 5.8 g/ L or dried cell weight, poor growth was found at 39 °C with 0.5 g/ L of dried cell weight (Figure 16 A). Glucose consumption rate of 6.4, 6.4, 5.8 and 5.0 g/ L (Figure 16 B), respectively, no growth and glucose consumption at 41 and 43 °C. It could be noticed that growth of the mutant strain was depended highly on the temperatures because the reduction of growth occurs after elevating of the cultivation temperatures. Under this condition, L-glutamate was produced by 0.4, 0.4, 3.7 and 2.4 g/ L (Figure 16 C). However, L-glutamate detected at 41 °C (1.3 g/ L) and 43 °C (0.3 g/ L), were expectedly caused by cell lysis due to high temperature (data not shown). The product yields under this condition were 0.06, 0.06, 0.64 and 4.80 g/ g dried cell as shown in Figure 16 D, which clearly depend on the temperature, that is, increasing temperature enhanced L-glutamate production. Comparing growth and the production, it was found that 39 °C is the best for production by the UV-BB9, giving highest product yield. In contrast, it was given lowest of dried cell weight (0.5 g/L) and glucose consumption (5.0 g/L) compared with other temperatures. Therefore, high volume of L-glutamate might be resulted from cell lysis but not from the active growing cells. Together with growth and L-glutamate production, it was found that 37°C appears to be the most suitable condition. Although the growth was slightly lower than that of 32 and 35 °C, but giving higher of L-glutamate (3.7 g/ L) and product yield (0.64 g/ g dried cell).

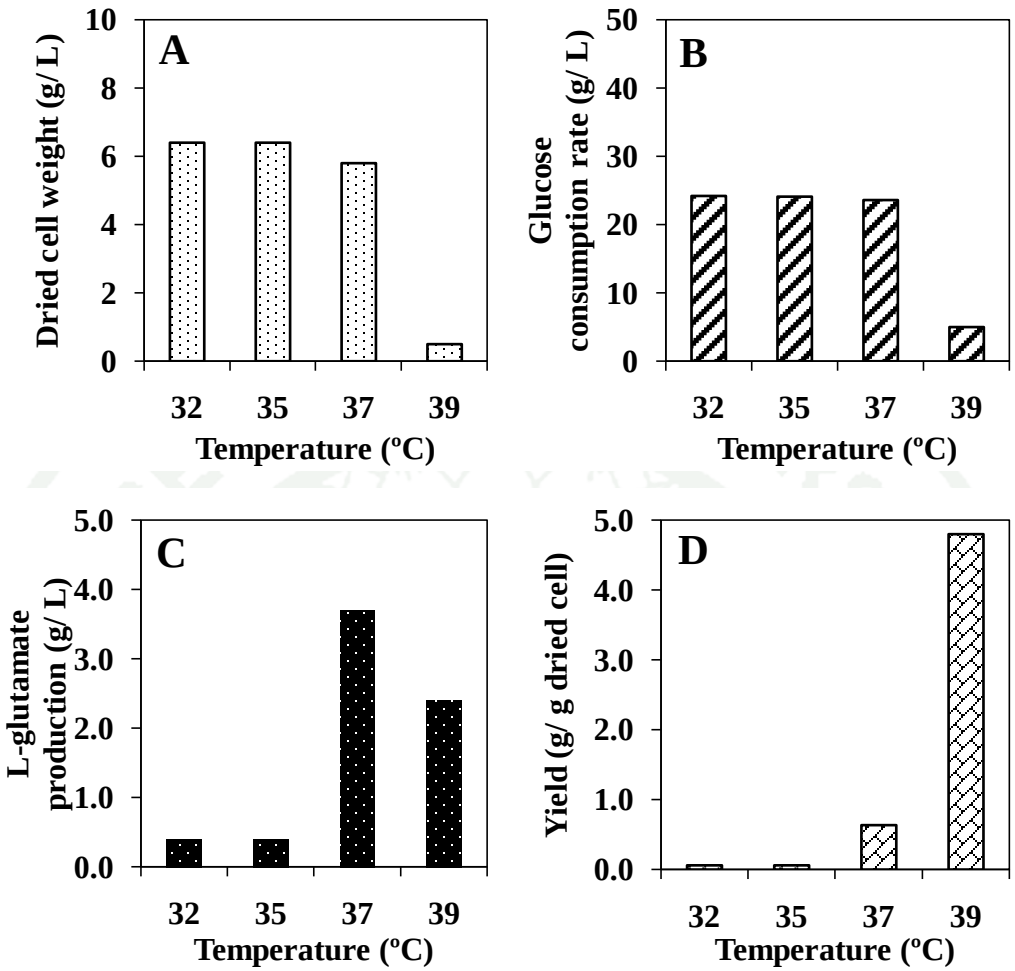


Figure 16 Growth as dried cell weight (A), glucose consumption rate B), L-glutamate production (C) and yield (D) of *C. glutamicum* UV-BB9 mutant strain. The cells were grown for 24 hours in 250 mL-Erlenmeyer flask containing 50 mL Basal Salt medium pH 7.2 with biotin 200 µg/ mL at various temperatures with shaking rate 220 rev min^{-1}.

From the experimental data obtained from both strains, it can be concluded that the optimum temperature for growth and L-glutamate production of the wild-type is the same as those of UV-BB9 at 37 °C. The mutant exhibited growth lower than that of wild-type. However, the mutant gave product yield 10.6 folds higher than that of the wild-type under the optimized temperature as summarized in the Table 4.

Table 4 Effects of temperature on growth and L-glutamate production of *C. glutamicum* DS50 wild type and UV-BB9 mutant strains. Cells were grown in 250 mL-Erlenmeyer flask containing 50 mL Basal Salt medium pH 7.2 in the presence of 200 µg/ L biotin at various temperatures with shaking rate 220 rev min^{-1}.

	DS50 (wild type)					UV-BB9 (mutant)			
	32°C	35°C	37°C	39°C	41°C	32°C	35°C	37°C	39°C
dried cell weight (g/ L)**	6.7	6.7	7.2	3.7	0.2	6.4	6.4	5.8	0.5
glucose consumption rate (g/ L)**	25.0	25.6	25.4	19.3	9.4	24.2	24.1	23.6	5.0
L-glutamate (g/ L)**	0.2	0.3	0.4	0.4	0.2	0.4	0.4	3.7	2.4
product yield (g L-glutamate /g dried cell)**	0.03	0.04	0.06	0.11	1.00	0.06	0.06	0.64	4.80

** calculated at 24 h

3.2 Optimum biotin contents for growth and L-glutamate production

The result previously obtained indicated that 37 °C was the most suitable for growth and L-glutamate production of both strains. This part of experiment was attempted to find out the optimum biotin contents for growth and L-glutamate production based on the optimum temperature at 37 °C. With the reason that biotin is required by *C. glutamicum* due to it is act as a growth factor, and under insufficient amount leading to induction of L-glutamate overproduce. Therefore, biotin limitation is become the basic strategy for L-glutamate excretion.

It was found that after cultured the wild-type strain in Basal Salt medium containing 200, 100, 60, 30, 5, 2.5 and 0 µg/ L biotin giving dried cell weight (calculated at maximum growth at 15 h) by 6.7, 6.9, 7.1, 6.4, 6.5, 6.7 g/ L (Figure 17 A), with glucose consumption of 23.6, 24.3, 25.0, 22.5, 22.9, 23.6 and 10.9 g/ L (Figure 17 B). L-glutamate production at each biotin concentrations were 0.4, 0.5, 0.6, 0.6, 1.0 and 6.2 g/ L (Figure 17 C), giving product yield by 0.06, 0.07, 0.08, 0.09, 0.09, 0.15 and 2 g/ g dried cell (Figure 17 D). It was noticed that biotin concentration in the range of 2.5-200 µg/ L have no remarkably effect on growth of the wild-type strain comparing with 0 µg/ L (Figure 19 A). Even increase or decrease amounts of biotin, the growth was slightly difference. However, it was exhibited the highest growth at 60 µg/ L of biotin. In case of the production, maximum value of L-glutamate production obtained by 0 µg/ L of biotin and then gradually decreased along with the enhancing of biotin contents. On the other hand, the reduction of product yield was directly proportional reversed to higher biotin contents. Due to the suitable condition for production must be deal with cell population and product gaining, Biotin at a concentration of 2.5 µg/ L appears to be the most suitable for growth and L-glutamate production by the wild-type strain.

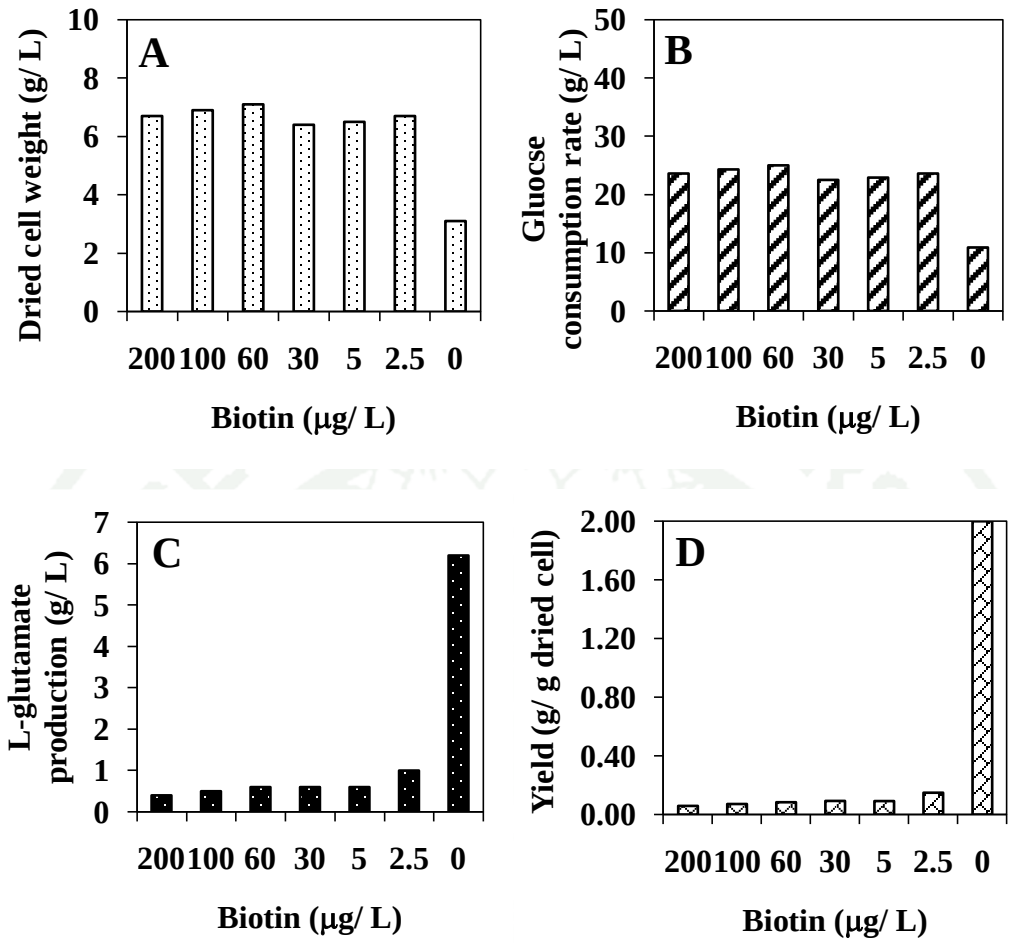


Figure 17 Growth as dried cell weight (A), glucose consumption (B), L-glutamate production (C) and yield (D) of *C. glutamicum* DS50 wild-type. The cells were grown in 250 mL-Erlenmeyer flask containing 50 mL Basal Salt medium pH 7.2 at various concentration of biotin contents with shaking rate 220 rev min^{-1}.

For UV-BB9 mutant strain, the highest growth as dried cell weight after cultivated for 15 h in the presence of biotin 200, 100, 60, 30, 5, 2.5 and 0 µg/ L were 6.2, 5.3, 5.3, 4.9, 4.6, 4.1 and 1.7 g/ L, respectively(Figure 18 A). Glucose consumption at each concentrations tested were 25.2, 21.5, 21.5, 19.9, 18.7, 16.6 and 6.9 g/ L, respectively (Figure 18 B). It was exhibited L-glutamate production by 3.7, 4.7, 4.9, 6.0, 6.8, 6.7 and 14.4 g/ L, respectively, (Figure 18 C), giving product yield by 0.60, 0.89, 0.92, 1.22, 1.48, 1.63 and 8.47 g/ g dried cell, respectively (Figure 18 D). It was also found that both of growth characteristic and the productivity of this strain highly dependent on biotin contents. Reducing of biotin contents affected sequentially decreased of dried cell weight (Figure 19 B) and glucose consumption, but enhanced L-glutamate production and product yield. On the other hand, highest growth (6.2 g/ L of dried cell weight) was observed at maximum volume of biotin (200 µg/ L), highest of L-glutamate production (14.4 g/ L) and product yield (2.08 g/ g dried cell) were observed in the absence of biotin (0 µg/ L). Based on the same criteria as that of the wild-type strain, highest productivity must be obtained by population growth of the active growing cell, not obtained by cell lysis. We expected that the highest product yield in the absence of biotin was achieved by cell lysis but not from the growing cell because of dried cell weight (1.7 g/ L) and glucose consumption (6.90 g/ L) which was the lowest value among the other concentrations. Therefore, we concluded that biotin 2.5 µg/ L was the most suitable for growth and L-glutamate production of the mutant strain. Dried cell weight, glucose consumption, productivity and yield under this condition were slightly difference from biotin 5 µg/ L.

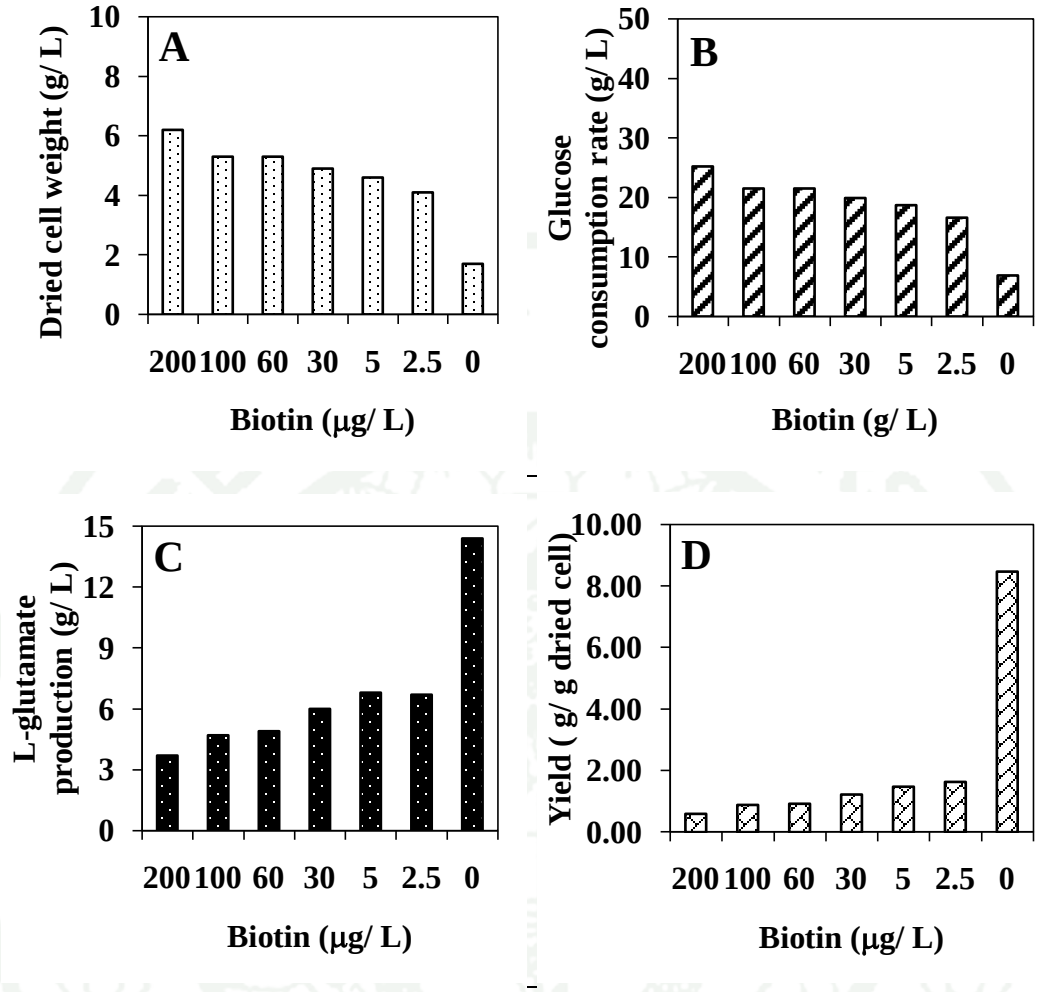


Figure 18 Growth as dried cell weight (A), glucose consumption (B), L-glutamate production (C) and yield (D) of *C. glutamicum* UV-BB9 mutant strain. The cells were grown in 250 mL-Erlenmeyer flask containing 50 mL Basal Salt medium pH 7.2 at various concentration of biotin with shaking rate 220 rev min^{-1}.

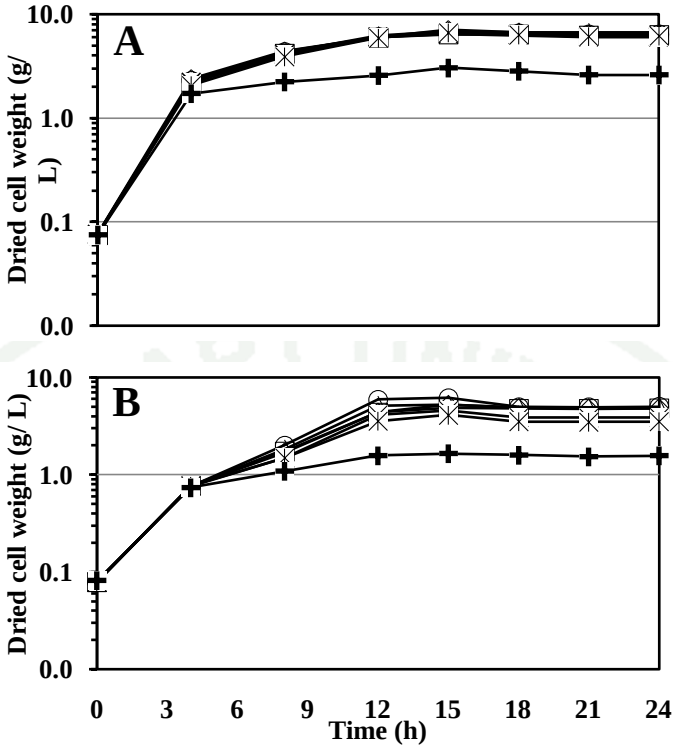


Figure 19 Growth profiles of *C. glutamicum* DS50 wild type strain (A) and *C. glutamicum* UV-BB9 mutant strain (B) under biotin at various concentrations. The cells were grown in 250 mL-Erlenmeyer flask containing 50 mL Basal Salt medium pH 7.2 under biotin 200 (○), 100 (△), 60 (◇), 30 (□), 5 (×), 2.5 (∗) and 0 (✚) µg/ L at 37 °C with shaking rate 220 rev min^{-1}.

Comparing between growth characteristics and the productivity, Basal Salt medium containing biotin 2.5 µg/ L was considered to be the most appropriate condition of the wild-type and the UV-BB9 not only for supporting growth but also for L-glutamate overproduce. Under this condition, the UV-BB9 exhibiting product yield by 10 folds higher than that of the wild-type strain. Moreover, the UV-BB9 required higher amounts of biotin for promote its growth. On the other hand, decreased of biotin contents in the culture medium affects reduction of growth, but result in overproduction of L-glutamate as summarized in the Table 5.

Table 5 Growth and L-glutamate production of *C glutamicum* DS50 wild-type strain and *C. glutamicum* UV-BB9 mutant strain. Cell were grown in 250 mL-Erlenmeyer flask containing 50 mL Basal Salt medium pH 7.2 with shaking rate 220 rev min^{-1} at 37°C.

	DS50 (wild type)							UV-BB9 (mutant)						
	biotin (µg/ L)							biotin (µg/ L)						
	200	100	60	30	5	2.5	0	200	100	60	30	5	2.5	0
maximum dried cell weight (g/ L) (calculated from OD_{660max} at 15 h)	6.7	6.9	7.1	6.4	6.5	6.7	3.1	6.2	5.3	5.3	4.9	4.6	4.1	1.7
glucose consumption (g/ L)**	23.6	24.3	25.0	22.5	22.9	23.6	10.9	25.2	21.5	21.5	19.9	18.7	16.6	6.9
L-glutamate (g/ L)**	0.4	0.5	0.6	0.6	0.6	1.0	6.2	3.7	4.7	4.9	6.0	6.8	6.7	14.4
product yield (g L-glutamate/ g dried cell)	0.06	0.07	0.08	0.09	0.09	0.15	2.00	0.60	0.89	0.92	1.22	1.48	1.63	8.47

** calculated at 24 h

4. Preliminary study on growth and L-glutamate production of the UV-BB9 in jar fermenter

The result obtained by optimization of biotin contents leads us to conclude that 2.5 µg/ L of biotin is the most suitable for growth and L-glutamate production under shake flask condition at 37 ºC. In addition, we also found that growth of the UV-BB9 was restricted to biotin contents. Therefore, the optimum biotin content in shake flask level might be insufficient for supporting growth and L-glutamate production in the jar fermenter. As we generally knew that factor influencing L-glutamate production by *C. glutamicum* is biotin contents. The appropriate amount for production is at sub-optimum level for growth, excess of biotin in the culture medium reduced L-glutamate excretion. To reduce the effect of excess biotin, the seed culture or inoculum should be washed prior to subsequently transferred to the main culture. Therefore, the aim of this part was to find out of growth characteristics and L-glutamate production capacity of the UV-BB9 using batch fermentation. The washed and non-washed seed culture were transferred to giving an approximately 0.8-1 initial OD_{660} in a 3 L-jar fermenter containing 1 L Basal Salt medium as a working volume in the presence of biotin at 400, 200 and 0 µg/ L. The fermentation process was operated with highly constant agitation speed at 300 rev min^{-1}, aeration rate of 0.5-1.0 vvm at 37 ºC.

4.1 Batch fermentation process operated with the aeration rate of 0.5 vvm using the non-washed cells

In the absence of biotin (0 µg/ L), cells growth took a long period to reach a maximum growth as dried cell weight 4.9 g/ L at 34 hours (Figure 20 A). Glucose was consumed by 9.5 g/ L (Figure 20 A). L-glutamate was remarkably detected as a first point at 6 hours (Figure 20 B). After that, the spot intensity was gradually increased until reaches the maximum growth and remained constant until the fermentation time is over. L-glutamate production at the maximum growth as quantified by enzymatic method was 8.7 g/ L (Figure 20 C) with the yield of 1.78 g/ g dried cell. This quantity remains quite stable until the end of fermentation period.

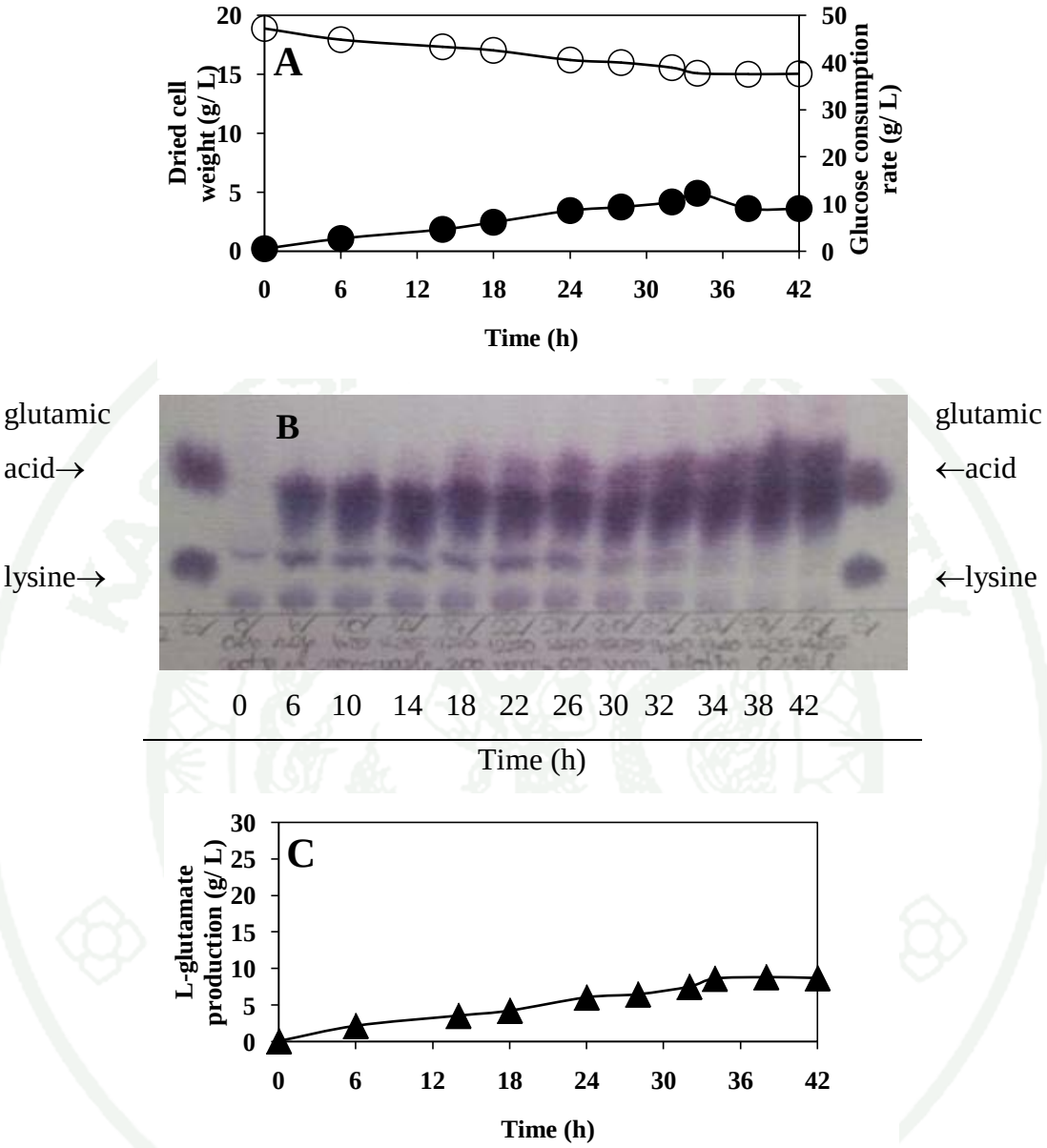


Figure 20 Time-course of growth as dried cell weight and glucose consumption (A; ● and ○), chromatogram (B) and L-glutamate production (C; ▲) of *C. glutamicum* UV-BB9 mutant strain. The non-washed inoculums were grown in a 3-L jar fermenter containing 1 L Basal Salt medium pH 7.2 in the absence of biotin. Batch fermentation was performed with agitation speed 300 rev min^{-1}, aeration rate of 0.5 vvm at 37 ºC.

In the presence of biotin 200 µg/ L, cells growth behavior remains slowly as that of biotin 0 µg/ L. To obtain a maximum dried cell weight 14.6 g/ L, cells were cultivated for 34 hours (Figure 21 A). Glucose consumption at maximum growth was 28.4 g/ L (Figure 21 A). The excretion pattern in this condition was slightly difference from the previous condition that is a relatively high amount of L-glutamate was initially appeared after cultivated for 6 hours and gradually decreased after reaching the maximum growth (Figure 21 B). L-glutamate production at the maximum growth was 13.9 g/ L, giving the product yield of 0.95 g/ g dried cell before reduction occured after reaching the maximum growth (Figure 21 C).

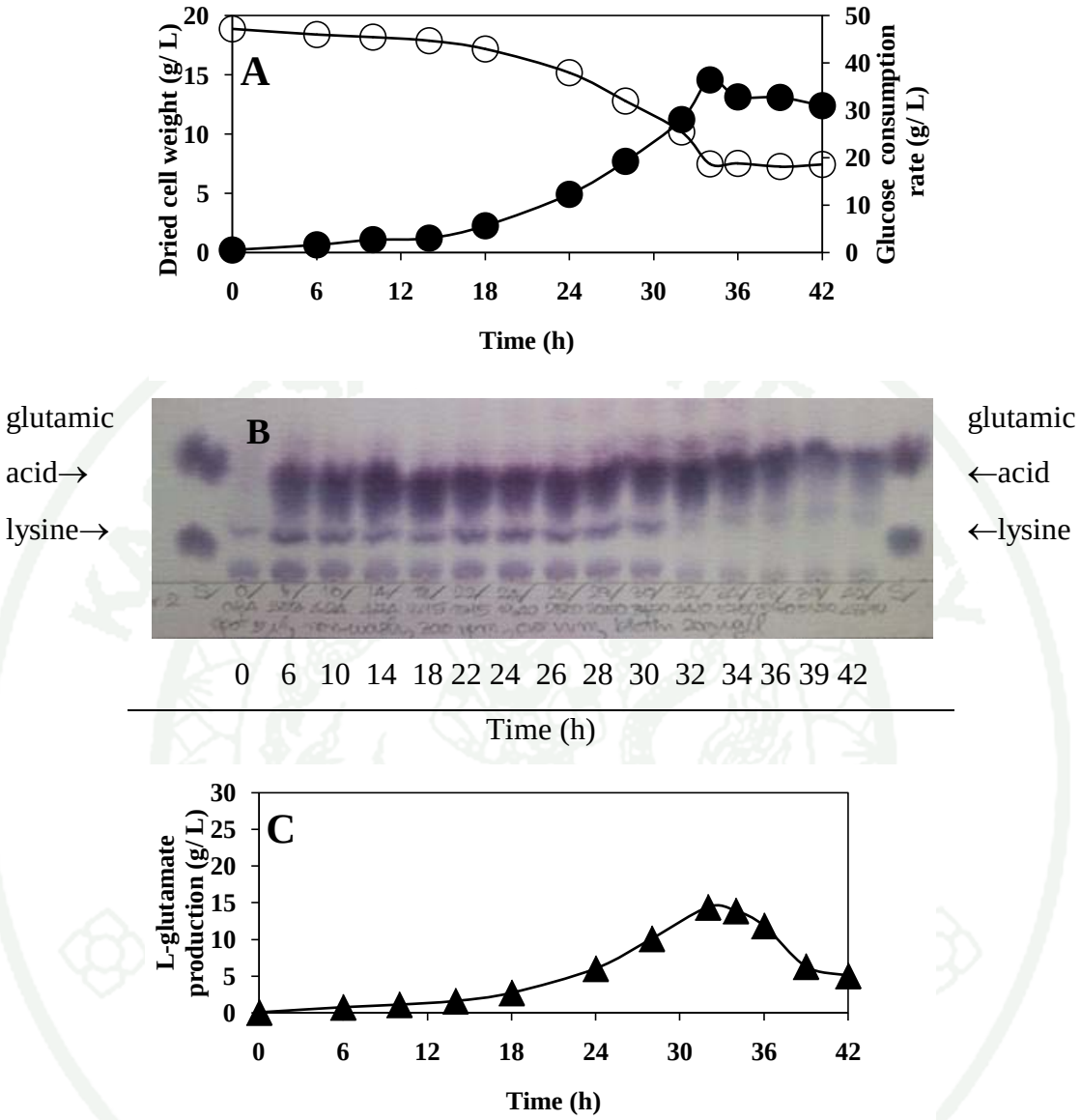


Figure 21 Time-course of growth as dried cell weight and glucose consumption (A; ● and ○), chromatogram (B) and L-glutamate production (C; ▲) of *C. glutamicum* UV-BB9 mutant strain. The non-washed inoculums were grown in a 3-L jar fermenter containing 1 L Basal Salt medium pH 7.2 in the presence of biotin 200 μg/ L. Batch fermentation was performed with agitation speed 300 rev min^{-1}, aeration rate of 0.5 vvm at 37 ºC.

Based on growth characteristics, L-glutamate excretion and L-glutamate production, biotin is one from another factors tested that promoted not only cell growth but also L-glutamate production. By batch fermentation process, the non-washed cells cultured in Basal Salt medium pH 7.2 containing biotin 200 µg/ L with the aeration rate of 0.5 vvm exhibiting L-glutamate production at maximum growth 1.59 folds higher than that of biotin 0 µg/ L. However, the product yield of 0.48 g/ g dried cell was lower than that of biotin 0 µg/ L for 0.53 folds (1.78 g/ g dried cell).

4.2 Batch fermentation process operated with the aeration rate of 1.0 vvm using the non-washed cells

We estimated that the aeration rate might be another critical factor affecting growth behavior and L-glutamate production. Although higher L-glutamate was achieved from cultivating the non-washed cells in main culture containing biotin 200 µg/ L but the product yield was lower than that of 0 µg/ L. Therefore, this part was conducted by increasing biotin contents up to 400 µg/ L, using higher aeration rate of 1.0 vvm at 37 ºC.

In the absence of biotin (0µg/ L), the lag phase was shorter than that of aeration rate 0.5 vvm. The UV-BB9 exhibiting maximum dried cell weight 5.9 g/ L after cultivation for 16 hours (Figure 22 A). Glucose consumption was achieved by 12.1 g/ L (Figure 22 A). A small amounts of L-glutamate that excreted as a first point was found after cultiation for 4 hours. The spot intensity was gradually increased until reaching the maximum growth point, and remains stable (Figure 22 B). The production at maximum growth was 11.0 g/ L (Figure 22 C), giving product yield of 1.86 g/ g dried cell.

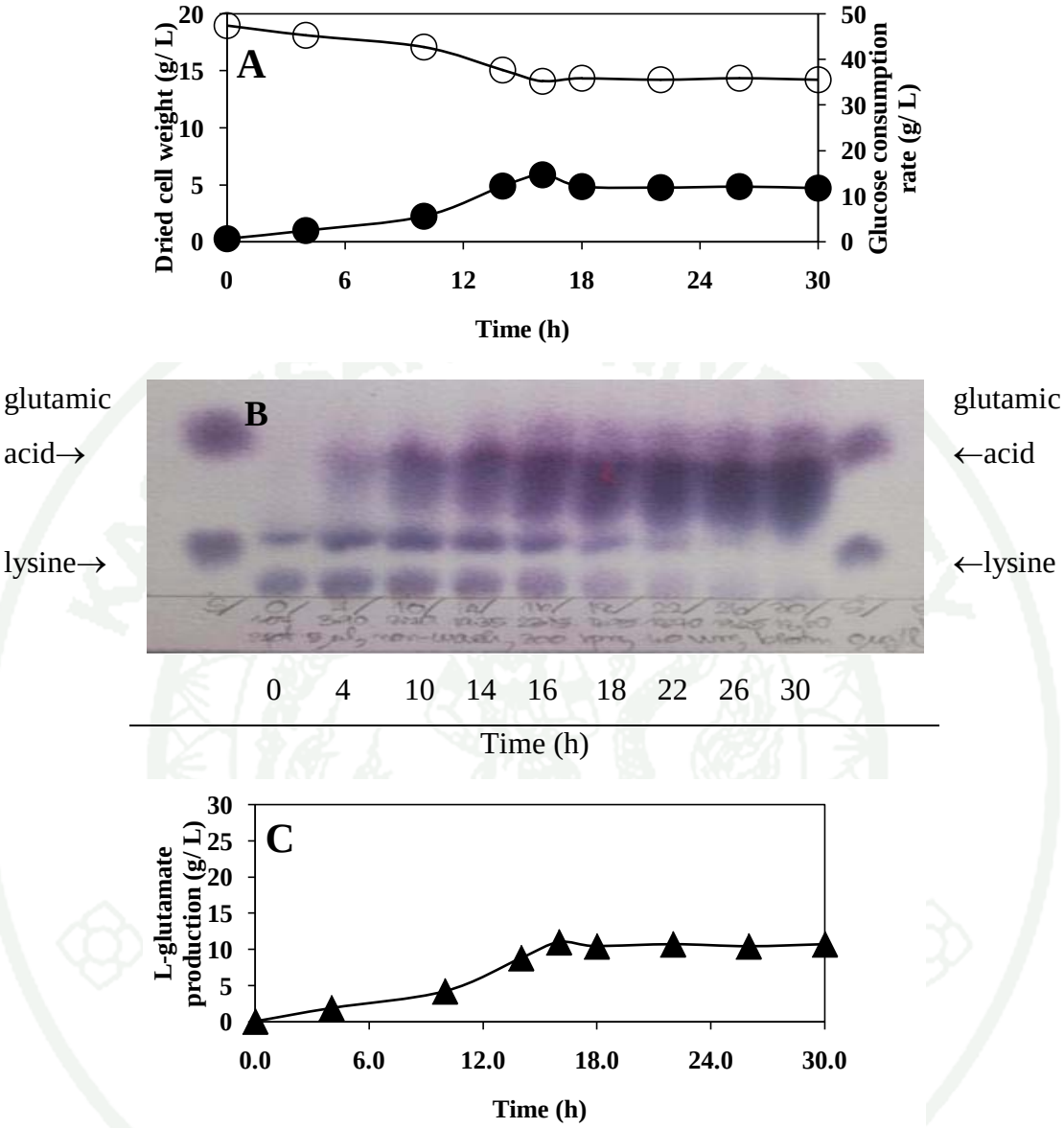


Figure 22 Time-course of growth as dried cell weight and glucose consumption (A; ● and ○), chromatogram (B) and L-glutamate production (C; ▲) of *C. glutamicum* UV-BB9 mutant strain. The non-washed inoculums were grown in a 3-L jar fermenter containing 1 L Basal Salt medium pH 7.2 in the absence of biotin. Batch fermentation was performed with agitation speed 300 rev min^{-1}, aeration rate of 1.0 vvm at 37 ºC.

In the presence of biotin 200 µg/ L, the UV-BB9 cells were exhibited a delay in logarithmic phase compared to that of biotin 0 µg/ L. It took 26 hours to reach a maximum dried cell weight 13.4 g/ L (Figure 23 A). A glucose was consumed by 26.6 g/ L (Figure 23 A). Same as that of biotin 0 µg/ L, a small amounts of L-glutamate that was initially excreted as a first point after cultivation for 4 hours was found. The spot intensity was sequentially increased until approaches the maximum growth. After that, the spot intensity was constant until fermentation process was over (Figure 23 B). The significantly highest amounts of L-glutamate more than the other conditions can be observed (Figure 23 C). It was exhibited 25.2 g/ L of L-glutamate and the product yield of 1.88 g/ g dried cell at a maximum growth was gained. Moreover, this highest amounts remained stable until the end of fermentation process.

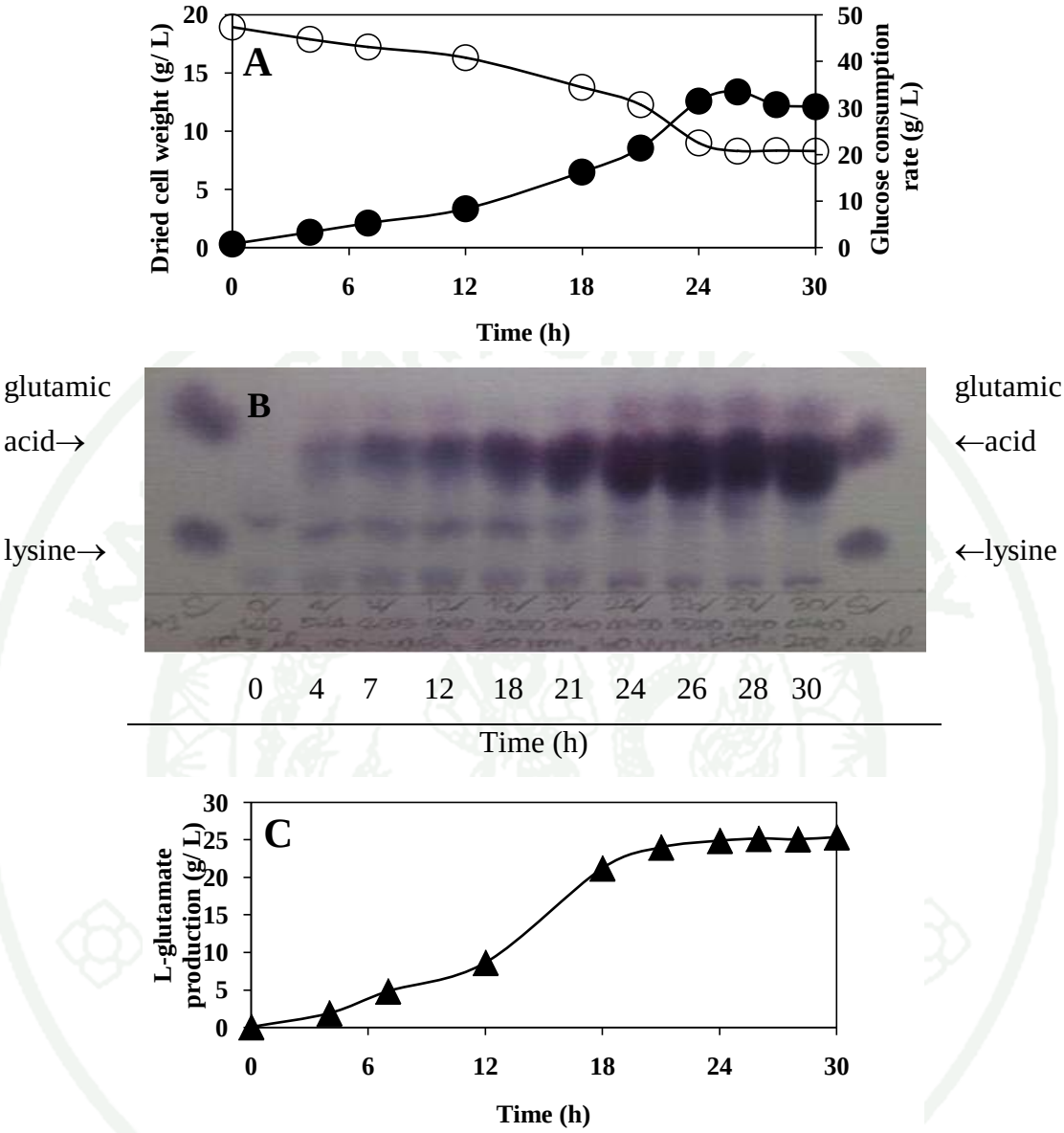


Figure 23 Time-course of growth as dried cell weight and glucose consumption (A; ● and ○), chromatogram (B) and L-glutamate production (C; ▲) of *C. glutamicum* UV-BB9 mutant strain. The non-washed inoculums were grown in a 3-L jar fermenter containing 1 L Basal Salt medium pH 7.2 in the presence of biotin 200 μg/ L. Batch fermentation was performed with agitation speed 300 rev min^{-1}, aeration rate of 1.0 vvm at 37 ºC.

In the presence of biotin-rich 400 µg/ L, The UV-BB9 was exhibited highest 14.8 g/ L of dried cell weight, 29.1 g/ L of glucose consumption after cultivation for 26 hours (Figure 24 A). As we expected, a few volume of L-glutamate was shown as a first point after cultivated for 4 hours. The intensity of amino acid bands were sequentially increased along with the propagation of cells and become saturation after towards the maximum growth (Figure 24 B). It was exhibited 19.4 g/ L of L-glutamate (Figure 24 C), and 1.31 g/ g dried cell1 of product yield at the maximum growth.

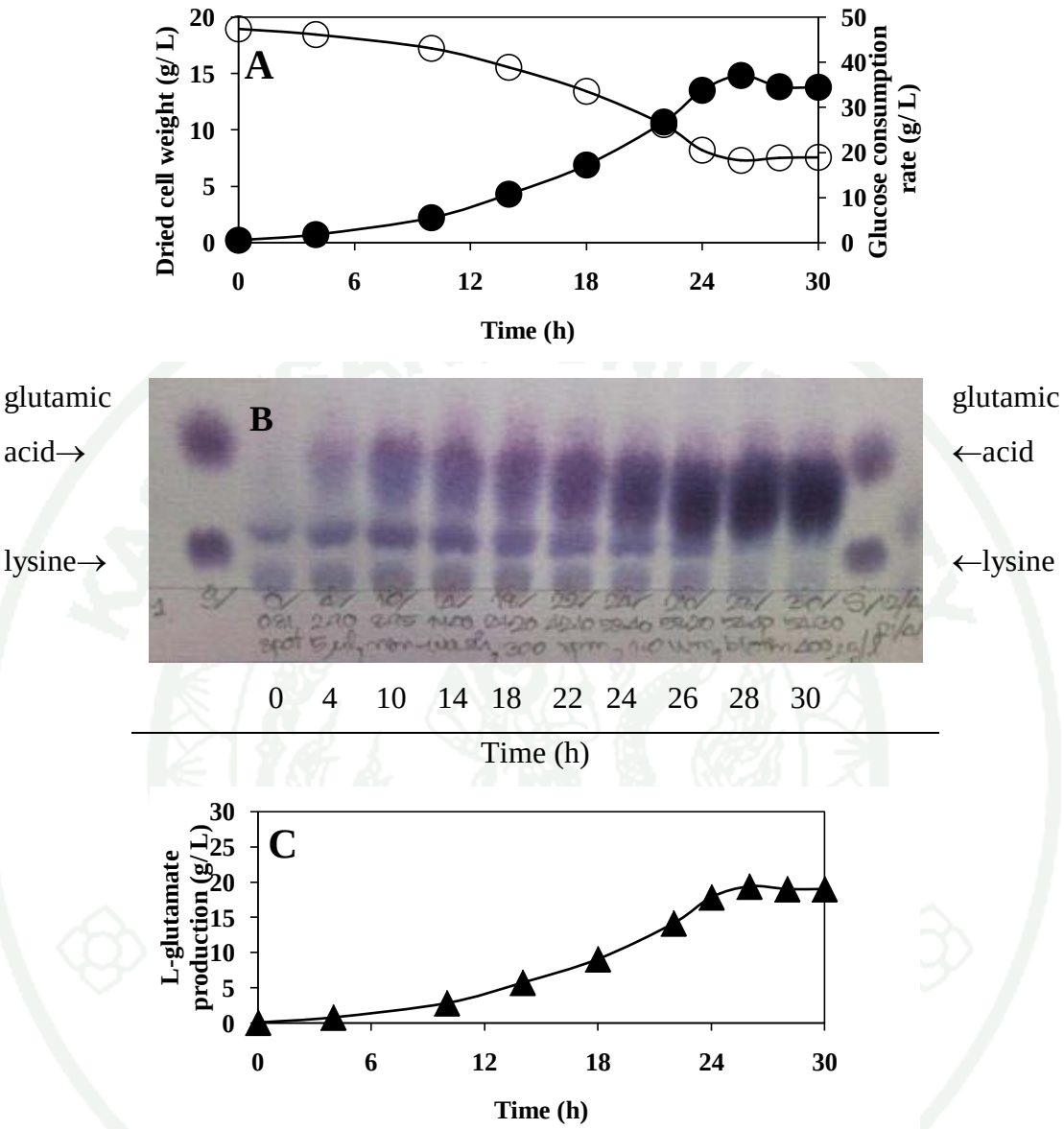


Figure 24 Time-course of growth as dried cell weight and glucose consumption (A; ● and ○), chromatogram (B) and L-glutamate production (C; ▲) of *C. glutamicum* UV-BB9 mutant strain. The non-washed inoculums were grown in a 3-L jar fermenter containing 1 L Basal Salt medium pH 7.2 in the presence of biotin 400 μg/ L. Batch fermentation was performed with agitation speed 300 rev min^{-1}, aeration rate of 1.0 vvm at 37 ºC.

The experimental results obtained by these range of biotin concentrations leads us to state that higher aeration rate of 1.0 vvm have a positive effect on growth of the UV-BB9. They spent a short time for lag phase before entering logarithmic phase compared with aeration rate of 0.5 vvm. However, the time-consuming of maximum growth in the presence of biotin 200 and 400 µg/ L was longer than that of 0 µg/ L. In the point of L-glutamate production, highest of L-glutamate was found in the presence of biotin 200 µg/ L, giving the product yield of 1.88 g/ g dried cell which 1.01 folds higher than that of biotin 0 µg/ L (1.86), and 1.44 folds higher than that of biotin 400 µg/ L (yield 1.31 g/ g dried cell).

4.3 Batch fermentation process operated with the aeration rate of 1.0 vvm using the washed cells

The results previously obtained by using the non-washed cells indicated that biotin content together with higher aeration rate is necessary for growth and L-glutamate production of the UV-BB9. Moreover, growth of the UV-BB9 was highly dependent on biotin contents (this information provided by optimization of biotin for growth and L-glutamate production). To confirm of such phenomena, the washed cells were used in a batch fermentation process.

In the presence of biotin 200 µg/ L, cells exhibited the maximum dried cell weight by 6.8 g/ L, after cultivated for 16 hours (Figure 25 A). Glucose consumption at maximum growth was 13.8 g/ L (Figure 25 A). A small amounts of L-glutamate was excreted after first 4 hours of fermentation time (Figure 25 B). L-glutamate production at maximum growth was 5.5 g/ L (Figure 25 C), yielding 0.80 g/g dried cell of the product yield.

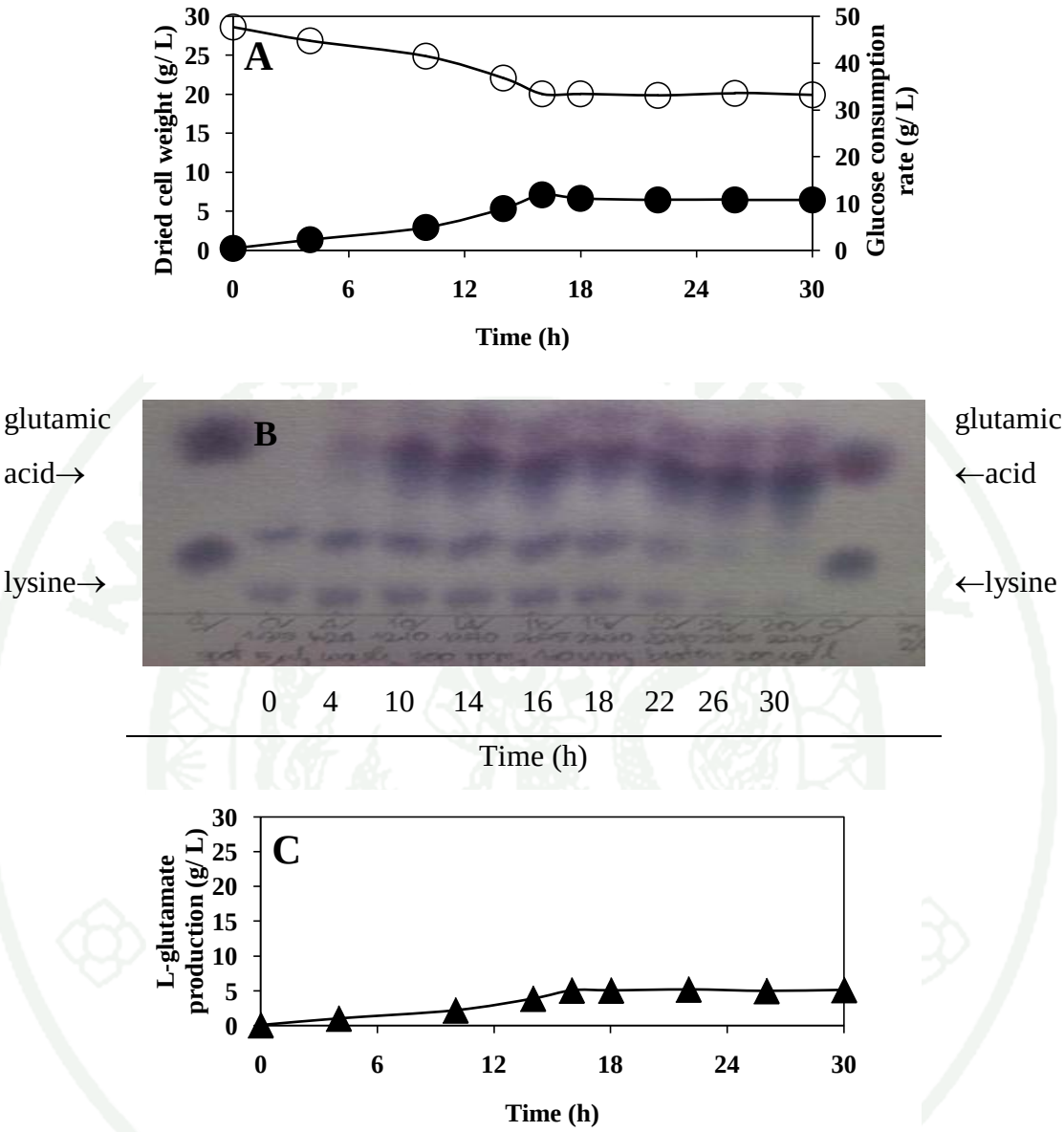


Figure 25 Time-course of growth as dried cell weight and glucose consumption (A; ● and ○), chromatogram (B) and L-glutamate production (C; ▲) of *C. glutamicum* UV-BB9 mutant strain. The washed inoculums were grown in a 3-L jar fermenter containing 1 L Basal Salt medium pH 7.2 in the presence of biotin 200 μg/ L. Batch fermentation was performed with agitation speed 300 rev min^{-1}, aeration rate of 1.0 vvm at 37 ºC.

In the presence of 400 µg/ L biotin-rich condition, cells took 16 hours in lag phase to reach 7.1 g/ L of maximum dried cell weight (Figure 26 A). Glucose was consumed by 14.3 g/ L (Figure 26 A). As the same as that of biotin 200 µg/ L, the amino acid was initially detected after cultivation for 4 hours (Figure 26 B). The band intensity was remarkably higher at the next point and remains stable until fermentation time was over. Maximum L-glutamate was produced by 5.1 g/ L (Figure 26 C), giving the yield by 0.72 g/ g dried cell.

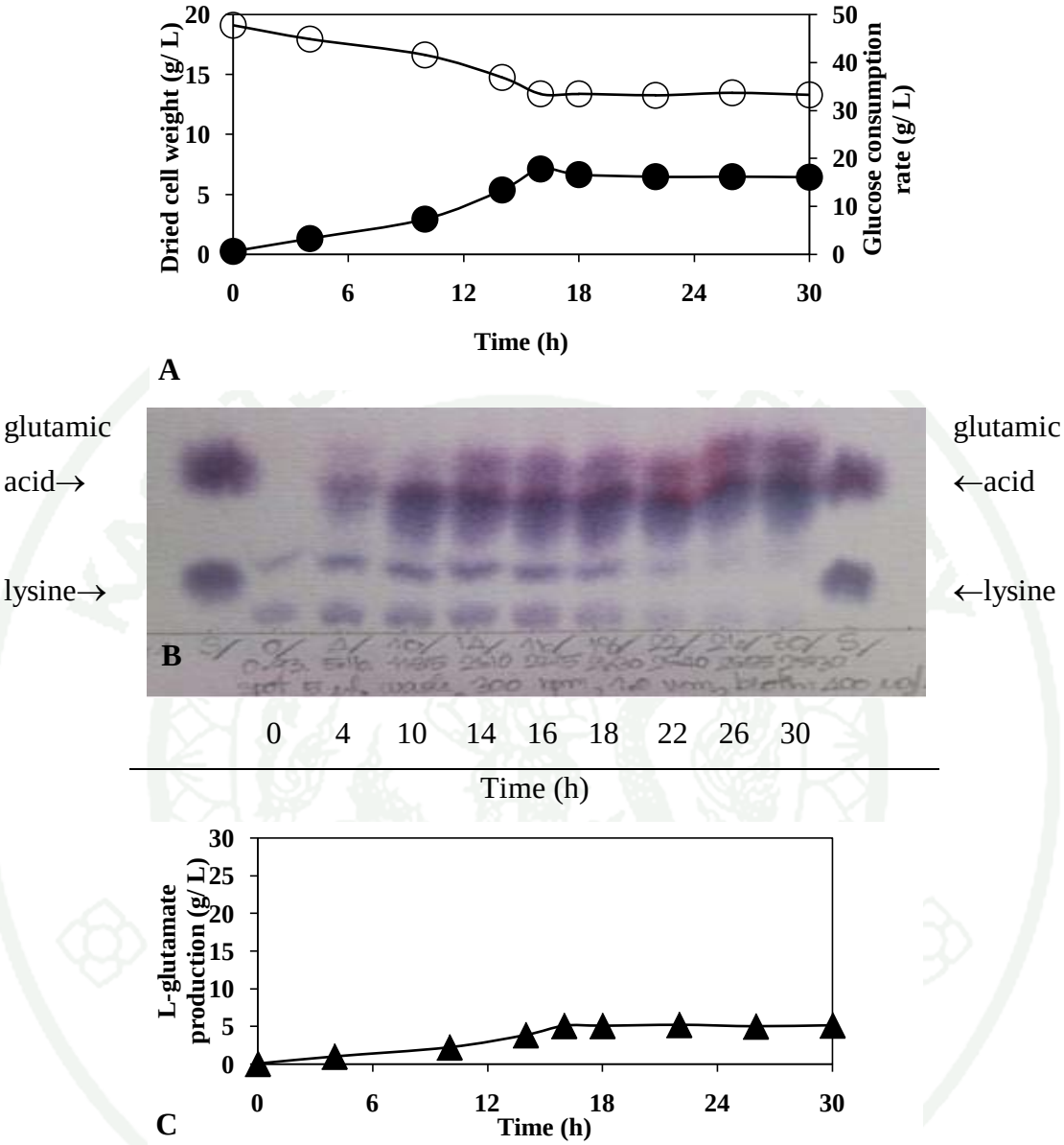


Figure 26 Time-course of growth as dried cell weight and glucose consumption (A; ● and ○), chromatogram (B) and L-glutamate production (C; ▲) of *C. glutamicum* UV-BB9 mutant strain. The washed inoculums were grown in a 3-L jar fermenter containing 1 L Basal Salt medium pH 7.2 in the presence of biotin 400 μg/ L. Batch fermentation was performed with agitation speed 300 rev min^{-1}, aeration rate of 1.0 vvm at 37 ºC.

The results informed that without biotin from the pre-culture, which removed by washing steps, only the volume provided by new medium might be insufficient for promoting growth and also for supporting the production. Cells growth was lower than that of the non-washed cells. However, cells growth could be enhanced with higher biotin contents. In the point of production, it was reccomended to the use of non-washed cell in which higher biotin contents affects the production of L-glutamate.

Comparing the overall data derived from the UV-BB9, highest of L-glutamate production was found in a batch fermentation process operated with the aeration rate of 1.0 vvm in the presence of biotin 200 μg/ L. The product yield was 1.98 folds higher than that of using non-washed cells, and 2.35 folds higher than that of 1.0 vvm using washed cells.

After obtained the best condition for growth and L-glutamate production of the UV-BB9 mutant strain, we used this condition to investigate the growth and L-glutamate production capacity of the DS50 wild-type strain.

The cells of DS50 wild-type was quickly grew and reaches the maximum growth, 17.8 g/ L of dried cell weight after fermentation process undergoes for 12 hours (Figure 27 A). Glucose was almost completely consumed by 35.3 g/ L (Figure 27 A). L-glutamate was initially excreted after cultivation for 8 hours and slightly increased till reaches the maximum growth. The highest intensity was found after cells entering stationary phase at 12-14 hours. After that, the amino acid bands were gradually disappeared (Figure 27 B). A very low volume of L-glutamate at maximum growth about 0.5 g/ L was produced (Figure 27 C), and gradually decreased at the rest of fermentation time, giving the lowest of 0.03 g/ g dried cell product yield.

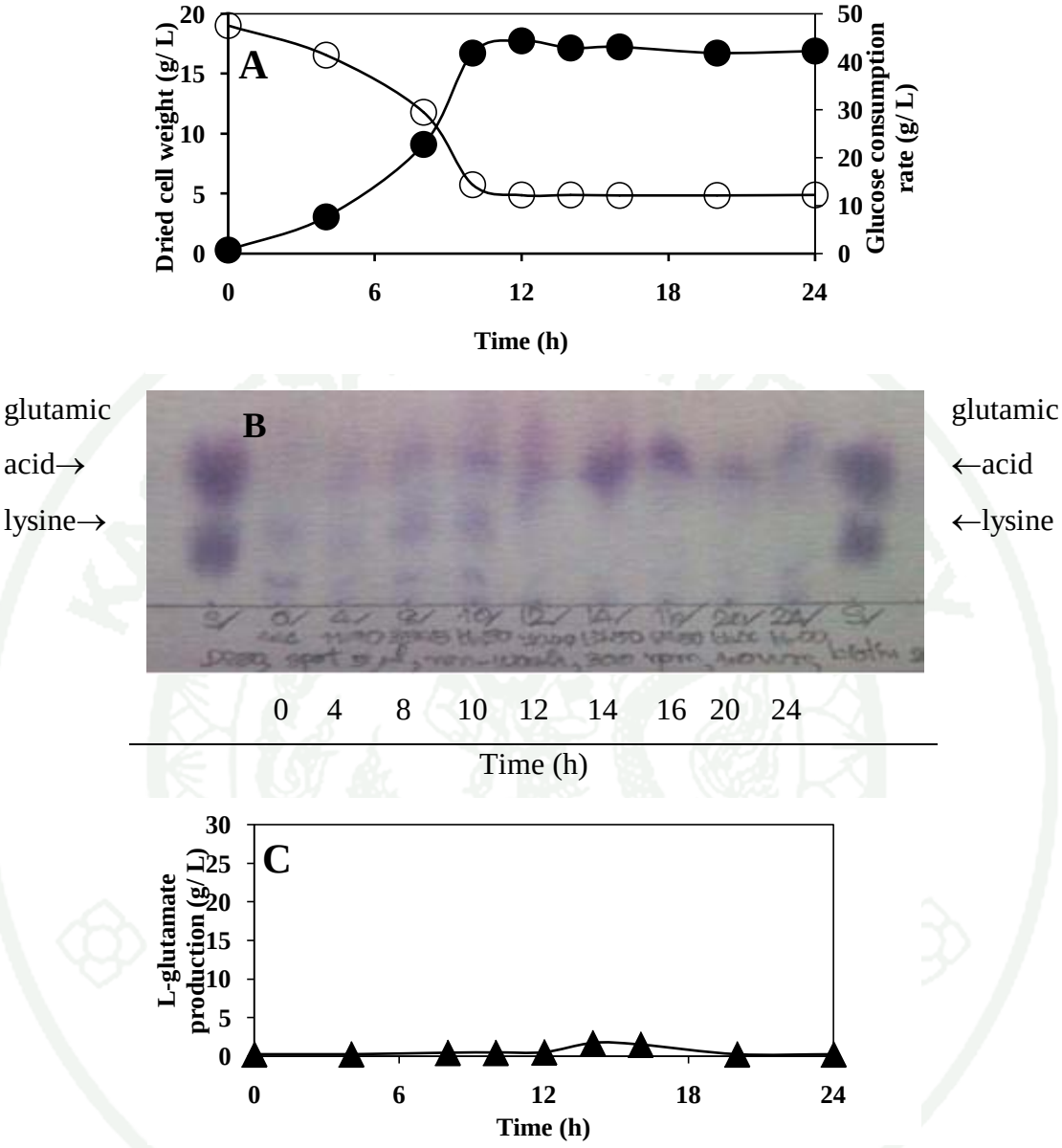


Figure 27 Time-course of growth as dried cell weight and glucose consumption (A; ● and ○), chromatogram (B) and L-glutamate production (C; ▲) of *C. glutamicum* DS50 wild-type strain. The washed inoculums were grown in a 3-L jar fermenter containing 1 L Basal Salt medium pH 7.2 in the presence of biotin 200 μg/ L. Batch fermentation was performed with agitation speed 300 rev min^{-1}, aeration rate of 1.0 vvm at 37 ºC.

Comparing between growth characteristics and production capacity of both strains, growth of the UV-BB9 was 1.32 folds lower than that of the DS50 wild-type strain. However, L-glutamate production and product yield were 50.4 and 62.6 folds higher than that of the wild-type strain as summarized in the Table 6.

Table 6 Growth and L-glutamate production of *C. glutamicum* DS50 wild-type strain and UV-BB9 mutant strain. Cells were grown in a 3 L-jar fermenter containing 1 L Basal Salt medium pH 7.2 in the presence of biotin 400, 200 and 0 µg/ L. Batch fermentation was performed with agitation speed 300 rev min^{-1} at 37 ºC.

Strain	Seed culture	Aeration rate (vvm)	Biotin content (µg/ L)	Time required for maximum growth (h)	Maximum dried cell weight (g/ L)	Glucose consumption at maximum growth (g/ L)	L-glutamate production at maximum growth (g/ L)	Product yield (g L-glutamate/ g dried cell)
UV-BB9	Non-washed	0.5	0	34	4.9	9.5	8.7	1.78
			200	34	14.6	28.4	13.9	0.95
		1.0	0	16	5.9	12.1	11.0	1.86
			200	26	13.4	26.6	25.2	1.88
			400	26	14.8	29.1	19.4	1.31
	Washed	1.0	200	16	6.8	13.8	5.5	0.80
			400	16	7.1	14.3	5.1	0.82
DS50	Non-washed	1.0	200	12	17.8	35.3	0.5	0.03

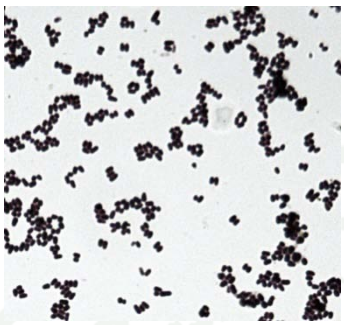
Discussions

Improvement of the commercial applicability of microbial strains has been practiced for centuries. The aim of this study is to improve strain of *Corynebacterium glutamicum* DS50, which isolated as a thermotolerant strain, by using random mutation with UV-mutagenesis. There are several researchers previously reported of the successfully used of whether UV radiation, chemical mutagens and/ or combination between UV-chemical mutagens to improve microbial strains, such as filamentous fungi (Zambare *et al*., 2010; Ahmed *et al*., 2007; Khattab *et al*., 2005; Huang *et al*., 2010; Xu *et al*., 2011; Chandra *et al*., 2012; Jun *et al*., 2009; Chand *et al*., 2004; Adsul *et al*., 2007; Bapiraju *et al*., 2004), yeast (Taloria *et al*., 2012, Shigetmatsu *et al*., 2010, Cao *et al*., 2011; Sridhar *et al*., 2002; Pasha *et al*., 2007; Li *et al*., 2007; Tan *et al*., 2003) and bacteria (Agrawal *et al*., 2011; Basavaraj *et al*., 2010; Haq *et al*., 2009; Joshi *et al*., 2010; Sachin *et al*., 2006; Zhong *et al*., 2011; Hui *et al*., 2011, Gao *et al*., 2012; Khaliq *et al*., 2009; Zhao *et al*., 2010; Ekwealor *et al*., 2006; Vaidya *et al*., 2003). Therefore, this conventional method is still considerably as an effective tool for microbial strain improvement. In general, particularly in bacteria, a good mutants are found among the induced-condition that giving lower survival rate about at least 0.1-1% or higher. In contrast, *C. glutamicum* UV-BB9 which has the ability to excrete extracellularly higher amount of L-glutamate compared to that of the wild-type strain was found at 16 % survival rate. It might be notice that low survival rate is not suitable for generating a desirable mutants of *C. glutamicum.* Moreover, there are still a number of research groups reporting the accomplishment of bacterial strain improvement with higher survival rate. For example, Zhao *et al* (2010) was successfully generated *Serratia marcescens* mutant with enhancing lipase production after treat cells with UV radiation for 30 second, giving 78% killing or 22% survival rate. Xu *et al* (2011) reported that higher acetoin-producing mutants of *Bacillus subtilis* were dominantly found with %killing ranging from 70-80% (20-30% survival rate) after UV-mutagenesis. Gao *et al* (2012) reported that after cells of *Clostridium acetobutylicum* were treated with NTG for 0.5 hours, resulting 72.1% killing (27.9% survival rate), the butanol-tolerant mutant strain No. 206 was found to produce 50% of butanol higher than that of the wild-type strain.

Agrawal *et al* (2012) successfully to generate the β-galactosidase hyperproducing mutant of *Bacillus subtilis* after 50% killing (50% survival rate) by UV-mutagenesis. Therefore, it might be state that 1) the screening steps should be undergoing whether lower or higher of %survival rate derived after mutagen treatment 2) lower of %survival rate or higher %killing not always directs the occurrence of desirable mutants.

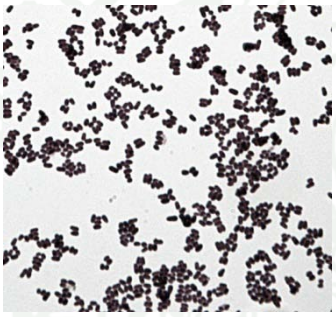
C. glutamicum exhibits highly tolerance to lysis by egg white lysozyme that catalyze the hydrolysis of β-1, 4 glycosidic bond between N-acetylglucosamine and N-acetylmuramic acid of peptidoglycan. Because of the outer layer mainly composed of mycolic acid, it makes this bacterium resist to cell lysis and/ or functions as permeability barrier (Hirasawa *et al*., 2000). The results obtained by primary and secondary screening leads us to assume that this ability is primarily concerned with the mechanism of L-glutamate production by *C. glutamicum*. As we expected, growth of the UV-BB9 was inhibited by treatment with 30 µg/ mL lysozyme and the turbidity gradually decreased after 2 hours of enzyme addition, indicating that the treated cells did not lyse. For the DS50 wild-type strain, growth was inhibited by lysozyme at a concentration of 45 µg/ mL. The turbidity of the culture gradually decreased after 2 hours as well as that of the mutant strain. It is indicating that the mutant strain slightly sensitive to lysozyme than that of the wild-type strain. However, light microscope shown that the wild-type exhibited a normal rod shape at 33 and 37 ºC. On the other hand, the UV-BB9 mutant strain exhibited a normal rod shape at 33 ºC and then became long-swollen rod at 37 ºC when growing in logarithmic phase (Figure 28). Moreover, optimum temperature of the UV-BB9 mutant strain became narrow (32-35 ºC) than that of the wild-type strain (32-37 ºC). The mutant obtained by this study is higher sensitive to lysozyme than *C. glutamicum* KY9714 (Hirasawa *et al*, 2000) which completely inhibited by 12.5 µg/ mL of lysozyme. We also found that the wild-type strain itself is highly sensitive to lysozyme than that of *C. glutamicum* KY9611 (Hirasawa *et al*, 2000) which inhibited by 400 µg/ mL of lysozyme.

A. wild-type strain at 33 ºC



B. wild-type strain at 37 ºC

C. mutant strain at 33 ºC



D. mutant strain at 37 ºC

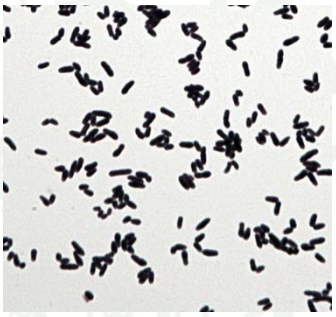


Figure 28 Cells morphology in a logarithmic phase of *C. glutamicum* DS50 wild-type strain at 33 and 37 ºC (A and B) and the UV-BB9 mutant strain at 33 and 37 ºC (C and D) in Basal Salt medium pH 7.2 containing biotin 200 µg/ L. Compound light micrographs are illustrated.

It had been believed for a long time that L-glutamate excretion occurred through the leak model, the injury of cell membrane, in which cell membrane permeability was changed by means of biotin limitation, addition of sub-lethal concentration of penicillin and addition of sub-optimal amounts of surfactant. But from the report of Shiratsuchi *et al* (1995), they found that large amounts of lysine and L-glutamate were simultaneously produced by cultivating a lysine-producing mutant of *Brevibacterium lactofermentum* with the addition of polyoxyethylenesorbitab monopalmitate (PESMP) or penicillin G. There results indicated that glutamate production of coryneform bacteria could not explain with only using the regulation of cell membrane permeability. They are also stated that a specific increase of L-glutamate production and reduction of lysine production in the

simultaneous fermentation process could not be explained by the leak model. These was explained in-deep by the report of Kawahara *et al* (1997) which they found that the activity of 2-oxoglutarate dehydrogenase was reduced during L-glutamate production in response to biotin limitation, PEMSP addition, while the glutamate dehydrogenase activity was remained unchanged in every induced condition. Also, 2-oxoglutarate dehydrogenase complex (ODHC) which catalyzes the first-step reaction for the conversion of 2-oxoglutarate to succinyl-Co A at a branch point of L-glutamate formation (as shown in the literature reviews) from the TCA cycle, and glutamate dehydrogenase (GDH) which catalyzes the reaction for the conversion of 2-oxoglutarate to L-glutamate were assumed to be important in the production of L-glutamate.

The result obtained from this thesis indicated that 2.5 μg/ L of biotin, or could be considered as the biotin-limited condition, was found to be suitable for L-glutamate production under shake flask level. It seems firmly supported by the report as mentioned above. On the other hand, it might be speculate that the UV-BB9 has a low level of 2-oxoglutarate dehydrogenase activity. The results obtained from batch fermentation as preliminary study shown that the UV-BB9 exhibited highest of L-glutamate under 200 μg/ L of biotin, not a biotin-limited condition, and obviously higher that of *Brevibacterium* sp.DSM 20411 (Nampoothiri *et al* (1999); *Corynebacterium glutamicum* KY9611wild-type, *C. glutamicum* KY9714 (*ltsA9714*), *C. glutamicum* KY9611 (*ltsA::kan*) (Hirasawa *et al*., 2000; Shimizu and Hirasawa, 2006);*Brevibacterium divaricatum* MTCC1529 (Jyothi *et al*., 2005); and *C. glutamicum* ATCC 13869 (Hasegawa *et al*., 2008) as summarized in the Table 7, might lead us to another story as compared with the previous researchers. However, Asakura Y *et al* (2007) have shown their perspective that metabolic flux change is expected to be very important for L-glutamate overproduction in *C. glutamicum*. Since ODHC is located at the branch point between the TCA cycle and L-glutamate formation, the reduction in ODHC activity capable to switch the metabolic flow from the TCA cycle to the L-glutamate formation. Nevertheless, the reduction in ODHC activity was not sufficient for the high L-glutamate production and did not always strictly corresponded to the level of L-glutamate production. To prove their

hypothesis, they constructed an *odhA* deletion mutant which completely lacked of ODHC activity and found surprisingly amounts of L-glutamate without any induction or any alteration of fatty acid composition. Their results indicated that the change in metabolic flux is directly effect L-glutamate production. Our results shown in this thesis might by firmly supported by the report of Nakamura *et al* (2007) which they found deeper than that of the former research groups. They found that the *odhA* disruptant had gained additional mutation in the Ncgl1221 gene which encodes a mechanosensitive channel protein, or L-glutamate exporter. This exporter was activated or opened under the condition that altering cell membrane tension to induce L-glutamate excretion. In point of temperature, Bokas *et al* (2007) reported that higher temperature affects the alteration of cytoplasmic membrane tension, corresponding to report of Nakamura *et al* (2007) which stated that change in membrane tension altered the structure of Ncgl1221 protein to export L-glutamate.

Table 7 L-glutamate production by various L-glutamate-producing strains.

Strain	Temp (ºC)	Triggering process	Production scale	L-glutamate production (g/ L)	Initial glucose (g/ L)	References
Brevibacterium sp. (DSM 20411)	30	No	Jar fermenter with batch process	21	50	Nampoothiri *et al* (1999)
Corynebacterium glutamicum KY9611 (wild-type)	30		Shake-flask	0.07	50	Hirasawa *et al* (2001) Shimizu and Hirasawa (2006)
	35			0.22		
	37			0.29		
C. glutamicum KY9714 (*ltsA9714*)	30			0.10		
	35	No		0.82		
	37			0.62		
C. glutamicum KY9611 (*ltsA::kan*)	30			0.47		
	35			1.10		
	37			2.45		
Brevibacterium divaricatum MTCC1529	30	No	Jar fermenter with batch process	5.6	20	Jyothi *et al* (2005)

Table 7 Continued

C. glutamicum ATCC13869	31.5	Biotin 3.0 µg/ L Tween 40 1.5 g/ L Penicillin G 2200 U/ L	Shake-flask	12 17 19	60	Hasegawa *et al* (2008)
C. glutamicum DS50 (wild-type) *C. glutamicum* UV-BB9 mutant (UV-derived mutant)	37	No	Jar fermenter with batch process	0.5 25.2	50	This work

CONCLUSION AND RECOMMENDATION

Conclusion

Cells of *Corynebacterium glutamicum* DS50 wild-type (10^7 cell/ mL) were subjected to random mutation by exposing to ultraviolet light 254 nm at a fixed distance of 30 cm for 6 minutes, yielding 16 % of survival rate. Through screening and selection steps, *C. glutamicum* UV-BB9, performed constitutively L-glutamate excretion which detected by paper chromatography using inhydrin, was found and selected as the potent strain among the 180 tested isolates from total 500 colonies of the mutant library, representing 36 % screen.

From the study on partial physiological characteristics, temperature sensitivity of the UV-BB9 was similar to, but growth was slightly lower than that of the wild-type strain. However, the UV-BB9 rather slightly sensitive to lysozyme than that of the wild-type strain. The mutant UV-BB9 and wild-type DS50 were inhibited by lysozyme at a concentration of 30 and 45 μg/ L, respectively.

The optimum temperature for growth and L-glutamate production of the UV-BB9 is at 37 ºC as same as that of the wild-type strain. It was exhibited 5.8 g/ L of maximum dried cell weight, and 0.64 g/ g dried cell of product yield. In the same condition, the DS50 wild-type strain exhibited 7.2 g/ L of maximum dried cell weight, and 0.06 g/ g dried cell of the product yield.

The UV-BB9 required higher biotin content for support its growth. Maximum dried cell weight of 6.2 g/ L was achieved after cultivation for 15 hours in the presence of biotin 200 μg/ L. Whereas biotin 60 μg/ L is sufficient for the wild-type strain to produce 7.1 g/ L after cultivation for 15 hours. Due to the fact that biotin plays an important role as a growth factor, optimum condition for L-glutamate production is not the same as that for growth. It was found that the UV-BB9 and the wild-type strain produced highest amount of L-glutamate by 6.7 and 1.0 g/ L in the

presence of biotin limited 2.5 µg/ L, giving product yield of 1.63 and 0.15 g/ g dried cell, which 11-folds higher than that of the wild-type strain.

The results obtained by preliminary study using a batch fermentation process indicated that aeration rate of 1.0 vvm in the presence of biotin 200 µg/ L have a positive effect on growth and L-glutamate production by the non-washed UV-BB9 cells. It was exhibited 13.4 g/ L of dried cell weight, 26.6 g/ L of glucose consumed, and highest 25.2 g/ L of L-glutamate, resulting 1.88 g/ g dried cell of the product yield. However, L-glutamate was decreased in the biotin-rich condition at 400 µg/ L. For the DS50 wild-type strain, it was produced a very low 0.5 g/ L of L-glutamate, giving the lowest product yield of 0.03 g/ g dried cell. Moreover, product yield of the UV-BB9 was 63-folds higher than that of the wild-type strain. The experimental results indicated potential of classical genetic method via UV mutagenesis as an effective tool for improve *Corynebacterium glutamicum* strain.

Recommendation

In order to improve L-glutamate production of the UV-BB9 mutant strain, various parameters involving in growth and L-glutamate production such as carbon and nitrogen source, C: N ratio, agitation speed, aeration, pH, and also fed-batch fermentation process should be further optimize.

LITERATURE CITED

Abe, S., K. Takayama and S. Kinoshita. 1967. Taxonomical study on glutamic acid producing bacteria. **Journal of General Applied Microbiology**. 13: 279-301.

Adsul, M. G., K. B. BAstawde, A. J. VArma and D. V. Gokhale. 2007. Strain improvement of *Penicillium janthinellum* NCIM 1171. **Bioresource Technology**. 98: 1467-1473.

Agrawal, R., A. Satlewal and A. K. Verma. 2012. Development of a β-glucosidase hyperproducing mutant by combined chemical and UV mutagenesis. 3 **Biotech**. 1: 1-8.

Ahmed, M., E. L. Bondkly and A. A. Keera. 2007. UV- and EMS-induced mutations affecting synthesis of alkaloids and lipase in *Penicillium roquefortii*. Arab **Journal of Biotechnology**. 10: 241-248.

Asakura, Y., E. Kimura, Y. Usuda, Y. Kawahara, K. Matsui, T. Osumi and T. Nakamatsu. 2007.Altered Metabolic Flux due to the Deletion of odhA causes L-glutamate Overproduction in *Corynebacterium glutamicum*. **Applied and Environmental Microbiology**. 73: 1308-1319.

Ault, A. 2004. The Monosodium Glutamate Story: The Commercial Production of MSG and Other Amino Acids. **Journal of Chemical Education**. 8: 347-355.

Baltz, R. H. 1999. Mutagenesis, pp 307-311. In M. C. Flickinger and S. W. Drew, eds. **Encyclopedia of Bioprocess Technology: Fermentation, biocatalysis and separation**. Wiley, New York.

Bapiraju, K. V. V. S. N., P. Sujatha, P. Ellaiah and T. Ramana. 2004. Mutation induced enhanced biosynthesis of lipase. **African Journal of Biotechnology**. 3: 618-621.

Basavaraj, S. H. and S. G. Gupta. 2010. Strain improvement of *Gluconacetobacter xylinus* 2526 for bacterial cellulose production. **African Journal of Biotechnology**. 9: 5170-5172.

Belanger, A. E., G. S. Bersa, M. E. Ford, K. Mikusova, J. T. Belisle, P. J. Brennan and J. M. Inamine. 1996. The embAB genes of *Mycobacterium avium* encode an arabinosyl transferase involved in cell wall arabinan biosynthesis that is the target for the antimycobacterial drug ethambutol. **Proceeding of the National Academy of Sciences of the United States of America**. 93: 11919–11924.

Bokas, D., Davin, U., Franck, G., Guy, D., Emmanuel, G., Stephane, D. and Jean-Louis, G. 2007. Cell envelop fluidity modification for an effective glutamate excretion in *Corynebacterium glutamicum* 2262. **Applied Microbiology and Biotechnology**. 76: 773-781.

Cao, W., M. Yang, Y. Xia, J. Dou, K. Chen, H. Wang, T. Xi and C. Zhou. 2011. Strain improvement for enhanced production of S-adenosyl-L-methionine in *Saccharomyces cerevisiae* based on ethionine-resistance and SAM synthetase activity. **Annals of Microbiology**. 62: 1395-1402.

Chand, P., A. Aruna, A. M. Maqsood and L. V. Rao. 2005. Novel mutation method for increased cellulase production. **Journal of Applied Microbiology**. 98: 318-323.

Chandra, M., A. Kalra, N. S.Sangwan, S. S. Gauray, M. P. Daroka and R. S. Sangwan. 2009. Development of a mutant of *Trichoderma citrinoviridae* for enhanced production of cellulase. **Bioresource Technology**. 100: 1659-1662.

Clement, Y., G. Laneele. 1986. Glutamate Excretion Mechanism in *Corynebacterium glutamicum*: Triggering by Biotin Starvation or by Surfactant Addition. **Journal of General Microbiology**. 132: 925-929.

Crueger, W. and A. Crueger. 1990. **BIOTECHNOLOGY: A TEXTBOOK OF INDUSTRIAL MICROBIOLOGY**. 2 nd ed. Sinauer Associates, Inc., Sunderland.

Delaunay, S., O. Gourdon, P. Lapujade, E. Mailly, E. Orial, J. M. Engasser, N. D. Lindley and J. L. Goergen. 1999. An improved temperature-triggered process for glutamate production with *Corynebacterium glutamicum*. **Enzyme Microbial and Technology**. 25: 762-768.

Delaunay, S., P. Lapujade, J. M. Eangasser and J. L. Goergen. 2002. Flexibility of the metabolism of Corynebacterium glutamicum 2262, a glutamic acid-producing bacterium, in response to temperature upshocks. **Journal of Industrial Microbiology and Biotechnology**. 28: 333-337.

Demain, A. L and J. L. Adrio. 2008. Strain improvement for production of pharmaceuticals and other microbial metabolites by fermentation. **Progress in Drug Research**. 65: 253-289.

Eakwealor, I. A. and J. A. N. Obeta. 2006. Screening of UV-irradiated and S-2-aminoethyl-L-cysteine resistant mutants of *Bacillus megaterium* for improved lysine accumulation. **African Journal of Bacteriology**. 22: 2312-2314.

Eikmanns, B. 2005. Central Metabolism: Tricarboxylic Acid Cycle and Anaplerotic Reactions, pp 241-276. *In* L. Eggelling and M. Bott, eds. **Handbook of *Corynebacterium glutamicum***. CRC press, Boca Raton.

Escuyer, V. E., M. A. Lety, J. B. Torrelles, K. H. Khoo, J. B. Tang, C. D. Rithner, C. Frehel, M. R. MNeil, P. J. Brennan and D. Chatterjee. 2001. The role of the *embA* and *emB* gene products in the biosynthesis of the terminal hexaarabinofuranosyl motif of *Mycobacterium smegmatis* arabinogalactan. **Journal of Biological Chemistry**. 276: 48854-48862.

Gao, K., Y. Li, S. Tian and X. Yang. 2012. Screening and characteristics of a butanol-resistant strain and butanol production from enzymatic hydrolysate of NaOH-pretreated corn stover. **World Journal of Microbiology and Biotechnology**. 28: 2963-2971.

Gonzalez, J. B., F. J. Fernandez and A. Tomasini. 2003. Microbial Secondary Metabolites Production and Strain Improvement. **Indian Journal of Biotechnology**. 2: 322-333.

Haq, I. U. H., S. Ali, A. Saleem and M. M. Javed. 2009. Mutagenesis of *Bacillus licheniformis* through ethyl methanesulfonate for alpha amylase production. **Pakistan Journal of Botany**. 41: 1489-1498.

Hasegawa, T., H. Ken-ichi, K. Hisashi and N. Tsuyoshi. 2008 Changes in Enzyme Activities at the Pyruvate Node in Glutamate-Overproducing *Corynebacterium glutamicum*. **Journal of Bioscience and Bioengineering**. 105: 12-19.

Hashimoto, K., H. Kawasaki, K. Akazawa, J. Nakamura, Y. Asakura, T. Kudo, E. Sakuradani, S. Shimizu and T. Nakamatsu. 2006. Changes in composition and content of mycolic acids in glutamate-overproducing *Corynebacterium glutamicum*. **Bioscience Biotechnology and Biochemistry**. 70: 22-30.

Hirasawa, T., M. Wachi and K. Nagai. 2000. A mutation in the *Corynebacterium glutamicum ltsA* gene causes susceptibility to lysozyme, temperature sensitive growth, and L-glutamate production. **Journal of Bacteriology**. 182: 2696-2701.

Hoischen, C. and R. Kramer. 1990. Membrane alteration is necessary but not sufficient for effective glutamate excretion in *Corynebacterium glutamicum*. **Journal of Bacteriology**. 172: 3409-3416.

Huang, L., P. Wei, R. Zang and Z. Xu. 2010. High-throughput screening of high-yield colonies of *Rhizopus oryzae* for enhanced production of fumaric acid. **Annual Microbiology**. 60: 287-292.

Joshi, D. S., M. S. Singhvi, J. M. Khire and D. V. Gokhale. 2010. Strain improvement of *Lactobaillus lactis* for D-lactic acid production. **Biotechnology Letter**. 32: 517-520.

Jun, H., Y. Bing, Z. Keying, D. Xuemei and C. Daiwen. 2009. Strain improvement of *Trichoderma reesei* Rut C-30 for increased cellulase production. **Indian Journal of Micro biology**. 49: 188-195.

Jyothhi, A N., K. Sasikiran, N. Bala and C. Balagopalan. 2005. Optimisation of glutamic acid production form cassava starch factory residues using *Brevibacterium divaricatum*. **Process Biochemistry**. 40: 3576-3579.

Kadam, S. R., S. S. Patil, K. B. Bastawde, J. M. Khire and D. V. Gokhale. 2006. Strain improvement of Lctobacillus delbrueckii NCIM 2365 for lactic acid production. **Process Biochemistry**. 41: 120-126.

Karla, M. S., R. K. Kulia and B. Ranganathan. 1973. Activation of Nisin Production by UV-Irradiation in a Nisin-Producing Strain of *Streptococcus lacti*s. **Specialia**. 5: 625-626.

Kasukabe, H., M. Yuichiro and F. Tetsuro. 1984. Method for Determinating L-glutamate in Soy Sauce with L-glutamate pxidase. **Agricultural and Biological Chemistry**. 48:181-184.

Katsumata, R., T. Oka and A. Furuya. 1991. Japan Patent H01-3475

Kawahara, Y., K. Takahashi-Fuke, E. Shimizu, T. Nakamatsu and S. Nakamori. 1997. Relationship between the glutamate production and the activity of 2-oxiglutarate dehydrogenase in *Brevibacterium lactofermentum*. **Bioscience Biotechnology and Biochemistry**. 61: 1109-1112.

Khaliq, S., K. Akhtar, M. A. Ghauri, R. Iqbal, A. M. Khalid and M. Muddassar. 2009. Change in colony morphology and kinetics of tylosin production after UV and gamma irradiation mutagenesis of *Streptomyces fradiae* NRRL-2702. **Microbiologicl Research**. 164: 469-477.

Khattab, A. A and W. A. Bazarra. 2005. Screening, mutagenesis and protoplast fusion of *Aspergillus niger* for the emhancement of extracellular glucose oxiase production. **Journal of Industrial Microbiology and Biotechnology**. 32: 289-294.

Kijima, N., D. Goyal, A. Takada, M. Wachi and K. Nagai. 1998. Induction of only limited elongation instead of filamentation by inhibition of cell division in *Corynebacterium glutamicum*. **Applied Microbiology and Biotechnology**. 50: 227-232.

Kitchakarn, S. 2007. **Isolation Screening and Characterization of Thermotolerant Glutamic Acid-Producing *Corynebacterium* Isolated in Thailand**. Master of Science Thesis, Kasetsart University.

Kole, M.M., B. G. Thompson, D. F. GErson and J. Senchai. 1986. Control of Nitrate Concentration in Fermentations of *Corynebacterium glutamicum*. **Biotechnology and Bioengineering**. 28: 659-662.

Levina, N., S. Totemeyer, N. R. Stokes, P. Louis, M. A. Jones and I. R. Booth. 1999. Protection of *Escherichia coli* cells against extreme turgor by activation of MscS and MscL mechanosensitive channels: identification of genes required for MscS activity. **European Molecular Biology Organization Journal**. 18: 1730-1737.

Li, W., S. Ye, K. Lou, F. Ge, L. Du, K. Wu and C. Ding. 2007. Isolation and characterization of *Candida* sp. Mutants enriched in S-adenosylmethionine (SAM). **Annals of Microbiology**. 57: 383-387.

Liebl, W. 2005. Corynebacterium Taxonomy, pp 9-34. *In* L. Eggeling and M. Bott, eds. **Handbook of *Corynebacterium glutamicum***. CRC press, Boca Raton.

Mitani, Y., X. Meng, T. Kamagata and T. Tamura. 2005. Characterization of *LtsA* from *Rhodococcus erythropolis*, an enzyme with glutamine amidotransferase activity. **Journal of Bacteriology**. 187: 2582-2591.

Momose, H. and T. Takagi. 1978. Glutamic acid production in biotin-rich media by temperature sensitive mutants of *Brevibacterium lactofermentum*, a novel fermentation process. **Agricultural Biotechnological and Chemistry**. 42: 1911-1917.

Nakamura, J., S. Hirano, H. Ito and M. Wachi. 2007. Mutations of the *Corynebacterium glutamicum* NCgl1221 Gene, Encoding a Mechanosensitive Channel Homolog, Induce L-glutamic Acid Production. Applied and **Environmental Microbiology**. 73: 4491-4498.

Nampoothiri, K. M. and P. Ashok. 1999. Fermentation and recovery of L-glutamic acid from cassava starch hydrolysate by ion-exchange resin column. **Revista de Microbiologia**. 30: 258-264.

Nampoothiri, K. M., C. Hoischen, B. Bathe, B. Mockel, W. Pfefforle, K. Krumback, H. Sahm and L. Eggeling. 2002. Expression of genes of lipid synthesis and alter lipid composition modulates L-glutamate efflux of *Corynebacterium glutamicum*. **Applied Microbiology and Biotechnology**. 58: 89-96.

Nara, T., H. Samejima and S. Kinoshita. 1964. Effect of penicillin on amino acid fermentation. **Agricultural Biological and Chemistry**. 28: 120-124.

Nelson, N. 1944. A photometric adaptation of Somogyi method for the determination of glucose. **Journal of Biological Chemistry**. 153: 375-380.

Onishi, J., M. Hayashi, S. Mitsuhashi and M. Ikeda. 2003. Efficient 40 ºC fermentationof l-lysine by a new *Corynebacterium glutamicum* mutant developed by genome breeding. **Applied Microbiology and Biotechnology**. 62: 69-75.

Parekh, S. 2000. Improvement of microbial strains and fermentation process. **Applied Microbiology and Biotechnology**. 54: 287-301.

Pasha, C., R. C. Kuhad and L. V. Rao. 2007. Strain improvement of thermotolerance *Saccharomyces cerevisiae* VS_3 strain for better utilization of lignocellulosic substrates. **Journal of Applied Microbiology**. 103: 1480-1489.

Radmacher, E., K. C. Stansen, G. S. Besra, L. J. Alderwick, W. N. Maughan, G. Hollweg, H. Sahm, V. F. Wendisch and L. Egelling. 2005. Ethambutol, a cell wall inhibitor of Mycbacterium tuberculosis, elicits L-glutamate efflux of *Corynebacterium glutamicum*. **Microbiology**. 151: 1359-1368.

Reershemius, H. K. 2008. **Production of L-methionine with *Corynebacterium glutamicum***. Ph.D. Thesis, Technischen Universitat.

Rubinder, K., B. S. Chadha, N. Singh, H. S. Saini and S. Singh. 2002. Amylase hyperproduction by deregulated mutants of the thermophilic fungus *Thermomyces lanuginosus*. **Journal of Industrial Microbiology and Biotechnology**. 29: 70-74.

Schleifer, K. H. and O. Kandler. 1972. Peptidoglycan types of bacterial cell walls and their taxonomic implications. **Bacteriology Review**. 36: 470-477.

Schultz, C., A. Niebisch and L. Gebel. 2007. Glutamate overproduction by *Corynebacterium glutamicum*: dependence on the oxoglutarate dehydrogenase inhibitor protein OdhI and protein kinase PknG. **Applied Microbiology and Biotechnology**. 76: 691-700.

Shigematsu, T., Y. Nasuhara, G. Nagai, K. Nomura, K. Ikarashi, M. Hirayama, M. Hayashi, S. Ueno and T. Fujii. 2010. Isolation and Characterization of Barosensitive Mutants of *Saccharomyces cerevisiae* Obtained by UV mutagenesis. **Journal of Food Science**. 75: 509-514.

Shiio, I and K. Ujigawa. 1980. Presence and regulation of α-ketoglutarate dehydrogenase complex in a glutamate producing bacterium, *Brevibacterium flavum.* **Agricultural Biological and Chemistry**. 42: 1897-1904.

Shiio, I., S. Otsuka and M. Takahashi. 1962. Effect of biotin on the bacterial formation of glutamic acid. I. Glutamate formation and cellular permeability of amino acids. **Journal of Biochemistry**. 51: 56-62.

Shiio, I., S. Otsuka and N. Katsuya. 1963. Cellular permeability and extracellular formation of glutamic acid in *Brevibacterium flavum.* **Journal of Biochemistry**. 30: 750-758.

Shimizu, H. and T. Hirasawa. 2006. Production of Glutamate and Glutamate-related Amino Acids: Molecular Mechanism Analysis and Metabolic Engineering. **Microbiology Monograph**. 5: 1-38.

Shingu, H. and G. Terui. 1971. Studies on process of glutamic acid fermentation at the enzyme level. I. On the change of α-ketoglutaric acid dehydrogenase in the course of culture. **Journal of Fermentation and Technology**. 49: 400-405.

Shiratsuchi, M., H. Karanuma, Y. Kawahara, Y. Yoshihara, H. Miwa and S. Nakamori. 1995. Simultaneous and high fermentative production of L-lysine and L-gluatamic acid using a strain of *Brevibacterium lacotofermentum.* **Bioscience Biochemistry and Biotechnology**. 59: 83-86.

Sridha, M., N. K. Sree and L. V. Rao. 2002. Effect of UV radiation on thermotolerance of *Saccharomyces cerevisiae* VS_1 and VS_3 strains. **Bioresource Technology**. 83: 199-202.

Sukarev, S. 2002. Purification of the small mechanosensitive channel of *Escherichia coli* (MscS): the subunit structure, conduction, and gating characteristics in liposomes. **Biophysical Journal**. 83: 290-298

Takinami, K., H. Yoshii, H. Tsuri and H. Okada. 1995. Biochemical effects of fatty acid and its derivatives on L-glutamic acid fermentation, part II. Biotin-Tween 60 relationship in the accumulation of L-glutamic acid and the growth of *Brevibacterium lactofermentum*. **Agricultural Biological Chemistry**. 29: 351-359.

Taloria, D., S. Samanta, S. Das and C. Pututunda. 2012. Increase in Bioethanol Production by Random UV Mutagenesis of Saccharomyces cerevisiae and by Addition of Zinc Ions in the Alcohol Production Media. **APCBEE Procedia**. 2: 43-49.

Tan, T., M. Zhang, B, Wang, C. Ying and L, Deng. 2003. Screening of high lipase producing *Candid*a sp. And production of lipase by fermentation. **Process Biochemistry**. 39: 459-465.

Telenti, A., W. J. Philipp, S. Sreevatsan, C. Bernasconi, K. E. Stockbauer, B. Wieles, J. M. Musser and W. R. Jacob.1997. The *emb* operon, a gene cluster of *Mycobacterium tuberculosis* involved in resistance to ethambutol. Nature **Medicine**. 3: 567-570.

Uy, D., S. Delaunay, P. Germain, J. M. Engasser and J. L. Goergen. 2003. Instability of glutamate production by *Corynebacterium glutamicum* 2262 in continuous culture using the temperature-triggered process. **Journal of Biotechnology**. 104: 173-184.

Vaidya, R. J., S. L. Macmil, P. R. Vyas and H. S. Chhatpar. 2003. The novel method for isolating chitinolytic bacteria and its application in screening for hyperproducing mutant of *Alcaligenes xylosoxydans*. **Letters in Applied Microbiology**. 36: 129-134.

Xu, F., J. Wang, S. Chen, W. Qin, Z. Yu, H. Zhao, X, Xing and H, Li. 2011. Strain Improvement for Enhanced Production of Cellulase in *Trichoderma viridae*. *Applied Biochemistry and Microbiology*. 47: 53-58.

Xu, H., S. Joa and J, Liu. 2011. Development of a mutant strain of *Bacillus subtilis* showing enhanced production of acetoin. **African Journal of Biotechnology**. 10: 779-788.

Yoshida, K., Y. Fujita and D. Ehrlich. 1999. Three asparagines synthetase genes of *Bacillus subtilis*. **Journal of Bacteriology**. 181: 6081-6091.

Zambare, V. 2010. Strain Improvement of Alkaline Protease from *Trichoderma reesei* MTCC-3929 by Physical and Chemical Mutagen. **Institute of Integrative Omics and Applied Biotechnology Journal**. 1: 25-28.

Zhao, L. L., X. X. Chen and J. H. Xu. 2010. Strain improvement of *Serratia marcescens* ECU 1010 and medium cost reduction for economic production of lipase. **World Journal of Microbiology and Biotechnology**. 26: 537-543.

Zhong, C. H. and Y. G. Zheng. 2011. Enhancement of 1,3-Dihydroxyacetone Production by a UV-induced Mutant of *Gluconobacter oxydans* with DO Control Strategy. **Applied Biochemistry and Biotechnology**. 165: 1152-1160.

APPENDICES

Appendix A

Culture media

The compositions per 1 litre were composed as followed:

1. A1 medium

Glucose	5	g
Yeast extract	10	g
Peptone	10	g
NaCl	1	g

Adjust pH to 7.2 with 0.1 N NaOH. Sterilized by autoclave at 121ºC for 15 minutes.

2. Basalt Salt (BS) medium

Glucose	50	g
$MgSO_4.7H_2O$	0.25	g
$(NH_4)_2SO_4$	5	g
KH_2PO_4	2	g
K_2HPO_4	2	g
C1 solution	10	mL
C2 solution	1	mL
Biotin	200	µg
$CaCl_2.2H_2O$	0.01	g
Urea	5	g

C1 solution were composed of:

$FeSO_4.7H_2O$	0.01	g/ L
$MnSO_4.5H_2O$	0.01	g/ L

C2 solution were composed of:

$ZnSO_4.7H_2O$	0.01	mg/ L
H_3BO_4	0.1	mg/ L

$CoCl_2.6H_2O$	0.07	mg/ L
$CuCl_2.2H_2O$	0.03	mg/ L
$NiCl_2.6H_2O$	0.01	mg/ L
$NaMo_2O_2.2H_2O$	0.1	mg/ L

Separately prepared into 6 portions as described below:

For the portion 1, dissolved glucose, $MgSO_4.7H_2O$ with distilled water and adjusted volume to 600 mL. Sterilized at 110ºC for 10 minutes to avoided Browning's reaction.

For the portion 2, dissolved $(NH_4)_2SO_4$, KH_2PO_4 and K_2HPO_4 with distilled water. Adjusted pH to 7.2 with 0.1 N NaOH and filled up to 160 mL. Sterilized at 121ºC for 15 minutes.

For the portion 3, dissolved glucose, C1 solution and C2 solution with distilled water. Adjusted volume to 160 mL and sterilized at 110 ºC for 10 minutes.

For the portion 4, preparing 1,000X of biotin stock solution by dissolved 0.2 g biotin with distilled water, adjusted volume to 1,000 mL resulting a final concentration at 200 µg/ L. Before used, transferring 1 mL/ L media from a stock solution and filled up to 20 mL with distilled water. Sterilized at 121ºC for 15 minutes.

For the portion 5, preparing a stock solution of $CaCl_2$ with a final concentration at 1 g/ L. Transferring 10 mL/ L media and making up to 20 mL. Sterilized at 121ºC for 15 minutes.

For the portion 6, preparing 12.5% urea stock solution by dissolved 12.5 g of urea with distilled water and adjusted volume to 100 ml. Sterilized by membrane filtration and kept at 4ºC. Transferring 40 mL/ L media giving a final concentration at 5 g/ L.

After mixed all of portions well, placed overnight on a static or shaking condition to proved no post-contamination before use.

3. Lennox (L) medium

Yeast extract	5	g
NaCl	10	g
Bacto peptone	10	g
Glucose	1	g

Adjusted pH to 7.0 with 0.1 N NaOH and sterilized at 121ºC for 15 minutes.

Appendix B

Calculation methods

1. Adjustment of initial optical density at 660 nm

The volume of inoculums or seed culture used to adjusted an initial OD_{660} in the media throughout this thesis was calculated by the following equation:

$$V_1 = (N_2 \times V_2)/ N_1$$

where, V_1 is the volume of a given inoculums or seed culture needed to be use.

V_2 is the total volume of a given culture media.

N_1 is the exactly OD_{660} of a given inoculums or seed culture.

N_2 is the initial OD_{660} of a given culture media needed to be adjust.

2. Calculation of the maximum specific growth rate (h^{-1})

The maximum specific growth rate in any conditions that appeared in this thesis calculated from a slope at an exponential growth by the following equation:

$$\mu_{max} = (\ln X_t - \ln X_0)/ t$$

where, μ_{max} is the maximum specific growth rate

X_t is the OD_{660} at a time t

X_0 is the OD_{660} at start

t is the time

3. Correlation between OD_{660} and the CFU/ mL

OD_{660}	**Dilution factor**	**Numbers of colony**	**CFU/ mL**
5	10^{-6}	127	1.27×10^{9}
4	10^{-6}	45	4.50×10^{8}
3	10^{-6}	20	2.0×10^{8}
2	10^{-5}	58	5.8×10^{7}
1	10^{-2}	43	4.3×10^{4}

Appendix C

Analysis protocol

1. Qualitative of L-glutamate by paper chromatographic method

1.1 Preparation of sample

Separating cells from the culture broth by centrifuge at 10,000 rev min^{-1} at 4ºC for 5 minutes. The cell-free supernatant were kept in a cleaned tube at -20ºC in the freezer for further analysis.

1.2 Analytical reagents

1.2.1 The mixture of a solvent solution (n-butanol: acetic acid: water) at ratios of 4: 1: 1 were allowed to saturate overnight before use in a chromatographic tank.

1.2.2 Ninhydrin developing solution prepared as a fresh solution before use by dissolved 0.2 g of ninhydrin powder with 100 mL of acetone and kept in a brown-bottle to avoiding the deterioration by sunlight.

1.2.3 Standard solution of amino acid mixture L-glutamate and lysine were prepared by dissolved L-glutamate and lysine powder 0.01 g with a sterile distilled water and making up to 10 mL with volumetric flask. The resulting solution with final concentration at 1 µg/ µL of each kinds of amino acid were kept at -20ºC for further use.

1.3 Analytical steps

Five microlitre of the cell-free supernate were spotted and drying with hot-air onto a size of 20×20 cm chromatography paper and put it in rectangular position in chromatographic tank. After allowed the solvent migrated almost approaches the edge, the paper was removed and dried at room temperature. The paper was then spraying with ninhydrin developing solution and drying at 70 ºC for 2-3 minutes and repeat this step until the intensity of amino acid band remains constant.

2. Quantitative of L-glutamate by enzymatic method

2.1 Reagents

2.1.1 Potassium phosphate buffer

Solution A: 0.16 M KH_2PO_4, Dissolved 21.77 g of KH_2PO_4 with distilled water and make volume up to 1000 mL

Solution B: 0.16 M K_2HPO_4, Dissolved 27.87 g of K_2HPO_4 and then make volume up to 1000 mL with distilled water.

Solution A (mL)	Solution B (mL)	pH
68.5	31.5	6.5
62.5	37.5	6.6
56.5	43.5	6.7
51.0	49.0	6.8
45.0	55.0	6.9
39.0	61.0	7.0
33.0	67.0	7.1
28.0	72.0	7.2
23.0	77.0	7.3
19.0	81.0	7.4
16.0	84.0	7.5

2.1.2 0.3 M phenol (MW=94.11), Dissolved 2.838 g phenol and make volume up to 100 mL with distilled water. When used, applies 10 µL into a mixture solution to give a final concentration at 0.3 M.

2.1.3 0.3 M 4-aminoantipyrine, Dissolved 6.097 g with distilled water and adjust the total volume to 100 mL. When used, applies 10 µL into a mixture solution to give final concentration at 0.3 M.

2.1.4 5 U mL^{-1} peroxidase, Enters 20 mL of a sterilized distilled water into lyophilized enzyme-bottle (25000 U/ mL) and then mix by vortex to made a final concentration at 5 U/ 4 µL.

2.1.5 0.05 U mL^{-1} L-glutamate oxidase, 10 mL of sterilized distilled water was added into lyophilized enzyme-bottle (2.5 U/ mL) and mix well by vortex to give a final concentration at 0.05 U/ 20 µL.

2.1.6 0.004 M L-glutamate (MW=187.13), Dissolved 0.0748 g of L-glutamate with 100 mL sterilized distilled water to made a final concentration at 0.004 M/ 1 µL.

2.2 Analytical steps

The analysis of extracellular L-glutamate was done according to Kasukabe, *et. al*., 1984.

2.2.1 Applies a corresponding volume of reagents as summarized in the Table below:

Reagents	Concentration in a stock solution	Volume used for the sample (µL)	Volume used for the blank (µL)
160 µmol mL^{-1} potassium phosphate buffer pH 7.4 *	0.16 M KH_2PO_4 (MW=136.09) and 0.16 M K_2HPO4 (MW=174.189)	906	906
300 µmol mL^{-1} phenol *	0.3 M (MW=94.11)	10	10
300 µmol mL^{-1} 4-aminoantipyrine *	0.3 M (MW=203.25)	10	10
5 U peroxidase *	25000 U mL^{-1}	4	4
0.05 U L-glutamate oxidase *	2.5 U mL^{-1}	20	20
L-glutamate or sample	-	20	-
Distilled water *	-	30	50
Total volume	-	1000	1000

Note * prepare as a pre-mix solution

2.2.2 After homogeneously mixed by vortex, the mixture solution was allowed for reaction in gently-shaking condition at 37 ºC for 20 minutes.

2.2.3 Measurement the absorbance at 505 nm against the blank.

2.3 Standard correlation curve of L-glutamate.

Pipette the following reagents as describe below:

Concentration of standard L-glutamate (µmol)	Stock L-glutamate (µL)	Sterilized distilled water (µL)	Volume of pre-mix solution (µL)
0.00	0	50	950
0.04	10	40	950
0.08	20	30	950
0.12	30	20	950
0.16	40	10	950
0.20	50	0	950

3. Analysis of reducing sugar

3.1 Reagents

3.1.1 Copper reagent (Somogyi reagent): Solution A

3.1.1.1 Dissolve 12 g of Sodium potassium tartrate, 24 g of Sodium carbonate in 250 mL of distilled water.

3.1.1.2 Added the solution of 5 g $CuSO_4$. $5H_2O$ in 5 mL distilled water and mix well.

3.1.1.3 Gently added 16 g of $NaHCO_3$

3.1.1.4 Added the solution of 180 g Na_2SO_4 in 500 mL distilled water.

3.1.1.5 Make volume up to 1000 mL and kept in brown-bottle to avoid day light. This solution must be previously prepared at least 1 week. In case of precipitated, filtration was applied before use.

3.1.2 Arsenomolybdate reagent (Nelson reagent): Solution B

3.1.2.1 Dissolve 25 g of Ammonium molybdate in distilled water 450 mL.

3.1.2.2 Added 21 mL of Conc. H_2SO_4 and the solution of 3 g $Na_2HASO_4.7H_2O$ in 25 mL distilled water.

3.1.2.3 After filled volume up to 100 mL, this solution was previously prepared at 24 hour before use and kept in brown-bottle to avoided the deterioration by sunlight.

3.2 Analytical steps

3.2.1 Added 1 mL of sample in which the appropriate concentration of sugar must be in the range of 20-160 µg/ mL in a cleaned test tube.

3.2.2 After added 1 mL of the Solution A, the mixture solution was boiling for 10 minutes and then placed on ice to stop reaction.

3.2.3 Added 1 mL of the Solution B, mixed well by vortex and put on a static condition for 15 minutes.

3.2.4 Added 10 mL of distilled water, mixed by vortex and measured the absorbance at 520 nm.

3.3 Standard correlation curve of glucose

Dissolved analytical grade of 1.000 g D-glucose (dried in hot air oven at 120 ºC for 2 hours) in a volumetric flask and make volume up to 100 mL. The resulting solution was used as a stock solution of standard glucose. To constructed a correlation curve, the solution was diluted as summarized in table below:

Concentrations of glucose standard (µg/ mL)	Volume from 1 mg/ mL of glucose stock solution (mL)	Distilled water (mL)
0	0	5.0
20	0.1	4.9
40	0.2	4.8
60	0.3	4.7
80	0.4	4.6
100	0.5	4.5
120	0.6	4.4

CURRICULUM VITAE

NAME : Mr. Suphoj Noisakul

BIRTH DATE : December 12, 1982

BIRTH PLACE : Phranakhon Si Autthaya, Thailand

EDUCATION :

YEAR	**INSTITUTE**	**DEGREE/ DIPLOMA**
2005	Phranakhon Si Ayutthaya Rajabhat Univ	B.S. (Applied Biology)
2013	Kasetsart Univ	M.Sc. (Microbiology)

POSITION/ TITLE : -

WORK PLACE : -

SCHOLARSHIP/ AWARD : -