รายงานการวิจัยฉบับสมบูรณ์

การพัฒนาการผลิตกรดอะมิโนและกรดอินทรีย์จากวัสดุลิกโนเซลลูโลสโดย *Corynebacterium sp.*ที่คัดแยกได้ในประเทศไทย

Development production of amino acid and organic acid from lignocellulosic materials by *Corynebacterium sp.* isolated from Thailand

โดย

อาจารย์ ดร. ศิริลักษณ์ ธีระดากร

สถาบันเทคโนโลยีชีวภาพและวิศวกรรมพันธุศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

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ชื่อผู้วิจัย อาจารย์ ดร. ศิริลักษณ์ ธีระดากร

เดือนและปีที่ทำวิจัยสำเร็จ มกราคม 2557

บทคัดย่อ

ทำการเก็บตัวอย่างแหล่งดินและมูลสัตว์จากจังหวัดสุรินทร์และจังหวัดสุพรรณบุรี รวมทั้งหมด 65 ตัวอย่าง เพื่อคัดเลือกสายพันธุ์จลินทรีย์ *Corynebactereium sp* ที่สามารถผลิตกรดอะมิโนและกรดอินทรีย์ พบว่ามี 33 สายพันธุ์ที่สามารถเจริญในอาหารแข็งสำหรับคุณสมบัติการผลิตกรดกลูตามิก และ 32 สายพันธุ์มที่สามารถเจริญเติบโตในอาหารแข็งสำหรับคุณสมบัติการผลิตกรดซัคซินิก โดยพบว่ามี 3 สายพันธุ์ที่มีศักยภาพในการผลิตกรดซัคซินิก (isolate SP-II/A1, SP-II/A3 and SP-II/A4) ทำการศึกษานำร่องในการผลิตกรดซัคซินิกจากวัสดุประเภทลิกโนเซลลูโลส โดยใช้ *Corynebacterium glutamicum* DSM 20300 พบว่าเมื่อใช้สารแหล่งไนโตรเจน เปปโตน10กรัม/ลิตรร่วมกับยีสต์สกัด10 กรัม/ลิตร การเจริญของจุลินทรีย์ มีค่าสูงสุด 9.62 กรัม/ลิตร และเมื่อใช้สารแหล่งคาร์บอนคือ กลูโคส 10 กรัม/ลิตร จุลินทรีย์มีการเจริญสูงสุด 14.34 กรัม/ลิตร และเมื่อใช้สารละลายย่อยสลายของชานข้าวฟ่าง (เทียบเท่าน้ำตาลรีดิวส์) 10 กรัม/ลิตร การเจริญของจุลินทรีย์จะมีระยะการเจริญเติบโตในช่วงระยะปรับตัวนานขึ้นและมีการเจริญสูงสุด 9.82 กรัม/ลิตร

Project Title Development production of amino acid and organic acid from lignocellulosic materials by *Corynebacterium sp.* isolated from Thailand

Name of the Investigators Dr. Siriluk Teeradakorn

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ABSTRACTS

The samples were collected from buffalo dung in Suphanburi province (SP-I, SP-II), soil sample in Suphanburi province (SP-III) and cow dung from Surin province (SR-I, SRII), Thailand. Thirty-three isolates with ability amino acid and thirty-two isolates with ability succinic acid were obtained. They were non-spore-forming, gram-positive or negative rod. The morphological characteristics colonies appearing on the screening agar plate after 24 h of incubation were circular, smooth and gray with 1–2 mm in diameter. Three of those isolates have a potential for succinic acid production (isolate SP-II/A1, SP-II/A3 and SP-II/A4). *Corynebacterium glutamicum DSM20300* was used as a representative for investigating growth and succinic acid production from agriculture biomass. The use of nitrogen source, combination of 10 g/l of peptone and 10 g/l of yeast extract, gave the highest amount of cell growth of 9.62 g/l. Using glucose as a carbon source exhibited better cell growth than using monosugar from SSS hydrolysate. A maximum cell growth of 14.34 g/l was obtained with 10 g/l of glucose. In case using SSS hydrolyzate as a carbon source, longer lag phase was found and the highest amount of cell growth of 9.82 g/l was obtained.

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Chapter I

Introduction

1.1 The lignocellulosic biomass

Lignocellulosic biomass is the most abundant material in the world. Its sources range from trees to agricultural residues. It is the major component of biomass, comprising around half of the plant matter produced by photosynthesis and representing the most abundant renewable organic resource in soil. It consists of four major components. They are cellulose, hemicellulose, lignin and extractives (e.g., chlorophyll and waxes) which are associated whith each other (Fengel and Wegener, 1984) (Figure 1).

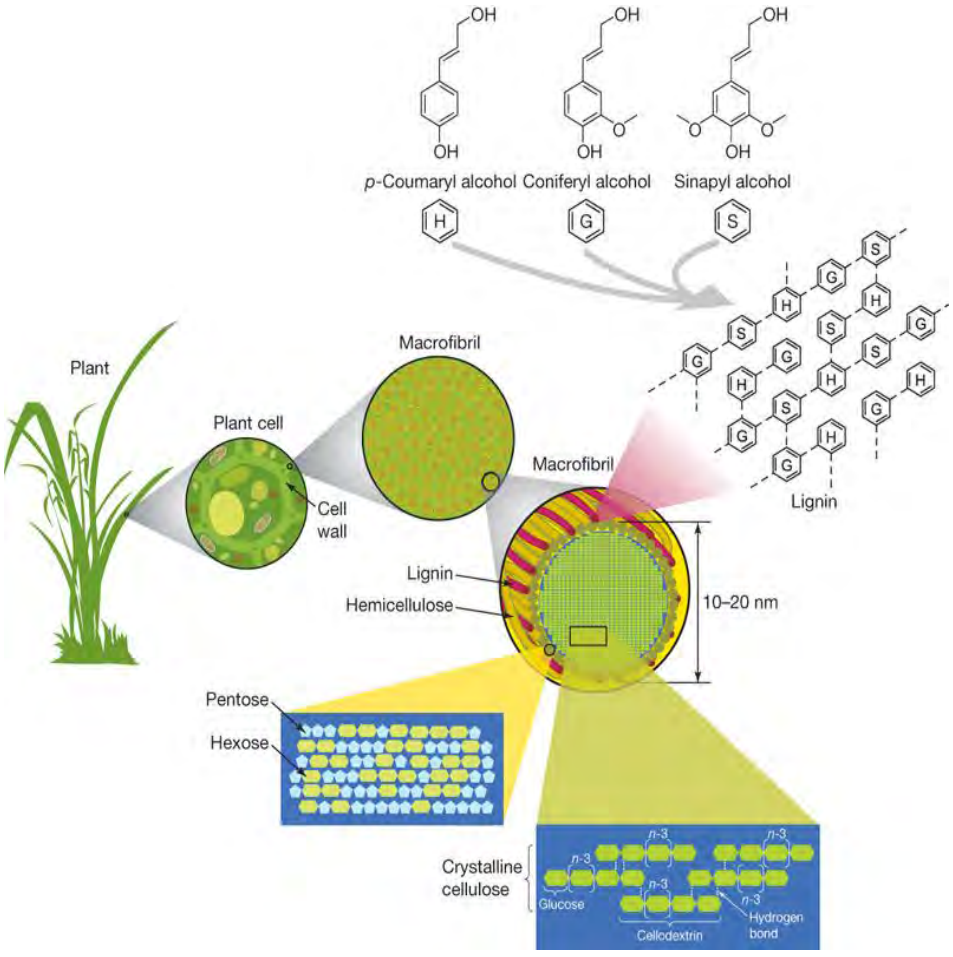


Figure 1 Composition of lignocellulosic material.

(Source: EM Rubin *Nature* 454, 841-845 (2008) doi: 10.1038/nature07190)

1.1.1 Cellulose

Cellulose in biomass usually is present in both crystalline and amorphous (Kumar et al., 2009). The coalescence of several polymer chains lead to the formation of microfibrils, which in turn are united to form fibers. In this way cellulose can obtain a crystalline structure (Sánchez, C., 2009).

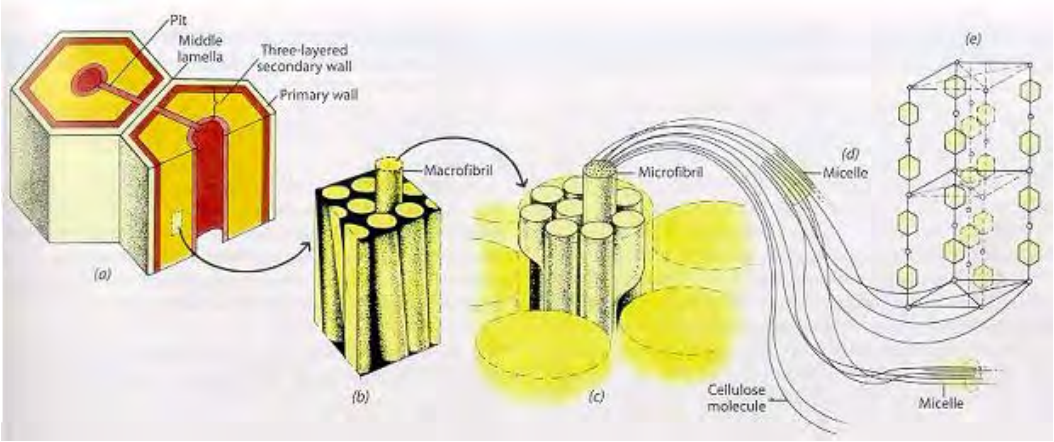


Figure 2 Formation of micro- and macro-fibrils of cellulose and their position in the plant cell wall (Source: Harmsen, P. F. H., 2010)

Figure 2 illustrates structure as well as the placement of cellulose in the plant cell wall. The cellulose content of wood varies between species in the range of 40-50 %. Some lignocellulosic material can have more cellulose than wood (Table 2.1). Cellulose is a linear polymer that is composed of D-glucose subunits linked by β-1, 4 glycosidic bonds (4-O-β-D-glucopyranosyl-D-glucose) forming the dimer cellobiose (Fengel and Wegener, 1984). These form long chains (or elemental fibrils) link together by hydrogen bonds and van der waals forces (Sánchez, 2009) (Figure 2.3). Many properties of cellulose depend on its degree of polymerization (DP), i.e. the number of glucose units that make up one polymer molecule. The DP of cellulose can extend to a value of 17,000, even though more commonly a number of 800-10000 units are encountered (Harmsen, P. F. H., 2010).

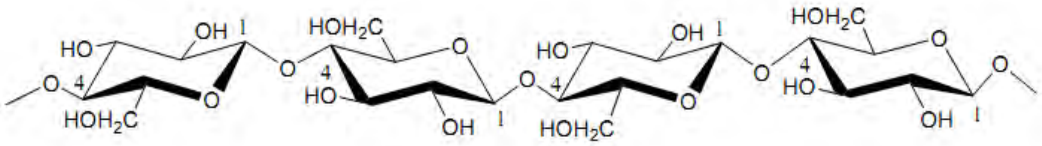


Figure 3 Cellulose structure $(C_6H_{12}O_6)_n$
(Source: Gardner, K. H. and Blackwel, J., 1974)

Cellulose has a strong tendency to form intra-molecular and inter-molecular hydrogen bonds between hydroxyl groups (OH-groups) within the same cellulose chain, which harden the straight chain and promote aggregation into parallel and form a crystalline supermolecular structure and give cellulose a multitude of partially crystalline fiber structures and morphologies. The cellulose strains are 'bundled' together and form so called cellulose fibrils or cellulose bundles. These cellulose fibrils are mostly independent and weakly bound through hydrogen bonding (Laureano-Perez *et al.,* 2005). This causes the cellulose to be formed crystalline structures and make them particularly difficult to digest. The linkages between glucose molecules in cellulose are most commonly broken by enzymes (Hendriks, A.T.W.M. and Zeeman, G., 2009).

1.1.2 Hemicellulose

Hemicellulose is a polysaccharide with a lower molecular weight than cellulose. It is a complex carbohydrate structure that consists of different polymers like pentoses (like D-xylose and L-arabinose), hexoses (like D-mannose, D-glucose and D-galactose), and sugar acids (4-O-methyl-glucuronic, D-galacturonic and D-glucuronic acids). Sugars are linked together by β-1,4 and sometimes by β-1,3 glycosidic bonds with α-(4-O)-methyl-D-glucuronopyranosyl units attached to anhydroxylose units (Sánchez, C., 2009). The backbone of the chains of hemicelluloses can be a homopolymer (generally consisting of single sugar repeat unit) a branched polymer chain that is mainly composed of five carbon sugar monomers, xylose and to a lesser extent six carbon sugar monomers such as glucose, a heteropolymer (mixture of different sugars) (Harmsen, P. F. H., 2010). Formulas of the sugar component of hemicelluloses are listed in Figure 4.

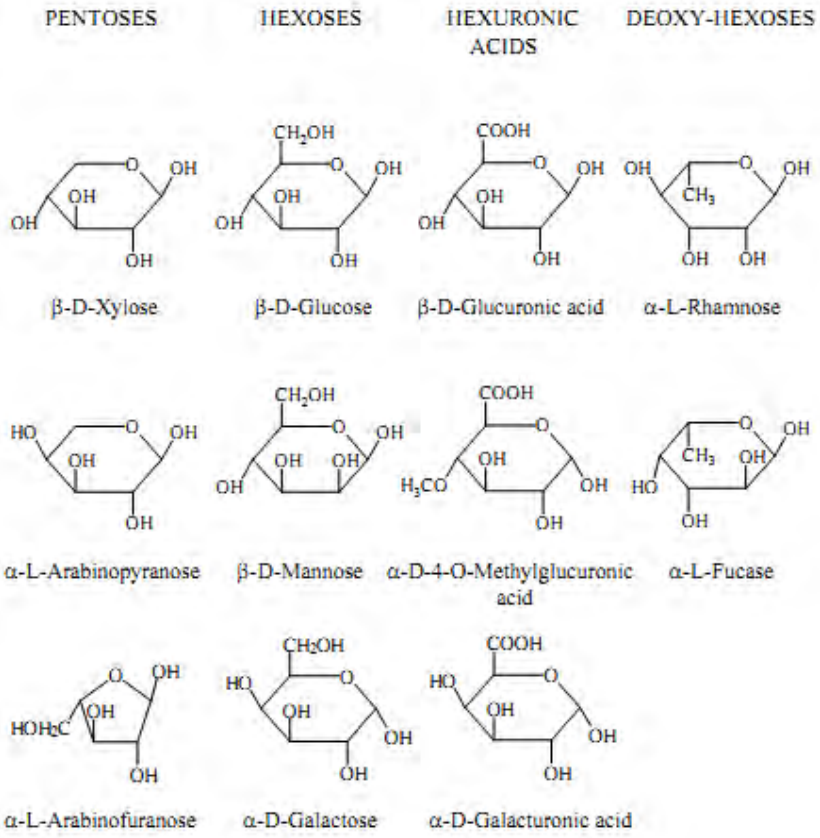


Figure 4 The monomeric building units of hemicellulose

(Source: Hansen and Plackett, 2008)

Important aspects of the structure and composition of hemicellulose are the lack of crystalline structure, mainly due to the highly branched structure, and the presence of acetyl groups connected to the polymer chain. Hemicellulose serves as a connection between the lignin and the cellulose fibers and gives the whole cellulose–hemicellulose–lignin network more rigidity (Laureano-Perez et al., 2005) as shown in Figure 5.

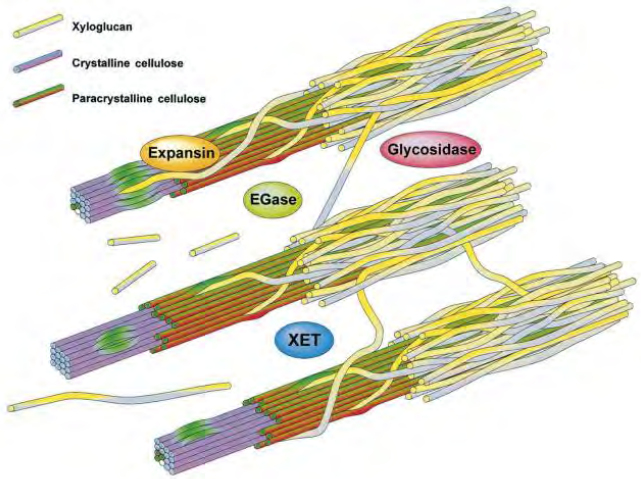


Figure 5 Cellulose and hemicellulose network
(Source: Rose, J.K.C. and Bennett, A.B. 1999)

The main difference between cellulose and hemicellulose is that hemicellulose has a lower molecular weight than cellulose, and branches with short lateral chains consisting of different monosacharide units (Fengel and Wegener, 1984). Hemicellulose has lower DP (only 50-300) with side groups on the chain molecule and is essentially amorphous. Because of the amorphous morphology, it is partially soluble or swellable in water (Sánchez, C., 2009). Hemicellulose is insoluble in water at low temperature. However, its hydrolysis starts at a temperature lower than that of cellulose, which renders its soluble at elevated temperatures. The presence of acid highly improves the solubility of hemicellulose in water (Harmsen, P. F. H., 2010). Hemicellulose can be extracted by alkali or hot water and hydrolyzed by diluted H_2SO_4. The solubility of the different hemicellulose compounds, the solubility increase with increasing temperature. The solubilization of hemicellulose compounds into the water starts around 180^{o}C under neutral conditions according to Bobleter (1994).

1.1.3 Lignin

Lignin is strongly intermeshed and chemically bonded by non-covalent forces and by covalent cross linkages (Sánchez, C., 2009). Lignin is a complex, cross-linked polymer that forms a large molecular structure. It gives mechanical strength to wood by gluing the fibers together (reinforcing agent) between the cell walls and it is linked to both hemicellulose and cellulose, forming a physical seal that is an impenetrable barrier in preventing penetration of solutions and enzymes (Howard R.L., 2003). This amorphous heteropolymer is synthesized by the generation of free radicals, which are released in the peroxidase-mediated dehydrogenation of three phenylpropane units (C9): coniferyl alcohol (guaiacyl propanol), coumaryl alcohol (p-hydroxyphenyl propanol) and sinapyl alcohol (syringyl propanol). The monomeric building units of lignin are shown in Figure 6.

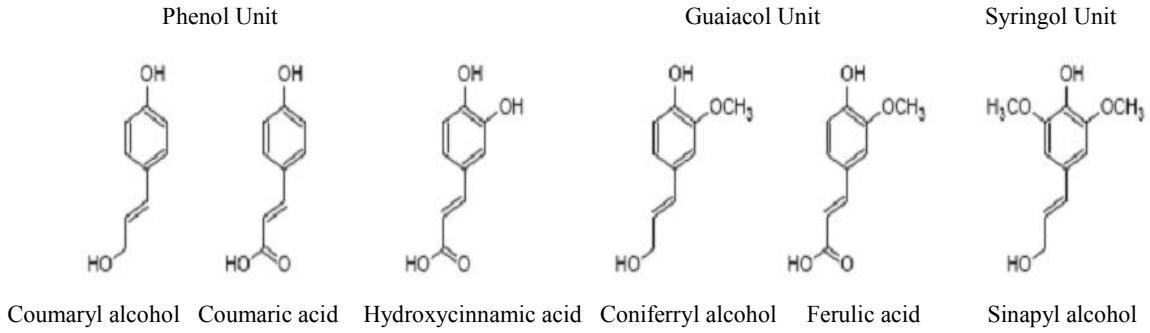


Figure 6 The monomeric building units of lignin
(Source: Boerjan at al., 2003 and Holladay et al., 2007)

This heterogeneous structure is linked by C–C and aryl-ether linkages, with aryl-glycerol β-aryl ether being the predominant structures (Sánchez, C., 2009). It is present in the cellular wall to give structural support, impermeability and resistance against microbial attack and oxidative stress. The model structures of lignin are shown in Figure 7.

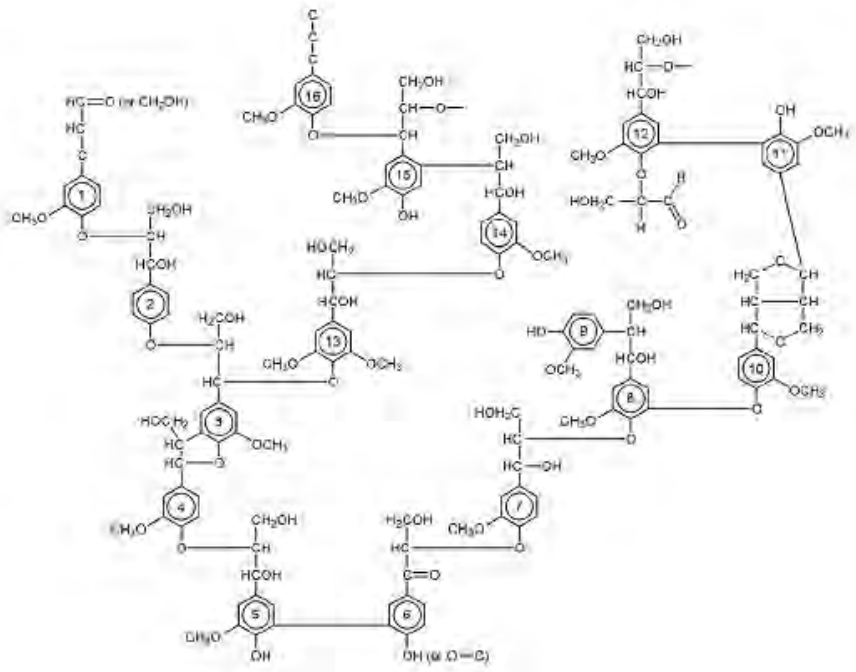


Figure 7 Model structure of spruce lignin

(Source: Harmsen, P.F.H., 2010)

Normally lignin starts to dissolve in water around 180^{o}C under neutral conditions (Bobleter, 1994). It plays an important role in the cell's endurance and development, as it affects the transport of water, nutrients and metabolites in the plant cell. It acts as binder between cells creating a composite material that has a remarkable resistance to impact, compression and bending. The solubility of the lignin in acid, neutral or alkaline environments depends on the precursor (p-coumaryl, coniferyl, sinapyl alcohol or combinations of them) of the lignin (Hendriks, A.T.W.M. and Zeeman, G., 2009). Solvents have been identified to significantly dissolve lignin include low molecular alcohols, dioxane, acetone, pyridine, and dimethyl sulfoxide. Furthermore, it has been observed that at elevated temperatures, thermal softening of lignin takes place, which allows depolymeristation reactions of acidic or alkaline nature to accelerate (Harmsen, P. F. H., 2010). The solubilization of lignocellulose components not only depends on temperature, but also on other aspects like moisture content and pH (Fengel and Wegener, 1984). Of cellulose, hemicellulose and lignin the hemicelluloses are the most thermal-chemically sensitive. During thermal–chemical pretreatment firstly the side groups of hemicellulose react, followed by the hemicellulose backbone (Hendriks, A.T.W.M. and Zeeman, G., 2009). Guo, G.L.*et al.* 2009 addressed are the physical properties of each of the components of

lignocellulose, and how each of these components supplies to the behavior of the complex structure as a whole.

The composition of lignocellulosic materials suchase wood, grass, forestry waste, agricultural residues (e.g., wheat straw, corn stover, sorghum straw and sugarcane bagasse) and municipal solid waste. Which is dependent on the source of feedstocks are shown in Table 1

Table 1 Lignocellulose contents of common agricultural residues and wastes.

Lignocellulosic materials	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Hardwood stems	40-55	24-40	18-25
Softwood stems	45-50	25-35	25-35
Nut shells	25-30	25-30	30-40
Corn cobs	45	35	15
Paper	85-99	0	0-15
Wheat straw	30	50	15
Rice straw	32.1	24	18
Sorted refuse	60	20	20
Leaves	15-20	80-85	0
Cotton seeds hairs	80-95	5-20	0
Newspaper	40-55	25-40	18-30
Waste paper from chemical pulps	60-70	10-20	5-10
Fresh bagasse	33.4	30	18.9
Solid cattle manure	1.6-4.7	1.4-3.3	2.7-5.7
Coastal Bermuda grass	25	35.7	6.4
Switch grass	45	31.4	12
Orchard grass (medium maturity)	32	40	4.7
Grasses (average values for grasses)	25-40	25-50	10-30

(Source: Howard R.L., 2003; Sun, Y. and Cheng, J., 2002)

NA, data not available

1.2 Sorghum

Sorghum *(Sorghum bicolor* (L.) Moench) is a cane-like plant with high sugar content. Stem is rich in sugar and juice which Brix between 15% and 23%. It is a high photosynthetic efficiency, with high biomass yield crop and it is an interesting annual plant because it can adapt to a wide range of climate from the tropics to cool temperate areas. It is also drought tolerant and has waterlogging resistant (resistant to flood land; soaked in the flood for one week, sorghum can quickly return to growth after flood), salinity resistant (between 0.5% and 0.9%, higher than maize, wheat and rice) and alkalinity resistance properties (Li, S.Z. and Halbrendt, C.C., 2009). This plant mainly composed of soluble (glucose and sucrose) and insoluble carbohydrates (cellulose and hemicellulose). It is an interesting renewable potential particularly important energy plant for the production of fuel biobased chemical production. Soluble carbohydrates are easily converted to organic acid, while insoluble carbohydrates (cellulose and hemicellulose) conversion to succinic acid involves acid or enzymatic hydrolysis of the biopolymer to soluble oligosaccharides followed by their fermentation to succinic acid (James *et al.* 2007). Moreover, the juice from its stalks also contains many essential trace elements for microbial growth and ethanol production (Laopaiboon et al. 2009). An alternative approach has been the direct process in which one or more microorganisms carry out simultaneous saccharification and fermentation (SSF) of cellulose to ethanol in the same bioreactor. In Thailand, sorghum can be planted in all regions especially in the central region, with currently available cultivars, yields of 3,500 to 4,000 L of ethanol per hectare of sorghum can be obtained from fermentable sugars (Nuanpeng, S. *et al.*, 2011).

Sweet sorghum growth period of sorghum is about 4 months and water requirements (8000 m^3 over two crops) are 4 times lower than those of sugarcane (12 to 16 months and 36000 m^3 $crop^{-1}$ respectively. Cost of cultivation of sweet sorghum is 3 times lower than sugarcane. Seed propagation is suitable for mechanized crop production. The succinic acid production process from sorghum is eco-friendly compared to that from molasses burning quality is superior-less sulphur than from sugarcane and high octane rating.

Agricultural straw is a good source for fermentable sugars despite of its low digestibility. After pretreatment with dilute acid, alkali or steam explosion, it can be enzymatically saccharified to fermentable sugars that are mainly a mixture of glucose and xylose. Therefore agricultural straw can also serve as an attractive low-cost feedstock for producing bio-based chemicals such as succinate, hydrogen, or other higher value products.

However, the use of sweet sorghum straw hydrolyzed as carbon source for the fermentative production of succinic acid by *A. succinogenes* has not been reported yet. It has been demonstrated that *A. succinogenes* CGMCC1593 could utilize various carbon sources including xylose with yeast extract as complex nitrogen source to produce succinic acid (Liu et al., 2008).

1.3 Succinic acid production from biomass

The main process of succinic acid production from lignocellulose was presented in Figure 8.

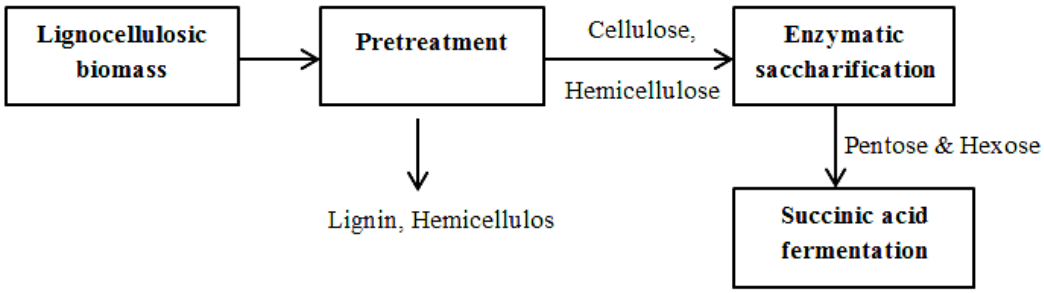


Figure 8 Generalized process of bioconversion of lignocellulose into succinic acid

Bioconversion of lignocellulosic residues to succinic acid, higher value products normally requires multi-step processes, which include:

(1) Pretreatment (mechanical, chemical or biological)

(2) Enzyme saccharification of the pretreated lignocellusic material to liberate readily fermentable sugars (e. g. hexose or pentose sugars)

(3) Monosugars utilization to support microbial growth for succinic acid production

Of the three components, lignin is the most recalcitrant to degradation whereas cellulose, with its highly ordered crystalline structure, is more resistant to hydrolysis than hemicellulose. Also the presence of lignin and hemicellulose make the accessibility of cellulase enzymes and acids to cellulose more difficult, thus reducing the efficiency of the hydrolysis process. Pretreatment is required to alter the size and structure of the biomass, as well as its chemical composition, so that the hydrolysis of the carbohydrate fraction to monomeric sugars can be achieved rapidly and with greater yields. The hydrolysis process

can be significantly improved by removal of lignin and hemicellulose, reduction of cellulose crystallinity, increament of porosity through pretreatment processes. In the hydrolysis process, the sugars are released by breaking down the carbohydrate chains the six carbon sugars or hexoses; glucose, galactose and mannose are readily fermented to succinic acid by microbial action. Details of each step are explained in the following content.

1.4 Pretreatment of lignocellulosic biomass

Pretreatment is a crucial process step for the biochemical conversion of lignocellulosic biomass into biosuccinate. It is required to alter the structure of cellulosic biomass to make cellulose more accessible to the enzymes that convert the carbohydrate polymers into fermentable sugars. The goals of the pretreatment process is to modification and remove of lignin structure, depolymerization and remove hemicellulose, disruption of the crystallinity of cellulose, removing acetyl groups from hemicelluloses and increase of the surface and the porosity of the lignocellulosic material (Figure 9).

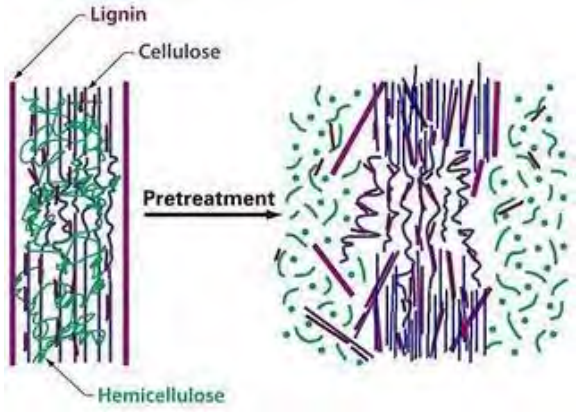


Figure 9 Schematic presentations of effects of pretreatment on lignocellulosic biomass (Source: Hsu, et al, 1980)

The pretreatment must meet the following requirements;

1) To improve the formation of sugars or the ability to subsequently of sugars by hydrolysis

2) To avoid the degradation or loss of carbohydrate (cellulose and hemicellulose)

3) To avoid the formation of inhibitors to the subsequent hydrolysis product that inhibits enzymatic saccharification and fermentation process (Sánchez, C., 2009)

4) An economical for pretreatment should use inexpensive chemicals and require simple process and equipment

However, these are actually the most important challenges of current pretreatment technologies. Pretreatment methods can be classified into 4 methods into different categories as shown in Figure 10.

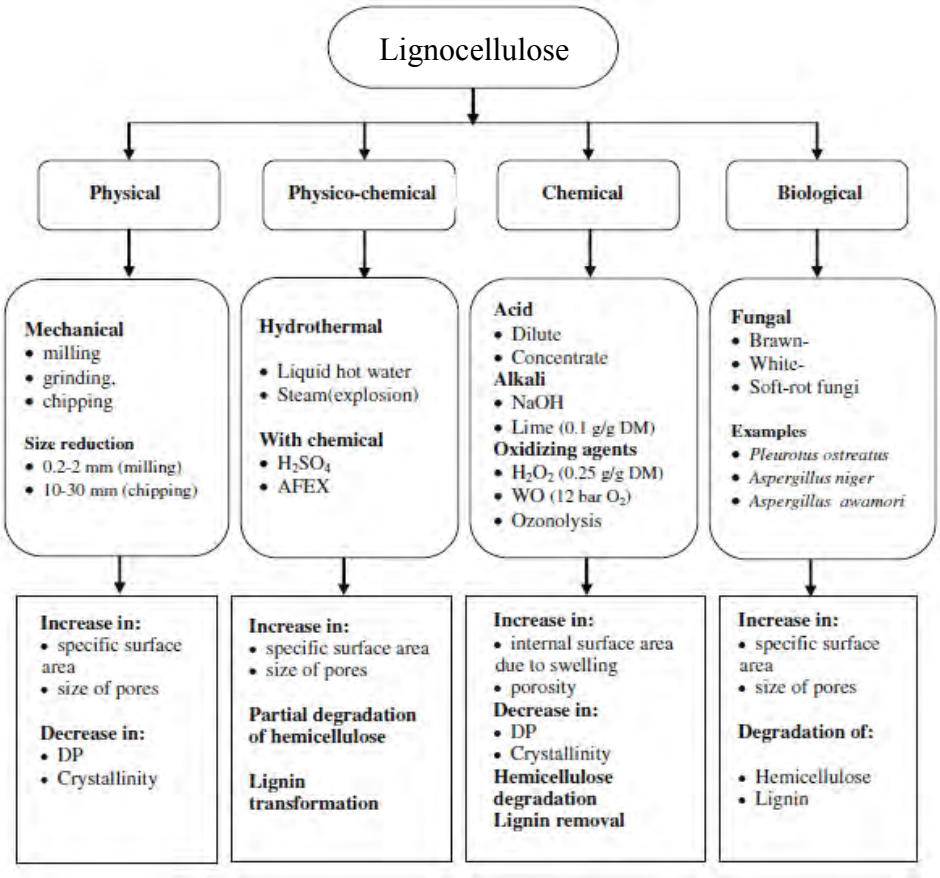


Figure 10 The most common pretreatment methods used on lignocellulose and their possible effects (DP, degree of polymerization; WO, wet oxidation)
(Source: Talebnia, F. *et al.,* 2010)

Among all these methods, the applied methods usually use combination of these methods, such as mechanical pretreatment together with chemical pretreatment effects in order to achieve high sugar release efficiencies, low toxicants production, and low energy consumption (Talebnia et al., 2010).

Dilute-acid hydrolysis has been successfully developed for pretreatment of lignocellulosic materials (Kumar, P. et al., 2009). In general there are two types of dilute acid hydrolysis; a high temperature and continuous flow process for low solids loading (T>160°C, 5-10 w/w% substrate concentration) and low temperature and batch process for high solids

loading (T≤160 °C, 10-40% substrate concentration). Dilute (mostly sulfuric) acid is sprayed onto the raw material and the mixture is held at 160-220 °C for short periods up to a few minutes. Sulfuric acid at concentrations usually below 4 %w/v has been of the most interest in such studies as it is inexpensive and effective. Hydrolysis of hemicellulose then occurs, releasing monomeric sugars and soluble oligomers from the cell wall matrix into the hydrolysate. The dilute H_2SO_4 pretreatment can achieve high reaction rates and significantly improve cellulose hydrolysis (Kumar, P. et al., 2009). Hemicellulose removal increases porosity and improves enzymatic digestibility with maximum enzymatic digestibility usually coinciding with complete hemicellulose removal to almost 100% for complete hemicelluloses hydrolysis (Sun, Y. and Cheng, J. 2002). As an alternative to inorganic acids, organic acids (e.g. maleic acid, fumaric acid) can be used for dilute acid pretreatment. The treatment offers good performance in terms of recovering hemicellulose sugars but there are also some drawbacks. The hemicellulose sugars might be further degraded to furfural and hydroxymethyl furfural, strong inhibitors to microbial fermentation. Furthermore, acids can be corrosive and neutralization results in the formation of solid waste. This method is suitable for biomass with low lignin content, as no lignin is removed from the biomass (Harmsen, P.F.H., 2010), while biological pretreatments are low energy requirement and mild operation conditions. The microorganisms such as white, brown and soft rot fungi that belong to class basidiomycetes are used to degrade lignin and hemicellulose in waste materials. Brown rots mainly attack cellulose, whereas white and soft rots attack both cellulose and lignin. Lignin degradation by white rot fungi occurs through the action of lignin degrading enzymes such as peroxidases and laccase. Nevertheless, the rate of biological hydrolysis is usually very low, so this pretreatment requires long residence times, the organisms predominantly responsible for lignocellulose degradation are fungi, and the most rapid degraders in this group are basidiomycetes (Sun and Cheng, 2002; Harmsen, P.F.H., 2010). The ability to degrade lignocellulose is thought to be associated with a mycelial growth habit that allows the fungus to transport lack nutrients such as nitrogen and iron, to a distance into the nutrient poor lignocellulosic substrate that constitutes its carbon source.

Several microorganisms, mainly fungi, have been isolated and identified as lignocellulolytic organisms. The most widely studied white-rot organism is *Phanerochaete chrysosporium*, which is one of the holobasidiomycetes. *Trichoderma reesei* and its mutants are the most studied ascomycete fungi, and are used for the commercial production of hemicellulase and cellulase (Esterbauer et al., 1991). Not even white rot fungi

are known to be capable of using lignin as a sole carbon and energy source and it is generally believed that lignin break down is necessary to gain access to cellulose and hemicellulose. Although white rot basidiomycetes have been shown to efficiently mineralize lignin, species differ gross morphological patterns of decay they cause *Phanerochaete chrysosporium* strains simultaneously degrade cellulose, hemicellulose and lignin. Brown rot mechanism has likely evolved independently multiple times from white rot decay fungi. Presumably, lignin breakdown is energetically unfavourable, selection has favoured a mechanismwhich can specifically attack the cellulose and hemicellulose components (Sánchez, C., 2009). White rot fungi seems to be the most effective microorganism for biological pretreatment of lignocellulosic materials (Kumar et al., 2009). The important microbial enzymes for lignocellulose hydrolysis are shown in Table 2 (Alper and Stephanopoulos, G., 2007).

Table 2 Important enzymes for hydrolysis lignocellulose

Enzyme type	Function	Typical sources
Cellobiohydrolase	Solubilizes crystalline cellulose	Fungal systems (especially *Trichoderma* and *Aspergillus* spp.)
Endoglucanase	Hydrolyses the β-(1,4) glycosidic bonds in cellulose	Fungal systems (especially *Trichoderma* and *Aspergillus* spp.)
β-glucosidase	Hydrolyses β-linked disaccharides into monosaccharides	Fungal systems (especially *Trichoderma* and *Aspergillus* spp.)
Xylanase	Hydrolyses β-1,4-xylan into xylose	Fungal systems (especially *Trichoderma* and *Aspergillus* spp.)
Lignin peroxidase	Oxidizes lignin molecules through an H_2O_2 donor	White rot and brown rot fungi
Laccase	Oxidizes phenol groups	White rot fungi

(Source: Alper, H. and Stephanopoulos, G., 2009)

Biological pretreatment offers some conceptually important advantages such as low chemical and energy used in pretreatment process. However, most of these processes are too slow limiting its application at industrial level (Sánchez et al., 2009). Chemical pretreatments have serious disadvantages in terms of the requirement for specialized corrosion resistant equipment, extensive washing, and proper disposal of chemical wastes. Biological pretreatment is a safe and environmentally friendly method for lignin removal from lignocellulose. The advantages and disadvantages of various pretreatment methods are also summarized in Table 3.

The choice of the pretreatment technology used for a particular biomass depends on its composition and the byproducts produced as a result of pretreatment. These factors significantly affect the costs associated with a pretreatment method. There have been some reports comparing various pretreatment methods for biomass.

Table 3 Comparison of the pretreatment method (Harmsen, P.F.H., 2010: Kumar, P. *et a*l., 2009)

Pretreatment	Mode of action (in addition to in-creasing the surface area)	Potential sugar yield	Inhibitor formation	Residue formation	Need for re-cycling chemicals	Low in-vestment costs	Low opera-tional costs	Applicable to various biomass	Proven at pilot scale	Additional remark
Mechanical	Reduces cellulose crystallinity	--	++	++	++	+	-	+	+	Power consumption usually higher thaninherent biomass energy
Liquid hot water	Removal of hemicellulose	++	-	++	++	+			++	
Weak acid	Removal of hemicellulose (major) Alteration lignin structure (minor)	++	-	-	-	+/-	+	+	++	Specially suitable for biomass with low lignin content
Strong acid	Hydrolysis of cellulose and hemi-cellulose to glucose, xylose and other sugars; alters lignin structure	++	-	-	-	-	+/-	++	++	High cost; equipment corrosion; hazardous, toxic and corrosive.
Alkaline	Removal of lignin (major) and hemicellulose (minor)	++	++	-	-	++		+/-	+/-	Long residence times required; irrecoverable salts formed and incorporated into biomass
Organosolv	Removal of lignin (major) Removal of hemicellulose (mi-nor), depending on solvent used	++	++	+	-	-	-	+	++	High quality lignin. Solvent used may be inhibitor for cell growth
Wet oxidation	Removal of lignin (major) Dissolve hemicelluloses Decrystallization cellulose	+/-	++	+	++	+			-	Tank requirement, pressure, temperature, cost of oxygen
Steam explosion	Removal hemicellulose (major) Alteration lignin structure (minor)	+	-	+	++	+	+	+/-	++	Low environ-mental impact
AFEX	Removal of lignin (major) and hemicellulose (minor) Decrystallization cellulose	++	++		-			-		No need for small particle size for efficacy
CO_2 explosion	Removal of hemicellulose Decrystallization cellulose	+	+	++	++	-			-	More cost effec-tive than AFEX
Ozonolysis	Reduces lignin content; does not produce toxic residues	+	+	+	-	-	-	+	-	Large amount of ozone required; expensive
Combined mechanical/ Alkaline	Removal of lignin (major) and hemicellulose (minor)	++	++	-	-	+/-	+/-	+	+	
Biological	Degrades lignin and hemicelluloses; low energy requirements	--	++	++	+	+/-	+/-	-	-	rate of hydrolysis is very low

+ = positive characteristic: E.g. high yield of fermentable sugars, no or low fermentation inhibitors, no residue formation, no or low need for recycling of chemicals, low investment costs, high applicability to different biomass types, proven at pilot scale, low operational costs

- = negative characteristic: E.g. low yield of fermentable sugars, high amount of fermentation inhibitors, high residue formation, need for recycling of chemicals, high investment costs, low applicability to different biomass types, not (yet) proven at pilot scale, high operational costs

1.5 Enzymatic saccharification of the pretreated lignocellulosic biomass

Cellulase can be divided into three major enzyme activity classes. These are endoglucanases or endo-1, 4-β-glucanase (EC3.2.1.4), cellobiohydrolase or exoglucanase (EC 3.2.1.91) and β-glucosidase (EC3.2.1.21).

1.5.1 Endoglucanases (EC 3.2.1.4)

Endoglucanases (EG), often called carboxymethylcellulase (because of the artificial substrate used for their detection), are thought to initiate attack randomly at multiple internal glycosidic bonds within the amorphous regions of the cellulose yielding glucose and cello-oligo-saccharides. Endoglucanases typically contain a relatively low amount of carbohydrate ranging from 1 to 12% Isoelectric points are usually acidic, between 2.6 and 4.9 (Wen, Z. *et al.,* 2005).

1.5.2 Exoglucanase (EC 3.2.1.91)

Exoglucanase, often called cellobiohydrolase, is the major component of the fungal cellulase system accounting for 40–70% of the total cellulase proteins, and can hydrolyze highly crystalline cellulose (Malherbe, S. and Cloete, T.E., 2002). Cellobiohydrolases which cuts the cellulose chain from either the reducing or non-reducing end, releasing either "cellobiose" and "cellooligosaccharides" or glucose as major product. The enzymes are monomeric with molecular masses typically between 50 and 65 kDa and the isoelectric points are acidic, typically between 3.6 and 4.9 (Wen, Z. *et al*., 2005).

1.5.3 β-glucosidase (EC 3.2.1.21)

β-glucosidase hydrolyzes glucose dimers (Malherbe, S. and Cloete, T.E., 2002) which cleaves the cellodextrins and cellobiose units to liberate glucose the end product (Jeya, M. et al., 2009). The enzymes are monomeric with molecular masses typically between 50 and 65 kDa and the isoelectric points are acidic, typically between 3.6 and 4.9 (Wen, Z. et al., 2005).

Generally, the endoglucanases and cellobiohydrolases work synergistically in the hydrolysis of cellulose but the details of the mechanisms involved in the process are still unknown (Rabinovich et al., 2002). The efficient hydrolysis of cellulose requires the concerted action of at least three enzymes that are required for the complete breakdown of cellulose to simple sugars (Kumar, P. et al., 2009) (Fig. 11).

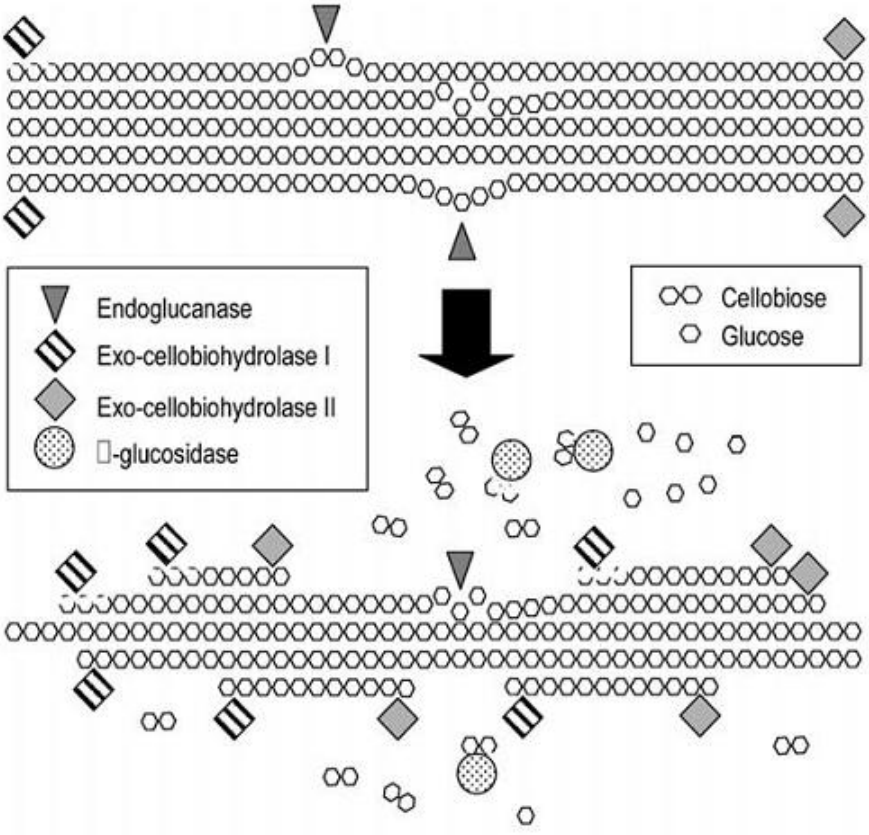


Figure 11 A simplified schematic representation of the process involved in complete enzymatic saccharification of a cellulose microfibril (Source: Malherbe, S. and Cloete, T.E., 2002)

Although the model described in Figure 11 is developed from data obtained from *Trichoderma koningii* and *Phanerochaete chrysosporium*, it does well to describe the general aspects of enzymatic saccharification of cellulose. The concerted actions of these enzymes are required for complete hydrolysis and utilization of cellulose. The rate limiting step is the ability of endoglucanases to reach amorphous regions within the crystalline matrix and create new chain ends, which exo-cellobiohydrolases can attack. Endoglucases attack amorphous regions within the crystalline microstructure, thereby creating new foci for attack by exocellobiohydrolases. Cellobiose dimers are cleaved by β-glucosidases to yield glucose monomers, which can be transported across the membrane to participate in energy generating metabolic reactions (Malherbe, S. and Cloete, T.E., 2002).

1.6 L-glutamic acid production by *Corynebacterium glutamicum*

Corynebacterium glutamicum belong to the family *Corynebacteriaceae*. The phenotypic analysis showed that this organism is an aerobic condition, Gram-positive rod, non-motile and non-sporulating. The process to gain L-glutamic acid with *Corynebacterium*

glutamicum by direct fermentation (Kinoshita et al. 1961) is very well investigated. Until today there is research about some aspects of this topic. Key factors for the cultivation process in order to reach high amounts of L-glutamic acid are the optimal concentration of biotin to influence and support cell growth and the secretion of the product in the extracellular environment (Stansen 2005). Another important factor to prevent side reactions and by-products is the oxygen supply. Under partially anaerobic conditions other additional products like lactic acid could be obtained (Kole et al. 1986).

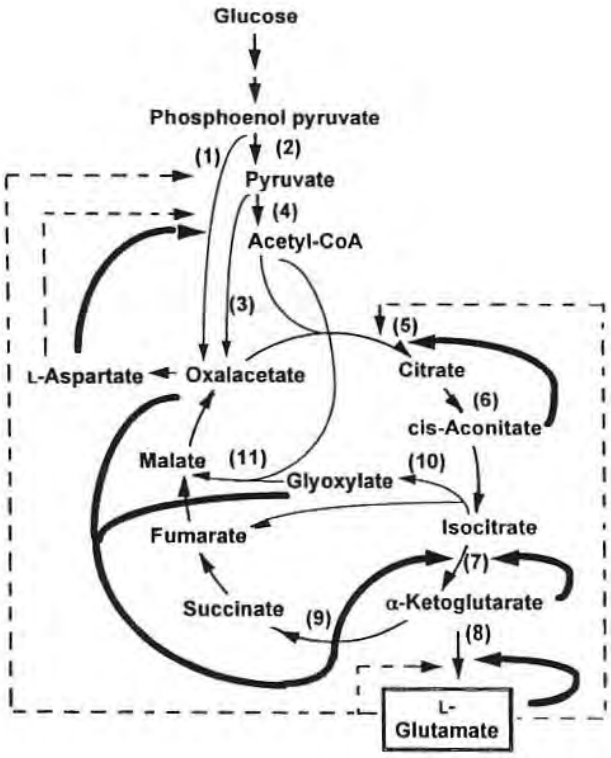


Figure 12 Regulation of L-glutamic acid biosynthesis in *Corynebacterium glutamicum*; straight lines represent feedback inhibition, dashed lines represent feedback repression.

Enzymes in Figure 12 (see numbers) 1: phosphoenolpyruvate carboxylase, 2: pyruvate kinase, 3: pyruvate carboxylase, 4: pyruvate dehydrogenase, 5: citrate synthase, 6: aconitase, 7: isocitrate dehydrogenase, 8: L-glutamate dehydrogenase (GDH), 9: α-ketoglutarate dehydrogenase (KDH), 10: isocitrate lyase, 11: malate synthethase

The most important factor for L-glutamate overproduction is the activity of the enzymes GDH and KDH (see Figure 12). In overproducers the conversion velocity of α-ketoglutarate to L-glutamic acid with GDH is 150 times higher than the side reaction of the substrate with KDH which leads back to the citric acid cycle (Shiio et al. 1980). The

problems in modifying metabolic fluxes in desired directions are quite obvious due to the complexicity and various connections in these metabolic cycles (Braunschweig 2008).

1.7 Succinic acid production

Succinic acid, an important four-carbon platform chemical, is mostly being produced by chemical processes using liquefied petroleum gas or petroleum oil as a starting material. However, it has been widely accepted that the current petroleum-based processes will be soon replaced by fermentation of renewable resources due to the limited nature of petroleum reserves and growing environmental concerns (Song and Lee, 2006)

Since Robert Knock, the Nobel Prize winner, proved that succinic acid has a positive influence on human metabolism and there is no risk of its accumulation in the human body, it has been used in food industries. Succinic acid is an intermediate in the tricarboxylic acid (TCA) cycle and is one of the essential metabolites found in living organisms. Thus, it is synthesized in almost all microbial, plant and animal cells. Those organisms suitable for the efficient production of succinic acid can be categorized into fungi and bacteria. Many researchers have made tremendous efforts to develop a biological process for the production of succinic acid by employing fungi such as *Aspergillus niger*, *Aspergillus fumigatus*, *Fusarium spp., Byssochlamys nivea*, *Lentinus degener*, *Paecilomyces varioti*, *Penicillium viniferum* and *Penicillium simplicissimum* are known to excrete the acid (Magnuson *et al.* 2004). Optimised yeast succinate production systems are rarely *Saccharomyces cerevisiae*. These organisms produce succinic acid as a metabolic byproduct under aerobic and/or anaerobic conditions. *S. cerevisiae* has been best studied among them to achieve high concentration of succinic acid in the manufacture of wine (Wakai *et al.* 1980). A series of its mutant strains were developed by the inactivation of undesired genes, and some of them showed the increased levels of succinic acid compared with the wild type strain (Arikawa *et al.* 1999). *A. niger* has been recognized as a very important organism for the production of various organic acids, especially citric acid and gluconic acid. This organism produces more than 78 g/l of citric acid with the yield of 65% (w/w) on sucrose (McIntyre *et al.* 1997). Furthermore, it shows an ability to utilize various carbon sources with a good yield (115%, w/w) on rapeseed oil (Elimer E. 1998). Recently, the central carbon metabolism of this organism and its metabolic network were deciphered by combining genomic, biochemical and physiological information. Based on them, a stoichiometric model composed of 284 metabolites and 335 reactions was on structed. Simulation of this stoichiometric model

suggested that this organism can produce 1.5 mol succinic acid from 1 mol glucose under microaerobic condition (David *et al.* 2003). However, the use of fungi has been mostly limited to the manufacture of food and beverages due to the difficulties in fermentation, separation and purification as well as low productivities. Succinic acid is generated by a number of anaerobic bacteria, including *Anaerobiospirillum succiniciproducens, Propionibacterium sp., Escherichia coli, Pectinatus sp., Bacteroides sp., Ruminococcus flavefaciens, Actinobacillus succinogenes, Bacteroides amylophilus, Prevotella ruminicola, Succcinimonas amylolytica, Succinivibrio dextrinisolvens, Wolinella succinogenes,* and *Cytophaga succinicans*. Among these strains, *A. Succiniciproducens* and *A. succinogenes* are known as the most efficient succinic acid-producing strains (Hong, 2007). Only few Gram-positive bacteria like *Corynebacterium glutamicum* and *Enterococcus faecalis* have been studied for succinic acid production. Several engineered *C. glutamicum* strains were created by disruption and replacement of genes, and their optimal culture conditions were developed. It was possible to increase the succinic acid production rate seven times and the glucose consumption rate five times under oxygen deprived condition (Inui *et al.* 2004). The metabolic pathways leading to the synthesis of succinic acid are diverse. Some bacteria mainly utilize the phosphoenolpyruvate (PEP) carboxylation reaction, while others use multiple pathways to form succinic acid. Many different succinic acid producing Gram-negative bacteria have been isolated in various anaerobic environments such as domestic sludge, cattle waste, rice paddy, marine shipworm, mouth of dog, rumen and gastro-intestines (Van der Werf *et al.* 1997). Most bacteria, which produce succinate naturally in significant titers, have been isolated in the rumen of ruminants. The anaerobic conditions, caused by carbon dioxide, methane and traces of hydrogen production, create the unique environment for microbial succinic acid production (Kamra 2005). *A.succinogenes, A. succiniciproducens, M. succiniciproducens* and *B. fragilis* are natural succinate producing strains, which all have been isolated in the rumen. They produce a mixture of volatile organic acids and as capnophiles they can cope with high carbon dioxide and use it as a carbon source together with sugars. In some cases carbon dioxide is essential for growth and adapted screening methods have to be employed to isolate novel capnophilic strains (Ueda *et al.* 2008). Most probably these efforts will lead to many more isolates that efficiently produce succinate. In Table 5 gave a comprehensive overview of the many succinate producing strains described in literature.

Table 4 Overview of the yields, rates and titers for different succinate production processes described in literature. (Song and Lee 2006)

Strain	Fermentation strategy/medium	Y [g/g glc]	q_{succ} [g/g CDW/h][d]	r_{succ} [g/l/h]	Titer [g/l][c]	Time/D [h/h^{-1}]	By-products[b]
A. succinogenes							
FZ6	an; B; Csl, Ye, Na_2CO_3	0.94	N.D.	1.01	63.7	62.8	Fo, Py, Pr, Ac
130Z	an; B; Csl, Ye, Na_2CO_3	0.79	N.D.	1.56	29–39	39–79	Fo, Py, Ac
FZ53	an; B; Glc, Csl, Ye, $MgCO_3$	0.82	N.D.	1.36	105.8	78	Fo, Py, Pr, Ac
130Z	an; B; Glc, Csl, Ye, Ac, $MgCO_3$	0.87	N.D.	0.18	17.4	96	Fo, Py, Ac
130Z	an; rB; Glc, Csl, Ye, Ac,	0.86	N.D.	0.88	33.9	38.5	Fo, Py, Ac
130Z	an; B; Glc, Def, $NaHCO_3$	0.46	0.47	0.28	4.2	15	Fo, Ac, Et
130Z	an; B; Glc, Def, $NaHCO_3$	0.5	0.19	0.3	4.1	15	Fo, Ac, Et
130Z	an; B; Glc, Ye, CO_2, $NaHCO_3$	0.62	0.3	1.35	33.8	30	Fo, Ac
CGMCC1593	an; B; Mo, Ye, CO_2, Na_2CO_3	0.79	N.D.	0.97	46.4	48	Fo, Ac
CGMCC1593	an; F; Mo, Ye, CO_2, Na_2CO_3	0.94	N.D.	1.15	55.2	48	Fo, Ac
CGMCC1593	an; F; Glc, Ye, CO_2, Na_2CO_3	0.75	N.D.	1.3	60.2	46.3	Fo, Ac
130Z	an; B; Wh, Ye, CO_2	0.57	N.D.	0.58	50	48	Fo, Ac
130Z	an; B; Whe, CO_2, $MgCO_3$	0.81	N.D.	1.19	64.2	65	Fo, Ac
CGMCC1593	an; B; CS, CO_2, $MgCO_3$	0.81	N.D.	0.95	45.5	48	Fo, Ac
CGMCC1593	an; F; CS, CO_2, $MgCO_3$	0.82	N.D.	1.21	53.2	44	Fo, Ac
A. succiniciproducens							
ATCC 29305[a]	an; B; Glc, Csl, CO_2, Na_2CO_3	0.79	N.D.	0.79	15.9	20	Fo, Ac, Et, La
ATCC 53488	an; B; Glc, Csl, CO_2, Na_2CO_3	0.91	N.D.	1.93	43.5	22.5	Fo, Ac
ATCC 53488	an; B; Glc, P, Ye, CO_2, Na_2CO_3	0.88	N.D.	0.87	33.2	38	Fo, La, Ac
ATCC 53488	an; 2sC; Glc, Csl, CO_2, Na_2CO_3	0.85	N.D.	2.03	39.1	D = 0.4–0.1 h^{-1}	Fo, Ac
FA-10	an; B; Glc, Csl, CO_2, Na_2CO_3	0.66	N.D.	0.77	34.1	44.5	Fo, Ac, Py
FA-10	an; B; Glc, Ye, P, CO_2, Na_2CO_3	0.7	N.D.	0.78	31.6	40.3	Fo, Ac
ATCC 53488	an; B; Glc, Ye, P, CO_2, Na_2CO_3	0.99	1.5	1.2	32.2	27	Fo, Ac
ATCC 53488	an; B; Glc, Ye, P, CO_2/H_2,	0.86	0.45	1.8	34.4	19	Ac
ATCC 53488	an; B; Wh, Csl, CO_2, Na_2CO_3	0.84	N.D.	N.D.	34.3	N.D.	Fo, La, Ac
ATCC 53488	an; F; Wh, Csl, CO_2, Na_2CO_3	0.91	N.D.	0.96	34.7	36	Fo, La, Ac
ATCC 53488	an; C; Wh, Csl, CO_2, Na_2CO_3	0.64	N.D.	3	19.8	D = 0.15 h^{-1}	Fo, La, Ac
ATCC 53488	an; B; Glc/Gl, P, Ye, CO_2,	0.97	0.29	1.35	29.6	22	Ac
ATCC 53488	an; B; Wo, Csl, CO_2, Na_2CO_3	0.88	0.37	0.74	23.8	32	Ac
ATCC 53488	an; mC; Glc, P, Ye, CO_2,	0.88	1.1	10.4	83	D = 0.93 h^{-1}	Ac
ATCC 53488	an; mC; Glc, P, Ye, CO_2,	0.71	0.51	3.3	14.3	D = 0.20 h^{-1}	Ac
ATCC 53488	an; B; Gal, P, Ye, CO_2, Na_2CO_3	0.87	1.32	1.46	15.3	10.5	Ac
ATCC 53488	an; B; Gal/Glc, P, Ye, CO_2,	0.87	3	0.97	14.7	15	Ac
M. succiniciproducens							
MBEL55E	an; B; Glc, P, Ye, CO_2	0.70	0.54	1.87	14	7.5	Fo, La, Ac
MBEL55E	an; B; Wh, Csl, Ye, CO_2	0.72	0.37	1.22	13.5	11	Fo, La, Ac
MBEL55E	an; C; Wh, Csl, Ye, CO_2	0.69	0.45	1.00	12	D = 0.1 h^{-1}	Fo, Ac
MBEL55E	an; C; Wh, Csl, Ye, CO_2	0.60	1.77	3.90	5	D = 0.7 h^{-1}	Fo, La, Ac
MBEL55E	an; B; Wo, Ye, CO_2	0.56	0.58	1.17	11.7	12	Fo, La, Ac
MBEL55E	an; C; Wo, Ye, CO_2	0.60	1.80	1.40	9.5	D = 0.2 h^{-1}	Fo, La, Ac
MBEL55E	an; C; Wo, Ye, CO_2	0.55	6.38	3.19	8	D = 0.4 h^{-1}	Fo, La, Ac
LPK7	an; F; Glc, Ye, CO_2	0.76	0.72	1.80	52.4	30	Py, Ma, Ac, La
MBEL55E	an; B; Glc, Ye, CO_2, $NaHCO_3$	0.59	0.52	1.75	10.5	6	Fo, La, Ac
LPK7	an; C; Glc (9 g/l), Ye, CO_2	0.71	0.64	1.29	12.9	D = 0.1 h^{-1}	Py, Ac, La
LPK7	an; C; Glc (9 g/l), Ye, CO_2	0.29	0.78	1.56	5.2	D = 0.3 h^{-1}	Py, Ac, La
LPK7	an; C; Glc (18 g/l), Ye, CO_2	0.28	0.53	1.07	10.7	D = 0.1 h^{-1}	Py, Ac, La
LPK7	an; C; Glc (18 g/l), Ye, CO_2	0.10	0.52	1.05	3.5	D = 0.3 h^{-1}	Py, Ac, La
LPK7	an; B; Glc, Def, CO_2	0.54	0.53	1.67	10.1	6	Fo, La, Ac
E. coli							
JCL1208pPC201	an; B; Glc, Ye, T, $MgCO_3$	0.29	N.D.	0.59	10.7	18	Fo, Ac, Et, La
NZN111pMEE1	d; B; Glc, Ye, T, CO_2/H_2, $MgCO_3$	0.64	N.D.	0.32	12.8	40	Ac, Et
MG1655/pUC18	an; B; Glc, Ye, T, CO_2	0.42	0.48	0.43	4.2	10	Fo, La
AFP111	d; B; Glc, Csl, CO_2, Na_2CO_3	0.54	N.D.	0.52	51	99	Ac
JCL1242-*pyc*	an; B; Glc, Ye, T, Na_2CO_3, CO_2	0.15	0.17	0.14	1.5	9.8	Fo, Ac, Et, La
NZN111pTrcML	d; F; Glc, Ye, T, CO_2/H_2	0.47	N.D.	0.08	9.4	120	Ma, Ac, Fo, La,
AFP400	an; B; Glc, Ye, T, CO_2, $MgCO_3$	0.61	N.D.	0.61	6.1	35	Fo, Ac, Et, La
AFP111-*pyc*	d; B; Glc, Ye, T, CO_2, Na_2CO_3	1.10	0.13	1.30	99.2	76	Ac, Et
NZN111pTrcML	d; B; So, Ye, T, CO_2	1.10	N.D.	0.13	10	75	Ma, Ac, Et
SS373	d; B; Glc, Ye, CO_2, Na_2CO_3	0.73	N.D.	0.32	11	34	Py
NZN111pTrcMLFu	d; B; Glc, Ye, T, CO_2/H_2	0.35	N.D.	0.06	7	120	Ac, Et
HL27615k	ae; B; Glc, Ye, T, Na_2CO_3	0.45	N.D.	0.16	5	49	Py, Ac
HL27659k-*pepc*	ae; F; Glc, Ye, T, $NaHCO_3$	0.62	0.09	0.72	58.3	59	Py, Ac
HL51276k-*pepc**	ae; B; Glc, Ye, T, $NaHCO_3$	0.71	0.05	0.14	8.3	59	Py, Ac
HL27659k	ae; C; Glc, Ye, T, $NaHCO_3$	0.59	0.20	0.70	7	D = 0.1 h^{-1}	Py, Ac
SBS550MG	an; F; Glc, Ye, T, $NaHCO_3$	1.06	0.21	0.42	40	95	Fo, Ac

Table 5 Overview of the yields, rates and titers for different succinate production processes described in literature (continues).

Strain	Fermentation strategy/medium	Y [g/g glc]	q_{succ} [g/g CDW/h][d]	r_{succ} [g/l/h]	Titer [g/l][c]	Time/D [h/h^{-1}]	By-products[b]
E. coli							
W3110GFA	an; F; Glc, Ye, $NaHCO_3$, CO_2	0.2	N.D.	0.03	2.1	80	Fo, Ac, Et, La
SBS110MG-*pyc*	an; B; Glc, Ye, T, $NaHCO_3$, CO_2	0.9	N.D.	0.65	16	24	Fo, Ac
TUQ19/pQZ6	d; B; Glc, Ye, T, $MgCO_3$, CO_2	0.8	N.D.	0.79	13	16	Ac, Et, La
TUQ2/pQZ6/pQZ5	d; B; Glc, Ye, T, $MgCO_3$, CO_2	0.8	1.2	0.59	12	20	Ac, Et, La
W3110	d; B; Sucr, Ye, T, CO_2, $MgCO_3$	1.2	N.D.	0.81	24	30	N.D.
SBS550 pHL314	an; F; Glc, Ye, T, $NaHCO_3$, CO_2	1.1	N.D.	0.42	40	95	Fo, Ac
AFP184	d; B; Glc, Csl, CO_2	0.8	N.D.	1.27	38	30	Py
AFP184	d; B; Fru, Csl, CO_2	0.7	N.D.	1.01	30	30	Py
AFP184	d; B; Xyl, Csl, CO_2	0.5	N.D.	0.78	23	30	Py
NZN111	d; F; Glc, Ac, $NaHCO_3$, CO_2	0.7	0.2	0.70	28	40	Py, Ac
W3110	d; B; Mo, Csl, CO_2, $MgCO_3$	0.52[e]	N.D.	0.87	26	30	N.D.
KJ060	an; B; Glc, def, $NaHCO_3$, CO_2	0.9	N.D.	0.90	87	120	Ma, Ac, La
KJ073	an; B; Glc, def, $NaHCO_3$, CO_2	0.8	N.D.	0.82	79	96	Ma, Ac, Py
KJ060	an; B; Glc, def, $NaHCO_3$, CO_2	1.1	N.D.	0.61	73	120	Ma, Ac, La, Py
KJ122	an; B; Glc, def, $NaHCO_3$, CO_2	0.9	0.4	0.88	83	93	Ma, Ac, Py
C. glutamicum							
R	µae; rF; Glc, Ye, Cas, $NaHCO_3$	0.19	0.13	3.8	23	6	Ac, La
R Δ*ldhA*-pCRA717	µae; rF; Glc, Ye, Cas, $NaHCO_3$	0.92	0.06	3.17	146	46	Ac, Ma, La, Py
Bacteroides fragalis							
MTCC1045	an; B; Glc, Ye, P, Na_2CO_3, CO_2	0.62	N.D.	0.42	12.5	30	N.D.
MTCC1045	an; B; Glc, Ye, P, Na_2CO_3, CO_2	0.57	N.D.	0.83	20	24	N.D.

Fermentation strategy: an: anaerobic, ae: aerobic, d: dual phase; µae: micro-aerobic; B: batch, rB: repeated-batch, F: fed-batch, rF: fed-batch with cell recycling, C: continuous culture, 2sC: two stage continuous culture (2 different dilution rates), mC: continuous culture with integrated membrane for cell recycling; Medium: Glc: glucose, Gl: glycerol, Sucr: sucrose, Fru: fructose Gal: galactose, So: sorbitol, P: peptone, T: tryptone, Ye: yeast extract, Cas: casamino acids, Csl: corn steep liquor,Wh: Whey, Wo: pretreated wood hydrolysate (extraction of inhibitory compounds), Mo: cane molasse, Whe: wheat hydrolysate, CS: corn stover hydrolysate, Ac: acetate, Def: defined medium; Symbols: D: dilution rate, rsucc: volumetric production rate, qsucc: specific production rate, Y: yield (S = carbon source indicated); By-products: Fo: formic acid, La: lactic acid, Ac: acetic acid, Py: pyruvate, Ma: malate, Pr: propionate, Et: ethanol.

[a] ATCC 29305 has been redeposited as ATCC 53488 in 1992.
[b] In most cases no carbon balance was reported, in some cases the balance does not close, indicating the presence of more by-products.
[c] Some of the titers are low due to low initial carbon source concentration.
[d] Most studies only report OD values, thus the specific production rate could not be calculated (N.D.).
[e] Approximated value based on an average molasse sugar content.

To date, the bacteria isolated from the rumen, including *A. succinogenes* and *M. succiniciproducens*. *Actinobacillus succinogenes* is a capnophilic, facultatively anaerobic, gram-negative bacterium that naturally produces high concentrations of succinate as a fermentation end product in addition to formate, acetate, and ethanol. *A. succinogenes* converts glucose to phosphoenolpyruvate (PEP), at which point metabolism splits into the following two branches: (i) the formate-, acetate- and ethanol-producing C3 pathway, and (ii) the succinate-producing C4 pathway (James *et al.* 2005) (Figure 13).

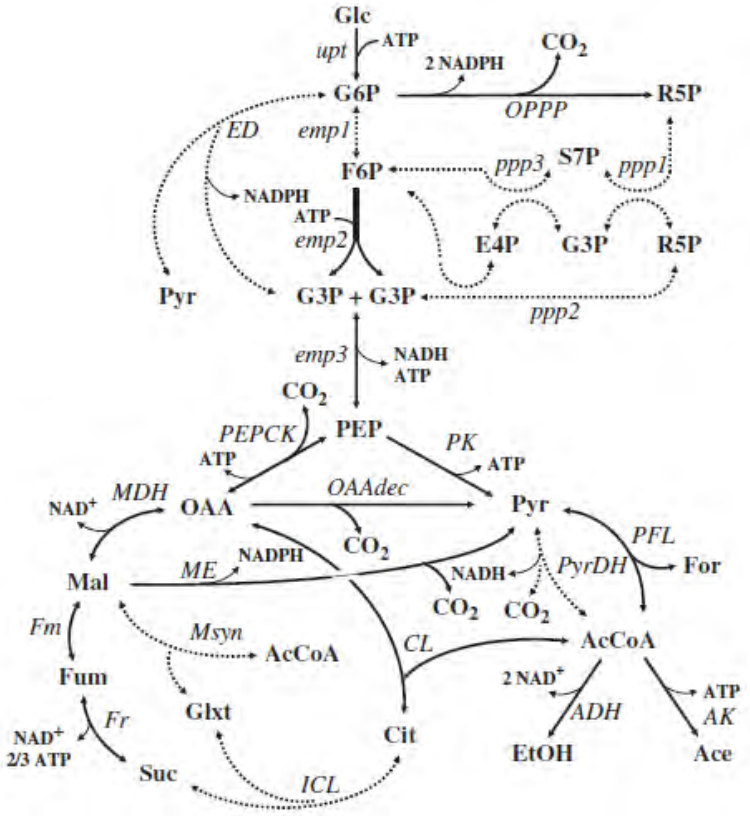


Figure 13 *A. succinogenes* metabolic pathways addressed in this study. Solid lines: pathways or reactions for which enzyme activity was detected in vitro; dotted lines: pathways or reactions where no activity or uncertain activity was detected in vitro (van der Werf *et al.*, 1997). Unidirectional arrows: fluxes considered to be unidirectional (all other fluxes are considered to be reversible). The C4 pathway is defined as: PEPOAA-Mal-Fum-Suc. The C3 pathway is defined as: PEP-Pyr-AcCoA-Ace-EtOH. Alternative PPP reactions, cysteine and methionine degradation pathways, and amino acid synthesis pathways are not shown (Supplementary material). It was assumed that 0.67 ATP is produced per fumarate reductase reaction based on data from Wolinella succinogenes.

Metabolites: AcCoA, acetyl–coenzymeA; Ace, acetate; Cit, citrate; EtOH, ethanol; E4P, erythrose–4– phosphate; For, formate; Fum, fumarate; F6P, fructose–6–phosphate; Glc, glucose; Glxt, glyoxylate; G3P, glyceraldehyde–3–phosphate; G6P, glucose–6–phosphate; Mal, malate; OAA, oxaloacetate; PEP, phosphoenolpyruvate; Pyr, pyruvate; R5P, pentose-phosphates, Suc, succinate; S7P, sedoheptulose–7–phosphate. Pathways and reactions: ADH, alcohol dehydrogenase; AK, acetate kinase; CL, citrate lyase; ED, Entner–Doudoroff pathway; emp1, 2, and 3, Embden–Meyerhoff–Parnas (EMP) or glycolytic reactions; Fm, fumarase; FR, fumarate reductase; ICL, isocitrate lyase and aconitase; MDH, malate

dehydrogenase; ME, malic enzyme; Msyn, malate synthase; OAAdec, oxaloacetate decarboxylase; OPPP, oxidative pentose phosphate pathway; PEPCK, PEP carboxykinase; PFL, pyruvate formate-lyase; PK, pyruvate kinase and PEP:glucose phosphotransferase system (PTS); ppp1 and 2, transketolase; ppp3, transaldolase; PyrDH, pyruvate dehydrogenase or PFL coupled with formate dehydrogenase; upt, glucose phosphorylation by hexokinase and PTS (James *et al.* 2007).

A. succinogenes and *M. succiniciproducens*, are the best candidates for succinic acid production as they produce succinic acid as a major fermentation product. This is most likely due to that the rumen is a highly efficient organ providing an environment to produce succinic acid. Many different succinic acid producing Gram-negative bacteria have been isolated in various anaerobic environments such as domestic sludge, cattle waste, rice paddy, marine shipworm, mouth of dog, rumen and gastro-intestines. To date, the bacteria isolated from the rumen, including *A. succinogenes* and *M. succiniciproducens*, are the best candidates for succinic acid production as they produce succinic acid as a major fermentation product. This is most likely due to that the rumen is a highly efficient organ providing an environment to produce succinic acid. The rumen is a unique microbial ecosystem found in many species of herbivorous mammals known as ruminants. The primary role of the rumen is to allow pre-gastric digestion of various polysaccharide materials, which is mediated by a great diversity of rumen microorganisms, consisting of 109–1010 bacterial, 105–106 protozoan and 103–104 fungal cells ml^{-1} of rumen fluid (Orpin 1984). The production of C4 dicarboxylic acids in the rumen reduces energy loss associated with methanogenesis (30–40 mol% of CH_4 is present in the ruminal gas) by increasing the amount of metabolizable energy available to the animal in the form of propionic acid. Although the C4 dicarboxylic compounds, such as oxaloacetic, malic, fumaric and succinic acids are not detected in the ruminal fluid, large amounts of these acids are produced by CO_2 fixation reactions, using 60–70 mol% of CO_2 present in the ruminal gas. The major C_3 compounds in the cell used for carboxylation reaction are PEP and pyruvate. In particular, succinic acid is converted to propionic acid, which can account for 20% (w/w) of total volatile fatty acids (VFAs) in the rumen, by succinic acid utilizing bacteria such as *Veillonella parvula* (Johns *et al.* 1951), *Selenomonas ruminantium* (Wolin *et al.* 1988) and *Succiniclasticum ruminis* (Van Gylswyk *et al.* 1995). Propionic acid produced this way is absorbed through the rumen wall for subsequent oxidation to provide energy and biosynthetic precursors for the animals.

Therefore, it is reasonable to think that some microorganisms present in the rumen will be a good succinic acid producer.

1.7.1 Actinobacillus succinogenes

A. succinogenes was originally isolated from bovine ruminal contents and belongs to the family *Pasteurellaceae* based on its 16S rRNA sequence analysis. A total of 2115 genes have been identified of which 1768 have a predicted function. In total only 404 genes have been connected to a KEGG pathway, which means a lot of research is still needed to fully understand the biochemistry of this organism (Markowitz 2008). The phenotypic analysis showed that this organism is a facultative anaerobic, capnophilic, mesophilic, non-motile, pleomorphic, and gram-negative rod or occasionally filamentous bacterium. *A. succinogenes* shows a distinctive ability to produce a relatively large amount of succinic acid from a broad range of carbon sources such as arabinose, cellobiose, fructose, galactose, glucose, lactose, maltose, mannitol, mannose, sorbitol, sucrose, xylose or salicin under anaerobic condition (Guettler *et al.* 1996).

Because *A. succinogenes* is capnophilic, it needs carbon dioxide to simulate growth and succinate production. A key enzyme for these organisms is phosphoenolpyruvate carboxykinase (PEPCK), which converts phosphoenolpyruvate with carbon dioxide and ADP into oxaloacetate and ATP. The increase in growth rate is thus linked to the increase in substrate level phosphorylation by this reaction. The difference with non-capnophilic bacteria PEPCK, such as *E. coli* PEPCK, can be found in the kinetic properties of the enzyme. In *E. coli* PEPCK functions as a part of gluconeogenesis, which means that the enzyme has a high affinity for ATP and oxaloacetate, but a low affinity for ADP and carbon dioxide or carbonate. The introduction of an *A. succinogenes* PEPCK in *E. coli* was described by Kim *et al.* and resulted in a 6.5-fold increase of succinate productions in anaerobic, CO_2-rich conditions, proving its difference in function (Beauprez *et al.* 2010).

Unlike *E. coli* or *A. succiniciproducens*, *A. succinogenes* is a moderate osmophile and has good tolerance to a high concentration of glucose, which is beneficial for fermentation. Extensive physiological and genetic studies relating to succinic acid production in *A. succinogenes* have been performed. Five key enzymes responsible for succinic acid production were identified to be PEP carboxykinase (*pck*), malate dehydrogenase (*mdh*), malic enzyme (*sfc*), fumarase (*fum*) and fumarate reductase (*frd*). Also, enzymatic analysis revealed the presence of pyruvate kinase (*pyk*), pyruvate ferredoxin oxidoreductase (*pfo*),

acetate kinase (*ack*), alcohol dehydrogenase (*adh*) and lactate dehydrogenase (*ldh*), which affect succinic acid flux in the central metabolic pathways. PEP carboxylation, which is the important committed step for succinic acid production in rumen bacteria, is strongly regulated by CO_2 levels. Theoretically, 1 mol of CO_2 is required to form 1 mol of succinic acid. The higher CO_2 level resulted in an increased succinic acid production at the expense of ethanol and formic acid. This is most likely due to the increased carboxylation of PEP to oxaloacetate rather than PEP conversion to pyruvate. Also, the addition of extra electron donors including hydrogen and electrically reduced neutral red resulted in the significant increase of succinic acid production. These observations are consistent with that the use of more reduced sugars such as arabitol, mannitol and sorbitol resulted in significant increases in the succinic acid and ethanol production compared with glucose (Van der Werf *et al.* 1997).

Although the variant strains produced less ethanol, acetic, formic and lactic acids, formation of these byproducts could not be completely avoided. Furthermore, the accumulation of propionic and pyruvic acids, which are not generally detected in the cultivation of other succinic acid producing bacteria, was observed. Considering the costs of separation and purification of succinic acid from fermentation broth containing mixed acids, the formation of byproducts should be minimized, or if possible, completely eliminated by metabolic engineering and fermentation process optimization. Although we interest to improve of the performance of *A.succinogenes* metabolite will be produce succinic acid and not produce an end product (James *et al.* 2005). Moreover, *A. succinogenes* can ferment a broad spectrum of carbon sources and these properties allow fermentation of cane molasses, whey and wheat hydrolysates that are much cheaper carbon sources than refined sugar and glucose (Du *et al.* 2008). A disadvantage of the same environment is the richness of different substrates. A lot of vitamins and amino acids are abundant in the rumen, which resulted in the loss of biosynthetic routes making the addition of these vitamins and amino acids to minimal medium necessary(McKinlay *et al.* 2005). The fact that glutamate is an essential amino acid and α-ketoglutarate can be used as a substitute indicates that two essential genes from the TCA cycle are missing or inactive during growth on glucose, isocitrate dehydrogenase and α-ketoglutarate dehydrogenase (Beauprez *et al.* 2010).

Indeed, researchers have screened several succinic acid-producing microorganisms. In order to develop a cost-competitive fermentative succinic acid production process, strain improvement maximizing the production of succinic acid but minimizing the formation of

by-products through rational metabolic engineering is of vital importance. We recently developed an improved succinic acid producer, *A. succinogenes*, by define optimum condition, resulting in much increased succinic acid production while reduced formation of by-products, such as acetic, formic, and lactic acids. In addition, the possibilities of cost-effective succinic acid production by *A. succinogenes* from inexpensive and abundant feedstocks, including lignocellulosic hydrolysates was also investigated (Song and Lee, 2006).

1.8 Succinic acid markets and applications

At present, succinic acid is mostly produced by the chemical process from *n*-butane through maleic anhydride. It is sold at the price of \$5.9 to 9.0 kg^{-1} depending on its purity. Its manufacturing cost is affected by several factors including succinic acid productivity and yield, the costs of raw materials, and recovery method. Particularly, the cost of maleic anhydride has been known to contribute most significantly to the overall cost of succinic acid production.

The overall economics still limits the bio-based succinic acid production also the assessment of raw material cost and the estimation of potential market size clearly suggest that the current petroleumbased succinic acid process will be replaced by the fermentative succinic acid production system in the near future (Li *et al.* 2011).

Succinic acid is a member of the C4-dicarboxylic acid family, and was originally referred to as spirit of amber, as it was classically procured from amber via pulverization and distillation, for use in a treatment for rheumatic aches and pains. Succinic acid is also employed in the preparation of bulk chemicals such as surfactants, ion chelators, and food additives, as well as in the preparation of fine chemicals, including supplements used in pharmaceuticals, antibiotics, and vitamins (Figure 14).

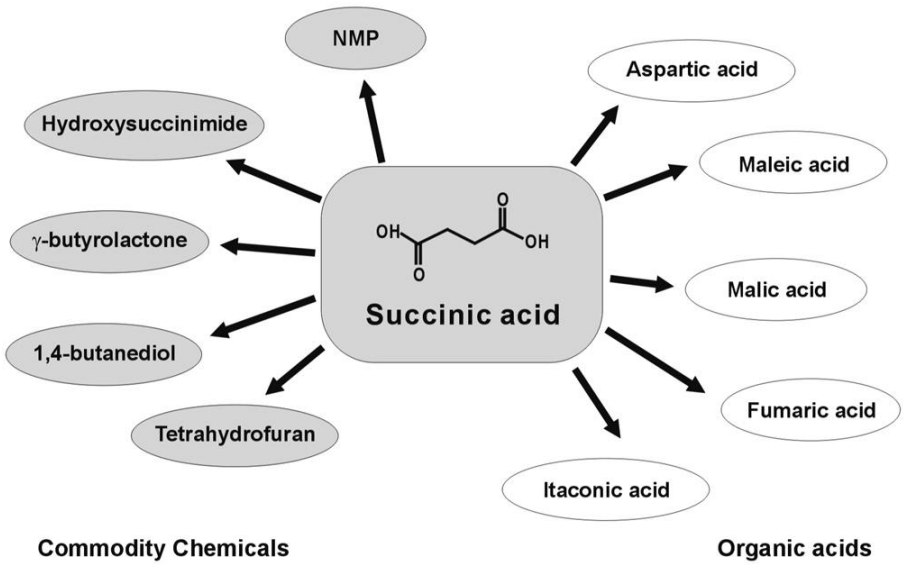


Figure 14 Applications of succinic acid

Succinic acid can be utilized not only s an end product, but also as a precursor for a variety of fine chemicals, including 1, 4-butanediol, tetrahydrofuran, γ-butyrolactone, and biodegradable polymers. This broadspectrum application characteristic makes succinic acid one of the most attractive green chemicals currently available, and has become the subject of significant concern to bioengineers, as well as chemical engineers.

Currently, succinic acid is primarily manufactured via the hydrogenation of maleic anhydride to succinic anhydride, followed by hydration to succinic acid, and only a small quantity of succinic acid is generated via microbial fermentation. However, considering finite petroleum resources and the increasing price of oil, much effort is currently being exerted to develop a system for the biological production of succinic acid and its derivatives, using renewable feedstocks (Zeikus *et al.* 1999). With the development of recombinant DNA technology, metabolic engineering has become the principal paradigm relevant to bioengineering research. Although the enhanced production of some metabolites has been achieved in metabolic engineering studies, many other attempts have failed due to a lack of rational strategies. In order to gain insight into the intracellular metabolic conditions of microorganisms, a variety of computational tools have been developed, including metabolic flux analysis (MFA). MFA allows the calculation of intracellular metabolic fluxes on the basis of the stoichiometry of intracellular reactions and mass balances around the intracellular metabolites (Edwards *et al.* 1999 and Nielsen *et al.* 2003). MFA has been applied to the calculation of the maximum theoretical yield of a desired metabolite to be produced, and to identify the rigidity of branch points within metabolic pathways. Another possible application

for this technology is the identification of alternative metabolic pathways resulting in a desired product (Lee *et al.* 2002) Recently, a global attempt to analyze the complex manners in which metabolic networks respond to environmental changes and artificial gene modification has been initiated, and this effort has been greatly facilitated by the development of high-throughput techniques (Hong, 2007). Biobased chemical production is a growing multibillion dollar industry converting renewable resources into valuable products (Wilke. 1999) A $15 billion market could be based on succinate for producing bulk chemicals such as 1, 4-butanediol (a precursor to "stronger-than-steel" plastics), ethylenediamine disuccinate (a biodegradable chelator), diethyl succinate (a green solvent for replacement of methylene chloride), and adipic acid (nylon precursor) (Zeikus *et al.* 1999). However, the cost of biobased succinate is not yet competitive with petrochemical-based alternatives such as maleic anhydride. The development of a cost-effective industrial succinate fermentation will rely on organisms able to produce high concentrations of succinate at high rates (James *et al.* 2005).

Chapter II

Material and Method

2.1 Sample collection

The samples were collected from buffalo dung in Suphanburi province (SP-I, SP-II), soil sample in Suphanburi province (SP-III) and cow dung from Surin province (SR-I, SRII), Thailand.

2.2 Screening for amino acid producing strain

One gram of sample was added into 3 ml of enrichment broth containing; glucose 20 g/l, K_2HPO_4 2 g/l, NaCl 1 g/l, $MgSO_4.7H_2O$ 0.2 g, yeast extract 0.5 g/l, biotin 0.02 µg/l, thiamine-HCl 4 µg/l, pH 7.0. Sodium azide (2 mg/l) was filter-sterilized and added to the autoclaved-mixture (Asano et al. 1989). Bacteria were then isolated from the positive tubes by subcultured to the enrichment agar plate and incubated at 37 ºC for overnight. The visibly isolated colonies were picked and streaked on fresh agar plates, this is called subculturing. The purpose is to create an agar plate with a "pure culture" of only one bacterial species on it. Then the plates were again incubated overnight at 37 ºC.

Screening agar plates used for the isolation of bacteria containing: glucose 50 g/l, $(NH_4)_2SO_4$ 20 g/l, $(NH_4)_2S_2O_3$ 10 g/l, urea 5 g/l, KH_2PO_4 1 g/l, K_2HPO_4 2 g/l, $MgSO_4 \cdot 7\ H_2O$ 0.25 g/l and agar 15 g/l. pH was adjust to 7.2. The medium was dissolved in distilled water and autoclaved at 121ºC for 15 minutes. A mineral elements contained; $CaCl_2$ 1.0 µg/l, $FeSO_4.7\ H_2O$ 1.0 µg/l, $MnSO_4 \cdot H_2O$ 1.0 µg/l $ZnSO_4 \cdot 7\ H_2O$ 0.1 µg/l, biotin 0.3 µg/l and thiamin 0.2 µg/l. Vitamins and amino acids were prepared by sterile membrane filtration (0.22 m nylon, Millipore Express, Ireland) and added to the medium (Braunschweig 2008).

2.3 Screening for succinic acid producing strain

General anaerobic cultivation techniques were used for the growth of this organism. Strict anaerobic conditions were ensured by using anaerobic pack (MGC, Japan). One gram of sample was added into 3 ml of enrichment broth containing; glucose 20 g/l, polypeptone 5 g/l, yeast extract 5 g/l, K_2HPO_4 3 g/l, NaCl 2 g/l, $(NH_4)_2SO_4$ 2 g/l, $CaCl_2 \cdot 2H_2O$ 0.2 g/l, $MgCl_2 \cdot 6H_2O$ 0.4 g/l, and $MgCO_3$ 15 g/l (Bryant 1972 and Hungate 1966.) Bacteria were then

isolated from the positive tubes by subculturing to the enrichment agar plate and incubated at 37 ºC for overnight. The visibly isolated colonies were picked and streaked on fresh agar plates and incubated overnight at 37 ºC.

Screening agar plates used for the isolation of bacteria containing: glucose 20 g/l, NaCl 1 g/l, Yeast Extract 5 g/l, K_2HPO_4 3 g/l, $(NH_4)_2SO_4$ 1 g/l, $CaCl_2.2H_2O$ 0.2 g/l $MgCl_2.6H_2O$ 0.2 g/l, Na_2CO_3 1 g/l and agar 15 g/l. pH was adjusted to 6.5. The medium was dissolved in distilled water and autoclaved at 121ºC for 15 minutes.

2.4 Determination of succinic acid

2.4.1 Thin layer chromatography (TLC)

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

2.4.2 High-performance liquid chromatography (HPLC)

Before injection into a column, all samples were centrifuged at 12,000 rpm for 15 min and then filtered through a cellulose membrane acetate filter (pore size 0.45 µm). The condition for analysis process was shown below.

Column	Bio-Rad Aminex®HPX-87H (300mm x 7.8mm)
Guard column	Carbo-H micro-guard cartridge
Eluent	5 mmol/L H_2SO_4
Temperature	45 ^oC
Flow rate	0.6 ml/min
Injection volume	20 µl
Detecter	Refrective Index detecter (Shimadzu Model RID-6A)
Retention time	20 min

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

standards (Tartaricacid, Formic acid, Malic acid, Citric acid, Oxalic acid and Succinic acid) (Appendix A).

2.4.3 Gram strain

The positive colonies on the agar plate were checked for the morphological characteristics by Gram stain method.

2.5 Sweet sorghum straw pretreatment

Sweet sorghum straw (SSS) was obtained from The Suphanburi Field Crops Research Center in Thailand. The SSS consisted of 44.51% cellulose, 38.62% hemicellulose, 6.18% lignin and 10.69% ash. Chopped SSS was dried in an oven at 70°C to a constant weight. Thirty grams of chopped SSS was suspended in 300 ml of 3% aqueous H_2SO_4 solution and incubated at 120°C for 10 minutes. After pretreatment, the hydrolyzate was neutralized with 40% NaOH, centrifuged and filtered through 0.45 μm filters before analyzing total reducing sugars with the DNS method and monomeric sugars (glucose, xylose, galactose, arabinose and mannose) by HPLC.

2.6 Succinic acid producing strain

The strain of *Corynebacterium glutamicum DSM20300* obtained from Leibniz Institute DSMZ-German Collection of Microorganisms and Cultures was used as a representative for investigating the succinic acid production from agricultural material.

2.6.1 Stock culture

Corynebacterium glutamicum DSM20300 was grown at 37°C, 200 rpm in Luria–Bertani (LB) broth containing the following constituents: sucrose 20 g/l, peptone 10 g/l, yeast extract 5 g/l, NaCl 5 g/l and autoclaved at 121ºC for 15 minutes then maintained in 30% glycerol at −70◦C. The cultures were also maintained on solid media (15 g/l agar) (James *et al.* 2005).

2.6.2 Stock medium

Cultures were maintained on solid media containing; glucose 5.0 g/l, casein peptone tryptic digest 10 g/l, yeast extract 5 g/l, NaCl 5 g/l and agar 15 g/l. The medium was dissolved in distilled water and adjust pH to 7.2-7.4 with 2N NaOH and 2M HCl. The medium was autoclaved at 121ºC for 15 minutes.

2.6.3 Inoculum medium / Growth medium

Inoculum medium containing: glucose 5 g/l, casein peptone tryptic digest 10 g/l, yeast extract 10 g/l, NaCl 5 g/l, K_2HPO_4 2.5 g/l and adjust pH to 7.1-7.4.The medium was autoclaved at 121ºC for 15 minutes.

2.6.3.1 Effect of various carbon sources on growth

Glucose and SSS hydrolyzed were used as a carbon source in growth medium. They were separately sterilized at 115 °C for 30 min and added to the growth medium to maintain the initial concentrations of 10 g/l. Ten percentage of the preculture was inoculated into 50 ml of growth medium. The cultures were incubated at 37°C with agitation 200 rpm for 0-24 h depending on the carbon source used.

2.6.3.2 Effect of substrate concentration on growth

Various concentration of carbon sources; 5-15 g/l of glucose or 5-15 g/l of total reducing sugars in SSS hydrolyzate was added in the growth medium

2.6.3.3 Effect of different nitrogen sources on growth

Ten percentage of the inoculum was inoculated into 50 ml of growth medium. Nitrogen sources used in the growth medium were shown in Table 6. Glucose was separately sterilized and added to the medium to maintain the initial concentrations of 20 g/l. The initial pH of the medium was adjusted to 7.0.

Table 6 Composition of nitrogen sources in the growth medium

Medium	Yeast extract (g/l)	Peptone (g/l)	$(NH_4)_2SO_4$ (g/l)	Total Nitrogen (g/l)
Formula 1	10	10	0	20
Formula 2	8	8	5	20
Formula 3	5	5	10	20
Formula 4	3	3	15	20
Formula 5	0	0	20	20

2.6.4 Production medium

Production medium containing: sugar 30 g/l, yeast extract 30 g/l, urea 2 g/l, $MgCl_2.6H_2O$ 2 g/l, $CaCl_2$ 1.5 g/l, $MnCl_2$ 0.07 g, Na_2HPO_4 4.4 g/l, NaH_2PO_4 3.3 g/l, $MgCO_3$ 30 g and adjust pH to 7. The medium was autoclaved at 121ºC for 15 minutes. Biotin 0.3µg/l and thiamin 0.2 µg/l were prepared by sterile membrane filtration (0.22 m nylon, Millipore Express, Ireland) and added (Braunschweig 2008).

2.7 Succinic acid production process

The two-phase cultivation was adopted in this study. The first phase was prepared by transferred *C. glutamicum* from inoculum medium to 50 ml of growth medium in a 250-mL flask and incubated for 15 hours under aerobic condition at 37°C with agitation 200 rpm. In the second phase, cell was transferred to production medium using glucose or sugars from SSS hydrolysate as carbon source and incubated under anaerobic condition at 37°C with agitation 200 rpm. Carbon source in the growth and production phase were summarized in Table 7.

Table 7 Carbon source in the growth and production phase

Microorganism	**Carbon sources**	
	Growth phase	**Production phase**
C. glutamicum	- 5 g/l glucose*	- 20 g/l glucose*
	-5 g/l glucose	- 20 g/l of RS from SSS Hydrolyzed
	-5 g/l of RS from SSS Hydrolyzed	- 20 g/l of RS from SSS Hydrolyzed
	-aerobic condition at 37 ◦C, 200 rpm,	-anaerobic condition at 37 ◦C, 200 rpm,

* mean control of the experiment

2.8 Analysis

2.8.1 Cell growth

Cell growth was monitored by measuring the absorbance at 660 nm (OD660) using a spectrophotometer (UV160, Shimadzu Corporation, Japan). Cultures were harvested during the log phase by centrifugation (8000 × g, 10 min at 4◦C), and the cell pellet was washed with sterile water to reduce the carry-over of nutrients and metabolic products. Dry cell weight (DCW) was calculated from a curve relating the OD660 to DCW. An OD660 of 1.0 was equivalent to 1.63 g of CDW/l.

2.8.2 Reducing sugars

The reducing sugars concentration was determined by the 3, 5-dinitrosalicylic acid (DNSA) method applied from Miller (1959), with D-glucose as the standard. In a typical reaction, 50 µl of sample and 150 µl of reagent are mixed and heated in a boiling water bath for 10 min, then cooled immediately on ice bath and added 1 ml of distilled water. At the end of the reaction, the absorbance was measured by spectrophotometer at 540 nm. Concentration of the released glucose was measured from a standard glucose curve (Appendix C).

2.9 Statistical analysis

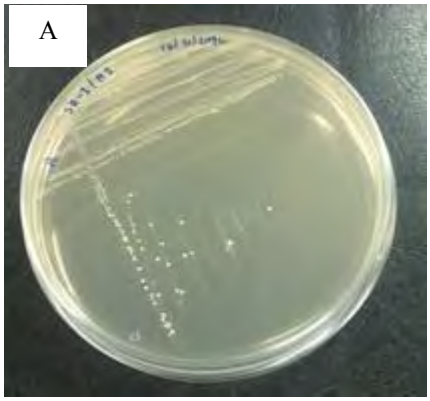
All experiments were carried out in triplicate and the results were shown as means±standard deviations. The experimental data were analyzed using SPSS software with one-way analysis of variance (ANOVA) followed by Tukey's multiple range method test to compare means. Differences in means were judged to be significant when p values for the null hypothesis were 0.05 or less.

Chapter III

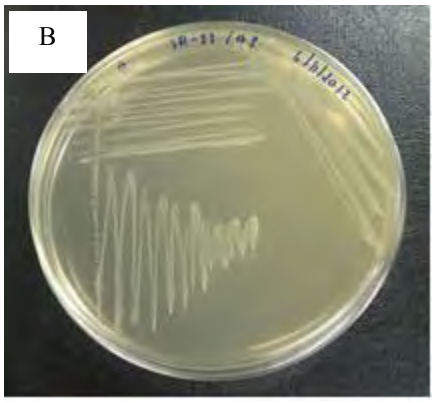
Results and Discussion

3.1 Screening for amino acid producing strain

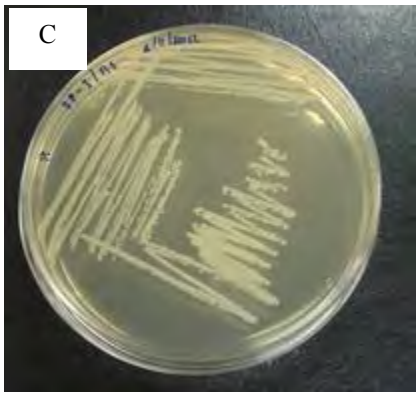
Microorganisms with ability amino acid first screened based on colony characteristics on the screening medium were shown in the Figure 15.



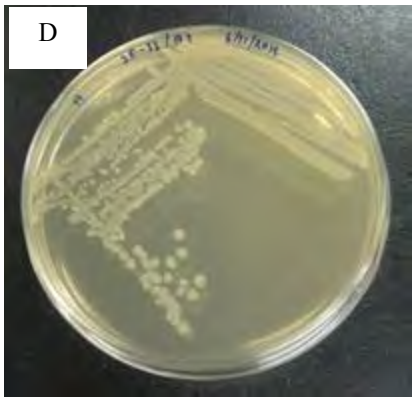
SR-I/A2



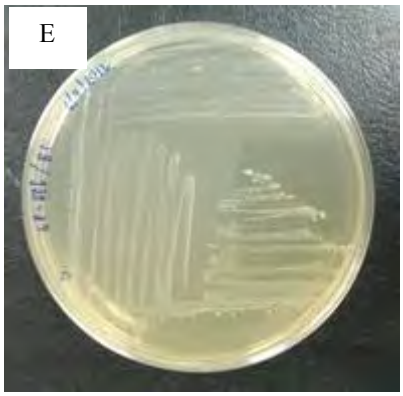
SR-II/A2



SP-I/A1



SP-II/A1



SP-III/B1

Figure 15 The colony characteristics of amino acid producing strain on the screening agar plate: (A) SR-I/A2; (B) SR-II/A2; (C) SP-I/A1; (D) SP-II/A1; (E) SP-III/B1.

3.2 Screening for succinic acid producing strain

Microorganisms with ability succinic acid first screened based on colony characteristics on the screening medium were shown in the Figure 16.

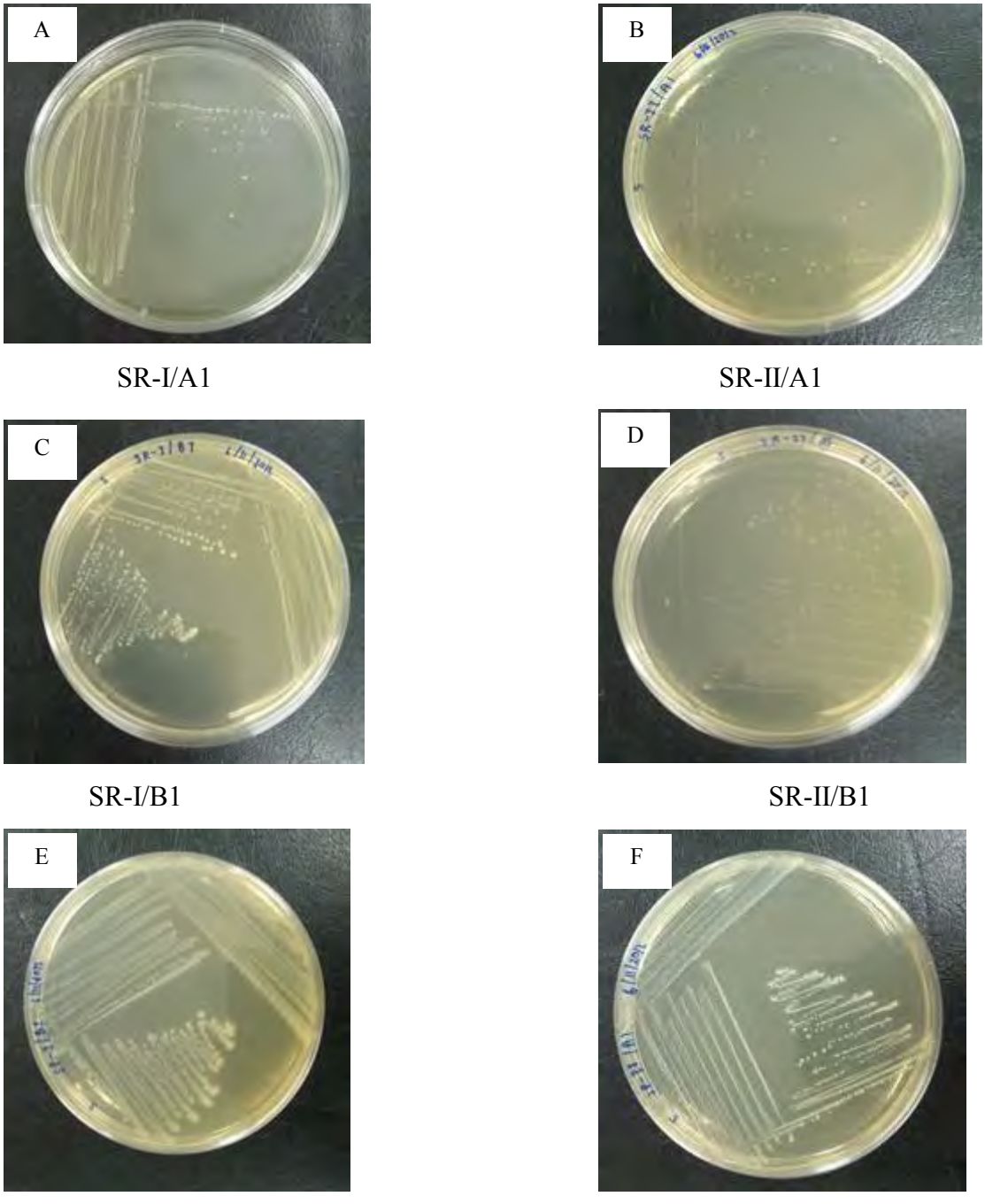


Figure 16 The colony characteristics of succinic acid producing strains on the screening agar plate: (A) SR-I/A1; (B) SR-II/A1; (C) SR-I/B1; (D) SR-II/B1; (E) SP-I/B1; (F) SP-II/A1.

Microorganisms were first screened based on colony characteristics on screening medium and gram staining. These results shown that morphological characteristics colonies appearing on the screening agar plate after 24 h of incubation were circular, smooth and gray with 1–2 mm in diameter. The bacterium was non-spore-forming and gram-negative rod and gram positive rod as shown in Figure 17, Table 8 and Table 9.

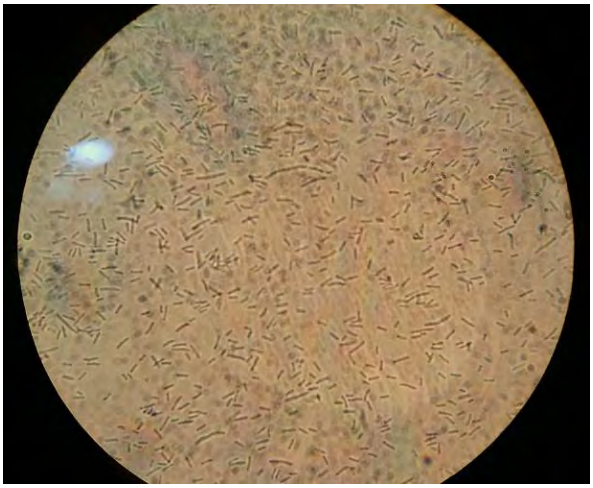


Figure 17 Characteristics of gram positive rod bacteria under a microscope.

Table 8 Preliminary screening for amino acid producing strains

Sample	Gram	Sample	Gram
SR-I/A1	+	SR-II/B3	+
SR-I/A2	+	SP-I/A1	+
SR-I/A3	+	SP-I/A2	+
SR-I/A4	+	SP-I/A3	+
SR-I/A5	+	SP-I/B1	+
SR-I/B1	+	SP-I/B2	-
SR-I/B2	-	SP-I/B3	-
SR-I/B3	-	SP-I/B4	-
SR-I/B4	-	SP-I/B5	+
SR-I/B5	+	SP-I/B6	-
SR-I/B6	+	SP-II/A1	+
SR-I/A.6	-	SP-II/A2	-
SR-II/A1	+	SP-II/A3	+

Table 8 Preliminary screening for amino acid producing strains (continuous)

Sample	Gram	Sample	Gram
SR-II/A2	+	SP-II/B1	+
SR-II/A3	+	SP-II/B2	+
SR-II/B1	+	SP-III/B1	+
SR-II/B2	-		

Table 9 Preliminary screening for succinic acid producing strains

Sample	Gram	Sample	Gram
SR-I/A1	+	SP-I/B1	+
SR-I/A2	+	SP-I/B2	-
SR-I/A3	+	SP-I/B3	-
SR-I/A4	+	SP-I/B4	-
SR-I/A5	+	SP-I/B5	+
SR-I/A6	+	SP-I/B6	+
SR-I/A7	+	SP-II/A1	+
SR-I/B1	-	SP-II/A2	-
SR-I/B2	-	SP-II/A3	+
SR-I/B3	+	SP-II/A4	+
SR-I/B4	+	SP-II/A5	+
SR-I/B.5	+	SP-II/A6	+
SR-II/A1	+	SP-II/B1	+
SR-II/A2	+	SP-II/B2	+
SP-I/A1	+	SP-II/B3	+
SP-I/A2	+	SP-II/B4	+

3.3 Analysis for succinic acid ability

The isolated strains with succinic acid ability were confirmed by Thin layer chromatography (TLC) and High performance liquid chromatography (HPLC) method.

3.3.1 Thin layer chromatography

The TLC method gave clear yellow spots (Fig. 18) with various standard organic acids (succinic acid, lactic, fumaric, malic, citric and α-ketoglutaric acid) with distinct retention factor (Rf) values (Table 10).

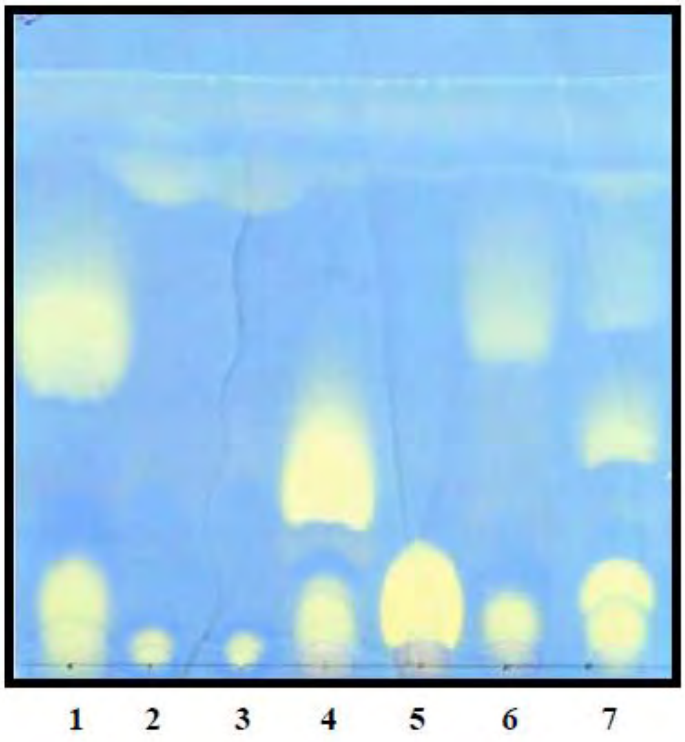


Figure 18 Resolution of different standard organic acids on TLC plate. (1) succinic acid; (2) lactic acid; (3) fumaric acid; (4) malic acid; (5) citric acid; (6) α-ketoglutaric acid; and (7) mixture of these organic acids.

Table 10 Analysis of standard organic acids on TLC plate

Organic acids	Rf values
Succinic acid	0.56

Lactic acid	0.81
Fumaric acid	0.81
Malic acid	0.33
Citric acid	0.16
α-ketoglutaric acid	0.58

Succinic acid was resolved for 30 min and showed a prominent yellow spot with an Rf value of 0.56. Among 32 isolated strains tested, 11 strains were found to produce succinic acid. Results of prominent succinic acid ability were presented in Tables 11.

Table 11 Analysis of isolated strains for succinic acid ability by TLC method

No.	**Sample**	**TLC plate**	**No.**	**Sample**	**TLC plate**
1	SR-I/A1	-	17	SP-I/B1	+
2	SR-I/A2	-	18	SP-I/B2	++
3	SR-I/A3	-	19	SP-I/B3	+
4	SR-I/A4	-	20	SP-I/B4	-
5	SR-I/A5	-	21	SP-I/B5	+
6	SR-I/A6	-	22	SP-I/B6	-
7	SR-I/A7	-	23	SP-II/A1	+++
8	SR-I/B1	++	24	SP-II/A2	-
9	SR-I/B2	-	25	SP-II/A3	+++
10	SR-I/B3	-	26	SP-II/A4	+++
11	SR-I/B4	-	27	SP-II/A5	-
12	SR-I/B.5	-	28	SP-II/A6	-
13	SR-II/A1	++	29	SP-II/B1	-
14	SR-II/A2	++	30	SP-II/B2	-
15	SP-I/A1	+	31	SP-II/B3	-
16	SP-I/A2	-	32	SP-II/B4	-

+++, Very bright yellow spot; ++, bright yellow spot; +, pale yellow spot; -, no spot.

3.3.2 High performance liquid chromatography

Table 12 Analysis of isolated strains for succinic acid ability by HPLC method

No.	Sample	Succinic acid conc. (g/l)	No.	Sample	Succinic acid conc. (g/l)
1	SR-I/A1	-	17	SP-I/B1	0.187
2	SR-I/A2	-	18	SP-I/B2	1.938
3	SR-I/A3	-	19	SP-I/B3	1.210
4	SR-I/A4	-	20	SP-I/B4	-
5	SR-I/A5	-	21	SP-I/B5	1.156
6	SR-I/A6	-	22	SP-I/B6	-
7	SR-I/A7	-	23	SP-II/A1	2.655
8	SR-I/B1	1.808	24	SP-II/A2	-
9	SR-I/B2	-	25	SP-II/A3	2.398
10	SR-I/B3	-	26	SP-II/A4	2.015
11	SR-I/B4	-	27	SP-II/A5	-
12	SR-I/B.5	-	28	SP-II/A6	-
13	SR-II/A1	2.026	29	SP-II/B1	-
14	SR-II/A2	1.866	30	SP-II/B2	-
15	SP-I/A1	1.590	31	SP-II/B3	-
16	SP-I/A2	-	32	SP-II/B4	-

3.4 Sweet sorghum straw pretreatment

Sweet sorghum straw has the potential feedstock as a carbon source for succinic acid production. Dilute sulfuric acid pretreatment was effective in solubilizing cellulose and hemicellulose in the biomass to fermentable sugars. In case of sweet sorghum straw, the maximum yield of glucose and xylose were 0.234 g glucose/g dry substrate and 0.208 g xylose/g dry substrate, respectively, at the pretreatment condition : 120ºC, 3%H_2SO_4 for 10 min. In this case, a total of 50.04% of glucan and 76.41% of xylan were converted to glucose and xylose, respectively (Poonsrisawat, A. 2010).

Table 13 Yield of monosugars liberated from the acid pretreatment of the sorghum straw

Conditions	**Yield $_{avg}$ (g monosugar /g dry substrate)**			
	Glucose	**Xyl**	**Gal, Man, Ara**	**Total sugars**
Acid pretreatment of sorghum straw 120°C, 3% H_2SO_4,10 min	0.234±0.079	0.208±0.073	0.235±0.164	0.676±0.230

* Untreated sorghum straw consists of 44.51% cellulose and 38.62% hemicellulose

** The acid pretreated sorghum straw consists of 69.5% cellulose and 0.44% hemicelluloses

3.5 Effects of nitrogen source on the growth of *Corynebacterium glutamicum*

The effect of nitrogen sources on cell growth was investigated at different cultivation times. The experimental results demonstrated in Figure 19.

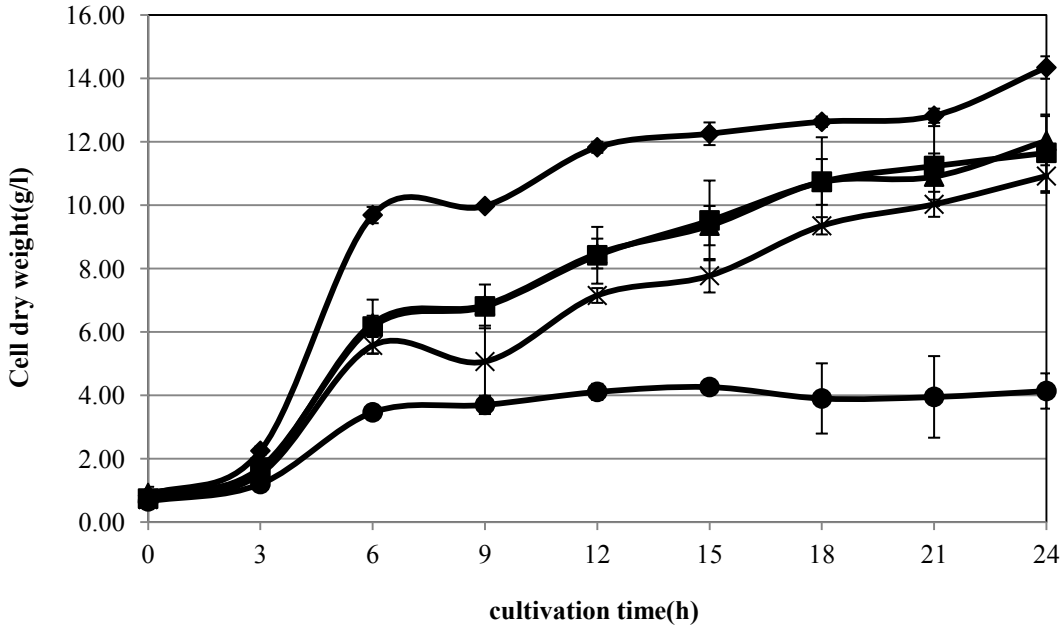


Figure 19 Effect of nitrogen sources on cell growth of *C.glutamicum*
Symbol key: (♦) formula 1; (■) formula 2; (▲) formula 3; (×) formula 4; (●) formula 5

The results indicated that the used of nitrogen source from formula 1 (combination of 10 g/l of peptone and 10 g/l of yeast extract) gave the highest amount of cell growth followed by nitrogen sources formula 2, 3, 4 and 5, respectively. The time courses of cell growth using difference nitrogen source revealed a significant increase on cell growth with extend cultivation time.

Table 14 ANOVA result of *C.glutamicum* cell growth using various nitrogen ratio

CDW

Tests of Between-Subjects Effects

Dependent Variable: CDW

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	2251.942[a]	44	51.180	134.616	.000
Intercept	6339.566	1	6339.566	16674.424	.000
Medium	579.584	4	144.896	381.107	.000
Cutivation_Time	1477.447	8	184.681	485.750	.000
Medium * Cutivation_Time	194.912	32	6.091	16.021	.000
Error	34.218	90	.380		
Total	8625.726	135			
Corrected Total	2286.160	134			

[a] R Squared = .985 (Adjusted R Squared = .978)

Table 15 Multiple comparison result of *C.glutamicum* cell growth using various difference nitrogen ratio

CDW

	Medium	N	Subset 1	2	3	4
Tukey HSD(a,b)	Formular 5	27	3.26407			
	Formular 4	27		6.45574		
	Formular 2	27			7.44681	
	Formular 3	27			7.47007	
	Formular 1	27				9.62689
	Sig.		1.000	1.000	1.000	1.000
Duncan(a,b)	Formular 5	27	3.26407			
	Formular 4	27		6.45574		
	Formular 2	27			7.44681	
	Formular 3	27			7.47007	
	Formular 1	27				9.62689
	Sig.		1.000	1.000	.890	1.000

Means for groups in homogeneous subsets are displayed.

Based on Type III Sum of Squares

The error term is Mean Square(Error) = .380

[a] Uses Harmonic Mean Sample Size = 27.000

[b] Alpha = .05

Table 16 Multiple comparison result of *C.glutamicum* cell growth on time course

CDW

	Cutivation_ Time	N	Subset						
			1	2	3	4	5	6	7
Tukey HSD(a,b)	0 h	15	.77147						
	3 h	15		1.67327					
	6 h	15			6.23560				
	9 h	15			6.47953				
	12 h	15				7.99747			
	15 h	15				8.63433			
	18 h	15					9.47353		
	21 h	15					9.78987		
	24 h	15						10.61940	
	Sig.		1.000	1.000	.975	.122	.893	1.000	
Duncan(a ,b)	0 h	15	.77147						
	3 h	15		1.67327					
	6 h	15			6.23560				
	9 h	15			6.47953				
	12 h	15				7.99747			
	15 h	15					8.63433		
	18 h	15						9.47353	
	21 h	15						9.78987	
	24 h	15							10.61940
	Sig.		1.000	1.000	.282	1.000	1.000	.163	1.000

Means for groups in homogeneous subsets are displayed.

Based on Type III Sum of Squares

The error term is Mean Square(Error) = .380.

a Uses Harmonic Mean Sample Size = 15.000.

b Alpha = .05.

The analyses of variance for all of the data using SPSS software indicated that the means of various ratio of nitrogen sources used for cell growth from *C. glutamicum* were statistically different with a 95% confidence interval with Tukey's test result at $\alpha = 0.05$. A maximal cell growth of 9.626 g/l was obtained with 10 g/l of peptone and 10 g/l of yeast extract were used to nitrogen source. However, reducing organic nitrogen source and adding inorganic source might be used as an economical and feasible nitrogen source for the organic acid production by *C.glutamicum.* Result shown that formula 2 and 3 were used to nitrogen source no significantly at $\alpha = 0.05$. Considering the time course of cell growth, the highest amount of cell was obtained at the 24^{th} h of cultivation. However the exponential cell culture

at 15 h was used as inoculum for further study of organic acid production

Many literatures reported that the key factor in order to reach high amounts of L-glutamic acid was the optimal concentration of biotin to influence and support cell growth and the secretion of the product in the extracellular environment (Stansen 2005). However, from an economic point of view, it was agreeable to choose the lowest possible nitrogen source and minimal addition vitamin and amino acid necessary for the highest possible amino acid production. An overall economic process must include the achievement of a high production rate at a minimum expensive medium and over short times. This study was shown 10 g/l of peptone and 10 g/l of yeast extract were used to nitrogen source gave a highest cell growth. Further study, an improvement growth rate of *C.glutamicum*, the effect of biotin concentration and the optimum condition for amino acid production will be investigated.

3.6 Effect of carbon source on the growth of *C. glutamicum*

The effect of carbon sources glucose and sugar from hydrolyzed SSS on cell growth was investigated at different cultivation times. The experimental results demonstrated in Figure 20.

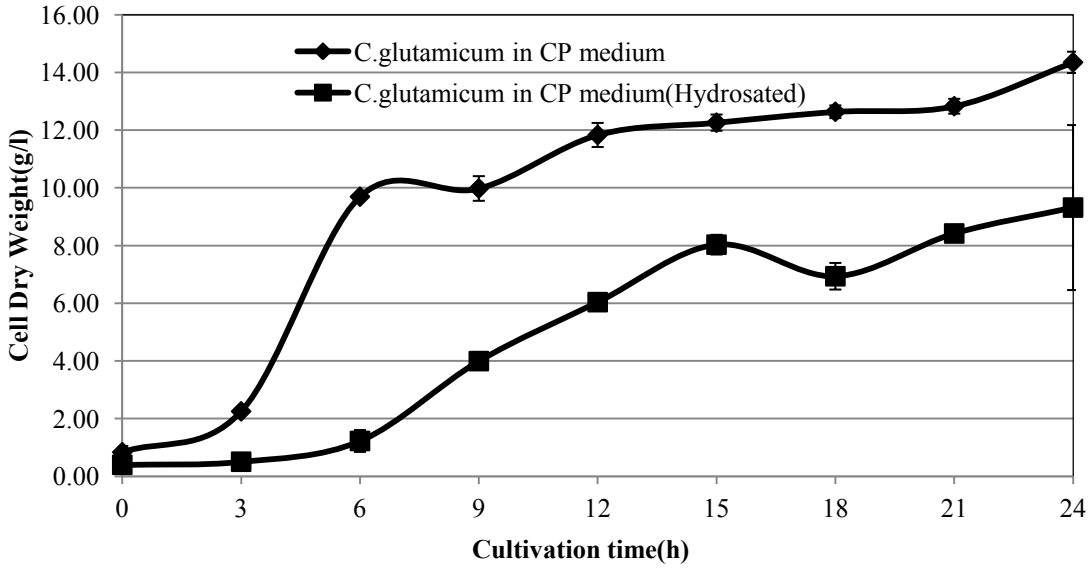


Figure 20 Effect of carbon sources on cell growth of *C.glutamicum*
Symbol key: (♦) glucose; (■) sugar from SSS hydrolyzed

The result was indicated that using glucose as a carbon source exhibited better cell growth than using monosugars from SSS hydrolyzate. The obvious profile of cell growth in SSS hydrolyzate, a longer lag phase was found with this inoculation, it might be the complexity of the composition of SSS substrate. Notwithstanding *C.glutamicum* can growth in hydrolyzate of SSS, suggesting that SSS hydrolyzate might be used as an economical and feasible carbon source for the growth of organic acid producer strain; *C.glutamicum*. The ongoing study is about the potential to use this carbon source for an economical succinic acid production.

The cell growth on time course study using glucose and SSS hydrolyzed as a carbon source revealed a significant increase on cell growth with extend cultivation time. The obvious profile of cell growth from glucose used as carbon source indicated that 0-3 h was a lag phase, 3-6 h was a log phase or exponential phase and 12 h approach to early stationary phase. In case using SSS hydrolyzate as a carbon source, result was shown that SSS hydrolyzed gave a longer lag phase than using glucose as a carbon source.

Table 17 ANOVA result of *C.glutamicum* cell growth using various carbon source

Tests of Between-Subjects Effects

Dependent Variable: CDW

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	2206.624(a)	17	129.801	35.852	.000
Intercept	3486.702	1	3486.702	963.051	.000
Medium	668.814	1	668.814	184.731	.000
Cultivation_time	1265.214	8	158.152	43.683	.000
Medium * Cultivation_time	272.595	8	34.074	9.412	.000
Error	325.843	90	3.620		
Total	6019.169	108			
Corrected Total	2532.466	107			

Table 18 Multiple comparison result of *C.glutamicum* cell growth on time course

	Cultivation_time	N	Subset				
			1	2	3	4	5
Tukey HSD(a,b)	3 h	12	.90708				
	0 h	12	.94533				
	6 h	12	2.69000	2.69000			
	9 h	12		4.06008	4.06008		
	12 h	12			6.37750	6.37750	
	15 h	12				7.96400	7.96400
	18 h	12				8.74275	8.74275
	21 h	12					9.06950
	24 h	12					10.38108
	Sig.		.356	.705	.083	.071	.060
Duncan(a,b)	3 h	12	.90708				
	0 h	12	.94533				
	6 h	12		2.69000			
	9 h	12		4.06008			
	12 h	12			6.37750		
	15 h	12				7.96400	
	18 h	12				8.74275	
	21 h	12				9.06950	9.06950
	24 h	12					10.38108
	Sig.		.961	.081	1.000	.184	.095

The analyses of variance for all of the data using SPSS software indicated that the means of various carbon sources used for cell growth of *C. glutamicum* were statistically different with a 95% confidence interval with Tukey's test result at $\alpha = 0.05$. A maximal cell growth of 14.347 g/l was obtained with 10 g/l of glucose used as a carbon source. Considering the time course of cell growth, the highest amount was obtained at the 24^{th} h of cultivation time but no significant on cell growth when extend cultivation time from 15 to 24 h, also 15 h approach to early stationary phase. However the exponential phase from this growth profile was 6-12 h, was used as inoculum to produce succinic acid in further study.

From this experiment, it was shown that using difference carbon source has effect on cell growth of *C.glutamicum*. Glucose was optimum for *C.glutamicum* cell growth. However, the possibilities of cost-effective in fermentation by *C.glutamicum* from inexpensive and abundant feedstock, including lignocellulosic biomass was available used as substrate since *C.glutamicum* could grow in a medium containing the hydrolyzate of SSS.

3.7 Effect of substrate concentration on the growth of *C. glutamicum*

The significant of substrate concentrations on cell growth has been investigated. The effect of various concentrations from 5-15 g/l of glucose and reducing sugars of SSS hydrolyzed in the inoculums medium were examined. The results demonstrated in Figure 21.

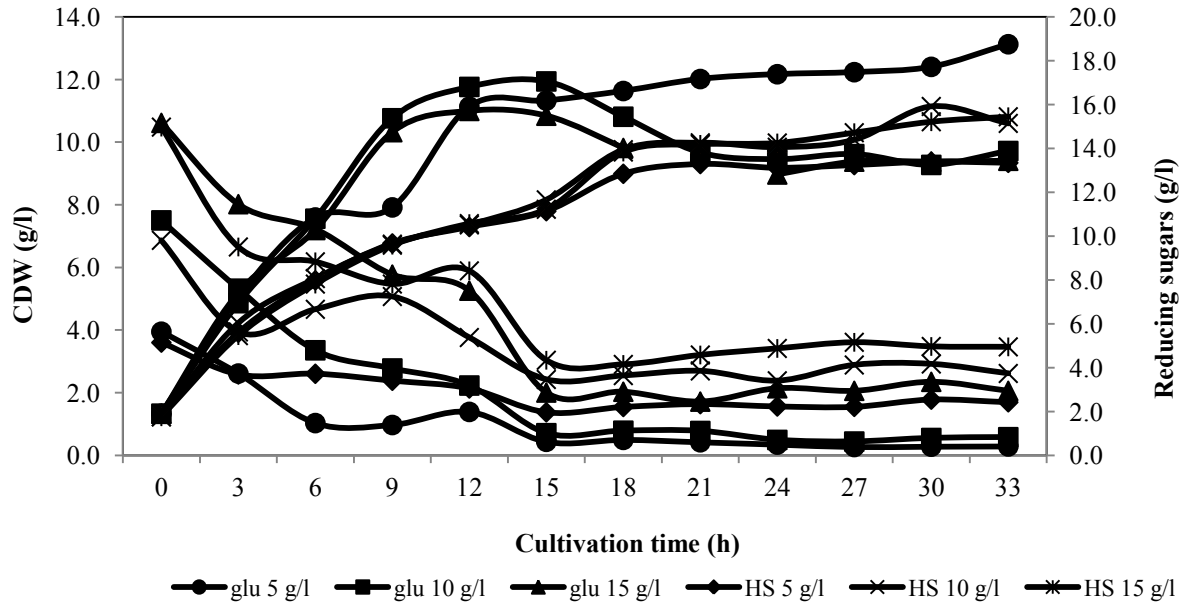


Figure 21 Effect of substrate concentration on cell growth of *C.glutamicum*

Table 19 ANOVA result of *C.glutamicum* cell growth using various substrate concentration

Tests of Between-Subjects Effects

Dependent Variable: CDW

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	1966.267(a)	71	27.694	63.731	.000
Intercept	15040.911	1	15040.911	34613.371	.000
Carbon_source	143.507	5	28.701	66.050	.000
Cultivation_time	1613.225	11	146.657	337.499	.000
Carbon_source * Cultivation_time	209.534	55	3.810	8.767	.000
Error	62.574	144	.435		
Total	17069.752	216			
Corrected Total	2028.841	215			

a R Squared = .969 (Adjusted R Squared = .954)

Table 20 Multiple comparison result of *C.glutamicum* cell growth using various substrate concentration

CDW

	Carbon_source	N	Subset 1	2	3	4	5
Tukey HSD(a,b)	5 g/l HS	36	7.34489				
	15 g/l HS	36		7.82097			
	10 g/l HS	36		7.89547	7.89547		
	15 g/l glucose	36			8.28161		
	10 g/l glucose	36				8.89656	
	5 g/l glucose	36					9.82864
	Sig.		1.000	.997	.135	1.000	1.000
Duncan(a,b)	5 g/l HS	36	7.34489				
	15 g/l HS	36		7.82097			
	10 g/l HS	36		7.89547			
	15 g/l glucose	36			8.28161		
	10 g/l glucose	36				8.89656	
	5 g/l glucose	36					9.82864
	Sig.		1.000	.632	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = .435.
a Uses Harmonic Mean Sample Size = 36.000.
b Alpha = .05.

Table 21 Multiple comparison result of *C.glutamicum* cell growth on time course

CDW

	Cultivation _time	N	Subset							
			1	2	3	4	5	6	7	8
Tukey HSD(a,b)	0 h	18	1.29506							
	3 h	18		4.51183						
	6 h	18			6.50778					
	9 h	18				8.20333				
	12 h	18					9.32450			
	21 h	18					9.57494	9.57494		
	15 h	18					9.65372	9.65372	9.65372	
	24 h'	18					9.93111	9.93111	9.93111	9.93111
	18 h	18						10.11617	10.11617	10.11617
	27 h	18						10.14628	10.14628	10.14628
	30 h	18							10.37144	10.37144
	33 h	18								10.50011
	Sig.		1.000	1.000	1.000	1.000	.209	.290	.059	.296
Duncan (a,b)	0 h	18	1.29506							
	3 h	18		4.51183						
	6 h	18			6.50778					
	9 h	18				8.20333				
	12 h	18					9.32450			
	21 h	18					9.57494	9.57494		
	15 h	18					9.65372	9.65372		
	24 h'	18						9.93111	9.93111	
	18 h	18							10.11617	10.11617
	27 h	18							10.14628	10.14628
	30 h	18							10.37144	10.37144
	33 h	18								10.50011
	Sig.		1.000	1.000	1.000	1.000	.160	.128	.068	.113

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = .435.
a Uses Harmonic Mean Sample Size = 18.000.
b Alpha = .05.

The low cost and high monosugars content of sweet sorghum straw hydrolazate made it an attractive potential substrate for cell growth of *C. glutamicum*. As shown in Figure 21, vigorous growth from various substrate concentrations with 10 g/l of peptone and 10 g/l of yeast extract were used as nitrogen sources. The analyses of variance for all of the data using SPSS software indicated that the means of various ratio of carbon source used for cell growth from *C. glutamicum* were statistically different with a 95% confidence interval with Tukey's test result at $\alpha = 0.05$. A maximal cell growth of 9.83 g/l of CDW when 5 g/l of glucose as a carbon source after 18 h but no significance change of cell growth from extend cultivation time to 33 h (10.50 g/l). This result showed that 5 g/l glucose is the optimal carbon source on

cell growth, follow by 10 g/l, 15 g/l of glucose and 10 g/l of SSS hydrolyzate, However, no distinct lag phase was seen with 10 g/l of SSS hydrolyzate so it has a potential used as a carbon source for succinic acid production.

3.8 Effects of carbon source on cell growth and succinic acid production of *C. glutamicum*

To study effect of carbon sources on cell growth and succinic acid production have been investigated by using different carbon source from glucose and SSS hydrolyzate in the inoculums medium then transfer to production medium as shown in Table 7. The result was examined by determine cell growth as shown in Figure 22.

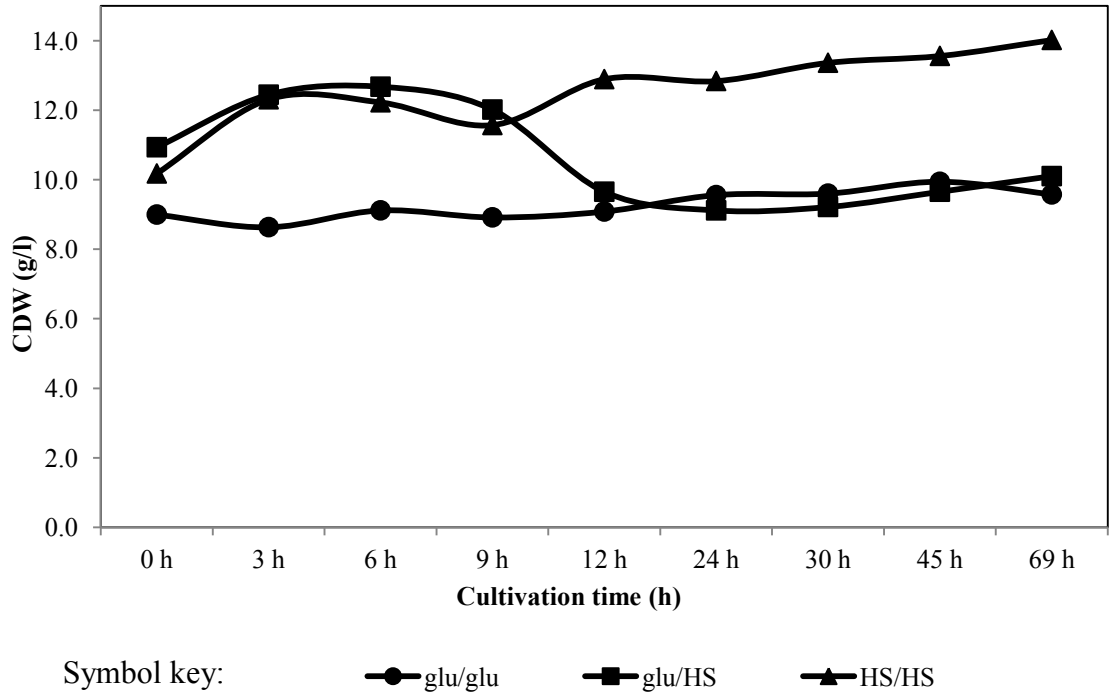


Figure 22 Effects of carbon sources on the growth of *C. glutamicum* in the production medium

The various carbon sources with 10 g/l of peptone and 10 g/l of yeast extract as nitrogen sources was used both in the inoculums and production medium. Figure 22 showed that cell growth of *C. glutamicum* in production medium had no significantly increased. The cells might use the carbon source in the production medium as a precursor for product formation. In this study, we have not yet determined the amount of succinic acid formation.

The analyses of variance for all of the data using SPSS software indicated that the means of various ratio of carbon source used for cell growth from *C. glutamicum* were statistically different with a 95% confidence interval with Tukey's test result at $\alpha = 0.05$, this result had no significance (data not show).

Chapter IV
Conclusion

The samples were collected from buffalo dung in Suphanburi province (SP-I, SP-II), soil sample in Suphanburi province (SP-III) and cow dung from Surin province (SR-I, SRII), Thailand. Thirty-three isolates with ability amino acid and thirty-two isolates with ability succinic acid were obtained. They were non-spore-forming, gram-positive or gram- negative rod. The morphological characteristics colonies appearing on the screening agar plate after 24 h of incubation were circular, smooth and gray with 1–2 mm in diameter. Three of those isolates have a potential for succinic acid ability (isolate SP-II/A1, SP-II/A3 and SP-II/A4).

Corynebacterium glutamicum DSM20300 was used as a representative for investigating growth and succinic acid production from agriculture biomass. Sweet sorghum straw hydrolyzate was used as a carbon source to replacement glucose for growth in succinic acid production. The used of nitrogen source from formula 1 (combination of 10 g/l of peptone and 10 g/l of yeast extract) gave the highest amount of cell growth of 9.626 g/l. Using glucose as a carbon source exhibited better cell growth than using monosugars from SSS hydrolyzate. A maximum cell growth of 14.347 g/l was obtained with 10 g/l of In case using SSS hydrolyzate as a carbon source, longer lag phase was found. Further study by using SSS hydrolyzate in production medium, the cell growth of *C. glutamicum DSM20300* had no significantly increased. The cells might use the carbon source in the production medium as a precursor for product formation. In this study, we have not yet determined the amount of succinic acid formation.

Future Perspectives

1. Investigate growth and succinic acid producing of the potential isolate
2. Improve the process economic by replacement carbon source in the fermentative medium with the sweet sorghum hydrolyzate

References

Alper, H. and Stephanopoulos, G. (2009) Engineering for biofuels: exploiting innate microbial capacity or importing biosynthetic potential. Nature review Microbiology 7: 705-723.

Arikawa Y., Kuroyanagi T., Shimosaka M., Muratsubaki H, Enomoto K, and Kodaira R. (1999) Effect of gene disruptions of the TCA cycle on production of succinic acid in *Saccharomyces cerevisiae*. J Biosci Bioeng. 87: 28–36.

Beauprez J.J., Mey M.D. and Soetaert W. K. (2010) Microbial succinic acid production: Natural versus metabolic engineered Producers. Process Biochemistry 45: 1103–1114.

Bobleter, O. (1994) Hydrothermal degradation of polymers derived from plants. Prog. Polym. Sci. 19: 797–841.

Braunschweig Z. (2008) Production of L-methionine with *Corynebacterium glutamicum.* Media for *Corynebacterium glutamicum* DSM20300. 29-31.

David H., Akesson M. and Nielsen J. (2003) Reconstruction of the central carbon metabolism of *Aspergillus niger*. *J Dairy Sci.* 270:4243–53.

Du C., Lin S.K.C., Koutinas A., Wang R., Dorado P., Webb C. (2008) A wheat biorefining strategy based on solid-state fermentation for fermentative production of succinic acid. Bioresour Technol 99:8310–5.

Edwards J. S., Ramakrishna R. Schilling C. H. and Palsson B. O. (1999) Metabolic flux balance analysis. pp. 13-57. Metabolic Engineering. Marcel Dekker, New York, NY, USA.

Elimer E. (1998) Citric acid production from rape seed oil by *Aspergillus niger*. *Food Technol Biotechnol.* 36: 189–92.

Esterbauer H, Steined W, Labudova I, Herman A, Hayn M. (1991) Production of *Thrichoderma* cellulase in laboratory and pilot scale. Bioresource Technology 36:51–65.

Fengel, D. and Wegener, G., (1984) Wood: Chemistry, Ultrastructure, Reactions. De Gruyter, Berlin.

Guettler M.V., Jain M.K. and Rumler D. (1996) Method for making succinic acid, bacterial variants for use in the process, and methods for obtaining variants. US Patent. 5: 573-931.

Guo, G.L., Hsu, D.C., Chen, W.H., Chen, W.H. and Hwang, W.S. (2009) Characterization of enzymatic saccharification for acid-pretreated lignocellulosic materials with different lignin composition. Enzyme and Microbial Technology 45: 80–87.

Harmsen, P. F. H., Huijgen, W.J.J., Bermúdez López, L.M. and Bakker, R.R.C. (2010) Literature review of physical and chemical pretreatment processes for lignocellulosic biomass. Netherlands: 8-30.

Hendriks, A.T.W.M. and Zeeman., G. (2009) Pretreatments to enhance the digestibility of lignocellulosic biomass. Bioresource Technology 100: 10–18.

Hong S.H. (2007) Systems Approaches to Succinic Acid-producing Microorganisms. Biotechnology and Bioprocess Engineering 12: 73-79.

Howard R.L., Abotsi, E., Rensburg E.L. J. and Howard, S. (2003) Lignocellulose biotechnology: issues of bioconversion and enzyme production. African Journal of Biotechnology 2: 602-619.

Inui M., Murakami S., Okino S., Kawaguchi H., Vert`es A.A., Yukawa H. (2004) Metabolic analysis of *Corynebacterium glutamicum* during lactate and succinate production under oxygen deprivation conditions. J Mol Microbiol Biotechnol. 7:182–96.

James B. McKinlaya, Shachar-Hillb Y., Zeikusa J.G., Vieille C. (2007) Determining *Actinobacillus succinogenes* metabolic pathways and fluxes by NMR and GC-MS analyses of ^{13}C-labeled metabolic product isotopomers. Metabolic Engineering 9: 177–192.

James B., McKinlay L.J., Zeikus G. and Vieille C. (2005) Insights into *Actinobacillus succinogenes* Fermentative Metabolism in a Chemically Defined Growth Medium. Applied and environmental microbiology. 6651–6656.

Kamra D.N. (2005) Rumen microbial ecosystem. Curr Sci. 89:124–35.

Kinoshita S., Udaka S. and Akita S. (1961) Method of producing l-glutamic acid by fermentation. United States Patent Office, number 3.003.925 (Patented Oct. 10. 1961).

Kole M.M., Thompson B.G., Gerson D.F. and Senechai J. (1986) Control of Nitrate Concentration in Fermentations of *Corynebacterium glutamicum*. Biotechnology and Bioengineering 28: 659–662.

Kumar, P., Barrett, D.M., Delwiche, M.J. and Stroeve, P. (2009) Methods for pretreatment of lignocellulosic biomass for efficient hydrolysis and biofuel production. Industial Engineering Chemistry Research. 48: 3713–3729.

Laureano-Perez, L., Teymouri, F., Alizadeh, H., Dale, B.E. (2005) Understanding factors that limit enzymatic hydrolysis of biomass. Appl. Biochem. Biotechnol. 1081–1099.

Li Q., Wang D., Huc G., Xing J. and Sua Z. (2011) Integrated bioprocess for high-efficiency production of succinic acid in an expanded-bed adsorption system. Biochemical Engineering Journal 56; 150– 157.

Li, H., Kim, N.J., Jiang, M., Kang, J.W. and Chang, H.N. (2009) Simultaneous saccharification and fermentation of lignocellulosic residues pretreated with phosphoric acid–acetone for bioethanol production. Bioresource Technology 100: 3245–3251.

Magnuson J.K., Lasure L.L. (2004) Organic acid production by filamentous fungi. In: Tkacz JS, Lange L, editors. Advances in fungal biotechnology for industry, agriculture, and medicine.NewYork: Kluwer Academic/Plenum Publishers. 307–340.

Malherbe, S. and Cloete, T.E. (2002) Lignocellulose biodegradation: fundamentals and applications. Environmental science and biotechnology 1: 105-114.

Markowitz V.M., Ivanova N.N., Szeto E., Palaniappan K., Chu K., Dalevi D. (2008) A data management and analysis system for metagenomes. Nucleic Acid Res 36: 534–538.

McIntyre M. and McNeil B. (1997) Effects of elevated dissolved CO_2 levels on batch and continuous cultures of *Aspergillus niger* A60: an evaluation of experimental methods. Appl Environ Microbiol 63: 4171–4177.

McKinlay J.B., Zeikus J.G., Vieille C. (2005) Insights into Actinobacillus succinogenes fermentative metabolism in a chemically defined growth medium. Appl Environ Microbiol. 71:6651–6.

Nielsen J. and Villadsen J. (1994) Analysis of reaction. 78: 97-161. In: J. Nielsen and J. Villadsen (eds.). Bioreaction Engineering Principles. Plenum press, New York, NY, USA.

Nuanpeng, S., Laopaiboon, L., Srinophakun, P., Klanrit, P., Jaisil, P. and Laopaiboon, P. (2011) Ethanol production from sweet sorghum juice under very high gravity conditions: batch, repeated-batch and scale up fermentation. Electronic Journal of Biotechnology 14: 1-12.

Orpin CG. (1984) The role of ciliate protozoa and fungi in rumen digestion of plant cell walls. Animal Feed Sci Technol 10:121–43.

Poonsrisawat, A. (2010) Optimization of chemical saccharification of sweet sorghum straw and bagasse for ethanol fermentation. Master's Thesis. Program in Biotechnology, Facultry of science, Chulalongkorn University.

Sánchez, C. (2009) Lignocellulosic residues: Biodegradation and bioconversion by fungi. Biotechnology Advances 27: 185–194.

Shiio I. and Ujigawa K. (1980) Presence and regulation of α-ketoglutarate dehydrogenase complex in a glutamate producing bacterium, *Brevibacterium flavum.* Agric.Biol.Chem. 42: 1897-1904.

Song H., Lee S.Y. (2006) Production of succinic acid by bacterial fermentation, Enzyme Microbial. Technol. 39:352–361.

Stansen K.C. (2005). Charakterisierung der Ausscheidung von L-Glutamat bei *Corynebacterium glutamicum.* Dissertation Forschungszentrum Jülich.

Sun, Y. and Cheng, J. (2002) Hydrolysis of lignocellulosic materials for ethanol production. Bioresource Technology 83: 1-11.

Talebnia, F., Karakashev, D. and Angelidaki, I. (2010) Production of bioethanol from wheat straw: An overview on pretreatment, hydrolysis and fermentation. Bioresource Technology 101: 4744–4753.

Ueda K., Tagami Y., Kamihara Y, Shiratori H., Takano H., Beppu T. (2008) Isolation of bacteria whose growth is dependent on high levels of CO2 and implications of their potential diversity. Appl Environ Microbiol.74: 4535–8.

Van der Werf M.J., Guettler M.V., Jain M.K. and Zeikus J.G. (1997) Environmental and physiological factors affecting the succinate product ratio during carbohydrate fermentation by *Actinobacillus* sp. 130Z. Arch Microbiol 168:332–42.

Van Gylswyk N.O. (1995) *Succiniciclasticum ruminis* gen-nov, sp.-nov, a ruminal bacterium converting succinate to propionate as the sole energyyielding mechanism. Int J Syst Bacteriol 45:297–300.

Wakai Y., Shimazaki T. and Hara S. (1980) Formation of succinate during fermentation of sake mash and grape must. Hakkokogaku 58:363–8.

Wen, Z., Liao, W. and Chen, S. (2005) Production of cellulase/β-glucosidase by the mixed fungi culture *Trichoderma reesei* and *Aspergillus phoenicis* on dairy manure. Process Biochemistry 40: 3087–3094.

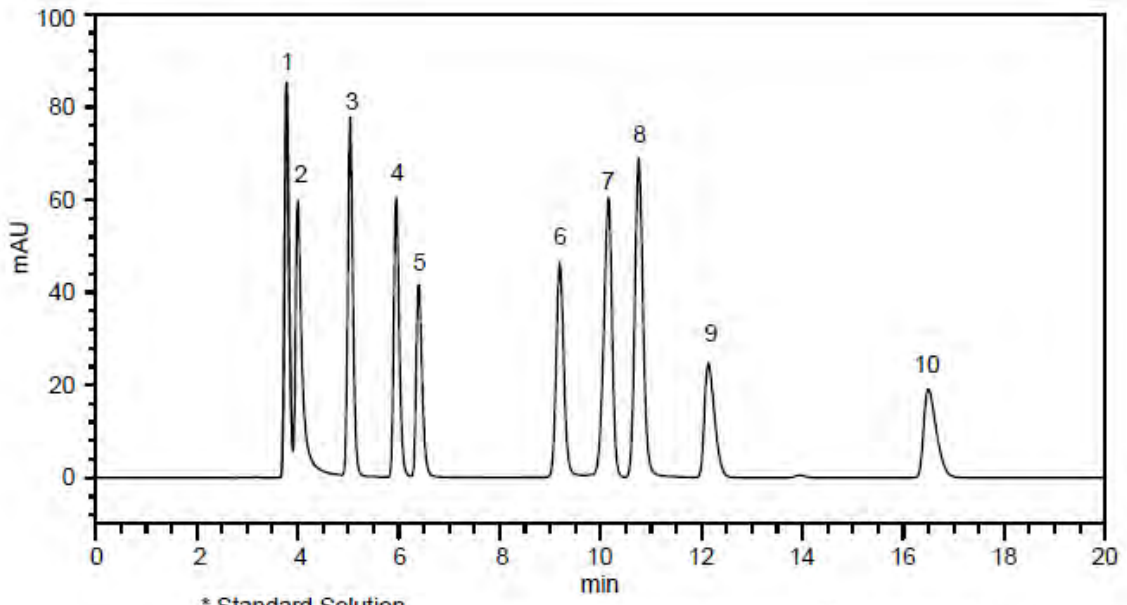
Wilke, D. 1999. Chemicals from biotechnology: molecular plant genetics will challenge the chemical and fermentation industry. Appl. Microbiol. Biotechnol. 52: 135–145.

Wolin M.J. and Miller T.L. (1988) Microbe-microbe interactions. In: Hobson PN, editor. The rumen microbial ecosystem. London: Elsevier Applied Science 343–359.

Zeikus J. G., Jain, M.K. and Elankovan P. (1999) Biotechnology of succinic acid production and markets for derived industrial products. Appl. Microbiol. Biotechnol. 51: 545-552.

APPENDIX A

Standard peaks of organic acid by HPLC (Aminex HPX-87H Column)



* Standard Solution

Component	Conc. (mg/L)	Component	Conc. (mg/L)
1. Tartaric acid	500	6. Pyroglutamic acid	100
2. Formic acid	1000	7. Citric acid	1000
3. Malic acid	1000	8. Fumaric acid	10
4. Lactic acid	1000	9. Succinic acid	1000
5. Acetic acid	1000	10. Propionic acid	1000

Figure A Standard peaks of organic acid on the Aminex HPX-87H Column (Formic acid, Malic acid, Lactic acid, Acitic acid, Citric acid, Fumaric acid and Succinic acid)

APPENDIX B

B1. Calibration curve for various concentration of glucose by DNS method

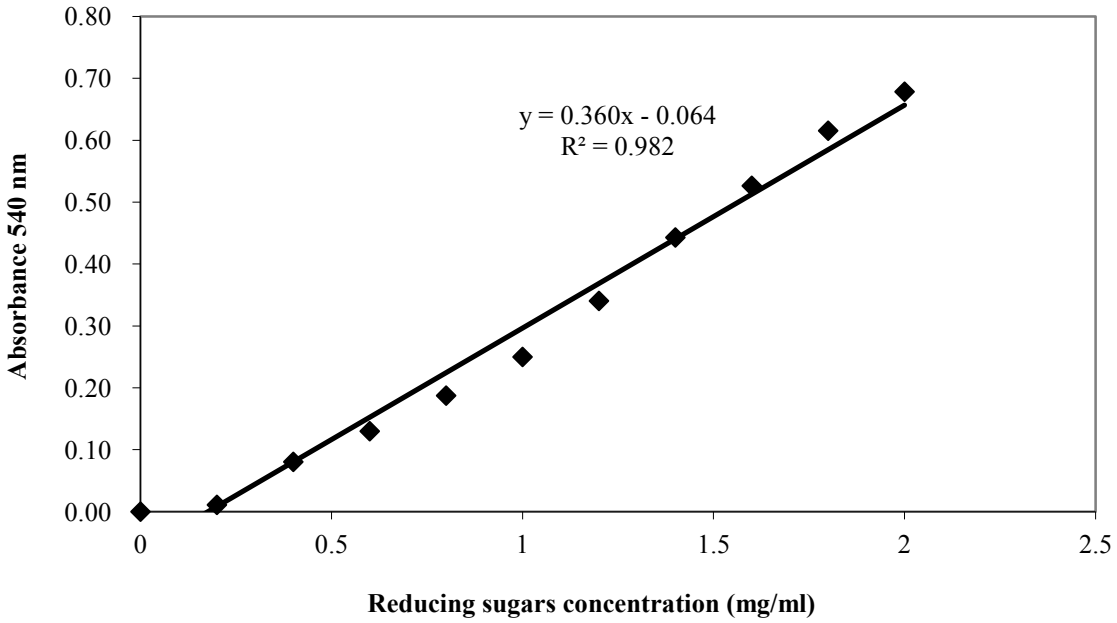


Figure B1 Calibration curve for various concentration of glucose by DNSA method

Equation; $Y = 0.360X - 0.064$

Glucose concentration (g/l) $= \dfrac{\text{OD } 540 + 0.064}{0.360}$

B2. Correlation between cell dry weight of *C. glutamicum* (g/l) and OD660

Cell dry weight (g)	cell (g/l)	OD660
0.0049	1.633	1.010
0.0041	1.367	0.862
0.0034	1.133	0.702
0.0027	0.900	0.495
0.0016	0.533	0.269
0.0001	0.000	0.000

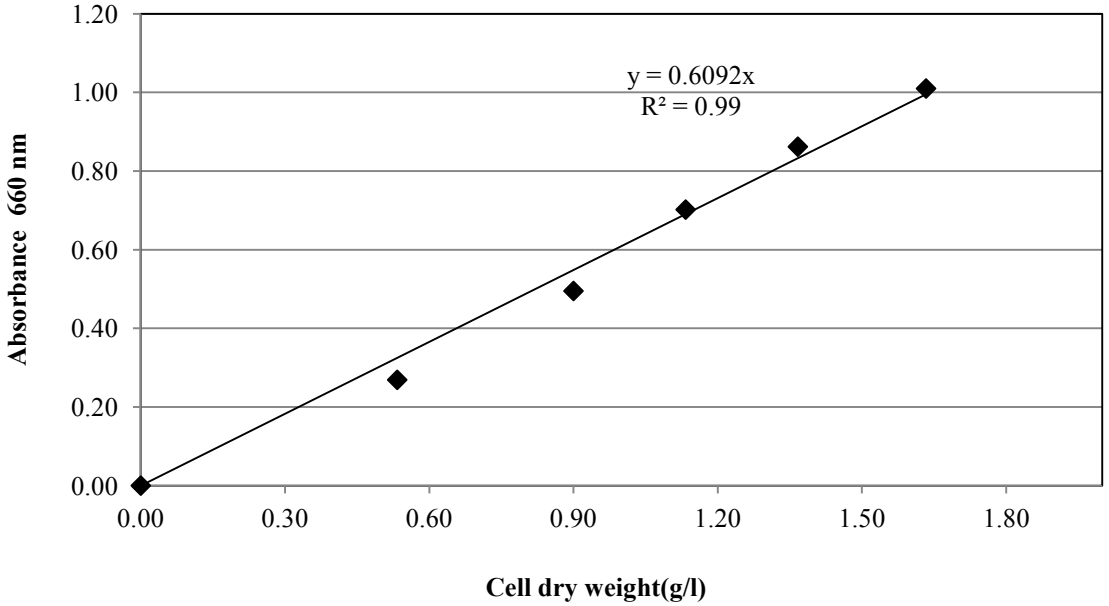


Figure B2 Correlation between cell dry weight (g/l) and OD660

Equation; $Y = 0.6092X$

$$\text{Cell dry weight (g/l)} = \frac{\text{OD 660}}{0.6092}$$

ประวัติคณะผู้วิจัย

1. ชื่อหัวหน้าโครงการ

1. ชื่อ

(ไทย) นางสาว ศิริลักษณ์ ธีระดากร

(อังกฤษ) Miss Siriluk Teeradakorn

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

3. ตำแหน่งปัจจุบัน นักวิจัย 7

4. หน่วยงาน สถาบันเทคโนโลยีชีวภาพและวิศวกรรมพันธุศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

ที่อยู่ อาคารสถาบัน3 ซอยจุฬาลงกรณ์62 ถนนพญาไท แขวงวังใหม่ เขตปทุมวัน กรุงเทพฯ 10330

โทรศัพท์ 02-2188073 โทรสาร 02-2533543

โทรศัพท์เคลื่อนที่ 089-1220626

E-mail tsiriluk@chula.ac.th

5. ประวัติการศึกษา

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

6. ความชำนาญ/ความสนใจพิเศษ

6.1 วิศวกรรมกระบวนการ

6.2 การพัฒนาเครื่องวัดการเจริญเติบโตแบบออนไลน์ของจุลินทรีย์ ในกระบวนการหมัก

2 ชื่อผู้ร่วมโครงการ

1. ชื่อ

(ไทย) นางสาว สาวิตร ตระกูลน่าเลื่อมใส

(อังกฤษ) Ms. SAVITR TRAKULNALEAMSAI

2. เลขหมายประจำตัวประชาชน

3. ตำแหน่งปัจจุบัน ผู้ช่วยศาสตราจารย์

4. หน่วยงาน ภาควิชาจุลชีววิทยา คณะวิทยาศาสตร์ มหาวิทยาลัยเกษตรศาสตร์

ที่อยู่ 50 พหลโยธิน แขวงลาดยาว เขตจตุจักร บางเขน กรุงเทพฯ 10900

โทรศัพท์ 02-5625555 ต่อ 4016 โทรสาร 02-5792081

โทรศัพท์เคลื่อนที่ 081-9063980

E-mail rdisat@ku.ac.th

5. ประวัติการศึกษา

ปริญญา	สาขาวิชา	มหาวิทยาลัย	ปีที่ได้รับ พ.ศ.
วิทยาศาสตร์บัณฑิต	จุลชีววิทยา	เกษตรศาสตร์	2526
วิทยาศาสตร์มหาบัณฑิต	จุลชีววิทยา	เกษตรศาสตร์	2530
วิทยาศาสตร์ดุษฎีบัณฑิต	เทคโนโลยีชีวภาพ	โอซาก้า (ประเทศญี่ปุ่น)	2538

6. ความชำนาญ/ความสนใจพิเศษ

6.1 DNA Technology, Enzyme Engineering, Molecular Systematics of Bacteria

……

(อ.ดร. ศิริลักษณ์ ธีระดากร)

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