การผลิตกรดซักซินิกจากแป้งมันสำปะหลังโดยการทำให้เป็นน้ำตาลควบคู่กับการหมักด้วยเชื้อเอสเชอริเชียโคไลที่ถูกดัดแปลงพันธุกรรม

นางสาวคีรีน โฆ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาเทคโนโลยีชีวภาพ
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SUCCINIC ACID PRODUCTION FROM CASSAVA STARCH BY SIMULTANEOUS SACCHARIFICATION AND FERMENTATION BY METABOLICALLY ENGINEERED *ESCHERICHIA COLI*

KIRIN KHOR

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

Suranaree University of Technology

Academic Year 2014

SUCCINIC ACID PRODUCTION FROM CASSAVA STARCH BY SIMULTANEOUS SACCHARIFICATION AND FERMENTATION BY METABOLICALLY ENGINEERED *ESCHERICHIA COLI*

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

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คีรีน โฆ : การผลิตกรดซักซินิกจากแป้งมันสำปะหลังโดยการทำให้เป็นน้ำตาลควบคู่กับกาหมักด้วยเชื้อเอสเชอริเชียโคไลที่ถูกดัดแปลงพันธุกรรม (SUCCINIC ACID PRODUCTION FROM CASSAVA STARCH BY SIMULTANEOUS SACCHARIFICATION AND FERMENTATION BY METABOLICALLY ENGINEERED *ESCHERICHIA COLI*.) อาจารย์ที่ปรึกษา : ผู้ช่วยศาสตราจารย์ ดร. เขมวิทย์ จันต๊ะมา, 96 หน้า.

แป้งมันสำปะหลังถูกนำมาประเมินการผลิตกรดซักซินิกด้วย การทำให้เป็นน้ำตาลควบคู่กับการหมัก (simultaneous saccharification and fermentation; SSF) ด้วยเชื้อเอสเชอริเชียโคไล สายพันธุ์ KJ122 ที่ถูกดัดแปลงพันธุกรรมแล้ว โดยเชื้ออีโคไลสายพันธุ์ KJ122 ที่ถูกดัดแปลงพันธุกรรมเพื่อปรับปรุงการผลิตกรดซักซินิกด้วยการกำจัดยีนบางยีนบางยีนออกไป (*ldhA adhE ackA* (*focA-pflB*) *mgsA poxB tdcDE citF aspC sfcA*) เชื้อสายพันธุ์ดังกล่าวนี้สามารถผลิตกรดซักซินิกในระดับผลผลิตที่ดีเยี่ยม (0.96 กรัมต่อกรัมกลูโคส) ความเข้มข้น และอัตราผลิตผลที่สูงในอาหารเลี้ยงเชื้อเกลือแร่ที่มีกลูโคสภายใต้สภาวะการหมักแบบกะแบบไร้ออกซิเจน อย่างไรก็ตามเชื้อสายพันธุ์ดังกล่าวไม่สามารถใช้แป้งได้อย่างมีประสิทธิภาพได้เนื่องจากการยับยั้งกระบวนการสลาย ด้วยเหตุนี้เอนไซม์กลูโคอะไมเลสถูกนำมาใช้เพื่อปลดปล่อยน้ำตาลจากแป้งมันสำปะหลัง ผลกระทบของอุณหภูมิ ค่าความเป็นกรดด่าง ความเข้มข้นของเอนไซม์กลูโคอะไมเลส ปริมาณหัวเชื้อเริ่มต้น และความเข้มข้นของแป้งมันสำปะหลังต่อการผลิตกรดซักซินิกถูกนำมาศึกษา การหมักถูกทดสอบด้วยการทำให้เป็นน้ำตาลควบคู่กับการหมัก (SSF) แบบกะในขวดเลี้ยงเชื้อขนาด 500 มิลลิลิตร ซึ่งบรรจุอาหารเลี้ยงเชื้อ 350 มิลลิลิตร และดำเนินการที่ค่าความเป็นกรดด่างของอาหารเลี้ยงเชื้อที่ 5.5, 6.0, 6.5 และ 7.0 อุณหภูมิ 37, 39, 41 และ 43 องศาเซลเซียส, ความเข้มข้นของแป้งสำปะหลังที่ 70, 90, 110, 130 และ 150 กรัมต่อลิตร, ความเข้มข้นของเอนไซม์กลูโคอะไมเลสที่ 300, 500, 700, 900, 1,100 และ 1,300 ยูนิตต่อกรัมแป้งมันสำปะหลัง, ปริมาณหัวเชื้อเริ่มต้นถูกทดสอบที่ 0.1, 0.2, 0.3, 0.6, 0.9, 1.2 และ 1.5 OD_{550}

ภายใต้สภาวะที่เหมาะสมคือ ค่าความเป็นกรดด่างที่ 6.5 อุณหภูมิ 41 องศาเซลเซียส ความเข้มข้นของแป้งมันสำปะหลังเท่ากับ 70 กรัมต่อลิตร ปริมาณหัวเชื้อที่ 0.6 OD_{550} และ 500 ยูนิตของเอนไซม์กลูโคอะไมเลสต่อกรัมแป้งมันสำปะหลัง กรดซักซินิกที่ความเข้มข้น 70.08±0.12 กรัมต่อลิตรถูกผลิตขึ้นหลังจาก 72 ชั่วโมงของการหมักในกระบวนการทำให้เป็นน้ำตาลควบคู่กับการหมัก (SSF) โดยผลผลิตเท่ากับ 1.01±0.013 กรัมต่อกรัมแป้งมันสำปะหลังที่ถูกใช้ไป ซึ่งเทียบได้เท่ากับ 0.91±0.013 กรัมต่อกรัมกลูโคสที่ถูกใช้ไป และผลิตผลเท่ากับ 0.97±0.001 กรัมต่อลิตรต่อชั่วโมง ดังนั้นผลผลิตที่ได้เป็นจริงตามผลผลิตทางทฤษฎีซึ่งเท่ากับร้อยละ 81.25 ความเข้มข้นของกรดซัก

ซินิกถูกผลิตขึ้นที่ความเข้มข้นสูงขึ้นที่ 82.46±0.51 กรัมต่อลิตร ผลผลิตที่ 1.03±0.010 กรัมต่อกรัมแป้งมันสำปะหลังที่ถูกใช้ไปซึ่งเทียบได้เท่ากับ 1.00±0.010 กรัมต่อกรัมกลูโคสที่ถูกใช้ไปและผลิตผลที่1.15±0.008 กรัมต่อลิตรต่อชั่วโมงถูกผลิตหลังจาก 72 ชั่วโมงในกระบวนการทำให้เป็นน้ำตาลควบคู่กับการหมัก (SSF) แบบกึ่งกะจากผลการทดลองพบว่ากระบวนการทำให้เป็นน้ำตาลควบคู่กับการหมัก (SSF) แบบกึ่งกะสามารถเพิ่มความเข้มข้น ผลผลิต และผลิตผลของกรดซักซินิกได้ถึงร้อยละ 17.67, 2.0 และ 18.56 ภายใต้สภาวะในกระบวนการทำให้เป็นน้ำตาลควบคู่กับการหมัก (SSF) ผลผลิตที่ได้เป็นจริงตามผลผลิตทางทฤษฎีซึ่งเท่ากับร้อยละ 89.3 ตามลำดับ ดังนั้นแป้งมันสำปะหลังจึงเป็นวัตถุดิบทางเลือกสำหรับการผลิตกรดซักซินิกที่มีประสิทธิภาพโดยใช้เชื้ออีโคไลสายพันธุ์ KJ122 ที่ถูกดัดแปลงพันธุกรรมแล้ว

สาขาวิชาเทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา_____

ปีการศึกษา 2557 ลายมือชื่ออาจารย์ที่ปรึกษา_____

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม_____

KIRIN KHOR : SUCCINIC ACID PRODUCTION FROM CASSAVA STARCH BY SIMULTANEOUS SACCHARIFICATION AND FERMENTATION BY METABOLICALLY ENGINEERED *ESCHERICHIA COLI.* THESIS ADVISOR: ASST. PROF. KAEMWICH JANTAMA, Ph.D., 96 PP.

SUCCINIC ACID/*E. COLI*/CASSAVA STARCH/SIMULTANEOUS SACCHARIFICATION AND FERMENTATIO

Succinic acid production by simultaneous saccharification and fermentation (SSF) or cassava starch using engineered *Escherichia coli* KJ122 strain was evaluated. The *E. coli* KJ122 was engineered to improve succinic acid production by elimination of some genes (Δ*ldhA* Δ*adhE* Δ*ackA* Δ*(focA-pflB)* Δ*mgsA* Δ*poxB* Δ*tdcDE* Δ*citF* Δ*aspC* Δ*sfcA*). It produced excellent succinate yield (0.96 g/g glucose), high titer and productivity in mineral salts medium containing glucose under simple-batch anaerobic conditions. However, this strain does not efficiently utilize starch due to catabolic repression, thus glucoamylase was used to release sugar from cassava starch. The effects of temperature, pH, glucoamylase load, inoculum size and cassava starch concentration on succinic acid production were investigated. The fermentation was performed by batch SSF process in a 500 mL vessel with 350 mL working volume. The processes were carried out with pH of the medium adjusted to 5.5, 6.0, 6.5 and 7.0; temperature at 37, 39, 41 and 43^{o}C; different cassava starch concentrations of 70, 90, 110, 130 and 150 g/L; various enzyme loading of 300, 500, 700, 900, 1100 and 1300 U/g-cassava starch. An initial biomass concentration was

provided at the different value of 0.1, 0.2, 0.3, 0.6, 0.9, 1.2 and 1.5 OD_{550}.

Under the optimum conditions (pH 6.5, 41^{o}C, 70 g/L of cassava starch concentration, 0.6 OD_{550} cell density, and 500 U/g-cassava starch of glucoamylase), 70.08±0.12 g/L of succinic acid was produced after 72 h of fermentation in SSF process, with a yield of 1.01±0.013 g/g cassava starch which was equivalent to 0.91±0.013 g/g glucose consumed and productivity of 0.97±0.001 g/L/h. Therefore, the theoretical yield of 81.25% was achieved. A higher succinic acid concentration of 82.46±0.51 g/L, with a yield of 1.03±0.010 g/g cassava starch which was equivalent to 1.00±0.010 g/g glucose consumed and productivity of 1.15±0.008 g/L/h was produced after 72 h in fed-batch SSF. The result showed that the fed-batch SSF procedure was able to increase succinate concentration, yield and productivity considerably by about 17.67%, 2.00% and 18.56%, respectively. Under fed-batch SSF condition, 89.30% of the theoretical yield was achieved. Therefore, cassava starch would be an alternative raw material for an efficient production of succinic acid by engineered *E. coli* KJ122.

School of Biotechnology

Academic Year 2014

Student's Signature_____

Advisor's Signature_____

Co-advisor's Signature_____

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Kirin Khor

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LIST OF ABBREVIATIONS

ACKA	=	Acetate kinase
ADHE	=	Alcohol dehydrogenase
Amy	=	Alpha amylase
AMG	=	Glucoamylase
AMP	=	Adenosine monophosphate
ASPC	=	Asparte aminotransferase
ATP	=	Adenosine triphosphate
cAMP	=	Cyclic Adenosine monophosphate
CDW	=	Cell dry weight
CITF	=	Citrate lyase
DMRT	=	Duncan's multiple-ranges test
DNSA	=	3, 5-dinitrosalicyclic acid
DP	=	Degree of polymerization
E. coli	=	*Escherichia coli*
FOCA	=	Formate-lyase
FPU	=	Filter paper unit
g/L	=	Gram (s)/liter
h	=	Hour (s)
HPLC	=	High performance liquid chromatography
kg	=	Kilo gram

LIST OF ABBREVIATIONS (Continued)

L	=	Liter
LB	=	Luria Bertani
LDHA	=	Lactate dehydrogenase
MGSA	=	Methylglyoxal synthase
mM	=	Milli-molar
mL	=	Milli-liter
min	=	Minute (s)
NADH	=	Nicotinamide adenine dinucleotide (reduced form)
NAD^+	=	Nicotinamide adenine dinucleotide (oxidative form)
^oC	=	Degree Celsius
OD	=	Optical cell density
pKa	=	The logarithmic constant
POXB	=	Pyruvate oxidase
p.s.i	=	Pounds per square inch
PCK	=	Phosphoenolpyruvate carboxykinase
PEP	=	Phosphoenolpyruvate
PPC	=	Phosphoenolpyruvate carboxylase
PTA	=	Phosphotransacetylase
RDS	=	Rapidly digestible starch
rpm	=	Revolutions per minutes
SFCA	=	Malic enzyme
SHF	=	Separate hydrolysis and fermentation

LIST OF ABBREVIATIONS (Continued)

SDS	=	Slowly digestible starch
SSF	=	Simultaneous saccharification and fermentation
TDCDE	=	Propionate kinase
U	=	Unit
UV	=	Ultraviolet
V	=	Volt
vvm	=	Gas volume flow per unit of liquid volume per minute
WIS	=	water-insoluble solids
w/v	=	Weight per volume
YE	=	Yeast extract
μL	=	Micro-liter

CHAPTER I

INTRODUCTION

1.1 Significance of the Study

Succinic acid, a four-carbon dicarboxylic acid produced as an intermediate of the tricarboxylic acid cycle or as an end product of anaerobic metabolism, has been used widely in the agricultural, food, green solvent, and pharmaceutical industries (Zeikus *et al.,* 1999). Currently, succinic acid is valued as one of the key platform chemical used directly in preparation of biodegradable polymers such as polybutylene succinate and polyamides and as a raw material to synthesize fine chemicals of the C4 family, including 1,4-butanediol, tetrahydrofuran, N-methyl pyrrolidinone, 2-pyrolidinone and gamma-butyrolactone (Song *et al.,* 2008; Song and Lee, 2006). Thus, development of technology for succinic acid production is very important to provide the requirement of industry. In recent years, biological production of succinic acid from renewable resources has attracted significant interest because succinic acid production from fermentation has more advantages over chemical processes (Du *et al.,* 2007). Additionally, succinic acid production by fermentation has distinct advantages over productions of other organic acids and would contribute to reduce greenhouse gas because CO_2 is consumed during fermentation. Thus, this process would provide further incentive for the microbial succinate production. Because of the requirement of succinic acid in many applications is high and increasing every year, interest in anaerobic fermentation has intensified especially as how it relates to the utilization

of cheap carbon sources to produce higher-value fermentation derived succinic acid. However, the realization of bio-based succinic acid industrial production strongly depends on utilization of cheaper renewable resources to reduce the cost of fermentation process, for insteance agricultural, dairy waste products and starch (Werpy *et al.*, 2006). Therefore, using inexpensive carbon source such as cassava starch can provide the fermentation process to produce succinic acid more economically competitive.

Simultaneous saccharification and fermentation (SSF) is the most promising process for the production of organic acids or value-added products from raw materials with multiple researchers focusing on the process (Klinke *et al.*, 2001; Öhgren *et al.*, 2006; Philippidis and Hatzis, 1997). In SSF, the risk of product inhibition is minimized as the sugars obtained in hydrolysis are simultaneously utilized by the microorganism to produce value-added products. Enzyme (glucoamylase) inhibition is reduced, thus increasing sugar production rates, concentrations, and yields and decreasing enzyme loading requirements. The drawbacks associated with this process include the different operating conditions required for enzymatic hydrolysis and fermentation. This process has many advantages over separate hydrolysis and fermentation (SHF).

Succinate can be produced by fungi and bacteria, only bacteria are used in fermentation for food and beverage (Litchfild, 1996). Investigations of succinic acid production have been carried out with many bacteria. For instance, propionate-producing bacteria such as *Propionibacterium* species, typical gastrointestinal bacteria such as *Pectinatus* sp., *Bacteroides* sp., rumen bacteria such as *A. succinogenes* and *Mannheimia succiniciproducens* (Guettler *et al.*, 1996; Lee *et al.*, 2006). However, these microorganisms require more nutritional including complex components which increase cost involving with the production, and difficult to be modified. Metabolically

engineered *E. coli* strain named KJ122, produces succinate under simple nutrients, antibiotics for maintenance and expression of heterologous genes and clear genetic background (Jantama *et al.,* 2008a).

Cassava is one of the most important crop and abundant renewable in Southeast Asian region. It provides major part of the daily calories needs of people and grows more widely than grains (Omemu *et al.,* 2005). Cassava starch is cheap and easy to find in the tropical and subtropical areas like in Asia, especially in Thailand. Moreover, cassava is believed the cheapest sources of starch compared the cereals, tubers and root crops (Lu *et al.,* 2011). Cassava contains almost 70-75% of starch; starch is one type of complex sugar known as polysaccharide. Similar to cellulose, starch molecule are glucose polymers linked with α-1,4 and α -1,6 glycosidic bonds. To produce glucose from starch, it is necessary to break down the chain of this carbohydrate. Starch can be directly convert to fermentable sugar via enzymatic hydrolysis without performing pretreatment any process. Enzymes act as catalysts for the reaction, produce better yield, less byproducts and easy to handle due to not having a corrosive problem. Also, utility cost of enzymatic hydrolysis has recently been found to be lower compared to acid hydrolysis (Balat, 2007). Therefore, cassava starch is likely to be promising alternative feedstocks for microbial succinic acid production.

There are many reports that considered the utilization of cassava starch as a substrate. Ado *et al.* (2009) tested a co-culture of *Aspergillus niger* and *Saccharmyces cerevisiae* to produce ethanol by using cassava starch hydrolysate as substrate. Lu *et al.* (2011) investigated on algae to ferment cassava starch for biodiesel production. Ayo (2012) found that the feed concentration of 25 g/L of cassava starch hydrolysate had the highest conversion rate of 97% yielding maximum productivity of 11.40 g/L/h of

lactic acid, for 8 h after starting up. Some of these works produced ethanol, and some other works produced lactic acid or other products. However, none of the reports have shown the production of succinic acid from cassava starch. Therefore, in this study, it is an interesting to investigate the production of succinic acid from cassava starch as starting material by fermentation processes using metabolically engineered *E. coli* KJ122 (Jantama *et al.*, 2008a) that produces succinic acid as a major fermentative product. Moreover, the cultivation conditions and fermentation medium for the succinic acid production were also optimized. Batch simultaneous saccharification and fermentation (SSF) and fed-batch SSF were performed to achieve high succinic acid production.

1.2 Research Objectives

To achieve bio-based succinate production for large scale and to decrease petrochemical succinate processing, the production of bio-based succinate was performed with optimization of the parameters such as cassava starch feeding rate, pH, temperature, inoculum size, and enzyme loading during the SSF process by metabolically engineered *E. coli* KJ122 which provided an efficient production of succinic acid. Additionally, an enzyme, glucoamylase, has an ability to break down α-(1→4) and α-(1→6) glycosidic bonds of glucose subunits containing in cassava starch. Thus, the purpose of this study was to evaluate the performance of metabolically engineered *E. coli* KJ122 strain for producing succinic acid from cassava starch through enzymatic hydrolysis in SSF process. Therefore, the objectives of this research were:

a) To evaluate the effect of external glucoamylase enzyme loading, cassava starch concentration, initial cell density, pH and temperature on the amount of glucose

released from enzymatic hydrolysis of cassava starch and the succinic acid production simultaneously by metabolically engineered *E. coli* KJ122.

b) To demonstrate the feasibility of succinic acid production from cassava starch under combined SSF and fed-batch processes by metabolically engineered *E. coli* KJ122 using the optimum condition obtained from above mentioned parameters.

1.3 Research Hypothesis

Starch, known as polysaccharide, is a type of complex sugar, whose molecule consists of glucose polymers joined by α-1,4 and α-1,6 glycosidic bonds. Therefore, the previously engineered *E. coli* KJ122 strain would promote high titer, yield and productivity of succinic acid production from cassava starch as a sole carbon source by using glucoamylase enzyme to break down the chain of the carbohydrate. The SSF process for treating starch and producing targeted succinic acid simultaneously has an advantage over SHF with regard to higher productivity, less glucose inhibition on both of enzymatic hydrolysis and fermentation as well as lower capital investment. Thus, SSF process was applied in this work.

1.4 Scope and Limitation of the Study

The optimization of bio-based succinic acid production was performed with metabolically engineered *E. coli* KJ122 strain under combined SSF with batch and fed-batch processes using cassava starch as substrate. Parameters such as cassava starch concentration, initial cell concentration, pH, temperature, and enzyme loading were optimized during the experiment for an efficient production of succinic acid. Therefore, cost-effectiveness in succinate production would be obtained by means of utilization of the cassava starch.

CHAPTER II

LITERATURE REVIEW

2.1 Overview of Succinic Acid

2.1.1 Succinic Acid and Its Properties

Succinic acid was firstly discovered in 1546 by Georgius Agricola. Succinic acid (IUPAC systematic name: butanedioic acid), also known as dicarboxylic acid (Diprotic acid) with a chemical formula of $HOOC(CH_2)_2COOH$, is a chemical compound that plays a role in several biochemical processes (Zeikus *et al.*, 1999). The name derives from Latin succinum, meaning amber, from which the acid may be obtained. The carboxylate anion is called succinate and esters of succinic acid are called alkyl succinates. In chemical reaction, succinic acid is manufactured through oxidation of n-butane or benzene followed hydrolysis and finally dehydrogenation. In its pure form, succinic acid occurs as colourless triclinic prisms (α-form) and monoclinic prisms (β-form) (Fumagalli, 2007). Important properties of succinic acid are summarized in Table 2.1.

Table 2.1 Properties of succinic acid (Fumagalli, 2007).

Property	Details	Value	Units
Acidity	pKa1	4.21	-
	pKa2	5.64	-
Boiling point		235	^{o}C
Melting point		189	^{o}C
Molar mass		118.09	g/mol
Solubility	20^{o}C	58	g/L
Specific gravity		1.57	g/cm^3

2.1.2 Commercialization of Succinic Acid

Interestingly, the total market size for uses of succinic acid is more than $400,000,000 per year and the large use of succinic acid and its derivatives is around 20,000-30,000 tons per year (Beauprez *et al.*, 2011; Kidwell, 2008) with the increasing rate by 10% per year. Currently, up to 270,000 tons of industrial succinic acid is sold at the price of $5.90-8.80/kg depending on its purity (Walke and Vorlop, 2004) and is mainly produced by chemical process from butane or oxidation of benzene through maleic anhydride. To obtain the lower price, many researchers are trying to produce succinic acid from renewable resources with utilizing cheap carbon sources such as corn, molasses, glucose and sugars. It is expected that natural succinate price will be decreased by $0.55/kg if production size is above 75,000 tons (Kidwell, 2008). Because of its economics promise, fermentation-derived succinic acid has the potential to become a large volume commodity chemical that is forming the basis for supplying many important intermediate and specialty chemicals. Moreover, succinic acid can

replace many chemicals derived from benzene and intermediate petrochemicals, resulting in large decline pollution from the manufacture and consumption of over 250 benzene-derived chemicals (Ahmed and Morris, 1994; Jantama *et al.*, 2008b). Additionally, succinic acid production by fermentation has distinct advantages over productions of other organic acids and the microbial succinic acid production incorporates CO_2, a primary greenhouse gas, providing further incentive for production by microorganism.

2.1.3 Benefit of Succinic Acid

Succinic acid is valued as one of the key platform chemicals used directly in preparation of biodegradable polymers such as polybutylene succinate and polyamides and as raw material to synthesize fine chemicals of the C4 family for which demand is growing rapidly. Succinic acid is a platform chemical for many commondity and industrial chemicals (Figure 2.1). There are four existing succinic acid markets for applications in many industries (Sado and Tajima, 1980). First of all, succinic acid acts as an antimicrobial agent, as a flavoring agent, and as an acidulation/pH modifier. In the food technology, Sodium succinate and dilysine succinate are recently introduced as flavor enhancers which can replace monosodium glutamate in low sodium food (Jain *et al.,* 1989). Second, succinic acid is used in the production of health relating agents including pharmaceuticals, antibiotics, amino acids and vitamins. Third, succinic acid is also served as an intermediate chemical which can synthesize variety of chemicals for industrial application such as butanediol, tetrahydrofuran, butyrolacton, and other four carbon chemicals (Darke *et al.*, 1987). Finally, it has also been applied in the chemical industry as a surfactant, extender, and foaming agent as well as, ion chelator

in which it is used in electroplating to prevent corrosion and pitting of metals. Therefore, it is evident that the technology had been developed for succinic acid production to supply the requirement of industries.

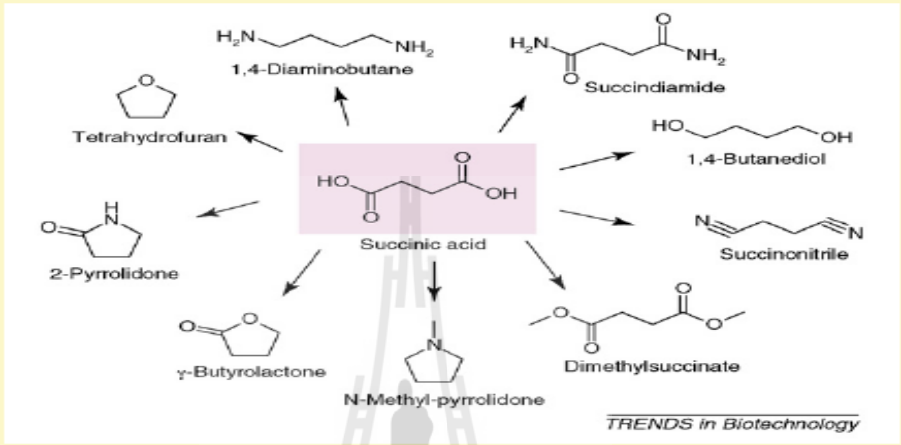


Figure 2.1 Various substances derived from succinic acid chemical conversion (Sauer *et al.*, 2008).

2.1.4 Succinate Production by Microbial Fermentation

Succinic acid is an intermediate of the tricarboxylic acid cycle and one of the fermentation end products of anaerobic metabolism by several anaerobic and facultative microorganisms (Song and Lee, 2006). Microorganism with a capability to produce succinic acid can be divided into two groups namely bacteria and fungi, only bacteria are used in fermentation for food and beverage (Litchfield, 1996). Investigations of succinic acid production were carried out with many bacteria. For instance, propionate-producing bacteria such as *Propionibacterium* species, typical gastrointestinal bacteria such as *E. coli*, *Pectinatus* sp., *Bacteroides* sp., rumen bacteria such as *Mannheimia succiniciproducens* (Lee *et al.*, 2001), *Actinobacillus*

succinogenes, and *Anaerobiospirillum succiniciproducens* (Guettler *et al.*,1996). It is noted that good succinate producers are isolated and cultured from the rumen.

There are many bacteria which have been found to produce high succinate as a major product in fermentation (Guettler *et al.*, 1996). Some of them including *A. succinogenes* (Du *et al.*, 2007; Guettler *et al.*, 1996; Li *et al.*, 2011; Meynial-Salles *et al.*, 2007), *Anaerobiospirillum succiniciproducens* (Lee *et al.*, 2001), and *M. succinoproducens* (Lee *et al.*, 2006; Song and Lee, 2006) are naturally produced as high productivity as 4 g/L/h with impressive titers of 300-900 mM and high yields more than 1.1 mol succinate/mol glucose. However, these bacteria have more nutritional requirements including complex components and are difficult to be modified, which increase cost involving in production, downstream processing and wastes (Jantama *et al.*, 2008a). Future development of succinate production needs two stages. First, fermentation needs strains cooperated with CO_2 in medium by using batch, fed batch, or continuous modes. In this case, medium should be cheap, microorganisms should require simple nutrients and the fermentation process should be simple (Sauer *et al.*, 2008). Second, product recovery are designed to purify succinate from mixed acids by using electrodialysis, ion exchange and amine-based on extraction (Song and Lee, 2006). Furthermore, *E. coli*, due to its fast growth, simple nutrients demands and clear genetic background, has been studied to produce succinic acid. Metabolically engineered *E. coli* KJ122 containing the knockout genes including Δ*ldhA* Δ*adhE* Δ*ackA* Δ*(focA-pflB)* Δ*mgsA* Δ*poxB* Δ*tdcDE* Δ*citF* Δ*aspC* Δ*sfcA*, was expected to be an excellent producer with excellent succinate yield (1.46 mol/mol glucose), high titer and productivity.

2.1.5 Succinate Production by *E. coli* KJ122

Among *E. coli* KJ derivatives, *E. coli* KJ122 was reported to be efficient for metabolizing glucose to succinic acid in high titer, yield and productivity. KJ122 was constructed to produce succinic acid in mineral salts medium containing glucose under anaerobic conditions by a combination of metabolic engineering and metabolic evaluation. This strain was engineered for improvements in succinic acid production by eliminating some genes such as Δ*ldhA* Δ*adhE* Δ*ackA* Δ*(focA-pflB)* Δ*mgsA* Δ*poxB* Δ*tdcDE* Δ*citF* Δ*aspC* and Δ*sfcA* (Jantama *et al.*, 2008a). The strain KJ122 significantly increased succinate yield (1.46 mol/mol glucose), titer (680-700 mM) and average volumetric productivity (0.9 g/L/h) with less other organic acids. Moreover, KJ122 produced 1.4-1.5 mol succinate per mol of glucose, 85% of the maximum theoretical yield (0.71 mol/mol glucose used) of succinate during simple, anaerobic, using mineral salts medium without the addition of plasmid or foreign gene in common fermentation (batch fermentation) and any complex nutrients. The increase in succinate yield and titer are presumed to result from increase in the availability of oxaloacetate and malate that allow a longer fraction of carbon to proceed to succinate (Figure 2.2). Therefore, the strain KJ122 is a potential biocatalyst for the economical production of succinic acid.

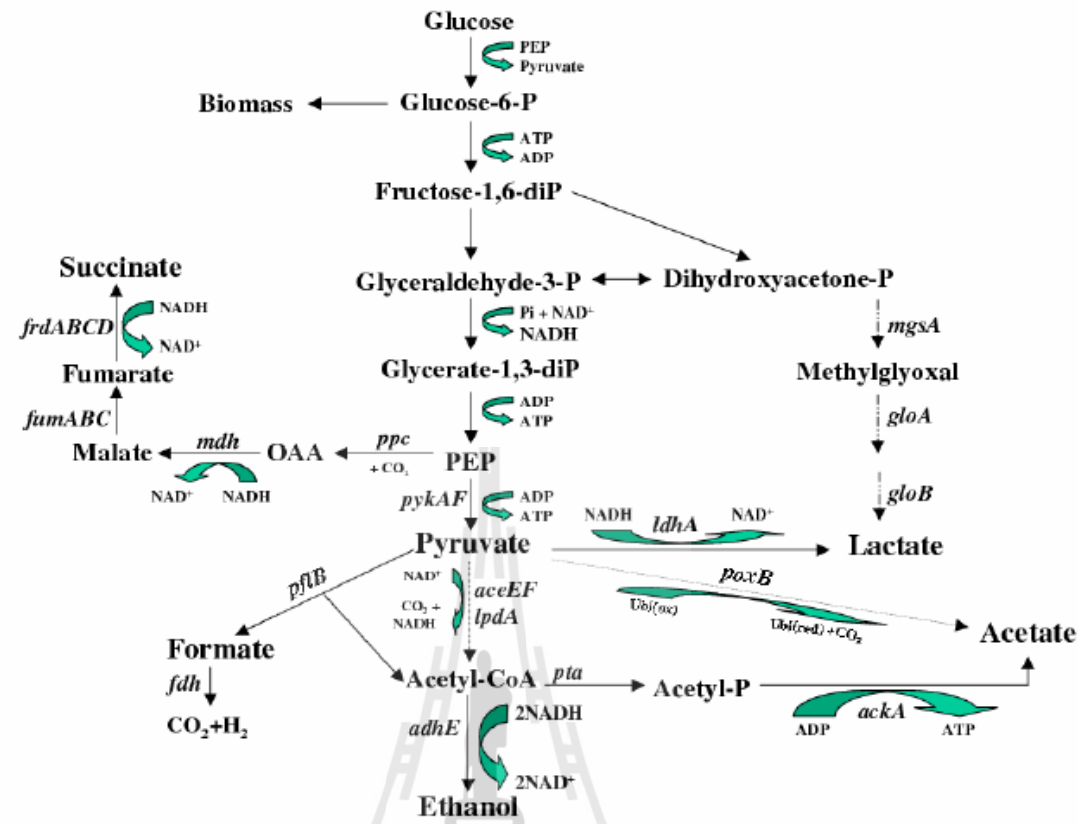


Figure 2.2. Central metabolic pathway of *E. coli*. Solid arrows represent central fermentative pathways. Dotted arrow represents microanaerobic pathway (*poxB*). Dash arrow represents minor lactate producing pathway (*mgsA*, *gloAB*). Genes: *pykAF*: pyruvate kinase, *ldhA*: lactate dehydrogenase, *pflB*: pyruvate formate-lyase, *pta*: phosphate acetyltransferase, *ackA*: acetate kinase, *adhE*: alcohol dehydrogenase, *ppc*: PEP carboxylase, *aceEF/lpdA*: acetyltransferase/ dihydrolipoamide acetyltransferase component of the pyruvate dehydrogenase complex, *mdh*: malate dehydrogenase, *fumABC*: fumarase, *frdABCD*: fumarate reductase, *fdh*: formate dehydrogenase, *mgsA*: methyglyoxal synthase, *gloAB*: glyoxylase, and *poxB*: pyruvate oxidase (Jantama *et al.*, 2008b).

2.1.6 Succinic Acid Production from Different Carbon Sources

Developing a cost effective culture medium with maximum yield production is one of the significant aspects in fermentation processes. Production of succinic acid has been reported from variety of carbon sources utilized by *E. coli* (Table 2.2). Recently, many researchers have focused on studying of conversion of agricultural waste products to succinate such as corn stalk (Li *et al.,* 2010), corn straw (Zhen *et al.,* 2010), cane molasses (Agarwal *et al.,* 2006; Liu *et al.,* 2008), glycerol (Lee *et al.,* 2001), whey (Whan *et al.,* 2008), sucrose (Liu *et al.,* 2008), corn cob hydrolysate (Yu *et al.,* 2010), soybean meal hydrolysate (Thakker *et al.,* 2011) and wheat-milling by-products (Dorado *et al.,* 2009). On the other hand, downstream processing is also considered as an importantly cost effective process to produce succinate (Jantama *et al.,* 2008a; Jantama *et al.,* 2008b; Sauer *et al.,* 2008). Therefore, the purity of substrates and less complex nutrient medium should be considered in the fermentation and purification process for succinic acid production.

Table 2.2 Comparison of succinate production from available carbon sources in *E. coli.*

Organism	Medium/condition	Succinate yield (g/L)	Reference
E. coli KJ122 (*ldhA, adhE, ackA, (focA-pflB), mgsA , poxB, tdcDE, citF, aspC, sfcA*)	Glucose AM1 (100 g/L) with 10g/L $NaHCO_3$,simple batch, 120h Incubation, pH maintained with 1:1Mixture of 6MKOH+3MK_2CO_3	108 [0.9][a]	Jantama et al., 2008b
E. coli SBS550MG (*ldhA, adhE, iclR, ackA-pta*), *L. latis pyc, Bacillus subtilis citZ*	Glucose (20g/L; 100 g total glucose) LB supplemented with 1 g/L $NaHCO_3$, 200 mg/L ampicillin, and 1mM IPTG, 100% CO_2 at 1 L/min STP headspace, repeated fed-batch fermentation , 95h incubation	39.9 [0.42]	Sanchez et al., 2005a; Cox et al., 2006
E. coli ATCC8739 (*pflB, ptsI*, and *pck* overexpressed)	Glycerol, NBS mineral salts medium supplemented with 5% (w/v) glycerol, 100mM potassium bicarbonate, pH controlled by 2.4M potassium carbonate in 1.2M potassium hydroxide, simple batch, 144h incubation time.	94.47 [0.65]	Zhang et al., 2010
E. coli AFP184 (*pflB, ldhA*, and *ptsG*)	Carbon source: glucose, fructose, xylose, glu/fru and glu/xyl supplemented with 5 g/L CSL, Dual phase aerobic growth and anaerobic production, sparging with air flow by CO_2,, 32 h incubation time.	30-40 [0.93-1.25]	Andersson et al., 2007
E. coli SBS550MG, SHL413, pUR400	Sucrose [NR], 20 g/L peptone, 10 g/LYE supplemented in medium, fed batch, dual phase, 100 mg/L ampicillin, 0.2 L/min, pH controlled by 2 M Na_2CO_3, 96 h incubation	32.65 [0.34]	Wang et al., 2011a
E. coli SD121 (*ldhA, pflB, ptsG*) *ppc* overexpression	Corn stalk hydrolysate, two-stage fermentation, 20 g/L tryptone, 10 g/L YE, pH controlled by 10M NaOH and 10% H_2SO_4 (v/v) in aerobic phase, and 4M Na_2CO_3 in anaerobic phase, 70 h incubation time	57.81 [0.508]	Wang et al., 2011b
E. coli K12, *pflB, ldhA, ppc*, Trc-*Bspck*	Corn stalk hydrolysate, LB medium, dual-phase fermentations, 16h incubation time	11.13 [0.69]	Liu et al., 2012
E. coli HL27659K, pKK313, pRU600 (*iclR, sdhAB, ackA-pta poxB, pstG*) *Rhizobium etli pyc* overexpressed	Soybean meal hydrolysate, LB medium, 10 g/L $MgCO_3$, appropriate antibiotics, Dual phase fermentation, 48h incubation time.	36.84 [0.76]	Thakker et al., 2013

E. coli is profoundly known to be able to metabolize all hexose and pentose sugars (Asghari *et al.*, 1996; Underwood *et al.*, 2004), to grow fast without a requirement of complex nutrients, and easily manipulated its metabolic pathways by genetic engineering. It can also be a potential target microorganism to improve strain

and design production process of succinic acid (Lin *et al.,* 2005). Furthermore, *E. coli* ATCC 8739 named KJ122 was possibly produced by a combination of metabolic engineer and metabolic evaluation for succinic acid production with glucose medium under anaerobic conditions with high yield, titer and average productivity. However, the succinic acid production by *E. coli* KJ122 from cassava starch has not yet been investigated. Also, this strain does not have strong ability to metabolize polysaccharide like starch that contain in raw material such as corn, potato, wheat and cassava. To provide this ability in KJ122 strain, glucoamylase should be added prior to fermentation. Therefore, the strain KJ122 was used throughout this research to study for production of succinic acid from cassava starch.

2.2 Factors Affecting the Succinate Production

There are several research that need to be addressed in order to produce succinic acid within the targeted cost, development of high performance succinic acid producing microorganisms and lowering the cost of the raw material. Many factors affecting the succinic acid production have been investigated. The optimization of fermentation processes requires profound knowledge of the factors determining microbial metabolism, and the influence of process parameters.

2.2.1 Effect of Substrate Concentration

At high concentrations, some substrates can also inhibit the enzyme activity. Thus, the initial sugar concentration plays an important role in the succinic acid fermentation. Uncompetitive inhibition is substrate inhibition which occurs at high substrate concentrations. It happens when two molecules of substrate can bind to the

enzyme, and thus block the activity. Some works showed that high initial glucose concentration in raw material reduced both the specific growth rate and substrate utilization rate due to the substrate inhibition phenomenon (Tango and Ghaly, 1999). At concentration above 80 g/L of substrate, considerable sugar remain unfermented, probably due to product inhibition (Madihah *et al.,* 2001), while 120 g/L of substrate the fermentation activity of culture is negligible (Qadeer *et al.,* 1980) due to substrate inhibition.

2.2.2 Effect of Temperature

Temperature is the key environmental parameter that affects the fermentation process (Yuwono and Kokugan, 2008). Low temperature has been reported to introduce the outgrowth of contaminating microorganism, thereby influencing the performance of the organic acid production (Neysens and De Vuyst, 2005). Hujanen and Linko (1996) reported that the highest organic acid production with low organic acid concentration and yield at one temperature whereas the low organic acid productivity obtained resulted in high organic acid concentration and yield at another temperature. For *E. coli*, which is known to grow at the optimal temperatures was 37^{o}C for maximum productivity and yield. Some previous reported investigated the cultivation temperature on the solid-state fermentation of organic acid production by controlling the growth temperatures at 22, 30, 35, and 40^{o}C. The results from measuring the residual starch and reducing sugar in 4 h and 8 h indicated that there was increased in starch hydrolysis and reducing sugar accumulation as the temperature increased from 22 to 30^{o}C, and a further increase in temperature from 30 to 40^{o}C resulted in a slight improvement for the saccharification by both *R. oryzae* 2062 and *R.*

oryzae 36017 cultures. Therefore, the organic acid production and biomass growth were affected by the temperature.

2.2.3 Effect of pH

The fermentation pH can be either set at the beginning and then left to decrease due to acid production or it is controlled by an addition of alkaline solutions. Moreover, the culture pH plays a crucial role in determining the outcome of sugar metabolism. The weak organic acids, produced as end-products of metabolism are, by nature, toxic to the cell, and are able to partition in the cell membrane in their undissociated form (Jones and Woods, 1986). At sufficiently high acid concentration, the pH gradient across the membrane collapses, resulting in total inhibition of cell metabolic functions within the cells. At low pH, the accumulation of acid end products, cause a decrease in growth rate which was eventually halted, although substrate utilization and cellular metabolism continue (Zhu and Yang, 2004). The optimal pH for succinic acid production varies between 6.0 and 7.0. A pH approximate 7 is optimal for *E. coli* strains. Previous studies investigated the influence of culture pH on organic acid fermentation from molasses where organic acid fermentation were performed in a jar fermentor at 38^{o}C and pH 5.0-9.0. Although the optimum pH for cell growth was seen to be 8.0, the organic acid fermentation at pH 7.0 was completed faster than that at pH 8.0. The cell growth at pH 5.0 almost completely ceased after 10 h of fermentation, the highest organic acid concentration was obtained at pH 7.0 with a comparable yield with pH 6.0 (Wee *et al.*, 2004). Moreover, some reported showed the effect of various initial pH on the lactic acid production of the immobilized microorganism during batch fermentation of liquid pineapple waste. At initial pH 6.5, cell started to utilize glucose

earlier and at a faster rate than at other initial pH. Maximum organic acid concentration was attained at initial pH 6.5. Further increase in initial pH beyond 6.5 does not improve the lactic acid production (Idris and Suzana, 2006). It is possible that the higher initial pH brought too much stress on the microorganism metabolic abilities.

2.2.4 Effect of Enzyme Loading

Non specific reactions may result in poor product yields. High chemicals and energy consumption as well as harmful by-products have a negative impact on the environment. In a number of cases, some or all of these drawbacks can be virtually eliminated by using enzymes. Enzyme reactions may often be carried out under mild conditions, they are highly specific, and involve high reaction rates. In addition, as only small amounts of enzymes are needed in order to carry out chemical reactions even on an industrial scale, both solid and liquid enzyme preparations take up very little storage space. Mild operating conditions enable uncomplicated and widely available equipment to be used, and enzyme reactions are generally easily controlled. They also reduce the impact of manufacturing on the environment by reducing the consumption of chemicals, water and energy, and the subsequent generation of waste. They are biological catalysts in the form of proteins that catalyze chemical reactions in the cells of living organisms. In general, these metabolic requirements can be defined as 1) Chemical reactions must take place under the conditions of the habitat of the organism; 2) Specific action by each enzyme; 3) Very high reaction rates. Nevertheless, the price of the enzyme is still the problem existing in the biosynthesis of succinic acid. The cost can be significantly reduced if the amount of enzyme loading in fermentation decreases. Many researchers have studied on how to conduct fermentation with less amount of

enzyme loading, but produce higher products (Absar *et al.*, 2009; Öhgren *et al.*, 2007). Wang *et al.* (2012) studied the efficient 2,3-Butanediol production from cassava powder by a crop-biomass-utilizer, *Enterobacter cloacae subsp. dissolvens* SDM, with variation of glucoamylase loading in the range from 100 U/g to 1000 U/g cassava powder. They found that at the dosage of 400 U glucoamylase/g cassava powder, glucose was completely released within 4 h, and this glucoamylase usage was selected for the research. Chen *et al.* (2011) optimized succinic acid production by using simultaneous saccharification and fermentation of acid-pretreated rapeseed meal with *Actinobacillus succinogense*. In this study, pectinase loading ranging from 0% to 3.0% was investigated in SSF and found that increasing the pectinase loading from 2% to 3% had no influence on succinic acid concentration or production rate and the low sugars concentration after culture for about 20 h, thus pectinase loading of 2% was optimal for succinic acid production. The results indicated that enzymatic hydrolysis was the limiting step in the SSF. Therefore, to reduce the product cost, it is not necessary to utilize excessive high amount of enzyme for fermented products in SSF.

2.2.5 Effect of Cell Density

High cell densities are prerequisite for high productivity and shorter fermentation time. In fermentation process, an adequate amount of cells is necessary for complete substrate utilization and adequate production yield (Agbogbo *et al.*, 2007). Souza *et al.* (2012) reported that the increase in the inoculum size led to an increase in the cell viability of a thermo tolerant mutant of *Saccharomyces cerevisiae* and the amount of product in the fermentation depend on the amount of cells in the initial inoculum. They also mentioned that the initial cell concentration affects the

fermentation rate, product concentrations, and yield but average specific cell growth rate decrease at high initial cell concentrations. To find the possible initial cell concentration, Shen *et al.* (2012) constructed the experiment in the range of initial cell concentration of 1.0-50 g/L. They found that the final product concentration was obviously increased from 14.8 to 21.2 g/L, when the initial cell inoculation increased from 1.0 to 3.0 g/L. However, the final product concentration was almost constant as initial cell inoculation varied from 3.0 to 5.0 g/L. Similar to this work, Palmqvist *et al.* (1996) suggested that increasing initial cell inoculation could accelerate the fermentation rate, the more initial cell density was inoculated, the shorter duration of glucose accumulation was appeared but product yield could not be significantly improved by inoculating very high cell density.

2.3 Overview of *E. coli* KJ122 Strain

Previously, some *E. coli* KJ strains were constructed from derivation of wide type *E. coli* ATCC 8739. The biocatalyst, KJ073 (Δ*ldhA::FRT* Δ*adhE::FRT* Δ*ackA::FRT* Δ*(focA-pflB)::FRT* Δ*mgsA* and Δ*poxB*) was constructed by combining metabolic engineering and metabolic evolution, produced 1.2 mol of succinic acid per mol of glucose (Jantama *et al.*, 2008a). However, this strain also accumulated FRT fragments. Development of a new strain (KJ097) was genetically improved by eliminated all FRT sites to achieve the maximum theoretical yield (1.71 mol succinate per mol glucose) (Jantama *et al.*, 2008b). The next strain, KJ098 contains any intervening sequences within the respective deleted regions (*tdc DE* and *aspC*) and is equivalent to KJ097 for producing succinate. Therefore, KJ122 was constructed and able to be a high succinic acid producer. This strain was knocked out some genes such

as Δ*ldhA* Δ*adhE* Δ*ackA* Δ*(focA-pflB)* Δ*mgsA* Δ*poxB* Δ*tdcDE* Δ*citF* Δ*aspC* Δ*sfcA* and produced excellent succinate yield (1.46 mol/mol glucose), high titer and productivity.

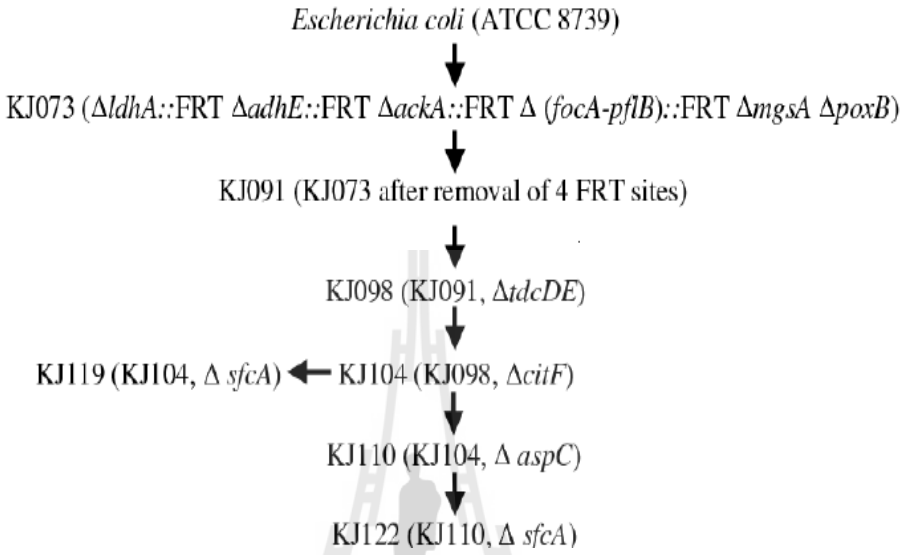


Figure 2.3 Strain Constructions (Jantama *et al.*, 2008b).

Brief of main events happened during metabolic engineering and evolution (Chan, 2010)

1. Elimination of lactate dehydrogenase (*ldhA*): This pathway was knocked out to conserve both NADH and carbon atoms and helps channeling carbon to acetyl-CoA pool.

2. Elimination of alcohol dehydrogenase (*adhE*): Mutation of the pathway had function to conserve both NADH for further succinate formation through the native fermentation pathway, and carbon atoms in the form of acetyl-CoA.

3. Elimination of acetate kinase (*ack*): The route conserved carbon atoms and prevented the conversion of acetyl-P to acetate accumulated during anaerobic conditions.

4. Elimination of formate-lyase (*focA-pflB*): The deletion of formate-lyase was expected to disrupt the formate production as reductant and the extremely production of acetyl-CoA, a potential source of acetate. Deletion of *focA-pflB* is well known to cause acetate auxotrophy under anaerobic conditions (Sawers *et al.*, 1988). Surprisingly, after selection (metabolic evolution), acetate was omitted and KJ strains were developed without acetate auxotrophy.

5. Elimination of methylglyoxal synthase (*mgsA*): The objective of knocking out of *mgsA* is to dissipate lactate and methylglyoxal, an inhibitor of both growth and glycolysis.

6. Elimination of pyruvate oxidase (*poxB*): Pyruvate oxidase plays role as a potential source of acetate and CO_2 during incubation under microaerophilic conditions. However, deletion of *poxB* did not reduce acetate production. The mutant resulted in unexpected changes in fermentation products, an increase in succinate and decrease in malate. In the strain, cell yield and succinate production were improved during medium transfers while malate, pyruvate and acetate were also increased.

7. Elimination of propionate kinase (*tdcDE*): Expression of *tdcD* could functionally replace *ackA,* increasing the production of ATP and acetate from acetyl-P and providing a competitive growth advantages. In contrast, deletion of *tdcD* and *tdcE* (adjacent genes) in KJ091 to construct KJ098 resulted in malate production omitted, actatate and pyruvate production reduced, and succinate formation increased.

8. Elimination of citrate lyase (*citF*): Under anaerobic conditions, OAA is partitioned between a reduced product, malate, and a more oxidized intermediate, citrate. Citrate represents a potential source of the acetate. Citrate can be converted to OAA and acetate by citrate lyase (*citDEF*), a mechanism proposed to recycle the intracellular OAA pool

for other metabolic function (Nilekani and Sivaraman, 1983). The *CitF* was knocked out in KJ098 to produce KJ104. There was no effect on succinate and acetate levels, even though; cell yield was decreased by 22%.

9. Eliminating the combination of asparte aminotransferase (*aspC*) and malic enzyme (*sfcA*): Aspartate aminotransferase (*aspC*) is a multiple enzyme that catalyzes the synthesis of aspartate, phenylanine and other compounds by transamination. One of its reactions, L-aspartate is synthesized from OAA by transamination with L-glutamate. Deletion of *aspC* was speculated to increase succinate production by reducing carbon flow into aspartate. However, mutation of *aspC* in KJ110 had no effect on succinate yield, cell yield, or acetate. Therefore, aspartate migh be formed by alternative pathways such as aspartate ammonia-lyase (*aspA*). The combination of *aspC* and *sfcA* (malic enzyme) deletion in KJ122 had improved succinate yield, titer and average productivity by 8%, 13% and 14%, individually even though single deletion of *aspC* or *sfcA* had no significant development in succinate production. It was presumed that the single deletion was inefficient in this step because they need to be compensated, in part by increasing flow through the remaining enzyme activity, malic enzyme or aspartate.

2.4 Overview of Fermentation Process

2.4.1 Simultaneous Saccharification and Fermentation (SSF)

SSF is the most promising process for organic acid or value-added products from raw materials with multiple researchers focusing on the process (Klinke *et al.,* 2001; Philippidis and Hatzis, 1997; Romani *et al.,* 2008; Teymuri *et al.,* 2005). SSF has many advantages over SHF. It eliminates the use of separate reactors for saccharification and fermentation, thus reducing equipment capital cost and

contamination risk due to the simple process. In addition, it decreases the total process time and increases saccharification yield (Li *et al.,* 2013). In SSF, the risk of product inhibition is minimized as the sugar accumulation, which is the end-product in separate hydrolyzation of substrates that are simultaneously utilized by the microorganism to produce value-added products. Enzymes inhibition is reduced thus increasing sugar production rates, concentrations, and yields and decreasing enzyme loading requirements. Nonetheless, the drawbacks associated with this process include the different operating conditions required for enzymatic hydrolysis and fermentation.

2.4.2 Previous Biobased-chemical Production by SSF Process

Several experiments have been conducted to obtain optimum yield of biobased-chemical by using SSF process. The results and other information of previous study are described below.

Srichuwong *et al.* (2009) constructed the SSF process with *S. cerevisiae* to produce ethanol from potato mash. The ethanol yield of 16.61% (v/v) was obtained which was equivalent to 89.7% of the theoretical yield.

Öhgren *et al.* (2007) reported on a comparison between simultaneous saccharification and fermentation and separate hydrolysis and fermentation using steam-pretreated corn stover. The enzymatic loading in these experiments was 10 FPU/g water-insoluble solids (WIS) and the yeast concentration in SSF was 1 g/L (dry weight) of a *S. cerevisiae* strain. SSF gave a 13% higher overall ethanol yield than SHF (72.4% versus 59.1% of the theoretical).

Wang *et al.* (2013) studied the effects of incubation duration on delignification efficiency and structural modification of cellulose, as well as the

digestibility of cellulose by SSF with white rot fungus *Trametes velutina* D10149. The result showed that the ethanol concentration of 5.16 g/L was detected in the fermentation broth from the cellulose sample biodegraded for 16 weeks after 24 h SSF.

Ethanol production by *S. cerevisiae* NRRL Y-2034 from wheat straw (WS) by SHF and SSF was studied by Saha (2013). This strain produced 13.3 g ethanol per liter in 30 h from biodetoxified wheat straw hydrolysate (WSH) by SHF. In SSF experiments, the strain produced 13.0 g ethanol per liter in 72 h from the non-detoxified WS but took 48 h to produce 13.1 g ethanol per liter from biodetoxified WS. By comparison, SSF of washed solid residues yielded 12.6 g ethanol per liter in 43 h. SSF offered advantage over SHF in reducing the total time of conversion of pretreated WS to ethanol by 53-57%.

Yee *et al.* (2012) examined the response of switchgrass lines to milder pretreatment conditions with yeast-based simultaneous saccharification and fermentation and a consolidated bioprocessing approach using *Clostridium thermocellum, Caldicellulosiruptor bescii* and *Caldicellulosiruptor obsidiansis*.

Ikwebe and Harwey (2011) applied SSF to study intensification of bioethanol production in an Oscillatory Baffled reactor (OBR). *S. cerevisiae* was employed as the fermenting organism at 38ºC and pH 4.8. In the first part of this work the use of the OBR resulted in a 7% increase in glucose yield compared with a shake flask, after 48 h of saccharification with 8.0 g/L ethanol in the OBR. This represented 89.8% of the theoretical yield, as compared with 7.7 g/L in the shake flask representing 81.29%, a difference of 9 percentage point.

Huang *et al.* (2005) employed SSF process to produce lactic acid by fungal species of *Rhizopus arrhizus* 36017 and *Rhizopus oryzae* 2062 with utilizing

potato starch as carbon source. Both *R. arrhizus* 36017 and *R. oryzae* 2062 had a capacity to carry out a single stage SSF process for lactic acid production from potato starch wastewater. A growth condition with starch concentration approximately 20 g/L at pH 6.0 and 30ºC was favourable for both starch saccharification and lactic acid fermentation, resulting in lactic acid yield of 0.85–0.92 g/g associated with 1.5–3.5 g/l fungal biomass produced in 36–48 h fermentation. Finally, SSF process has been applied successfully in the production of bioethanol and other bioproducts.

2.4.3 Succinic Acid Production by SSF Process

Up to date, there are a few works applying SSF process to produce succinic acid. Zhang *et al.* (2010) have only reported about a pre-treated corn stover with diluted alkaline that could be potentially used to produce succinic acid by *A. succinogenes* in SSF process. The result showed that the maximum value of succinic acid concentration and yield were obtained 47.4 g/L and 0.72 g/g, respectively. In the experiment, batch SSF process was constructed in optimization condition within initial substrate concentration of 70 g/L, enzyme loading supplied 20 FPU cellulase with 10 U cellubiase/g substrate at 38ºC. Additionally, succinic acid production by *A. succinogenes* with utilizing acid-pretreated rapeseed meal as a substrate in fed-batch SSF process was established by Chen *et al.* (2011). Succinic acid concentration, yield and productivity was reached 23.4 g/L, 11.5 g/100 g dry mater and 0.33 g/L/h, respectively in SSF process at a culture pH 6.4 with pectinase loading 2% without yeast extract supplementation. Among these researches, utilization of cheap starchy material such as cassava as a substrate to produce succinic acid has never been reported. Furthermore, the present work was be expected to develop an efficient strategy for

simultaneous saccharification and fermentation of cassava starch for succinic acid production using *E. coli* KJ122 with providing high titer, yield and productivity.

2.5 Overview of Starch

Starch is a main source of energy in human diet and animal feed. Besides being an energy source, starch is widely used as a functional texturizer in food industries. Starch found naturally in granules form most plants. It is also found that cereal seeds in maize, wheat, rice, barley and in tubers/roots from potato are especially rich in starch. It is the most abundant and universally distributed forms of storage polysaccharide in plants, and occurs as granules in the chloroplast of green leaves and amyloplast of seeds, pulses and tubers (Tester and Debon, 2000). Starch granule organization is very complicated and depends strongly on the botanical origin. Starch exists naturally in the form of discrete granules within plant cells. The starch granule is mainly composed of a mixture of two polymers (α-glucan polymers): an essential linear polysaccharide called *amylose* (20-30%; water soluble linear polymer) and highly branched polysaccharide called *amylopectin* (70-80%; water insoluble branched polymer).

2.5.1 Amylose

Amylose is essential a linear chain of (1→4)-linked α-D-glucopyranosyl units and very few α-(1→6) branches, may occur once in every 180-320 units, or 0.3-0.5% of the linkages, which causes it to be hydrolyzed more slowly but have higher density (Figure 2.4). The average molecular weight of amylose molecule is about 106 g/mol and degree of polymerization (DP) of 6000. The chains can easily form single or double helices. The amylose chain gives the molecules a right-handed spiral or helical

shape. The inside of the helix contains predominantly hydrogen atom and is lipophilic, while the hydrophilic hydroxyl groups are positioned on the outside of coil (Whistler and BeMiller, 1997).

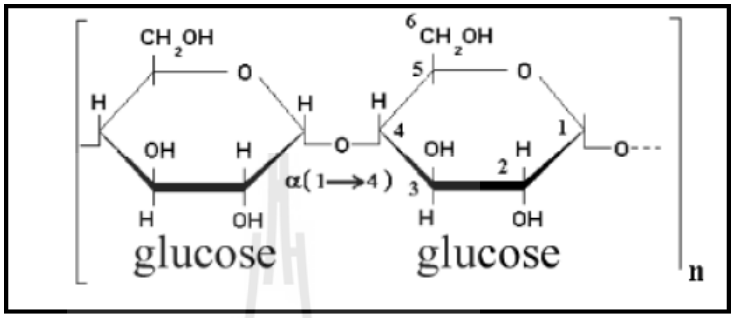


Figure 2.4 Chemical structures of α-amylose macromolecule (Oates, 1997).

2.5.2 Amylopectin

In contrast, amylopectin is a soluble and highly branched polymer of glucose found in plants and has an average molecular weight range from 10^7 to 5×10^8 g/mol and DP of 2×10^6. This makes it one of the largest polymers in nature, the other being amylose. Glucose units are linked in the consistency of thousands of short linear chain of (1→4)- linked α-D-glucopyranosyl units, linked to each other by α-(1→6) linkages occurring every 24 to 30 glucose units. The branch point linkages constitute 4-5% of the total linkages (Whistler and BeMiller, 1997). The most important characteristics of native starch are white in color, absence in unpleasant odor, and provide a high peak viscosity which is very useful in many applications (Sriroth, 1999). The amylopectin structure is described by a cluster model (Figure 2.5). An amylopectin molecule consists of main chain, called the C chain, which carries the one reducing end-group and numerous branches, in termed A chains and B chains. Short chains (A chains)

of DP 12-16 that can form double helices are arranged in clusters. The clusters comprise 80% to 90% of the chain and are linked by longer chains (B chains) that form the other 10% to 20% of the chains. Most B chains extend into 2 (DP about 40) or 3 clusters (DP about 70), but some extend into more clusters (DP about 110) (Thompson, 2000).

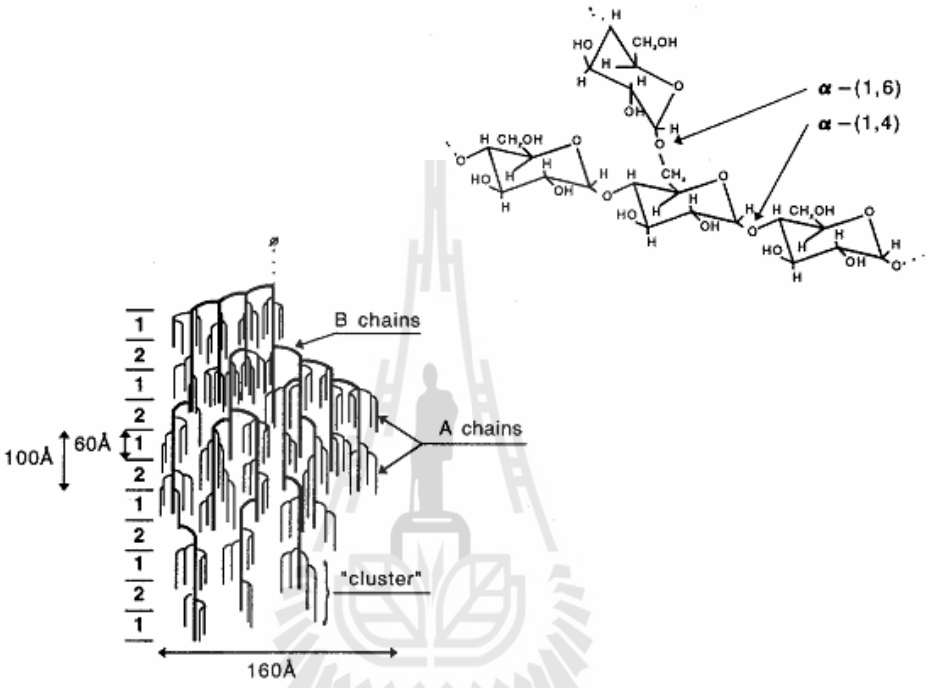


Figure 2.5 Cluster model of amylopectin (Whistler and BeMiller, 1997).

The way of starch granules being stored suits to its role. Although it is insoluble in water and densely packed but it still allows for plant's metabolic enzymes. Starch technologists who are interested in the hydrolysis of the component macromolecules noted that once the granule has been destroyed, it will be useful. Effect of hydrolysis enzyme on native starch granules has been prevailed by closer examination by many researchers (Tonukari, 2004). The chemical composition of starch sources are shown in Table 2.3.

Table 2.3 Chemical composition of starch sources.

Chemical composition (% dry basis)	Starch sources			
	Cassava root	Wheat (whole grains)	Potato (whole grains)	Maize (whole grains)
Moisture	70.0	68.3	75.8	75.0
Starch	24.0	26.2	19.9	19.0
Fiber	2.00	2.00	1.10	2.70
Protein	1.00	1.20	1.80	3.20
Other substances	3.00	2.30	1.40	0.10

Source : (Parigi-Bini and Chiericato, 1976; Tonukari, 2004)

2.6 The Classification of Starch

Starch can be classified as nutritional propose. The classification is based on the extent of digestibility of starch as follows: (Englyst and Hudson, 1996; Englyst and Hudson, 1997).

2.6.1 Rapidly Digestible Starch

Rapidly digestible starch (RDS) consists mainly of amorphous and dispersed starch. It is digested quickly in the small intestine. In *vitro* testing, it is hydrolyzed to the constituent glucose molecules in 20 min. RDS is best exemplified by freshly cooked starchy foods, such as cassava starch, mashed potatoes. In this case, starch granules have been gelatinized and are more accessible to enzymatic digestion.

2.6.2 Slowly Digestible Starch

Slowly digestible starch (SDS) likes RDS, is expected to be completely digestion in the small intestine but it is digested more slowly than RDS. During in vitro hydrolysis, SDS is converted to glucose during 20 and 110 min. This category consists of physically inaccessible amorphous starch and raw starch with a type A and type C crystalline structure, such as cereals.

2.7 Overview of Glucose

Glucose ($C_6H_{12}O_6$) contains six carbon atoms, one of which is part of an aldehyde group, and therefore known as an aldohexose (Figure 2.6). Glucose commonly presents in a form of white substance or a solid crystal. Glucose also known as confectioners' syrup and can be dissolved in water as an aqueous solution (Van Der Marrel *et al.*, 2002). The molar mass and density of glucose is 180, 16 g/mol and 1.54 g/cm^3, respectively. The melting point of α-D-glucose and β-D-glucose is 146^{o}C and 150^{o}C, respectively. Glucose, a monosaccharide also known as grape sugar, is an important carbohydrate in biology. The living cells use it as a source of energy and metabolic intermediate. It is one of the main products of photosynthesis in both prokaryotes and eukaryotes.

From two stereoisomers of the aldohexose sugars, only one of its form in which D-glucose is biologically active. D-glucose is often referred to as dextrose monohydrate or, especially in the food industry, simply dextrose (from dextrorotatory glucose). Glucose is produced commercially via enzymatic hydrolysis of starch. Many crops can be used as the source of starch. Maize, rice, wheat, potato, cassava, arrowroot, and sago are all used in various parts of the world.

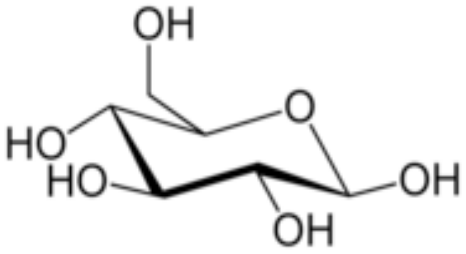


Figure 2.6 Glucose structure (Van Der Marrel *et al.*, 2002).

2.8 Enzymatic Hydrolysis

The carbohydrates in the starchy materials must be pretreated in order to hydrolyze the starch to simple sugar. Hydrolysis process for fermentable sugar involved two methods which are enzymatic and acid hydrolysis. Acid hydrolysis uses acid as a catalyst for the reaction while enzymatic hydrolysis uses enzyme as its catalyst. Enzyme hydrolysis was preferred compared with acid hydrolysis because acid is very corrosive and difficult to handle. Moreover, utility cost of enzymatic hydrolysis is recently found to be lower compared with acid hydrolysis (Balat, 2007) as enzymatic hydrolysis is usually conducted at mild condition. Hence, enzymatic hydrolysis is more preferable in this study.

Starch is degraded by enzyme called α-amylase and glucoamylase which are derived from bacteria (Nigam and Singh, 1995). The degradation products would be oligosaccharide fragments such as glucose, maltotetraose, maltose, maltoriose as well as oligosaccharide containing α-1,6-branches. All of the components are known as dextrin mixture. However, the percentage of glucose is very low and needs further enzymatic treatment. The oligosaccharides formed from amylase activity are further hydrolyzed by glucoamylase. Glucoamylase also known as amyloglucosidase, is an exoacting hydrolase, which releases single glucose molecules from the non-reducing

end of α-(1,4) oligo- or polysaccharides and is being used in the saccharification step. Meanwhile, the glucoamylase breaks the α-(1,6) glycosidic bonds in the liquefied starch chains and completing the hydrolysis of starch. Saccharification leads to about 96% yield of glucose, and about 4% byproducts (Nigam and Singh, 1995; Wu and Lee, 1997).

2.8.1 Liquefaction Step

In liquefaction step, gelatinization is required to increase the rate of hydrolysis as the native starch is slowly degraded by α-amylase (Shariffa *et al.*, 2009). Therefore, gelatinization and swelling are needed to make the starch easy to breakdown by enzyme (Aehle, 2007). Gelatinization is achieved by heating starch with water which occurs automatically when starchy materials are cooked. Under this condition, the pores of the starch become larger than usual and the enzyme can easily penetrate into the starch polymer and interrupt the hydrogen bond between the polymer chains to become weak (Shariffa *et al.*, 2009). Liquefaction process is employed to loosen the structure of starch polymer and reduces the viscosity of the gelatinized starch and ease the next hydrolysis processing. α-amylase enzyme, which is thermostable enzymes (Liu *et al.*, 2008; Nigam and Singh, 1995) is being used in liquefaction step where it will initially attack the interior bonds of starch granules which composed of long chain of glucosyl residues linked by α-(1,4) glycosidic bond. Additionally, α-amylase operates optimally at 90^{o}C and pH 6 (Liu *et al.*, 2008). Again, the optimum liquefaction pH was reported to be 6.0 by Van Der Marrel *et al.* (2002). The optimum α-amylase action and reducing sugar production in continuous enzymatic hydrolysis was obtained at pH 6.0 and 30^{o}C.

2.8.2 Saccharification Step

Saccharification step is important to further hydrolyze the liquefied starch. Glucoamylase also known as amyloglucosidase is being used in the saccharification step. The glucoamylase breaks the α-(1,6) glycosidic bonds in the liquefied starch chains. Saccharification leads to about 96 % yield of glucose, and about 4% byproducts. Saccharification of corn starch has been reported to be performed at 55^{o}C and pH 5 while the optimum saccharification has been conducted at 60^{o}C and pH 4.5 (Van Der Marrel *et al.,* 2002). In separate study, Agarwal *et al.* (2001) has found that at high temperature, the rate of saccharification reduced substantially at the optimum condition for saccharification were at 45^{o}C and pH 5.0. Figure 2.7 describes the action of hydrolytic enzymes on amylose and amylopectin. α-amylase is an exo-acting enzyme cleaving β-maltose molecules from the non-reducing end of amylose or from the outer branches of amylopectin. Meanwhile, α-amylase is an endo-acting enzyme hydrolyzing α-(1,4) bonds at random, producing the malto-oligosaccharides (linear or branched). Furthermore, amyloglucosidase is an exo-acting hydrolase, which releases single glucose molecules from the non-reducing end of α-(1,4) oligo- or polysaccharides. This enzyme is unique because it can hydrolyse α-(1,6) branching points, and completing the hydrolysis of starch (Tester *et al.*, 2004).

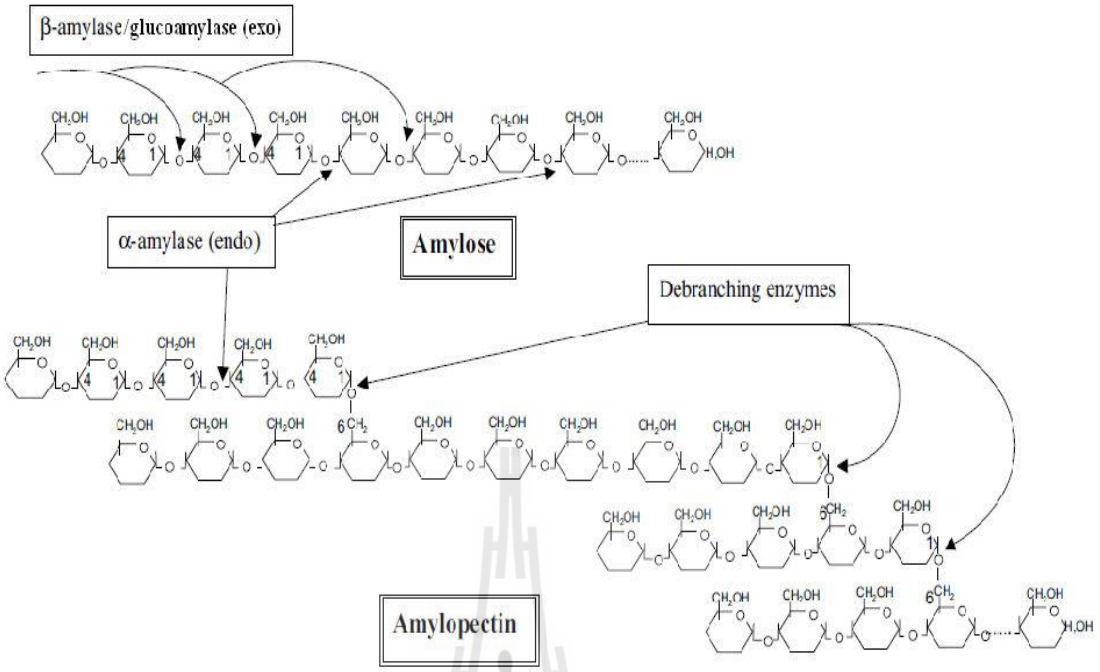


Figure 2.7 Mode of action of enzymatic hydrolysis (Tester *et al.*, 2004).

CHAPTER III

MATERIALS AND METHODS

3.1 Fermentation Methods

3.1.1 Cassava Starch and Enzymes

Cassava starch was purchased from Korat starch industry (Nakhon Ratchasima, Thailand). The cassava starch was stored in desiccator until used. The moisture content of this substrate was 9.65±0.33% and, glucose and maltose content were 115.6±3.47% and 12.16±1.19% (90 g/L cassava starch concentration), respectively. Commercials enzymes including α-amylase (Amy; 42,169 U/g) and glucoamylase (AMG; 21,300 U/mL) were used. These enzymes were obtained from Siam Victory Chemicals Co., Ltd., Bangkok, Thailand.

3.1.2 Growth Condition of Strains and Media

E. coli KJ122 was kindly provided by the Department of Microbiology and Cell Science, University of Florida, Gainesville, Fl, USA. A new low salts medium, AM1 (4.2 g/L total salts; Martinez *et al.*, 2007) (Table 3.1) was used in the fermentation with KJ122 strain. Media was sterilized by autoclave at 15 psi for 15 min. Seed cultures and fermentation were grown at 37°C; 200 rpm in AM1 mineral salts medium containing 3% (w/v) glucose, 100 mM $KHCO_3$ and 1 mM betaine HCl. These seed cultures were maintained at pH 7.0 by HCl during initial experiments. Subsequently,pH was maintained by automatic pumping 6M KOH. Fermentations were inoculated at

an initial OD_{550} of 0.1, the calculation based on 3 OD which is equivalent to 1 g/L CDW (cell dry weight) and carried out in 500 mL small in-house built fermentator vessels with a working volume of 350 mL. Anaerobiosis was rapidly achieved during growth phase by bicarbonate served to ensure an atmosphere of CO_2. To estimate the amount of cell density, three milliliter of each sample was taken during fermentation every 8 h.

Table 3.1 Composition of AM1+1mM betaine mineral low salts media (excluding carbon source).

Component	**Concentration (mmol/L)**
$(NH_4)_2HPO_4$	19.92
$NH_4H_2PO_4$	7.56
Total PO_4	27.48
Total N	47.93
[a]Total K	1.00
$MgSO_4$ $7H_2O$	1.50
Betaine-KCl	1.00
	(µmol/L) [b]
$FeCl_3$ $6H_2O$	8.88
$CoCl_2$ $6H_2O$	1.29
$CuCl_2$ $2H_2O$	0.88
$ZnCl_2$	2.20
Na_2MoO_4 $2H_2O$	1.24
H_3BO_3	1.21
$MnCl_2$ $4H_2O$	2.50
Total Salts	4.1g/L

[a]KOH is used to neutralize betaine-HCl stock.

[b]Trace metal stock (1000X) was prepared in 120 mM HCl.

3.1.3 Optimization of Culture Conditions

In the experiment of the batch SSF process to produce succinic acid, 500 mL small in-house-built fermenter vessel with 350 mL working volume was used, the glucoamylase filtrated through 0.20 μm filter membrane was added in portions. The cell culture, glucoamylase and AM1 medium were added in small in-house-built fermenter vessel containing above mentioned components. The fermentation was initiated by inoculation with a seed culture (5%, v/v), and carried out at 37ºC with initial pH 7.0. The pH was controlled by automatic addition of mixture of the optimized metal carbonate solution and KOH when pH was decreased.

To study the effect of pH on succinic acid production, the fermentation was carried out with the initial pH adjusted to 5.5, 6.0, 6.5 and 7.0. The effect of temperature was studied by carrying out fermentation at temperature in the range of 37ºC, 39ºC, 41ºC and 43ºC. In order to determine the kinetics parameters of the *E. coli* KJ122 strain, different concentration of cassava starch such as 70 g/L, 90 g/L, 110 g/L, 130 g/L and 150 g/L was applied for succinic acid fermentation. Additionally, various enzyme mixtures loading such as 300, 500, 700, 900, 110 and 130 U/g-cassava starch were used to determine concentration of succinic acid production. Furthermore, an initial cell biomass was provided with the different value of 0.1, 0.2, 0.3, 0.6, 0.9, 1.2 and 1.5. Subsequently, the length of fermentation time was extended to 72 h at the optimum culture condition. The experiment was performed twice.

3.1.4 Succinic Acid Production with Batch SSF in 2 L Bioreactor

In the experiment of the batch SSF process to produce succinic acid, 2 L bioreactor with 350 mL working volume was used. The optimized parameters obtained

from the previous experiments were used for succinic acid production. For cassava starch liquefaction, cassava starch (70 g/L) and 56.91 µL of α-amylase were added to 73 mL deionized water before autoclave. The cell culture (0.6 OD), glucoamylase (500 U/g cassava starch) and AM1 medium were added in to fermenter containing above mentioned components. The fermentation was initiated by inoculation with a seed culture (20% v/v), and carried out at 39ºC with initial pH 6.5. The pH was controlled by automatic addition of mixture of the optimized metal carbonate solution and KOH when pH was decreased. In order to understand whether the liquefied starch was completely hydrolyzed under this condition, the broth of fermentation was enzymatically hydrolyzed by addition of glucoamylase (See Appendix A).

3.1.5 Succinic Acid Production by Fed-batch SSF in 2 L Bioreactor

In fed-batch SSF, the experiment was carried out with the same procedure as SSF and feeding strategy was done intermittent by feeding in which 50 g/L cassava starch concentration was used as the initial substrate concentration. Liquefied cassava starch solution (300 g/L) was added simultaneously with AMG when the residual glucose concentration in the fermentation broth was reduced to 15 g/L, in order to keep an appropriate glucose concentration. The experiments were repeated twice. Samples were removed from the fermenter every 8 h until 72 h for further analyses.

3.2 Analytical Methods

Three milliliter of samples were removed during fermentation every 8 h and centrifuged at 15,000 rpm for 3 min. The supernatant and the precipitant (cells) were used for further analysis.

The moisture content of the cassava starch was estimated by drying at 130ºC. The reducing sugar which was resulted from enzymatic hydrolysate was measured using the 3, 5-dinitrosalicyclic acid (DNSA) method (Miller, 1959). At time zero and 8 h cell concentration from starch fermentation was determined by using a modified method of Tang *et al.,* (2010). From 12 h to 72 h, cell biomass was estimated from the optimal density at 550 nm (0.33 g of cell dry weight/L) for appropriately diluted culture samples. Organic acids and sugars were determined by using high performance liquid chromatography, HPLC, (Agilent, 2009) equipped with UV and refractive detectors with Bio-Rad Aminex HPX-87H ion exclusion culumn. The supernatant was filtrated through 0.2 µm filter membrane prior to injecting to HPLC. Yield of product was calculated base on the potential glucose in medium with an assumption that 1 g starch was converted to 1.1 g glucose and 1 g maltose was assumed to produce 1.053 g glucose (Liew *et al.,* 2006). Reducing sugars (glucose and maltose) resulting from starch hydrolysis during the fermentation was determined by HPLC.

3.2.1 Determination of Fermentation Products

Organic acids and sugars were determined by HPLC. Sulfuric acid is used as mobile phase at concentration of 4 mM. The culture from the bioreactor was centrifuged at maximum speed for 3 min to separate cells and supernatant. The supernatant will be further filtrated through 0.2 µm filter membrane before injecting to HPLC. Yield of product was calculated based on the potential glucose in medium with assumption that 1 g starch was converted to 1.1 g glucose and 1 g maltose was assumed to produce 1.053 g glucose (Liew *et al.,* 2006). Moreover, specific productivity was determined as shown in the equation below (Clarke *et al.,* 2011).

$$Q_p \text{ (g/biomass/h)} = \left[\frac{titre\ 2 - titre\ 1}{density\ 2 - density\ 1}\right] \times daily\ growth\ rate \quad \textbf{(1)}$$

where

$$\text{Daily growth rate} = \left(\frac{\ln(density\ 2) - \ln(density\ 1)}{time\ 2 - time\ 1}\right)/24 \quad \textbf{(2)}$$

3.2.2 Determination of Biomass Concentration

Biomass was estimated from the optical density at 550 nm from appropriately diluted culture samples. Three units of OD_{550} was equivalent to gram CDW per liter. Cell concentration (g cell dry weight per 1 ml of fermentation broth) of the fermentation broth was determined using a modified method of Tang *et al*., (2010). A portion of 20 µL α-amylase was added to 1 mL of culture medium and then incubated at 90ºC for 2 h to hydrolyze starch in the medium to soluble dextrin. Samples, which were contained in pre-weighted Eppendorf tubes, were centrifuged at 15,000 rpm for 10 min at 4ºC. The supernatant was discharged and the remaining solid was redissolved with water and once centrifuged. After discharging the supernatant, the cells, which was free from starchy substances, was dried at 105ºC for 4 h to determine for dry cell weight.

3.2.3 Determination of Sugar Content in Starch

The reducing sugar presenting in the enzymatic hydrolysate was measured using DNSA method (Miller, 1959). A standard curve was prepared using standard glucose solution. A blank solution containing 1 mL of deionized water was heated for 5 min in boiling water with 3 mL of DNSA reagent. In order to prepare a standard curve, standard solution containing anhydrous D-glucose (0.1-1.0 mg/mL in deionized water) was heated with 3 mL of DNSA reagent for 5 min in a boiling water bath followed by measuring optical density at 540 nm and plot of glucose concentration

(as reducing sugar) versus optical density was established. In order to measure the reducing sugar concentration in the hydrolysate cassava, 250 mg of hydrolysate was suspended in 15 mL of deionized water in a 50 mL beaker. One milliliter of the diluted suspension was heated prior to measuring optical density as mentioned above (with DNSA reagent). The reducing sugar was read from the standard curve using least squares linear regression (See Appendix B).

3.2.4 Determination of Starch Hydrolysis Products

Starch concentration of the samples was determined using a modified method of Thang *et al.* (2010). A portion of 20 μL α-amylase was added to 1 mL of culture medium and the mixture was incubated at 90°C for 3 h to hydrolyze starch to soluble dextrin. Then, 8,880 μL of 0.1 M acetate buffer at pH 4.5 and 100 μL of AMG were added to the solution and the mixture was further incubated at 58°C for 4 h. The solution was allowed to cool down to room temperature and then transferred to a 10 mL volumetric flask followed by filling it with distilled water to the volume. Glucose concentration of this solution was determined using the HPLC method. Starch concentration in fermentation broth was calculated as follows:

$$\text{Starch concentration (g/L)} = \text{Glucose concentration (g/L)} \times 10^{a} \times 0.9^{b} \quad \textbf{(3)}$$

where *a* is dilution factor and *b* is correction factor for glucose to starch. The calculation of starch concentration in accordance with the glucose concentration is shown in Appendix C.

Reducing sugars (glucose and maltose) resulting from starch hydrolysis during the fermentation was determined by HPLC. The temperature of the column was operated at 45-55°C with a flow rate of 0.4 mL/min.

3.2.5 Statistical Analysis

Analysis of variance (ANOVA) was conducted using SPSS software (SPSS) 17.0 for windows; SPSS Inc., Chicago, IL). Triplicate determinations were performed on each test and average values were used for reporting. The differences among mean values were established using Duncan's multiple-ranges test (DMRT) at 95% significant confidence.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Factors Effect on Succinic Acid Production

4.1.1 Effect of Cassava Starch Concentration on Succinic Acid Production in SSF

At high substrate concentration, uncompetitive-substrate inhibition is usually observed which resulting in deactivating enzymatic reactions and inhibiting microbial growth during SSF process (Tango and Ghaly, 1999). To achieve a suitable concentration of cassava starch during SSF, various concentrations of cassava starch from 70 to 150 g/L were investigated for succinate production by *E. coli* KJ122 at 37^{o}C, 500 U/g-cassava starch, pH 6.5 and 0.1 OD. The cultivations were stopped at approximately 72 h. The different concentrations of cassava starch, biomass, succinic acid and by-product concentrations are presented in Table 4.1.

Table 4.1 Performance of *E. coli* KJ122 with different cassava starch concentrations

Starch (g/L)	Max CDW (g/L)	Glucose residual (g/L)	Succinate (g/L)	Yield [a,b]		Productivity		By-product
				Gross (g/g)	Observed (g/g)	Volumetric (g/L/h)[c]	Specific (g/g CDW/h)	Acetate (g/L)
70	2.67±0.05[d]	8.40±0.43	63.51±0.19 $^{\beta,\gamma}$	0.93±0.02	0.94±0.01	0.99±0.003$^{\beta}$	0.372±0.02$^{\beta}$	8.78±0.13
90	2.30±0.06	32.09±1.47	59.32±0.94$^{\beta}$	0.67±0.04	1.13±0.05	0.93±0.044$^{\beta}$	0.404±0.03$^{\beta}$	7.03±0.04
110	2.01±0.10	58.96±5.99	63.09±7.92$^{\beta,\gamma}$	0.60±0.06	1.07±0.01	1.01±0.099$^{\beta}$	0.504±0.08$^{\beta}$	9.05±1.46
130	2.20±0.07	78.89±4.05	63.66±8.20$^{\beta,\gamma}$	0.50±0.05	0.98±0.03	1.01±0.097$^{\beta}$	0.462±0.07$^{\beta}$	8.11±1.14
150	2.05±0.11	82.95±7.30	64.54±0.75$^{\gamma}$	0.44±0.001	0.75±0.09	1.00±0.011$^{\beta}$	0.496±0.06$^{\beta}$	5.85±1.66

[a] Yield was calculated as product concentration divided by the amount of cassava starch provided during fermentation

[b] Yield was calculated as product concentration divided by the amount of glucose consumed during fermentation (based on 1 g cassava starch equivalent to 1.1 g glucose conversion factor)

[c] Productivity calculated at the end of fermentation time

[d] All data represent the average of two replicates with standard deviation. Value bearing different Greek symbol are significantly different ($P < 0.05$).

During fermentation, saccharification was occured, but it was not necessary for the starch to be completely hydrolyzed to glucose as it can cause inhibition to cell growth. Therefore, the optimum amount of cassava starch concentration was also important for the process. As shown in Figure 4.1, the highest succinate concentration of 64.54±0.75 g/L was obtained when initial cassava starch concentration was 150 g/L. However, this highest value of succinate concentration was not significant difference compared with the succinate concentration (63.51±0.19 g/L) when initial cassava starch concentration was 70 g/L. The cassava starch concentration (70 g/L) also produced the lowest residual glucose at the concentration of 8.40±0.43 g/L while cassava starch in the range of 90 to 150 g/L introduced the residual glucose ranging from 32 to 83 g/L. The observed yields for cassava starch in the range 90 to 130 g/L are slightly decreasing, but all values are higher than that of 70 g/L of cassava starch (Figure 4.1). This indicates that the consumption of glucose by *E.coli* KJ22 still occurs, so it is likely that there is no occurrence of glucose inhibition within the cassava starch range 90 to 130 g/L. On the other hand, it was observed that the observed yield was dramatically decreased at 150 g/L of cassava starch. This implies that glucose inhibition occurs at higher concentration of cassava starch. In addition, glucose was still remained in the fermentation broth in all conditions even the incubation time was prolonged to 72 h. The remaining glucose concentration was significantly higher when more cassava starch was provided to the fermentation broth.

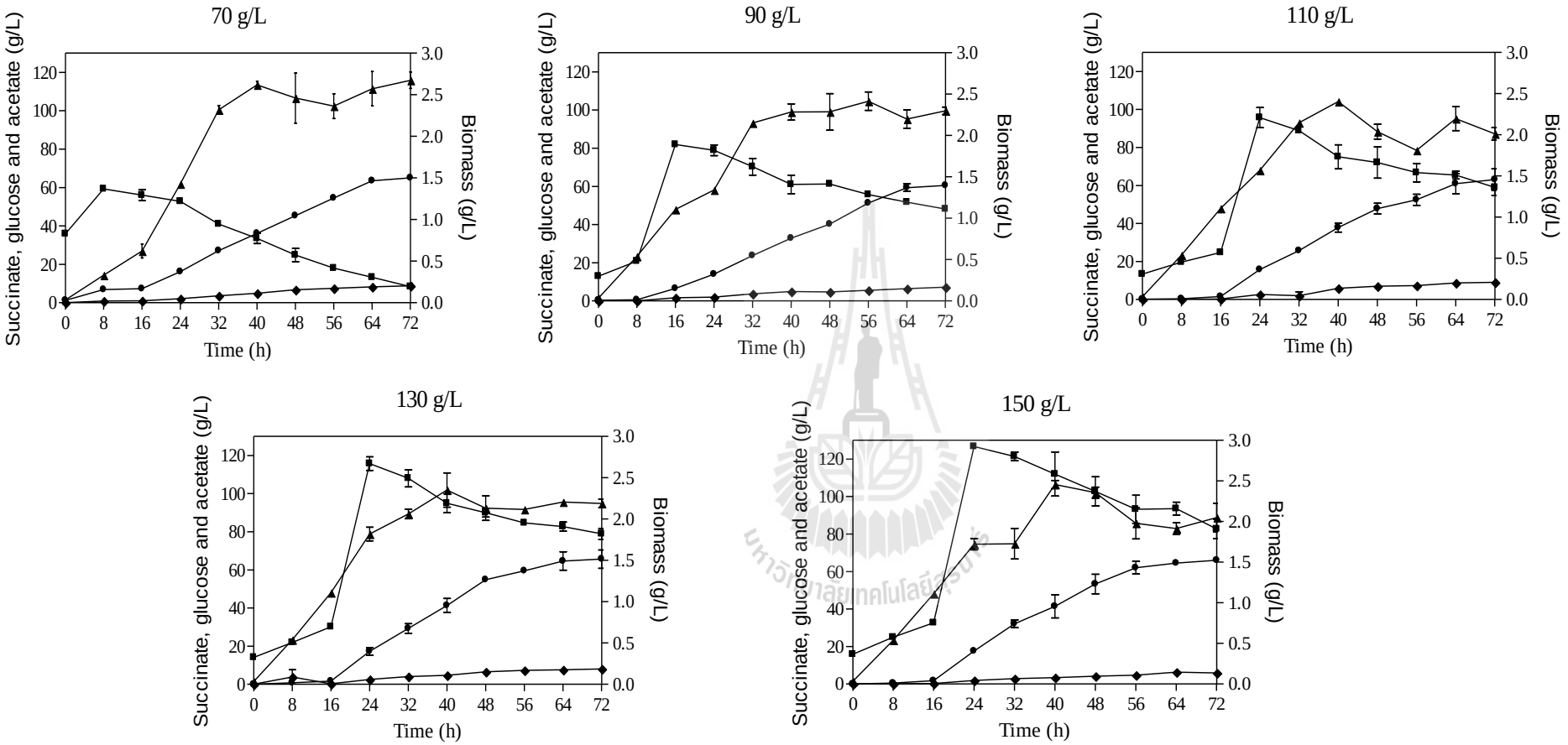


Figure 4.**1** Time course of cassava starch fermentation to succinic acid by *E. coli* KJ122 under 500 U/g-cassavas starch, 200 rpm, pH 6.5, 37^{o}C, and 0.1 OD initial cell density in batch SSF culture. (●) Succinate, (■) Glucose, (▲) Biomass, (♦) Acetate

This probably caused by the high initial glucose concentration which could inhibit the cell growth and succinate production as well as sugar utilization accordingly. The inhibition of high sugar concentration to the cell growth and succinic acid production was probably resulted from the fall of water activity that affects the metabolic rate (Wang *et al.*, 2012).These results thus reflected that the higher the initial cassava starch concentrations, the lower the succinate yield. Similar finding was also observed by Chan *et al*. (2012) and Agarwal *et al*. (2006) in which the production of succinate were performed in *E. coli* using sucrose and sugarcane molasses, respectively. In addition, Wang *et al*. (2012) have proposed that the inhibition of high sugar concentration on the cell growth and 2,3-butanediol production may probably result from the fall in water activity affecting the microbial metabolic rate. Additionally, Zhu *et al.* (2012) pointed out that ethanol concentrations and yields by *Saccharomyces cerevisiae* had significantly decreased when cassava pulp concentrations were provided to the medium during SSF process. Furthermore, Wang *et al*. (2010) studied L-lactic acid production from cassava powder by *Lactobacillus rhamnosus* under SSF process. They found that high amount of cassava powder provided to the bioreactor partly inhibited the cell growth and caused lower L-lactic acid production. They also suggested that some toxics components including cyanides commonly found in fresh cassava root had adversely affecting the cell growth and microbial biochemical productions. According to this experiment, the optimal concentration of 70 g/L cassava starch for succinate production using SSF process by the strain KJ122 was selected for subsequent study.

4.1.2 Effect of pH on Succinic Acid Production in SSF

For bacteria in the family of Enterobacteriaceae, the optimal pH for growth is about 7.0. However, the optimal enzymatic activity of AMG is between pH 3.5 and 5.5 according to the manufacturer's specification. Therefore, the cultivation of *E. coli* KJ122 at pH as low as 3.5 during SSF process for the best condition for saccharification and succinate production is impossible. Thus, the effect of pH of medium on succinic acid production was also considered in this study. The results showed that an improvement of growth and biomass was observed when pH was gradually increased from 5.5 to 7.0 (Table 4.2). As expected, the succinate production was significantly enhanced when pH was increased from 5.5 to 6.5. The highest succinate concentration of 63.51±0.19 g/L was obtained at pH 6.5 that was considered the same level as that obtained at pH 7.0 (62.84±2.33 g/L). In addition, the succinate production yield of 0.93 and 0.92 g/g-cassava starch provided were observed at pH values of 6.5 and 7.0, respectively while the yields at pH 5.5 (0.38±0.02 g/g-cassava starch provided) and pH 6.0 (0.74±0.009g/g-cassava starch provided) were significantly lower. Glucose was also remained at the least concentration of 7.85±0.03 g/L when the strain KJ122 was cultivated at pH 7.0 comparing with those at lower pH values (Figure 4.2). The results suggest that decreases in growth and biomass production at the pH values less than 6.0 resulted from a sufficient high acid concentration collapsing the pH gradient across the cell membrane. Consequently, the total inhibition of cellular metabolic functions within the cells at low pH including substrate utilization negatively affected succinate production by the strain KJ122.

Table 4.2 Performance of *E. coli* KJ122 with different controlled pH

pH	Max CDW (g/L)	Glucose residual (g/L)	Succinate (g/L)	Yield[a,b]		Productivity		By-product
				Gross (g/g)	Observed (g/g)	Volumetric (g/L/h)	Specific (g/g CDW/h)	Acetate (g/L)
5.5	1.78±0.15	54.03±6.77	26.83±1.33$^{c,\beta}$	0.38±0.024	1.22±0.091	0.42±0.021$^{\beta}$	0.238±0.027$^{\beta}$	3.43±0.17
6.0	2.38±0.17	20.99±0.81	50.32±0.26$^{\gamma}$	0.74±0.009	0.96±0.059	0.79±0.004$^{\mu}$	0.333±0.043$^{\theta}$	6.83±0.59
6.5	2.67±0.05	8.40±0.43	63.51±0.19$^{\pi}$	0.93±0.016	0.94±0.013	0.99±0.003$^{\theta}$	0.372±0.020$^{\theta}$	8.78±0.13
7.0	2.55±0.09	7.85±0.03	62.84±2.33$^{\pi}$	0.92±0.002	0.92±0.002	0.98±0.001$^{\theta}$	0.385±0.013$^{\theta}$	8.92±0.14

[a] Yield was calculated as product concentration divided by the amount of cassava starch provided during fermentation

[b] Yield was calculated as product concentration divided by the amount of glucose consumed during fermentation (based on 1 g cassava starch equivalent to 1.1 g glucose conversion factor)

[c] All data represent the average of two replicates with standard deviation. Value bearing different Greek symbol are significantly different ($P < 0.05$).

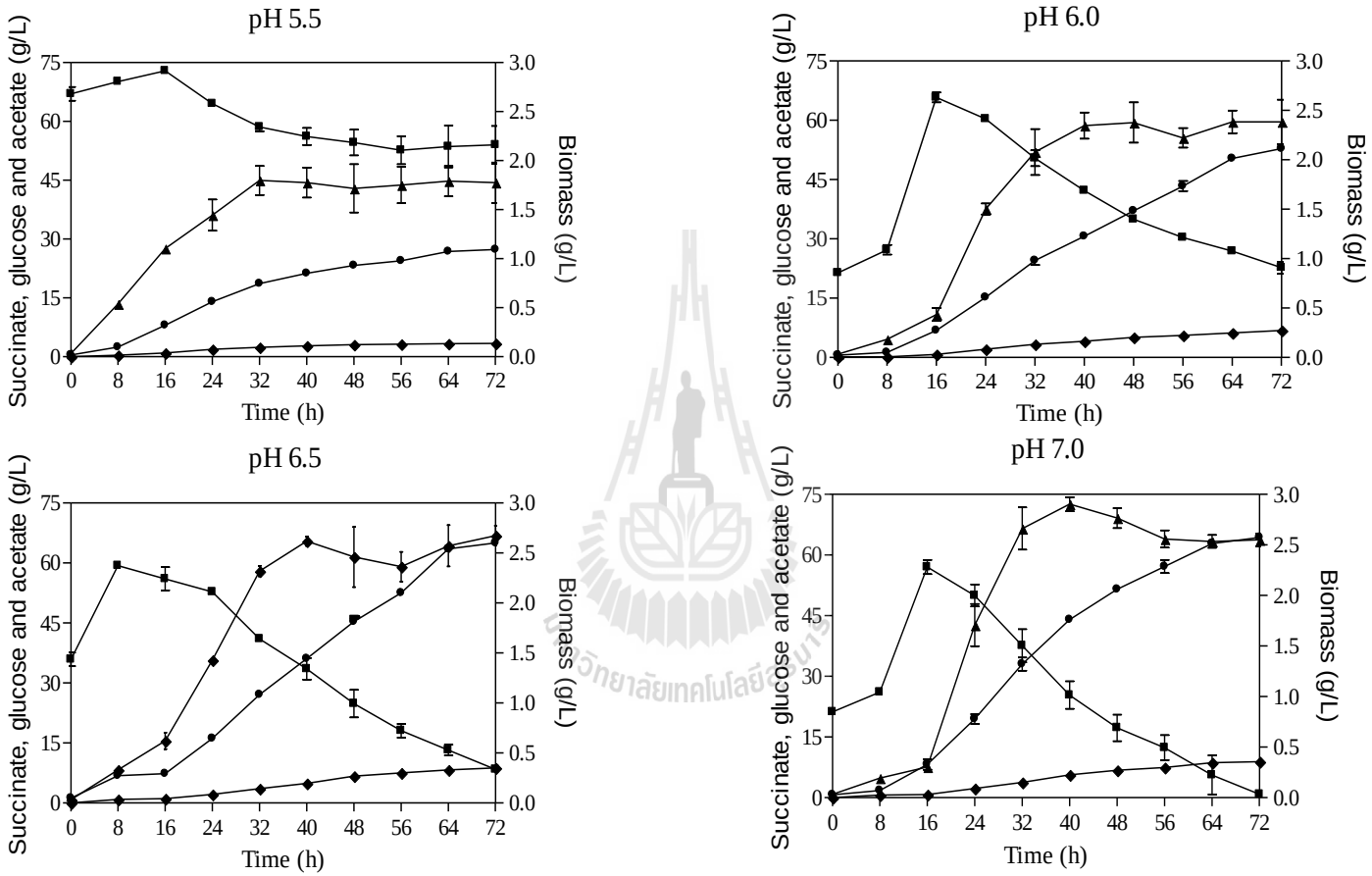


Figure 4.2 Time course of cassava starch fermentation to succinate by *E. coli* KJ122 with different controlled pH under 70 g/L of cassava starch concentration, 500 U/g-cassavas starch, 200 rpm, 37ºC, and 0.1 OD initial cell density in batch SSF culture. (●) Succinate, (■) Glucose, (▲) Biomass, and (♦) Acetate.

The maintenance of medium at high pH value is usually involved with an addition of higher amounts of base solution. However, the higher amount of base is essential to maintain higher level of pH, thus a higher cost due to the extra base addition was consequently increased. In our experiment, no significant effect on yields and titers was observed when the pH was increased from 6.5 to 7.0. Therefore, the lower pH at 6.5 is preferential for the process, and was applied for further investigation. This phenomenon was also observed during succinate and other biochemical productions during SSF process. Recently, Sawisit *et al*. (2014) utilized cassava pulp with cellulase enzyme to obtain fermentable sugars during succinate production using SSF process by the strain KJ122. They found higher sugars residual at pH lower than 6.5. However, no significant difference of succinate titers and yields was found at pH between 6.5 and 7.0. In addition, Wang *et al*. (2012) investigated an initial pH value in SSF of cassava powder for 2,3-Butanediol production by *Enterobacter cloacae*. They also found that the bacterium started utilizing cassava powder at a faster rate than at other initial pH values, but 2,3-butanediol production was continually enhanced to the highest level at pH around 6.0 to 6.5. Moreover, Agarwal *et al.* (2006) investigated a cost effective fermentative production of succinic acid from cane molasses and corn steep liquor by wild type *E. coli*. The results revealed that pH has important role affecting both cell growth and growth-associated production of succinate. They reported that the optimal pH for succinic acid production was also at 6.5.

4.1.3 Effect of Glucoamylase on Succinic Acid Production in SSF

The price of AMG used during SSF process may still be problematic for succinic acid production in large scale. Nevertheless, the cost can be significantly reduced if the amount of an enzyme loading during SSF process decreased. Liquefied cassava starch (70 g/L) was saccharified by adding AMG at the concentrations ranging from 300 to 1300 U/g-cassava starch during SSF process at pH 6.5. At 300U AMG/g-cassava starch, only 55.56±0.31 g/L of succinic acid was achieved with the yield of 0.81±0.026 g/g cassava starch provided. A further increase in enzyme loading from 500 to 1300 U AMG/g-cassava starch resulted in significant improvement in concentration of succinic acid. However, an increase in AMG loading in this range had no influence on succinic acid production in terms of both titer and yield. Succinate concentrations produced were ranged from 62 to 64 g/L with yields of 0.90 - 0.95 g/g cassava starch provided. Although, the use of enzyme in the range of 300 to 500 U AMG/g-cassava starch resulting in negative effect on the remaining glucose concentrations (about 8.5 g/L) comparing with those at higher enzyme loading greater than 500 U AMG/g-cassava starch (1.0 to 1.50 g/L). It was presumed that the lower AMG loading affected the saccharification efficiency of starch resulted in higher degree of polymerization of glucose in the liquefied cassava starch than the conditions that contained higher enzymatic loadings (700 to 1300 U AMG/g-cassava starch). The strain KJ122 might delay or retard in consumption of higher degree of polymerized glucose chains. This led to accumulation of higher amounts of glucose residual in the fermentation broth. Surprisingly, the use of enzyme loading at any levels did not affect the growth of the strain KJ122 (Table 4.3).

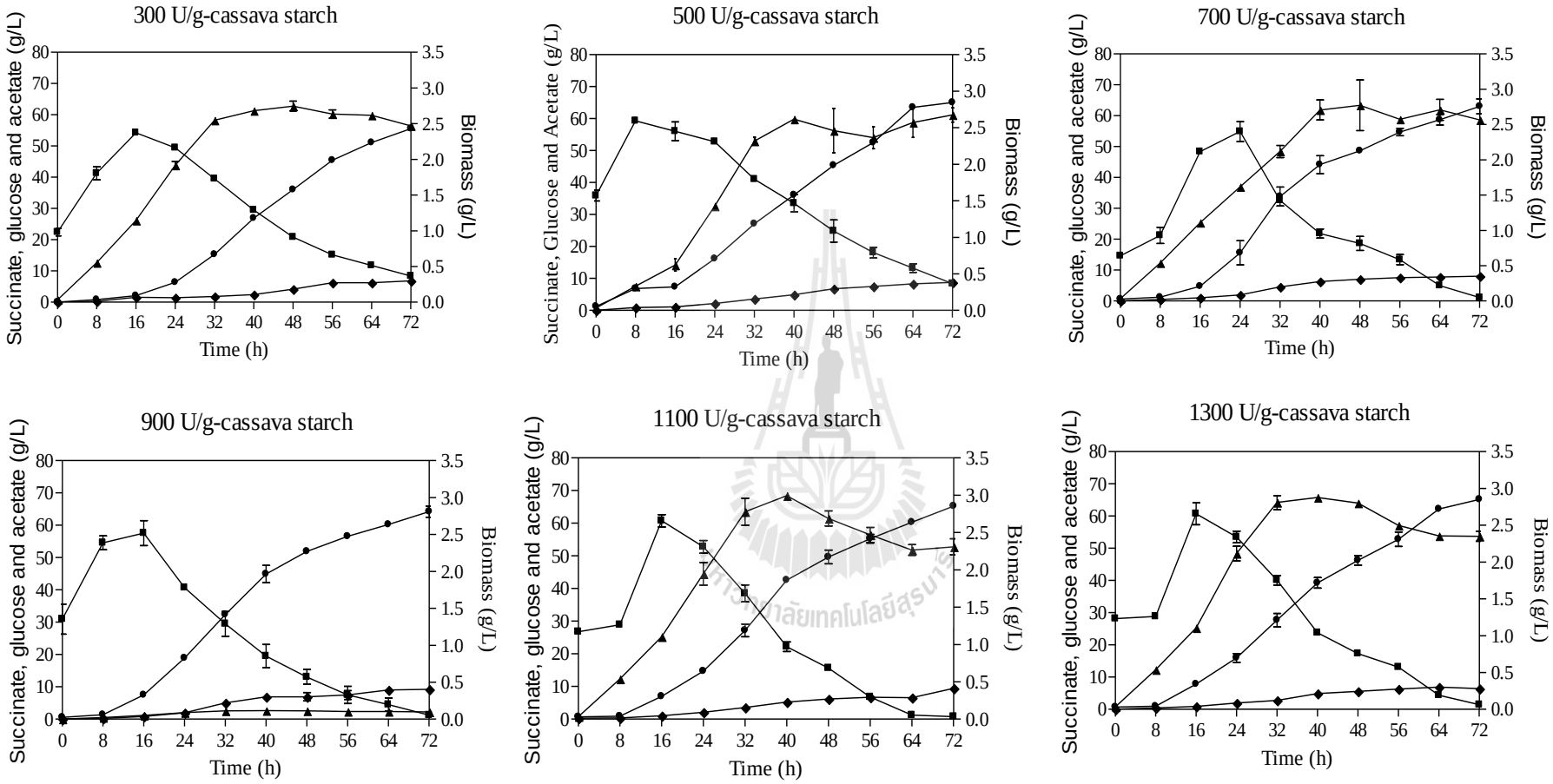


Figure 4.3 Time course of cassava starch fermentation to succinic acid by *E. coli* KJ122 with different enzyme glucoamylase loading under 70 g/L of cassava starch concentration, pH 6.5, 200 rpm, 37ºC , and 0.1 OD initial cell density in batch SSF culture. (●) Succinate, (■) Glucose, (▲) Biomass, and (♦) Acetate

Finding an optimal enzyme loading is essential in the SSF process to achieve high titer of microbial production, yield and productivity, and to reduce the cost of production by lowering amount of enzyme dosage. In Figure 4.3, the use of enzyme loading between 500-1300 U AMG/g-cassava starch did not influence titers and production yields of succinate by the strain KJ122. In addition, providing a lower amount of AMG at 300 U/g-cassava starch resulted in decreasing succinate titer and yield. Thus, a suitable AMG loading of 500 U/g-cassava starch was considered optimum for succinate production by *E. coli* KJ122 in SSF from cassava starch. Chen *et al.* (2011) investigated the optimal enzyme loading in SSF for succinate production using *Actinobacillus succinogenes*. The pectinase enzyme loading varied from 0% (v/w) up to 3% (v/w) was investigated for hydrolysis of acid-pretreated rapeseed meal for succinate production using SSF. They found that the increased level of pectinase loading from 2% to 3% (v/w) had no influence on succinate concentration and its production yield. Furthermore, Wang *et al*. (2012) also revealed that the utilization of AMG at the dosage of 400 U/g-cassava powder could completely release glucose from cassava powder within 4 h and resulted in the highest production of 2,3-butanediol by *En. cloacae*. In contrast, while the enzyme loading higher or lower than 400 U AMG/g-powder resulting in negative effect on 2,3-butanediol production by the strain in SSF process. Sawisit *et al*. (2014) also found that the highest efficiency of cassava pulp hydrolysis with cellulase complex loading at 3% (v/w) on dry pulp combined with 2% (v/w on dry pulp) AMG was achieved during succinate production by the strain KJ122 in SSF. No further improvement in sugars and succinate productions from cassava pulp was observed when more concentrations of cellulase complex were provided. Based on our and others' results, it suggests that enzymatic hydrolysis is the rate limiting step in SSF process. In addition, it is not necessary to utilize an excessive level of enzyme in SSF process, thus reducing the cost of production of microbial products

Table 4.3 Performance of *E. coli* KJ122 with different glucoamylase loading

AMG (U/g)	Max CDW (g/L)	Glucose residual (g/L)	Succinate (g/L)	Yield[a,b]		Productivity		By-product
				Gross (g/g)	Observed (g/g)	Volumetric (g/L/h)	Specific (g/g CDW/h)	Acetate (g/L)
300	2.26±0.28	8.38±000	55.56±0.3$^{c,\beta}$	0.81±0.026	0.82±0.032	0.87±0.006$^{\beta}$	0.351±0.007$^{\beta}$	6.85±0.13
500	2.67±0.05	8.40±0.43	63.51±0.1$^{\gamma}$	0.93±0.016	0.94±0.012	0.99±0.003$^{\theta}$	0.372±0.020$^{\beta,\mu}$	8.78±0.13
700	2.57±0.03	1.09±0.20	63.03±3.35$^{\gamma}$	0.93±0.030	0.84±0.028	0.98±0.052$^{\theta}$	0.384±0.025$^{\beta,\mu,\theta}$	8.00±0.70
900	2.23±0.07	1.24±0.31	64.23±0.74$^{\gamma}$	0.95±0.004	0.85±0.024	1.00±0.037$^{\theta}$	0.394±0.028$^{\beta,\mu,\theta}$	9.21±0.90
1100	2.31±0.15	0.80±0.12	65.23±1.03$^{\gamma}$	0.93±0.01	0.87±0.002	1.01±0.016$^{\theta}$	0.442±0.035$^{\mu,\theta}$	8.53±1.30
1300	2.35±0.09	1.47±0.81	62.16±1.44$^{\gamma}$	0.90±0.019	0.82±0.026	0.97±0.022$^{\theta}$	0.413±0.093$^{\theta}$	6.41±1.52

[a] Yield was calculated as product concentration divided by the amount of cassava starch provided during fermentation

[b] Yield was calculated as product concentration divided by the amount of glucose consumed during fermentation (based on 1 g cassava starch equivalent to 1.1 g glucose conversion factor)

[c] All data represent the average of two replicates with standard deviation. Value bearing different Greek symbol are significantly different ($P < 0.05$).

4.1.4 Effect of Temperature on Succinic Acid Production in SSF

The efficiencies of bioprocesses are strictly temperature dependent owing to strong dependence of enzymatic activity and cellular maintenance upon temperature. The optimum temperature of AMG enzyme which permits total hydrolysis of starch to fermentable glucose is between 58 and 60ºC according to the manufacturer's recommendation. However, *E. coli* KJ122 has the optimal temperature for growth in the range 37 to 43ºC. The growth of *E. coli* KJ122 is usually stalled if the temperature exceeds 45ºC. In this experiment, it is essential to compromise between two optimal temperatures for bacterial growth and saccharification by AMG during SSF process at the temperature ranging from 37 to 43ºC. The highest succinate concentration of 66.93±5.08 g/L with yield 0.97±0.016 g/g cassava starch provided was observed at this temperature. Nevertheless, temperatures ranging from 37 to 41ºC seemed to exert a little effect on the succinate concentration and yield. It was observed that glucose remaining at temperature 37ºC was higher than those of temperatures 39-41ºC. At the higher temperature, the growth (0.27 g/L), and succinate titer (3.08±0.18 g/L) and yield (0.04 g/g-cassava starch provided) were minimized at 43ºC. It suggested that the temperature higher than 41ºC was not suitable for growth due to less substrate consumption by *E. coli* KJ122 strain, thus resulting in the very low succinate production (Figure 4.4).

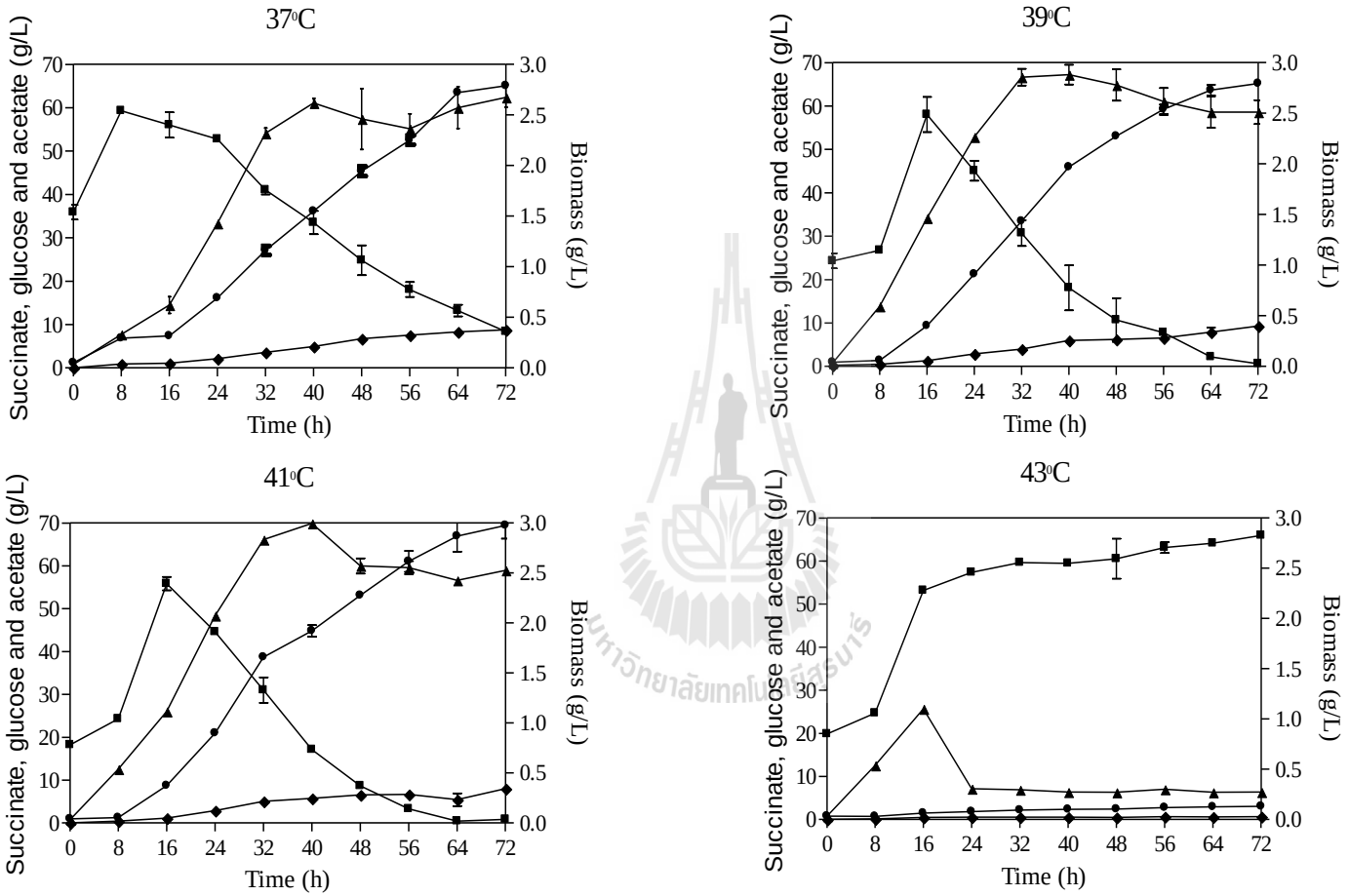


Figure **4.4** Time course of cassava starch fermentation to succinic acid by *E. coli* KJ122 with different temperature under 70 g/L of cassava starch concentration, pH 6.5, 200 rpm, 500 U/g-cassava starch, and 0.1 OD initial cell density in batch SSF culture. (●) Succinate, (■) Glucose, (▲) Biomass, and (♦) Acetate

Table 4.4 Performance of *E. coli* KJ122 with different temperature

Temp (ºC)	Max CDW (g/L)	Glucose residual (g/L)	Succinate (g/L)	Yield[a,b]		Productivity		By-product
				Gross (g/g)	Observed (g/g)	Volumetric (g/L/h)	Specific (g/g CDW/h)	Acetate (g/L)
37	2.67±0.05	8.40±0.43	63.51±0.19$^{c,\beta}$	0.93±0.016	0.94±0.012	0.99±0.003$^{\theta}$	0.372±0.020$^{\theta}$	8.78±0.13
39	2.51±0.17	0.61±0.13	63.68±1.75$^{\beta}$	0.93±0.032	0.84±0.031	0.99±0.027$^{\theta}$	0.397±0.015$^{\theta}$	9.31±0.08
41	2.53±0.03	0.85±0.16	66.93±5.08$^{\beta}$	0.97±0.080	0.88±0.075	1.04±0.079$^{\theta}$	0.414±0.035$^{\theta}$	8.78±0.13
43	0.27±0.00	65.92±1.20	3.08±0.18$^{\gamma}$	0.04±0.002	0.29±0.001	0.04±0.002$^{\beta}$	0.176±0.010$^{\beta}$	0.66±0.09

a Yield was calculated as product concentration divided by the amount of cassava starch provided during fermentation

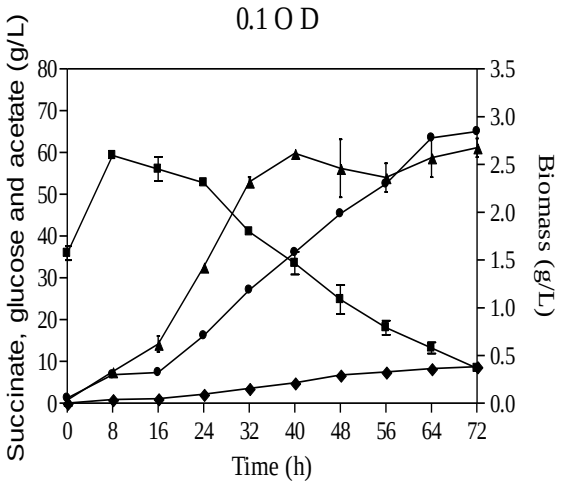
b Yield was calculated as product concentration divided by the amount of glucose consumed during fermentation (based on 1 g cassava starch equivalent to 1.1 g glucose conversion factor)

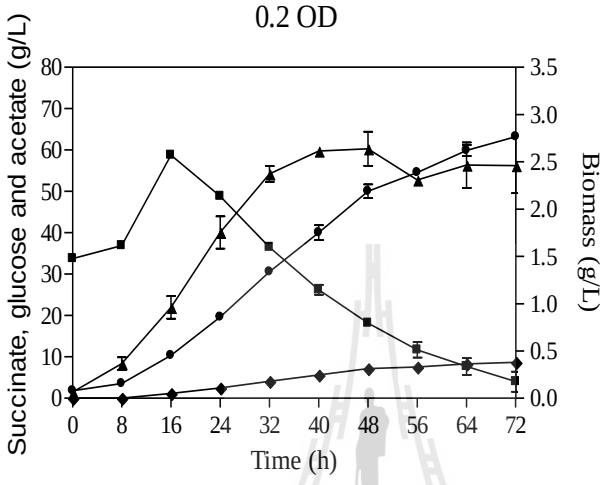
c All data represent the average of two replicates with standard deviation. Value bearing different Greek symbol are significantly different ($P < 0.05$).

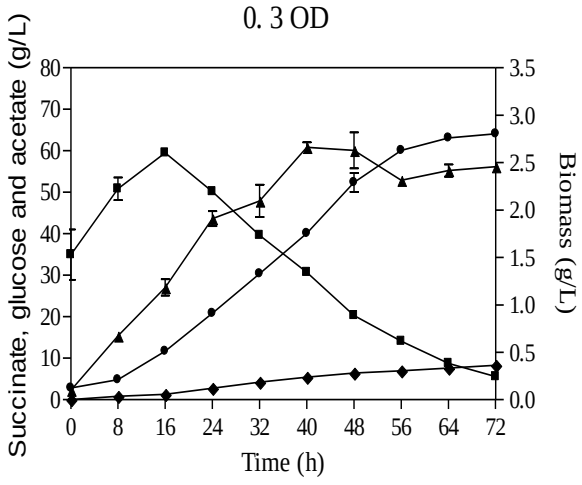
The balance of temperatures for fermentation and enzyme hydrolysis is also the key factor for many microbial biochemical productions using SSF process (Zhu *et al.* 2012). Optimal temperatures for microbial growth and enzymatic activity for substrate utilization should be compromised. Table 4.4 summarizes the results of different temperatures ranging from 37 to 41ºC on the production parameters no effect of temperature on growth and succinate production of the strain KJ122. However, the temperature at 43ºC adversely affected the growth and succinate production by the strain. Additionally, at 37ºC the strain KJ122 seemed to consume glucose at slower rate than those of temperatures 39ºC and 41ºC. Therefore, the temperature at 39ºC was the optimum temperature to produce succinate from cassava starch using SSF process with *E. coli* KJ122. Our result was similar to other published works. Sawisit *et al*. (2014) had also performed succinate production from cassava pulp using SSF process. They revealed that no significant difference in succinate titers and yields was observed when the fermentation performed at temperatures ranging from 37 to 41ºC. Zhu *et al.* (2012a) also demonstrated that neither a decrease nor an increase in the temperatures ranging from 30 to 40ºC induced a significant decrease in ethanol production efficiency from cassava pulp by *Saccharomyces cerevisiae*. Moreover, Zheng *et al.* (2010) conducted succinate production from corn stover under SSF process by *Actinobacillus succinogenes* at temperatures of 38, 40 and 42ºC. Their result showed that with an increase in temperature higher than 38ºC both succinate concentrations and yields were significantly decreased. In addition, Martinez *et al.* (2011) observed no remarkably difference in yield, productivity and final titer of succinate production by *E. coli* SBS550MG at temperatures ranging from 37 to 42ºC.

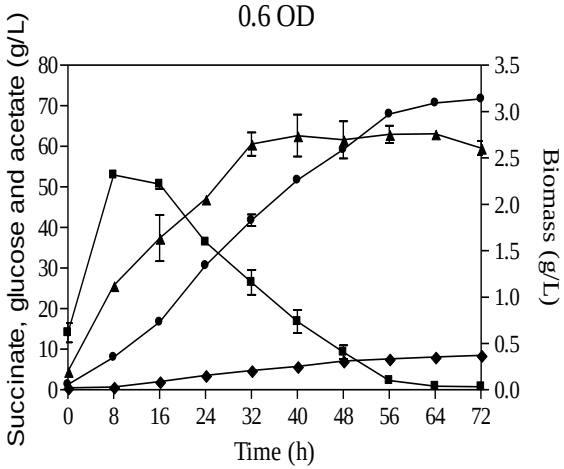
4.1.5 Effect of Inoculum Size on Succinic Acid Production in SSF

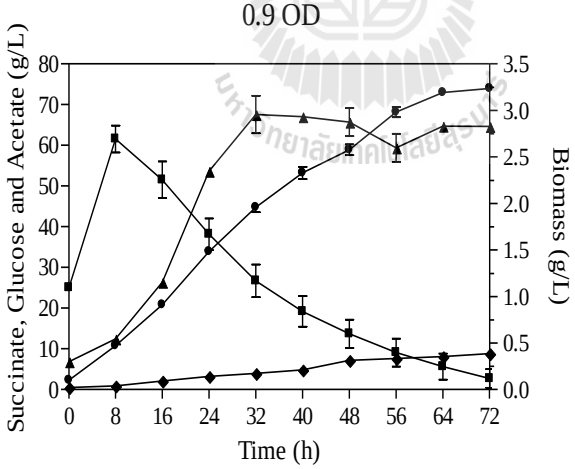
Zhu *et al.* (2012) and Martinez *et al.* (2011) reported that inoculum size can affect the productivity and yield of many microbial products. In the present work, the initial inoculum size was investigated in the range 0.1 to 1.5 OD which was equivalent to 0.033 to 0.5 g/L cell concentration. It was indicated by Figure 4.5 that the highest succinic acid concentration of 76.64 g/L was obtained at the inoculum size of 1.2 OD.

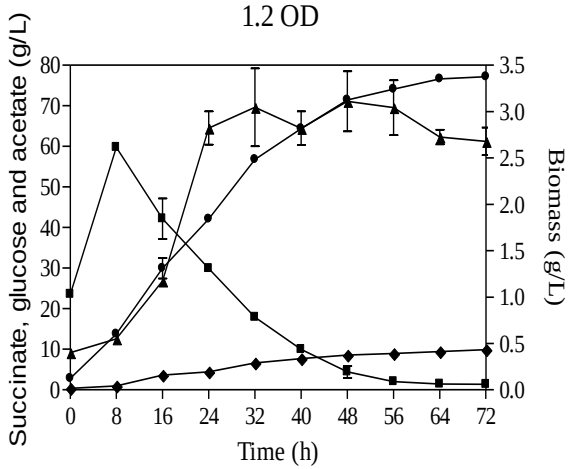

0.1 O D
Succinate, glucose and acetate (g/L)
Biomass (g/L)
Time (h)


0.2 OD
Succinate, glucose and acetate (g/L)
Biomass (g/L)
Time (h)


0. 3 OD
Succinate, glucose and acetate (g/L)
Biomass (g/L)
Time (h)


0.6 OD
Succinate, glucose and acetate (g/L)
Biomass (g/L)
Time (h)


0.9 OD
Succinate, Glucose and Acetate (g/L)
Biomass (g/L)
Time (h)


1.2 OD
Succinate, glucose and acetate (g/L)
Biomass (g/L)
Time (h)

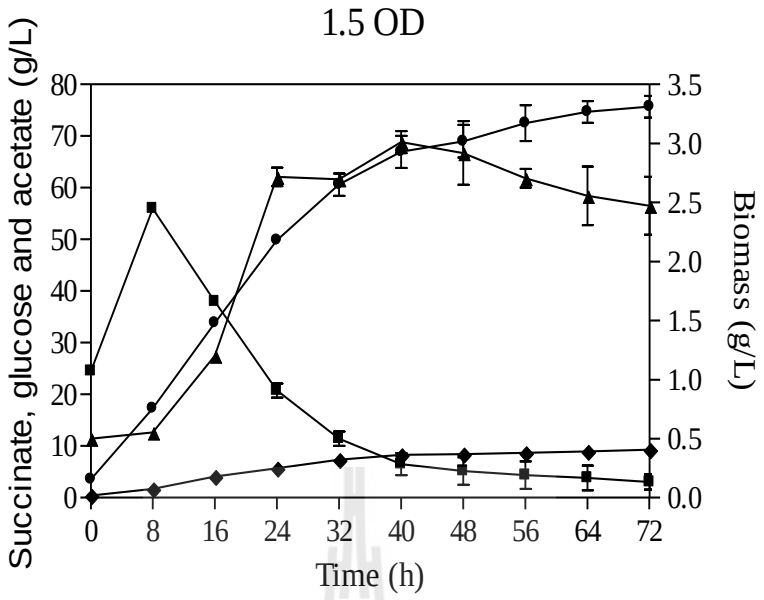


Figure 4.5 Time course of cassava starch fermentation to succinic acid by *E. coli* KJ122 with different cell concentrations under 70 g/L of cassava starch concentration, pH 6.5, 200 rpm, 39°C, and 500 U/g-cassava starch in batch SSF culture. (●) Succinate, (■) Glucose, (▲) Biomass, (♦) Acetate.

As summarized in Table 4.5, the value of titer, yield and productivity of succinic acid noticeably increased from 0.6 to 1.5 OD. This result indicated that cell density has a significant effect on both productivity and titer of succinic acid. Nevertheless, the increasing in the cell density showed no significant difference in succinic acid concentration in the range of 0.6 to 1.5 OD. Martinez *et al.* (2011) pointed out that the increasing cell density resulted in higher succinate production, and overcome glucose inhibitory effect. However, higher cell concentration (60 to 80 OD) showed decrease in product concentration and specific productivity. In the present study, succinic concentration and specific productivity kept increasing from 63.09 to 76.64 g/L and 0.372 to 0.478 g/g CDW/h, respectively when initial cell density was in

the range 0.1 to 1.5 OD. This implies that the initial cell density range used in this study was not high enough to allow decreasing in succinic acid concentration and specific productivity to occur. On the other hand, succinic production with 0.6 OD initial cell density produced observed yield 0.94 g/g-glucose consumed very close to 0.96 g/g-glucose consumed resulted from *E.coli* KJ122 that used glucose as substrate (Jantama *et al.*, 2008). Therefore, further experiments were performed with the initial cell density 0.6 OD.

Table 4.5 Performance of *E. coli* KJ122 with different initial cell density

Initial OD	Max CDW (g/L)	Glucose residual (g/L)	Succinate (g/L)	Yield[a,b]		Productivity		By-product
				Gross (g/g)	Observed (g/g)	Volumetric (g/L/h)	Specific (g/g CDW/h)	Acetate (g/L)
0.1	2.67±0.05	8.40±0.43	63.51±0.19$^{c,\beta}$	0.93±0.016	0.94±0.012	0.99±0.003$^{\beta}$	0.372±0.020$^{\theta}$	8.78±0.13
0.2	2.46±0.27	4.03±2.45	63.59±1.08$^{\beta}$	0.92±0.015	0.88±0.027	0.99±0.008$^{\beta}$	0.407±0.071$^{\theta}$	8.73±0.36
0.3	2.54±0.03	5.58±1.08	63.09±1.9 $^{\gamma}$	0.92±0.001	0.90±0.015	0.99±0.001$^{\beta}$	0.401±0.001$^{\theta}$	8.33±0.09
0.6	2.61±0.11	0.75±0.42	70.68±0.57$^{\Upsilon}$	1.03±0.004	0.94±0.018	1.10±0.002$^{\mu}$	0.424±0.018$^{\theta}$	8.51±0.20
0.9	2.83±0.06	2.72±3.27	72.98±1.32$^{\pi,\Upsilon}$	1.05±0.005	0.99±0.038	1.14±0.020$^{\mu,\theta}$	0.404±0.016$^{\theta}$	8.81±0.23
1.2	2.68±0.11	1.35±0.31	76.64±0.73$^{\pi}$	1.10±0.016	1.02±0.019	1.19±0.011$^{\theta,\gamma}$	0.448±0.039$^{\theta}$	9.91±0.37
1.5	2.47±0.25	2.98±0.22	72.72±1.73$^{\pi,\Upsilon}$	1.09±0.052	1.02±0.020	1.17±0.046$^{\gamma}$	0.478±0.034$^{\theta}$	9.31±0.53

[a] Yield was calculated as product concentration divided by the amount of cassava starch provided during fermentation

[b] Yield was calculated as product concentration divided by the amount of glucose consumed during fermentation (based on 1 g cassava starch equivalent to 1.1 g glucose conversion factor)

[c] All data represent the average of two replicates with standard deviation. Value bearing different Greek symbol are significantly different ($P < 0.05$).

4.2 Succinic Acid Production with Batch SSF in 2 L Bioreactor

Batch SSF experiments were performed in a 2 L bioreactor with working volume 1.5 L. The optimized parameters obtained from the previous experiments were used for succinate production as follows: 70 g/L cassava starch, 500 U AMG/g-cassava starch, pH 6.5, 39ºC and 0.6 OD_{550} cell density at 200 rpm for 72 h. Figure 4.6 showed succinate production using liquefied cassava starch as carbon source under anaerobic conditions during batch SSF by *E. coli* KJ122. Liberated glucose was a main product from cassava starch hydrolyzed after adding AMG into the bioreactor. The maximum concentration of the glucose was at 8 h incubation time. After that, it started continuously decreasing and was completely consumed by the strain KJ122 after 72 h of incubation.

The stoichiometric equation for succinic acid formation from glucose can be formulated as follows (Martinez *et al.*, 2011).

$$\text{Glucose} + 0.86\ HCO_3^- \rightarrow 1.71\ \text{Succinate}^{2-} + 1.74\ H_2O + 2.58\ H^+ \quad \textbf{(4)}$$

During batch fermentation in 2 L fermenter, *E. coli* KJ122 could produce succinate at an impressive titer and yield of 70.08±0.12 g/L and 1.01±0.013 g/g cassava starch provided or 0.91±0.007 g/g glucose consumed, respectively with the overall productivity of 0.97±0.001 g/L/h. The succinate yield and productivity obtained from cassava starch were in good agreement with the previous report with glucose by the strain KJ122 (Jantama *et al*. 2008). From the Equation 4, Jantama *et al.* (2008) have also revealed that the theoretical yield of succinate production by microbial fermentation from glucose is 1.71 mol/mol glucose consumed (1.12 g/g glucose consumed). The strain KJ122 produced succinate at the yield of 1.4 to 1.5 mol/mol

glucose or 0.92 to 0.98 g/g glucose consumed. In this present study, the yield of succinate production from cassava starch by the strain KJ122 was achieved at 1.01±0.013 g/g cassava starch provided or 0.91±0.013 g/g glucose consumed (1g cassava starch is equivalent to 1.1 g glucose). Therefore, the theoretical yield of 81.25% was achieved. This indicated no significant difference in the succinate production yield from glucose and cassava starch by the strain KJ122 in batch fermentation. In addition, the specific productivity 0.422±0.004 g/g CDW/h was obtained by the strain KJ122 (Table 4.6). Chen *et al*. (2014) revealed that the highest specific productivity of 0.171 g/g CDW/h was achieved when glucose was used as carbon sources by *E. coli* NZN111. Sawisit *et al*. (2014) had also found that the specific productivity of succinate of 0.235 g/g CDW/h was achieved in batch fermentation of cassava pulp by the strain KJ122. The results suggested that *E. coli* KJ122 exhibited high efficiency in the specific production of succinate from glucose-based and even cassava-derived substrate during batch fermentation compared with those previous reports.

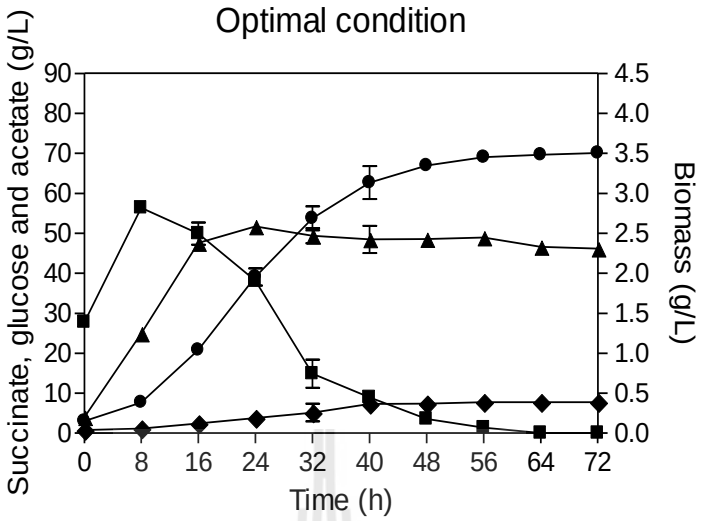


Figure 4.6 Time course of cassava starch fermentation to succinic acid by *E. coli* KJ122 under optimal condition, 70 g/L of cassava starch concentration, pH 6.5, 200 rpm, 500 U/g-cassava starch, 39ºC and 0.6 OD initial cell density in batch SSF culture. (●) Succinate, (■) Glucose, (▲) Biomass, and (♦) Acetate

Generally, cost of a bulk chemical production is affected by the price of raw materials as substrates, fermentation, and product separation process. Thus, using low cost raw materials can partially reduce the cost of the overall production. Cassava starch is considered as the low cost raw materials for some fermentation productions. Using Cassava starch as substrate can possibly decrease the total production cost by 30% (personal communication, Apple Flavor and Fragrance Group Co. Ltd, Shanghai, China). Moreover, the cost of enzyme used in this study for processing a ton of cassava starch was estimated to be about 5% of that of cassava starch (Wang *et al.*, 2012). The results implies that cassava starch hydrolyzed by enzyme could be used as an alternative economic substrate for succinic acid production using batch SSF based on the simplicity of the operation.

4.3 Succinic Acid Production with Fed-batch SSF in 2 L Bioreactor

SSF process should be performed at high substrate concentrations to lowering the overall demand of process energy and water consumption, thus decreasing the production cost (Sassner *et al.* 2006). However, batch SSF processes are difficult to perform with high dry matter content due to high viscosity of medium. Fed-batch is the preferred production strategy when high nutrient concentrations affect the productivity and yield. To increase higher succinate production efficiency, fed-batch SSF was performed in this study. In fed-batch SSF, the experiments were started with the optimized condition as performed in a batch SSF. AMG was added in portions simultaneously together with the liquefied cassava starch. The liquefied solution of cassava starch (300 g/L) was added to the bioreactor in order to keep an appropriate glucose concentration at 30 g/L. Figure 4.7 represents the succinate production after two supplementations of liquefied cassava starch at 20 h and 28 h. The maximum concentration of succinate was enhanced to 82.46±0.51 g/L with the yield of 1.03±0.010 g/g cassava starch provided or 1.00±0.010 g/g glucose consumed and productivity of 1.15±0.008 g/L/h. Also, the specific productivity of 0.456±0.002 g/g CDW/h was observed. The results confirmed that succinate concentration, yield and productivity could be improved by the fed-batch process in comparison to batch SSF process.

In fed-batch mode, the viability of the microorganism due to increase in the efficiencies of mass and heat transfer was improved, causing the hydrolysis and fermentation reaction to be faster and more efficient. Our results showed that the fed-batch SSF procedure also increased yield, productivity, succinate concentration considerably by about 2.00%, 18.56%, and 17.67%, respectively, comparing with those

of batch SSF process. Chen *et al.* (2011) could enhance the succinic acid concentration from 15.5 g/L to 23.4 g/L at the yield 11.5 g per 100 g dry matter and productivity from 0.22 to 0.33 g/L/h by applying fed-batch SSF procedure from rapeseed meal with *A. succinogenes* strain. Additionally, Bretz and Kabasci (2012) could increase the succinate production yield by *An. succiniciproducens* from 0.60 to 0.88 g/g glucose consumed in comparison to batch process with similar glucose concentrations. Sawisit *et al*. (2014) also mentioned that higher succinate concentration and average productivity were significantly observed during fed-batch SSF than those observed in batch experiments by *E. coli* KJ122. Therefore, the results confirmed that succinate concentration, yield and productivity could be efficiently improved in the fed-batch process in comparison to batch SSF process.

In this present study, the yield of succinate production obtained from fed-batch SSF was equivalent to 1.03 g/g cassava starch provided or 1.00 g/g glucose consumed. Therefore, the theoretical yield of 89.3% was achieved. Chen *et al*. (2014) produced succinate at high concentration by high cell density in rich medium containing cassava powder and starch. However, succinate yields of 0.6 to 0.7 g/g substrate provided obtained from their processes were significantly lower than those of ours (1.01 to 1.03 g/g cassava starch provided). Also, the very high specific productivity of 0.456 g/g CDW/h was observed in the strain KJ122 while lower specific productivities (0.259 and 0.339 g/g CDW/h were observed in cassava powder and starch, respectively) were obviously observed during succinate production by *E. coli* NZN111. Therefore, *E. coli* KJ122 would be one of the microbial strains for the cost effective production of succinate with cassava starch as a sole carbon source.

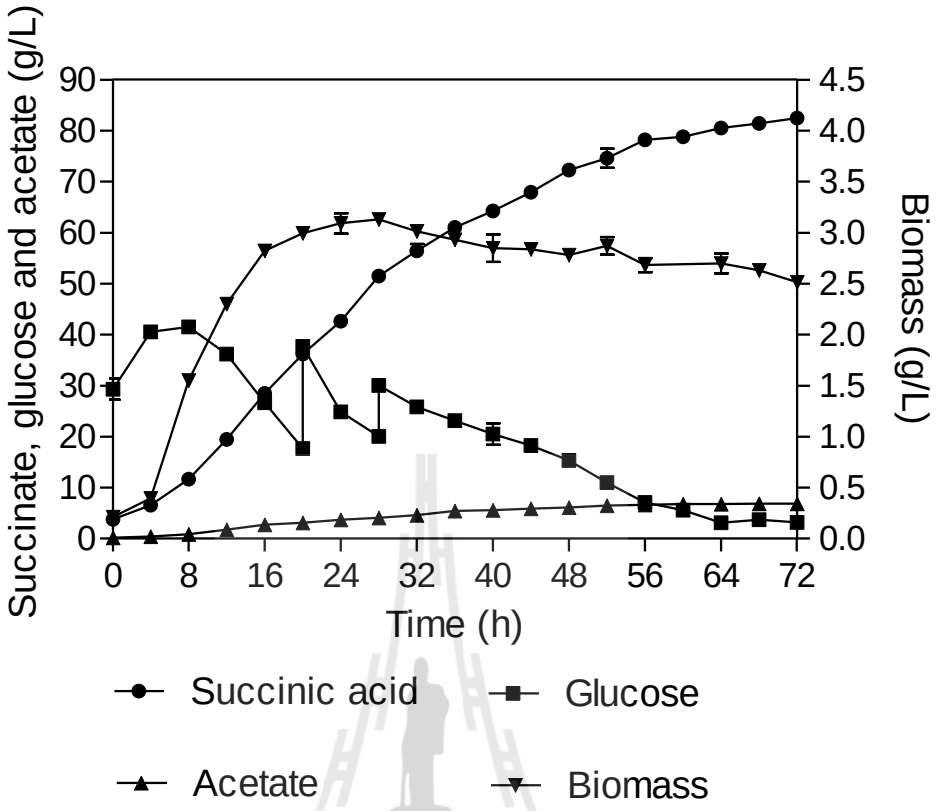


Figure 4.7 Time course of cassava starch fermentation to succinic acid by *E. coli* KJ122 under optimal condition with initial cassava starch concentration 50 g/L, pH 6.5, 200 rpm, 500 U/g-cassava starch, 39^{o}Cand 0.6 OD cell density in Fed-batch SSF culture. (●) Succinate, (■) Glucose, (▲) Biomass, and (♦) Acetate

Table 4.6 Comparison of succinic acid production to other researches

Substrate	Microorganism	Fermentation strategy	Cell concentration (g/L)	Succinate concentration (g/L)	Yield[a,b]		Productivity		References
					Gross yield (g/g)	Observed yield (g/g)	Volumetric (g/L/h)	Specific (g/g CDW/h)	
Cassava powder	*E. coli* NZN111	Fed-batch SSF	10.08	106.17	0.66[d]	0.99[d]	2.54	0.259	Chen *et al.*,2014
Cassava starch	*E. coli* NZN111	Fed-batch SSF	17.08	127.13	0.71[e]	1.02[e]	1.77	0.339	Chen *et al.*,2014
Cassava pulp	*E. coli* KJ122	Batch SSF	3.58±0.32	80.86±0.49	0.70±0.37[f]	0.87[f]	0.84±0.01	0.235	Sawisit *et al.*, 2014
		Fed-batch SSF	ND[c]	98.63±0.12	0.72±0.97[g]	1.08[g]	1.03±0.01	ND	
Cassava starch	*E. coli* KJ122	Batch SSF	2.30±0.009	70.08±0.12	1.01±0.013[β,h]	0.91±0.013[β,h]	0.97±0.001[β]	0.422±0.004[β]	This study
		Fed-batch SSF	2.51±0.006	82.46±0.51	1.03±0.010[β,h]	1.00±0.010[θ,h]	1.15±0.008[θ]	0.456±0.002[θ]	

[a] The succinate yield was calculated as product concentration divided by the amount of cassava starch provided during fermentation
[b] The succinate yield was calculated as product concentration divided by the amount of glucose consumed during fermentation
[c] The results have not shown
[d] The amount of succinate produced from 161.16 g cassava powder provided during the fermentation
[e] The amount of succinate produced from 178.34 g cassava starch provided during the fermentation
[f] The amount of succinate produced from 100.00 g cassava pulp provided during the fermentation
[g] The amount of succinate produced from 70.00 g cassava starch provided during the fermentation
[h] The amount of succinate produced from 80.00 g cassava starch provided during the fermentation

CHAPTER V

CONCLUSION

This study has shown that *E. coli* KJ122 can ferment cassava starch to produce succinic acid with low amount of acetic acid (as the only by-product) by utilizing glucoamylase as an enzymatic hydrolysis. Hydrolysis with glucoamylase was an efficient method to obtain fermentable sugars with glucose as the main product of hydrolysate. To reduce the cost of production, cassava starch was used as an efficient substrate for an efficient succinic acid production.

A high succinic acid concentration of 70.08 g/L, with a yield of 1.01 g/g cassava starch provided and productivity of 0.97 g/L/h was obtained from batch SSF in a 2 L bioreactor with the optimum conditions of pH 6.5, 200 rpm, 39^{o}C, 70 g/L of cassava starch, a glucoamylase loading of 500 U/g-cassava starch and biomass 0.6 OD. The optimal dosage of cassava starch was also explored to achieve high yield and efficient utilization of substrate. Cell density had a significant effect on the productivity and specific productivity of succinic acid. A reduction in the amount of enzyme used for cassava starch hydrolysis could improve the process economy. The strategies for decreasing enzymes loading and feeding substrate in this study were suitable for improvements in succinic acid titer, yield and production rate. The yield and productivity from starch fermentation seemed to not differ from those from glucose fermentation. Cost estimation including raw material and enzyme would help prove the. succinic acid fermentation from starch providing more merits than glucose fermentation

In fed-batch SSF with the optimized condition from batch SSF and utilizing low initial substrate concentration, the results of succinic acid titer, yield and productivity were improved to 82.46 g/L, 1.03 g/g cassava starch provided and 1.15 g/L/h, respectively. Under fed-batch SSF condition, 89.30% of theoretical yield was achieved. The results in this study indicated that in SSF experiments, cassava starch could be used as a carbon source for efficient production of succinic acid. Furthermore, fed-batch SSF could further resolve the initial mixing problems at high glucoamylase loading, enabling high succinic acid production.

In summary, a more efficient succinic acid production from cassava starch investigated by using SSF with glucoamylase supplementation could be feasible. Moreover, *E. coli* KJ122 would be a potential strain for the cost effective production of succinic acid using cassava starch, a low cost and widely available material in Southeast Asian region, especially in Thailand.

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APPENDICES

APPENDIX A

GLUCOSE CONCENTRATION UNDER DIFFERENT CONDITIONS

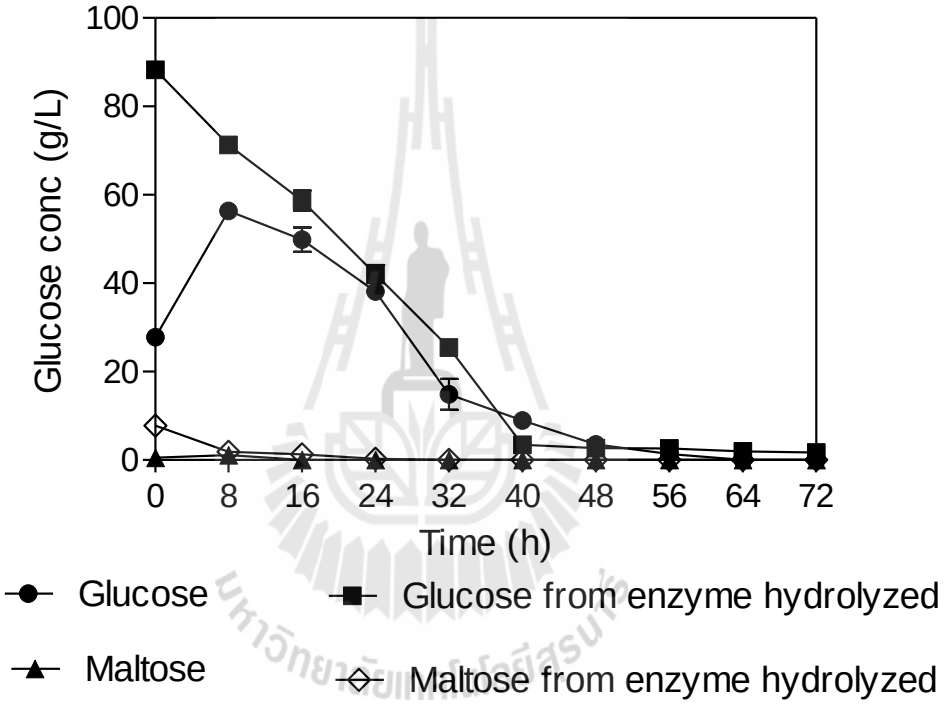

Figure 1A Comparison of glucose concentration from enzymatically hydrolyzed by glucoamylase and glucose concentration remain in the broth at each time point

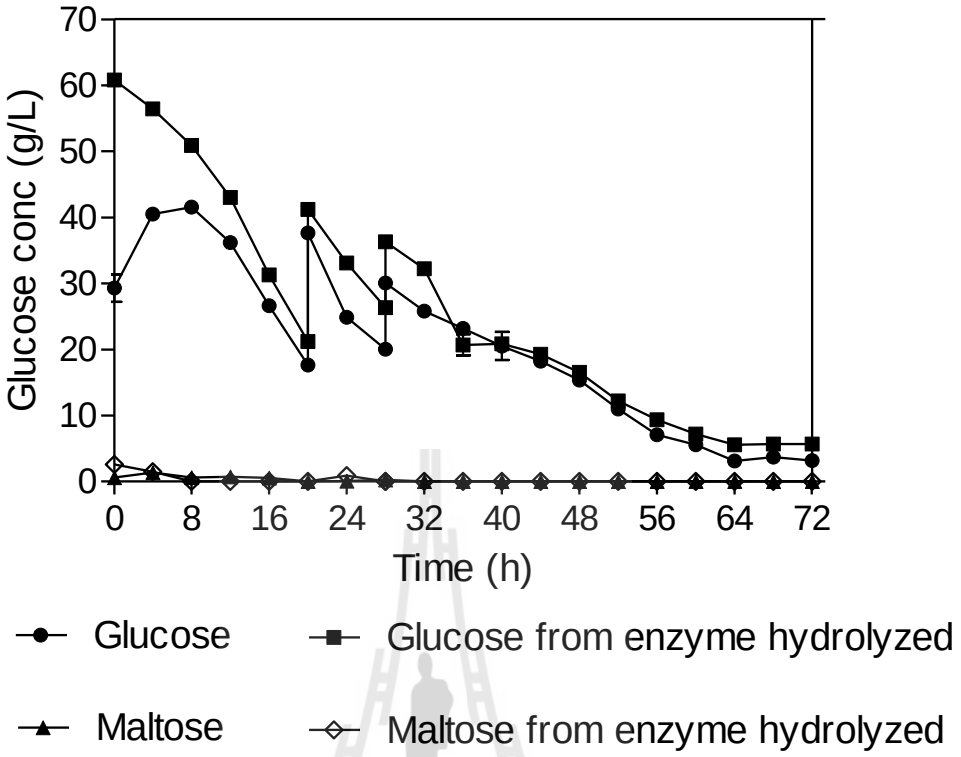

Figure 2A Comparison of glucose concentration from enzymatically hydrolyzed by glucoamylase and glucose concentration remain in the broth at each time point

APPENDIX B

STANDARD CALIBRATION CURVE

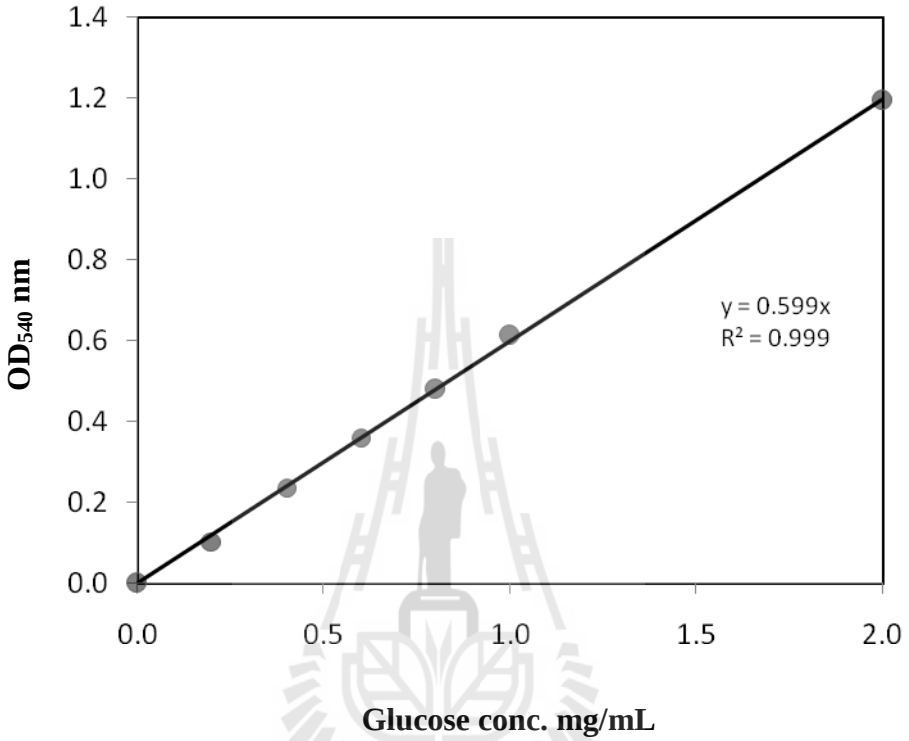

Figure 1B Standard calibration curve of sugar concentration by measurement density using a spectrophotometer at 540 nm by DNSA metho

APPENDIX C

CORRELATIONS BETWEEN GLUCOSE AND STARCH

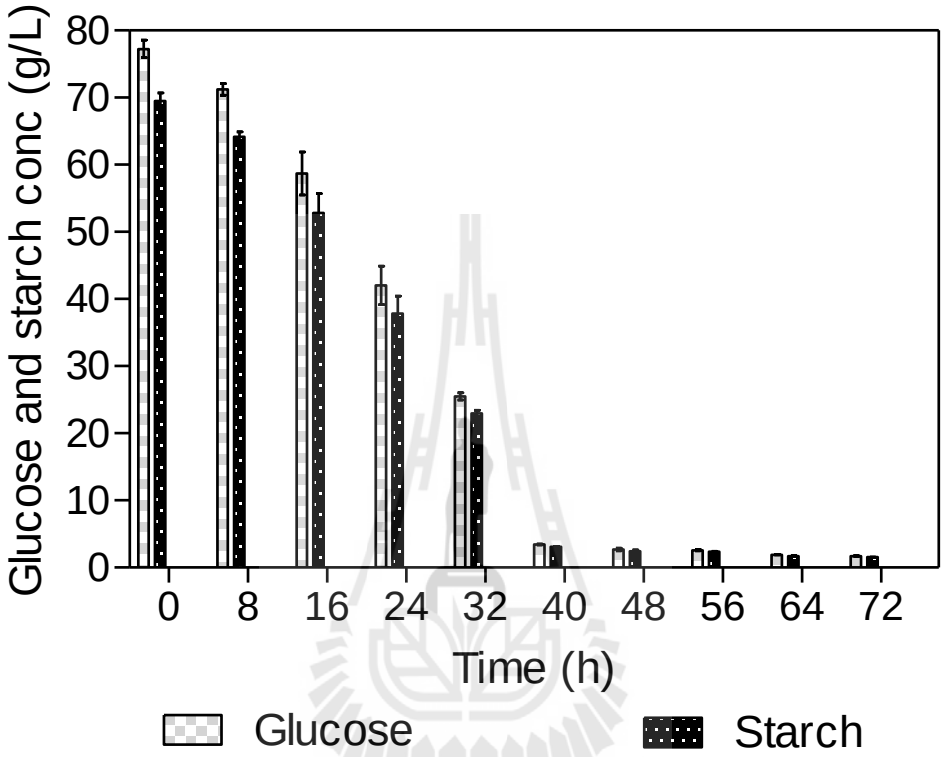

Figure 1C Correlations between glucose and starch in batch SSF with optimal condition such us 70 g/L cassava starch, 39ºC, pH 6.5, 0.6 OD cell density, 200 rpm and 500 U/g-cassava starch

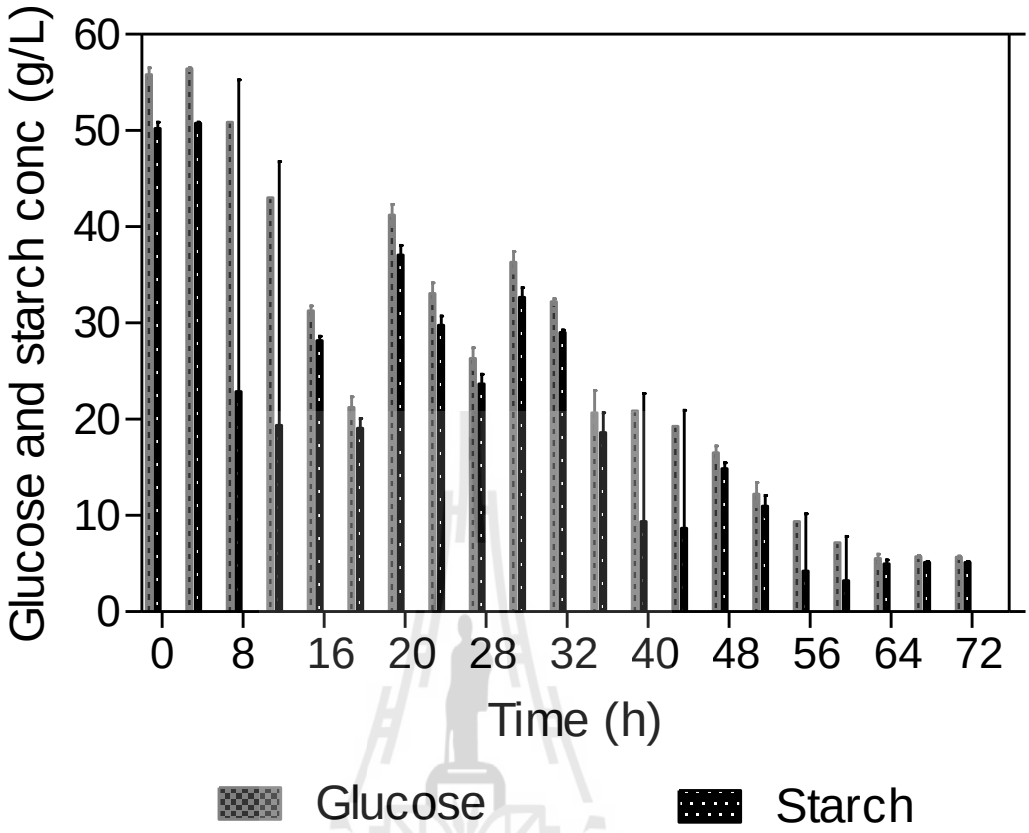

Figure 2C Correlations between glucose and starch in fed-batch SSF with optimal condition such us 50 g/L cassava starch, 39ºC, pH 6.5, 0.6 OD cell density, 200 rpm and 500 U/g-cassava starch

BIOGRAPHY

Miss Kirin Khor was born on March 28th, 1985 in Banteay meanchey province, Cambodia. She graduated from Bakan High School, Pursat province, in 2003. In 2004, she won a government scholarship program to further study an undergraduate program in the field of chemistry at Royal University of Phnom Penh, Phnom Penh, Cambodia. In 2008, she successfully completed her undergraduate program with Bachelor Degree in Chemistry. In 2009, she passed a teacher training national exam for pedagogy study to become a teacher at National Institute of Education, Phnom Penh. In 2010, she completed her training and became a high school chemistry teacher at Bakan High school. In 2012, she obtained a financial support from National Research Council of Thailand, Suranaree University of Technology, Nakhon Ratchasima, Thailand to continue her graduate study in the field of biotechnology under School of Biotechnology, Institute of Agriculture. Her graduate study was under the supervision of Asst. Prof. Dr. Kaemwich Jantama, and her research interest was in "Succinic acid production from cassava starch by simultaneous saccharification and fermentation by metabolically engineered *E. coli*". Results of her study were presented in the TSB International Forum 2014 held in September 16th-19th, 2014 at BITEC Bang Na, Bangkok, Thailand. When she graduates from Suranaree University of Technology, she will serves as a lecturer or researcher at Royal University of Phnom Penh or Royal Academy of Cambodia, Cambodia.