PRODUCTION OF SUCCINIC ACID FROM SUCROSE AND SUGARCANE MOLASSES BY METABOLIC ENGINEERED *ESCHERICHIA COLI*

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การผลิตกรดซักซินิกจากน้ำตาลซูโครส และกากน้ำตาลอ้อย จากเชื้อ *ESCHERICHIA COLI* ที่ดัดแปลงวิถีกระบวนการสร้างและสลาย

นางสาวสิธา จัน

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

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สิธา จัน : การผลิตกรดซักซินิกจากน้ำตาลซูโครส และกากน้ำตาลอ้อย จากเชื้อ*ESCHERICHIA COLI* ที่ดัดแปลงวิถีกระบวนการสร้างและสลาย (PRODUCTION OF SUCCINIC ACID FROM SUCROSE AND SUGARCANE MOLASSES BY METABOLIC ENGINEERED *ESCHERICHIA COLI*) อาจารย์ที่ปรึกษา : อาจารย์ ดร. เขมวิทย์ จันต๊ะมา, 90 หน้า.

เชื้อเอสเชอริเชีย โคไล สายพันธุ์ KJ122 ได้ถูกดัดแปลงพันธุกรรมเพื่อให้สามารถผลิตซั กซิเนตในระดับความเข้มข้น และ ผลได้ที่สูงในอาหารเลี้ยงเชื้ออย่างง่ายที่มีกลูโคส ภายใต้สภาวะการหมักแบบไร้ออกซิเจนอย่างง่าย อย่างไรก็ตามสายพันธุ์นี้ไม่สามารถใช้น้ำตาลซูโครสอย่างมีประสิทธิภาพเนื่องจากการยับยั้งกระบวนการสลาย ดังนั้นเพื่อที่จะเพิ่มประสิทธิภาพการใช้น้ำตาลซูโครสของสายพันธุ์ KJ122 ยีนที่สร้างเอนไซม์สลายซูโครส (*cscA* and *cscKB*) ที่มีส่วนของโปรโมเตอร์ของเชื้ออีโคไลสายพันธุ์ B ซึ่งความสามารถในการใช้น้ำตาลซูโครสได้ตามธรรมชาติถูกโคลนและแสดงออกในอีโคไลสายพันธุ์ KJ122 โดยที่สายพันธุ์ KJ122 ที่มีพลาสมิดลูกผสมที่ชื่อว่า pKJSUC อยู่จะถูกคัดเลือกการใช้น้ำตาลซูโครสอย่างมีประสิทธิภาพในอาหารเลี้ยงเชื้อชนิด Phenol Red ที่มีน้ำตาลซูโครสผสมอยู่ทั้งอาหาร เลี้ยงเชื้อแบบเหลว และอาหารเลี้ยงเชื้อ แบบอาหารวุ้น โคลนที่ได้สามารถแสดงอาณาเขตโคโลนีสีเหลือง ใสที่ใหญ่กว่าเมื่อเทียบกับสายพันธุ์ KJ122 ที่ไม่มีพลาสมิดลูกผสมอยู่บนอาหารเลี้ยงเชื้อแบบวุ้น และยังแสดงความสามารถในการเจริญ และการสร้างกรดอย่างร วดเร็วในอาหาร เลี้ยงเชื้อแบบเหลว หลังจากการทำวิวัฒนาการของกระบวนการสร้างและสลาย สายพันธุ์ KJ122-pKJSUC สามารถใช้น้ำตาลซูโครสอย่างมีประสิทธิภาพในการผลิตกรดซักซินิก ที่มีความเข้มข้นสูงโดยมีการสะสมของผลิตภัณฑ์อันไม่พึงประสงค์ ในระดับต่ำ ในอาหารเลี้ยงเชื้อแบบ AM1 อาหารเลี้ยงเชื้อแบบเหลวชนิด ที่มีความเข้มข้นเหมาะสม ที่ 70 กรัมต่อลิตรของน้ำตาลซูโครส ให้กรดซักซินิกที่ความเข้มข้น 50.52 ± 1.8 กรัมต่อลิตร (ผลิตผล 1.05 กรัมต่อลิตร ต่อชั่วโมง) ในขวดเลี้ยงเชื้อขนาด 500 มิลลิลิตร ภายใต้สภาวะไร้ออกซิเจน และที่ความเข้มข้น 46.59 ± 1.23 กรัมต่อลิตร (ผลิตผล 0.97 กรัมต่อลิตร ต่อชั่วโมง) ในถังปฏิกรณ์ชีวภาพขนาด 10 ลิตร ภายในเวลา 48 ชั่วโมง อย่างไรก็ตาม พบว่ายาปฏิชีวนะไม่มีผลกระทบต่อการผลิต กรดซักซินิก จากเชื้อสายพันธุ์ KJ122-pKJSUC ภายใต้สภาวะการหมักแบบกะ นอกจากนั้นกรดซักซินิกถูกผลิตที่ความเข้มข้น 47.69 ± 3.94 กรัมต่อลิตร (ผลิตผล 0.99 กรัมต่อลิตร ต่อชั่วโมง) จากกากน้ำตาล อ้อย 150 กรัมต่อลิตร ในขวดเลี้ยงเชื้อขนาดเล็กภายใต้สภาวะไร้ออกซิเจน ในเวลา 48 ชั่วโมง จากการเพิ่มขนาดการหมักในถังปฏิกรณ์ชีวภาพขนาด 10 ลิตรพบว่ากรดซักซินิกถูกผลิตขึ้นที่ความเข้ มข้น 35.14 ± 7.53 กรัมต่อลิตร และ 65.01 ± 0.64 กรัมต่อลิตร ภายในเวลา 48 และ 96 ชั่วโมง ตามลำดับ จากผลการทดลอง ดังกล่าวแสดงให้เห็น ว่าเชื้ออี โคไล KJ122-pKJSUC น่าจะเป็นสายพันธุ์ที่มีศักยภาพในการผลิต กรดซักซินิกจากน้ำตาลซูโครส และกากน้ำตาลอ้อยที่มีความคุ้มค่าทางเศรษฐกิจ

สาขาวิชาเทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา_____

ปีการศึกษา 2553 ลายมือชื่ออาจารย์ที่ปรึกษา_____

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม_____

SITHA CHAN : PRODUCTION OF SUCCINIC ACID FROM SUCROSE AND SUGARCANE MOLASSES BY METABOLIC ENGINEERED *ESCHERICHIA COLI*. THESIS ADVISOR : KAEMWICH JANTAMA, Ph.D., 90 PP.

SUCCINATE/ *ESCHERICHIA COLI*/ ANAEROBIC CONDITIONS/ SUCROSE/ SUGARCANE MOLASSES

Escherichia coli KJ122 was engineered to produce high titer and yield of succinate in mineral salt medium containing glucose under simple-batch anaerobic conditions. However, this strain does not efficiently utilize sucrose due to catabolic repression. To enhance the sucrose utilization of KJ122, sucrose utilizing genes (*cscA* and *cscKB*) containing their native promoters from *E. coli* B, which is able to naturally utilize sucrose, were cloned and functionally expressed in KJ122. The transformants harboring a recombinant plasmid named pKJSUC were selected for the efficient sucrose utilization on the phenol red agar and broth supplemented with sucrose. The clones exhibited a larger clear yellow zone on the agar compared to KJ122 without the plasmid, and showed a high ability in fast growth and acid production in cultivation broth. After performing metabolic evolution, KJ122-pKJSUC efficiently consumed sucrose to produce high succinate concentration with less accumulation of by-products in AM1 medium. The optimal concentration of sucrose at 70 g/L could produce succinate at 50.52 ± 1.80 g/L (productivity at 1.05 g/L/h) in 500 mL small anaerobic bottles and at 46.59 ± 1.23 g/L (productivity at 0.97 g/L/h) in 10 L bioreactor within 48 hours. In addition, it was found that antibiotics had no effects on succinate production by KJ122-pKJSUC under batch conditions. Succinate at concentration of 47.69 ± 3.94 g/L (productivity at 0.99 g/L/h) was produced from 150 g/L molasses in a small anaerobic bottle after 48 hours. In 10 L bioreactor, succinate at concentration of 35.14 ± 7.53 g/L (0.73 g/L/h) and 65.01 ± 0.64 g/L (0.67 g/L/h) was

produced within 48 hours and 96 hours, respectively. These results demonstrated that KJ122-pKJSUC would be a potential strain for the economic bio-based succinate production from sucrose and sugar cane molasses.

School of Biotechnology

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Student's Signature_____

Advisor's Signature_____

Co-advisor's Signature _____

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Sitha Chan

CONTENTS

Page

CONTENTS (Continued)

Page

CONTENTS (Continued)

Page

LIST OF TABLES

Table **Page**

LIST OF FIGURES

Figure **Page**

LIST OF FIGURES (Continued)

Figure **Page**

LIST OF FIGURES (Continued)

Figure **Page**

LIST OF FIGURES (Continued)

Figure **Page**

LIST OF ABBREVIATIONS

ACKA	=	Acetate kinase
ADHE	=	Alcohol dehydrogenase
AMP	=	Adenosine monophosphate
ASPC	=	Asparte aminotransferase
ATP	=	Adenosine triphosphate
ATCC	=	American type culture collection
bps	=	Base pairs
cAMP	=	Cyclic Adenosine monophosphate
CFU	=	Colony form unit
CITF	=	Citrate lyase
CRP	=	Catabolic repression protein
csc	=	Chromosomally encoded sucrose catabolism
CSL	=	Corn steep liquor
E. coli	=	*Escherichia coli*
FruR	=	Fructose repressor
GalP	=	Galactose permease
h	=	Hour
HPLC	=	High performance liquid chromatography
IPTG	=	Isopropyl-β-D-thiogalactoside
kg	=	Kilo gram
L	=	Liter
LB	=	Luria Bertani

LIST OF ABBREVIATIONS (Continued)

LDHA	=	Lactate dehydrogenase
MGSA	=	Methylglyoxal synthase
mM	=	Milli-molar
N	=	Normality
NADH	=	Nicotinamide adenine dinucleotide (reduced form)
NAD^+	=	Nicotinamide adenine dinucleotide (oxidative form)
^oC	=	Degree Celsius
OD	=	Optical cell density
p.s.i	=	Pounds per square inch
PCK	=	Phosphoenolpyruvate carboxykinase
PCR	=	Polymerase chain reaction
PEP	=	Phosphoenolpyruvate
PFLB	=	Pyruvate formate-lyase
POXB	=	Pyruvate oxidase
PPC	=	Phosphoenolpyruvate carboxylase
PTA	=	Phosphotransacetylase
PTSG	=	Glucose-specific transporter of the phosphotransferase system
rpm	=	Revolutions per minutes
SFCA	=	Malic enzyme
TBE	=	Tris-borate-EDTA
TDCDE	=	Propionate kinase
UV	=	Ultraviolet
V	=	Volt
vvm	=	Gas volume flow per unit of liquid volume per minute

LIST OF ABBREVIATIONS (Continued)

w/v	=	Weight per volume
X-gal	=	Bromo-chloro-indolyl-galactopyranoside
YE	=	Yeast extract
μL	=	Micro-liter

CHAPTER I

INTRODUCTION

Regarding to the world-wide oil demand for fuel and commodity chemicals, a use of crude oil would be reduced in the near future due to declining in reserves, increasing in price and green house gas pollution. Shifting the fossil-based chemicals to the bio-based chemicals, biotechnology industries have been pushed for sustainable production (Mckinlay *et al*., 2007). Moreover, biotechnology pays attention in hazardous reduction, safety chemical promotion, biodegradable chemical, and life cycle perspective (Hatti-kaul *et al*., 2007; Sauer *et al*., 2008). In common, biocatalysts have been applied in the industries, especially, in food and pharmaceutical. Microorganisms, so far, have been genetically engineered to produce a target end product with high yield but less by-products. *E. coli* is well known to be modified in metabolic pathway for production of variety of value organic acids and ethanol derivatives (Yu *et al*., 2010).

Succinic acid is considered as a high value organic acid which could be manufactured form feedstocks (Werpy *et al*., 2004). Succinic acid is a precursor of many specialty chemicals for food, pharmaceuticals, green solvent and biodegradable plastics. These chemicals include 1, and 4-butanediol, tetrahydrofuran, and N-methyl pyrolidinone. Currently, succinic acid has been mainly supplied by petrochemical process through the conversion of maleic anhydride. Unlike chemical process, succinic acid fermentation is based on renewable resources and CO_2 consumption. Therefore, the succinic acid fermentation has more benefits over the chemical process (Sauer *et al*., 2008).

1.1 Application of Succinic Acid

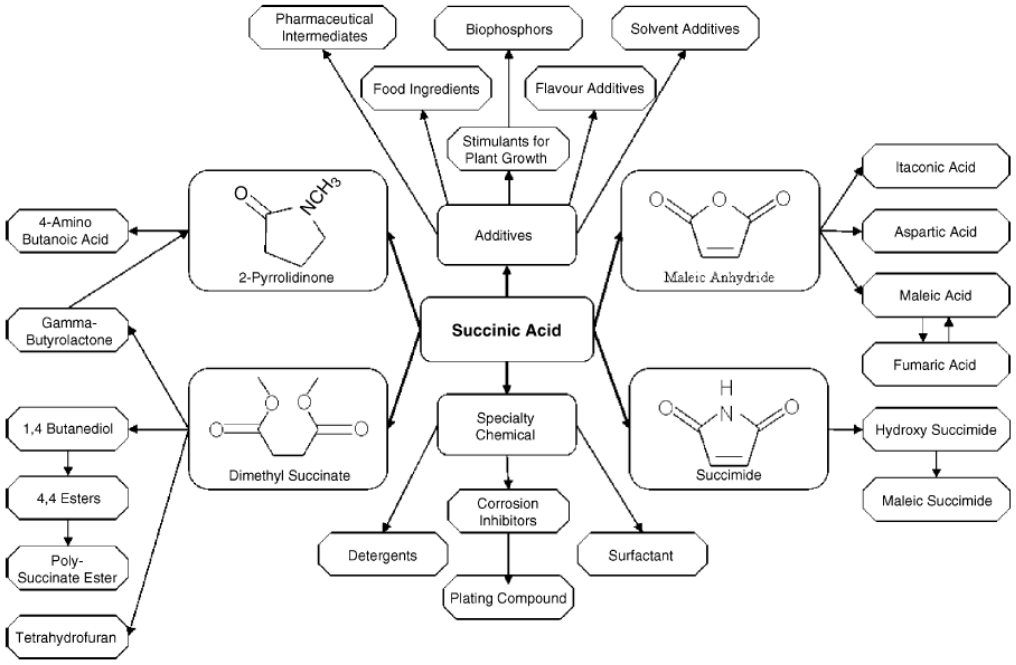

Figure 1.1 Various chemicals derived from succinic acid (Song *et al*., 2006).

Succinic acid is a platform chemical for many commodity and industrial chemicals. In figure 1.1, principal applications of succinic acid were described (Song *et al*., 2006). First, succinic acid is applied as a surfactant, an additive as a detergent and foaming agent. Second, it acts as ion chelator in which it is used in electroplating for preventing the corrosion of metal. Third, succinic acid performs as an acidulant, a pH regulator and flavoring agent in the food industry. For example, sodium succinate and dilysine succinate were introduced as flavoring enhancers which could replace monosodium glutamate in low sodium foods (Jain *et al*., 1989). Fourth, succinic acid is used in pharmaceutical products such as antibiotics, amino acids, and vitamins. In addition, succinic acid was a precursor of specialty chemical including adipic acid, 1,4-butanediol, tetrahydrofuran, *N*-methyl pyrrolidinone, pyrrolidinone, succinic acid esters, and succinate salts. More interestingly, succinic acid derivatives which have diamines and diols group, can be used as monomer of bio-degradable plastics such as polyester, polyamides, and polyester amides (Bechthold *et al*., 2008).

1.2 Commercial of Succinic Acid

Currently, the large use of succinic acid and its derivatives is around 20,000-30,000 tones per years with the potential price of $400,000,000 per year (Kidwell *et al*., 2008; Zeikus *et al*., 1999). This rate increases by 10 % per year and the market size is estimated to be more than 270,000 tons per year (Willke and Vorlop *et al*., 2004). A commercialized succinic acid is mainly produced by chemical process from butane or oxidation of benzene through maleic anhydride. The price of succinic acid is reported to be in the range of $5.9 – 9.0 /kg depending on its purity. Confronted with the rising price of petroleum and pollution, there are many researchers tending to make succinic acid from renewable resources using microorganism and green biotechnology. The large scale of fermentative succinate was produced in early 1980 (Zeikus *et al*., 1980). Fermentative succinate production is about 5,000 ton per year and is sold at $2.20/kg to the food market. As expected, natural succinate price would be decreased by $0.55/kg if production size would be above 75,000 tones due to utilizing cheap carbon substrates such as corn, starch, molasses, and sugars (Zeikus *et al*., 1999; Kidwell *et al*., 2008). Bio-based succinate needs consumption of CO_2 during fermentation so this process would contribute to reduce green house gas. Moreover, it declines pollution from the manufacturer by constituting many commodities based on benzene and intermediate petrochemical of over 250 bezene-dirived chemicals (Ahmed and Morris *et al*., 1994). In this decade, fermentative succinate has much been developed in commercial scale using engineering *Escherichia coli* strains as a biocatalyst. In 2011- 2012, hundred tones of succinic acid is expected to be produced using *E. coli* as a catalyst and glucose as a feedstock in a administration plant which was developed by DSM and France's Roquette Frères.

Meanwhile, Myriant from the USA tested the scale-up production of succinic acid in the 20,000 L bioreactor, also used *E. coli* as bio-catalyst but unrefined sugar as feedstock (http://www.rsc.org/chemistryworld/News/ 2010/January/21011003.asp). In Japan, Mitsubishi has

also attempted to industrialize fermentation production of succinic acid, which will be used as monomer units of the company's biodegradable plastics (Xu *et al*., 2010). For industrial scale of succinate production, the costs of substrates and downstream processing are crucial constraints to become economically viable for succinate production. Purification has cost around 60%-70% of total bio-production process (Sauer *et al*., 2008).

Therefore, this study focused on succinate production by metabolic engineered *E. coli* in simple batch fermentation under anaerobic conditions. In this study, cheap sugar substrates such as sucrose or sugarcane molasses will be utilized to reduce cost of production. More interestingly, the pollution occurring from wastes of sugar production (sugarcane molasses) would be reduced.

1.3 Research Objectives

Up to date, succinic acid is mainly formed by chemical process. To achieve bio-based succinate production for large scale and to decrease pretochemical succinate processing, the production of bio-based succinate would be performed with cheap carbon substrate in which the lower of production cost would be achieved. Agricultural resources are renewable and abundant on market. In South-east-Asia, sucrose is one of the most prevalence and inexpensive carbon substrates. Moreover, sugarcane molasses, by-product during sugar production, is rich in sugars for succinate production. A strain KJ122 was derived from *E. coli* ATCC 8739, and was developed to produce high level of succinate in mineral salt medium containing glucose as carbon source under anaerobic conditions by a combination using metabolic engineering and metabolic evolution. Compared to theoretical yield, 85% succinate was produced by this strain with the high yield, and titer average productivity but less by products. This strain might be a good producer for commercial production of succinate because it produces succinate under simple conditions (anaerobic and batch fermentation), and simple nutrients (glucose with mineral salts medium) without additional of plasmid or foreign genes.

However, KJ122 strain is not able to consume sucrose efficiently to produce succinate due to the catabolic repression. To improve succinate market economically, the production of succinate from metabolic engineered *E. coli* KJ22 would be performed in a mineral medium comprising sucrose or cane molasses as a sole carbon source to lower the cost of medium. Therefore, this project studied the succinate production from sucrose and sugarcane molasses in mineral salts medium by metabolic engineering *E. coli* under simple batch conditions. Additionally, the characteristic of heterologous gene expression, pKJSUC that harbored the sucrose utilizing gene (*cscBKA*) in KJ122, was investigated to elucidate the effect of the recombinant plasmid on cell growth and succinate production in this strain. The other parameters such as optical density (OD), sugar concentrations, effect of antibiotic, and effect of large scale were demonstrated in the study. Meanwhile, the study indicated efficient succinate production from sucrose and sugarcane molasses in terms of titer and productivities in KJ122 carrying the cloned plasmid.

1.4 Scope of this study

This research work emphasized on the study of succinate production in the previously engineered *E. coli* KJ122 that produces succinate as a major fermentative product during anaerobic-batch fermentation from mineral salts medium containing sucrose or sugarcane molasses as a sole carbon source. Batch fermentation of succinate was conducted with KJ122 harboring a newly constructed plasmid, pKJSUC, containing sucrose-utilizing genes from *E. coli* KO11. Metabolic evolution of KJ122-pKJSUC was performed to select the clones that produce succinate with the high titer, yield, and productivities from medium containing sucrose and the cheap sucrose sources such as sugarcane molasses. Therefore, the best representative clone were used for testing the parameters affecting the succinate production such as types and concentration of sugars supplemented to the medium, types of mediums (LB and AM1), the initial cell concentrations and the larger production.

CHAPTER II

LITERATURE REVIEW

2.1 Succinate Producers

Succinic acid is an intermediate in the tricaboxylic acid cycle and one of the fermentation end products of anaerobic of metabolism by several anaerobic and facultative microorganisms (Song *et al*., 2006). Even though succinate can be produced by fungi and bacteria, only bacteria are used in fermentation for food and beverages. Various bacteria have been reported to produce succinic acid including typical gastrointestinal bacteria, rumen bacteria, some lactobacillus strains and *E. coli* strain (Kaneuchi *et al*., 1988; Agarwal *et al*., 2006).

There are many bacteria which have been found to produce high succinate as a major product in fermentation (Guettler *et al*., 1998). Efficient strains such as *Actinobacillus succinogenes* (Guettle *et al*., 1996 a, b). *Anaerobiospirillum succiniciproducens* (Glassner and Datta *et al*., 1992), and *Mannheimia succinoproducens* (Lee *et al*., 2006) naturally produce as high productivity as 4 g/L/ h with impressive titers at 300-900 mM and high yields more than 1.1 mol succinate/mol glucose. Even though, both microorganisms have ability to produce succinate at high rate, they require complex media ingredients in which the process increases production cost involving 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 models. In this case, medium should be cheap and fermentation process should be simple (Saure *et al*., 2008). Second, product recovery would be designed to purify succinate from mixed acids by using electro-dialysis, ion exchanger

and amine-based extraction (Song *et al*., 2006), simulated moving bed (SMB) chromatographic process (Wang *et al*., 2010) and nanofiltration (Kang *et al*., 2005).

2.2 Succinate Producing Pathways in Microorganisms

Succinic acid producing bacteria produce not only succinate but also mixed acid such as lactic acid, acetic acid and formic acid including ethanol during fermentation. The rumen bacterium, *A. succiniproducen*, is reported to produce succinate up to 120 mol/100 mol glucose, accompanied with by-products of acetate, pyruvate, formate and ethanol. Unlike rumen bacteria, *E. coli* produces succinate as a minor product as low as 12 mol/ 100 mol glucose (Zeikus *et al*., 1999). In theory, proximally 1.71 mol succinate is produced per mol glucose consumed plus CO_2 (Equation 1). Based on redox balance, 24 electrons in glucose are converted to 14 electrons in succinate (Mckinlay *et al.,* 2007).

$$\textbf{Glucose + 0.86 } HCO_3^- \longrightarrow \textbf{1.71 succinate}^{2-} \textbf{ + 1.74 } H_2O \textbf{ + 2.58 } H^+ \text{ (Eq. 1)}$$

There are several pathways to form succinate. One pathway involves phosphoenolpyruvate (PEP) carboxylation that is catalyzed by PEP carboxylase or PEP carboxykinase. Under anaerobic conditions, succinate is normally produced by the PEP carboxylation pathway. *An. succiniciproducens* and *A. succinogenes* have been known to be the best succinic acid producing strains. The strains produce succinic acid through four reactions catalyzed by PEP carboxykinase, malate dehydrogenase, fumarase and fumarate reductase, respectively. However, *E. coli* metabolizes glucose to primarily produce acetate, formate and ethanol as well as minor concentration of lactate and succinate under anaerobic fermentation (Figure 2.1).

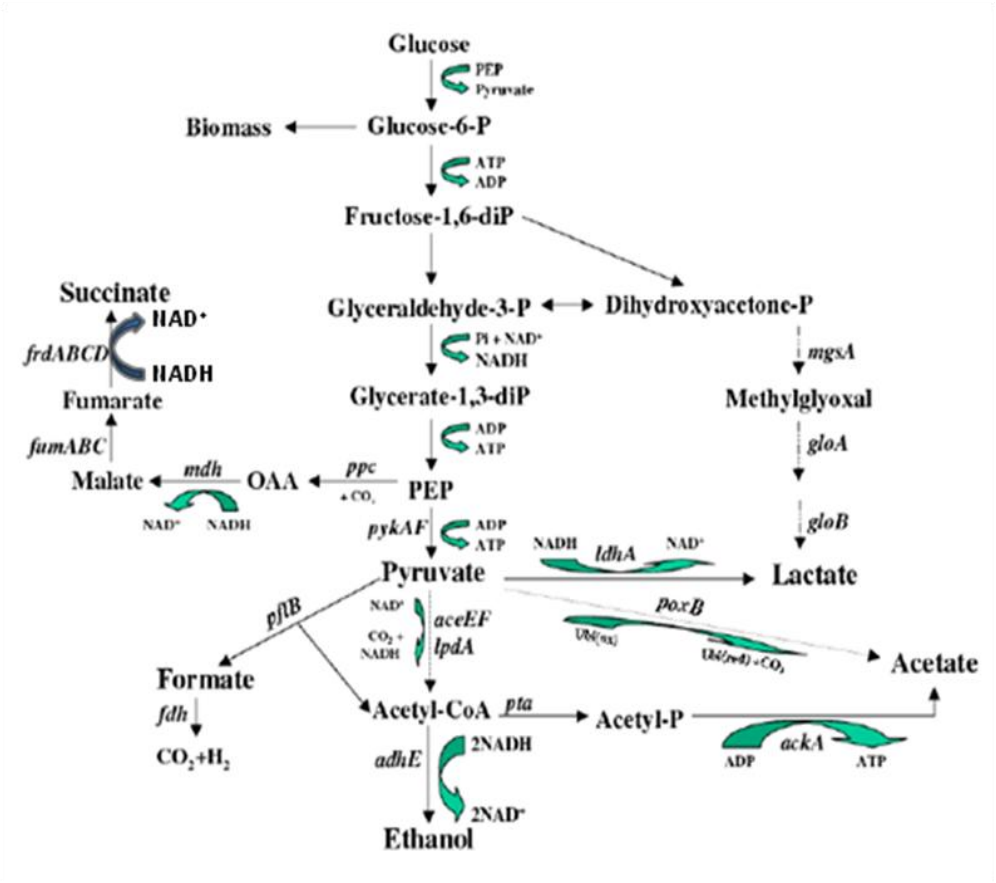

Figure 2.1 Central metabolic pathway of *E. coli*. Solid arrows represent central fermentative pathways. Dotted arrow represents microanaerobic pathway (*poxB*). Dash arrow represents minor lactate producing pathway (*mgsA, gloAB,*). Genes: *pykAF*: pyruvate kinase, *ldhA*: lactate dehydrogenase, *pflB*: 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*., 2008a).

2.3 Development of Succinic Acid Producing Strains from *E. coli*

E. coli has been considered as the best studied bacterium because the microorganism has an ability to grow fast without a requirement of complex nutrients and it is easy to manipulate its metabolic pathways by genetic engineering. Many researchers studied extensively on pathways for constructing *E. coli* strains. Succinate can be produced under both aerobic and anaerobic conditions. Since aerobic system provided the maximum theoretical yield of sucinate less than that of anaerobic system, and CO_2 production was result in aerobic route. Therefore, anaerobic conditions were considered as dominant pathway for engineering *E. coli* (Mckinlay *et al*., 2007). Glucose was fermented to PEP and the carboxylation of PEP to oxaloacetate by *ppc* is the primary fermentative route for succinate (Figure 2.2). Meanwhile NADH is generated in glycolysis pathway for cell growth and being as intermediate (co-enzyme) in succinic acid production.

Since rumen bacteria had natural ability to produce high succinate, genetically modified metabolic pathway was developed to engineer some key enzymes to be closed to the nature pathway. Succinic acid production from glucose by *E. coli* was increased overexpressing of endogenous phosphoenolpyruvate carboxylase (*ppc*, PEPPC), while overexpression of endogenous phosphoenolpyruvate carboxykinase (*pck*, PEPCK) had no effect (Millard *et al*., 1996). However, overexpression of

A. succinogenes PEPCK increased the production of succinic acid as much as 6.5-fold (Kim *et al*. 2004). Moreover, analysis of metabolism *in silico* was used to modify *E. coli* pathway to be similar to rumen bacteria pathway. However, the result showed that succinate produced was low (Lee *et al*., 2005). The highest level of succinate production is 339 mM by engineered *E. coli* containing only native genes (Andersson *et al*., 2007).

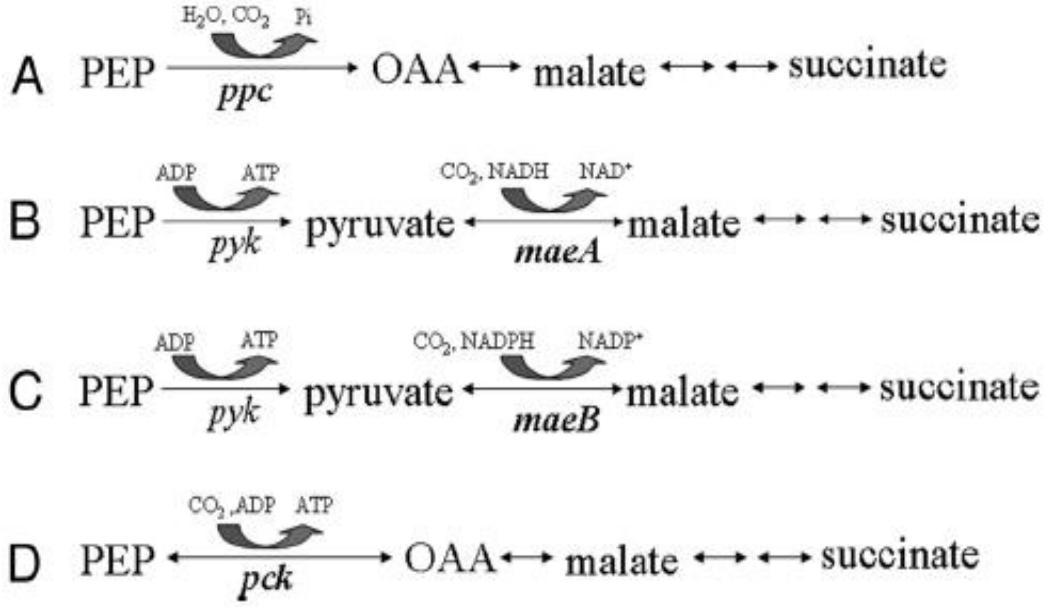

Figure 2.2 Carboxylation pathways potentially available for succinate production in *E. coli*. (A) PEP carboxylase (primary fermentation route). (B) NADH linked malic enzyme (gluconeogenic). (C) NADPH-linked malic enzyme (gluconeogenic). (D) PEP carboxykinase (gluconeogenic). Genes encoding carboxylation activities are shown in bold (Zheng *et al*., 2009).

In order to convert pyruvate to succinate, pyruvate carboxylase plays an important enzyme in one of available pathways for succinate production in *E. coli* (Figure 2.2). Overexpression of pyruvate carboxylase in AFP11 (AFP111/pTrc99A-*ppc*) extremely increased succinate titer (99.2 g/L) and productivity (1.3 g/L/h). Pyruvate could be reconverted to PEP by ATP with the formation of PPi and AMP, but the energy is wasted by this process (Zhang *et al*., 2009). Since *E. coli* excretes succinate in small amount, gene knockouts in succinnic acid competition pathway were done to improve its yield. For example, AFP111, a mutant in *ptsG* system (glucose phosphotransferase), pyruvate-formate lyase, and lactate dehydrogenase, could produce succinate at concentration of 36 g/L

(Chartterjee *et al*., 2001). Next, gene inhibition was intensively studied in central pathway of *E .coli*. Sanchez *et al*., 2005 reported that SBS550MG was efficient in succinate production at 1.6 mol/mol glucose consumed with productivity of 10 mM/h. This strain was deactivated the fermentation by-products genes such as *ldhA* encoding lactae dehydrogenase, *adhE* encoding alcohol dehydrogenase , *ack-pta* encoding acetate kinase and phosphate acetyl transferase and the targeting glyoxylate shunt flux by deleting the transcriptional repressor of the glyoxylate shunt (*iclR*) and over expressing *Bacillus subtilis citrate synthase* and *B. retli pyc* from a multi-copy plasmids. The overexpressions of some key enzymes for succinate development were observed. Overexpressed fumurate reductase (*frdABCD*) activated fumarate to succinate in recombinant *E. coli* with the conversion rate at 93% (Goldberge *et al*., 1983; Wang *et al*., 1998). Carbonic anhydrase from *Anabaena* sp.7120 was overexpressed in *E. coli* to provide bicarbonate for improved succinate production (Wang *et al*., 2009).

For an alternative carboxylation pathway, malate dehydrogenase (*mdh*) was expressed in JCL1208 strain to dissipate accumulated pyruvate to succinate resulting in 108 mM succinate with a molar yield of 0.98 mol succinate per mol of fermented glucose (Millard *et al*., 1996). In 2009, Wang observed that the specific glucose consumption rate and specific productivity were increased with overexpressing *mdh* in the *ldhA* double mutant NZN111 resulting in 1.14 mol succinate produced per mol glucose consumed (Figure 2.2).

Also, *E. coli* was genetically engineered to produce succinic acid under aerobic condition. In 2005, Lin et al. constructed strain HL27659K that was eliminated in succinate dehydrogenase (*sdhAB*), phosphate acetyltransferase-acetate kinase (*pta-ackA*), pyruvate oxidase (*poxB*), glucose transporter (*ptsG*), *iclR* and overexpressed phosphorenolpyruvate

carboxylase (*ppc*) to promote the glyoxylate shunt and to make succinate as a main product. This strain produced less than 100 mM succinate with yield (0.91 mol/ mol of glucose consumed) and required oxygen in fermentation (Lin *et al*., 2005a, b, and c).

Considering the cost of materials (medium), purification, and waste disposal, novel strain of *E. coli* C was genetically engineered and developed by growth-based selection (metabolic evolution) for increased succinate production in a mineral salts medium supplemented to 1 mM betaine as an osmoprotectant (Jantama *et al*., 2008b). KJ122 was reported to be efficient in metabolizing glucose to succinate in high titer, yield, and productivity but excreting less by-products. Hence, this strain can be comparable to the best succinate producing rumen bacteria.

Examples of succinate fermentation in two stages fermentation are improved in downstream processing. High succinate productivity (1.77 g/L/h) was maintained by resuspending cells in fresh-non complex media (Andersson *et al*., 2009). Recovered cell produced succinic acid in medium containing only glucose and $MgCO_3$. This method provided succinate acid mass yield of 0.85 g/g and average productivity of 1.81 g/L/h (Ma *et al*., 2010). Dual phase fermentation was noted to cooperate with sparging of O_2, CO_2 or H_2 (Andersson *et al*., 2007 and 2009; Ma *et al*., 2010).

2.4 KJ122 and its derivatives

KJs' strains were developed to be not only efficient strains, but they also acted as an ideal biocatalyst for industrial succinate production based on fast growth, no requirement of expensive nutrients, but less mixed organic acids under simple batch conditions. There are some dominant KJs' strains which are noticeable in high succinate production. Stain KJ073, derived from wide type *E. coli* ATCC 8739, was constructed by combination of metabolic engineering and metabolic evolution. Strain KJ073 was knockout in genes encoding

alternative NADH oxidizing pathway such as Δ*ldhA::FRT* Δ*adhE::FRT* Δ*ackA::FRT* Δ*(focA-pflB)::FRT* Δ*mgsA* and Δ*poxB.* The strain produced succinate with molar yield of 1.2 per mole of glucose consumed; however, after genes deletion, there are all FRT fragments remain (Jantama *et al*., 2008a). Strain KJ091 was genetically improved by removing all FRT fragments in which this strain was similar to KJ073 for producing succinate (Jantama *et al*., 2008b). Further development, KJ122 was constructed and able to be a high succinate producer (Figure 2.3). Strain KJ122 (Δ*ldhA* Δ*adhE* Δ*ackA* Δ*(focA-pflB)* Δ*mgsA* Δ*poxB* Δ*tdcDE* Δ*citF* Δ*aspC* Δ*sfcA*) produced excellent succinate yield (1.46 mol/mol glucose), succinate titer (80 g/L) and average volumetric productivity (0.9 g/L/h) at 96 hours.

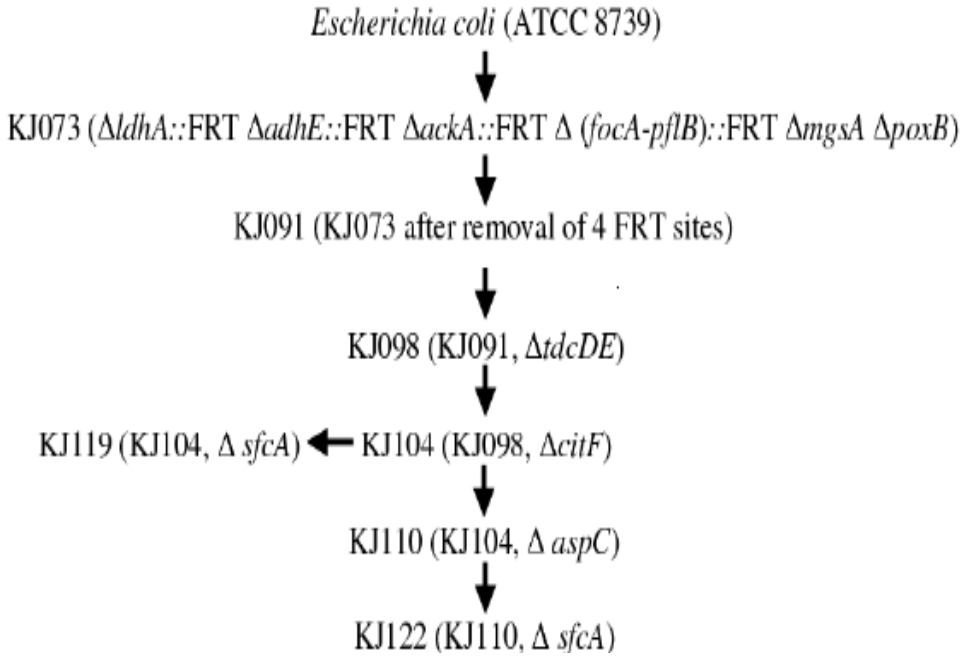

Figure 2.3 Strain construction of KJ122 (Jantama *et al*., 2008a, b).

Brief summary of main events happened during metabolic engineering and evolution for constructing KJ122 was explained as below.

1. **Elimination of lactate dehydrogenase (*ldhA*)**: This pathway was knocked out to conserve both NADH and carbon atoms, not loss to the production of lactate under anaerobic conditions. The elimination of this enzyme helps channeling carbon skeletons to PEP pool.
2. **Elimination of alcohol dehydrogenase (*adhE*)**: Deletion of the pathway had function to conserve both NADH for further succinate formation through the native fermentation pathway, and carbon atoms for formation of OAA.
3. **Elimination of acetate kinase (*ack*):** The route conserved carbon atoms and in which the deletion of this enzyme 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 production of formate as reductant and the extremely production of acetyl-CoA, a potential source of acetate. Deletion of *focA-pflB* is well known in causing acetate auxotrophy under anaerobic conditions (Sawers and Bock *et al*., 1988). Surprisingly, after selection by metabolic evolution, acetate was omitted and KJs' strains are grown without acetate.
5. **Elimination of methylglyoxal synthase (*mgsA*):** The objective of knocking out of *mgsA* is to dissipate lactate and to reduce an accumulation of methylglyoxal, an inhibitor of both growth and glycolysis (Egyud *et al*., 1966; Grabar *et al*., 2006; Hopper *et al*., 1971).
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 amounts also increased.

7. **Elimination of propionate kinase (*tdcDE*):** Expression of *tdcD* could functionally replace *ackA* thus 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 eliminating malate production, reducing actatate and pyruvate production, and increasing succinate formation.
8. **Elimination of citrate lyase (*citF*)**: Under anaerobic conditions, OAA partition is 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*) in which a mechanism is proposed to recycle the intracellular OAA pool for other metabolic functions (Nilekani and Sivaraman *et al*., 1983). CitF was knocked out in KJ098 to produce KJ104. There is no effect on succinate and acetate levels, even though; cell yield was decreased by 22 %.
9. **Eliminating the combination of aspartate 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 to construct KJ122 had no effect on succinate yield, cell yield, or acetate. Therefore, aspartate might be formed by alternative pathways such as aspartate ammonia-lyase (*aspA*). The high level of malate to pyruvate could result from the decarboxylation of malateto pyruvate by malic enzyme (*sfcA*). This pathway is well known to occur during gluconeogenisis. There is no improvement in succinate production and cell growth after *sfcA* deletion in KJ119. This result is opposite to what Stole and Donnely *et al*., 1997 found that over expression of

malic enzyme is an available route for succinate production. Surprisingly, the combination of *aspC* and *sfcA* deletions 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 is 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 decarboxylase.

In 2009, Zhang found that increased succinate production in KJs' strains were due to increasing expression of PEP carboxykinase (*pck*) and inactivation of the glucose phosphoenolpyruvate-dependent phosphotransferase sytem (PTS) after metabolic evolution. In addition, the glucose uptakes system was replaced by an alternative pathway, galactose permease (GalP) in KJ strains. GalP was noted to use ATP rather than PEP for phosphorylation. This provided an energy mechanism to increase the pool size of PEP and to facilitate redox balance. Furthermore, improved succinate yield made the succinate producing pathway in *E. coli* that was closed to succinate producing rumen bacteria (Zhang *et al*., 2010).

2.5 The demands and concepts for developing bio-based succinate

The biological production processes of succinate need to be economically feasible including a yield around 0.88 g/g, a rate between 1.8 and 2.5 g/L/h and a titer around 80 g/L (Beauprez *et al*., 2010). To date, none of the developed microbial strains which have been reported has reached all of these standards. However, developments in the metabolic engineering methods mentioned above showed great promise for further improvements in the near future. Next efforts should be done to optimize the current metabolic engineering towards succinic acid rather than to set up new metabolic routes (Yu *et al*., 2010). To make the fermentation process competitive, researchers are attempting to find more productive

microbial strains that can resist high concentration of succinic acid and utilize cheap feedstock and to develop novel separation and purification technology with low cost (Xu *et al*., 2010).

2.6 Availability of various carbon sources to produce succinic acid

One of the key aspects in the fermentation process is the development of a cost effective culture medium to obtain maximum product yield. Production of succinic acid has been reported from variety of carbon sources utilized by *E. coli* (Table 2.1) and rumen bacteria (Table 2.2). In general, glucose was known as an appropriate substrate for *E. coli* and rumen bacteria in succinate production. Considering the cost of substrate, varieties of agricultural sources and wastes including sugarcane molasses (Agarwal *et al*., 2006; Liu *et al*., 2008), glycerol (Lee *et al*., 2001; Zhang *et al*., 2010), whey (Samuelov *et al*., 1999; Lee *et al*., 2003a; Wan *et al*., 2008), sucrose (Liu *et al*., 2008; Oh *et al*., 2009), lignocelluloses including wood hydrolysate and straw hydrolysate (Kim *et al*., 2004, Lee *et al*., 2003b), corn stalk (Li *et al*., 2010), wheat miling by-product (Dorado *et al*., 2009), corn straw hydrolysate (Zheng *et al*., 2009), and corncob hydrolysate (Yu *et al*., 2010) have been tested to produce succinic acid by fermentation. It was noted that many researchers have focused on studying of conversion of agricultural waste products to succinate, however, downstream processing is crucial in cost-effectiveness to produce succinate. Therefore, the purity of substrates and less complex nutrient medium should be considered (Sauer *et al*., 2007; Jantama *et al*., 2008).

Table 2.1 Comparison of succinate production from carbon sources[a] in *E. coli*.

Organism	Medium/condition	Succinate titer (g/L)	Reference
E. coli AFP111 (*pflAB, ldhA, ptsG*) *Rhizobium* etli *pyc* overexpressed.	Glucose (40 g/L; 90 g/L total glucose) in medium supplemented to 20 g/L tryptone , 10 g/L YE and 40 g/L $MgCO_3$,dual phase fed batch, 76 h incubation.	99.31 [1.31][b]	Vemuri *et al*., 2002 a,b
E. coli KJ122 (*ldhA, adhE, ackA, (focA-pflB), mgsA , poxB, tdcDE, citF, aspC, sfcA*)	Glucose (100 g/L) supplemented to AM1 medium and 10 g/L $NaHCO_3$, simple batch, pH maintained with 1:1Mixture of 6 M KOH+3 M K_2CO_3, 120 h Incubation.	82.66 [0.9]	Jantama *et al*., 2008b
E. coli HL27659K/pKK313 (*iclR, sdhAB ackA-pta poxB, pstG*) Sorghum vulgare *pepc* expressed	Glucose (106 g/L) in medium supplemented to 20 g/L tryptone, 32 g/L YE and 2 g/L, $NaHCO_3$ fed batch fermentation under complete aerobic condition, 59 h incubation.	58.92 [1.00]	Lin *et al*., 2005d
E. coli SBS550MG (*ldhA, adhE, iclR, ackA-pta*), *L. latis pyc, B. subtilis citZ*	Glucose (20 g/L; 100 g /L total glucose) LB supplemented to 1 g/L $NaHCO_3$, 200 mg/L ampicillin, and 1mM IPTG, 100% CO_2 at 1 L/min headspace, repeated fed-batch fermentation , 95h incubation.	40.03 [0.42]	Sanchez *et al*., 2005; Cox *et al*., 2006

E. coli AFP184 (*pflB, ldhA*, and *ptsG*)	Carbon source: glucose, fructose, xylose, glu/fru and glu/xyl with 5 g/L CSL, Dual phase aerobic growth and anaerobic production, sparging with air flow by CO_2, 32 h incubation time.	25-40 [0.78-1.25]	Andersson *et al*., 2007
E. coli SBS550MG, pHL413, 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.	32.65 [0.34]	Wang *et al*., 2011
E. coli from the rumen of buffalo	Sugarcane molasses (10% (v/v)) supplemented to 10% CSL, 20 mM $NaCO_3$, CO_2 sparging, incubation time 72 h.	17 [0.56]	Agarwal *et al*., 2006
E. coli ATCC8739 (*pflB, ptsI*, and *pck* overexpressed)	Glycerol 5 % (w/v), NBS medium supplemented to 5% (w/v) glycerol, 100 mM $KHCO_3$, pH controlled by 2.4 M K_2CO_3 with 1.2 M KOH, simple batch, 144 h incubation time.	12.04 [0.083]	Zhang *et al*., 2010

[a] Abbreviation CSL, corn steep liquor; YE, yeast extract; NR, not reported.

[b] Average volumetric productivity is shown in brackets [g L^{-1} h^{-1}].

Table 2.2 Comparison of succinate production from carbon sources in rumen bacteria.

Organism	Medium/ condition	Succinate titer (g/L)	Reference
A. awamori 2B 361 U2/1	62.1 g/L of wheat milling by-product, semi-defined medium with YE, 10 g/L $MgCO_3$, pH controlled by 10 M NaOH, batch fermentation, and 50 h total incubation time.	62.1 [1.24]	Dorado *et al*., 2009
A. succinogenes CGMCC1593	Sugarcane molasses (64.4 g/L of sugar mixture) supplemented to AS medium, 10 g/L YE, $MgCO_3$ (varied), 0.05 vvm CO_2 sparging rate, pH controlled by 3 M Na_2CO_3, batch fermentation, 48 h total incubation.	46.4 [0.96]	Liu *et al*., 2008
A. succinogenes CGMCC1593	58 g/L of corn straw hydrolysates, 15 g/L YE, $MgCO_3$ (varied), some minerals, CO_2 sparging at 0.1vvm, batch fermentation, incubation time 48 h.	45.5 [0.94]	Zheng *et al*., 2009
A .succinogenes 130Z (ATCC 55618)	100 g/L cheese whey, 5 g/L YE, 10 g/L peptone, some minerals, 0.5 vvm CO_2 flow rate, batch	27.9 [0.58]	Wan *et al*., 2008

A. succinogenes	30 g/L Corncob hydrolysate, 20 g/L YE, 40 g/L $MgCO_3$, some minerals, batch fermentation, 48 h total incubation time.	23.64 [0.49]	Yu *et al*., 2010
A. succinogenes BE-1	30 g/L Corn stalk, 30g/L YE, 2g Urea, 30g $MgCO_3$, some minerals, pH controlled by 10 M NaOH, batch fermentation, incubation time 72 h.	15.8 [0.22]	Li *et al*., 2010
M. succiniciproducens MBEL55E (KCTC 0769BP)	Pre-treated wood hydrolysate (16.09 g/L glucose and 7.1 g/L xylose) 5 g/L YE, some minerals, pH controlled by 5 N NaOH and 7 g/L Na_2CO_3, CO_2 spargering at 0.25 vvm, batch fermentation.	11.73 [1.17]	Kim *et al*., 2004
Anaerobiospirillum succiniciproducens (ATCC 29305)	6.5 g/L glycerol supplemented in AnS1 medium, 5 g/L YE, 10 g/L polypeptone, pH controlled by 1.5 M Na_2CO_3, CO_2 spargering at 0.25 vvm.	4.9 [NR]	Lee *et al*., 2001

It is well known that *E. coli* has a native ability to metabolize all hexoses and pentose sugars (Asghari *et al*., 1996; Underwood *et al*., 2004); however, less than 50% of *E. coli* is reported to uptake sucrose (Holt *et al*., 1994). Most of sucrose enteric bacteria such as *Klebsiella* ssp, *E. coli* and *Salmonella* take up and phosphorylate sucrose by PEP-dependent sucrose in PTS system, which generates intracellular sucrose-6-phosphate. Sucrose-6-phosphate is cleaved by a sucrose-6-phosphate hydrolase (invertase) into D-glucose-6-phoshate and D-fructose, which itself is phosphorylated by an ATP-dependent fructokinase (Postma *et al*., 1993 and 1996).

On the other hand, some of *E. coli* such as wide-type strain EC3132 and *E. coli* B (ATTC11303) are capable of metabolizing sucrose by non-phosphotransferase dependent system. Sucrose is transported in to the cells by a sucrose:H^+ symporter named CSC-B (encoded by *cscB*) (Jahreis *et al*., 2002; Moniruzzaman *et al*., 1997; Shukla *et al*., 2004). The transporter looks like other well-studied permeases, a lactose permease (LacY) from *E.coli* K-12 (Marger *et al*., 1993). The sucrose gene cluster in *E. coli* B consists of 3 operons (Figure 2.4), which are an operon encoding a repressor protein, CSC-R (encoded by *cscR*), an operon encoding invertase, CSC-A (*cscA*), and a bicistronic operon encoding fructokinase, CSC-K (encoded by *cscK*) and an anion symport (*cscB*) for sucrose, respectively (Shukla *et al*., 2004) . The genes are divergently arranged, and are negatively regulated by the CSC-R repressor, and are positively controlled by catabolite repression via cAMP-CrpA (Sharon *et al*., 2005).

Figure 2.4 Map of the *csc* regions in *E. coli* KO11 genome.

Since *E. coli* KJ122 ferments glucose to succinate, sucrose should be considered for next common substrate. In addition, sugarcane molasses, byproduct of sucrose, which

consists of water, 50% (w/w) total sugars (sucrose, glucose and fructose), suspended colloid, heavy metals, vitamins and nitrogen compounds, etc (Roukas *et al*., 1998; Kotzamanidis *et al*., 2002) would be the next target substrate to produce succinate. In 2008, USDA reported that raw cane sugar and sugarcane molasses were sold at the price of $0.28 per Kg and $0.1 per Kg, respectively, (www.ers.usda.gov/Briefing/Sugar/Data.htm). However, this strain has no native ability to metabolize sucrose. In 2005, Agarwal reported that 7.1 g/L of succinic acid was produced from sugarcane molasses and corn steep liquor (CSL) within 36 h by *E. coli*. Based on this study, wide-type *E. coli* cannot metabolize sugarcane molasses to succinate efficiently. Furthermore, succinate production from sugarcane molasses was improved in titer (46.4 g/L) and productivity (0.97 g/L/h) by *A. succinogenes* (Lui *et al*., 2008). However, *A. succinoigenes* required yeast extract and external sparging rate which made cost in fermentation process and purification. Therefore, sugarcane molasses would be used as one of interesting substrates to produce succinic acid by KJ122 under anaerobic conditions with functional expression of *cscBKA*.

CHAPTER III

MATERIALS AND METHODS

3.1 Genetic methods

3.1.1 Strains, media and growth condition

The details of the strains, plasmids, and primers used in this study were summarized (Table 3.1). KJ122 does not contain any foreign genes or plasmids. Cultures were grown at 37^{o}C, 200 rpm in modified Luria Bertani (LB) broth containing in one liter: 10 g Difco peptone, 5 g Difco yeast extract, 5 g NaCl and sucrose (2% (w/v)). Cultures were maintained on solid medium (20 g agar). Ampicillin or kanamycin (50 µg/mL), and chloramphenicol (34 µg/mL) were included as appropriate.

Table 3.1 Strains, plasmids and primers were used in this study.

	Relevant Characteristics	
	***E. coli* Strain**	
		Source
KO11	*E. coli* B, *pdc* and *adhB* integration	Ohta *et al*., 1991
KJ122	*E. coli* ATCC 8739, Δ*ldhA* Δ*adhE* Δ*ackA* Δ(*focA-pflB*) Δ*mgsA* Δ*poxB* Δ*tdcDE* Δ*citF*Δ*aspC* Δ*sfcA*	Jantama *et al*., 2008b
KJ122-pKJSUC	*E. coli* KJ122 harboring pKJSUC	This study
	Plasmid	
pCR2.1-TOPO	Cloning plasmid, *bla kan*	Invitrogen
pKJSUC	*cscKBA* harbored in pCR2.1-TOPO	This study
	Primers	
EC-*cscKBA*[a]	5’AATCTAGAGACCGTGATACACGGGACAG 3’ 5’AATCTAGAAGAGTGACGCGGATCCCTTG 3’	This study
M13[b]	5’ TGTAAAACGACGGCCAGT 3’ 5’ CAGGAAACAGCTATGACC 3’	This study
EC-*adhE*[c]	5’CCGCTGTCTGATAACTGGTC 3’ 5’GCATAAGCGGATGGTCACTG 3’	Jantama *et al*., 2008b
EC-*ackA*[d]	5’CGGGACAACGTTCAAAACAT 3’ 5’ATTGCCCATCTTCTTGTTGG 3’	Jantama *et al*., 2008b
EC-*aspC*[e]	5’ TCCATCGCTTACACCAAATC 3’ 5’TGGGGGATGACGTGATATTT 3’	Jantama *et al*., 2008b
EC-*focA*[f]	5’ AGATCGCCAGCCGCTGCAAT 3’ 5’ AACCGTTGGTGTCCAGACAG 3’	Jantama *et al*., 2008b
EC-*ldhA*[g]	5’ ATGAAACTCGCCGTTTATAG 3’ 5’ TTAAACCAGTTCGTTGCCC 3’	Jantama *et al*., 2008b
EC-*sfcA*[h]	5’ CTATGCTTGATCGGCAACCT 3’ 5’ ACGATCGCCTGGTTTTAATG 3’	Jantama *et al*., 2008b
EC-*tdcDE*[i]	5’ CGCCGACAGAGTAATAGGTT 3’ 5’ TGATGAGCTACCTGGTATGG 3’	Jantama *et al*., 2008b

[a,b] : Primers were used for analyzing plasmid pKJSUC.

[c,d,e,f,g,h,i] : Primers were used for confirming KJ122.

The ability to utilize sucrose was screened using Phenol red sucrose agar (1% (w/v) sucrose) and phenol red broth in aerobic and anaerobic tubes (Table 3.2).

Table 3.2 Composition of Phenol red sucrose agar. Final pH at 25^{o}C is 7.4 ± 0.2.

Component	**Concentration (g/L)**
Protease Peptone	10.00
Beef extract	1.00
Sodium chloride	5.00
Sucrose	10.00
Phenol Red	0.025
Agar	15.00

A new low salt medium, AM1 (4.2g/L total salts; Martinez *et al*., 2007) was used in fermentations with KJ122 and KJ122-pKJSUC. This medium was supplemented with 100 mM $KHCO_3$ and 2% (w/v) sucrose and includes 1 mM betaine (Table 3.3). The base used in the anaerobic fermentation experiments is a mixture of 3 N potassium hydroxide and 1.5 M potassium carbonate. Potassium carbonate is not only used for neutralization; it is also provides carbon dioxide indirectly for the phosphorenolpyruvate (PEP) carboxylation in the succinate production pathway. Media were sterilized by autoclave at15 p.s.i for 17 minutes.

Table 3.3 Composition of AM1 medium supplemented to 1 mM betaine (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
$CoCl_2$ $6H_2O$	1.26
$CuCl_2$ $2H_2O$	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	**4.1 g/L**

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

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

3.1.2 General genetic engineering techniques

3.1.2.1 Extraction of Genomic DNA from *E .coli*

Genomic DNA from *E. coli* KO11 was extracted by kit (Macherey-Nagel). One milliliter of culture was centrifuged at 12,000 rpm in a 1.5 mL micro-centrifuge tube for 5 minutes. Supernatant was removed and the pellet was resuspended in 180 µL of Buffer T1. Amount of 25 µL of Proteinase K were added and mixed vigorously and then incubated at 56^{o}C for overnight (vortex occasionally during incubation).Volume of 200 µL of Buffer B3 were added and mixed briefly then, incubated at 70^{o}C for 10 minutes. Two hundred and ten micro-liters of ethanol (96-100 %) were added to the sample and

mixed vigorously. A Nucleospin® Tissue Column was placed in a collection tube and the sample was applied to the column. The sample was centrifuged at 11,000 rpm and the flow-through was discarded. Next, 500 µL of Buffer BW were added and centrifuged for 1 minute to discard a excess flow-through. After that, 600 µL of Buffer were added to the column and centrifuged at 11,000 rpm. The flow-through was removed and column was centrifuged for 1 minute at 1,000 rpm to remove an excess ethanol. The column was placed on to a 1.5 mL micro-centrifuged tube and 100 µL of pre-warmed Buffer BE (70^{o}C) were added to the column. The column was incubated for 1 minute and centrifuged for 1 minute at 11,000 rpm.

3.1.2.2 DNA Amplification by Polymerase Chain Reaction (PCR)

The standard PCR reaction was performed using 10x PCR Master Mix solutions (Qiagen, Valencia, CA) in a PCR reaction of 50 µL. Twenty five micro-liters of master mix containing 10 mM of each dNTP (dATP, dGTP, dCTP and dTTP), PCR reaction buffer (20 mM Tris-HCl pH 8.8, 10 mM KCl, 10 mM $(NH_4)_2SO4$, 2 mM $MgSO_4$, 1% (v/v) Triton® X-100, 1 mg/mL nucleasefree BSA, and *Taq* polymerase enzyme), 40 pmole of each primer (forward and reverse strand primers), and 50 ng of either plasmid or chromosomal DNA template and distilled water, were added to the mixture. The reaction was performed in automated Flexcycler PCR machine. The PCR condition was shown in Table 3.4. After the amplification reaction was finished, an aliquot of the PCR reaction mixture was examined on 1.0% (w/v) agarose gel electrophoresis.

Table 3.4 PCR parameters for the amplification of specific genes. The extension time is depended on the length of the genes (1 kb/min).

PCR profile to amplify genes				
Step	**Period**	**Temperature (^{o}C)**	**Time (min)**	**Number of cycles**
1	Pre-denaturing	95	5 min	1
2	Denaturing	95	30 sec	
	Annealing	55	30 sec	35
	Extension	72	Vary	
3	Extra-extension	72	10 min	1

3.1.2.3 Agarose Gel Electrophoresis of DNA

To analyze the size of DNA fragments and restriction patterns, the PCR products and DNA fragments was subjected to agarose gel electrophoresis. The appropriate amount of agarose powder was dissolved in 0.5x TBE buffer [89 mM Tris-HCl, 89 mM boric acid, 25 mM EDTA pH 8.0] or 1x TAE buffer [40 mM Tris-HCl, 40 mM acetic acid, 25 mM EDTA pH 8.0] under boiling temperature to ensure the homogeneity of the gel solution. Five microliters of loading dye [0.1% (w/v) bromophenol blue, 40% (w/v) Ficoll and 5 mM EDTA)] were added and mixed well to the DNA samples before loading into the wells of the solidified gel. The electrophoresis was performed at a constant voltage, 80 V, for 1 hour. After completion of electrophoresis, the gel was stained with 2 μg/mL ethidium bromide for 2 to 4 minutes and destained in distilled water for 10 minutes. The DNA bands were visualized under UV light and photographed by a gel documentation system.

3.1.2.4 PCR Clean-up Gel Extraction

PCR products were excised from 1% agarose gel (after detection of the expected size of DNA fragment). The reagents and protocol from the Kit (Macherey-Nagel) were used to purify the DNA fragments. The weight of gel slice was determined and transferred to a clean tube. The hundred milligrams of agarose gel were added in 200 μL Buffer NT. The sample was incubated at 50^{o}C until the gel slices were dissolved (5 - 10

minutes). Vortex was applied every 2 - 3 minutes until the gel slices were dissolved. A NucleoSpin® Extract II Column was placed into a collection tube (2 mL) and loaded the sample and then, centrifuged for 1 minute at 11,000 rpm. A flow-through was discarded. Then, 600 µL of Buffer NT3 were added to the column and centrifuged for 1 minute at 11,000 rpm. The flow-through was discarded. The tube was centrifuged for 2 minutes at 11,000 rpm to remove an excess buffer NT3. The column was placed into a clean 1.5 mL micro-centrifuged. Finally, 20 µL of Elution buffer NE were added and incubated at room temperature for 1 minute and then centrifuged for 1 minute at 11,000 rpm.

3.1.2.5 Restriction Endonuclease Digestion of DNA

In 20 µL reaction mixture, 0.2 to 1 µg of plasmid DNA, 1x restriction endonuclease buffer, approximately 5U restriction endonuclease enzyme, , and sterile distilled water were composed. The restriction endonuclease buffer, the amount of restriction endonuclease used and the optimum condition for digestion were selected according to the manufacturer's instructions.

3.1.2.6 DNA Ligation

DNA ligation reaction was conducted by using TOPO TA cloning kit (Invitrogen). The plasmid vector (pCR®2.1-TOPO®) is supplied linearized with single 3'-thymidine (T) overhang for TA cloning® and Topoisomerase I covalently bound to the vector (referred to as "activated" vector). The principal for this cloning kit is that *Taq* polymerase has a nontemplate-dependent terminal transferase activity that add a single deoxyadenosine (A) to the 3'ends of PCR products and the linearized vector has single overhang 3' deoxythymidine (T) residues. Topoisomerase I from *Vaccina* virus binds to duplex DNA at specific sites and cleave the phosphodiester backbone after 5'-CCCTT in one strand. The energy from the broken phosphodiester backbone is converted by formation of a covalent bond between the 3' phosphate of the cleaved strand and a tyrosyl residue (Tyr-274) of topoisomerase I. The phospho-tyrosyl bond between the DNA and enzyme can

subsequently be attacked by the 5’ hydroxyl of the original cleaved strand, reversing the reaction and releasing topoisomerase.

The ligation mixture contained 4 µL fresh PCR products, 1 µL of salt solution (50 mM NaCl, 2.5 mM $MgCl_2$) and 1 µL TOPO® vector. The ligation mixture was incubated at room temperature for 30 minutes before transformation into *E. coli* was performed.

3.1.2.7 Preparation of *E. coli* Competent Cells

A single colony of *E. coli* was inoculated into 5 mL of LB broth and incubated at 37°C, 200 rpm for overnight. Then, 0.5 mL of culture were inoculated into 50 mL LB broth and incubated at 37°C with shaking until the OD_{600} was 0.4. The culture was centrifuged at 4,000 rpm, 4°C for 8 minutes. The pellet was re-suspended and washed in 15 mL of ice-cold 10% (v/v) glycerol for 3 times. After washing the cell, the white cell pellet was re-suspended and placed on ice for 10 minutes. For long cell storage, 500 µL of GYT medium (10% (v/v) glycerol, 0.12% (w/v) yeast extract and 0.25% (w/v) tryptone) were added into the cell suspension then 100 µL aliquots were stored at –80°C.

3.1.2.8 Transformation of Competent Cells by Electroporation

A recombinant plasmid, 100 ng to 10 µg (1 to 2 µL), was mixed with electroporated competent cells to obtain final concentration at 100 µL of the competent cells, and the mixture was transferred to an ice-cold 0.2 cm Bio-red® cuvette. The cuvette was incubated on ice for 5 minutes. The cells were pulsed by using Electroporator 2510 under the conditions used with *E. coli* 1,800 V for 5 seconds. Then 1 mL of ice-cold LB broth was added to the cuvette immediately and the solution was transferred to a sterile 15 mL tube. The tube was incubated at 37°C with 200 rpm shaking for 1 hour. Two hundred micro-litters of transformed *E. coli* TOP10, were spread on LB agar plates containing 100 mL of IPTG (0.1 M), 20 mL of X-gal (20 mg/mL), 50 µg/mL ampicillin, and incubated overnight at 37°C for a blue white screening test.

3.1.3 Construction of plasmid containing a sucrose utilizing genes derived from *E. coli* KO11

Nucleotides of *csc* gene were searched from NCBI (Accession Number AY314757) for *E. coli* KO11 (Shukla *et al*., 2004). PCR was performed to amplify the sucrose-utilizing gene (*csc*) with the size of 4145bp. The DNA fragment is included two operons, an operon encoding invertase (*cscA*), and a bicistonic operon (*cscKB*) encoding fructokinase and an anion symport for sucrose. However *cscR"* was excluded due to its function as a repressor for sucrose metabolism. Genomic extraction was performed to obtain genomic DNA of *E. coli* KO11. The pair primers of EC-*cscKBA* (Table 3.1) were used to amplify the fragment under PCR parameters (Table 3.4). Gel purification was performed to obtain the pure PCR fragment. The pCR2.1-TOPO plasmid was used as a vector for ligating the PCR fragments. The recombinant plasmids were transformed into *E. coli* TOP10 competent cells for constructing plasmid library. *E. coli* TOP10 carrying the recombinant plasmid was selected by the blue-white screening test. The recombinant plasmids named pKJSUC in *E. coli* TOP10 cells were harvested by plasmid extraction kit and checked by PCR, agarose gel electrophoresis, set of restriction enzymes and DNA sequence analyzing. Then pKJSUC was extracted and further transformed into *E. coli* KJ122.

3.1.4 Phenol red sucrose test

In this step, the ability in sucrose utilization of transformed *E. coli* KJ122 harboring pKJSUC was screened by a Phenol red 1% (w/v) sucrose agar and confirmed by Phenol red sucrose broth in aerobic and anaerobic tubes at 37^{o}C. Phenol red is a pH indicator. Broth changes from red color to yellow when strains produce succinic acid and other mixed organic acids.

3.2 Metabolic Evolution

Under strong pressure such as high temperature, strong and sole substrate limitation, microorganisms have mutation occurred in DNA genes and then selection occurred in the phenotype which naturally change in proteins, especially, substrate specific proteins. This directs the evolution of new function. The target gene of evolution in all variants occurs as point mutations in the DNA sequence changing the function of enzymes. Many studies on the ability of the whole new metabolic pathway have been exposed for metabolic engineering *E. coli* in novel environments. Only microorganisms existing target enzyme can survive and enhance reactions in the new resource. This is a selection of strongly genes within microorganism for survival under strong pressure of selection (Jantama *et al*., 2008a, b). The selection was conducted in KJ122-pKJSUC for production of high succinate from sucrose and sugar cane molasses. Starting at initial OD_{550} of 0.1 (Dry cell weight 0.033 g/L = OD_{550} 0.1), cells were grown in fresh AM1 medium. The culture was subsequently transferred in fresh AM1 medium when the OD_{550} of culture was reached the range of 1 to 2. The transfers were performed until achievement of a clone thus exhibiting fast cell growth, rapid consumption of sucrose and high production and productivity of succinic acid, with less production of other organic acids.

3.3 Anaerobic Fermentation

3.3.1 Anaerobic fermentation in small-scale bottle using LB medium

KJ122 and KJ122-pKJSUC inocula were prepared by inoculating seeds into modified LB medium containing 2% (w/v) sucrose. Inocula were grown at 37^{o}C, 200 rpm. After 16-18 h, cells were harvested by centrifugation at 4,000 g for 10 minutes. Fermentations were inoculated at OD_{550} of 0.1 of inocula and carried out in a 500 mL container with 350 mL working volume at 37^{o}C, 150 rpm. LB medium containing 5% (w/v)

sucrose supplemented to 100 mM $KHCO_3$ and 1 mM betaine was used. Kanamycin (50 µg/mL) was also added in fermentation broth of KJ122 harboring pKJSUC and pH was controlled at 7 by the mixture of 3 N potassium hydroxide and 1.5 M potassium carbonate. The total incubation time was 96 hours and the experiments were performed in duplicate.

3.3.2 Anaerobic fermentation in small scale bottle using AM1 medium

KJ122 inocula were prepared by inoculating seeds into modified LB medium containing 2% (w/v) sucrose (100 mL in 250 mL flask). KJ122-pKJSUC inocula were prepared by inoculating seeds into AM1 medium containing 5% (w/v) sucrose (350 mL in 500 mL bottle). Two kinds of inocula were grown at 37^{o}C, 150 rpm. After incubation time 16-18 h, cells were harvested by centrifugation at 4,000 g for 10 minutes. Fermentations were inoculated at OD_{550} of 0.1 (0.033 g/L dry cell weight) and carried out in a 500 mL bottle with 350 mL working volume at 37^{o}C, 100 rpm. Initial OD_{550} (0.01, 0.1, and 0.6), concentrations of sucrose, mixed sugars (35.4 g/L sucrose, 7.3 g/L glucose and 7.3 g/L fructose), sugarcane molasses, and mixture of sucrose and glucose (the ratio of 42.7 g/L sucrose and 7.3 g/L glucose) were used. Kanamycin (50 µg/mL) was also added in fermentation broth of KJ122 harboring pKJSUC as appropriate. The pH was controlled at 7 by the mixture of 3 N potassium hydroxide and 1.5 M potassium carbonate. The total incubation time was 96 hours and the experiments were performed in triplicate.

3.3.3 Anaerobic fermentation in 10 L bioreactor

Fermentations were performed at 37^{o}C, 100 rpm with 7.5 L working volume in 10 L bioreactor. The pH was controlled at 7 and AM1 was also used as a basal medium. Optimal substrates concentrations (sucrose concentration at 70 g/L and sugarcane molasses at 150 g/L) and initial OD_{550} at 0.1 were used for large scale. The total incubation time was 96 hours and the experiments were performed in duplicate.

3.4 Colony Count

Numbers of KJ122 harboring pKJSUC cells were counted on LB agar supplemented with kanamycin (50 µg/mL) every 24 h during fermentations. The agar plate was incubated at 37^{o}C for 24 hours. For cultures containing sugarcane molasses, amount of cells were counted on LB plate. The LB plate was incubated at 37^{o}C for 16-18 hours.

3.5 Analytical Methods

Five milliliters of samples were removed during fermentation every 24 hours for measurement of cell mass. Cell mass was estimated from the optical density at 550 nm (0.033 g/L of dry cell weight = OD_{550} 0.1) with a spectrophotometer Spekol®1500. Organic acids and sugars were determined by using high performance liquid chromatography, HPLC, (Agilent, 2009) equipped with UV and refractive detectors (RID) with a Bio-Rad Aminex HPX-87H ion exclusion column. Sulfuric acid is used as mobile phase at concentration of 4 mM with quality pump at 0.4 mL/min. RID and column temperatures were at 35^{o}C and 45^{o}C respectively. Each sample was injected at concentration of 10 uL for 45 minutes. The culture was withdrawn and centrifuged at maximum speed for 4 minutes to separate cells and supernatant. The supernatant was filtered by 0.2 µm filter before injecting to HPLC.

CHAPTER IV

RESULTS AND DISCUSSIONS

4.1 Construction of plasmid containing a sucrose utilizing genes derived from *E. coli* KO11

Sucrose utilizing genes (*cscKBA*) were used to construct a gene library in pCR2.1-TOPO plasmid (Figure 4.1).

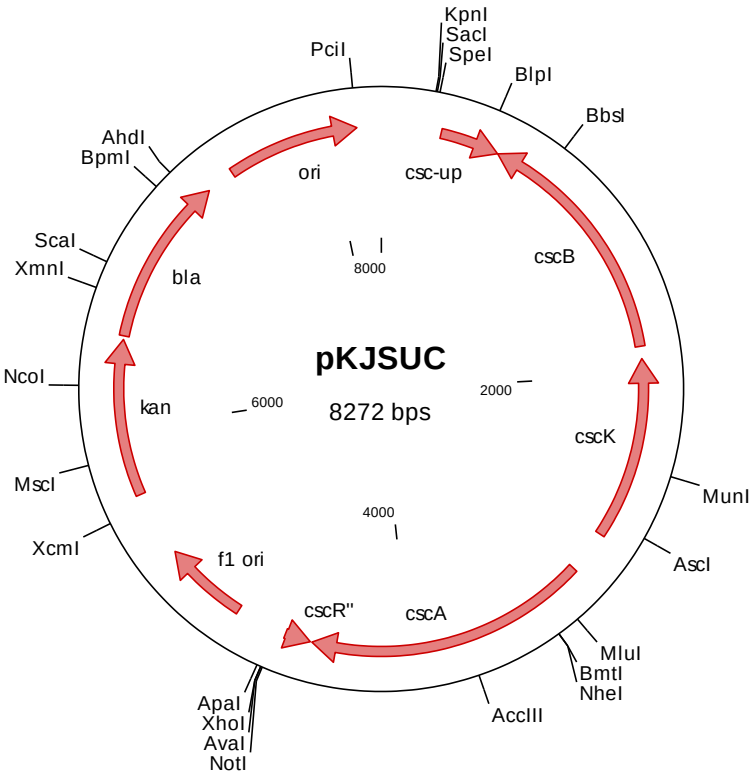

Figure 4.1 The recombinant plasmid pKJSUC. Arrows indicate directions of transcription.

The recombinant plasmids were transformed into *E. coli* TOP10 and the insertion of *cscBKA* gens in this plasmid was confirmed by the PCR analysis using M13 primers. The result showed that the PCR product was in size of 4145 bps as expected (Figure 4.2). One, designed pKJSUC was sequenced fully in both directions by DNA sequence analysis and the sequences were corrected as expected (see Appendix).

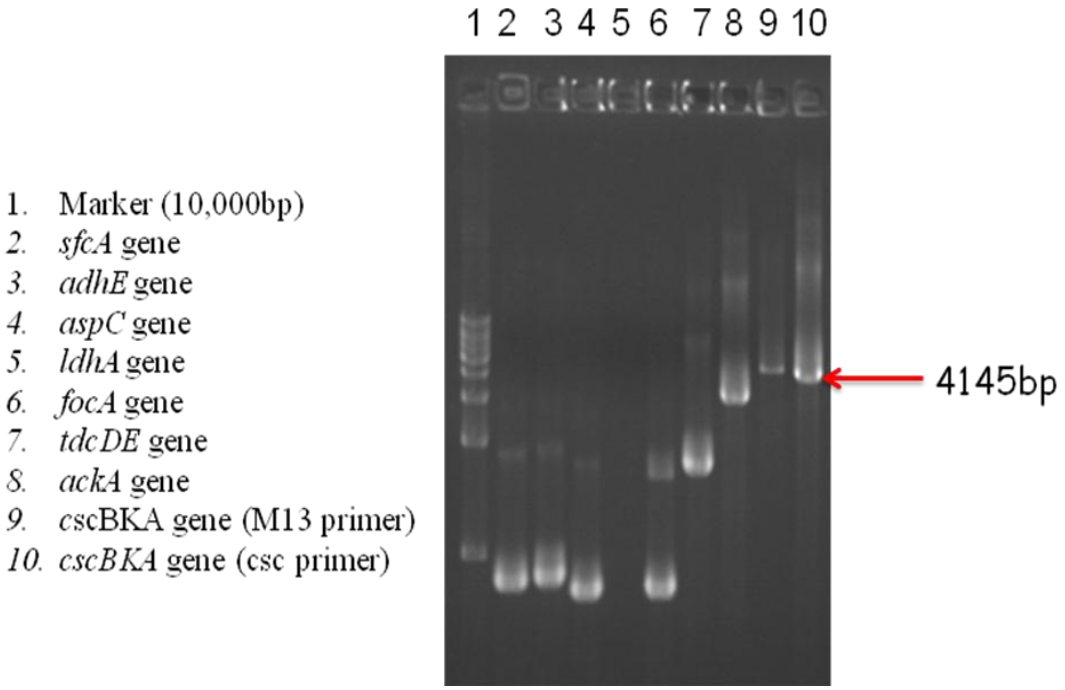

Figure 4.2 Gel electrophoresis performed to confirm strain KJ122 and the plasmid pKJSUC. From lane 2 to 8, the PCR bands were represented for deleted genes in genome of KJ122. From lane 9 to 10, PCR bands were represented for inserted gene (*cscKBA*).

The transformants of KJ122 harboring pKJSUC were selected for ampicillin resistance and screened for sucrose fermentation using phenol red plates containing 1% (w/v) sucrose (Figure 4.3A). Phenol red is a pH indicator that changes color based on pH values. When the indicator molecule is in the low pH solution or medium (pH is lower than 7), it changes color from red to yellow. In contrast, phenol red changes color from red to pink when it is in the high pH medium. Sucrose containing phenol red agar was in red color (control).

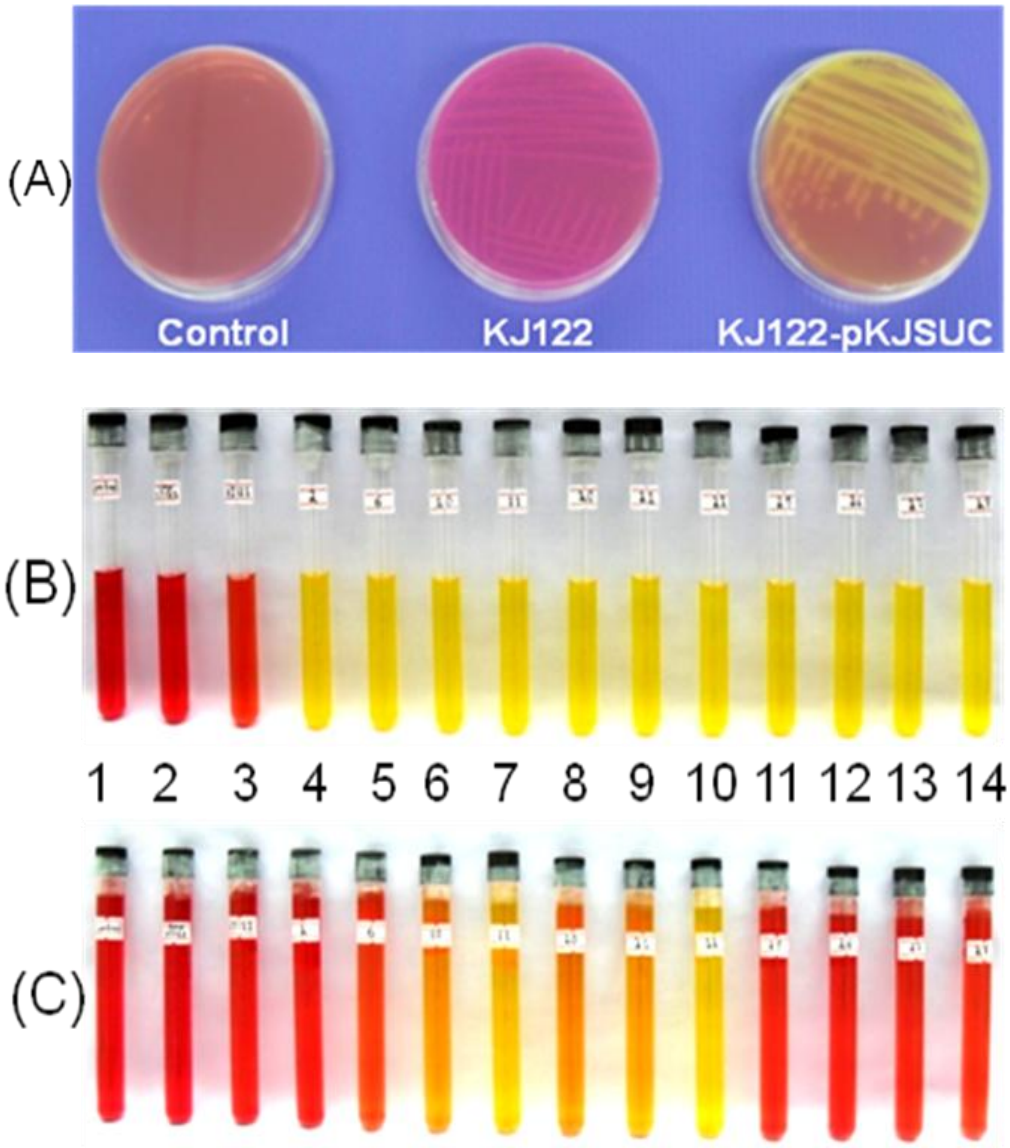

Figure 4.3 Screening and selection for KJ122-pKJSUC by sucrose phenol red agar and broth. (A) Screening KJ122 and KJ122-pKJSUC on phenol red sucrose agar. Control consists of only phenol sucrose agar without strain inoculation. (B) KJ122-pKJSUC in aerobic tubes containing phenol red sucrose broth. Number 1 was control (broth only), number 2 was a medium incubated with KJ122 (broth with kanamycin), number 3 is a medium incubated with KJ122 and number 4 to 14 were the medium incubated with KJ122-pKJSUC from colonies grown on agar plates. (C) KJ122-pKJSUC selection in anaerobic tubes containing phenol red sucrose broth.

For KJ122, it could not consume sucrose efficiently then the strain did not produce any acids from sucrose as a carbon substrate. This indicated that the strain utilized amino acids in the medium as a nitrogen source for growth instead of sucrose then the ammonium was generated from amino acid degradation process resulting in higher the pH of the medium. Therefore, the phenol red agar was in pink color. However, KJ122-pKJSUC could have an ability to utilize sucrose contained in the medium thus producing succinic acid resulting in changing to a yellow color of phenol red agar.

Twenty-eight colonies were picked up from screened plates and were inoculated in phenol red broth containing 1% (w/v) sucrose. Of the 28 colonies inoculated, 11 colonies were able to change the broth color from red to yellow after 2 nights incubation time in aerobic tubes (Figure 4.3B), when 2 colonies were able to change broth color to yellow in anaerobic tubes (Figure 4.3C). One of two colonies grown in anaerobic tubes was confirmed that the clone harbored the plasmid by using PCR analysis (Figure 4.2; lane 9 and 10), and was chosen as a representative clone for the fermentation experiment in which it exhibited rapid sucrose consumption and high succinate production in LB medium under anaerobic conditions. However, KJ122 without plasmid pKJSUC, consumed sucrose inefficiently due to an absence of sucrose utilizing genes.

4.2 Sucrose metabolism in KJ122-pKJSUC

Sucrose is the most abundant sugar due to its origin in higher plant tissues. Sucrose is known as a disaccharide, composed of a D-glucose unite and a D-fructose unite via (1α-2β) glycosidic linkage. The cleavage of glycosidic linkage could be beneficial to provide glucose and fructose as substrates for efficient conversion to high valued chemicals such as succinate. KJ122 has been engineered to produce succinic acid at high yield and titer; however, it has no ability to metabolize sucrose. *E. coli* KO11 is an ethanologenic derivative of the *E. coli* B strain and is able to ferment sucrose. In this research, we

hypothesized that a functional expression of sucrose utilizing gene derived from the genome of *E. coli* KO11 would enhance the succinate production in strain KJ122 harboring the sucrose utilizing genes in the plasmid pKJSUC.

The protein encoded from the recombinant plasmids, pKJSUC, have functions to cleave glycosidic linkage between glucose and fructose subunits and to covert fructose to fructose-1-phosphate before the strain can utilize glucose and fructose to produce succinate (Figure 4.4). Sucrose was expected to permeate into cells by non PTS-dependent system, which was a function of CSC-B with H^+ symport. CSC-A converts sucrose to glucose and fructose by an invertase activity. Fructose is phosphorylated to fructose-1-phosphate by CSC-K (fructokinase). These are all key enzymes functioning in the recombinant plasmid pKJSUC. Glucose and fructose metabolism were occurred in glycolysis pathway. KJ122 is a metabolic engineered strain and it has some mutations of key enzymes such as glucokinase and phosphoenolpyruvate carboxykinase (PCK). PCK was recognized as a vital enzyme for caboxylation and conservation of energy in succinate production pathway (Zhang *et al.*, 2009).

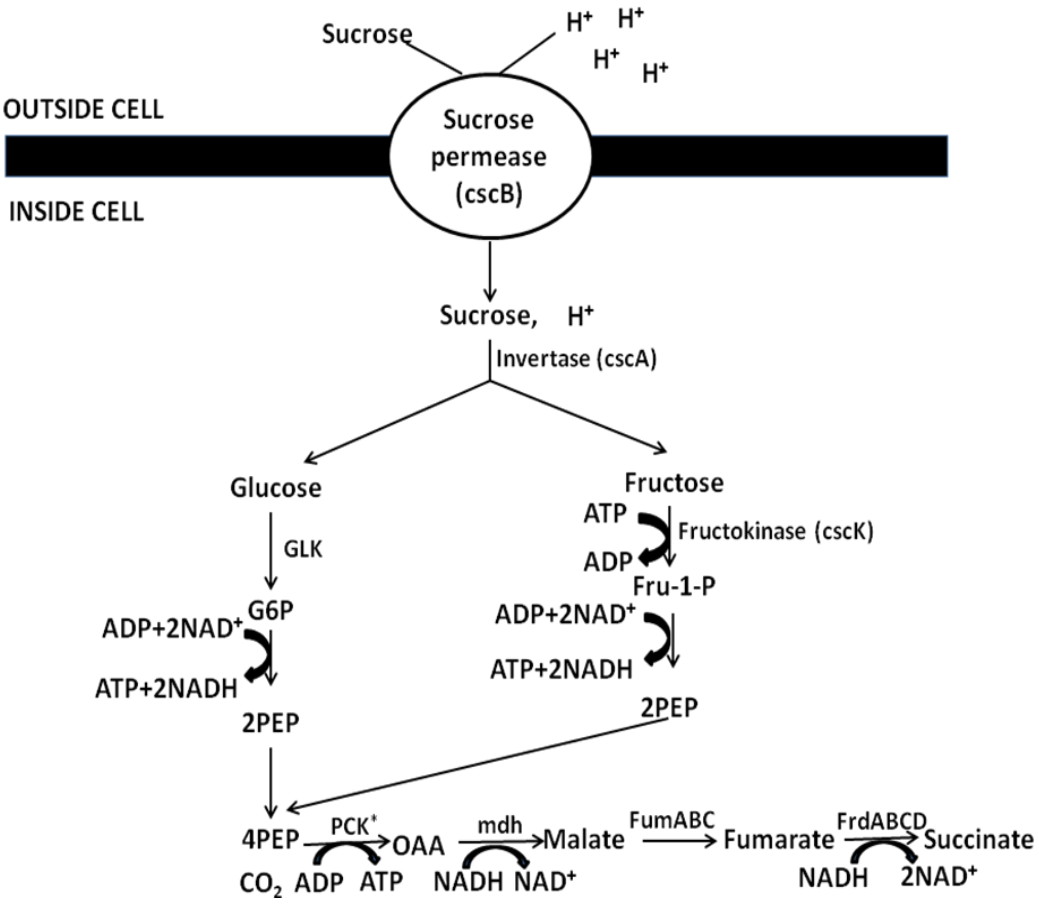

Figure 4.4 Proposed mechanism of sucrose metabolism in KJ122-pKJSUC.

4.3 Production of succinic acid in small bottles

4.3.1 Metabolic evolution

Growth based selection (metabolic evolution) and adaptation of KJ122-pKJSUC in AM1 medium supplemented with sucrose were done by sub-culturing into the new fresh medium. The best representative clone resulted in high biomass, rapid sucrose consumption, high titer and productivity of succinate but less by-products under high concentration of sucrose. The cultures were subsequently inoculated 32 times in fresh AM1 medium supplemented to 10 % (w/v) sucrose. From the transfer number of 1 to 21, the lower succinate production was produced along with low biomass (Figure 4.5A and B). During these transfers, accumulation of lactate, formate, acetate and ethanol

were detected (data not shown). From the transfer number of 22 to 28, the improvements of biomass and succinate production were obtained. The strain from the transfer of 24 could produce high succinate concentration (7.21 g/L) and high biomass (0.86 g/L) within 24 hours. A few amounts of lactic acid and formic acid were detected but no ethanol was observed during these sub culturing. From the transfer number of 29 to the end of metabolic evolution, it was observed that succinate and biomass were dramatically decreased with high concentration of by-products, including ethanol accumulation. The accumulation of ethanol might result from the effect of sucrose involving in the spontaneous mutations of some iso-enzymes in the ethanol producing pathway. Acetaldehyde dehydrogenase2 (encoded from *mhpF*) might be activated to generate acetaldehyde from acetyl-CoA (www.ecocyc.org). This reaction requires NADH and the gene expression of this enzyme is induced by cAMP-CRP. Acetaldehyde is converted further to ethanol by ethanol dehydrogenase (encoded by *adhP*). This reaction also requires NADH. Therefore, the activation of these enzymes might regenerate NAD^+ simultaneously with the accumulation of ethanol. However, from these results, we decided to select the best strain for the succinate production in which this strain did not produce ethanol. The culture of KJ122-pKJSUC from the transfer number of 24 was used as starter in the further step of fermentation with pH control.

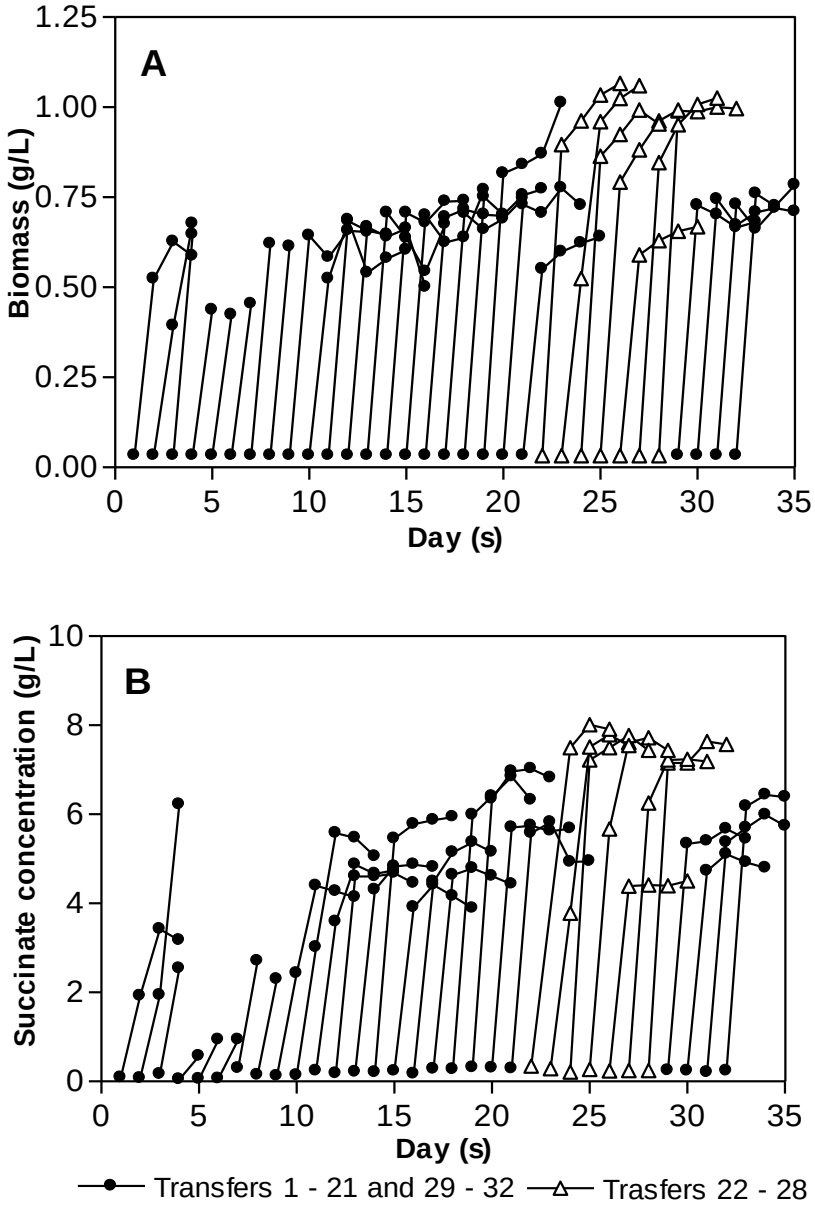

Figure 4.5 Metabolic evolution of KJ122-pKJSUC in AM1 medium containing 10% (w/v) sucrose. (A) Biomass, (B) Succinate concentration. Transfers were made at first 24 hours intervals. Fermentations were done for 4 days from transfers 9 - 32.

4.3.2 Production of succinic acid from sucrose in LB broth

Succinate production was performed in LB medium containing 5 % (w/v) sucrose. The fermentations with KJ122 and with KJ122-pKJSUC were compared in 5% (w/v) sucrose LB medium to evaluate the effect of recombinant plasmid, pKJSUC, on the succinate production, cell growth and sucrose metabolism. After 72 hours, about 46.1 ± 0.19

g/L succinate was produced by KJ122-pKJSUC whereas about 0.44 ± 0.2 g/L succinate were produced by KJ122 (Figure 4.6A and B). This result indicated that the plasmid pKJSUC affected the sucrose utilization in KJ122 due to relief the catabolic repression and enhancing sucrose metabolism thus resulting in higher growth cell and the succinate production. Acetate was also increased during the cell growth and succinate production. The acetate concentration was attained about 5.84 ± 0.23 g/L after 72 hours by KJ122-pKJSUC (Figure 4.6A).

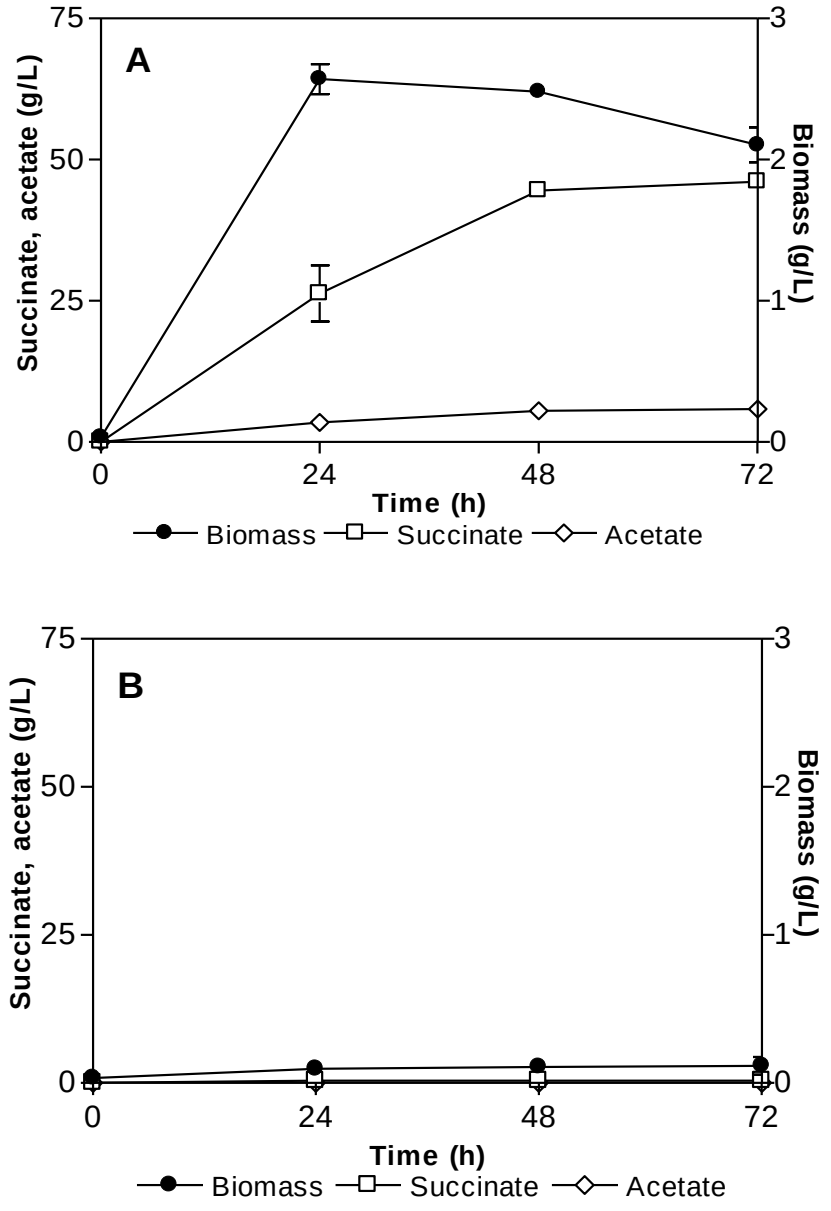

Figure 4.6 Succinate, acetate, and biomass from KJ122-pKJSUC (A) and KJ122 (B) in LB medium containing 5% (w/v) sucrose.

All of sucrose molecules were metabolized to glucose and fructose during 48 hours by KJ122-pKJSUC. Glucose molecules were efficiently all consumed by the strain after 48 hours simultaneously with sucrose utilization. However, fructose molecules were slowly metabolized and fructose consumption was started after glucose was exhausted (Figure 4.7). This finding was similar to the report of Shukla (2004). Even though fructose was almost all metabolized, its residue was still detected after fermentation at concentration of 1.14 ± 0.21 g/L. This result implied that fructose utilization was delayed by the catabolic repression and the mutation in PTS^{fruc}-PEP dependent system of KJ122 (unpublished data). Moreover, fructose residue clearly demonstrated that invertase (*cscA*) had function to metabolize sucrose both inside and outside the cells. Therefore, fructose is a less efficient substrate for cell growth and the succinate production by KJ122-pKJSUC.

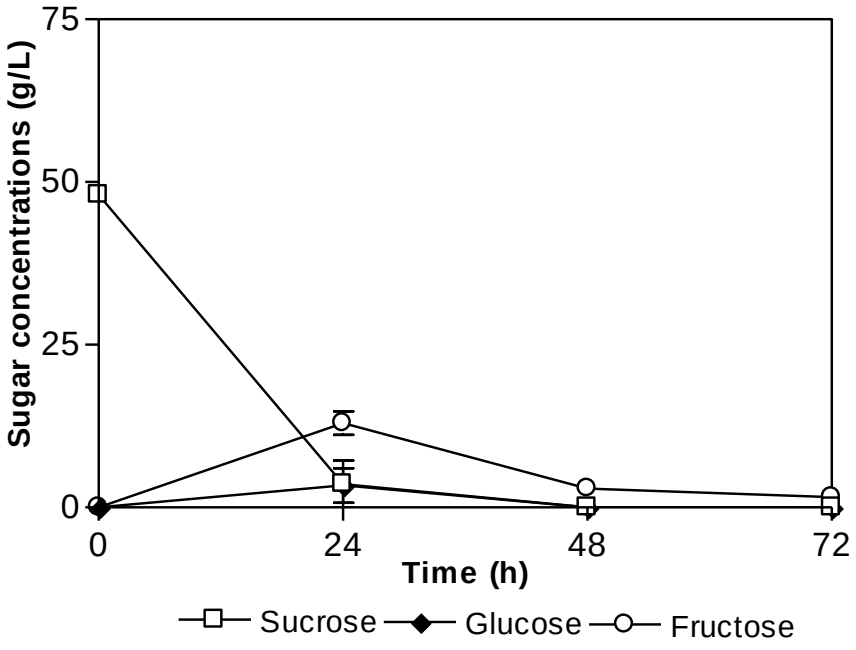

Figure 4.7 Sucrose metabolism in KJ122-pKJSUC from LB medium containing 5% (w/v) sucrose.

4.3.3 Production of succinic acid from sucrose in AM1 medium

To make cost effectiveness in bio-based succinate production, mineral salt medium was used in this study. AM1 medium is simple mineral salt medium, and cost effective medium as well as a good medium for purification step because it does not contain complex nutrients such as yeast extract. After 72 hours, succinate concentration of 44.6 ± 0.92 g/L was produced along with acetate at 5.71 ± 0.2 g/L from 5% (w/v) sucrose in AM1 medium (Figure 4.8) whereas succinate was produced at 46.1 ± 0.19 g/L with acetate at 5.84 ± 0.23 g/L from 5% (w/v) sucrose in LB medium. This result demonstrated that there is no significant deference between LB medium, and AM1 medium for the succinate production by the strain KJ122-pKJSUC. In anaerobic small bottles of *A. succinogenes* CGMCC1593, succinate (40.3 ± 0.8 g/L), acetate (5.2 ± 0.3 g/L) and sugar residue (0.5 ± 0.3 g/L) were attained when grown in 50 g/L sucrose supplemented to medium and 15 g/L yeast extract after 60 hours (Lui *et al*., 2008). Compared with *A. succinogenes* CGMCC1593, succinate (42.04 ± 0.56 g/L), acetate (5.44 ± 0.09 g/L) and fructose residue (3.97 ± 0.09 g/L) was produced from 50 g/L sucrose supplemented to AM1 medium by KJ122-pKJSUC after 48 hours in anaerobic small bottles. This result demonstrated that KJ122-pKJSUC was as an efficient strain as *A. succinogenes* CGMCC1593 for economic bio-based succinate production.

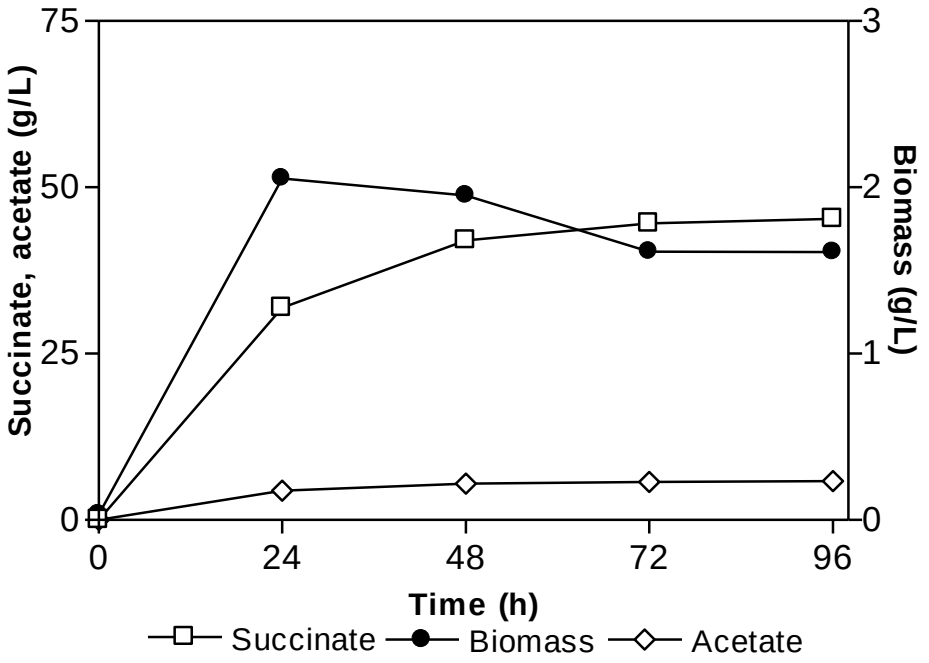

Figure 4.8 Production of succinate from 50 g/L sucrose supplemented to AM medium by KJ122-pKJSUC.

Furthermore, sucrose concentration was optimized by comparison of biomass, succinate concentration and residual sugar (Figure 4.9). It is noted that succinate concentration was increased when substrate concentration was increased while the maximum biomass produced slightly decreased (from initial sucrose concentration at 70 g/L to 100 g/L). The optimal concentration of sucrose was found at 70 g/L due to the low fructose residue.

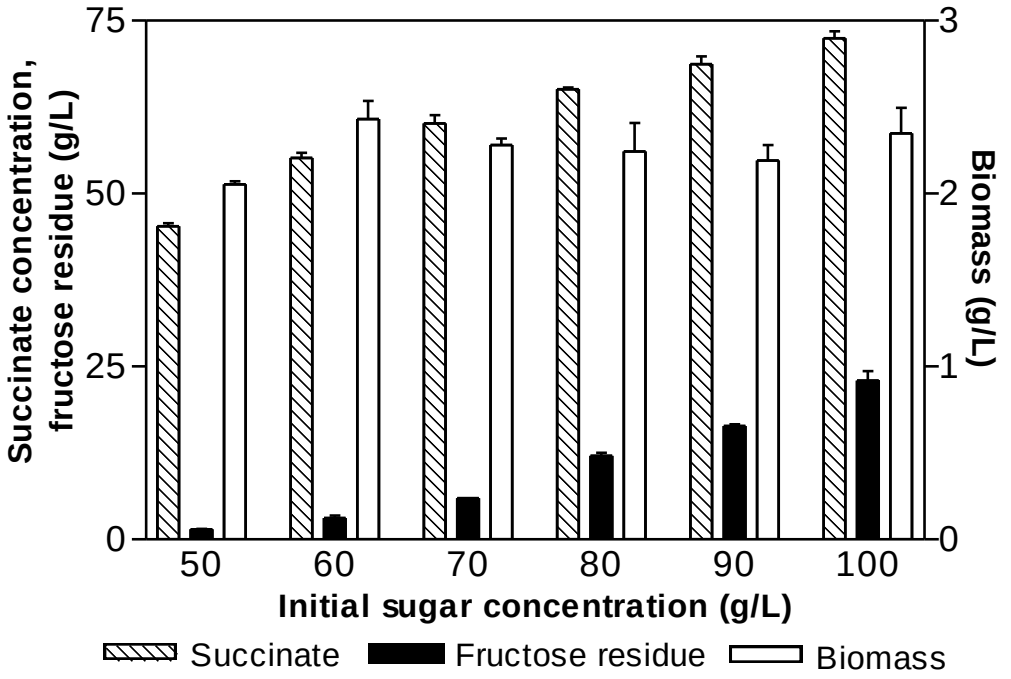

Figure 4.9 Optimization of sucrose concentration in AM1 medium. Succinate and fructose residue were presented after 96 hours incubation time. Biomass of KJ122-pKJSUC was in 24 hours.

At 48 hours, succinate was produced at 50.52 ± 1.8 g/L with productivity of 1.05 g/L/h. After 96 hours, succinate at 60.18 ± 2.02 g/L was produced (Figure 4.10). Since the more initial concentration of sucrose was greater than 70 g/L, the more residue sugars were obtained in the medium after 96 hours incubation time. In addition, the biomass obtained from the broth contained the sucrose greater than 70 g/L was not significantly different. The initial sucrose concentration at 70 g/L was chosen as an optimal concentration.

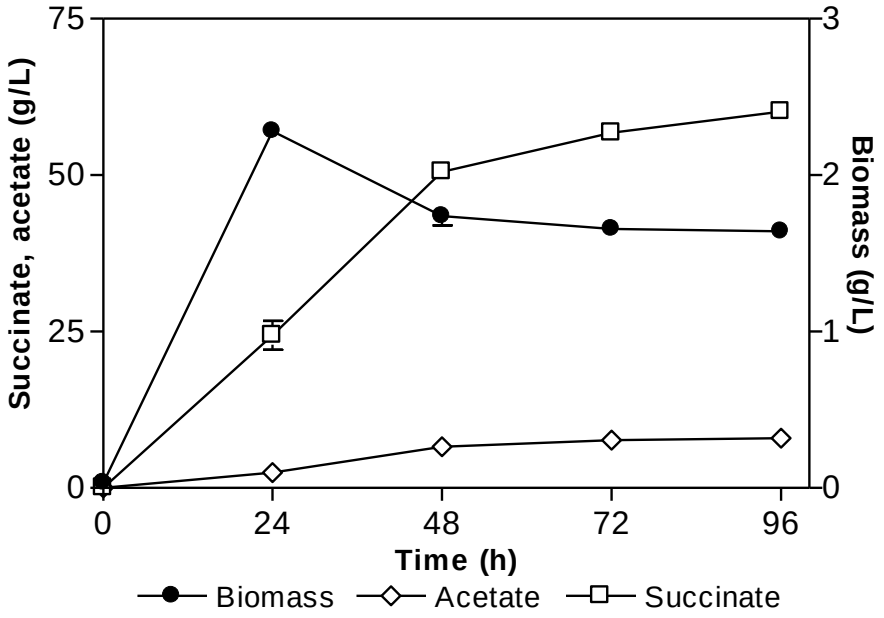

Figure 4.10 Succinate production from 70 g/L sucrose supplemented to AM1 medium by KJ122-pKJSUC.

The maximum biomass was obtained at 24 hours in all fermentations of various sucrose concentrations. In addition, titers and productivities of succinate from 70 g/L to 90 g/L sucrose were not significant different (around 50 g/L) within 48 hours if the product would be economically harvested when compared with 96 hours incubation (Figure 4.11). Moreover, ratio conversion of succinate was around 90 % in all various sucrose concentrations at 48 hours (Table 3.5). Even though high succinate (57.44 ± 1.08 g/L) was produced from sucrose (100 g/L) within 48 hours, the high residual fructose (37.45 ± 1.58 g/L) was obtained compared with the residue sugars of 14.89 ± 0.37 g/L (from the initial concentration of 70 g/L sucrose). Thus when considering economically, the optimal concentration of sucrose would be at 70 g/L.

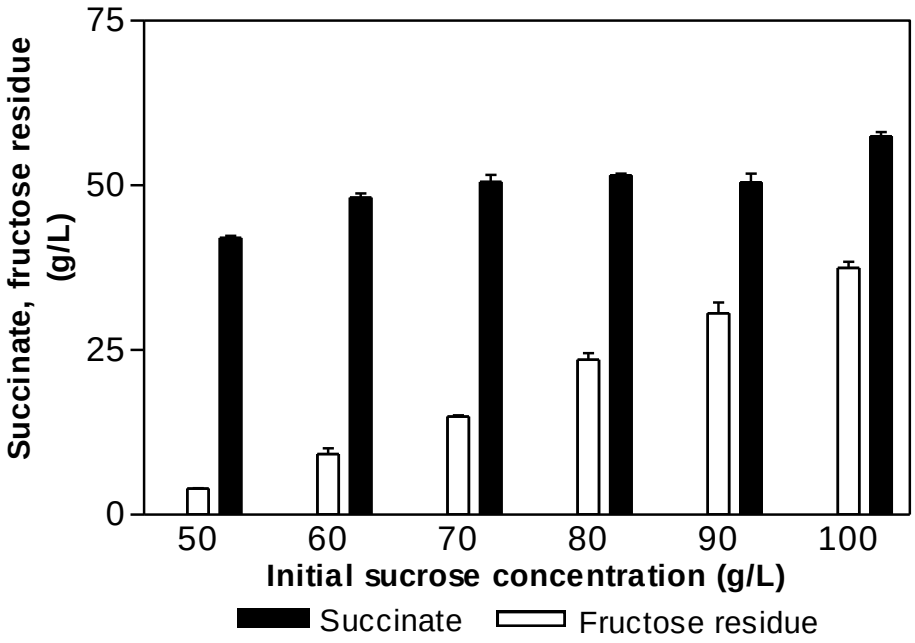

Figure 4.11 Optimization of sucrose concentration in AM1 medium. Succinate and fructose residues were presented after 48 hours incubation time from KJ122-pKJSUC.

Table 3.5 Fermentation results of KJ122-pKJUC using various concentrations of sucrose supplemented to AM1 medium.

Sucrose (g/L)	**Succinate (g/L)**		**Fructose residue (g/L)**		**Percentage* conversion (%)**	
	48 h	**96 h**	**48 h**	**96 h**	**48 h**	**96 h**
50	42.04 ± 0.56	45.27 ± 0.75	3.97 ± 0.09	1.45 ± 0.13	91.33	93.24
60	46.30 ± 1.05	55.16 ±1.28	9.21 ± 1.49	3.09 ± 0.67	91.15	96.92
70	50.52 ± 1.88	60.18 ± 2.02	14.89 ± 0.37	5.97 ± 0.01	91.67	93.98
80	51.52 ± 0.44	65.10 ± 0.46	23.53 ± 1.72	12.10 ± 0.72	91.23	95.87
90	50.46 ± 2.22	68.74 ± 1.89	30.60 ± 2.77	16.37 ± 0.53	84.94	93.35
100	57.44 ± 1.08	72.45 ±1.76	37.45 ± 1.58	22.99 ± 2.34	91.83	94.07

*Percentage conversion = [succinate] x 100 / [initial sucrose - fructose residue]

This result suggested that it was not economical for cultivating KJ122-pKJSUC with high initial sucrose concentration. What we found was that fermentation of sucrose concentration up to 100 g/L (double concentration of 10 g/L glucose) increased succinate titer and prolonged log phase but lower cell density. This finding was contrasted to Lin (2008) while they reported that the high level of glucose concentration over 100 g/L was

lower succinate level. The increasing of succinate was not proportional to the carbon substrate supplied to the broth and this caused the increase of fructose residue (product and substrate inhibitions). High fructose residue might raise the cost of bio-based succinate involving in a downstream processing and waste disposal.

Strain KJ122-pKJSUC could produce succinate at 72.45 ± 1.76 g/L from 100 g/L sucrose after 96 hours (Figure 4.9). Initial sucrose concentration at 50 g/L to 60 g/L were metabolized all after 24 hours but the concentration of sucrose at 70 g/L to 100 g/L were done all at 48 hours. Small amount of glucose was detected at 48 hours and it was not detected at all after 72 hours. In 1997, Moniruzzaman reported that 90 g/L sucrose was completely fermented by *E. coli* KO11 after 60 to 72 hours for ethanol production. This implied that the CSC-KBA proteins were efficiently expressed in KJ22-pKJSUC for sucrose metabolism to glucose and fructose. Moreover, the exclusion of repression protein (CSC-R) might enhance sucrose metabolism. The genes (*cscBK*, and *cscA*) are also positively controlled by cAMP-CrpA that the binding sites are presented at the -35 regions in both operons (Shukla *et al*., 2004; Reid *et al*., 2005). Meanwhile, strain KJ073, the parental stain of KJ122, showed the increasing of cAMP and CRP due to catabolic repression protein even it grew on glucose (Zhang *et al*., 2010). Therefore, the rapid sucrose metabolism would result from the absence of *cscR* in pKJSUC and high level expression of cAMP in KJ122, thus all affecting the rapid growth of KJ122-pKJSUC. However, enzymatic assay for the enzymes and real time RT-PCR should be performed to confirm this hypothesis.

KJ122-pKJSUC exhibited to maximum biomass at 24 hours and the succinate production (50.52 ± 1.88 g/L; productivity at 1.05 g/L/h) at 48 hours (Figure 4.12). From 48 to 96 hours, succinate productivity was rapidly declined and small amount of succinate was excreted. Based on previous study of KJ122 strain, around 44.83 g/L of succinate (productivity at 0.97 g/L/h) was produced from 10 % (w/v) glucose and high biomass was obtained within 48 hours (Jantama *et al*., 2008b). At the end of fermentation time (96

hours), around 80 g/L succinate was produced from glucose. Even though, glucose provides high yield and titer of succinate when fermentation time was longer, the succinate productivities were not different no matter glucose or sucrose was used as substrate. It would reflect that fermentation time for production of succinic acid to obtain the high productivity from KJ22-pKJSUC was only the first 48 hours of incubation in AM1 medium supplemented to sucrose.

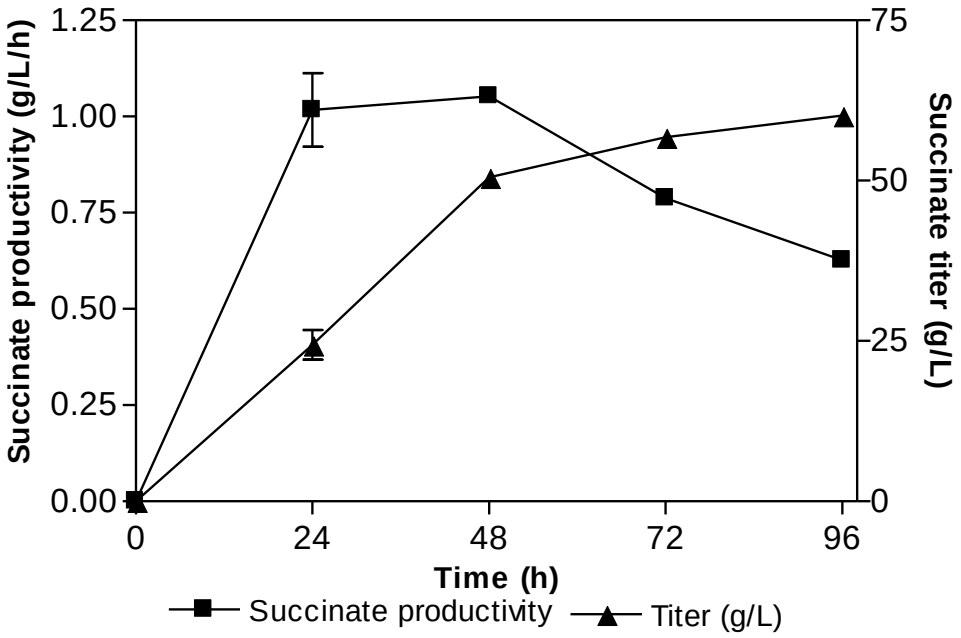

Figure 4.12 Succinate titer and productivity from 70 g/L sucrose supplemented to AM1 medium by KJ122-pKJSUC.

Acetic acid is the main by-product by KJ122 and KJ122-pKJSUC. High excretion of acetate in KJ122 might result from that the strain was not deleted phosphotransacetylase (*pta*) (Jantama *et al*., 2008b). Acetate accumulation was observed in various concentrations of sucrose fermentation by KJ122-pKJSUC. At 96 hours, high acetate accumulation (7.94 ± 0.51 g/L) was observed in 70 g/L sucrose fermentation (Figure 4.13). Compared with KJ122-pKJSUC, KJ122 produced acetate at 7.32 g/L from 10 % (w/v) glucose (Jantama *et*

al., 2008b). The differences between substrates and concentrations might have small effect on the acetate production.

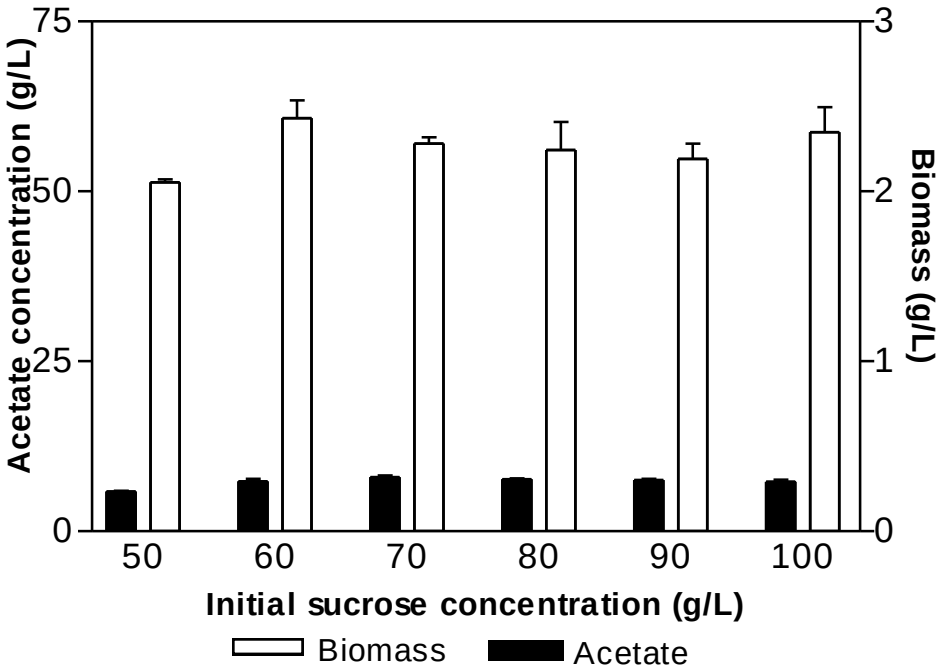

Figure 4.13 Acetate productions by KJ122-pKJSUC in various sucrose concentrations at 96 hours incubation time. Biomass was represented at 24 hours.

To make bio-based succinate more economic, succinate production without kanamycin addition in the fermentation broth for plasmid maintenance would be performed. The result showed that there is slightly significant in the absent or present kanamycin for the succinate production in term of cell growth, titer, and productivity of succinate (Figure 4.14).

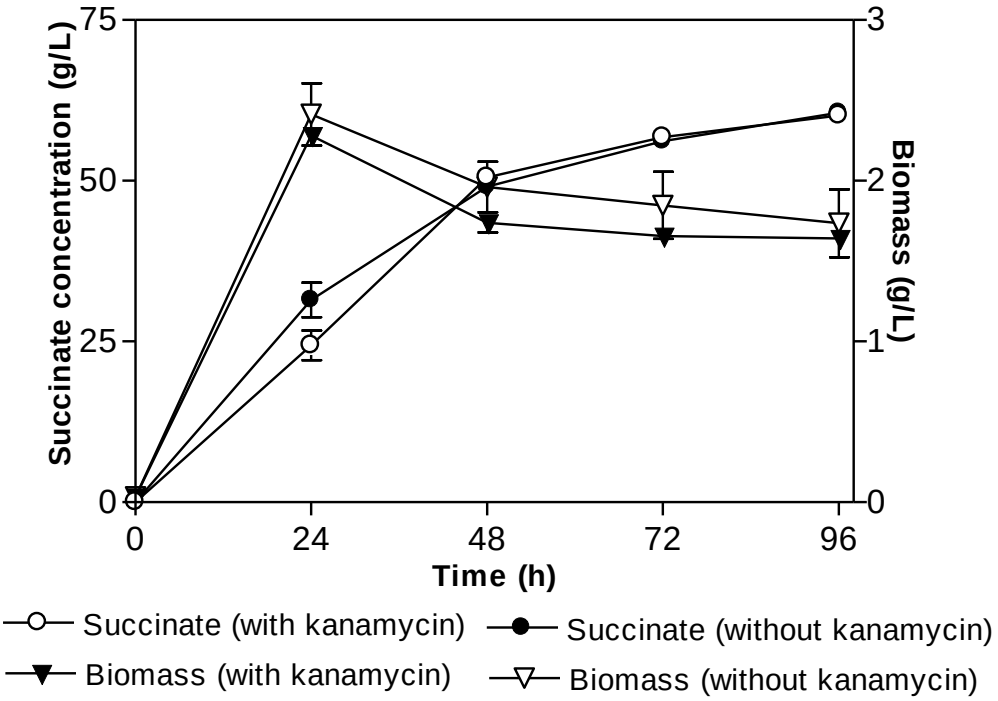

Figure 4.14 Effect of antibiotic on succinate production. Initial sucrose concentration was at 70 g/L supplemented with AM1 medium for succinate production by KJ122-pKJSUC.

At the end of fermentation time (96 hours), succinate (60.59 ± 0.97 g/L), acetate (8 ± 0.74 g/L), fructose residue (7.53 ± 0.23 g/L) were obtained in 70 g/L sucrose fermentation without kanamycin (Figure 4.15). Based on this result, antibiotic did not have effect on the succinate production by KJ122-pKJSUC in batch fermentation. Completely rapid sucrose consumption at 48 hours might be benefit for sucrose metabolism even though; recombinant plasmids were lost during cell divisions or structural instability of plasmid material due to mutations (Manbouquette *et al*., 1986). Antibiotic was recommended to not use in batch fermentation only in this study. For other models such as continuous fermentation, effect of fermentation and regulatory parameters on expression under a better condition should be studied in case that antibiotic will not be used.

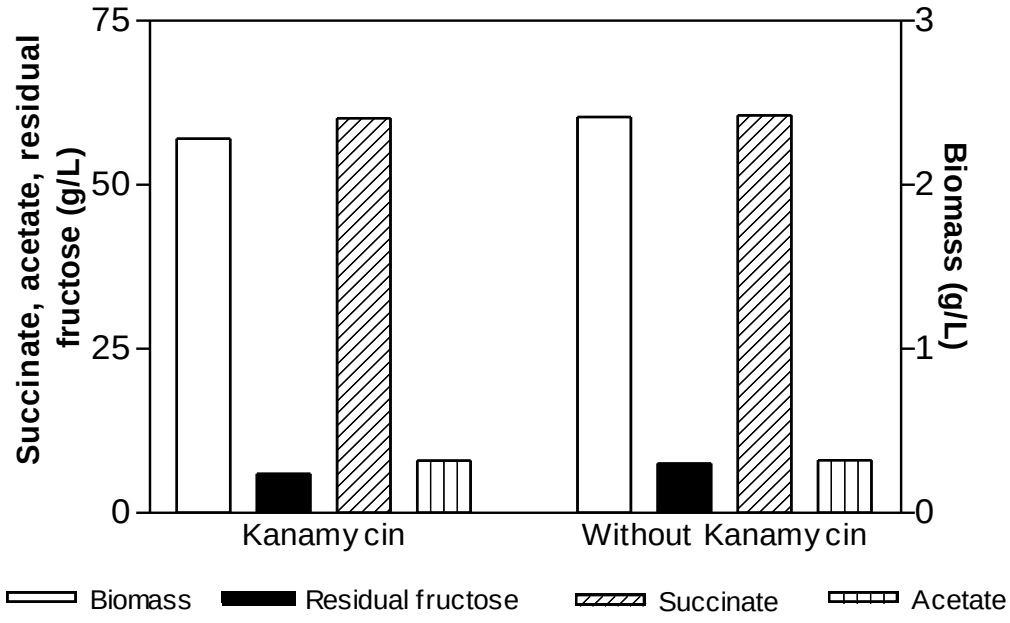

Figure 4.15 Effect of antibiotic on succinate production by KJ122-pKJSUC from 70 g/L sucrose supplemented to AM1 medium. Concentrations of succinate, acetate, and fructose residue were presented as products after 96 hours incubation time. Biomass was presented after 24 hours.

4.3.4 Effect of different initial OD_{550} on succinate production

Initial inocula were studied to investigate the effect of cell growth on the succinate production. Inocula at OD_{550} of 0.6, 0.1 and 0.01 were inoculated into AM1 medium containing 70 g/L sucrose to produce succinate. The succinate concentrations obtained were at 62.43 ± 0.77 g/L, 60.18 ± 2.02 g/L and 59.64 ± 1.4 g/L individually at 96 hours, respectively (Figure 4.16).

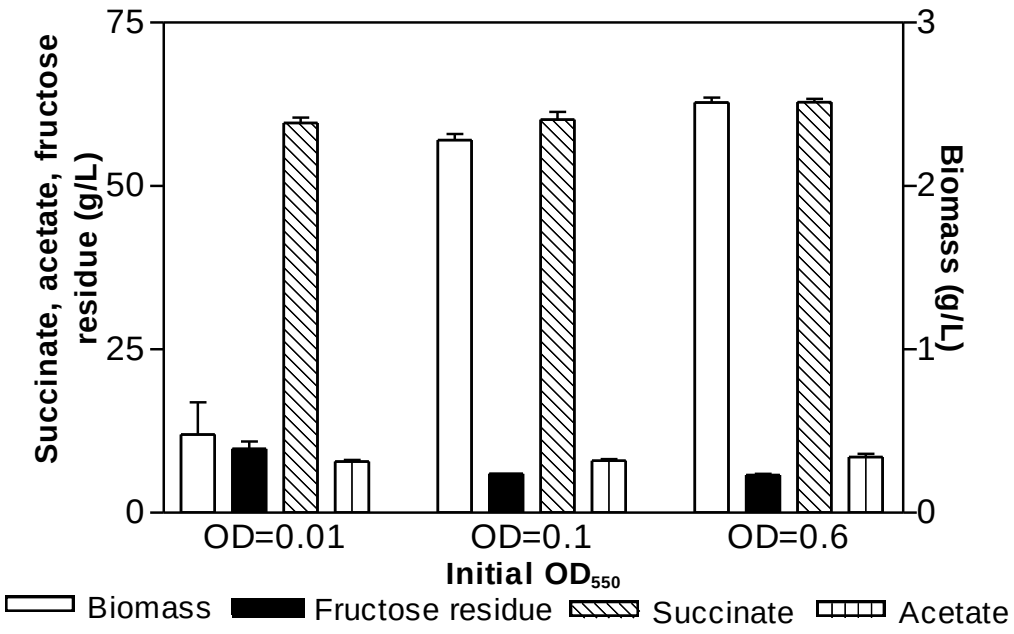

Figure 4.16 Effect of different initial OD_{550} on succinate production from 70 g/L sucrose supplemented to AM1 medium by KJ122-pKJSUC in fermentation time (96 hours). Concentrations of succinate, acetate, and fructose residue were presented as products after 96 hours incubation time. Biomass was presented after 24 hours.

At 24 hours, initial OD_{550} at 0.6 could produce succinate at 41.59 ± 0.92 g/L (productivity at 1.73 g/L/h) while initial OD_{550} 0.1 and 0.01 produced succinate at 24.4 ± 3.97 g/L (1.01 g/L/h) and 2.52 ± 2.37 g/L (0.1 g/L/h), respectively. Because succinate is a growth associated product, increasing inoculums size improved productivity. However, initial OD_{550} at 0.6 and 0.1 provided the succinate production at 53.56 ± 0.27 g/L and 50.52 ± 1.88 g/L at 48 hours, respectively (Figure 4.17). Small inoculum size at 0.01 caused lower succinate production in terms of titer and productivity. Succinate level (54.27 ± 2.87 g/L), and acetate (6.66 ± 0.75 g/L) were attained at 72 hours. Considering to the cost of inoculums preparation and succinate titer, initial OD_{550} at 0.1 could be the optimal inoculum for the succinate production at concentration of 50.52 ± 1.88 g/L with productivity of 1.05 g/L/h.

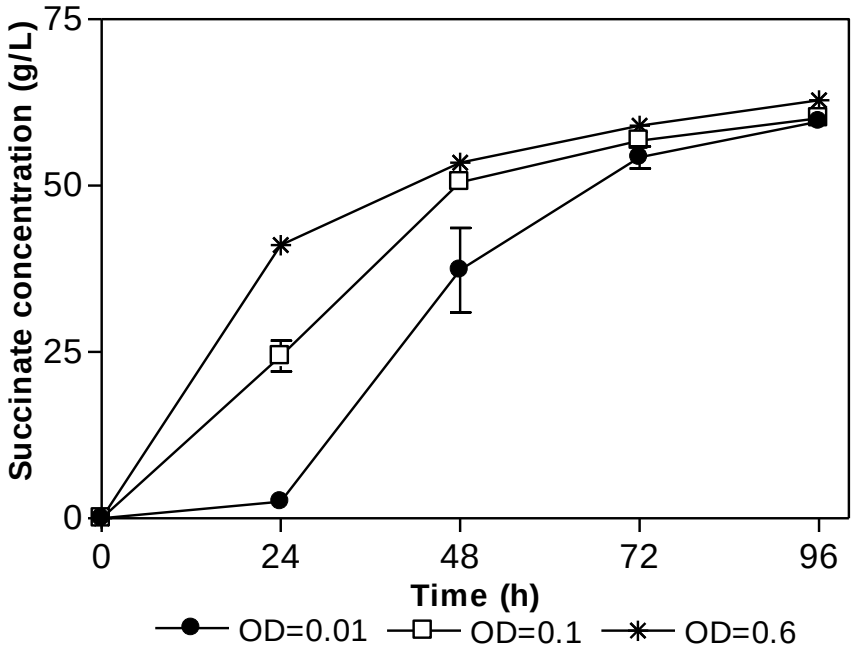

Figure 4.17 Time course of succinate production by KJ122-pKJSUC at different initial OD_{550}.

4.3.5 Production of succinate from mixed sugars of sucrose, glucose, and fructose

Mixed sugars of sucrose, glucose and fructose were used as substrate to study succinate production prior to molasses fermentation. Mixed sugars were composed of 35.4 g/L sucrose, 7.3 g/L glucose and 7.3 g/L fructose. At 96 hours, succinate (43.09 ± 1.36 g/L), fructose residue (1.26 ± 0.32 g/L), and acetate (6.41 ± 0.48 g/L) were obtained in mixed sugar fermentation by KJ122-pKJSUC. Maximum biomass was reached at 48 hours (Figure 4.18). At 48 hours, succinate productivity (0.78 g/L/h) in mixed sugars fermentation is lower than (0.87 g/L/h) in 5 % (w/v) sucrose fermentation. Exponential phase was longer in mixed sugars than that in sucrose. It seems likely that the mixture of glucose and fructose did not improve succinate productivity. This contrasted to what Andersson (2007) found in which glucose and fructose mixture increased the succinate productivity per viable cell as a function of time rather than using glucose or fructose alone. This result could explain the differences between glucose and fructose uptake pathway in KJ122-pKJSUC and *E. coli*

AFP184. Galactose permease (GalP) and glucokinase (GLK) was subjected to mutate in KJ122 whereas; glucose PTS mutation is occurred in AFP184. In KJ122-pKJSUC, sucrose uptakes into cells were via sucrose permease (CSC-B). Metabolism of sucrose to glucose and fructose by invertase was occurred both inside and outside the cells (previous discussion in sucrose metabolism). Therefore, glucose and fructose uptake pathway would be involved to galactose permease (GalP) and PTS^{fru} dependent system, respectively, if sucrose was cleaved outside the cells. Fructose was not an efficient substrate because PTS system was mutated in KJ122. Unlike fructose, glucose was an efficient substrate for GalP. The phosphorylation of glucose is by glucokinase (GLK), one enzyme which was activated in mutation of KJ122. In 1997, Meyer indicated that besides the *glk* encoded enzyme, there is no ATP-dependent glucose phosphorylating activity. Moreover, the researcher proved that *glk* appeared to be constitutively expressed and only weakly repressed by growth on glucose. Only FruR have small effect on GLK repression. FruR has been considered as a repressor for genes encoding enzymes in glycolytic pathway and as an activator for genes encoding enzymes in gluconeogenic pathway (Ramseier *et al*., 1993). However, how glucose was phosphorylated to glucose-6-phosphate by GLK is still not known. To summarize, glucose is an efficient substrate for GalP and GLK which might be an ATP conserving enzyme for succinate production.

In contrast, AFP184 used only PTS system for glucose and fructose uptakes (Adersson *et al*., 2007). Normally, *E. coli* fermented glucose first before other sugars were initiated to consume. However, AFP184 might have mutation in PTS system which causes fructose to be metabolized slightly faster than glucose during the aerobic phase. For the fructose fermentation by AFP184, PEP is converted to pyruvate by the PTS with metabolic imbalance. This imbalance prevents a balanced redistribution of reducing equivalent and led to accumulation and excretion of pyruvate. Glucose and fructose mixture might provide an

increased pool of PEP to balance the pyruvate generated by fructose transport and result in higher productivity or even yield than fructose alone.

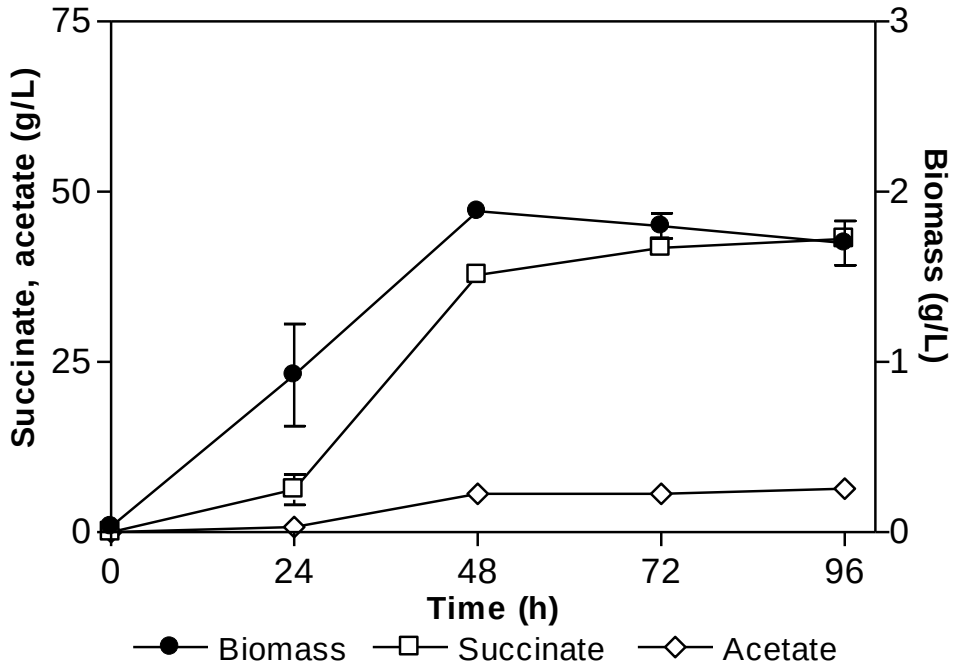

Figure 4.18 Succinate production from mixed sugars supplemented to AM1 medium by KJ122-pKJSUC.

Sugars metabolism in mixed sugars was similar to sucrose in which sucrose and glucose were simultaneously and completely consumed at 48 hours, but fructose was slowly metabolized after glucose exhausted and left-over after the end of incubation (Figure 4.19). At the end of incubation time (96 hours), fructose residue at concentration of 1.26 ± 0.32 g/L and 1.14 ± 0.21 g/L were attained from mixed sugars and 50 g/L sucrose fermentation, respectively.

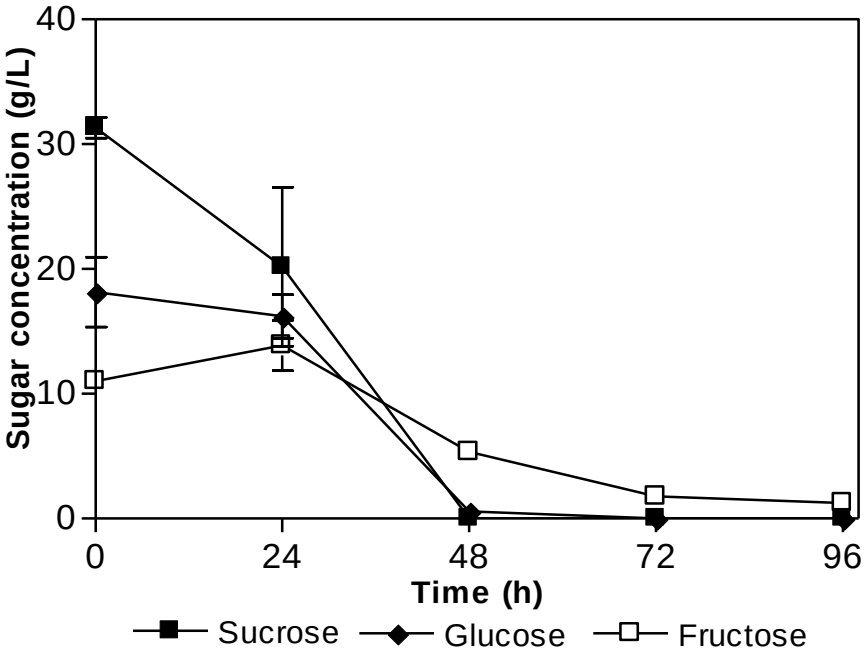

Figure 4.19 Sugars metabolism in mixed sugars supplemented to AM1 medium by KJ122-pKJSUC.

In KJ122, succinate (8.43 ± 0.23 g/L), and acetate (0.88 ± 0.06 g/L) were produced (Figure 4.20). At 96 hours, KJ122 fermented only glucose to succinate; however, sucrose, glucose and fructose residues were detected (data not shown). Concentration of sucrose might inhibit KJ122 growth, even though; glucose and fructose were provided in the fermentation broth.

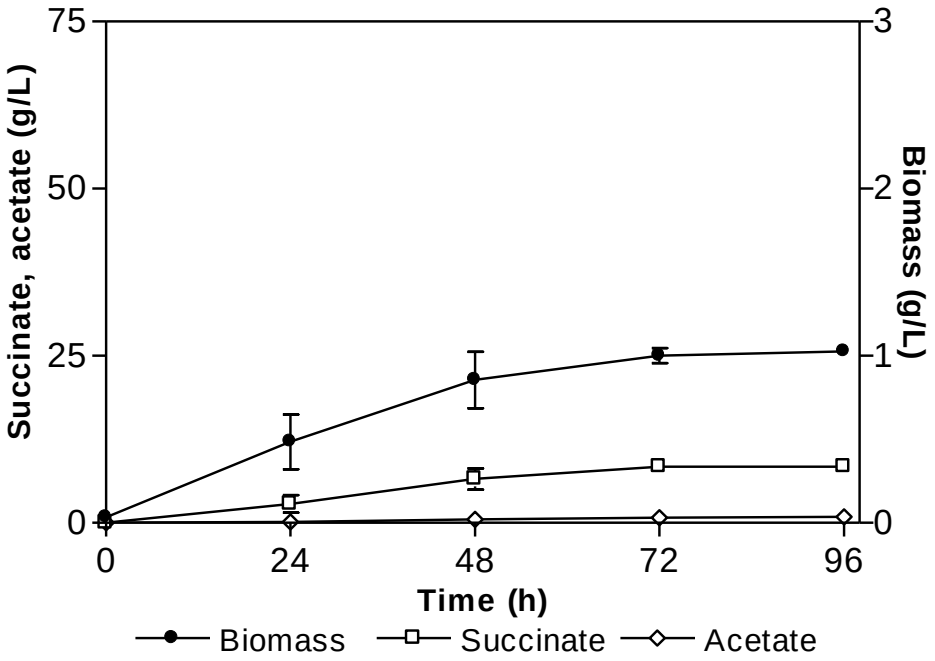

Figure 4.20 Succinate production from mixed sugars supplemented to AM1 medium by KJ122.

4.3.2 Production of succinate from sugarcane molasses

Sugarcane molasses is an inexpensive carbon source for next target bio-conversion of fermentative succinate. The production of succinate was significantly increased when the number of viable cells increased (Figure 4.21 and 4.22). At 24 hours, high viable cells were obtained and succinate concentrations were produced from 11.27 g/L to 20.5 g/L by using sugarcane molasses concentration from 50 g/L to 100 g/L. The highest amount of viable cells reached in 15 % (w/v) sugarcane molasses to produce succinate (44.6 ± 2.57 g/L) at 48 hours. However, maximum cell count in 17.5 % (w/v) sugarcane molasses was lowest and succinate was produced at 56.78 ± 6.5 g/L within 72 hours. The optimal concentration of sugarcane molasses was found at 150 g/L to produce succinate. It was observed that the succinate productivity was decreased in 175 g/L sugarcane molasses, while increasing sucrose concentration up to 100 g/L did not decrease succinate productivity in KJ122-pKJSUC. Succinate (44.6 ± 2.57 g/L), acetate (3.31 ± 0.26 g/L) and fructose residue (28.21 g/L ± 3.3 g/L) were obtained at 48 hours from 150 g/L sugarcane molasses

supplemented to AM1 medium (approximately 75 g/L sugars mixtures) by KJ122-pKJSUC in small anaerobic bottles (Figure not shown). In 2008, Liu reported that succinate (44.2 ± 1 g/L), acetate (6.2 ± 0.3 g/L) and sugar residue (9.5 ± 0.7 g/L) were obtained at 48 hours from sugarcane molasses (65 g/L sugar mixture) supplemented to 15 g/L yeast extract in anaerobic bottles by *A. succinogenes* CGMCC1593. KJ122-pKJSUC did not require complex nutrients for growth. Therefore, the high nutrients in molasses would not have affected on cell growth in which it was different from what found with *A. succinogenes* CGMCC1593 (Liu *et al*., 2008).

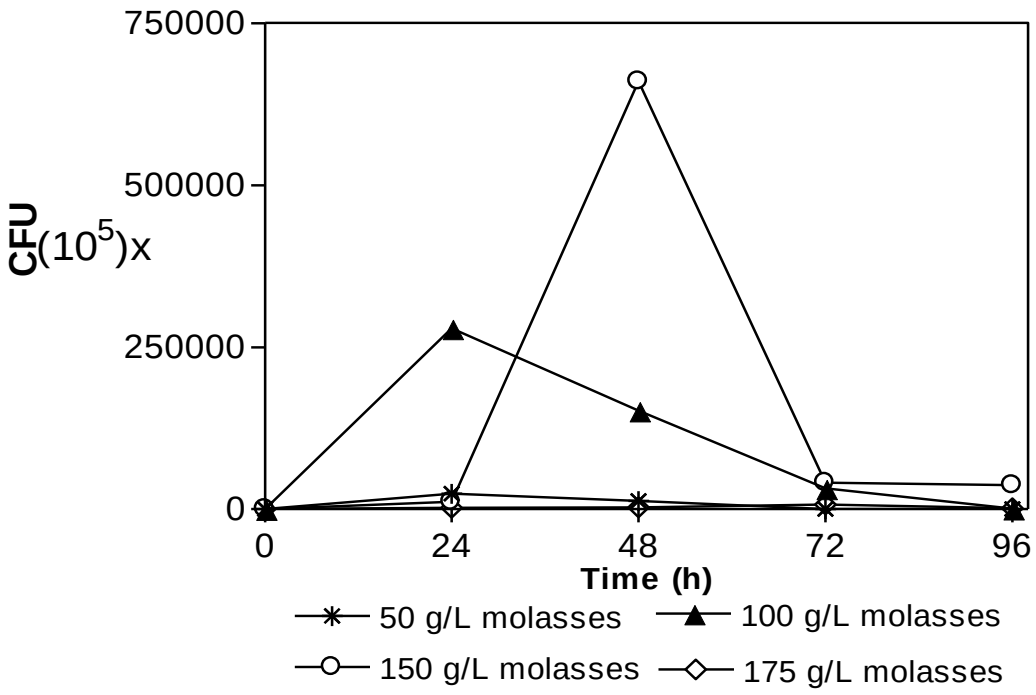

Figure 4.21 Cell growth was represented as colony form unit (CFU) of KJ122-pKJSUC and KJ122 combination in various sugarcane molasses concentrations supplemented to AM1 medium.

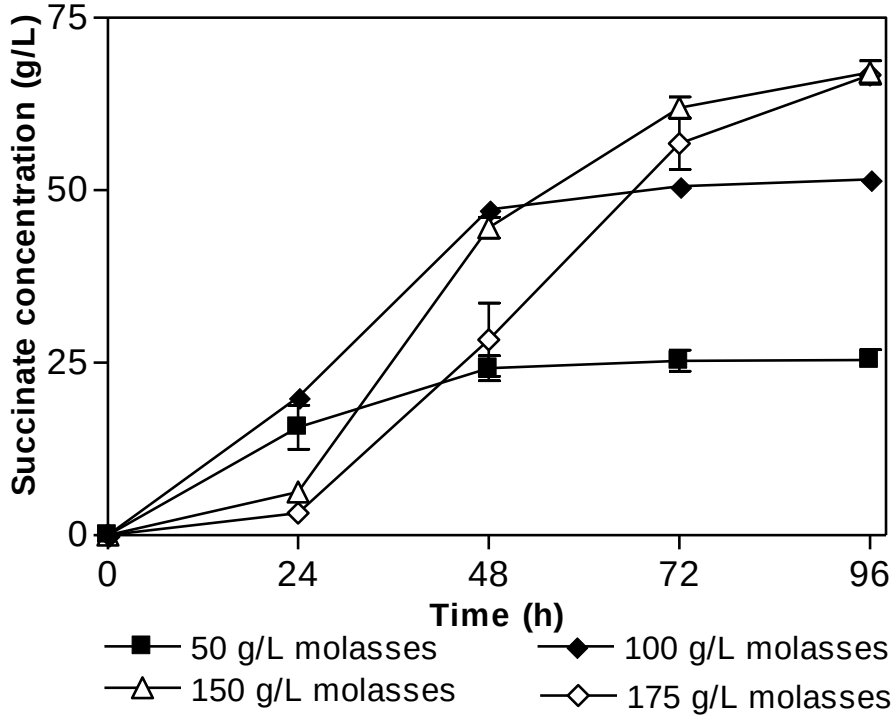

Figure 4.22 Succinate productions from different concentrations of sugarcane molasses supplemented to AM1 medium by KJ122-pKJSUC.

Comparison of succinate concentrations at 96 hours demonstrates that 150 g/L sugarcane molasses could be the optimal concentration (Figure 4.23) but the succinate production from sugarcane molasses was slower than that from sucrose alone at 24 hours (Figure 4.24). It could imply that cells need longer time to grow in sugarcane molasses than that in sucrose. This result was supported by shukla (2004) that the slowly sugarcane molasses fermentation was caused by the contribution of existing inhibitors in sugarcane molasses or additional inhibitors that might be produced during sterilization.

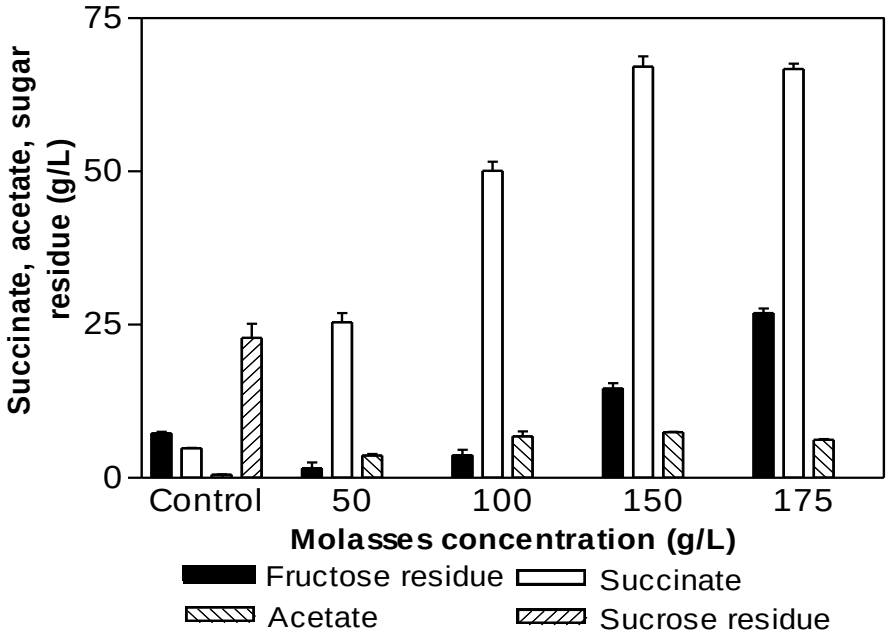

Figure 4.23 Products of sugarcane molasses fermentations by KJ122-pKJSUC and KJ122 in AM1 medium. Product concentrations were presented at 96 hours incubation time. For control, 50 g/L sugarcane molasses was fermented by KJ122.

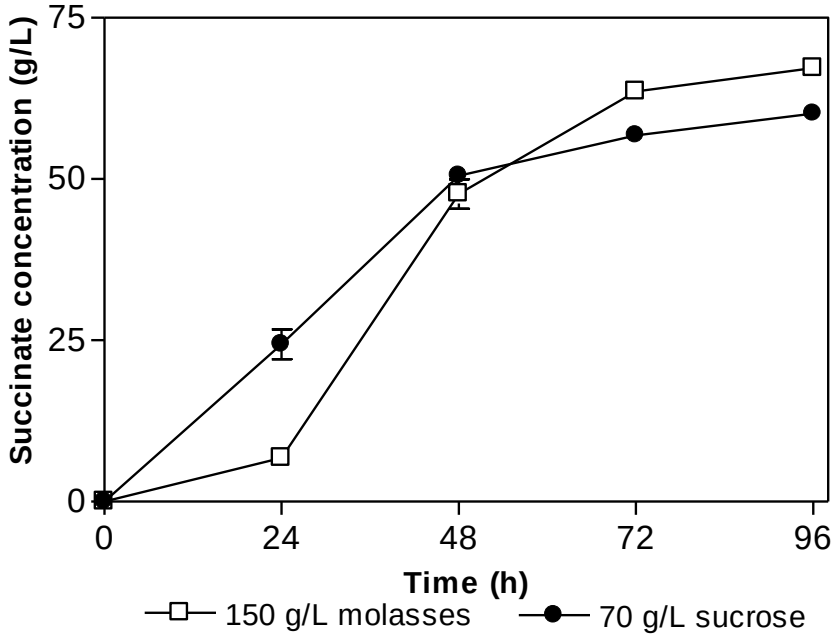

Figure 4.24 Comparison of succinate production by KJ122-pKJSUC in sucrose and sugarcane molasses. Initial sucrose and sugarcane molasses concentration at 70 g/L and 150 g/L (approximately 75 g/L total mixed sugars of sucrose, glucose and fructose), respectively, were supplemented to AM1 medium with initial OD_{550} at 0.1.

Antibiotic has not affected on the succinate production in term of titer and productivity, using 150 g/L sugarcane molasses as a substrate (Figure 4.25). At 48 hours, succinate (47.69 ± 3.94 g/L), acetate (4.27 ± 0.94 g/L) and fructose residue (24.8 ± 2.81 g/L) were attained from 150 g/L sugarcane molasses with succinate productivity of 0.99 g/L/h in AM1 medium without kanamycin. After 96 hours incubation time, succinate (67.2 ± 0.67 g/L), acetate (8.02 ± 0.2 g/L) and fructose residue (13.76 ± 2.01 g/L) was produced; however, succinate productivity (0. 7 g/L/h) was dramatically decreased.

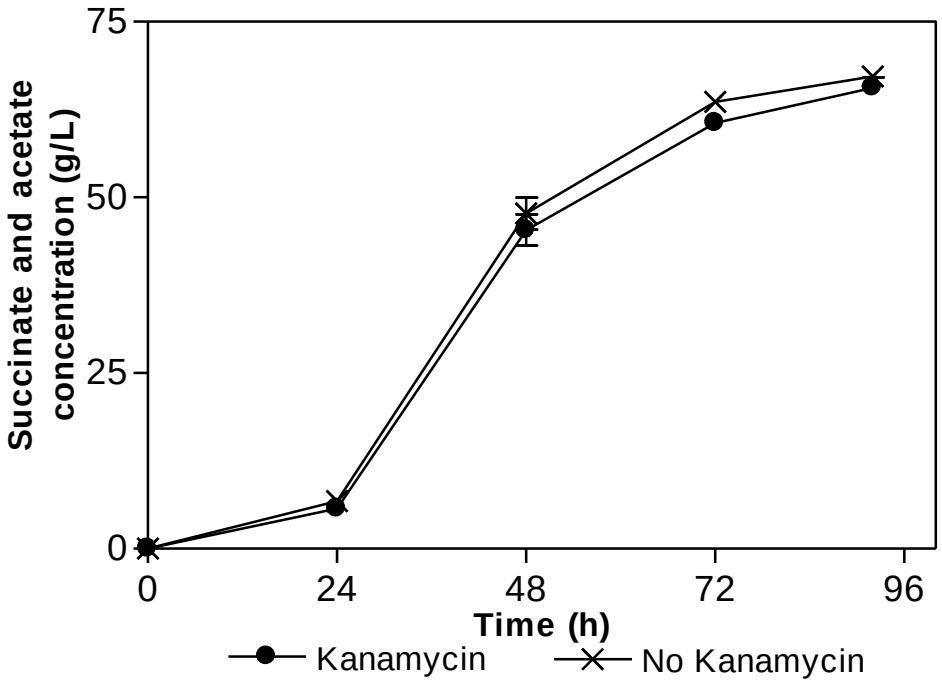

Figure 4.25 Comparison of succinate production by KJ122-pKJSUC in AM1 medium in the presence and absence of kanamycin.

4.3.2 Effect of glucose on succinate production

Glucose is an efficient substrate for KJ122 as well as KJ122-pKJSUC. As previous discussion in sucrose and artificial molasses fermentation, sucrose and glucose were simultaneously metabolized. Therefore, the effect of combination of sucrose and glucose in AM1 medium was expected to enhance cell growth and succinate production. To study this effect, mixture of glucose and sucrose at 50 g/L consisted of 42 g/L sucrose and 7.3 g/L glucose was used. Initial sugar mixture in the same ratio starting at 50 g/L (42 +7.3 g/L) , 60 g/L (51.42 + 8.76 g/L), 70 g/L (59.78 +10.22 g/L), 80 g/L (68.32 + 11.68 g/L), and 90 g/L (76.86 + 13.14 g/L) could produce succinate at 46.23 ± 1.56 g/L, 53.38 ± 2.76 g/L, 59.35 ± 0.82 g/L, 62.53 ± 0.53 g/L and 67.18 ± 1.27 g/L after 96 hours, respectively (Figure 4.26).

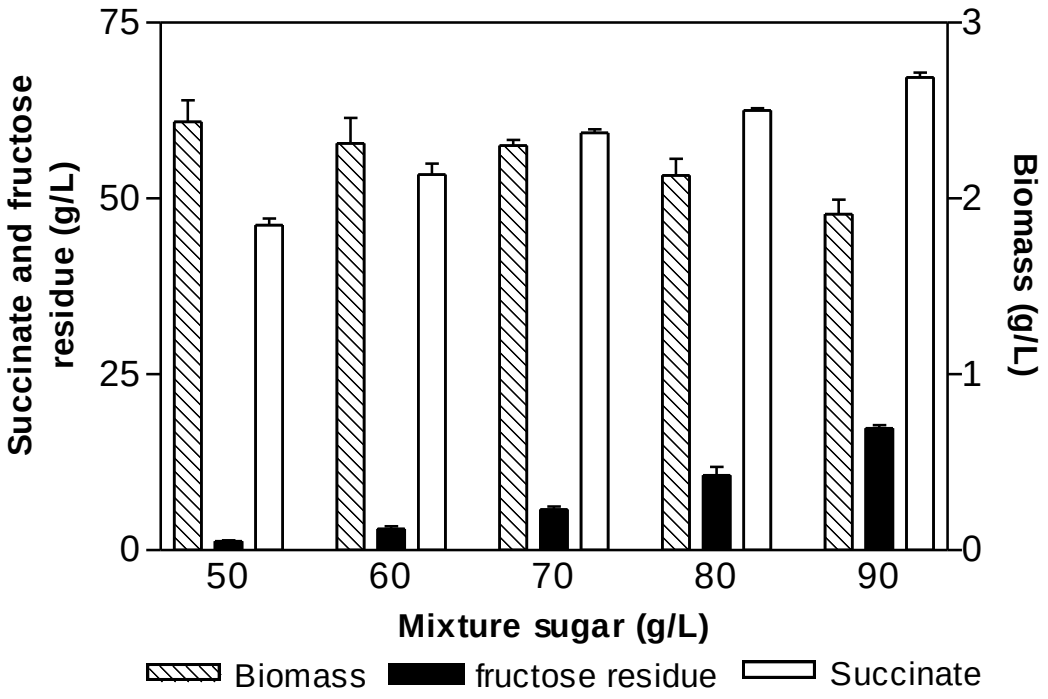

Figure 4.26 Optimized mixture of sucrose and glucose in AM1 medium for succinate production by KJ122-pKJSUC.

At 48 hours, sugar mixture at 70 g/L could produce succinate at 49.9 ± 0.7 g/L with productivity of 1.04 g/L/h (Figure 4.27). Sucrose molecules were metabolized all at 24 hours for sugar mixture fermentation at concentrations of 50 g/L, 60 g/L, and 70 g/L and at

48 hours for sugar mixture at 80 g/L, and 90 g/L. It seemed that glucose could not improve cell growth, succinate titer and productivity in the mixture of sucrose and glucose fermentation by KJ122-pKJSUC.

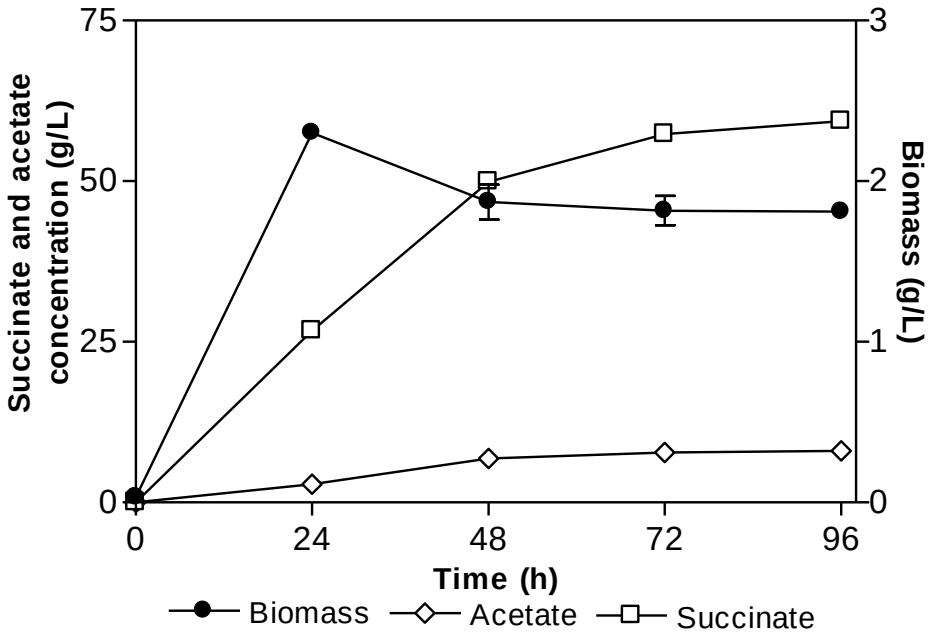

Figure 4.27 Production of succinate from 70 g/L of sucrose and glucose mixture in AM1 medium by KJ122-pKJSUC.

To take more consideration, effect of glucose in succinate production from mixture of sucrose and glucose was investigated in the medium without antibiotics. Maximum biomass was reached at 24 hours and succinate was attained at 51.55 g/L within 48 hours (productivity at 1.07 g/L/h). Therefore, glucose might not directly affect on cell growth and succinate production in KJ122-pKJSUC since low amount of glucose in the mixture of sugars did not also promote cell growth and succinate production in the broth with or without antibiotics. Since the ratio of mixture sucrose and glucose in this study did not improve succinate titer and productivity, the sugars mixture would not use as a substrate for large scale succinate production.

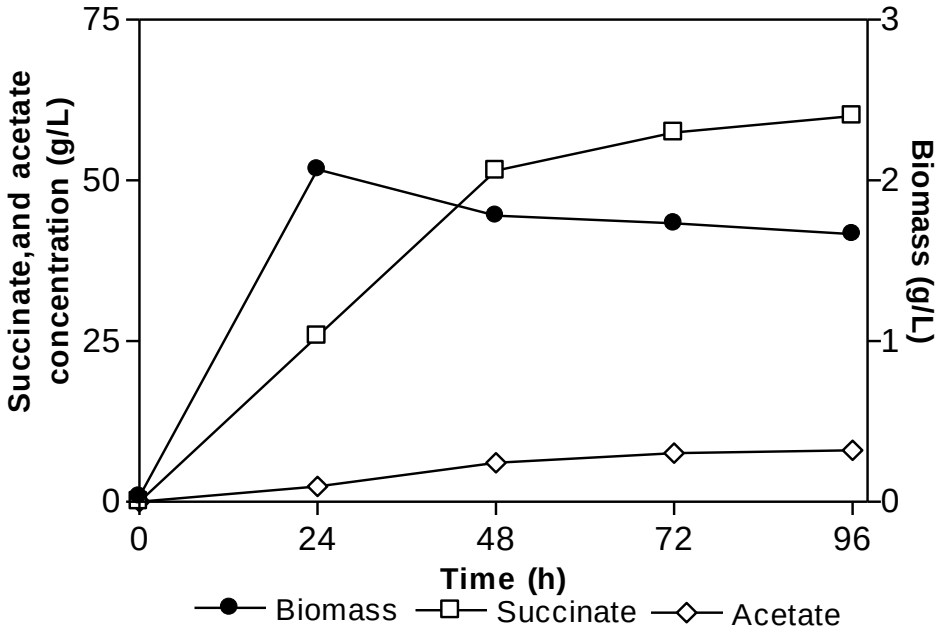

Figure 4.28 Production of succinate from 70 g/L of sucrose and glucose mixture supplemented to AM1 medium by KJ122-pKJSUC in the absence of antibiotics.

4.4 Production of succinic acid in 10 L bioreactor

Succinate production was enlarged to the working volume of 7.5 L in a 10 L bioreactor, with the optimal sugarcane molasses concentration at 150 g/L and initial OD_{550} at 0.1. At 96 hours, concentration of succinate, acetate, and fructose residue were 65.01 ± 0.64 g/L, 7.13 ± 0.13 g/L, and 15.32 ± 0.14 g/L respectively (Figure 4.29) while a 48 hours, KJ122-pKJSUC produced succinate (35.14 ± 7.53 g/L), acetate (2.03 ± 0.62 g/L) from 150 g/L untreated sugarcane molasses (approximately 75 g/L total sugars mixture) supplemented to AM1 medium under batch condition in 10 L bioreactor. *A. succinogenes* CGMCC1593 produced succinate (45.3 g/L), and high concentrations of acetic acid and formic acid more than 5 g/L and 1.5 g/L, respectively, from pre-treated sugarcane molasses (64.4 g/L total sugar concentration) supplemented to 10 g/L yeast extract in medium under batch condition in 5L bioreactor (Liu *et al.*, 2008). The result would imply that KJ122-pKJSUC had an

ability to produce succinate from sugarcane molasses comparable to what produce from *A. succinogenes* CGMCC1593 in the large scale fermentation.

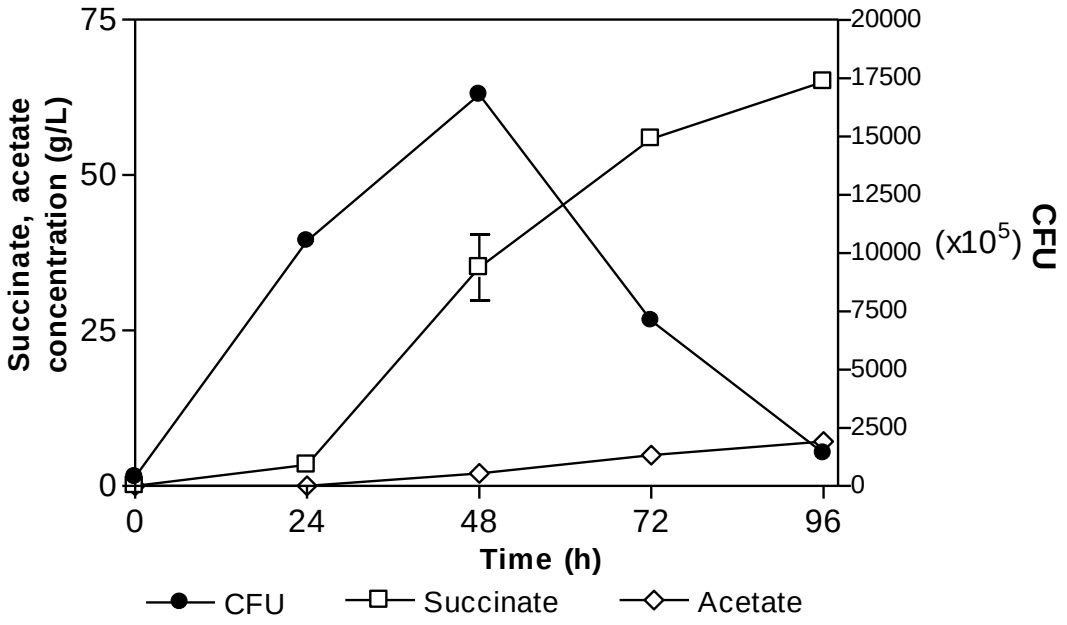

Figure 4.29 Production of succinate versus time (96 hours) in 10 L bioreactor under optimal conditions of sugarcane molasses at 150 g/L and initial OD_{550} at 0.1.

Colony counts in LB and LB containing kanamycin plates were observed to be highest amount at 48 hours (Figure 4.30A and B). Comparison of living cells in LB plate and LB (kanamycin) showed that the host cell (KJ122) lost some recombinant plasmids (pKJSUC) in fermentation. From the results, it could conclude that the loss of some recombinant plasmids, pKJSUC, did not affect on the succinate production. However, small accumulation of lactic acid was detected in fermentation.

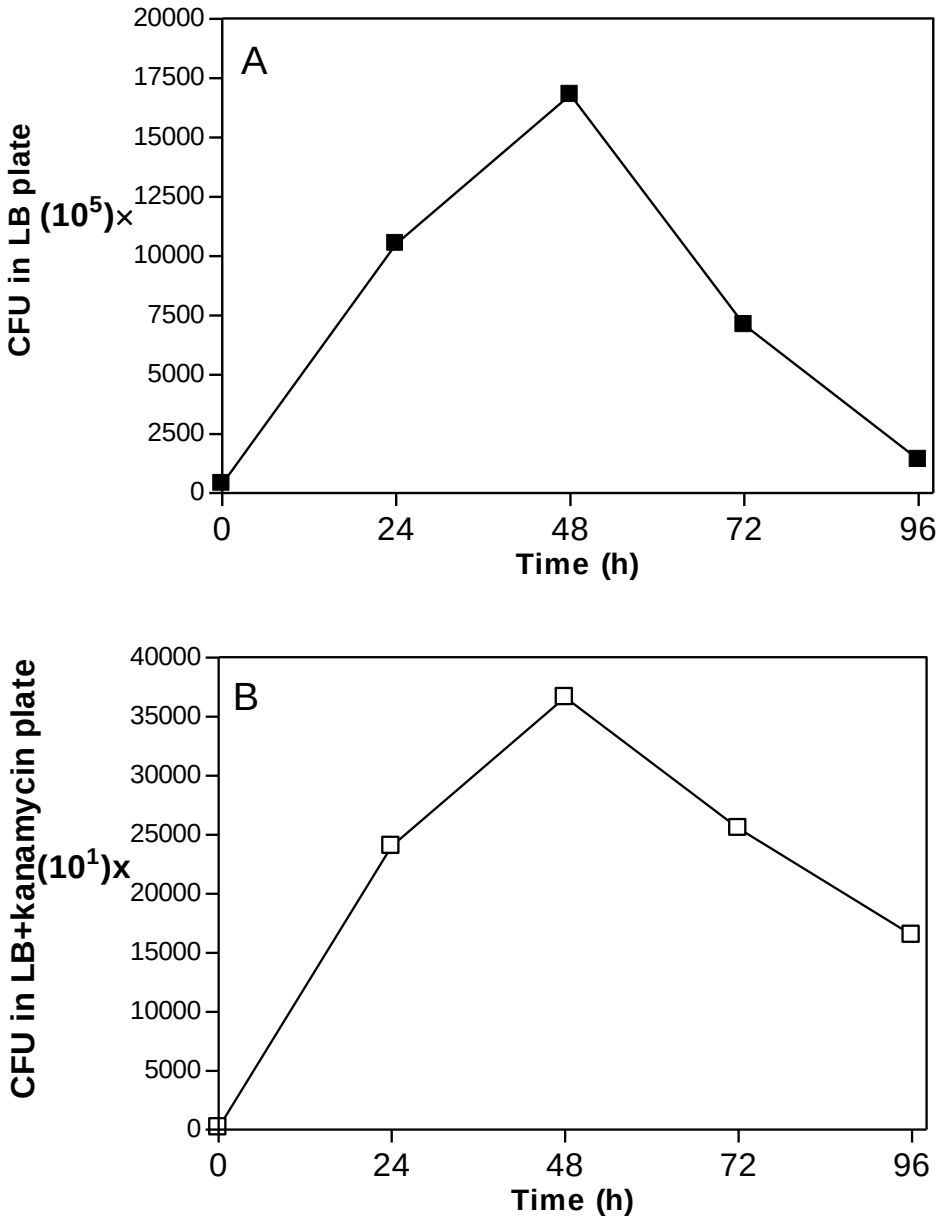

Figure 4.30 Colony form unit for expression of plasmid stability test in KJ122-pKJSUC. (A): CFU in LB plate and (B): CFU in LB plate supplemented to kanamycin.

Succinate production from 70 g/L sucrose was also performed in 10 L bioreactor (Figure 4.31). Maximum biomass (1.95 ± 0.09 g/L), succinate (46.59 ± 1.23 g/L), lactate (0.43 ± 0.15 g/L), acetate (4.94 ± 0.35 g/L), and fructose residue (16.18 ± 0.01 g/L) were obtained at 48 hours. However, succinate (57.26 ± 1.51 g/L), lactate (0.52 ± 0.47 g/L), acetate (7.28 ± 0.25 g/L), and fructose residue (7.86 ± 0.11 g/L) were obtained at 96 hours. Increasing incubation time led to the increase of small amount succinate but high amount of

acetate even though fructose was consumed. Succinate concentration and biomass were lower in bioreactor than those of the smaller scale. Succinate concentration slightly decreased in scale up production. Succinate concentration at 46.59 ± 1.23 g/L and productivity of 0.97 g/L/h were the highest concentration and productivity that has ever been reported using simple mineral medium and sucrose as a carbon sucrose under simple batch model in 10 L bioreactor.

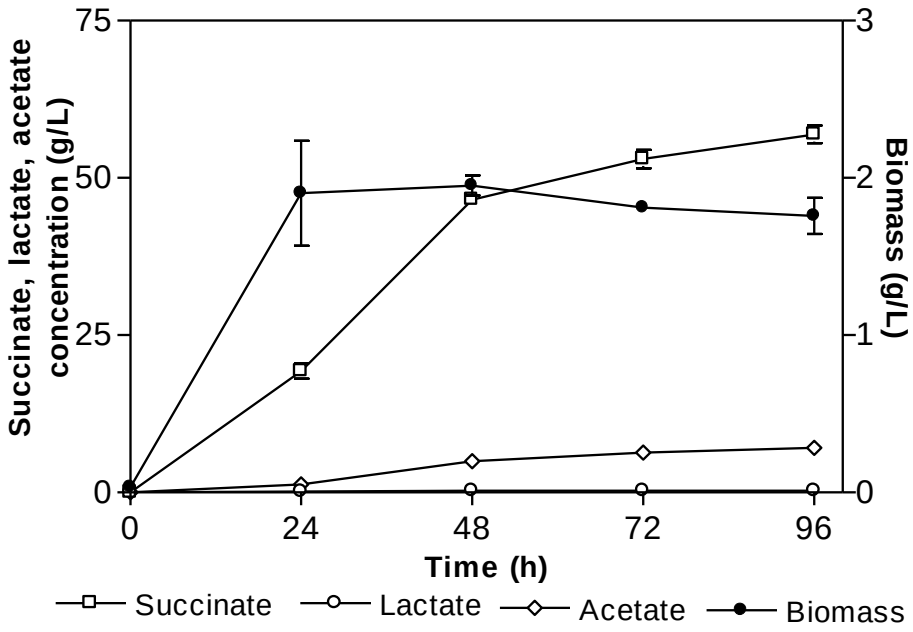

Figure 4.31 Production of succinate under optimal conditions of initial OD_{550} at 0.1 and 70 g/L sucrose supplemented to AM1 medium by KJ122-pKJSUC in 10 L bioreactor.

CHAPTER V

CONCLUSION

In this study, the growth and succinic acid production of *E .coli* KJ122 harboring pKJSUC were demonstrated in the simple mineral salts medium AM1 containing sucrose, artificial molasses and molasses as carbon substrates under simple-batch anaerobic conditions. The outcome of this study was focused on the cost effectiveness of bio-based succinic acid production from cheap medium and substrates as well as simple batch operation. The succinate concentration of 50 g/L from small anaerobic bottles could be the highest titer with the high productivity (1.05 g/L/h) within 48 hours produced among of the succinate producing bacteria by using sucrose as a sole substrate. In small anaerobic bottles, sugarcane molasses, an inexpensive carbon source at concentration of 150 g/L could provide the succinate titer around 47 g/L with a maximal productivity around 0.97 g/L/h. Moreover, KJ122-pKJSUC showed a high ability to produce succinate in AM1 medium supplemented with or without antibiotics. Considering the cost of substrates, medium, and downstream processing, it could clearly demonstrate that KJ122-pKJSUC would be an efficient strain for an economic succinate production in the near future. Scale up of succinate production by KJ122-pKJSUC was performed in 10 L bioreactor to study the effect of large scale production on succinate production. Around 35 g/L and 65 g/L of succinate (productivity at 0.72 g/L/h and 0.67 g/L/h) were produced from 150 g/L of molasses at 48 hours and 96 hours, while, 46 g/L and 57 g/L of succinate (productivity at 0.95 g/L/h and 0.59 g/L/h) were produced from 70 g/L of sucrose at 48 hours and 96 hours, respectively. The succinate production price would include the cost of carbon sources and medium, operation and bioprocess, as well as downstream processing (60% - 70% of the succinate production cost).

In conclusion, KJ122 could be a potential strain for the economic succinate production using abundant and feasible renewable substrates such as sucrose and molasses, which are readily available in Thailand or even the South-East Asia countries.

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APPENDICES

APPENDIX 1

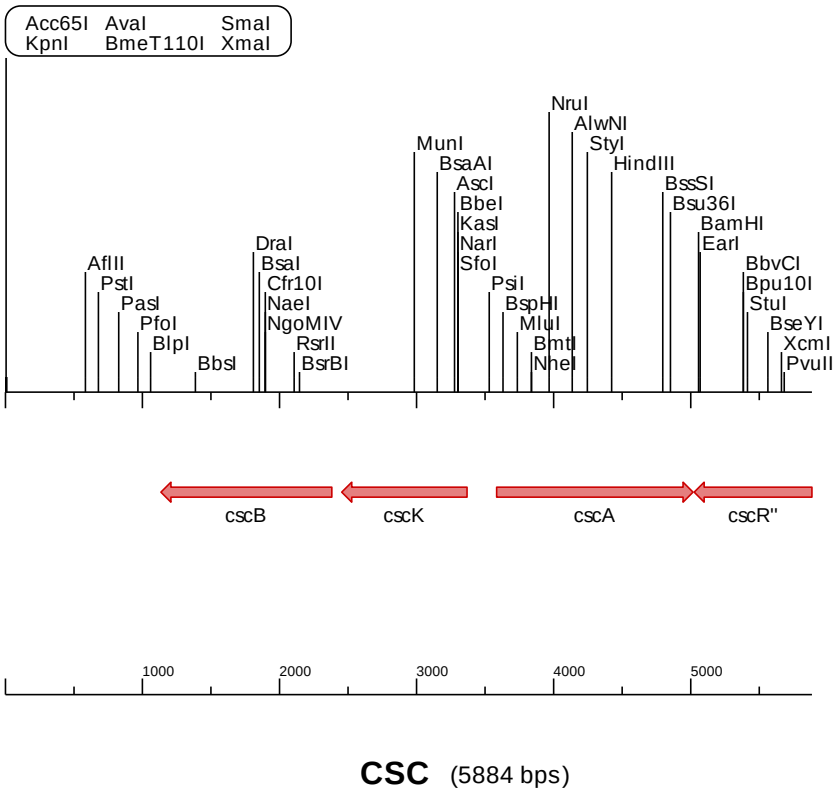

Figure 5.1 Gene *csc* arrangement in *E. coli* KO11 from Accession Number AY314757.

Inserted Gene (*cscBKA*) in pKJSUC

GCCGGGCGATGTAACCATCACACAGAATCCTGATAGCGAAATATGGCGTGACTC

GATACTTCACTCCGCAATGCATTCCTTGATGAATTCGCAGGACCGTGATACACG

GGACAGGTCACTGAATGACGACAATGTCCTGGAAATCAGCGACCGCGCATCTG

AAGTACATTTGAGCGACTGTACCAGAACATGAATGAGGCGTTTGGATTAGGCG

ATTATTAGCAGGGCTAAGCATTTTACTATTATTATTTTTGAGGGATATAGAGCTA

TCGACAACAACCGGAAAAAGTTTACGTCTATATTGCTGAAGGTACAGGCGTTTC

CATAACTATTTGCTCGCGTTTTTTACTCAAGAAGAAAATGCCAAATAGCAACAT

CAGGCAGACAATACCCGAAATTGCGAAGAAAACTGTCTGGTAGCCTGCGTGGT

CAAAGAGTATCCCAGTCGGCGTTGAAAGCAGCACAATCCCAAGCGAACTGGCA

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CAACTTCACTAATGAAATAATCCAGGGGTTAACGAACAGCGCGCAGGAAAGGA

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GATTCACAAAGAAAGGAATAATCGCCATGCACAGCGCTTCGAGTACCACCTGG

AATGAGTTGAGATAACCATACAGGCGCGTTCCTACATCGTGTGATTCGAATAAA

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CGTCCCCACAATAAATATGACGAAAACCCAGAAGTTTCGATCCTTGAAAACTGC

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AGCCAAAAGATCCCCAGGCGCGCGCTGTTCCATATTCGAAATGAAAATTTCGCGCCA

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TACTTCTCACTATTCCAGTTCTTGTCGACATGGCAGCGCTGTCATTGCCCCTTTCGCC

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CATCTGTAGATAATCCCGTAGAGGACAGACCTGTGAGTAACCCGGCAACGAACGCAT

CTCCCGCCCCCGTGCTATCGACACAATTCACAGACATTCCAGCAAAATGGTGAACTT

GTCCTCGATAACAGACCACCACCCCTTCTGCACCTTTAGTCACCAACAGCATGGCGA

*TCTCATACTCTTTTGCCAGGGCGCATATATCCTGATCGTTC***TGTGTTTTTCCACTGA**

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CCTGCCGCAAACACAAGCGGAGCAAATGCTCGTCTTGCCATAGATCTTCAC

GAATATTAGGATCGAAGCTGACAAAACCTCCGGCATGCCGGATCGCCGTC

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CAGAGATGTAACCATTCGCCATGTCGCCAGCAGGGCAAGTCTGTCGTCTCT

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CTTCAGATACGTGATATCGACTCCCTCAGTTAGCAGCGTTCTTTGCATTAA

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ATAGTGATAAACCTCTTTTTCGCAAAATCGTCATGGATTTACTAAAACATGC

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GCATGACGCTGGCGCGCAGCTATCAGAGAGCAATGGCAAACTTCTACAAC

GCCCGGTACACGAAGCTGAGTCGTTACGCCAGCAGCATCAATCTGTCTCTC

CCCGCACAATCAGCAATAAATATGTTTTGCAGGAAAACGCGCAAGCAGTTG

AGATTCAGTTGCAGTGGGCGCTGAAGAACAGTGATGC*CGAACATTACGGAT*

TACAGCTCGGCACTGGAATGCGGCTGTATATTGATAACCAATCTGAGCGACTTG

TTTTGTGGCGGTATTACCCACACGAGAATTTAGACGGCTACCGTAGTATTCCCC

TCCCGCAGCGTGACACGCTCGCCCTAAGGATATTTATCGATACATCATCCGTGG

AAGATTTATTAACGACGGGGAAGCGGTGATGAGTAGTCGAATCTATCCGCAGC

CAGAAGAACGGGAACTGTCGCTTATGCCTCCCACGGAGTGGCTGTGCTGCAAC

ATGGAGCACTCTGGCTACTGGGTTAACATAATATCAGGTGGAACAACGGATCA

ACAGCGGGCAAGGGATCCGCGTCACTCTTCCCCCTTCACGACCTTCAATAATAT

GCAATGCAGCTTCCCGCCCGATAATGTCATGTGGAAGCTGAATTGTGGTCAGCG

GCGG

Note: Normal letters are *cscB* gene, italic letters are *cscK* gene, bold letters are *cscA* gene, and underline letters are *cscR'* gene (incomplete gene).

APPENDIX 2

ABSTRACT

Proceeding of 17th National Genetic Conference on 7-9 April, 2011, Chiang Mai, Thailand: Functional Expression of Sucrose-Utilizing Gene in Metabolic Engineered *Escherichia coli* to Enhance Succinate Production from Sucrose.

Sitha Chan, Kaemwich Jantama

E. coli KJ122 was engineered to produce high titer and yield of succiate in mineral salt medium containing glucose under simple batch anaerobic fermentation. However, this strain does not efficiently utilize sucrose due to catabolic repression. To enhance the sucrose utilization of KJ122, sucrose utilizing genes (*cscA* and *cscKB*) containing their promoter regions from *E. coli* B, which naturally utilizes sucrose, was cloned and functionally expressed in KJ122. The transformants harboring a recombinant plasmid named pKJSUC were selected for the efficient sucrose utilization on the phenol red agar and broth supplemented with sucrose. The clones exhibited a larger clear yellow zone on the agar compared to KJ122 without the plasmid, and showed a fast ability in growth and acid production in cultivation broth. Succinate production was performed in LB broth supplemented with 50 g/L sucrose under anaerobic condition. At 72 hours, about 39 g/L of succinic acid was produced with less acetate at 5 g/L by KJ122-pKJSUC. This result showed that this strain was able to efficiently produce the high level of succinate from sucrose.

Keywords: Succinate, *E. coli,* Anaerobic Fermentation, Sucrose

BIOGRAPHY

Miss Chan Sitha was born on June 8th, 1986 in Battambong province, Cambodia. She attended and finished high school from the ProssMonivong High School in Battambong province. In 2004, she obtained a scholarship to further participate an undergraduate study at the Royal university of Phnom Penh. She finished her Bachelor Degree in Biochemistry in 2008. In 2009, she pursued her Master Degree in Biotechnology at Suranaree University of Technology (SUT), Thailand. During her attendance, she was fully financial funded under the SUT-Thailand-European Cooperation Facility (SUT-TECF) Project. Her research interest was entitled of "Production of succinic acid from sucrose and cane molasses". Some parts of results from this study had been presented as an oral presentation in the 3rd SUT Graduate Conference 2010 at Suranaree University of Technology, Nakhon Ratchasima, Thailand during November 21st-23rd, 2010 and as oral presentation with the proceedings of the 17th National Genetic Conference 2011, Chiang Mai during April 7th-9th, 2011. After she graduates from Suranaree University of Technology, she serves as a faculty member at Chemistry Department, Faculty of Science, the Royal University of Phnom Penh, Cambodia.