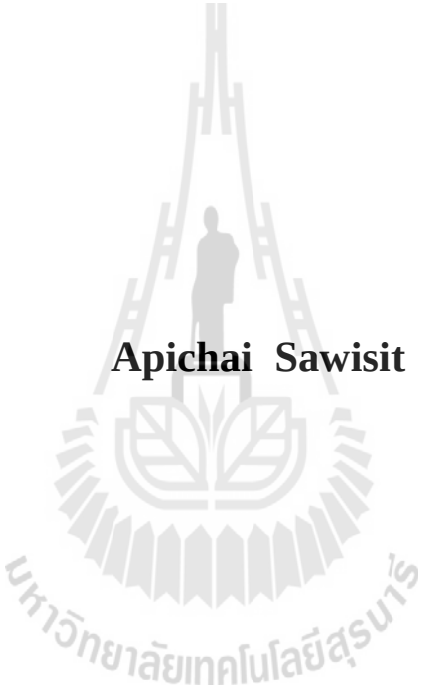
SUCCINATE PRODUCTION FROM CASSAVA PULP AND SUGARCANE BAGASSE BY METABOLICALLY ENGINEERED *ESCHERICHIA COLI* KJ122 AND ITS DERIVATIVES

Apichai Sawisit



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การผลิตซักซิเนตจากกากมันและชานอ้อยด้วยเชื้อที่ผ่านวิศวกรรมเมแทบอลิซึมสายพันธุ์เอสเชอริเชีย โคไล KJ122 และเชื้ออนุพันธ์

นายอภิชัย สาวิสิทธิ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

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อภิชัย สาวิสิทธิ์ : การผลิตซักซิเนตจากกากมันและชานอ้อยด้วยเชื้อที่ผ่านวิศวกรรมเมแทบอลิซึมสายพันธุ์เอสเชอริเชีย โคไล KJ122 และเชื้ออนุพันธ์ (SUCCINATE PRODUCTION FROM CASSAVA PULP AND SUGARCANE BAGASSE BY METABOLICALLY ENGINEERED *ESCHERICHIA COLI* KJ122 AND ITS DERIVATIVES) อาจารย์ที่ปรึกษา : ผู้ช่วยศาสตราจารย์ ดร.เขมวิทย์ จันต๊ะมา, 239 หน้า

เชื้อเอสเชอริเชีย โคไล KJ122 ถูกใช้สำหรับการผลิตซักซิเนตจากกากมันสำปะหลังและชานอ้อย เมื่อใช้กากมันสำปะหลังเป็นวัตถุดิบ เชื้ออีโคไลสายพันธุ์ KJ122 สามารถใช้กากมันสำปะหลังได้อย่างมีประสิทธิภาพ และผลิตซักซิเนตความเข้มข้น 41.46±0.05 กรัมต่อลิตร ในระหว่างกระบวนการหมักที่การย่อยเป็นน้ำตาลและการหมักแยกจากกัน (SHF) ส่วนกระบวนการหมักแบบกะที่มีการย่อยเป็นน้ำตาลพร้อมกันกับการหมัก (SSF) ที่สภาวะเหมาะสมของการใช้กากมันสำปะหลังความเข้มข้น 12 เปอร์เซ็นต์ (ร้อยละน้ำหนักต่อน้ำหนัก) ย่อยด้วยเอนไซม์ผสมระหว่างอะไมโลกลูโคซิเดส 2 เปอร์เซ็นต์ และเซลลูเลสเชิงซ้อน 3 เปอร์เซ็นต์ (ร้อยละปริมาตรต่อน้ำหนัก) ควบคุมค่าความเป็นกรด-ด่างที่ 6.5 และอุณหภูมิที่ 39 องศาเซลเซียส สามารถผลิตซักซิเนตได้ 82.33±0.14 กรัมต่อลิตร ทั้งนี้กระบวนการหมักแบบกึ่งกะโดยใช้กระบวนการหมักที่มีการย่อยเป็นน้ำตาลพร้อมกันกับการหมัก ผลิตซักซิเนตเพิ่มขึ้นอย่างมีนัยสำคัญเป็น 98.63±0.12 กรัมต่อลิตร

เชื้อกลายพันธุ์ได้ถูกคัดเลือกโดยการถ่ายเชื้ออย่างต่อเนื่องในอาหารเลี้ยงเชื้ออย่างง่าย AM1 ที่มีน้ำตาลไซโลสความเข้มข้น 10 เปอร์เซ็นต์ (ร้อยละปริมาตรต่อน้ำหนัก) หลังจากถ่ายเชื้อ 16 ครั้งในอาหารที่มีน้ำตาลไซโลส เชื้อที่เกิดการปรับตัวให้สามารถใช้ไซโลสได้ดีที่สุดจะถูกคัดแยกแล้วตั้งชื่อเป็นอีโคไลสายพันธุ์ AS1600a ซึ่งเชื้ออีโคไลสายพันธุ์ AS1600a สามารถผลิตซักซิเนตจากน้ำตาลไซโลสได้ 84.26±1.37 กรัมต่อลิตร นอกจากนี้เชื้อสายพันธุ์ AS1600a ยังให้ค่าอัตราการผลิต (0.96 กรัมต่อลิตรต่อชั่วโมง) ที่ได้จากการหมักน้ำตาลไซโลส 10 เปอร์เซ็นต์ (ร้อยละน้ำหนักต่อปริมาตร) ปรับปรุงจากเชื้อสายพันธุ์ KJ122 (0.31 กรัมต่อลิตรต่อชั่วโมง) ถึง 3 เท่า จากนั้นเชื้อสายพันธุ์ AS1600a ได้ถูกนำไปตรวจวิเคราะห์ลำดับนิวคลีโอไทด์ และพบว่าเชื้อดังกล่าวมีการกลายพันธุ์ในระบบนำเข้าน้ำตาลของโปรตีนกาแล็กโทสเพอมิเอส (GalP, G236D) ซึ่งการกลายพันธุ์ใน GalP นี้ ได้พิสูจน์แล้วว่าเกี่ยวข้องกับการปรับปรุงฟีโนไทป์ในการใช้น้ำตาลไซโลสของเชื้อกลายพันธุ์สายพันธุ์ AS1600a ทั้งนี้ยังพบว่ายีนทนต่อเฟอฟูรอล เช่น ยีนกลายพันธุ์ของ *fucO* (*fucO**) และ *puuP* เป็นประโยชน์ในการเพิ่มความสามารถในการทนต่อเฟอฟูรอลในเชื้ออีโคไลสายพันธุ์ AS1600a ซึ่งเชื้อที่มียีน *fucO** และ *puuP* ยีนใดยีนหนึ่ง สามารถสันดาปเฟอฟูรอลความเข้มข้น 20

มิลลิโมลาร์ ได้อย่างสมบูรณ์ภายในเวลา 72 และ 96 ชั่วโมง และผลิตซักซิเนต 70.21±0.93 และ 67.18±2.13 กรัมต่อลิตร ตามลำดับ ยิ่งไปกว่านั้นเชื้อดังกล่าวยังสามารถใช้น้ำตาลที่มีในไฮโดรไลเสตจากชานอ้อย (ปรับสภาพด้วยการปรับค่าความเป็นกรด-ด่างให้ได้ 9.0 และเติมเกลือไบซัลไฟต์) เพื่อผลิตซักซิเนตได้สูงกว่าเมื่อเปรียบเทียบกับเชื้อที่ไม่มียีนทนต่อเฟอฟูรอลถึง 37 เปอร์เซ็นต์ (72.76±0.98 เทียบกับ 46.05±1.34 กรัมต่อลิตร) หลังจากนั้นเชื้ออนุพันธ์ของอีโคไล AS1600a ที่ทนต่อสารที่ไม่ใช่เฟอฟูรอล ได้ถูกคัดเลือกโดยการถ่ายเชื้ออย่างต่อเนื่องในไฮโดรไลเสตจากชานอ้อยที่ปรับสภาพด้วยการปรับค่ากรด-ด่างให้ได้ 6.3 ร่วมกับการใช้การระเหยสุญญากาศและการเติมเกลือไบซัลไฟต์ แล้วเสริมด้วยน้ำตาลไซโลส 5 เปอร์เซ็นต์ (ร้อยละน้ำหนักต่อปริมาตร) หลังจากการถ่ายเชื้อ 145 ครั้ง ก็ทำการคัดเลือกโคโลนีจากประชากรเชื้อระหว่างการถ่ายเชื้อ หนึ่งในโคโลนีเหล่านั้นเชื้อสายพันธุ์ AS2003 สามารถเจริญในอาหารที่มีไฮโดรไลเสตจากชานอ้อย 70 เปอร์เซ็นต์ (ร้อยละปริมาตรต่อปริมาตร) (METSO; 185 องศาเซลเซียส; 7.5 นาที) ที่ปรับสภาพโดยการปรับค่าความเป็นกรด-ด่างให้ได้ 6.3 ร่วมกับการระเหยในสภาวะสุญญากาศและการเติมเกลือไบซัลไฟต์ และในกระบวนการหมักที่มีการควบคุมค่าความเป็นกรด-ด่าง เชื้อสายพันธุ์ AS2003 สามารถผลิตซักซิเนตได้สูงกว่าเมื่อเปรียบเทียบกับเชื้อสายพันธุ์ตั้งต้น (AS1600a) ราวๆ 15 เปอร์เซ็นต์ (85.46±1.69 ต่อ 72.66±0.59 กรัมต่อลิตร) จากการใช้ไฮโดรไลเสตจากชานอ้อย 50 เปอร์เซ็นต์ (ร้อยละปริมาตรต่อปริมาตร) ที่ปรับสภาพโดยการปรับค่าความเป็นกรด-ด่างให้ได้ 9.0 ร่วมกับการระเหยสุญญากาศและการเติมเกลือไบซัลไฟต์ นอกจากนี้เชื้อสายพันธุ์ AS2003 ที่มียีน *fucO** สามารถใช้ไฮโดรไลเสตที่ไม่ผ่านการปรับสภาพเลย ผลิตซักซิเนตได้มากกว่า 80 กรัมต่อลิตร หลังจากนั้นเชื้อสายพันธุ์ AS2003 ถูกนำไปตรวจวิเคราะห์ลำดับนิวคลีโอไทด์ ซึ่งพบว่ามียีนกลายพันธุ์ถึง 8 ยีนเมื่อเปรียบเทียบกับเชื้อตั้งต้นสายพันธุ์ AS1600a

สาขาวิชาเทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา_____

ปีการศึกษา 2558 ลายมือชื่ออาจารย์ที่ปรึกษา_____

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม _____

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม _____

APICHAI SAWISIT : SUCCINATE PRODUCTION FROM CASSAVA PULP AND SUGARCANE BAGASSE BY METABOLICALLY ENGINEERED *ESCHERICHIA COLI* KJ122 AND ITS DERIVATIVES. THESIS ADVISOR : ASST. PROF. DR. KAEMWICH JANTAMA, Ph.D., 239 PP.

SUCCINATE PRODUCTION/*ESCHERICHIA COLI*/CASSAVA PULP/ SUGARCANE BAGASSE/METABOLIC EVOLUTION

Escherichia coli KJ122 was utilized for succinate production from cassava pulp and sugarcane bagasse. With cassava pulp as the substrate, the *E. coli* KJ122 efficiently utilized cassava pulp and produced succinate at a concentration of 41.46±0.05 g/L during separate hydrolysis and fermentation (SHF). In batch simultaneous saccharification and fermentation (SSF), the optimization of 12% (w/w) cassava pulp with an enzyme loading of 2% amyloglucosidase + 3% cellulase complex (v/w) at pH 6.5 at 39°C provided the succinate concentration of 80.86±0.49 g/L. Fed-batch SSF significantly enhanced succinate concentration to 98.63±0.12 g/L.

Mutants were also selected by serial transfers in AM1 medium with 10% (w/v) xylose. After 16 serial transfers in xylose containing medium, the xylose-evolved strain was isolated and assigned as the *E. coli* AS1600a strain. The AS1600a strain produced 84.26±1.37 g/L succinate from xylose. The *E. coli* AS1600a strain also exhibited a 3-fold improvement in productivity with 10% (w/v) xylose (0.96 g/L/h) as compared with KJ122 strain (0.31 g/L/h). The *E. coli* AS1600a strain was sequenced and found to contain a mutation in galactose permease (GalP, G236D). This mutation in GalP was proved to be responsible for improvement in xylose

utilization in the *E. coli* AS1600a strain. Additionally, furfural resistant genes such as a mutated *fucO* (*fucO**), and *puuP* beneficially improved the furfural tolerance in the *E. coli* AS1600a strain. The strain individually harboring the *fucO** and *puuP* gene entirely metabolized 20 mM furfural within 72 and 96 h, and produced 70.21±0.93 and 67.18±2.13 g/L succinate, respectively. These strains could consume all sugars contained in sugarcane bagasse hydrolysate (pH 9.0 treatment + bisulfite addition) to produce about 37% succinate concentration higher than that of the strain harboring the empty vector control (72.76±0.98 versus 46.05±1.34 g/L). Further, non-furfural hydrolysate resistant derivatives of the *E. coli* AS1600a were also selected by serial transfers in the vacuum bisulfite-treated hydrolysate supplemented with 5% (w/v) xylose medium. After 145 serial transfers, clones from this population were isolated. One of these, the *E. coli* AS2003 strain, grew on 70% (v/v) of the pH 6.3 vacuum bisulfite-treated hydrolysate (METSO, 185ºC, 7.5 min). In pH-controlled fermentation, the AS2003 strain was able to produce about 15% higher succinate (85.46±1.69 g/L) when compared with the parental strain (AS1600a) from the use of the 50% (v/v) vacuum bisulfite-treated hydrolysate (pH 9.0 treated). Moreover, the *E. coli* AS2003 strain harboring a *fucO** gene could utilize non-detoxified sugarcane bagasse hydrolysate to produce succinate up to 80 g/L. The AS2003 strain was sequenced and found to contain 8 mutations when compared with the parental strain, AS1600a.

School of Biotechnology

Academic Year 2015

Student's Signature_____

Advisor's Signature_____

Co-Advisor's Signature_____

Co-Advisor's Signature_____

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LIST OF ABBREVIATIONS

AceF	=	Lipoate acetyltransferase
AceEE1	=	Subunit of pyruvate dehydrogenase complex
ACKA	=	Acetate kinase
ADHE	=	Alcohol dehydrogenase
ADP	=	Adenosine diphosphate
AM1	=	Alfredo Mertinez medium version 1
AMG	=	Amyloglucosidase
Amy	=	Amylase
ANOVA	=	Analysis of variance
ATCC	=	American type culture collection
ATP	=	Adenosine 5’-tri-phosphate
AOAC	=	Association of Official Analytical Chemists
ASPC	=	Aspartate aminotransferase
bp	=	Base pair
$^{\circ}$C	=	Degree Celsius
cAMP	=	cyclic adenosine 3′, 5′-monophosphate
CCR	=	Carbon catabolite repression
CDW	=	Dry cell weight
Cel	=	Cellulase
CFU/mL	=	Colony forming unit per Milliter (s)

LIST OF ABBREVIATIONS (Continued)

CITF	=	Citrate lyase
CMC	=	carboxymethyl cellulose
CoA	=	Coenzyme A
CSL	=	Corn steep liquor
E1	=	Subunit of PDH complex; AceE dimer
E2	=	Subunit of PDH comlex; AceF core
E3	=	Subunit of PDH complex; LpdA dimer
EV	=	Empty vector
Fe-S	=	Ferrous-sulfur compound
FNR	=	Fumarate-nitrate reductase regulatory protein
g	=	Gram (s)
GalP	=	Galactose permease
Glu	=	Glucose
g/L	=	Gram (s) per liter
g/L/h	=	Gram (s) per liter per hour
h	=	Hour (s)
HMF	=	Hydroxymethylfurfurral
HPLC	=	High performance liquid chromatography
IC_{50}	=	Concentration of inhibitor that reduce organic acids production by 50%
IPTG	=	Isopropyl-β-D-thiogalactoside
IU/g	=	International unit (s) per gram

LIST OF ABBREVIATIONS (Continued)

Kg	=	Kilogram (s)
K_m	=	Michalis constant
Kbp	=	Kilo base pair
L	=	Liter (s)
LB	=	Luria-Bertani
LpdA	=	Lipoamide dehydrogenase
LDHA	=	Lactate dehydrogenase
L+SScF	=	Liquefaction plus simultaneous sacharification and co-fermentation
M	=	Molar
MGSA	=	Methylglyoxal synthase
MIC	=	Minimum inhibitory concentration
mM	=	Millimolar
mg	=	Milligram (s)
mg/g	=	Milligram (s) per gram
mg/L	=	Milligram (s) per liter
min	=	Minute (s)
mL	=	Milliliter (s)
mm	=	Millimeter (s)
N	=	Normality
NAD	=	Nicotinamide adenine dinucleotide (Oxidized form)
NADH	=	Reduced form of Nicotinamide adenine dinucleotide

LIST OF ABBREVIATIONS (Continued)

NADP	=	Nicotinamide adenine dinucleotide phosphate (Oxidized form)
NADPH	=	Nicotinamide adenine dinucleotide phosphate (Reduced form)
NR	=	Not reported
NM	=	Not measured
OD_{550}	=	Optical density at 550 nm
pNG	=	p-nitrophenyl-b-D-glucopyranoside
% (v/v)	=	Percentage volume by volume
% (v/w)	=	Percentage volume by weight
% (w/v)	=	Percentage weight by volume
% (w/w)	=	Percentage weight by weight
PCK	=	Phosphoenolpyruvate carboxykinase
PCR	=	Polymerase chain reaction
PEP	=	Phosphoenolpyruvate
PFLB	=	Pyruvate fomate-lyase
POXB	=	Pyruvate oxidase
PPC	=	Phosphoenolpyruvate carboxylase
PTS	=	Phosphotransferase system
PYC	=	Pyruvate carboxylase
PYKAF	=	Pyruvate kinases A and F
ROS	=	Reactive oxygen species

LIST OF ABBREVIATIONS (Continued)

rpm	=	Revolutions per minute
RSD	=	Raw starch-degrading enzyme
SFCA	=	Malic enzyme
sp.	=	Species
TCA	=	Tricarboxylic acid
TDCD	=	Propionate kinase
U	=	Unit
U/g	=	Unit (s) per gram
μL	=	Microliter (s)
μM	=	Micro molar (s)
vvm	=	Gass volume flow per unit of liquid volume per minute
SHF	=	Separate hydrolysate and fermentation
SSF	=	Simultaneous sacharification and fermentation
Xyl	=	Xylose, Xylanase
YCH	=	Yeast cell hydrolysate
YE	=	Yeast extract

CHAPTER I

INTRODUCTION

1.1 Significance of the study

In recent years, high crude oil price and increasing concern over global warming have stimulated the search for alternative energy sources and also for alternative biochemical processes. The key to success in the development of profitable industrial biochemical conversion technologies is the choice of target fermentations that can compete with the efficiency of the petrochemical industry. For this purpose, it is essential to develop fermentations that produce molecular building blocks, which can be used as precursors for the production of a number of high-value chemicals or materials. The building block concept follows much of the same strategy that is used by the petrochemical industry, i.e., production of high-value chemicals from a limited number of chemical intermediates. Succinate has been identified by the U.S. Department of Energy as one of the top 12 building block chemicals that could be produced from renewable feedstocks (Werpy and Petersen, 2004).

Succinate is a number of the C4-dicarboxylic acid family. It is used as specialty chemical in food, agricultural, and pharmaceutical industries (McKinlay et al., 2007). More importantly, succinate could serve as the starting material for producing bulk chemicals such as 1,4-butanediol (a precursor to “strong-than-steel” and biodegradable plastics), ethylenediamine disuccinate (a biodegradable chelator), diethyl succinate (a “green” solvent replacement for methylene chloride), and adipic

acid (a nylon precursor) (Wang et al., 2010). It is evidenced that it is important to develop technologies for the production of succinate to supply the industrial needs. Currently, succinate is traditionally manufactured from maleic anhydride through *n*-butane using petroleum as raw material. It could also be produced by biochemical conversion of biomass using microbial fermentation. The fermentative production of succinate has many advantages over chemical processes owing to its simplicity and environment friendly nature. However, the industrial scale production of succinate requires a decrease in its production cost. One possible way to reduce the cost of the fermentation process is to use agricultural and dairy waste products and other lignocellulosic biomass rather than refined carbohydrates (Lynd et al., 1999). Therefore, the utilization of inexpensive carbon substrates, lignocellulosic hydrolysate like cassava pulp and sugarcane bagasse, can make the fermentation process for succinate production more economically competitive (Lee et al., 2001).

Cassava pulp, a fibrous by-product of the cassava processing industry, has recently become attractive as a cellulosic biomass due to its nature as a cheap, abundant, and renewable agricultural product (Sriroth et al., 2000). Cassava pulp contains reasonably high organic substances which later becomes rot and causes environmental concerns (Virunanona et al., 2013). At present, cassava pulp is generally used as low-value animal feed. Due to its rich organic matters, the utilization of cassava pulp for succinate production is not only adding a great value to cassava pulp but may solve the environmental problem.

A few studies on succinate production from cellulosic and hemicellulosic materials have been reported. Lee et al. (2003) demonstrated that *Anaerobiospirillum succiniciproducens* could be grown on the medium containing wood hydrolysate and

produced 24 g/L succinate (yield equal 88% w/w glucose). The wood hydrolysate was prepared with enzymatic hydrolysis of steam explosive oak wood chips with Cellulase (Novozymes Co.). Kim et al. (2004) used NaOH-treated wood hydrolysate as the carbon source to culture *Mannheimia succiniciproducens* MBEL55E, and have obtained 1.17 g/L/h and 3.19 g/L/h of succinate productivity in batch and continuous fermentation, respectively. Hodge et al. (2009) tested a metabolically engineered *E. coli* to ferment the detoxified softwood dilute sulfuric acid hydrolysate. Zheng et al. (2009) applied corn stover hydrolysate as carbon source in batch fermentation by *Actinobacillus succinogenes* CGMCC1593. A succinate concentration of 45.5 g/L was attained at the initial reducing sugar concentration in hydrolysate of 58 g/L. Liu et al. (2012) applied the dual phase fermentation to produce succinate from sugarcane bagasse hydrolysate by a metabolically engineered *E. coli*. A succinate concentration of 18.88 g/L with a yield of 0.96 g/g total sugars after 24 h of anaerobic fermentation was obtained.

All of the above reported cases are basically lignocellulosic materials hydrolysis and fermentation separated processes (SHF), which involved with two steps, namely hydrolysates preparation process and succinate fermentation process. Whereas, another technique called simultaneous saccharification and fermentation (SSF) process has been developed (Takagi et al., 1977). It can simultaneously perform the linocellulosic biomass enzymatic hydrolysis and fermentation at the same time and thus is considered as the most promising alternative way for the conversion of renewable raw materials into bio-based chemicals like succinate (Chen et al., 2011; Zheng et al., 2010). As SSF process is capable of treating lignocellulosic biomass and producing targeted product simultaneously in the same vessel, SSF has the

advantages over SHF in aspects such as higher productivity, less glucose inhibition on enzymatic hydrolysis and fermentation as well as lower capital investment. Based on this result, succinate production was performed in a SSF process with cassava pulp as the fermentation material and the previously engineered *E. coli* KJ122 (Jantama et al., 2008b) as the production strain.

Sugarcane bagasse is a fibrous residue after the sugarcane stalks are crushed to extract their juice. In some 80 developing countries, the sugar industrial currently produced some 1,100 million ton of sugar per years (Botha and Blottnitz, 2006). Per 1,000 kilograms of sugarcane provided 125 kilograms of bagasse, thus over 100 million tons of bagasses are produced annually throughout the world (Botha and Blottnitz, 2006). Several processes and products have been reported that sugarcane bagasse is utilized as a raw material electricity for generation, pulp and paper production, and products based on fermentation (Pandey et al., 2000). As this information, sugarcane bagasse is seemed to be the most promising alternative feedstock for microbial production of bio-based chemicals such as succinate. Sugarcane bagasse, like lignocellulosic materials in general, is resistant to enzymatic hydrolysis, because of both the tight network in the lignocellulose complex and the crystalline structure of the native cellulose. These difficulties can be overcome by employing a suitable pre-treatment. Unfortunately, the pretreatment processes also cause the breakdown of lignin and dehydration of the sugars, producing the inhibitory compounds such as organic acids (acetate and formate), furan derivatives (furfural and 5-hydroxymethylfurfurral; HMF) and phenolic compounds (Keating et al., 2014). These compounds, especialy furfural are known to have a negative impact with the growth of microorganisms and reduce product yields.

Several approaches have been reported to reduce the diluted acid hemicelluloses hydrolysate toxicity. The evaporation under vacuum showed to reduce levels of furfural, hydroxymethyl furfural, acetic acid and vanillin (Chandel et al., 2013). Sodium meta-bisulfite has also been demonstrated to decrease diluted acid hydrolysate toxicity (Nieves et al., 2011). Additionally, increasing the pH to 9.0 with ammonium hydroxide (NH_4OH) was investigated to decrease diluted acid hydrolysate toxicity with minimal sugar loss (Geddes et al., 2013). More recently, Geddes et al. (2015) evaluated that the combining of several treatments (vacuum evaporation, laccase, high pH, bisulfite, and microaeration) completely eliminated all inhibitory activity presenting in diluted hemicelluloses hydrolysate. However, the removing of those inhibitors prior to fermentation may increase the process cost and the potential loss of fermentable sugars (Keating et al., 2014).

The numbers of genes that are beneficial for furfural tolerance in *E. coli* with directly or indirectly promote the NADH-dependent reduction of furfural to the less toxic compound furfuryl alcohol have been reported. They included a *fucO*, an NADH-dependent propanediol (and furfural) oxidoreductase (Wang et al., 2011c), *pntAB*, a cytoplasmic NADH/NADPH transhydrolgenase (Miller et al., 2009), and a cryptic gene (*ucpA*) adjustment to sulfur assimilation operon (Wang et al., 2012). Additionally, an overexpression of *thyA*, thymidylase syntase, has also been investigated to increase furfural resistant in ethanologenic strain (Zheng et al., 2012). More recently, plasmid-based expression of polyamine transporters, *puuP* and *potE* have been evaluated to increase the metabolism of furfural and decreased the time required for xylose fermentation by ethanologenic strain (Geddes et al., 2014).

However, increasing furfural tolerance using plasmid-based expression of furfural resistant genes in succinate-producing strain have not much been studied so far.

1.2 Objectives

To become more attractive and more promising in the succinate production industry, the production of succinate by metabolically engineered *E. coli* KJ122 using cheap carbon substrates, cassava pulp and sugarcane bagasse, were emphasized. The maximum utilization of all sugar fractions in hemicellulose hydrolysate is essential to obtain an economic and viable conversion technology for succinate production. No such strains are currently available for the simutaneously co-fermentation of sugars mixture into succinate. Hence, this study focused on:

1) Demonstrating the feasibility of producing succinate from cassava pulp by metabolically engineered *E. coli* KJ122 using separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF).

2) Developing a metabolically engineered *E. coli* KJ122 that was able to efficient convert xylose, sugar mixtures, and diluted acid hydrolysate of sugarcane bagasse into succinate using a metabolic evolution.

3) Improving a succinate-producing strain that resist to inhibitor compounds in diluted acid hydrolysate of sugarcane bagasse.

3.1 Enhancing a strain resistance to furfural by introducing the beneficial furfural resistant genes from ethanologenic *E. coli* strain into succinate-producing strain.

3.2 Developing a strain resistant to non-furfural hydrolysate inhibitors by applying a metabolic evolution.

CHAPTER II

LITERATURE REVIEW

2.1 Succinate and its applications

Succinate, a dicarboxylic acid with the molecular formula $C_4H_6O_4$, was first discovered in 1546 by Georgius Agricola when he dried distilled amber. Currently, succinate is manufactured through oxidation of *n*-butane or benzene followed by hydrolysis and finally dehydrogenation. The four existing succinate markets are the detergent/surfactant market, the ion chelator market, food market (e.g. acidulants, flavours or antimicrobials) and the pharmaceutical market (Figure 2.1). These markets have high added value and do not require very cheap feedstock. However, commodity chemicals are mostly low cost bulk chemicals (Zeikus et al., 1999). Three succinate derivatives with major applications are obtained through hydrogenation routes. These are gamma-butyrolactone (GBL), 1, 4-butanediol (BDO) and tetrahydrofuran (THF). BDO has three main branches-polymers, tetrahydrofuran (THF) derivatives and butyrolactone (GBL) derivatives (Zeikus et al., 1999, Delhomme et al., 2009). The second group of succinate derived molecules is the pyrrolidones. Their applications are mainly in the solvent and polymer industry. Ammonium succinate and succinimides are alternative reactants for pyrrolidone production. Through reductive amination, succinic anhydride or maleic anhydride can be converted in an aqueous environment (Cukalovic and Stevens, 2008). Fumarate, malate and its aconate form the third group of potential succinate derivatives. The

chemical conversion of succinate to these three compounds involves high temperatures and pressures, in some cases in a multistep process. These high energy consuming processes can be avoided by direct fermentative production. All three of these compounds are naturally produced by microorganisms and production systems are being developed for industrial production (Beauprez et al., 2010).

2.2 Commercial succinate production

Currently, the large use of succinate and its derivatives is around 20,000-30,000 tones per years with the potential price of $400,000,000 per year (Beauprez et al., 2010; Kidwell et al., 2008). This rate increases by 10% per year and the market size is estimated to be more than 270,000 tons per year (Wallke and Vorlop et al., 2004). The commercialized succinate is mainly produced by chemical process from butane or oxidation of benzene through maleic anhydride. The price of succinate 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 succinate 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 tones per year and it 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 per year due to utilizing cheap carbon substrates such as corn, starch, molasses, and sugars (Kidwell et al., 2008). Bio-based succinate needs consumption of CO_2 during fermentation so this process would contribute to reduce greenhouse gas. Moreover, it declines pollution from the manufacturer by constituting many commodities based on

benzene and intermediate petrochemical of over 250 bezene derived chemicals (Ahmed and Morris, 1994). As a result, the demand of succinate in many applications is high and increasing every year, interest in anaerobic fermentation has intensified especially as how it relates to the utilization of cheap carbon source to produce succinate or even higher-value chemicals derived succinate.

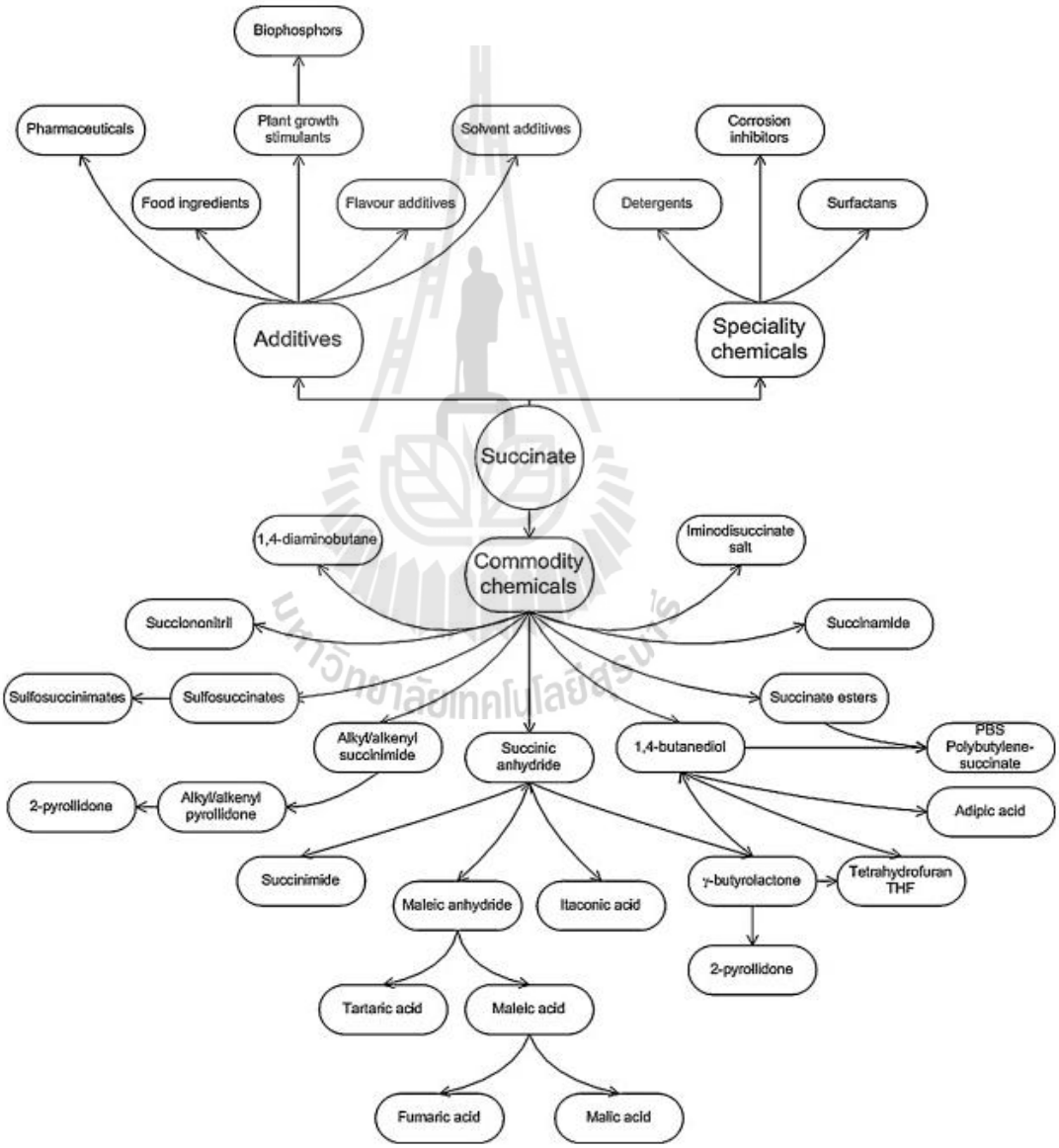

Figure 2.1 Overview of applications and products derived from succinate (Source; Beauprez et al., 2010).

2.3 Current production of succinate

Succinate produced by using petrochemical resource is derived from maleic anhydride, which is produced from *n*-butane through oxidation over vanadium-phosphorous oxide catalysts (Gascon et al., 2006; Liu et al., 2008). A simplified reaction pathway of *n*-butane to maleic anhydride is shown in Figure 2.2.

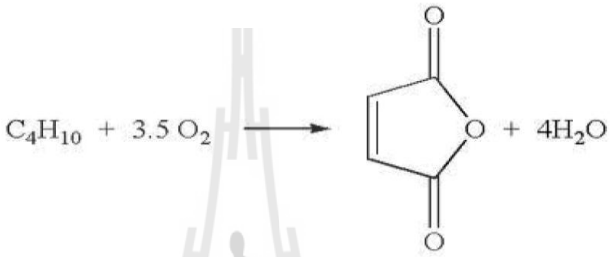

Figure 2.2 Reaction pathway from *n*-butane to maleic anhydride

(Source: Gascon et al., 2006).

The reaction from maleic anhydride to succinate begins by hydrolysis, breaking one of the single bonds between carbon and oxygen, forming maleic acid. The addition of hydrogen breaks the carbon-carbon double bond and completes the reaction, forming succinate. Succinate produced from fossil fuels is what gives it the distinction of not being a natural product (Song and Lee, 2006). While this method of production is currently cheaper than processing by fermentation, there are some very large drawbacks (Wan et al., 2008). As the term petrochemical processing implies, succinate is made using non-renewable resources such as natural gas which will become more difficult to find as time passes. As this raw material becomes harder to locate and demand continues to increase, it will become increasingly expensive (Isar et al., 2006). In addition, the removal of oil and gas from deposits, transportation and

processing require a lot of energy and generate a large amount of emissions (McKinlay et al., 2007). This process is not sustainable in the long term and another solution is required if industries want to continue producing succinate with increasing demand (Wan et al., 2008).

2.4 Succinate production through fermentation

Succinate, when produced through fermentation, converts glucose to succinate along a portion of the reductive cycle of the tricarboxylic acid (TCA) cycle (Lee et al., 2002). Figure 2.3 depicts the reactions and enzymes in a typical fermentation process that transforms glucose to succinate. In the central anaerobic metabolic pathway, pyruvate is assimilated to re-oxidize NADH via lactate dehydrogenase and alcohol dehydrogenase activities resulting in lactate and alcohol productions, respectively. In the simplest method of hydrogen disposal, pyruvate is reduced to lactate at the expense of NADH. The reaction is catalyzed by a cytoplasmic lactate dehydrogenase encoded by *ldhA*. The enzyme is jointly induced by acid pH and anaerobiosis. Lactate can be produced from dihydroxyacetone-phosphate (DHAP). DHAP is converted to methylglyoxal byproduct of *mgsA* (methyglyoxal synthase) and is subsequently converted to lactate by glyoxalase activities encoded by *gloAB* (Clark, 1989). Pyruvate formate-lyase encoded by *pflB*, which is responsible for anaerobic conversion of pyruvate to acetyl-CoA and formate. Pyruvate formate-lyase is posttranslationally interconverted between active and inactive forms. The enzyme synthesis is increased by anaerobiosis and can be raised further by pyruvate (McKinlay and Vieille, 2008). Acetyl-CoA produced from pyruvate can be used to generate ATP from ADP by conversion to acetate or to dispose off extra reducing

equivalents by conversion to ethanol. The first process depends on the consecutive action of phosphate acetyltransferase probably which is encoded by *pta* and acetate kinase encoded by *ackA*. Synthesis of these enzymes is not significantly changed by the respiratory condition of the cell. Consequently, most of the acetyl-CoA is excreted as acetate by cells growing on glucose under aerobic condition. In the absence of glucose, external acetate is mostly utilized by reversal of the pathway catalyzed by acetyl-CoA synthethase, encoded by *acs* (McKinlay et al., 2007). Acetyl-CoA is also converted to ethanol under anaerobic fermentation. The pathway involves a consecutive reduction of the acetyl group of acetyl-CoA to acetaldehyde, and acetaldehyde to ethanol at the expense of NADH. The reactions are catalyzed by a single polypeptide, which is alcohol dehydrogenase, encoded by *adhE*. The propinquity of the two sites of reduction might minimize escape of the acetaldehyde, which is chemically reactive. ADHE protein has dual enzyme activities, which are alcohol dehydrogenase and coenzyme-A-linked acetaldehyde dehydrogenase. However, alcohol dehydrogenase is more sensitive to inactivation by the aerobic metabolism (Clark, 1989).

The assimilation of PEP also occurs via carboxylation in which it generates succinate. For PEP carboxylation, fumarate reductase is activated and re-oxidizes NADH using fumarate as an electron acceptor. Endogenous or exogenous carbon dioxide is combined with PEP by phosphoenolpyruvate carboxylase encoded by *ppc*. The oxaloacetate formed is reduced to malate by the activity of malate dehydrogenase encoded by *mdh*. Malate is then dehydrated to fumarate by fumarase enzymes encoded by *fumABC*, whose anaerobic induction depends on FNR regulation. Fumarate is finally reduced to succinate by fumarate reductase. The net result is

disposal of four reducing equivalents ($4H^+ + 4e^-$). Fumarate reductase encoded by *frdABCD* can accept electrons from various primary donor enzymes through menaquinone. Fumarate reductase is induced anaerobically by fumarate but is repressed by oxygen or anaerobically by nitrate (Lee et al., 2002).

Pyruvate dehydrogenase multi-enzyme complex is composed of products of *aceEF* and *lpdA* genes. The reaction is the gateway to the TCA cycle, producing acetyl-CoA for the first reaction. The enzyme complex is composed of multiple copies of three enzymes: E1, E2 and E3, in stoichiometry of 24:24:12, respectively. The E1 dimers (encoded by *aceE*) catalyze acetylation of the lipoate moieties that are attached to the E2 subunits. The E2 subunits (encoded by *aceF*) are the core of pyruvate dehydrogenase complex and exhibit transacetylation. The E3 component is shared with 2-oxoglutarate dehydrogenase and glycine cleavage multi-enzyme complexes. Pyruvate is channeled through the catalytic reactions by attachment in thioester linkage to lipoyl groups carrying acetyl group to successive active sites. This enzyme complex is active under aerobic condition (Clark, 1989). Pyruvate can be converted to CO_2, acetyl-CoA, and NADH via the enzyme complex.

Under micro-aerobic condition, pyruvate oxidase encoded by *poxB* is responsible for generating C2 compounds from pyruvate during the transition between aerobic and strict anaerobic growth condition. This enzyme couples the electron from pyruvate to ubiquinone and decarboxylates pyruvate to generate carbon dioxide and acetate (McKinlay and Vieille, 2008). Under both aerobic and anaerobic respirations, the versatility of the electron transport system for generating proton motive force is made possible by employing ubiquinone or menaquinone in the plasma membrane as a diffusible electron carrier or adaptor to connect a donor

modular unit functionally to an acceptor modular unit. The types of electron carrier and donor modulars used for electron transport depend on the pattern of gene expression in response to the growth conditions. In anaerobic conditions, the electron donor modular units are primary dehydrogenases of the flavoprotein kind. The acceptor modular units consist of terminal reductases requiring various components, such as Fe-S. In general, when the terminal acceptor has a relatively high redox potential such as oxygen, ubiquinone is used as the redox adaptor i.e. pyruvate oxidase case. When the terminal acceptor has a relatively low redox potential such as fumarate and menaquinone is used instead for reduction of fumarate to succinate (Clark, 1989). The theoretical yield of succinate from glucose plus carbon dioxide should be 1.71 moles per mole of glucose based on stoichiometry (McKinlay and Vieille, 2008).

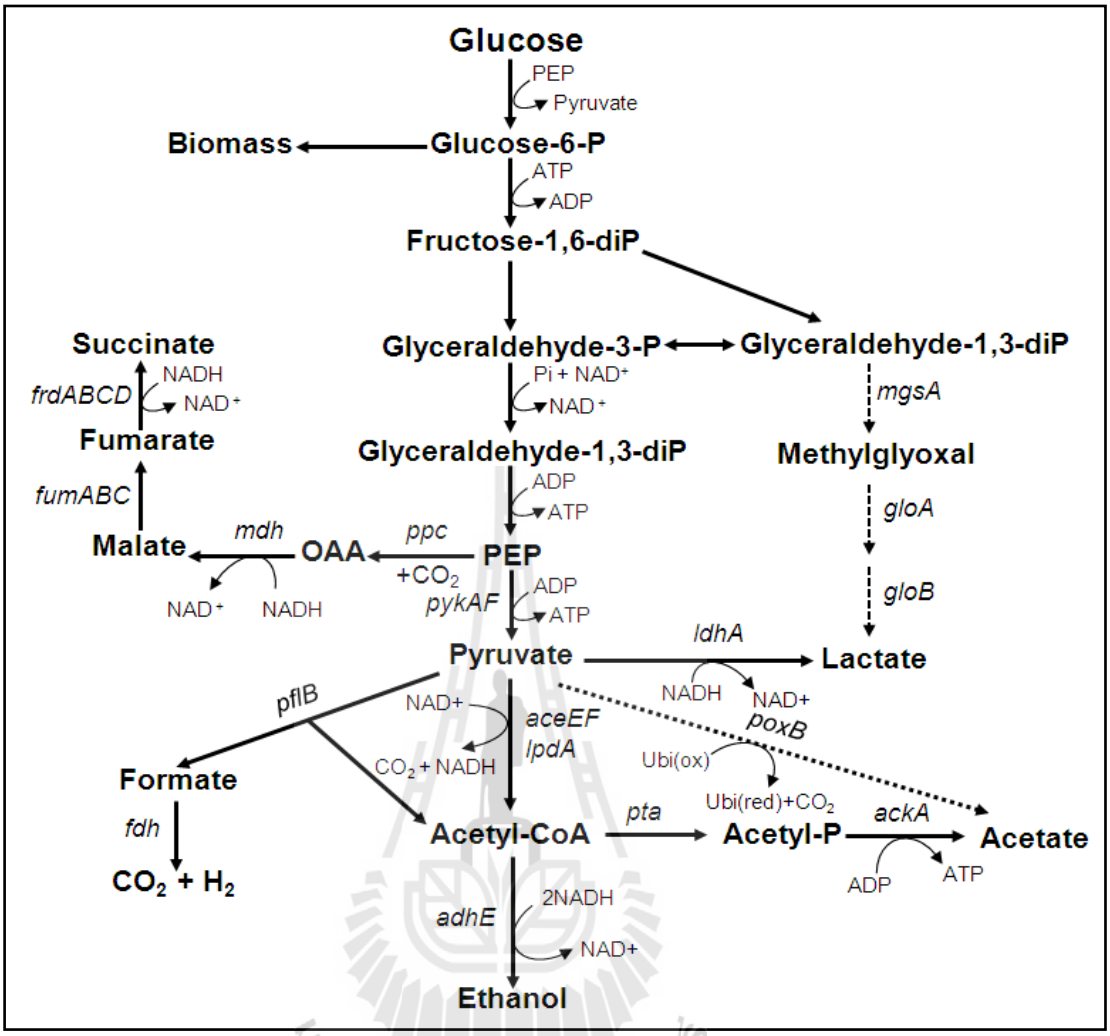

Figure 2.3 Central metabolic pathway of *E. coli*. Solid arrows represent central fermentative pathways. Dotted arrow represents microanaerobic pathway (*poxB*). Dash arrow represents minor lactate producing pathway (*mgsA*, *gloAB*). Genes: *pykAF*: pyruvate kinase, *ldhA*: lactate dehydrogenase, *pflB*: pyruvate formate-lyase, *pta*: phospho 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 (adapted from Clark, 1989).

2.5 Succinate producing strain

Succinate is an intermediate product of the Krebs cycle and a fermentative end-product, microorganisms lend themselves perfectly as production hosts. The choice of production host is very diverse, although most natural production hosts described in literature are capnophilic microorganisms. The non-natural production hosts, on the other hand, are chosen on the basis of their genetic accessibility. Various bacteria have been reported to produce succinate 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. Some of these such as *A. succinogene*s (Du et al., 2008; Lin et al., 2008; Li et al., 2010; Li et al., 2011), *An. succiniciproducens* (Lee et al., 2001), and *M. succinoproducens* (Kim et al., 2004) 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., 2008b). Future development of succinate production needs two stages. First, fermentation needs strains cooperated with CO_2 in the medium by operating either batch, fed batch, or continuous modes. In this case, medium should be cheap and fermentation process should be simple (Saure et al., 2008). Second, product recovery should be designed to purify succinate from mixed acids by using electro-dialysis, ion exchanger.

2.6 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 with productivity between 1.8 and 2.5 g/L/h and 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. Efforts should be done to optimize the current metabolic engineering towards succinate 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 succinate and utilize cheap feedstock in paralle with developing novel separation and purification technology with low cost (Xu et al., 2010).

2.7 Availability of various carbon sources to produce succinate

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 succinate has been reported from variety of carbon sources utilized by some derivatives *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), whey (Wan et al., 2008), sucrose (Liu et al., 2008) lignocelluloses including wood hydrolysate and straw hydrolysate (Kim et al., 2004), wheat milling

by-product (Dorado et al., 2009), corn straw hydrolysate (Zheng et al., 2009), corp stalk (Li et al., 2010), corncob hydrolysate (Yu et al., 2010), corn stover hydrolysate (Li et al., 2011), rapeseed meal hydrolysate (Chen et al., 2011) sugarcane bagasse hydrolysate (Liu et al., 2012), Beachwood xylan hydrolysate (Zheng et al., 2012), sake lees hydrolysate (Chen et al., 2012) and soybean meal hydrolysate (Thakker et al., 2013) have been tested to produce succinate by microbial 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., 2008).

It has been reported that *E. coli* has an ability to grow fast without requirement of complex nutrients, and the strain ability is easy to manipulate its metabolic pathways by genetic engineering, it has potential to become a target microorganism for strain improvement and process design for succinate production (Lin et al., 2005a). In the last decade, many research groups have been studying extensively to obtain high production yield of succinate by metabolic engineering of *E. coli* strain. However, all the method for producing succinate from *E. coli* published have involved rich media such as Lurie-Bertani (LB) broth, which contains sources of amino acids, proteins and other chemicals from yeast extract and peptone. Contaminating proteins and cells byproducts would have to be removed from the final product. Thus, the separation process requires removal of impurities including cells, proteins, organic acids, and other impurities. Moreover, antibiotics and isopropyl-β-D-thiogalactoside (IPTG) used for maintaining plasmid and inducing gene over expression increased the cost of succinate production.

2.8 Overview of *E. coli* KJ122 and its derivatives

Previously, 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 2.4).

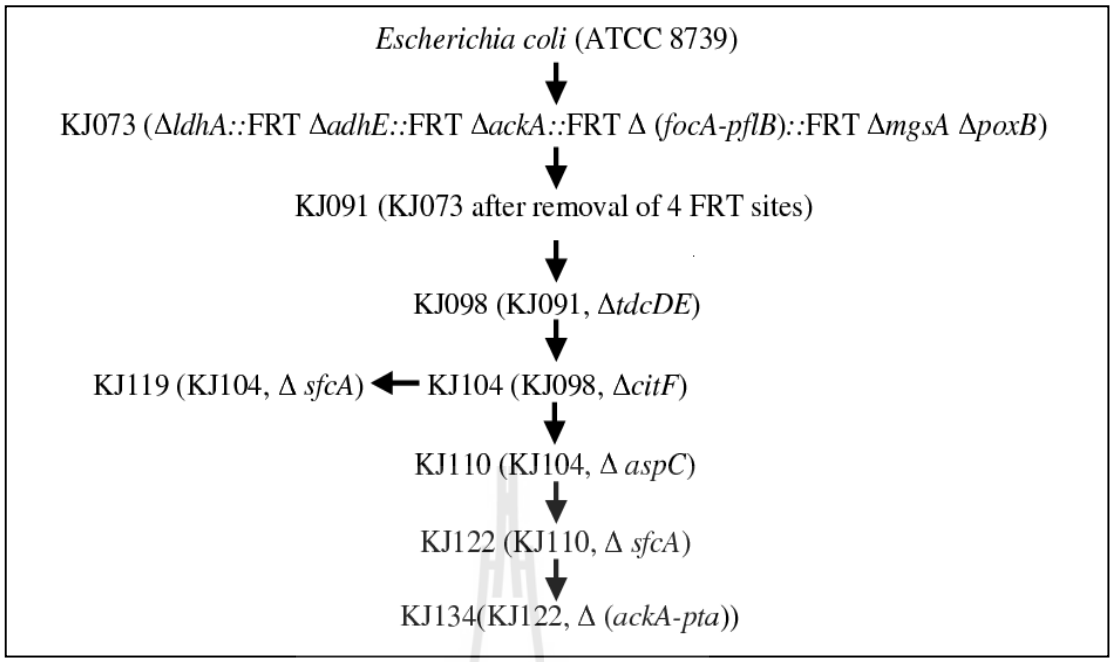

Figure 2.4 Strain construction of KJ122 (Jantama et al., 2008a).

Chan, (2010) has summaried the main events happening during metabolic engineering and evolution for constructing KJ122 as listed 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).

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, 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 Stols and Donnely, (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. However, 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).

In summary, the strain KJ122 (Δ*ldhA*, Δ*adhE*, Δ*ackA*, Δ(*focA-pflB*), Δ*mgsA*, Δ*poxB*, Δ*tdcDE*, Δ*citF*, Δ*aspC* and 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 h with less other organic acids under anaerobic fermentation from glucose. The KJ122 strain is a potential biocatalyst for the economical production of succinate. However, the succinate production by KJ122 from lignocellulosic materials has not yet been investigated. Therefore, strain KJ122 was used throughout this research to study the production of succinate from lignocellulosic materials, cassava pulp and sugarcane bagasse that are readily available, abundant and low-cost carbon sources in our country.

Table 2.1 Comparison of succinate production from various carbon sources by *E. coli.*

Organism	Medium/condition	Titer (g/L) Productivity [g/L/h][a]	References
E. coli AFP111 (Δ*pflAB,* Δ*ldhA,* Δ*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 time.	99.31 [1.31]	Vemuri et al., 2002
E. coli HL27659K/pKK313 (Δ*iclR,* Δ*sdhAB,* ΔackA-*pta* Δ*poxB,* Δ*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 absolutely aerobic condition, 59 h incubation time.	58.92 [1.00]	Lin et al., 2005b
E. coli from the rumen of buffalo	Sugarcane molasses (10%, v/v) supplemented to 10% CSL, 20 mM $NaCO_3$, CO_2 sparging, 72 h incubation time.	17 [0.56]	Agarwal et al., 2006
E. coli SBS550MG (Δ*ldhA,* Δ*adhE,* Δ*iclR,* Δ*ackA-pta*), *L. latis pyc, B. subtilis cit*Z	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 , 95 h incubation time.	40.03 [0.42]	Sanchez et al., 2005
E. coli AFP184 (Δ*pflB,* Δ*ldhA,* and Δ*ptsG*)	Carbon source: glucose, fructose, xylose, glucose/fructose and glucose/xylose 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 KJ122 (Δ*ldhA,* Δ*adhE* ,Δ*ackA,* Δ*(focA-pflB),* Δ*mgsA* , Δ*poxB,* Δ*tdcDE,* Δ*citF,* Δ*aspC,* Δ*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 time.	82.66 [0.9]	Jantama et al., 2008a
E. coli ATCC8739 (Δ*pflB,* Δ*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 1.2M KOH, simple batch, 144 h incubation time.	12.04 [0.083]	Zhang et al., 2010

Table 2.1 (continued).

E. coli SD121 (Δ*ldhA*, Δ*pflB*, Δ*ptsG*) *ppc* overexpression	Corn stalk hydrolysate, two-stage fermentation, 20 g/L tryptone, 10 g/L YE, pH controlled by 10 M NaOH and 10% H_2SO_4 (v/v) in aerobic phase, and 4 M Na_2CO_3 in anaerobic phase, 70 h incubation time.	57.81, [0.508]	Wang et al., 2011a
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, 96 h incubation time.	32.65 [0.34]	Wang et al., 2011b
E. coli K12, Δ*pflB*, Δ*ldhA*, Δ*ppc*, pTrc-*Bspck*	Corn stalk hydrolysate, LB medium, dual-phase fermentations, 16 h incubation time.	11.13 [0.69]	Liu et al., 2012a
E. coli Z6373 (endoxylanase XynC-A, xylosidase XyloA), Δ*lpp*, and *dsbA* overexpression	Beach wood xylan hydrolysate, AM1 medium, 0.1 M $NaHCO_3$ and 0.1 M $MgCO_3$, batch fermentation, 120 h incubation time.	14.44 [NR]	Zheng et al., 2012
E. coli BA204 (Δ*pflB*, Δ*ldhA*, Δ*ppc*) overexpressing PEPCK form *B. subtilis* 168	Sugarcane bagasse hydrolysate, 10 g/L tryptone, 5 g/L YE, 20 g/L $MgCO_3$, 0.3 mM IPTG. Dual phase aerobic growth and anaerobic production, sparging with air flow by CO_2, 24 h incubation time.	18.88 [0.78]	Liu et al., 2012b
E. coli HL27659K, pKK313, (pRU600) (Δ*iclR*, Δ*sdhAB*, ΔackA-*pta* Δ*poxB*, Δ*pstG*) *Rhizobium etli pyc* overexpressed	Soybean meal hydrolysate, LB medium, 10 g/L $MgCO_3$, appropriate antibiotics, Dual phase fermentation, 48 h incubation time.	36.84 [0.76]	Thakker et al., 2013
E. coli SD121 (Δ*ldhA*, Δ*pflB*, Δ*ptsG*) *ppc* overexpression	Xylose mother liquor, 42.27 g/L $MgCO_3$, 17.84 g/L YE, batch fermentation, 84 h incubation time.	52.09 [0.62]	Wang et al., 2014

Abbreviation: CSL, corn steep liquor; YE, yeast extract; YCH, yeast cell hydrolysate, NR, not reported. IPTG, isopropyl-b-Dthiogalactopyranoside. [a]Average volumetric productivity is shown in brackets [g/L/h].

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

Microbial strains	Medium and condition	Titer (g/L) Productivity [g/L/h][a]	References
An. succiniciproducens ATCC29305	6.5 g/L glycerol, 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
An. succiniciproducens ATCC 29305	Oak wood hydrolysate, 10 g/L CSL, 2.5 g/L polypeptone, 2.5 g/L YE, pH controlled by 2 M Na_2CO_3, CO_2 sparging at 0.25 vvm, batch fermentations, 28 h incubation time.	23.8 [0.85]	Lee et al., 2003
M. succiniciproducens MBEL55E	Oak wood hydrolysate (16.09 g/L glucose and 7.1 g/L xylose), 5 g/L YE, pH controlled by 5 N NaOH and 7 g/L Na_2CO_3, CO_2 sparging at 0.25 vvm, batch fermentation. 10 h incubation time.	11.73 [1.17]	Kim et al., 2004
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 fermentation, 48 h incubation time.	27.9 [0.58]	Wan et al., 2008
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 incubation time.	46.4 [0.96]	Liu et al., 2008
A. succinogenes 130Z	50 g/L wheat hydrolysate, $MgCO_3$ (varied), batch fermentation, 54 h incubation time.	64.2 [1.19]	Du et al., 2008
A. succinogenes CGMCC1593	Corn straw hydrolysates, 15 g/L YE, $MgCO_3$ (varied), some minerals, CO_2 sparging at 0.1 vvm, batch fermentation, 48 h incubation time.	45.5 [0.94]	Zheng et al., 2009
	Corn core, 15 g/L YE, $MgCO_3$ (varied), batch fermentation, 48 h incubation time.	32.07 [0.66]	
	Rice straw, 15 g/L YE, $MgCO_3$ (varied), batch fermentation, 48 h incubation time.	17.64 [0.37]	
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 incubation time.	62.1 [1.24]	Dorado et al., 2009

Table 2.2 (Continued).

A. succinogenes BE-1	Crop stalk wastes, 30 g/L YE, 2 g urea, 30 g $MgCO_3$, some minerals, pH controlled by 10 M NaOH, batch fermentation, 72 h incubation time.	15.8 [0.22]	Li et al., 2010
A. succinogenes	Corncob hydrolysate, 20 g/L YE, 40 g/L $MgCO_3$, batch fermentation, 48 h incubation time.	23.64 [0.49]	Yu et al., 2010
A. succinogenes CGMCC1593	Corn stover hydrolysate, 20 g/L CSL, 40 g/L $MgCO_3$, batch fermentation, 48 h incubation time.	47.4 [0.99]	Zheng et al., 2010
A. succinogenes CIP 106512	An acid hydrolysate of sugarcane bagasse, 10 g/L $NaHCO_3$, 3 g/L $MgSO_4$, 2 g/L YE, CO_2 sparging at 0.05 vvm, batch fermentation, 24 h incubation time.	22.5 [1.01]	Borges and Pereira, 2011
A. succinogenes ATCC 55618	Rapeseed meal hydrolysate, 10 g/L YE, 5 g/L CSL, pH controlled by 2 M Na_2CO_3, CO_2 sparging at 0.1 vvm, fed-batch fermentation, 24 h incubation time.	23.4 [0.33]	Chen et al., 2011a
A. succinogenes NJ113	70 g/L corn fiber hydrolysate, YCH (concentration equivalent to 15 g/L of YE), 150 µg/L biotin, pH controlled by 2.5 M Na_2CO_3, CO_2 sparging at 0.5 vvm, 75 h incubation time.	47.27 [0.63]	Chen et al., 2011b
A. succinogenes NJ113	Corn stover hydrolysate, biotin-supplementation, mixture of $Mg(OH)_2$ and NaOH as a neutralizer base at ratio 1:1, batch fermentation, 52 h incubation time	56.4 [1.08]	Li et al., 2011
A. succinogenes 130Z	Sake lees hydrolysate, 2.5 g/L YE and 0.2 mg/L biotin, CO_2 sparging at 0.5 vvm, pH controlled by 2 M Na_2CO_3, batch fermentation, 30 h incubation time.	52.3 [1.21]	Chen et al., 2012
A. succinogenes NJ113	50 g/L glucose, 10 g/L YE, 5.0 g/L CSL, 1 mg/L heme, CO_2 sparging at 0.5 vvm, pH controlled by 2 M Na_2CO_3, batch fermentation, 50 h incubation time.	37.9 [0.75]	Xi et al., 2013

Abbreviation: CSL, corn steep liquor; YE, yeast extract; YCH, yeast cell hydrolysate, NR, not reported.
[a]Average volumetric productivity is shown in brackets [g/L/h].

2.9 Lignocelluloses component

Lignocelluloses is a structural component in different plant cells, both woody plants and nonwoody (grass) (Howard et al., 2003). Based on its origin, the material can be divided into four major groups; forest residues, municipal solid waste, waste paper and crop residues. A common categorisation is also to separate them as softwood, hardwood and agricultural residues. All types of lignocellulosic material consist primarily of three components; cellulose, hemicellulose and lignin (Figure 2.5). These three segments constitute to approximately 90% of the total dry mass and together they build a complex matrix in the plant cell wall. The resisting part of the lignocellulose is ash and extractives (Chandel and Singh, 2011). The amount of cellulose, hemicellulose and lignin varies between species as shown in Table 2.3.

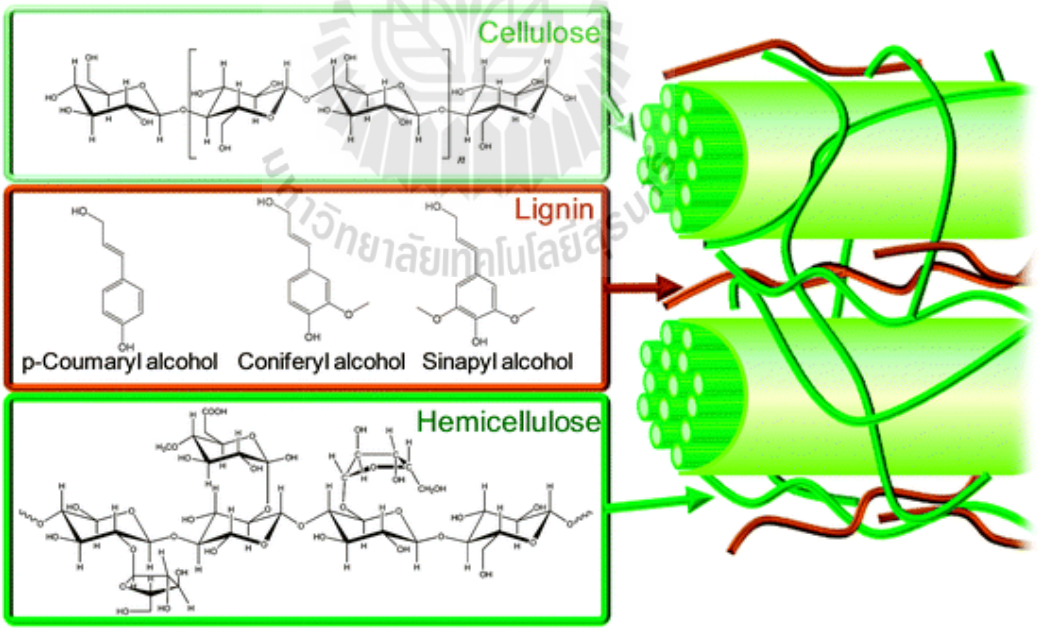

Figure 2.5 Structure of cellulose, hemicellulose and lignin in lignocellulosic materials (Source: Alonso et al., 2012).

Table 2.3 Cellulose, hemicellulose, and lignin contents in various lignocellulosic materials.

Lignocellulosic materials	**Cellulose (%)**	**Hemicellulose (%)**	**Lignin (%)**
Hardwood stems	40-55	24-40	18-25
Softwood stems	45-50	25-35	25-35
Nut shells	25-30	25-30	30-40
Corn cobs	45.0	35.0	15
Corn stalks	35.0	16.8	7.0
Grasses	25-40	35-50	10-30
Paper	85-99	0.0	0-15
Wheat straw	30.0	50.0	15.0
Sorted refuse	60.0	20.0	20.0
Newspaper	40-55	25-40	18-30
Waste papers from chemical pulps	60-70	10-20	5-10
Oat straw	39.4	27.1	17.5
Switch grass	45.0	31.4	12.0
Sunflower stalks	42.1	29.7	13.4
Sugarcane bagasse	40-50	25-35	10-15
Cassava pulp	8.1-15.6	2.8-4.6	2.2-2.8
Barley straw	33.8	21.9	13.8
Rice straw	36.2	19.0	9.9
Rye straw	37.6	30.5	19.0
Rice hull	36.0	15.0	19.0
Soya stalks	34.5	24.8	19.8

Adapted from Jørgensen et al. (2007), (Singh nee Nigam et al., 2009), and Chandel and Singh, (2011).

2.9.1 Cellulose

Cellulose, approximately 40-45%, is a major component of wood. It consists of linear polymers of glucose units and has the chemical formula $(C_6H_{10}O_5)_X$ (Balat, 2011). The D-glucose subunits are linked together by *β*-1,4-glycosidic bonds, forming cellobiose components, which then form the polymer. The chain, or elementary fibril, is linked together by hydrogen bonds and van der Waals forces (Perez et al., 2002). These forces, together with the orientation of the linkage, lead to a rigid and solid polymer with high tensile strength (Balat, 2011). Elemental fibrils are packed together and nearby chains are linked by hydrogen bonds, forming microfibrils. Microfibrils are covered by hemicellulose and lignin, which function as a complex matrix around the cellulose polymer. Through this, cellulose is closely associated with hemicellulose and lignin and therefore requires intensive treatments before isolation (Chandel and Singh, 2011). The cellulosic polymers can either be in crystalline or amorphous form, however, the crystalline form is more common. The highly crystalline structure is generally non-susceptible to enzymatic activities, while the amorphous regions are more susceptible to degradation (Perez et al., 2002).

2.9.2 Hemicellulose

Usually, lignocellulosic materials contain 20-30% hemicelluloses, which is a short (100-200 units) and highly branched polymer consisting of different carbohydrates, both hexoses and pentoses. The polysaccharide consists mainly of: D-xylose, D-mannose, D-galactose, D-glucose, L-arabinose, 4-O-methylglucuronic, D-galacturonic and D-glucuronic acids. The different sugars are linked together mainly by *β*-1,4-glycosidic bonds, but also with *β*-1,3 linkages (Perez et al., 2002). Mannose

and galactose are the other six-carbon sugars apart from glucose. The major sugar varies from species to species and represents of xylose in hardwood and agriculture residues and mannose in softwood (Balat, 2011). The branched structure of hemicellulose, and thereby an amorphous nature, makes it more susceptible for enzymatic degradation than cellulose (Perez et al., 2002).

2.9.3 Lignin

Lignin supports structure in the plant cell wall and has also a functional role in the plant resistance to external stress (Perez et al., 2002). The lignin molecule is a complex of phenylpropane units linked together forming an amorphous, non-water soluble structure. It is primarily synthesised from precursors consisting of phenylpropanoid and the three phenols existing in lignin are: guaiacyl propanol, *p*-hydroxyphenyl propanol and syringyl propanol. Linked together, these form a very complex matrix with high polarity (Balat, 2011). The lignin amount varies between different materials, but in general hardwood and agriculture residues contain less lignin than softwood. In comparison, cellulose and hemicellulose, lignin is the compound with least susceptibility for degradation. The higher amount of lignin in the material is, the higher the resistance to degradation. This resistance found in lignin is one major drawback when using lignocellulosic material for fermentation (Taherzadeh and Karimi, 2008).

2.9.4 Extractive

Extractive is a minor fraction of wood. Nevertheless, it is a large variety of components. The extractive comprises an extraordinarily large number of

individual compounds of both lipophilic and hydrophilic types such as terpenoids and steroids, fats and waxes, and phenolic constituents. Various parts of the plants differ in their amount and types of extractive. For example, fats and waxes are in the ray parenchyma cells while phenolic extractives are present mainly in the bark. The most of extractives are soluble in organic solvents, thus the quantitative determination is carried out by extraction with organic solvents. Extractive content is usually less than 10% in wood (Taherzadeh and Karimi, 2008).

2.9.5 Ash

Ash is an inorganic component present in wood or plant at rather low amounts. This ash is originated mainly from a variety of metal salts, carbonates, silicates, oxalates and phosphates, deposited in cell walls and lumina (Balat, 2011).

2.10 Pre-treatment of lignocellulosic materials

Lignocellulosic feedstock is protected by lignin, pre-treatment is needed to open up the structure and expose the cellulose. Today, lots of different possible pre-treatment methods exist. Overall generalized classifications of types of pre-treatments are physical, biological and chemical. Combinations of those such as physical treatment (high pressure/temperature) followed by a chemical treatment are often more effective. The goal of pre-treatment is to prepare the feedstock for enzymatic hydrolysis resulting in an increase in sugar conversion. The composition of different kind of biomass varies. The digestibility of a given feedstock depends on properties like lignin content, the accessibility of cellulose and its crystallinity. Other important factors that determine the digestibility are the degree of polymerization of cellulose,

porosity (available surface area), hemicelluloses covering cellulose and fibre strength. The ideal pre-treatment results in a disrupted biomass structure, ready for hydrolysis but with no formation of sugar degradation products or compounds that inhibit the fermentation (Agbor et al., 2011). If enzymatic hydrolysis is performed without any pretreatments, only about 20% of the available sugars are hydrolysed, but with pre-treatment 90% of the available sugar can be obtained and made available for fermentation (Narayanaswamy et al., 2011). However, the best method and conditions of pre-treatment depend greatly on the type of lignocelluloses (Mohammud et al., 2008).

Many methods have been introduced for pre-treatment of lignocellulosic materials prior to enzymatic hydrolysis or digestion. The methods of pre-treatment of lignocellulosic materials and the comparison of advantages and disadvantages of different pre-treatment options for lignocellulosic materials are summarized in Table 2.4. Among the different existing pre-treatment methods, steam explosion is one of the most commonly used for fractionation of biomass components. In steam explosion pre-treatment, biomass is exposed to pressurized steam followed by rapid reduction in pressure. The treatment results in substantial breakdown of the lignocellulosic structure, hydrolysis of the hemicellulosic fraction, depolymerization of the lignin components and defibration. Therefore, the accessibility of the cellulose components to degradation by enzymes is greatly increased (Moniruzzaman et al., 1996). Compared with alternative pre-treatment methods, the superior advantages of steam explosion include a significantly lower environmental impact, lower capital investment and less hazardous process chemicals (Garrote et al., 1999). On the other hand, the presence of degradation products from sugar and lignin is unavoidable and

must be taken into account in order to minimize the potential negative effect on subsequent steps (Tengborg et al., 2001). Nevertheless, steam explosion could still be considered as a feasible and cost effective pre-treatment process for the various chemical productions like succinate.

2.10.1 Steam explosion

Steam provides an effective means to rapidly heat up materials to the target temperature without excessively diluting the resulting sugars (Mosier et al., 2005). Steam pre-treatment is one of the most widely used methods for pretreating lignocellulose. This method was formerly known as 'steam explosion' because it was believed that an explosive action on the fibers was necessary to render the material amenable to hydrolysis. However, it is more likely that the hemicellulose is hydrolyzed by the acetic acid and other acids released during the steam pre-treatment (Mosier et al., 2005; Galbe et al., 2007). The biomass is subjected to high pressure saturated steam (0.69-4.83 MPa) at a temperature of 160-260°C which is typically maintained for a few seconds to a few minutes, after which the pressure is released (Sun and Cheng, 2002). The process causes solubilisation of the hemicelluloses and lignin transformation, thus improving the accessibility of the cellulose fibrils to the enzymes during hydrolysis (Mosier et al., 2005; Sun and Cheng, 2002). During steam pre-treatment, parts of the hemicellulose hydrolyze and form acids, which could serve as a catalyst for further hydrolysis of the hemicellulose. This situation in which the acids formed *in situ* catalyze the process itself, is known as 'auto-cleave' steam pre-treatment (Hendriks and Zeeman, 2009). Sometimes an acid catalyst such as H_2SO_4 or SO_2 can also be directly added to produce an effect similar to dilute acid hydrolysis,

thereby increasing the hemicellulose sugar recovery and digestibility of the solid residue (Galbe et al, 2007).

The use of catalysts are particularly important for softwoods (typically less acetylated), resulting in lower treatment temperatures and shorter reaction times, thereby improving hemicellulose recovery and reducing the formation of sugar degradation products (Galbe et al, 2007). Steam pre-treatment has a low energy requirement when compared with mechanical methods such as biomass comminution. The conventional mechanical methods require 70% more energy than steam pre-treatment to achieve the same degree of size reduction. Furthermore, steam pre-treatment neither incurs recycling costs nor does it have a negative impact on the environment (Sun and Cheng, 2002). However, limitations of steam pre-treatment include destruction of a portion of the xylan fraction which decreases sugar recovery, incomplete disruption of the lignin carbohydrate matrix and formation of inhibitory compounds. After pre-treatment, the biomass needs to be washed to remove the inhibitory materials along with water soluble hemicellulose. About 20-25% of the initial dry matter is removed by the washed water, resulting in a decrease in the overall sugar yield after saccharification (Sasaki et al., 2012).

Shorter residence times and lower temperatures have been shown to be more favorable because the sugars, especially those from the hemicellulose, do not degrade into products that inhibit the subsequent fermentation (Monavari et al., 2010). However, a high degree of severity is required to enhance the enzymatic digestibility of the cellulose fibers, especially in softwood. The maximum yields of sugars from hemicellulose and cellulose are not reached at the same degree of severity in the pre-treatment and hence an optimum severity can be found for

different systems since the proportions of hemicellulose and cellulose change depending on the type of biomass (Galbe et al, 2007).

2.11 Inhibitory compounds produced during lignocelluloses conversion

The pre-treatment processes release the sugars (C6/C5) and lignin, however, these processes also cause the breakdown of lignin and dehydration of the sugars, producing the inhibitory compounds that greatly reduces the overall efficiencies of the microorganism growth in the lignocelluloses hydrolysate. There are many inhibitory compounds produced during dilute acid hydrolysis of lignocelluloses, some have been proven to be present at higher levels and/or are particularly inhibitory to the fermenting organism (Ibraheem and Ndimba, 2013). The inhibitors can be classified into 3 major classes: organic acids, furan derivatives, and phenolic compounds (Ibraheem and Ndimba, 2013). As shown in Figure 2.6, furans are produced by the dehydration of pentose and hexose sugars resulting in furfural and hydroxymethyl furfural production, respectively (Palmqvist and Hahn-Hagerdal, 2000; Ibraheem and Ndimba, 2013; Keating et al., 2014). Organic acids such as acetate, formate and levulinic acid can be produced by the degradation of furans and by the cleavage of acid side chains present in the hemicellulose structure. Various phenolic compounds are produced from the breakdown of lignin polymer. The different classes of biomass (hardwood, softwood, and agricultural residue) may produce slightly different concentrations of these inhibitory compounds (Palmqvist and Hahn-Hagerdal, 2000; Ibraheem and Ndimba, 2013; Keating et al., 2014).

Table 2.4 Comparison of advantages and disadvantages of different pre-treatment options for lignocellulosic materials.

Pre-treatment process	Sugar yield	Inhibitor formation	By product generation	Reuse of chemical	Applicability to different feedstock's	Equipment cost	Success at pilot scale	Advantages	Disadvantages and limitations
Mechanical comminution	L	Nil	No	No	Yes	H	Yes	Reduces cellulose crystallinity	Power consumption usually higher than inherent biomass energy
Steam explosion	H	H	L	NR	Yes	H	Yes	Causes hemicellulose degradation and lignin transformation, cost-effective, low environmental impact	Destruction of a portion of the xylan fraction, incomplete disruption of the lignin-carbohydrate matrix, generation of compounds inhibitory to microorganisms
Ammonia fiber explosion (AFEX)	H	L	NR	Yes	NR	H	NR	Increases accessible surface area, removes lignin and hemicellulose to an extent, does not produce inhibitors for downstream processes	Not efficient for biomass with high lignin content
CO_2 explosion	H	L	L	No	NR	H	NR	Increases accessible surface area; cost-effective; does not cause formation of inhibitory compounds	Does not modify lignin or hemicelluloses
Ozonolysis	H	L	H	No	NR	H	No	Reduces lignin content; does not produce toxic residues	Large amount of ozone required, expensive
Acid hydrolysis	H	H	H	Yes	Yes	H	Yes	Hydrolyzes hemicellulose to xylose and other sugars; alters lignin structure	High cost, equipment corrosion, formation of toxic substances
Alkaline hydrolysis	H	L	H	Yes	Yes	Nil	Yes	Removes hemicelluloses and lignin, increases accessible surface area	Long residence times required, irrecoverable salts formed and incorporated into biomass
Organosolv	H	H	H	Yes	Yes	H	Yes	Hydrolyzes lignin and hemicelluloses	Solvents need to be drained from the reactor, evaporated, condensed, and recycled; high cost
Wet oxidation	H/L	Nil	L	No	NR	H	NR	Removal of lignin, dissolves hemicelluloses and causes cellulose decrystallization	NR
Liquid hot water	H	H	L	No	NR	NR	Yes	Removal of hemicelluloses making access to cellulose	Long residence time, irrecoverable salt formed
Ionic liquids	H/L	L	NR	Yes	Yes	NR	NR	Dissolution of cellulose, increased amenability to cellulase	Still in initial stages

Abbreviations; H; high, L; low and NR; not reported. Adapted from Kumar et al. (2009) and Menon and Rao, (2012).

2.12 Inhibitory mechanisms of hydrolysate compounds

The fine balance between the harsh conditions required during pretreatment due to the recalcitrance of lignocellulose and the protection of solubilized sugars from further exposure to those conditions is very difficult to maintain. Consequently, hydrolysates are rarely devoid of compounds that inhibit subsequent fermentation processes. Substantial research has been devoted to the study of key inhibitors present in hydrolysates, their method of toxicity and detoxification strategies. Hydrolysates vary in their degree of inhibition and biocatalysts respond differently to the inhibitors (Palmqvist and Hahn-Hagerdal, 2000; Martinez et al., 2001; Alriksson et al., 2011). Biocatalysts experience an aggregate inhibition effect imposed by the specific combinations of inhibitors present in the hydrolysates.

2.12.1 Organic acids

The major organic acids produced during the pretreatment and hydrolysis processes of lignocellolose are lactate, succinate, formate and acetate. Among them, acetate is the most abundant inhibitors in hydrolysates, present at concentrations ranging from 2-15 g/L (Takahashi et al., 1999). The organic acids can be derived from the dehydration of released sugars and/or decomposition of acetylxylan, a byproduct of hemicellulose degradation (Figure 2.5). Zaldivar and Ingram (1999) investigated the effects of organic acids (acetic, ferulic, gallic, 4-hydroxybenzoic, syringic, vanillic, furoic, formic, levulinic, caproic) on the growth of ethanologenic *E. coli* strain LY01. They found a high correlation between the hydrophobicity of the organic acids and their inhibitory effect (pH 7.0). The order of inhibition was: caproic > ferulic > feroic > 4-hydroxybenzoic > formic > acetic >

levulinic. In general, higher initial fermentation pH (*e.g.* pH 7.0 versus pH 6.0) and higher temperatures (*e.g.* 40°C versus 30°C) were observed to result in improved growth during organic acid stress. Palmqvist et al. (1996) and Roe et al. (1998) suggested that organic acids penetrate the cell membrane in an undissociated form and dissociate after entering the cytoplasm. This event collapses the cell's proton gradient resulting in a decrease of ATP, the cells primary energy source. Under acidic growth environments, a higher fraction of undissociated acids can readily penetrate more to the cell membrane. Increasing the initial cell density up to 40-fold did not overcome acid inhibition. Binary combinations of organic acids displayed an additive toxicity that more closely mimic hydrolysate conditions (Zaldivar et al., 1999). Interestingly, the combination of the two most abundant inhibitors in dilute acid hydrolysates – furfural and acetate – resulted in a synergistic inhibition of growth (80% reduction). Membrane leakage was not found to be a major inhibitory mechanism for organic acids.

2.12.2 Furan derivatives

2-furaldehyde (furfural) and 5-hydroxymethylfurfural (HMF) are dehydration products of pentose and hexose sugars, respectively, produced during acid pre-treatment and hydrolysis of lignocellulose (Ibraheem and Ndimba, 2013; Keating et al., 2014). Furfural has been identified as one of the key inhibitors affecting fermentation of dilute acid hydrolysates (Almeida et al., 2009). The presence of furfural and furan derivatives prolongs the lag phase during the initial 24 h of fermentation in both bacteria and yeast (Martinez et al., 2000; Almeida et al., 2009). In addition, the extent of the lag was a function of the initial furfural

concentration in *S. cerevisiae* hydrolysate fermentations (Palmqvist et al. 1996; Almeida et al., 2009). It was hypothesized that *S. cerevisiae* (Almeida et al., 2009) and *E. coli* (Zaldivar et al., 1999; Martinez et al., 2000) were metabolizing and detoxifying furfural to a less toxic compound via an unknown mechanism. Miller et al. (2009) isolated furfural-resistant strains of *E. coli* that were deficient in furfural reduction by the NADPH-dependent oxidoreductases YqhD and DkgA. This finding was counterintuitive as these enzymes can catalyze the reduction of furfural to the less toxic compound furfuryl alcohol. The authors performed 53 serial transfers in pH-controlled vessels under constant furfural stress. The resulting strain was able to produce the same level of ethanol as its parent strain (no furfural), in the presence of up to 1.0 g/L furfural. A 72 h lag was observed in the parent strain under similar conditions (1.0 g/L furfural).

Comparison of mRNA profiles revealed 12 oxidoreductases that were up or down regulated at least two fold following the addition of 0.5 g/L furfural (Miller et al., 2009). Of particular interest were four oxidoreductase genes that were downregulated in the presence of furfural. Three of these, *yqhD, dkgA,* and *yqfA,* decreased furfural tolerance when overexpressed from plasmids. Deletion of *yqhD* as well as expression of the NADH/NADPH transhydrogenase, PntAB, increased furfural tolerance. Similar global transcript analysis in *S. cerevisiae* identified a 5-hydroxymethylfurfural reductase, ADH6, involved in tolerance to HMF (Miller et al., 2009).

The effect of varying carbon (*e.g.* glucose and xylose) and nutrient source (*e.g.* yeast extract) have also been investigated (Palmqvist et al., 1999). The use of glucose and yeast extract improved the growth of *E. coli* in the presence of

furfural. The proposed mechanism of glucose and yeast extract benefit was the generation of more NADPH available for biosynthesis and reduction in the biocatalysts' biosynthetic needs, respectively.

Subsequent studies by Miller et al. (2009) using the parent strain LY180, revealed that sulfur assimilation was one of the targets of furfural inhibition. The NADPH pools required for amino acid and other biosynthetic reactions were being depleted during the reduction of furfural by oxidroreductases with a low K_m for NADPH resulting in reduced growth. It was showed that a total of 22 genes were upregulated ≥5-fold and approximately 400 genes were upregulated ≥2-fold when *E. coli* was exposed to 0.5 g/L furfural. The genes were divided up by functional groups. Amino acid and nucleotide biosynthesis genes were particularly altered (>20% of total genes in the functional group) in response to furfural. Most of the genes were downregulated. Growth of *E. coli* was improved by supplementation with 0.1 mM of 5 out of 20 amino acids tested. The order of benefit was: cysteine > methionine > serine and arginine > histidine. In addition, expression of genes involved in sulfur assimilation was generally increased. The authors hypothesized that *E. coli* cells were deficient in sulfur containing amino acids during furfural stress due to the inhibition of sulfate metabolism. Adding sulfur containing amino acids, amino acid precursors as well as sulfur compounds increased furfural tolerance.

Later on, Wang et al. (2011c) identified a beneficial oxidoreductase, FucO, capable of reducing furfural to the less toxic furfuryl alcohol. The unique feature of this enzyme was that it did not compete with biosynthetic NADPH pools like the previously characterized YqhD (Miller et al., 2009) but rather it used the anaerobically abundant NADH as the reductant. FucO was known as 1, 2-propanediol

reductase, however, *in vitro* studies and crystal structure analysis revealed that it can also reduce furfural using NADH as a cofactor. Purified FucO also exhibited 5-hydroxymethyl furfural reductase acitivity. Furfural tolerance was increased by 50% in the presence of 15 mM furfural when *fucO* was overexpressed from a plasmid in ethanologenic *E. coli* LY180 (Wang et al., 2011c). Global transcript analysis was used to search for additional unknown oxidoreductases that can use NADH as a cofactor (Wang et al., 2012). Four oxidoreductases, *aldA*, *ydhABC*, *yeiTA*, and *ucpA*, were upregulated at least 3-fold and had potential NADH binding domains. The expression of *ucpA* improved growth by 50% in the presence of 15 mM furfural. However, none of the oxidoreductases exhibited furan reductase acitivity *in vitro* with either NADH or NADPH (Wang et al., 2012). The exact function of UcpA and its mechanism for conferring furfural tolerance is still not clear.

The search for a genetic basis to furfural tolerance has also uncovered genes related to DNA biosynthesis. Zheng et al. (2012) isolated the thymidylate synthase gene, *thyA*, from genomic libraries of three bacterial species: *Bacillus subtilis*, *E. coli* and *Zymomonas mobilis*. The *thyA* containing plasmids were enriched when transformants were grown on medium containing furfural. The growth of *E. coli* in the presence of furfural is improved by overexpression of *thyA* and supplementation with thymine, thymidine or 5, 10-methylenetetrahydrofolate. Increased tolerance was proposed to be due to increased pyrimidine deoxyribonucelotides involved in DNA repairing (Zheng et al., 2012). The exact target(s) of furfural inhibition via pyrimidine deoxribonucleotide depletion remains unknown. However, Zheng et al. (2012) proposed several possible mechanisms:

competition for NADPH pool required for deoxyribonucleotide de novo synthesis, direct DNA damage and inhibition of enzymes involved in the folate cycle.

More recently, Geddes et al. (2014) reported that plasmid-based expression of polyamine transporters increased the metabolism of furfural and decreased the time required for xylose fermentation by ethanologenic strain. They suggested that polyamine transporters such as PotABCD, PlaP, PotE, and PuuP and polyamine supplements in the medium protect cellular processes from furfural damage and allow cells to complete the reduction of furfural to the less toxic furfuryl alcohol (Geddes et al., 2014).

Ibraheem and Ndimba, (2013) reported that furfural can inhibit the glycolytic and fermentative enzymes essential to central metabolic pathways (such as pyruvate, acetaldehyde and alcohol dehydrogenases), protein-protein cross linking and DNA degradation into single strands. Their high hydrophobicity allows furfural and HMF to compromise membrane integrity leading to extensive membrane disruption/leakage, which will eventually cause reduction in cell replication rate, ATP production, and consequently lower ethanol production (Zaldivar et al., 2000). *In-vitro* incubation of furfural with double stranded lambda phage DNA led to single-strand breaks, primarily at sequence sites with three or more adenine or thymine bases (Ibraheem and Ndimba, 2013). Furan derivatives are furthermore known to act synergistically with other inhibitors including phenolic and aromatic compounds as well as acetic, formic and levulinic acids (Ibraheem and Ndimba, 2013).

2.12.3 Phenolic compounds

Phenolic compounds released by partial solubilization of lignin and dehydration of sugars during dilute acid pretreatment are insoluble or partially soluble in the hydrolysate and include acids (ferulic acid, vanillic acids, 4-hydroxybenzoic acid and syringic acid), alcohols (guaiacol, catechol and vanillyl alcohol) and aldehydes (vanillin, syringic aldehyde and 4-hydroxylbenzaldehyde) (Ibraheem and Ndimba, 2013). These compounds have varying degrees of toxicity to the fermenting biocatalyst. Toxicity is related to the substituents on the phenol ring structure. Phenolic aldehydes have been found to be particularly toxic to biocatalysts (Palmqvist et al., 1996; Zaldivar et al., 1999). The types of phenolic compounds present in the pretreatment liquors are dependent on the biomass used due to differences in lignin structures of softwood, hardwood and grasses (Ibraheem and Ndimba, 2013). Phenolic compounds are known to partition into biological membranes altering the permeability and lipid/protein ratio, which thus increases cell fluidity, leading to cell membrane disruption, dissipation of proton/ion gradients and compromising the ability of cellular membranes to act as selective barriers (Heipieper et al., 1994). This membrane disruption, allows the release of proteins, RNAs, ATP, Ions, out of the cytoplasm, consequently causing reduced ATP levels, diminished proton motive force and impaired protein function and nutrient transport (Heipieper et al., 1994). Furthermore, they enhance the generation of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), super oxides (O_2^-) and super hydroxyl (OH^-) that interact with proteins/enzymes, which results in their denaturation, they damage cytoskeleton and other hydrophobic intracellular targets, cause DNA mutagenesis, and induce programmed cell death (Ibraheem and Ndimba, 2013).

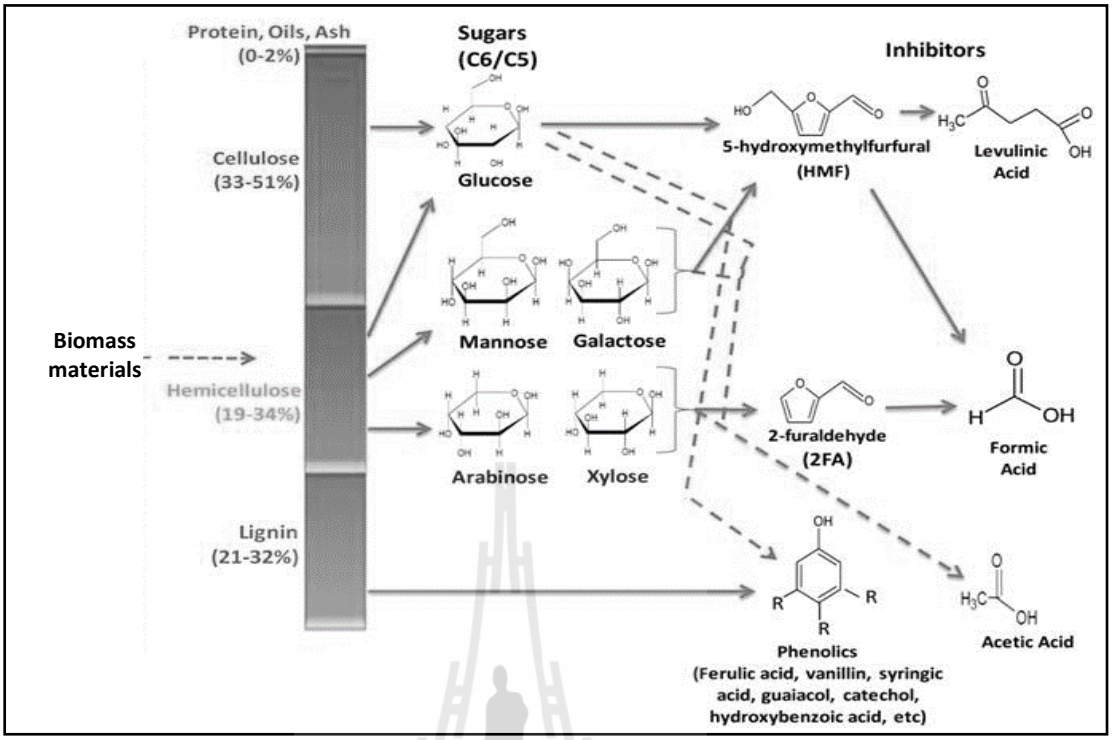

Figure 2.6 Production of inhibitors by dehydration of 6-carbon sugars and 5-carbon sugars to hydroxymethylfurfural and furfural, respectively, during dilute acid pretreatment of lignocellulosic biomass. Adapted from Ibraheem and Ndimba, 2013.

2.13 Detoxification processes

2.13.1 Biological methods

Treatment of willow hemicellulose hydrolysate with peroxidase and laccase enzymes from *Trametes versicolor* was found to increase maximum ethanol production of *S. cerevisae* (Jonsson et al., 1998). They also noticed that laccase selectively removed phenolic monomers and acids to almost completion. Laccase detoxified the hydrolysate by the oxidative polymerization of low molecular weight phenolic compounds. When *Trichoderma reesei* is added to the hemicellulose

hydrolysates it can remove acetic acid, furfural, and benzoic acid (Palmqvist et al., 1997). Instead of having a separate organism to detoxify the hydrolysate, such as *T. versicolor*, the peroxidase and laccase genes could be cloned into the fermenting organism. This would eliminate the need for separating the detoxification and fermentation steps and the maintenance of a separate organism.

2.13.2 Chemical methods

Extraction of a spruce hydrolysate with ether at a pH of 2 increased ethanol yield to that of a reference fermentation (Palmqvist and Hahn-Hagerdal, 2000). The inhibitory compounds extracted by the ether were acetic acid, formic acid, levulinic acid, furfural, hydroxymethylfurfural and phenolic compounds. Extraction by ethyl acetate had similar effects on hydrolysate fermentation and removed acetic acid, vanillin, 4-hydroxybenzoic acid and completely removed furfural (Palmqvist and Hahn-Hagerdal, 2000).

Alkaline pH treatement of hemicellulose hydrolysate with calcium hydroxide (over-liming), sodium hydroxide, potassium hydroxide or ammonium hydroxide has been shown to reduce toxicity of hydrolysates using yeasts and ethanologenic *E. coli* (Martinez et al., 2000, 2001; Mohagheghi et al., 2006). In most cases, however, high pH treatment also resulted in significant sugar destruction (Martinez et al., 2000, 200; Mohagheghi et al., 2006). Increasing the pH to pH 9.0 with ammonium hydroxide was demonstrated to decrease hydrolysate toxicity with minimal sugar loss (Alriksson et al., 2005; Geddes et al., 2013). The detoxification is believed to be due to precipitation of toxic compounds. Overliming decreases the

concentration of Hibbert's ketones, furfural and hydroxymethylfurfural in spruce hemicellulose hydrolysates (Palmqvist and Hahn-Hagerdal, 2000).

Some researchers found that the detoxification of willow hemicellulose hydrolysate was the most effective when they used a combination of overliming and with heated sulfite treatment (Palmqvist and Hahn-Hagerdal, 2000). Also, treatment with ion-exchange resins and charcoal can effectively remove inhibitors (Carvalho et al., 2006). Detoxification of sugarcane bagasse hydrolysate with anion resins effectively removed 84% of the acetic acid (Chandei, 2007). Kim et al. (2013) determined that the removal of phenolic compounds through activated charcoal or ethyl acetate enhanced fermentation and relieved enzyme inhibition.

2.13.3 Physical method

Volatile inhibitory compounds can be removed from hydrolysate by evaporation under vacuum (Palmqvist and Hahn-Hagerdal, 2000; Chandel et al., 2013; Frazer et al., 1989). The resulting hydrolysate is more concentrated in sugar and non-volatile inhibitors but has reduced levels of furfural, hydroxymethyl furfural, acetic acid and vanillin (Carvalho et al., 2006; Chandel et al., 2013). The addition of small amounts of air to the culture or headspace has been shown to promote the fermentation of hemicellulose hydrolysates. Low levels of aeration results in increased cell mass and ethanol productivity but reduce yields (Alfenore et al., 2004; Nieves et al., 2011). In addition, a combination of treatments (vacuum evaporation, laccase, high pH, bisulfite, microaeration) completely eliminated all inhibitory activity present in hydrolysate (Geddes et al., 2015). With this combination, fermentation of hemicellulose sugars (90% hydrolysate) to ethanol was completed

within 48 h, identical to the fermentation of laboratory xylose (50 g/L) in AM1 mineral salts medium (without hydrolysate) (Geddes et al., 2015).

2.13.4 Adaptive evolution

An additional strategy in combating the inhibitory effects of hemicellulose hydrolysate compounds is to adapt cells to the inhibitors. This can be done by maintaining continuous cultures to metabolically evolve the cells in the presence of hydrolysate and select for cells that are more robust having a high cell and product yield. *E. coli* can naturally remove the inhibitory effects of furfural by reducing it to furfuryl alchohol in anaerobic conditions or to furic acid in aerobic conditions. An evolved *E. coli* mutated to silence NADPH-dependent oxidoreductase genes, *yqhD* and *dkgA*, which are responsible for detoxifying furfural (Miller et al., 2009). Yeasts resistant to benzoic acid showed lower uptake rates of benzoic acid. The change in uptake rate could be due to changes in membrane permeability (Piper et al., 2001). The identification of potential genes involved in the conferred resistance to inhibitors in hydrolysate could lead to a genetic approach to engineer a more robust organism for hydrolysate fermentations with minimal or no prior detoxification.

2.14 Overview of fermentation processes

2.14.1 Simultaneous saccharification and fermentation (SSF)

Simultaneous saccharification and fermentation (SSF) is a process in which hydrolysis of cellulose and fermentation takes place in a single reactor. Unlike separate hydrolysis and fermentation (SHF), the risk of product inhibition is minimized as the sugars obtained in hydrolysis are simultaneously utilized by the

microorganism to produce value-added products. SSF reduces both capital cost and risk of contamination since the glucose released is quickly utilized by the microorganisms (Tomas-Pejo et al., 2009). SSF has been widely studied with various microorganisms to produce value-added products, like ethanol (Wingren et al., 2003; Ohgren et al., 2007), lactate (Romani et al., 2008), and hydrogen (Li and Chen, 2007).

So far only a few succinate productions using SSF process have been studied. For instance, Zheng et al. (2010) demonstrated that the pre-treated corn stover with diluted alkaline could be potentially used for succinate production by *A. succinogenes* CGMCC1593 using SSF process. The maximum succinate concentration and yield were 47.4 g/L and 0.72 g/g substrate, respectively when batch SSF process optimization condition was as follows: initial substrate concentration of 70 g/L, enzyme loading of 20 FPU cellulase supplemented with 10 U cellubiase per gram substrate, and fermentation temperature 38°C was employed.

Chen et al. (2011a) applied fed-batch SSF process in succinate production by *A. succinogenes* ATCC 55618 using acid-pretreated rapeseed meal as main substrate. A succinate concentration of 23.4 g/L with a yield of 11.5 g/100 g dry matter and productivity of 0.33 g/L/h was obtained from SSF of diluted acid-pretreated rapeseed meal at a culture pH of 6.4 and a pectinase loading of 2%, without supplementation of yeast extract.

2.14.2 Advantages and limitations of SSF

Advantages of SSF include the decrease of enzymatic inhibition due to glucose released in the saccharification of biomass and reduction in process steps and reactors (Deshpande et al., 1983). Also, the immediate conversion of sugars produced

decreases the instances in which contamination could become an issue within the conversion reaction. This is due to the lack of sufficient time for a contaminating organism to grow to a significant concentration in order to compete with the desired fermenting organism. This reduction in contamination decreases the number of undesirable products, potentially increasing the overall yield with less necessity for sterility. The disadvantages of such reactions include the inability of fermenting organisms to grow and convert glucose to fermentation products efficiently at optimal temperatures for enzymatic hydrolysis. The fermentation products can also inhibit the hydrolysis of biomass as it can cause uncompetitive inhibition to cellulases (Ghosh et al., 1982; Wu and Lee, 1997). Even with these problems, recent models comparing SSF with SHF have shown SSF reactions to be the superior conversion technology, even at lower temperatures (37°C) (Drissen et al., 2009). With this regard, the SSF process could be applied for the production of succinate from previously metabolically engineered *E. coli* KJ122 using lignocelluloses hydrolysate as carbon substrate.

CHAPTER III

GENERAL PROCEDURES

3.1 Growth medium and culture conditions

Seed cultures and fermentations were grown at 37°C, 150 rpm in AM1 mineral salt medium (4.2 g/L total salts; Martinez et al., 2007) containing sugars, 100 mM $KHCO_3$ and 1 mM betaine HCl. The composition of AM1 mineral medium is shown in Table 3.1. Fermentation was inoculated with an initial OD_{550} of 0.1 (0.33 mg of CDW/mL/OD). Anaerobiosis was rapidly achieved during growth with added bicarbonate serving to ensure an atmosphere of CO_2. During the fermentation process, the pH of the medium was maintained by automatic addition a mixture of the metal carbonate solution (3M K_2CO_3) and 6N KOH.

3.2 Fermentations with 500-mL small anaerobic vessel

The fermentations were carried out in a container with a 300 mL working volume out of 500 mL total volume. Temperature was controlled by means of submersion of containers in a thermo-regulated water bath. A magnetic stirrer beneath the bath mixed the cultures continuously. Fermentation vessels were sealed except for 16-gauge needle, which served as a vent for sample removal. Samples were removed from the containers during fermentation aseptically by syringe connected to the vessels (Figure 3.1).

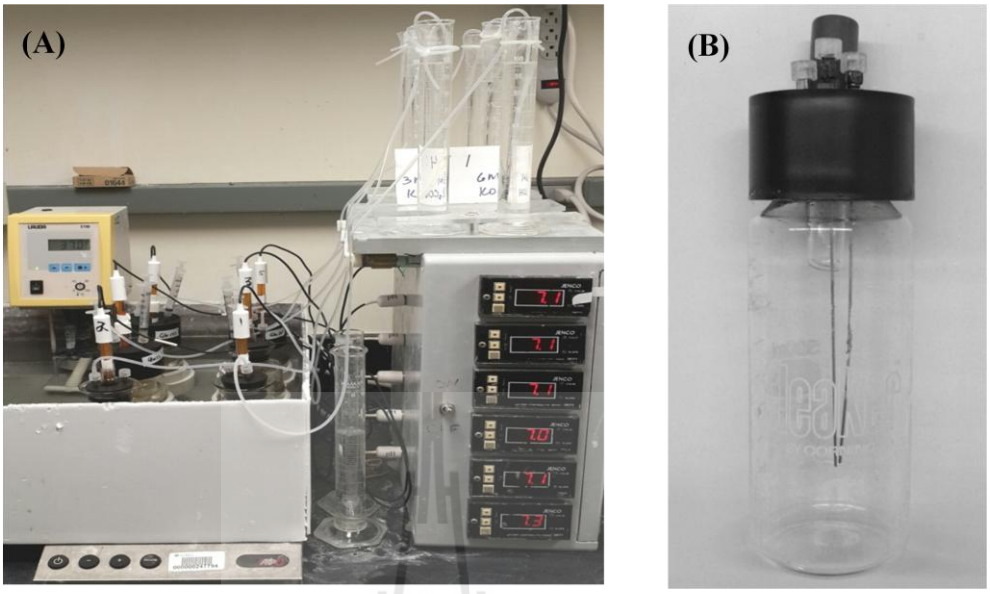

Figure 3.1 (A) The in-house-built small anaerobic vessels. (B) The 500-mL small anaerobic vessel (Fleaker).

3.3 Fermentation with 2-L bioreactor

The basic bioreactor was a Microferm Labolatory Fermenter (New Brunswick Scientific Co., New Brunswick, New Jersey, USA.) equipped with 2-L glass vessel with 1.2-L working volume. Continuous pH measurement and one-way control of pH were performed using an automatic pH control system consisting of a pH controller module, a pump module (Figure 3.2). The fermenter vessel, containing medium, was removed from the autoclave and attached to the fermenter apparatus while still hot (80-90°C). Cooling was initiated by means of cold water flowing though hollow baffles, controlled by an electronic thermostat. During ferementation the speed of stirrer was control at 200 rpm using an automatic stirrer control system.

Table 3.1 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-HCl	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.

Figure 3.2 A 2-L bioreactor apparatus.

3.4 Analytical methods

Fermentation samples were removed during fermentation for the measurement of cell mass, organic acids, and sugars. Cell mass was estimated from the optical density at 550 nm (0.33 mg of CDW/mL/OD) with a Bausch & Lomb Spectronic 70 spectrophotometer (Jantama et al., 2008a). Optical density (OD) was not measured in hydrolysate fermentations due to color (Geddes et al., 2014, 2015). Sugars, furans, and organic acids were analyzed by two high-performance liquid chromatography (HPLC) systems (Agilent Technologies 1200) as described previously (Geddes et al., 2014). Sugars and furan were analyzed using a BioRad (Hercules, CA) Aminex HPX-87P ion exclusion column (80ºC; nano-pure water as the mobile phase, 0.6 mL/min). Organic acids were analyzed using a BioRad Aminex HPX-87H column (45ºC; 4 mM H_2SO_4 as the mobile phase, 0.4 mL/min). Cultures collected from the fermenter were centrifuged to separate cells and supernatant. The supernatant was further filtrated

passing through a 0.2 μm filter prior to injecting to the HPLC. The 10 μL- injection volumes were automatically analyzed. Organic acids were separated in the column according to their molecular weight and structure.

CHAPTER IV

EFFICIENT UTILIZATION OF CASSAVA PULP FOR SUCCINATE PRODUCTION BY METABOLICALLY ENGINEERED *ESCHERICHIA COLI* KJ122

4.1 Introduction

Succinate is identified as having great promise in a bio-based economy, being a potential precursor for the synthesis of highly valuable products of commercial importance, including polymers, surfactants, green solvents, detergents, flavors, and fragrances (Zeikus et al., 1999). To efficiently produce a bio-based succinate, it is important that a low cost fermentation medium is used for the cultivation of microbial strains, and that these strains are able to utilize a wide range of low-cost sugar feedstock to produce succinate with impressive yields and productivities (Jantama et al., 2008a).

Cassava pulp, a fibrous by-product of the cassava processing industry, has recently become attractive as a cellulosic biomass due to its nature as a cheap, abundant, and renewable agricultural product. In Thailand, the cassava starch industry is estimated to generate at least one million tons of cassava pulp annually from 10 million tons of fresh tubers (Sriroth et al., 2000). Cassava pulp contains reasonably high organic substances which later becomes rot and causes environmental concerns (Virunanona et al., 2013). Presently, cassava pulp is

generally used as low-value animal feed. Due to its rich organic matters, the utilization of cassava pulp for succinate production is not only adding a great value to cassava pulp but may also solve the environmental problems.

A few studies had been reported on succinate production from cellulosic and hemicellulosic materials. For example, Chen et al. (2011a) produced succinate from an acid-pretreated rapeseed meal by *A. succinogenes* ATCC55618. Zheng et al. (2010) also produced succinate from corn stover by *A. succinogenes* CGMCC1593. More recently, Wang et al. (2011a) and Li et al. (2013) reported the utilization of bio-wasted cotton stalks into succinate by *A. succinogenes* 130Z. In 2008, Jantama et al. (2008a) reported the development of a metabolically engineered *E. coli* strain named KJ122. The strain produces succinate under simple anaerobic conditions in a mineral salts medium without any requirements of rich nutrients for the promotion of growth and antibiotics for the maintenance and expression of heterologous genes. The strain is able to produce succinate close to the theoretical maximum yield from glucose (1.71 mol/mol glucose used) and may be useful as a biocatalyst for the commercial production of succinate using cellulosic and lignocellulosic materials as carbon sources. Therefore, this study investigated the feasibility of succinate production from cassava pulp by metabolically engineered *E. coli* KJ122. To the best of authors' knowledge, this is the first report on the use of cassava pulp for succinate production by metabolically engineered *E. coli* using simple batch fermentation operations thus yielding impressive succinate titers and yields.

4.2 Materials and methods

4.2.1 Raw material

Cassava pulp was kindly provided by Korat Flour Industry Co., Ltd., Thailand. The step of cassava pulp power preparation was illustrated in Figure 4.1. Briefly, the cassava pulp was sun dried until the moisture content was less than 10% (w/w) on dry basis. The sundried cassava pulp was grounded into fine powder using a Cross-Beater mill (Glen Mill Corp., Maywood, NJ, USA) equipped with a 2 mm sieve screen. The grounded cassava pulp was kept in desiccator until further used.

Figure 4.1 Preparation of cassava pulp powder.

4.2.2 Microorganisms, media, and culture conditions

A metabolically engineered *E. coli* KJ122 (Jantama et al., 2008b) was kindly provided by the Department of Microbiology and Cell Sciences, University of Florida, Gainesville, Fl, USA. This strain was used for succinate production throughout this study. A low salts medium, AM1 (4.2 g/L total salts; Martinex et al., 2007) was used as a fermentation medium. This medium was supplemented with 100 mM $KHCO_3$ and 1 mM betaine. For seed culture preparation, one or two fresh colonies grown on LB medium were inoculated in AM1 medium containing sugars, and grown for 16–18 h at 37ºC with 150 rpm shaking. Fermentation was inoculated with an initial OD_{550} of 0.1 (0.33 mg CDW/mL/OD). Anaerobiosis was rapidly achieved during growth with added bicarbonate serving to ensure an atmosphere of CO_2. During the fermentation process, the pH of the medium was maintained by automatic addition of a mixture of 3 M KOH and 1.5 M K_2CO_3. The agitation speed was fixed at 150 and 200 rpm for small anaerobic bottle experiments and 2-L stirred bioreactor experiments, respectively.

4.2.3 Enzymes

Commercial enzymes including cellulase complex (Cel; Endoglucanase activity of 2,800 CMC-U/g and β-glucosidase activity of 775 pNG-U/g with minor hemicellulase as xylanase activity), xylanase (Xyl; 3,900 CMC-U/g), amylase (Amy; 42,169 IU/g), and amyloglucosidase (AMG; 21,300 IU/g) were purchased from Siam Victory Chemicals Co., Ltd., Bangkok, Thailand. The activities of enzymes reported here are as claimed by the description of the supplier's product sheet.

4.2.4 Cassava pulp hydrolysis

Enzymatic hydrolysis of cassava pulp was carried out in a 250 mL shaking flask at 40ºC with 200 rpm shaking for 48 h (Figure 4.2). The dried cassava pulp powder was soaked in AM1 medium (pH 6.0) at a concentration of 10% (w/w) on dry basis. The cellulase complex (Cel), xylanase (Xyl), α-amylase (Amy), and amyloglucosidase (AMG) enzymes were added into the cassava pulp slurry at a total enzyme loading of 2% (volume of enzyme per weight of dry pulp) for each enzyme depending on experiments. After 48 h, the enzymatic reactions were terminated by boiling for 10 min. The concentration of sugars liberated from cassava pulp was determined by high-performance liquid chromatography (HPLC).

The diluted acid-pretreated hydrolysate of cassava pulp was prepared according to the modified method described in Thongchul et al. (2010). Dried cassava pulp was hydrolyzed by diluted HCl (1 g of the dried matter was mixed with 9 mL of 1 M HCl) and heated at 121°C for 15 min. After the hydrolysis, the solid fraction was separated by centrifugation (4,000 rpm for 15 min). The sugars present in the hydrolysate were analyzed by HPLC. The pH of the acid-treated cassava pulp hydrolysate was adjusted to 6.0 by concentrated KOH prior to the fermentation step.

Figure 4.2 Incubation of cassava pulp during enzymatic hydrolysis.

4.2.5 Separate hydrolysis and fermentation (SHF)

An enzymatic-treated cassava pulp hydrolysate containing total sugars at the concentration of 50 g/L was used as a carbon substrate for succinate production. The model synthetic sugars mixture derived from sugars presenting in enzymatic-treated cassava pulp hydrolysate was also investigated for succinate production as a control experiment.

Batch separate hydrolysis and fermentation (SHF) experiment was carried out in a 500 mL in-house-built small fermenter vessel with a working volume of 350 mL. The fermentation was initiated after inoculation of the seed culture into the medium under anaerobic conditions. The experiments were performed for 96 h

and repeated at least two times. Samples were withdrawn after every 12 h till 96 h for further analysis.

4.2.6 Simultaneous saccharification and fermentation (SSF)

Batch simultaneous saccharification and fermentation (SSF) experiments were carried out in a 500 mL small in-house-built vessel with a working volume of 350 mL for 96 h. The dried cassava pulp powder was used as a substrate for SSF. The anaerobic bottle containing the cassava pulp slurry was autoclaved at 121ºC for 15 min. AM1 medium was added into the slurry after sterilization. The enzyme mixture (2% AMG + 3% Cel complex) was added to the sterile medium after the seed culture was inoculated. The effects of culture pH (6.0, 6.5, and 7.0) and fermentation temperatures (37, 39, and 41ºC) were also investigated with 7% (w/w) cassava pulp. All experiments were conducted in triplicate.

Batch SSF experiments were also performed in a 2-L stirred bioreactor with an initial volume of 1.2-L. The optimum concentrations based on dry basis of dried cassava pulp (5, 7, 10, 12, and 15%, w/w) for succinate production by *E. coli* KJ122 strain were also determined using the optimized fermentation parameters including culture pH, temperature, and enzyme mixture loading obtained from previous experiments. All experiments were conducted in triplicate.

In fed-batch SSF experiments, the concentration of cassava pulp at 5% (w/w) was initially supplemented in the fermentation medium with the enzyme mixture (2% AMG + 3% Cel). The cassava pulp slurry and the optimized enzyme mixture were intermittently added to yield a final concentration of cassava pulp of 15% (w/w) at the incubation times of 24 and 40 h. The cassava pulp slurry was prepared by adding of α-amylase prior to the autoclave at 121ºC for 20 min. The

medium and culture condition were exactly the same as in the batch SSF condition. All experiments were conducted in triplicate.

4.2.7 Fermentation products and cell mass measurement

Fermentation broth was removed during fermentation for the measurement of cell mass, organic acids, and sugars. Cell mass was estimated from the optical density at 550 nm (0.33 mg of cell dry weight/mL/OD) with a Bausch & Lomb Spectronic 70 spectrophotometer. Organic acids and sugars were determined using HPLC (Agilent Technology, Japan) equipped with an ion exclusion column (BIO RAD, Aminex, HPX-87H, USA) with a column temperature of 45ºC using 4 mM H_2SO_4 as a mobile phase with a flow rate of 0.4 mL/min. Culture collected from the fermenter was previously centrifuged to separate cells and supernatant. The supernatant was further filtered through a 0.2 µm filter membrane prior to injecting to HPLC. Ten microliters of injection volume were automatically analyzed. Organic acids were separated in the column depending on their retention times according to their molecular weight and structure.

4.2.8 Starch content analysis

Starch concentration of the cassava pulp was determined using a modified method explained by Thang et al. (2010). A portion of 20 µL α-amylase was added to 1 mL of culture medium and the mixture was incubated at 90ºC for 3 h to hydrolyze starch to soluble dextrin. After that, 8,880 µL of 0.1 M acetate buffer at pH 4.5 and 100 µL of AMG were added to the solution and the mixture was further incubated at 58ºC for 4 h. The solution was allowed to cool down and then transferred

to a 10 mL volumetric flask. The flask was then filled to the volume with de-ionized water. The glucose concentration of this solution was determined using the HPLC method, while the starch concentration in the fermentation broth was calculated as glucose concentration in gram per liter multiplies by a correction factor for glucose to starch (0.9).

4.2.9 Proximate analysis

The composition (ash, fat, and crude fiber) and moisture content of the cassava pulp were evaluated using the standard Association of Official Analytical Chemists method (AOAC, 1990). The crude protein content was calculated by converting nitrogen content determined by the micro-Kjeldahl method (Nx6.25). Carbohydrate content was calculated from the differences of those components.

4.2.10 Cell viability

Cell viability was determined by the total plate count technique. The viable cell counts were performed every 8 h during fermentations. LB agar plates were incubated at 37ºC for 24 h before counting grown cells. The total numbers of viable *E. coli* KJ122 cells were expressed as a colony forming unit per milliliter of the culture (CFU/mL).

4.2.11 Statistical analysis

Analysis of variance (ANOVA) was conducted using SPSS software (SPSS 17.0 for Windows; SPSS Inc., Chicago, IL, USA). Triplicate determinations were performed on each test and averages were used in the report. The differences

among mean values were established using Duncan's multiple range test at 95% significance level.

4.3 Results and discussion

4.3.1 Chemical composition of cassava pulp

Table 4.1 shows the composition of the dried cassava pulp powder used in this study. Starch was the main component in the dried cassava pulp, accounting for 58.74±0.15% w/w on dry basis. Carta et al. (1999) also revealed that cassava pulp generally contains about 50% (w/w on dry basis) starch that was considered as the main component determined as carbohydrates. However, the starch composition of cassava pulp varied drastically from place to place due to different crop varieties, growing locations and seasons, harvesting methods, and processing conditions (Sriroth et al., 2000; Pandey et al., 2000). Starch is usually composed of amylose and amylopectin that consist of a large number of glucose repeating units joined by α-1 → 4, and both α-1 → 4 and α-1 → 6 glycosidic bonds, respectively. Therefore, starch is one of the potential substrates for fermentative production of many chemicals including succinate.

Crude fiber (14.08±0.03% w/w on dry basis) was found as the second major component in the dried cassava pulp (Table 4.1). Crude fiber is typically composed of cellulose, hemicellulose, and lignin (Rattanachomsri et al., 2009). It is likely that glucose, xylose, arabinose, mannose, and galactose sugar units can be released from crude fiber by either chemical or enzymatic hydrolysis. Accordingly, sugar units liberated from starch and crude fiber in the dried cassava pulp can be utilized by *E. coli* KJ122 to produce succinate. The dried cassava pulp also contained

low levels of fat (0.41±0.01% w/w on dry basis), protein (1.77±0.23% w/w on dry basis), and ash content (1.87±0.03% w/w on dry basis). Pandey et al. (2000) suggested that the low ash content in cassava pulp could provide numerous benefits and was suitable for usage in bioconversion processes by means of microbial fermentation. On the contrary, other crop feedstock, such as rice straw (17.5% w/w on dry basis), wheat straw (11.0% w/w on dry basis), and rapeseed meal (7.1% w/w on dry basis), contains higher ash contents (Pandey et al., 2000; Thanaseelaan, 2013). This implies that high ash content may cause lower biomass conversion yield to fermentable sugars compared with the dried cassava pulp, resulting in lower overall production yields of succinate from the above mentioned crops by microorganisms.

Table 4.1 Comparison of several components of cassava pulp powder used in this study.

Main components	**(%, w/w on dry basis)**
Fat	0.41 ± 0.01
Protein	1.77 ± 0.23
Ash	1.87 ± 0.03
Crude fiber	14.08 ± 0.03
Moisture content	7.56 ± 0.13
Carbohydrate	74.31 ± 0.24
Starch content (Enzyme method)	58.74 ± 0.15

Values represent the mean of triplicate experiment ± SD between samples.

4.3.2 Components of sugars in hydrolysates of the dried cassava pulp

Figure 4.3A shows sugar compositions in cassava pulp hydrolysates treated with acid and various enzymes. The results revealed that glucose, cellobiose, xylose, maltose, and arabinose were present in both hydrolysates treated with diluted acid and enzymes. Glucose was the main sugar monomer liberated in any hydrolysates due to high starch content in the dried cassava pulp when AMG or Amy was used during saccharification. The total sugars yield from cassava pulp by Amy or AMG treatment was about 60.50% (w/w on dry basis). Furthermore, combined Amy and AMG treatment significantly increased total sugars production yield up to 65.21±0.12% (w/w on dry basis). On the contrary, treatments of cassava pulp with Cel or Xyl or Cel + Xyl enzymes yielded cellobiose as a major sugar component. The total sugars production was only in the range of 12.77–39.50% (w/w on dry basis). Cel and Xyl enzymes contain non-starch polysaccharide hydrolyzing activities, so it is expected that Cel and Xyl can hydrolyze β-1→4-D-glucose and linear β-1 →4-xylan linkages situated in cellulose and hemicelluloses structures found in crude fiber of the dried cassava pulp. As a result, cellobiose and glucose were released with moderate amout of xylose and maltose. This was corresponded with the study of Thongchul et al. (2010) who found that cellobiose was generally present in hydrolysate of cassava pulp treated with only cellulase enzyme but glucose yield of 0.25 g/g dry pulp (less than 50% of the theoretical yield) was only obtained. In addition, Rattanachomsri et al. (2009) and Zhu et al. (2012) also reported that very low yields of glucose and other reducing sugars released from cassava pulp were attained when only cellulase or xylanase or polygalacturonase was individually supplied for cassava pulp hydrolysis. It was clear that a high proportion of non-

reducing sugars and oligosaccharides might be present in the cassava pulp hydrolysates treated only with non-starch hydrolyzing enzymes.

For starch degrading (RSD) amylolytic enzymes (Amy and AMG), neither Amy nor AMG alone, nor combined Amy and AMG, hydrolyzed the cassava pulp effectively (Figure 4.3A). Amy and AMG enzymes degraded only released starch granules containing α-1→4-D-glucose and α-1→6-D-glucose linkages but no other polysaccharides presented in cassava pulp, thus yielding mostly glucose and partly maltose. However, when either Cel or Xyl or even their mixture was added to the reaction in combination with Amy and AMG, the sugars content was greatly improved. Cel and Xyl additionally hydrolyzed cellulose and hemicelluloses structures. The total sugars yield reached up to 85.50±0.69% (w/w on dry basis). The total sugars content was comparable to that contained in an acid hydrolyzed cassava pulp (86.36±0.18% w/w on dry basis). The results suggested that the addition of Cel and Xyl synergistically degraded cellulose and hemicelluloses thus enhancing enzymatic efficacy of both Amy and AMG. Therefore, it is clear that treatments with Cel and Amy or AMG are beneficial in increasing sugar yield from cassava pulp. Zhu et al. (2012) also reported that the highest released sugar yield was achieved (up to 0.46 g/g dry pulp) when combined cellulase, Amy, and AMG were used to hydrolyze cassava pulp. Only sugar yield of 0.20–0.28 g/g dry pulp was obtained when individual Amy or AMG and their combinations were used for saccharification. Rattanachomsri et al. (2009) also revealed that the use of pectinase and β-glucosidase combined with cellulase and RSD enzymes could maximize the release of reducing sugar (up to 0.57 g/g dry pulp) during non-thermal enzymatic saccharification of cassava pulp.

As Cel contained a minor hemicellulase as xylanase activity, an increasing amount of Cel loadings reflected an increased hemicellulase activity. Consequently, Xyl could be omitted. In the treatments of AMG + Cel or Amy + Cel, the sugars content obtained was not significantly different (Figure 4.3A). It may be concluded that the use of AMG or Amy with Cel for hydrolyzing the dried cassava pulp was equivalent. Therefore, the efficacy of cassava pulp hydrolysis with different Cel loading (1, 2, 3, 4, and 5% v/w on dry pulp) combined with only 2% (v/w on dry pulp) AMG was investigated. The results showed that total sugars content after enzymatic cassava pulp hydrolysis with Cel loading up to 3% (v/w on dry pulp) was considerably improved. Beyond this concentration, a slight decrease in total sugars content was observed (Figure 4.3B). The combined action of 3% (v/w on dry pulp) Cel and 2% (v/w on dry pulp) AMG in enzymatic cassava pulp hydrolysis yielded the highest total sugars content. The total sugars yield of 86.48±0.24% (w/w on dry basis) was comparable to those obtained either from diluted acid hydrolysis (86.36±0.18% w/w on dry basis) or from a combination of Amy + AMG + Cel + Xyl (85.48±0.69% w/w on dry basis). However, our result was in contrast to those reported by Thongchul et al. (2010). A much higher sugar yield (up to 85% theoretical yield) was obtained from cassava pulp with acid hydrolysis while the highest sugar yield of only 40.7% was obtained with cellulase for 24 h followed by Amy for 1 h and then AMG for 3 h. This results indicated an efficient enzymatic hydrolysis of starch and non-starch fibrous structure contained in the dried cassava pulp with a combination of 3% (v/w on dry pulp) cellulase complex (endoglucanase: 562 CMC-U/g dry pulp and β-glucosidase: 10 pNG-U/g dry pulp) and 2% (v/w on dry pulp) AMG (284 IU/g dry pulp) (Table 4.2). In conclusion, our enzymatic hydrolysis conditions were superior to

other previously published reports in which cassava pulp was used to produce other products including lactic acid and ethanol (Thongchul et al., 2010; Rattanachomsri et al., 2009; Zhu et al., 2012).

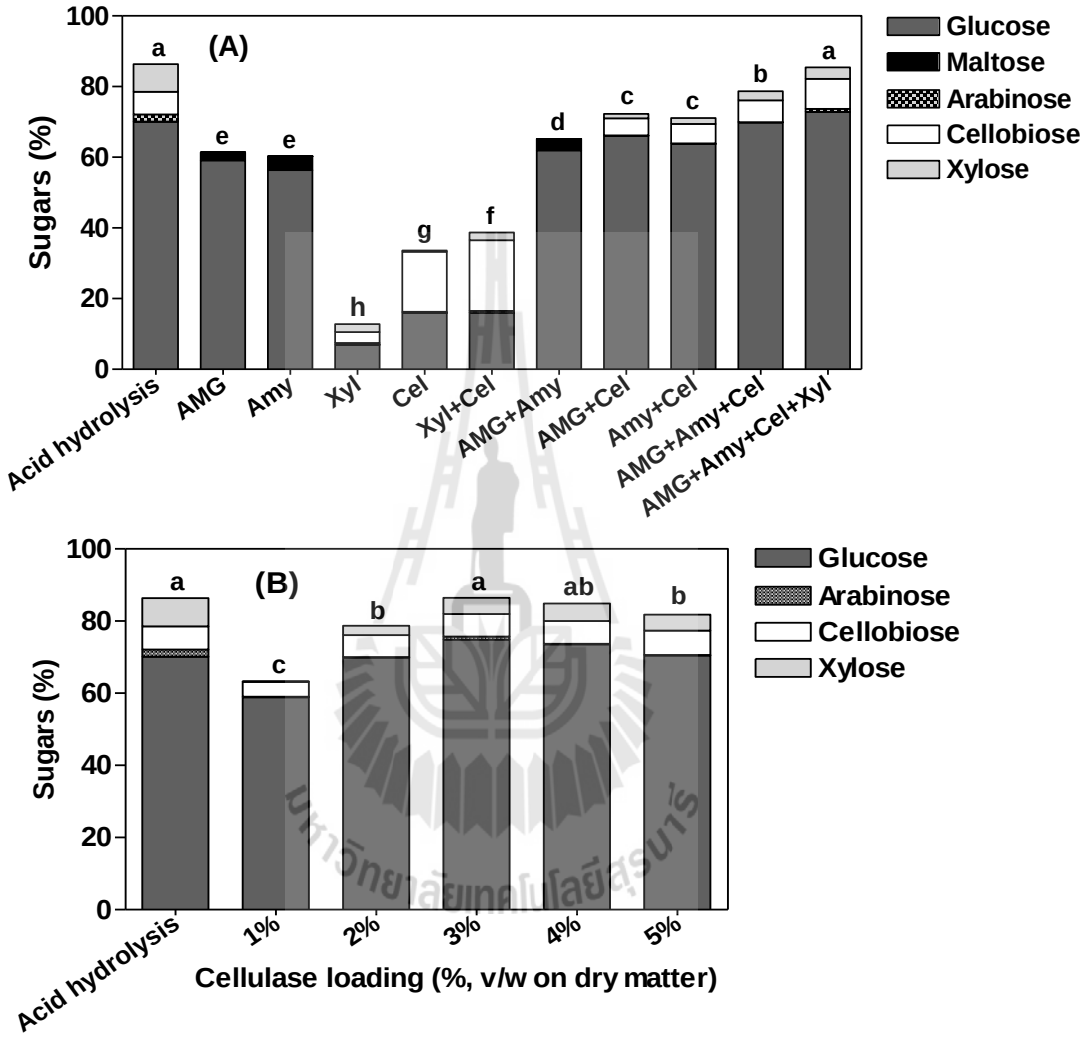

Figure 4.3 Composition of sugars in various cassava pulp hydrolysates. (A) Effect of various enzymes used during saccharification of the dried cassava pulp (B) Effect of cellulase complex loading combined with AMG to cassava pulp hydrolysis. All enzymes used were fixed at 2% (v/w, on dry matter). Lower case letters (a-h) represent a significant difference between mean value of treatments ($p<0.05$). AMG, Amy, Xyl, Cel strand for amyloglucosidase, α-amylase, xylanase and cellulase complex, respectively. The experiments were performed in triplicate and each value expressed as the mean value.

Table 4.2 Enzymes activity used during hydrolysis of cassava pulp with various combinations of enzymes.

Enzymes	Enzymes activity					Total sugars (%, w/w on dry basis)
	Amy (IU/g)	AMG (IU/g)	Xyl (CMC-U/g)	Cel[A] (CMC-U/g)	B (CMC-U/g)	
Amy	562	–*	–	–	–	60.26±0.74[e]
AMG	–	284	–	–	–	61.39±0.34[e]
Xyl	–	–	52	–	–	12.66±1.18[h]
Cel	–	–	–	37	10	33.60±0.64[g]
Amy+AMG	562	284	–	–	–	65.21±0.12[d]
Amy+Cel	562	–	–	37	10	71.08±0.66[c]
AMG+Cel	562	284	–	37	10	72.27±0.24[c]
Cel+Xyl	–	–	52	37	10	38.73±1.30[f]
Amy+AMG+Cel	562	284	–	37	10	78.69±1.04[b]
Amy+AMG+Cel+Xyl	562	284	52	37	10	85.48±0.69[a]
Acid hydrolysis**	–	–	–	–	–	86.36±0.18[a]

Hydrolysis was conducted at 40°C at an initial pH of 6.0 with shaking at 200 rpm for 48 h with 10% (w/w) cassava pulp.

[A]Cellulase complex contains endoglucanase and *β*-glucosidase activity and minor xylanase activity. All enzymes used were fixed at 2% (v/w, on dry matter).

*–, not added. AMG, Amy, Xyl, Cel, and B strand for amyloglucosidase, α-amylase, xylanase, cellulase complex, and *β*-glucosidase, respectively.

**Acid hydrolysis reaction contained 10% (w/w) cassava pulp in 1M HCl and heated at 121°C for 15 min. Lower case letters (a-h) represent a significant difference between mean value of treatments ($p<0.05$).

4.3.3 Succinate production by separate hydrolysis and fermentation (SHF)

E. coli KJ122 strain did not grow and produce succinate in the medium containing only alkaline neutralized-acid-treated cassava pulp hydrolysate as a sole carbon source. It was likely that the acid-treated cassava pulp hydrolysate contained undesirable by-products, such as furfural derivatives, organic acids, and phenolic compounds. Cantarella et al. (2004) suggested that the hydrolysates from acid hydrolysis must be neutralized with alkaline before being used in the fermentation. High concentrations of salts after neutralization and other inhibitory by-products could severely harm the microorganism and inhibit the fermentation. Therefore, the enzymatic hydrolysate of the dried cassava pulp was considered as an alternative carbon source in succinate production by *E. coli* KJ122 without any additional treatments. An enzymatic hydrolysate of cassava pulp containing total sugars at the initial concentration of 50 g/L was used as a carbon source for succinate production in this study. The utilization of sugar in a medium containing a synthetic sugars mixture equivalent to those found in the cassava pulp hydrolysate was also evaluated. The results revealed that KJ122 strain was able to considerably utilize sugar contained in the enzymatic and synthetic cassava pulp hydrolysates, and simultaneously produced succinate under anaerobic conditions (Figure 4.4 and Table 4.3). The succinate concentrations obtained from the synthetic sugar mixture and the enzymatic cassava pulp hydrolysates were 40.73±0.23 and 41.46±0.05 g/L, respectively, with yields of 0.84±0.01 and 0.82±0.01 g/g dry pulp, respectively. The succinate productivities obtained were substantially equal in both hydrolysates (about 0.84 g/L/h). The maximum succinate production yields and productivities obtained in this experiment were comparable to those previously reported by Jantama et al. (2008b) when only

glucose was used as sole carbon source. Zheng et al. (2009) performed succinate production from corn straw by *A. succinogenes* CGMCC1593 using batch and fed-batch SHF. The succinate concentrations of 45–53 g/L with yields of 80.4–82.5 g/100 g corn straw were obtained. Wang et al. (2011a) also utilized corn stalk using batch SHF and produced succinate by *E. coli* SD121. The succinate concentration of 57 g/L with a yield of 87 g/100 g corn stalk was attained. Jiang et al. (2014) also reported succinate production of 21.1 g/L with a yield of 76 g/100 g corn stalk using SHF operation by *E. coli* DC115. These results confirmed that *E. coli* KJ122 strain could produce succinate in comparison amount with other previously published works that reported the utilization of agricultural waste products using SHF for succinate production. However, all abovementioned works used rich medium for microbial cultivation and fermentation thus resulting in slightly higher productivities. Accordingly, the production cost would be increased due to the high cost of rich nutrients and high operating cost for purification and separation processes. After 48 h incubation, glucose, xylose, and arabinose were exhausted and succinate production reached its maximum and remained constant until the end of fermentation. Xylose and arabinose were simultaneously consumed along with glucose consumption without catabolite repression (Figure 4.4). In general, xylose and arabinose are usually transported to *E. coli* cells by xylose-ABC transporter and arabinose: H^+ symporter, respectively. Whilst, expression levels of both transporters are regulated upon a catabolite repression mechanism. Zhang et al. (2009) revealed that *E. coli* KJ122 contained mutations in the normal PTS system and dramatically increased expression levels of adenylated cyclase and CRP (cAMP-receptor protein) proteins comparable to its parental strain (*E. coli* ATCC8739). Increased levels of cAMP-CRP

protein could be resulted from an inactivation of the native glucose PTS system thus de-repressing a catabolite repression, which in turn prevents glucose from repressing uptake of other sugars including xylose and arabinose in *E. coli* KJ122. Andersson et al. (2007) reported that xylose was co-metabolized with glucose at a substantially higher rate than that in the medium containing merely xylose by *E. coli* AFP184 that contained a mutation in PTS system thus affecting $EIIB^{glc}$. The overall succinate productivity by *E. coli* AFP184 was significantly higher during glucose/xylose co-metabolism than that obtained when xylose was individually metabolized. Jiang et al. (2014) revealed that *E. coli* DC115, a mutant of AFP111 that produces mainly succinate, co-transported glucose and arabinose, and utilized xylose only after glucose/arabinose co-metabolism was depleted. In addition, the co-metabolism of glucose and xylose was initiated when arabinose was absent. Unlike glucose, xylose, and arabinose, *E. coli* KJ122 strain could not efficiently utilize cellobiose and even the fermentation was prolonged till 96 h. KJ122 strain did not co-utilize cellobiose with other sugars, but started consuming cellobiose after all the sugars were exhausted. Nevertheless, the cellobiose consumption rate was very low and stalled. About fifty percent of cellobiose was utilized (Figure 4.4). This result was in accordance with that reported by Gokarn et al. (1997) who demonstrated succinate production from cellobiose by two anaerobic ruminal succinate-producing bacteria, *Fibrobacter succinogenes* S85 and *Ruminococcus flavefaciens* FD-1. The results showed that much less cellobiose was consumed at a lower rate compared with that of glucose by these two bacteria. The maximum succinate productivities by these two organisms reached only 9 mg/L/h for cellobiose while a productivity of up to 60 mg/L/h was attained for glucose. The low consumption rate of cellobiose in some

bacteria could be explained by cryptic mechanisms of *ascFB* genes (encoding a phosphotransferase system II and a phosphor-β-glucosidase, respectively) and chb (chitobiose/cellobiose-PTS permease) operon presenting in most *E. coli* strains and other related microorganisms. Ishida et al. (2009) revealed that most β-glucoside metabolic operons are cryptic to serve as a protective device against toxic β-glucosides found naturally, including cellobiose, arbutin, and salicin. When cultivated with the enzymatic cassava pulp hydrolysate, biomass produced by *E. coli* KJ122 was maximized at a concentration of 3.35 g/L, and the strain could prolong the growth in an exponential phase until 36 h of incubation. But the exponential growth of *E. coli* KJ122 strain was finished after 24 h of incubation for synthetic sugars mixture hydrolysate (Figure 4.4). Biomass produced in the medium containing cassava pulp hydrolysate was 1.4 times higher than that obtained by the synthetic sugar mixture hydrolysate. It was likely that cassava pulp hydrolysate contained an extra nitrogen source as protein content presenting in dry cassava pulp (Table 4.1) for promoting growth and succinate production in *E. coli* KJ122. The dry cassava pulp may not only contain nitrogen sources but also various amino acids, vitamins, minerals, and other growth factors that could also activate the growth of microorganisms. Most succinate-producing bacteria, such as *A. succinogenes*, *Anaerospirillum succinoproducens*, and *F. succinogenes* are the fastidious microorganism requiring special growth factors to support their growth and synthesize enzymes involved in succinate production (Liu et al., 2008). Chen et al. (2011a) and Leung et al. (2012) demonstrated succinate production using hydrolysates from rapeseed meal and wasted bread, respectively. They revealed that free amino nitrogen (FAN) contained in both rapeseed meal and wasted bread was released to hydrolysates at the concentrations of 200–500 mg/L,

and was essential to support the growth of *A. succinogenes*. Both studies suggested that wasted bread and rapeseed meal would be alternative substrates for efficient succinate production without additional nitrogen source supplementation such as yeast extract. Based on the study, it may be implied that cassava pulp could be also substituted other cellulosic or hemicellulosic substrates for the promotion of growth and succinate production in other succinate-producing bacteria.

Table 4.3 Fermentation parameters of *E. coli* KJ122 under separate hydrolysis and fermentation (SHF) using different carbon sources.

Kinetic parameters	**Model sugars mixture**	**Enzymatic cassava pulp**
Glucose residual (g/L)	0.00±0.00[a]	0.00±0.00[a]
Xylose residual (g/L)	0.00±0.00[a]	0.15±0.01[a]
Arabinose residual (g/L)	0.00±0.00[b]	0.08±0.00[a]
Cellobiose residual (g/L)	2.79±0.15[a]	1.88±0.05[b]
Succinate (g/L)	40.73±0.23[a]	41.46±0.05[a]
Acetate (g/L)	5.54±0.18[a]	5.55±0.25[a]
Maximum biomass	2.45±0.01[b]	3.35±0.32[a]
Succinate yield (g/g)	0.84±0.01[a]	0.82±000[a]
Succinate productivity (g/L/h)	0.42±0.00[a]	0.43±0.00[a]

All data represent the averages of at least two fermentations with standard deviations. [(a-b)] The values with different symbols in the same row are significantly different ($p<0.05$).

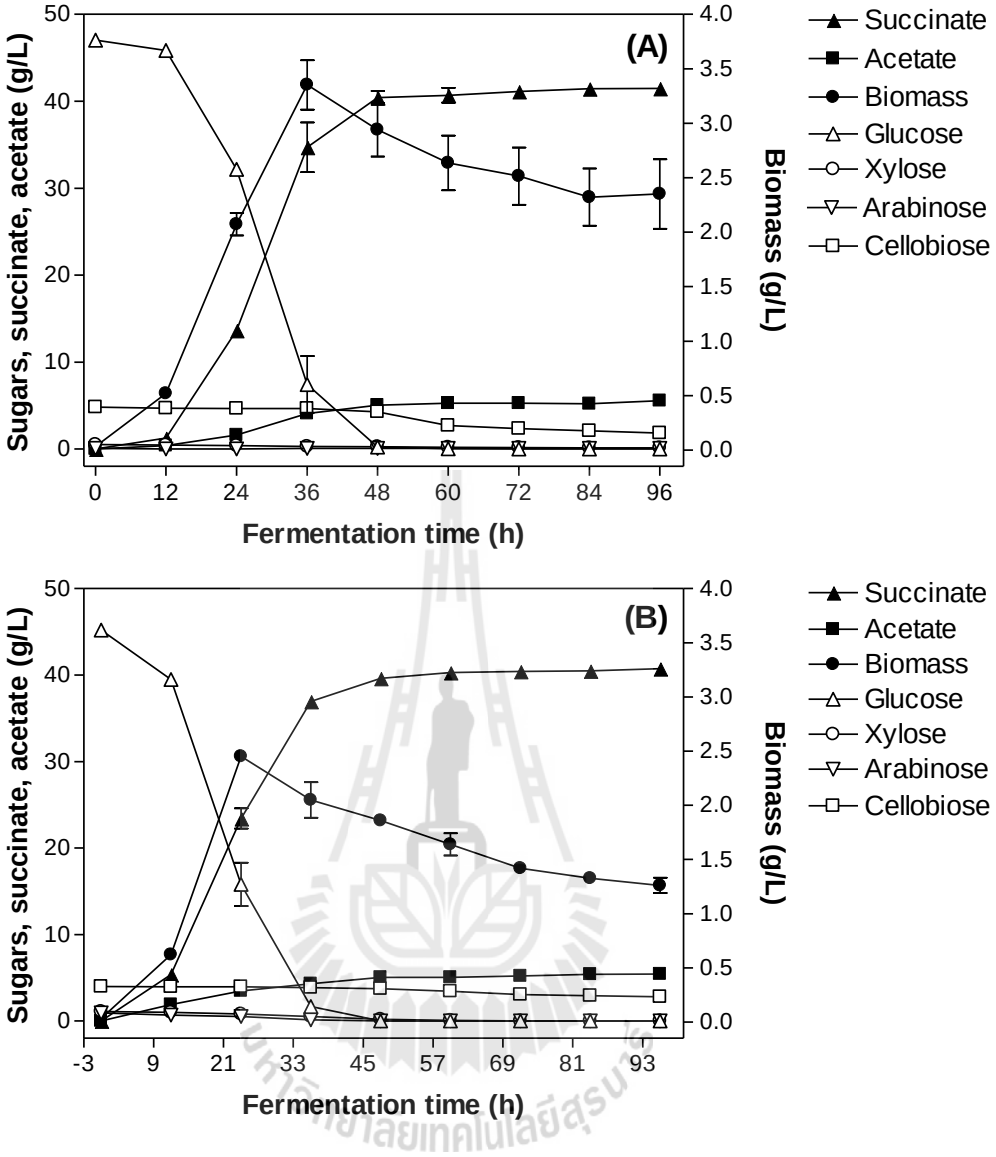

Figure 4.4 Time course of succinate production under separate hydrolysis and fermentation (SHF) using *E. coli* KJ122 in an in-house-built small fermentation. Cells were grown with an initial sugar concentration of 50 g/L supplemented with AM1 medium. (A) Succinate production from enzymatic hydrolysis of cassava pulp. (B) Succinate production from synthetic sugars mixture.

4.3.4 Succinate production by simultaneous saccharification and fermentation (SSF)

4.3.4.1 Effect of culture pH

The cultivation of *E. coli* at pH lower than 6.0 usually affects growth and sugars consumption. Therefore, succinate production by *E. coli* KJ122 at very low pH as optimal for enzymatic activities (especially for Cel at pH 5.0) during SSF is not possible. As a consequence, the effects of culture pH during SSF on succinate production were investigated in *E. coli* KJ122 strain. The highest succinate concentration of 52.54±0.16 g/L with a yield of 76.19±0.08 g/100 g dry pulp observed at pH 6.5. Nevertheless, the final concentrations of succinate and production yields showed no significant difference between pH 6.5 and 7.0 (52.00±0.20 g/L with a yield of 75.04±0.04 g/100 g dry pulp). In addition, a decrease of the culture pH from 6.5 to 6.0 resulted in a significant reduction in succinate production (48.35 ± 0.71 g/L) and yield (71.74±0.28 g/100 g dry pulp) along with a lower sugar utilization rate. Furthermore, sugars were left over at pH 6.0 at the highest concentration comparable to those at pH 6.5 and 7.0 (Figure 4.5A, Table 4.4). Agarwal et al. (2006) reported the highest growth and succinate production at pH 6.5 from sugarcane molasses and corn steep liquor by isolated *E. coli* strain. The growth and succinate production of this strain were significantly reduced at pH below and beyond this point. Li et al. (2013) had also demonstrated a conversion of cotton stalk to succinate by SSF using *A. succinogenes* 130Z at pH 7.0. In addition, Van der Werf et al. (1997) showed that the growth and succinate production of *A. succinogenes* 130Z were adversely affected at pH less than 7.0. With regard to the above mentioned, the

preferred controlled pH for succinate production by *E. coli* KJ122 during SSF process was 6.5, and this was applied for further investigation.

4.3.4.2 Effect of temperature

E. coli KJ122 did not grow well at temperatures over 42ºC, but the optimal temperature for enzymes used for cassava pulp hydrolysis recommended by manufacturer was around 50ºC. It is important to compromise between two optimal temperatures for both bacterial growth and enzymatic activities ranging from 37–41 to 50ºC, respectively. Figure 4.5B reveals the effect of temperature during SSF on succinate production by *E. coli* KJ122 strain. The highest succinate concentration of 55.42±0.53 g/L with a yield of 79.86±0.77 g/100 g dry pulp was achieved at 41ºC. However, this was not significantly different from the results obtained at 39ºC in which the succinate concentration of 54.54±0.69 g/L with a yield of 78.77±1.02 g/100 g dry pulp was attained. At 37ºC, a slight reduction in succinate production and yield, 52.54±0.16 g/L and 76.19±0.08 g/100 g dry pulp, respectively, was observed (Table 4.4). This result could be explained by the fact that an increase in temperature might elevate enzymatic activities and release more sugars resulting in higher conversion of sugars to succinate during SSF. Zheng et al. (2010) had also reported that with an elevation in temperature from 38 to 42ºC, higher concentrations of residual sugars from pretreated corn were found at higher temperatures during SSF for succinate by *A. succinogenes*. Therefore, the optimal temperature for succinate production by *E. coli* KJ122 strain during SSF was observed in the range of 39 - 41ºC.

Regardless of enzymatic activity, Agarwal et al. (2006) demonstrated that the highest succinate production was observed at 37 - 39ºC, but the

production was significantly lower when temperatures were below 35ºC. This results were in contrast to the work of Zheng et al. (2010) who reported that both succinate titer and yield were dramatically decreased with higher temperatures ranging from 37 to 42ºC for *A. succinogenes* CGMCC1593. Moreover, Zhu et al. (2012) demonstrated that neither a decrease nor an increase in the temperature ranging from 30 to 40ºC during SSF induced significant amount of ethanol production by *Saccharomyces cerevisiae*. These results suggest that optimal temperatures affecting products formation and fermentation kinetics depended upon microbial strains used during SSF.

4.3.4.3 Effect of cassava pulp concentration

It is generally accepted that substrate concentration for productions of various fermentative chemicals must be enhanced for industrial scale application to reduce operating costs (Zhu et al., 2012). Highly concentrated cassava pulp represents a challenge as a result of its resistance to heat and mass transfer leading to a decrease in the efficiency of hydrolysis. Thus, it is important to find a suitable substrate concentration of dried cassava pulp for succinate production under SSF by *E. coli* KJ122. The results showed that the level of succinate production significantly increased with an increase of dried cassava pulp concentrations from 5 to 12% (w/w). At a dried cassava pulp concentration of 15% (w/w), the succinate concentration was at the highest of 81.66±0.78 g/L (yield of 68.5±0.53 g/100 g dry pulp), nearly 2.5 times higher than that obtained from dried cassava pulp concentration of 5% (w/w). However, the succinate concentration was not significantly different when compared with that obtained from dried cassava pulp

concentration of 12% (w/w), at which a succinate concentration of 80.86 ± 0.49 g/L (yield of 70.34±0.37 g/100 g dry pulp) was obtained (Figure 4.5C and Table 4.4). In addition, when cassava pulp concentration was increased over 15% (w/w), the operation for SSF was problematic due to difficulties in mixing, thus lowering the efficiency of heat and mass transfer at high solid loading. As a result, it significantly decreased the succinate production by SSF (data not shown). A similar result was observed in the study of Zhu et al. (2012) who found that using cassava pulp concentration over 16% in SSF batch ethanol fermentation significantly decreased ethanol production and fermentation efficiency. Moreover, the highest concentration of residual sugar was found at 30.54±1.46 g/L for dried cassava pulp concentration of 15% (w/w), compared with 5.04±0.09 g/L for dried cassava pulp concentration of 12% (w/w) (Table 4.4). It is noteworthy that residual sugars left over in the broth can interfere and cause complications during purification and separation of succinate, production process which may result in reducing the efficiency of downstream processing process.

Table 4.4 Fermentation profile of succinate production from cassava pulp during SSF by *E. coli* KJ122.

Parameters	Residual sugars (g/L)	Succinate (g/L)	Acetate (g/L)	Yield (g/100 g dry pulp)
pH				
6.0	2.60±0.72[a]	48.35±0.71[b]	5.95±0.20[d]	71.74±0.28[b]
6.5	1.03±0.15[b]	52.54±0.16[a]	7.16±0.44[c]	76.19±0.08[a]
7.0	0.70±0.06[b]	52.00±0.20[a]	7.11±0.14[c]	75.04±0.04[a]
Temperature (ºC)				
37	1.03±0.15[a]	52.54±0.16[b]	7.16±0.44[c]	76.19±0.08[b]
39	0.72±0.03[ab]	54.54±0.69[a]	7.22±0.06[c]	78.77±1.02[a]
41	0.60±0.01[b]	55.42±0.53[a]	7.32±0.10[c]	79.86±0.77[a]
Cassava concentration (%, w/w on dry basis)				
5	0.27±0.01[c]	38.91±0.24[d]	4.11±0.07[e]	78.26±0.49[a]
7	0.72±0.03[c]	54.54±0.69[c]	7.22±0.06[c]	78.77±1.02[a]
10	1.05±0.01[c]	70.72±1.86[b]	11.37±0.18[b]	71.47±1.89[b]
12	5.04±0.09[b]	80.86±0.49[a]	13.36±0.10[a]	70.34±0.37[bc]
15	30.54±1.46[a]	81.66±0.78[a]	13.61±0.06[a]	68.36±0.53[c]

An enzyme loading was 2% AMG and 3% (v/w, on dry matter) cellulase complex.

All data represent the averages of at least two fermentations with standard deviations.

[(a-e)] The values with different symbols in the same row are significantly different ($p<0.05$).

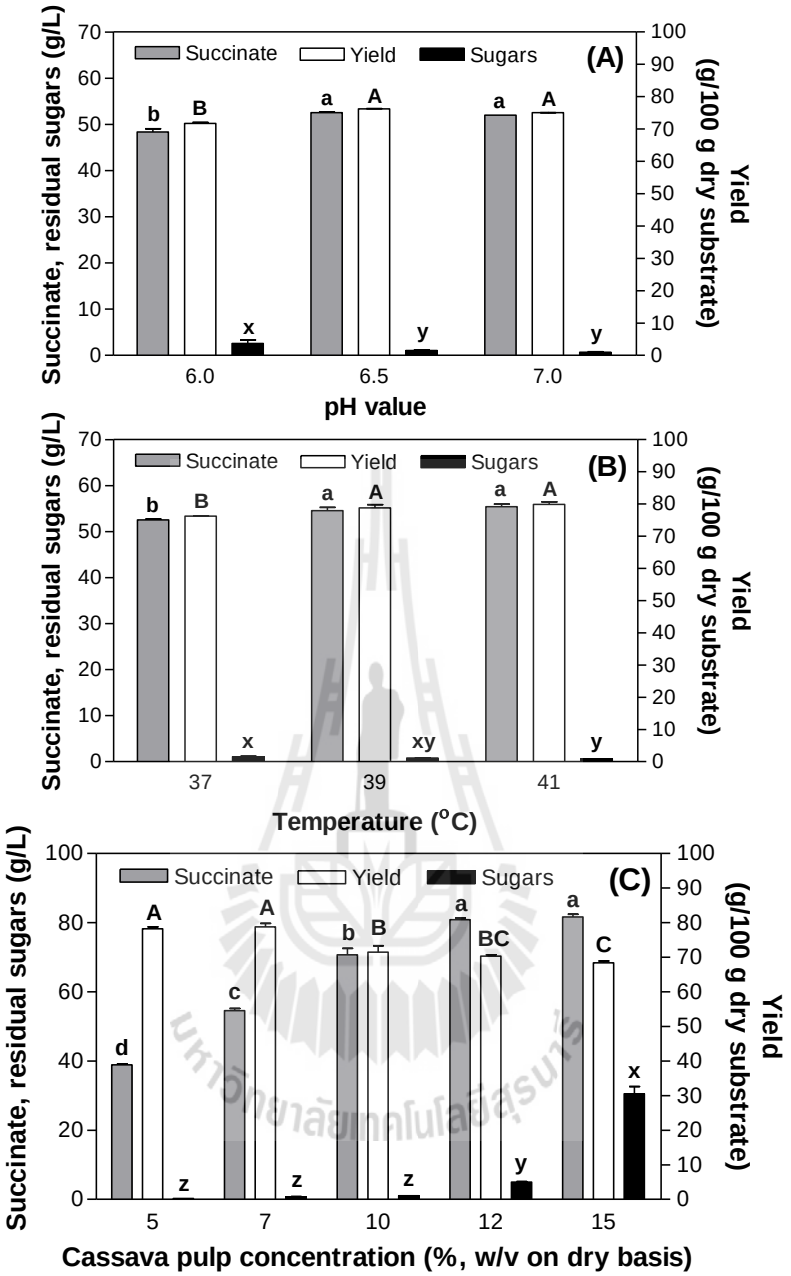

Figure 4.5 Effect of culture pH at 37°C (A), temperatures at pH 6.5 (B) at 70 g/L cassava pulp, and different cassava pulp concentrations at pH 6.5 and 39°C (C) on succinate production during SSF process by *E. coli* KJ122. An enzyme loading was 2% AMG and 3% (v/w, on dry matter) cellulase complex. Bars with different letters (a–c succinate, A–C yield, x–z residual sugars) show a significant difference between mean value of parameters ($p<0.05$). The experiments were performed in triplicate and each bar expressed as mean value.

4.3.5 Succinate production by batch simultaneous saccharification and fermentation (SSF) in a 2-L bioreactor

Figure 4.6A represents a time course of succinate production during batch SSF using 12% (w/w) dried cassava pulp by *E. coli* KJ122 strain. Cassava pulp was immediately hydrolyzed after adding enzymes into the reactor yielding fermentable sugars, mainly glucose. The maximum of liberated glucose was found around 16 h prior to decreasing and it was completely consumed by the microorganism within 72 h. Meanwhile the organism started growing as early as 8 h and reached its maximum at exponential growth within 24 h with viable cells count of 3.58±0.32 x 10^9 CFU/mL. At the end of fermentation, succinate concentration of 80.86±0.49 g/L was attained, corresponding to a yield of 70.34±0.37 g/100 g dry pulp and productivity of 0.84±0.01 g/L/h, respectively. The specific productivity for succinate production was attained at 272 mg/g CDW/h. Many reports were published in relation to succinate production by *A. succinogenes* by batch SSF operation of various carbon sources. Zheng et al. (2010) showed succinate production of 47.4 g/L, a yield of 72 g/100 g corn stover, and productivity of 0.98 g/L/h by batch SSF. Leung et al. (2012) reported succinate production of 47.40 g/L with only a yield of 55 g/100 g sugars used by batch SSF of waste bread. Chen et al. (2011) also demonstrated succinate production by SSF of rapeseed meal, in which a succinate concentration of 15.5 g/L and a yield of 12.4 g/100 g rapeseed meal were obtained (Table 4.5). The results suggested that dried cassava pulp concentration of 12% (w/w) was suitable for succinate production using batch SSF based on succinate production and the simplicity of the operation. It is interesting to note that xylose and arabinose were quickly co-metabolized with glucose, and arabinose was completely consumed after

56 h incubation. Xylose was almost entirely utilized in the presence of glucose during the first 56 h of incubation, but after 64 h of incubation the liberation of xylose from cassava pulp increased and reached the concentration of 7 g/L. After that, xylose was gradually consumed. It was even more pronounced when glucose was exhausted. This can be explained by the fact that *E. coli* KJ122 is de-repressed a catabolite repression due to inactivation of native glucose PTS system resulting from an overexpression of cAMP-CRP complex (Zhang et al., 2009). In addition, higher ATP requirement is expected for xylose transport by xylose-ABC transporter. Jiang et al. (2014) revealed that succinate production from xylose accumulated ATP 1.67 mol per xylose, while about 2.67 ATP per xylose was required for its metabolism by *E. coli* producing succinate strains. It is important for the strains to generate more ATP for xylose metabolism. Therefore, *E. coli* KJ122 might utilize ATP generated during glucose metabolism in an efficient consumption of xylose. Without glucose, ATP was not efficiently generated resulting in lower consumption of xylose. In addition, cellobiose was also slowly metabolized after glucose completion due to a cryptic mechanism of most β-glucoside metabolic operons. This phenomenon was similar to what was observed during SHF (Figure 4.5).

4.3.6 Succinate production during fed-batch simultaneous saccharification and fermentation (SSF) in a 2-L bioreactor

SSF is usually performed at a high substrate concentration to lower the overall process energy demand and water consumption, thus decreasing the production cost (Sassner et al., 2006). However, a batch SSF experiment is difficult to conduct with high dry matter content due to the high viscosity of substrates. A fed-

batch SSF experiment could provide a way to handle the initial mixture problems at high substrate loading. Therefore, a fed-batch SSF experiment with cassava pulp was carried out in a 2-L bioreactor in this study. As shown in Figure 4.6B, after two additions of 5% (w/w) cassava pulp at 24 and 40 h, succinate concentration was enhanced to 98.63±0.12 g/L, with a yield of 71.64±0.97 g/100 g dry pulp and productivity of 1.03 ± 0.01 g/L/h. Compared with batch SSF, fed-batch SSF significantly improved the succinate productivity and yield by 21.98 and 22.62%, respectively. However, yields obtained from batch and fed-batch SSF were comparable. Similarly, Chen et al. (2011) proved that fed-batch SSF provided an improvement in succinate production from 15.5 to 23.4 g/L, and productivity from 0.22 to 0.33 g/L/h from acid-pretreated rapeseed meal by *A. succinogenes*. The probable reason for this may be due to the fact that the fed-batch SSF process reduced the solid concentration in the initial broth and thus enhanced the efficiencies of mass and heat transfer, causing the hydrolysis and fermentation reaction to be faster and more efficient. Recently, Chen et al. (2014) showed succinate production from cassava starch with very high titer and yield by *E. coli* NZN111. The succinate concentration of 105.13 g/L with a yield of 78 g/100 g substrate and the overall productivity of 1.38 g/L/h was obtained. However, Chen et al. (2014) performed succinate production by two-stage batch fermentation. High-cell-density cultures were generated under aerobic conditions followed by anaerobic production of succinate. Acetate was used as a carbon source for biomass generation. Even though higher succinate concentration (up to 127 g/L) was achieved with higher cell density of *E. coli* NZN111, succinate yield was significant lower. Only 71 g succinate per 100 g substrate was obtained when higher starch concentration was provided and

higher cell density was also achieved. In addition, Chen et al. (2014) claimed that the specific succinate productivity at 338 mg/g CDW/h was obtained. In contrast to Chen et al. (2014) and other published works (Table 4.5), our fermentation condition differs in the use of inorganic salts medium rather than complex media, the lack of carbon dioxide gas providing and auxotrophic requirements, and the use of simple batch operation under anaerobic conditions. Growth and succinate production occur concurrently in a single step. Our single step succinate production was performed with lower cell density at maximum cell dry weight (CDW) of 2.52 g/L. No other carbon sources rather than cassava pulp were supplied during biomass generation. This resulted in a maximum specific succinate productivity of 409 mg/g CDW/h by *E. coli* KJ122 during fed-batch fermentation of cassava pulp. The specific succinate productivity by *E. coli* KJ122 was higher than those obtained from two-stage fermentation, which was reported by Chen et al. (2014). Therefore, our fermentation conditions would be expected to lower the cost of succinate production due to the simple use of carbon sources for both growth and succinate production, and purification and waste disposal by the strain with cassava pulp as carbon substrate.

The succinate titer and yield from cassava starch in the report of Chen et al. (2014) were higher than those from cassava pulp fermentation in our study. This was due to the fact that an enzymatic hydrolysis of starch is more efficient and yields more fermentable sugars, mostly glucose, leading to higher succinate production. In contrast, cassava pulp used in this study had fiber content of about 14.08±0.03 (%, w/w) which mainly comprised of cellulose, hemicelluloses, and lignin (Table 4.1). The lignocellulosic fibers in cassava pulp hindered the hydrolysis efficiency increasing the difficulty in enzymatic hydrolysis (Martinez et al., 2007). Also,

fermentable sugars including cellobiose released from cassava fiber is usually less efficient in utilization than that of glucose by *E. coli* KJ122. However, cassava pulp is known as agricultural waste and it is sold at a relatively lower price (about $0.016/kg pulp) when compared with cassava root or cassava starch, and the use of this material is limited to low-value animal feed (Sriroth et al., 2000; Virunanona et al., 2013). Cel and AMG enzymes cost $16.17 and $7.57/kg respectively, according to the manufacturer's price. Also, the cost of AM1 medium is estimated to be around $0.43/L. Based on our study, the production cost of succinate from cassava pulp was roughly estimated at approximately $5.53–6.44/kg succinate. This price is lower than succinate produced by petro-chemically, which is at the price of $5.9–9.0/kg (Zeikus et al., 1999). It is likely that the utilization of cassava pulp in succinate production may add a great value to cassava pulp. Consequently, this may turn low-value agricultural products into high-value-added chemicals.

4.4 Conclusion

Succinate production from cassava pulp by *E. coli* KJ122 was reported in this study. Succinate was produced with impressive titers, yields, and productivities under simple anaerobic conditions in a low-cost medium using fed-batch SSF and favorably compared with other published works (Table 4.5). Due to the high prices of commercial enzymes, a reduction in the amount of enzymes used for cassava pulp hydrolysis would improve the overall production process economy. The strategies for decreasing enzymes loadings and for feeding substrate need to be further investigated in conjunction with improvements in succinate titer, yield, and productivity.

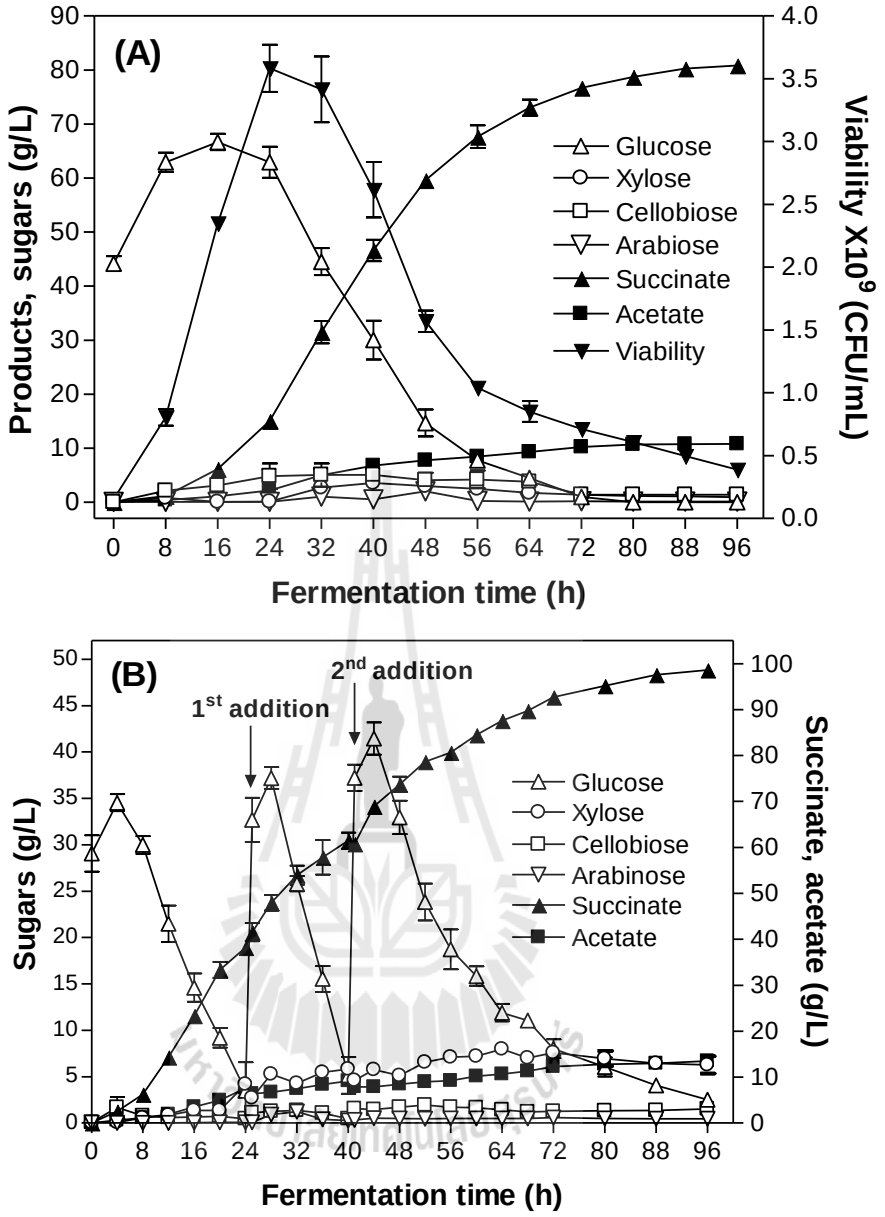

Figure 4.6 Time course of succinate production under (A) batch (B) fed-batch SSF using *E. coli* KJ122 in a 2-L stirred bioreactor. Batch SSF was conducted with 12% (w/w) dried cassava pulp hydrolyzed at 39°C with 2% AMG + 3% (v/w, on dry matter) Cel. Fed-batch SSF was conducted with initial 5% (w/w) dried cassava pulp hydrolyzed with 2% AMG + 3% (v/w, on dry matter) Cel. After culture of 24 and 40 h, cassava pulp and optimal enzymes mixture were intermittently added to yield a dry matter of cassava pulp at 15% (w/w). The culture was controlled at pH 6.5 and at temperature 39°C. The total incubation time was 96 h.

Table 4.5 Comparison of succinate from various cellulosic substrates by different microorganisms.

Biomass resource	Microorganism	Media/condition	Mode of process	Succinate (g/L)	Yield[a] (g/100 g substrate)	Productivity[b] (g/L/h)	References
Corn straw	*A. succinogenes* CGMCC1593	Complex medium supplemented with 5 g/L yeast extract, 5 g/L corn steep liquor, CO_2 gas sparging at 0.1 vvm, 20-80 g/L $MgCO_3$, pH maintained with 10 M NaOH, single step fermentation.	Batch SHF	45.5	80.7	0.95	Zheng et al., 2009
			Fed-batch SHF	53.2	82.5	1.21	
Corn stover	*A. succinogenes* CGMCC1593	Complex medium supplemented with 20 g/L corn steep liquor, pH maintained with 40 g/L $MgCO_3$, single step fermentation.	Batch SSF	47.4	72	0.98	Zheng et al., 2010
Waste bread	*A. succinogeness*	Complex medium supplemented with 200 mg/L free amino acid corresponding to 4 g/L yeast extract, CO_2 gas sparging at 0.5 vvm. pH maintained with 10 M NaOH, single step fermentation.	Batch SSF	47.3	55	1.12	Leung et al., 2012
Rapeseed meal	*A. succinogenes* ATCC55618	Complex medium supplemented with 15 g/L yeast extract, CO_2 gas sparging at 0.1 vvm, pH maintained with 2 M Na_2CO_3, single step fermentation.	Batch SSF	15.5	12.4	0.22	Chen et al., 2011
			Fed-batch SSF	23.4	11.5	0.33	
Corn stalk	*E. coli* SD121 (*ptsG* mutation and *ppc* expression)	Complex medium supplemented with 10 g/L yeast extract, 20 g/L peptone, pH maintained with 4 M Na_2CO_3, single step fermentation.	Batch SHF	57.81	87	0.96	Wang et al., 2011a
Cotton stalk	*A. succinogenes* ATCC 55618	Complex medium supplemented with 30 g/L yeast extract, pH maintained with 4 M Na_2CO_3, single step fermentation.	Batch SSF	63	64	1.17	Li et al., 2013

Table 4.5 (Continued).

Biomass resource	Microorganism	Media/condition	Mode of process	Succinate (g/L)	Yield[a] (g/100 g substrate)	Productivity[b] (g/L/h)	References
Cassava starch	*E. coli* NZN111	Salt medium (SM2) supplemented with 1% (w/v) vitamin B_1 and 30 mg/L kanamycin, two-state fermentation.	Fed-batch SSF	105.13	78	1.38	Chen et al., 2014
Cassava pulp	*E. coli* KJ122	A low salt medium (AM1) supplemented with 100 mM $KHCO_3$, pH maintained with 1:1 mixture of 3M KOH+1.5M K_2CO_3, single step fermentation.	Batch SHF	41.46	82.33	0.84	This study
			Batch SSF	80.86	70.34, [81.23][c]	0.84	
			Fed-batch SSF	98.63	71.64, [85.64]	1.03	

[a] The succinate yield was calculated based on the amount of succinate produced from 100 g dry substrate provided during the fermentation.

[b] The succinate productivity was calculated as the maximum concentration of succinate in the medium divided by the incubation time.

[c] The succinate yield in the brackets was calculated based on the amount of succinate produced from 1 g sugars consumed, and was expressed as a percentage. Assumption that the enzymatic hydrolysis of 1 g dry cassava pulp is generated 0.85 g of total sugars.

CHAPTER V

MUTATION OF GALP IMPROVED FERMENTATION OF MIXED SUGARS TO SUCCINATE USING ENGINEERED *ESCHERICHIA COLI* AS1600a AND AM1 MINERAL SALTS MEDIUM

5.1 Introduction

Sugars derived from lignocellulosic biomass represent an attractive feedstock for the fermentative production of chemical and plastics, replacing petroleum and eliminating competition for food (starch-based products). However, harsh pretreatments are required to soften this structure and increase access to cellulase enzymes (Keating et al., 2014). Dilute acid pretreatment can provide quantitative hydrolysis of hemicellulose polymers into monomeric sugars, but also creates toxic side products from sugars and lignin. Side products such as furfural, a dehydration product of pentose sugars, strongly inhibit growth and fermentation (Miller et al., 2009).

Several approaches are available to reduce toxicity of dilute acid hydrolysates for ethanol production. Including evaporation of volatiles under vacuum (Frazer and McCaskey, 1989; Chandel et al., 2013; Geddes et al., 2015), addition of sodium metabisulfite (Nieves et al., 2011), and base-treatments with ammonia or lime (Martinez et al., 2000; Geddes et al., 2013). Recently, Geddes et al. (2015) evaluated

that effectiveness of combining treatments (vacuum evaporation, laccase, high pH, bisulfite, and micro-aeration) to eliminate all inhibitory activity in dilute hemicelluloses hydrolysate. Removing all toxins in this way, however, may increase process cost and reduce yields of fermentable sugars (Keating et al., 2014).

Genetic improvement of biocatalysts arguably offers the most cost-effective approach to mitigate inhibitors in lignocellulose hydrolysates and to improve performance during fermentation. Many useful genes have been described for furfural tolerance in *E. coli* (Wang et al., 2013; Miller et al., 2009) during ethanol production. Optimal biocatalysts must be able to co-ferment sugar mixtures (primarily glucose and xylose) within a single vessel, eliminating the need for liquid/solid separation or 4 expensive treatments to mitigate toxins. No such strains are currently available for the production of succinate, a commercial intermediate for plastics, surfactants, green solvents, and detergents (Zeikus et al., 1999).

E. coli KJ122 was originally developed to ferment pure glucose streams from starch into succinate using GalP for glucose uptake (Jantama et al., 2008b; Zhang et al., 2009). However, previous studies have reported that this strain performs poorly with xylose as a substrate (Wang et al., 2013). Xylose is the most abundant sugar in dilute acid hydrolysates of sugarcane bagasse. Effective use of xylose, glucose, and other sugars by biocatalysts is desirable for commercialization of lignocellulosic feedstocks. In this study, we describe a derivative of KJ122 (strain AS1600a) that ferments xylose to completion as the sole carbon source, in sugar mixtures, and in a dilute acid hydrolysate of sugarcane bagasse using a simple mineral salts medium.

5.2 Materials and methods

5.2.1 Strains and plasmids

Strains, plasmids, and primers used in this study are listed in Table 5.1. All chromosomal modifications were made in *E. coli* KJ122. Luria Bertani (LB) medium was used for plasmid constructions (Top10F' host), *galP* gene deletion (KJ122) and plasmid transformation of succinate-producing strains. After genetic manipulations, succinate biocatalysts were grown in AM1 mineral salts medium (Jantama at al., 2008b; Martinez et al., 2007).

5.2.2 Isolation, sequencing, and comparison of chromosomal DNA

After 16th serial transfers, the xylose-evovled colonies were isolated from solid medium and tested for succinate production in pH-controlled fermentations with 10% (w/v) xylose. One of the best xylose-evolved colonies exhibiting a fast cell growth and produced highest succinate from 10% (w/v) xylose was assigned, named as *E. coli* AS1600a. Genomic DNA samples from *E. coli* (ATCC 8739), KJ122, and AS1600a were purified according to the bacterial genomic DNA protocol from the DOE Joint Genome Institute (http://jgi.doe.gov). Next-generation sequencing was performed using Illumina paired-end technology (150 bp read length). Sequencing and bioinformatics was provided by the Tufts University Core Facility (Boston, MA). Sequences were aligned and compared (CLC Sequence Viewer, Qiagen, Valencia, CA) using *E. coli* ATCC 8739 (Accession number NC_010468.1, NCBI) as the template.

5.2.3 Construction of expression vector pLOI5883.

Many plasmids that are stable in LB medium are less stable in AM1 mineral salts medium. A stable expression vector (pLOI5883) was constructed by replacing the pBR322 *oriR* replicon in pTrc99a with the RSF1010 replicon from pLOI707EH (Arfmann et al., 1992). Primer pairs RSF1010rep and pTrc99aΔ*oriR* were used to amplify the RSF1010 replicon and the pTrc99a backbone, omitting *oriR*. After amplification, both fragments were digested with *Spe*I and ligated (Figure 5.1). The resulting plasmid, pLOI5883, was confirmed by Sanger sequencing (Interdisciplinary Center for Biotechnology Research, University of Florida). After 5 serial transfers of KJ122 (pLOI5883) in AM1 glucose (50 g/L) broth without antibiotics, 100% of colonies retained the plasmid.

5.2.4 Cloning *galP* and *galP** into expression vectors

The *galP* gene was amplified from *E. coli* KJ122 (*galP*) and AS1600a (*galP**) using primer pair *galP*3 (Table 5.1) and Phusion polymerase (New England Biolabs, Ipswich, 6 MA). Amplified fragments were cloned into pLOI5883 between the *Nde*I and *Pst*I sites. Resulting plasmids were designated pLOI5746 and pLOI5747 (Figure 5.1), expressing the mutated *galP** and native *galP* genes, respectively. Constructions were confirmed by Sanger sequencing. Expression was induced by adding 10 µM IPTG.

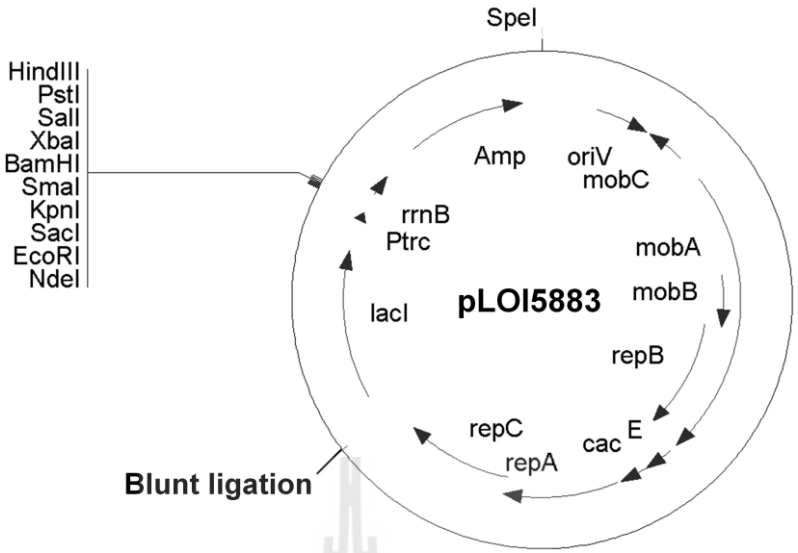

Figure 5.1 Expression Vector. Native *galP* and mutated *galP** were inserted between the *Nde*I and *Pst*I sites to produce pLOI5747 and pLOI5746, respectively and used to complement a derivative of KJ122 in which *galP* had been deleted.

5.2.5 Construction of vector (pLOI5899) for chromosomal integration

Previous studies used pLOI4162 (Jantama et al., 2008b) containing a *cat-sacB* cassette for selection of integration (chloramphenicol resistance) and counter selection (resistance to sucrose). Expression of *sacB* (dextran-sucrase) is lethal for *E. coli* in the presence of sucrose. The native *sacB* terminator was added to this cassette to reduce transcription of downstream genes. The backbone of pLOI4162 was amplified using primer pair 4162 (omitting the *sacB* gene). The native *sacB* gene including transcriptional terminator was amplified from *Bacillus subtilis* YB886 using primer pair 5899. After digestion with *Xba*I and *Sac*I, the two amplified

fragments were ligated to make pLOI5899 (Figure 5.2). Construction was confirmed by Sanger sequencing.

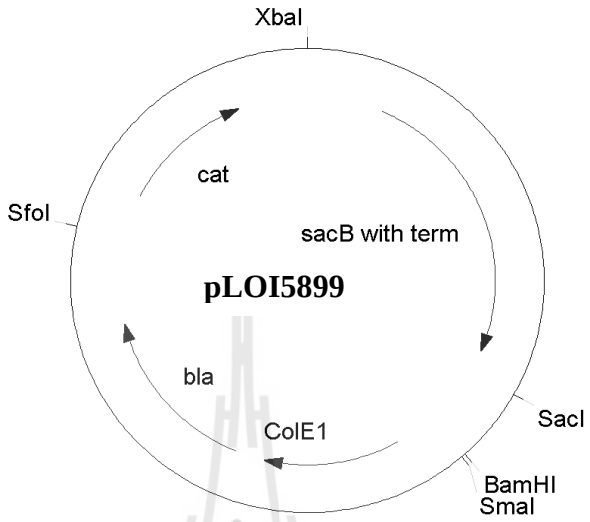

Figure 5.2 Vector containing the *cat-sacB* cassette for selection during integration. *SacB* without transcriptional terminator was replaced with a longer *sacB* fragment (*Xba*I-*Sac*I fragment) that included the terminator. This change was made to reduce read through from the *sacB* promoter and may also stabilize the message for improved selection (sucrose resistance).

Table 5.1 Strains, plasmids and primers used in this study.

Strains and plasmids	Relevant characteristics or sequence	Source or references
Strains		
E. coli KJ122	*E. coli* ATCC 8739 (Δ*ldhA*, Δ*adhE*, Δ*ackA*, Δ(*focA-pflB*) ΔmgsA, Δ*poxB*, Δ*tdcDE*, Δ*citF*, Δ*aspC*, Δ*sfcA*, *pck**, *ptsI**)	Jantama et al., 2008b; Zhang et al.,2009
E. coli KJΔgalP	KJ122 Δ*galP::cat-sacB*	This study
E. coli AS1600a	Clone from 16th transfer of *E. coli* KJ122 in 10% xylose containing point mutations in *galP* (*galP**) and an *rhs*-like gene	This study
E. coli Top10F'	Host used for plasmid construction	Thermo Fisher
Bacillus subtilis	Strain YB886; Source of *sacB* gene with native terminator	Laboratory collection
Plasmids		
pTrc99a	pTrc *bla oriR rrnB lacI^q*	Laboratory collection
pCR2.1-TOPO	*ori bla lacI^q* , vector for constructions	Thermo Fisher
pLOI707EH	Source of RSF1010 replicon	Arfman et al., 1992
pLOI4162	Integration vector	Jantama et al., 2008b
pLOI5899	Improved integration vector with *sacB* terminator	This study
pLOI5883	Expression vector, RSF1010 *rep* pTrc *bla rrnB lacI^q*	This study
pLOI5746	pLOI5883, expression of *galP** (mutant)	This study
pLOI5747	pLOI5883, expression of *galP* (native gene)	This study
Primers		
RSF1010 *rep*	Forward: GGAGCAGAAGAGCATACATCTGG Reverse: GGACTAGTCTGAAAGCGACCAGGTGCTCG	This study
pTrc99a Δ*oriR*	Forward: CGCTTACAGACAAGCTGTGACCG Reverse: GGACTAGTGTCTGACGCTCAGTGGAACGAA	This study
*galP*3	Forward: AGACATATGCCTGACGCTAAAAAACAGGGGCGGTC Reverse: AACTGCAGGCAGAGGATAGAGCGAAGAA	This study
4162	Forward: CTAGTCTAGACGTTCATGTCTCCTTTTTTATGTAC Reverse: CTAGGAGCTCTGCCGATATTGACTACCGGA	This study
5899	Forward: CTAGTCTAGAGCTTATGCCCATGCAACAGAAAC Reverse: CTAGGAGCTCTTAATTAGCCATTTGCTGC	This study
*galP*del	Forward:GAAAATCTTCGAACTGGCGGGTTATACCAAC ACTACCGAGCAAATGTGGGGTCGAGTGTGACGGAAG ATCACTTCGC Reverse:CGCGCAGTTTACGACCTTTCATCAGATTACGT TCAATATGTTCCAGCGAAACTCCGGTAGTCAATATCG GCAGAGCTC	This study

5.2.6 Deletion of *galP* in KJ122

Methods for chromosomal deletions have been previously described using Red recombinase technology (Datsenko and Wanner, 2000; Jantama et al., 2008b). Briefly, the *cat-sacB* cassette in pLOI5899 was amplified using primers that bridged the *cat-sacB* cassette and chromosomal region 5' and 3' to *galP* (primer pair *galP*del). The resulting amplified fragment was integrated into KJ122 by double homologous recombination with selection for chloramphenicol resistance (KJ122 ΔgalP::*cat-sacB*), designated strain KJ122Δ*galP*. Correct integration was confirmed by amplification and sequencing.

5.2.7 Fermentation

Fermentations were conducted in pH-controlled vessels (500 mL) with a 300 mL working volume (37°C, 150 rpm). Medium was maintained (pH 7.0) by automatic addition of a mixture of 6 N KOH and 3 M K_2CO_3 (1:4 ratio) (Jantama et al., 2008b). A low salts medium, AM1 (4.2 g/L total salts, Martinez et al., 2007) supplemented with 1 mM betaine, 100 mM $KHCO_3$ (Jantama et al., 2008b), and sugar (as indicated) was used for fermentation. Seed cultures were grown overnight (16 h) in AM1 medium supplemented with 5% (w/v) sugar. Fermentations were run with glucose, xylose, or a mixture of the two at either 5% (w/v) total sugar or 10% (w/v) total sugar. This total sugar concentration (100 g sugar/L) can be readily achieved from a 15% (w/v) slurry of sugarcane bagasse by the combination of dilute acid pretreatment and cellulase. Fermentations were inoculated to an initial OD_{550nm} of 0.1 (0.33 mg DCW/mL/OD). These were sampled and monitored for up to 120 h in some cases.

5.2.8 Metabolic evolution to select for improved xylose utilization

Strain KJ122 was repeatedly sub-cultured in AM1 medium containing 10% xylose until performance after 24 h was equivalent to KJ122 with 10% glucose (Figure 5.3; Figure 5.5A and Figure 5.5B) using pH-controlled fermenters (500 mL) with a 300 mL working volume (pH 7.0, 37°C, and 150 rpm) (Jantama et al., 2008a). After 16 serial transfers, the broth was spreaded on solid medium. Clones were tested individually for xylose utilization. One was selected for further work (designated AS1600a) and stored at -80°C as glycerol stocks (40% glycerol).

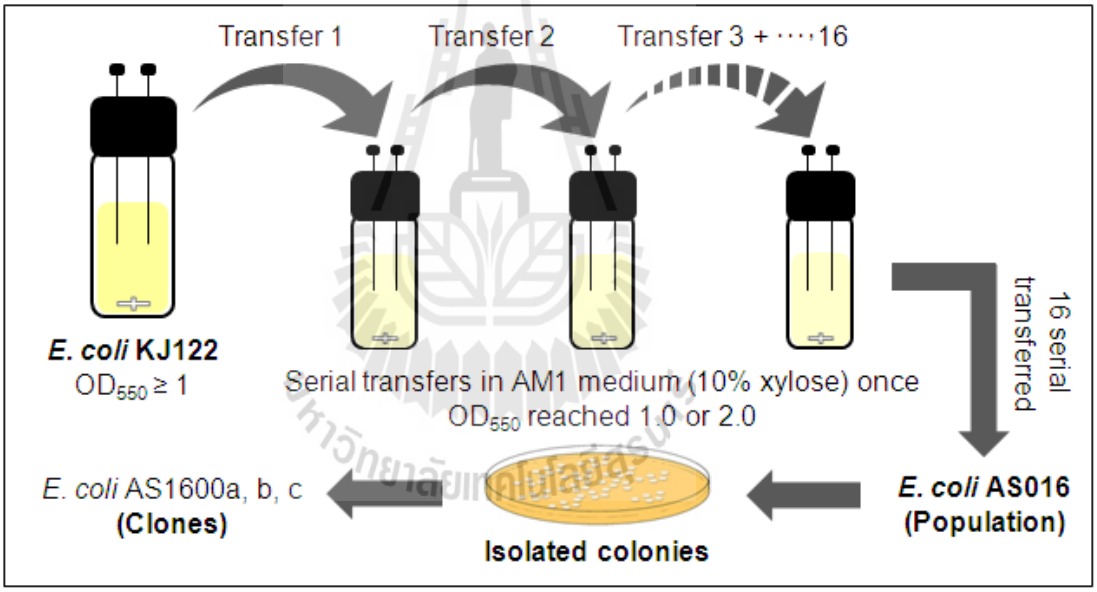

Figure 5.3 Metabolic evolution of *E. coli* KJ122 in xylose for succinate production

5.2.9 Preparation of sugarcane bagasse hydrolysate

Dilute acid hydrolysates of sugarcane bagasse were prepared at the University of Florida Biofuels Pilot Plant using steam gun (Figure 5.4) as described previously (8 kg phosphoric acid per tonne bagasse, 5 min, 190°C; Nieves et al.,

2011). Hemicellulose syrup (hydrolysate) was recovered by using a screw press, discarding solids. After removal of fine particulates with a Whatman GF/D glass fiber filter, clarified hydrolysate was stored at 4°C (pH 3.0) (Figure 5.5A). The average composition of sugarcane bagasse hydrolysates was 44 g/L xylose, 5 g/L glucose, 8 g/L arabinose, 4 g/L galactose, 5 g/L acetate, 2 g/L furfural, and trace amounts of hydroxymethyl furfural.

Figure 5.4 Showing the steam gun that used for production of hemicellulose hydrolysate.

5.2.10 Detoxification of sugarcane bagasse hydrolysate

Unless indicated otherwise, half of the weight of clarified bagasse hydrolysate was evaporated under vacuum at 55°C to remove volatile compounds by using a rotary evaporator (Buchi Rotavapor R110 evaporator, Flawil Switzerland) equipped with a Cole Palmer aspirator pump Model 7049-00 (Chicago, Illinois) (Geddes et al., 2015) (Figure 5.5B). The resulting concentrate was restored to its

original weight by adding sterile deionized water. Vacuum-treated hydrolysate was adjusted to pH 9.0 by addition of ammonium hydroxide (5N NH_4OH) and allowed to remain at room temperature for 16 h before inoculation. During this period, the broth pH declines to near pH 7. Stock solutions of bisulfite were freshly prepared and added immediately prior to inoculation (2 mM sodium metabisulfite, final concentration) (Nieves et al., 2011).

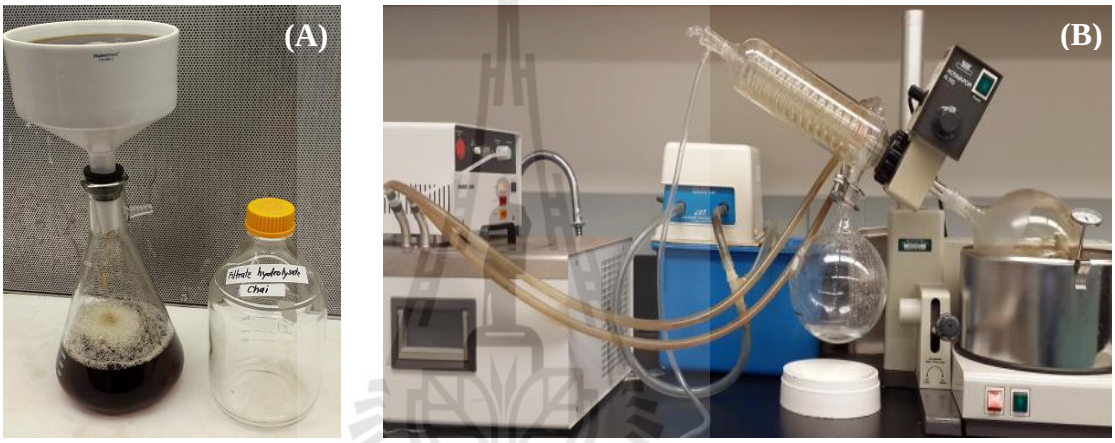

Figure 5.5 Filtration of hemicellulose hydrolysate (A). Rotary vacuum evaporator apparatus (B).

5.2.11 Analyses

Cell mass, organic acids, furfural, and sugars were measured during fermentation. Cell mass was estimated from OD_{550nm} (0.33 mg of cell dry weight/mL/OD) using a Bausch & Lomb Spectronic 70 spectrophotometer (Jantama et al., 2008b). OD was not measured in hydrolysate fermentations due to color interference (Geddes et al., 2014; 2015). Sugars, furans, and organic acids were analyzed by two high-performance liquid chromatography (HPLC) systems (Agilent

Technologies 1200) as described previously (Geddes et al., 2014). Sugars and furan were analyzed by a BioRad (Hercules, CA) Aminex HPX-87P ion exclusion column (80ºC; nano-pure water as the mobile phase, 0.6 mL/min). Organic acids were analyzed by a BioRad Aminex HPX-87H column (45ºC; 4 mM H_2SO as the mobile phase, 0.4 mL/min).

5.2.12 Statistical methods

Analysis of variance (ANOVA) was conducted using SPSS software (SPSS 17.0 for Windows; SPSS, Inc., Chicago, IL, USA). Results from at least 3 tests are reported as averages with standard deviations. Differences among mean values were established using Duncan's multiple range tests at 95% significance level.

5.3 Results and discussion

5.3.1 Fermentation of glucose and xylose by strain KJ122

Strain KJ122 was developed from *E. coli* ATCC8739 for the fermentation of pure glucose to produce high titers of succinate (Jantama et al., 2008b), however, it has been reported to ferment xylose slowly (Wang et al., 2013). This problem was confirmed using 10% (w/v) sugars and served as a starting point for strain improvement (Figure 5.7A and 5.7B). Strain KJ122 exhibited a lag time of 48 h with 10% (w/v) xylose with half of the xylose left unfermented after 120 h of fermentation (Figure 5.6A and 5.6B; Table 5.2). In contrast, strain KJ122 fermented 10% (w/v) glucose to completion without noticeable lag period (Figure 5.6A, 5.6B, and 5.7B). Reducing the xylose concentration from 10 to 5% (w/v) improved fermentation by reducing the lag time to 24 h (Figure 5.7C) and fermenting 5% (w/v) xylose to

substantial completion within 96 h of fermentation. After fermentation for 120 h, 44.17±0.78 g/L and 85.46±1.78 g/L succinate were produced from 5 and 10% (w/v) glucose, respectively. Succinate production from 10% (w/v) glucose (85.46±1.78 g/L) was 2.5 times higher than succinate production from 10% (w/v) xylose (37.49±1.72 g/L). Under xylose fermentation by wild type *E. coli*, the ABC transporter consumes one ATP to transport a xylose molecule and other ATP is needed for phosphorylation of xylulose. While conversion of xylose to pyruvate only yielded 0.67 net ATPs per mole xylose (Hasona et al., 2004). However, wild type *E. coli* produces equimolar of acetate and ethanol from two pyruvates. Therefore, the conversion of xylose to acetate and ethanol increases the overall redox balance and the net ATP yield from 0.67 to 1.5 per mole xylose, which meet the requirement for xylose metabolism (Hasona et al., 2004). Because of inactivation of pyruvate formate lyase (PFL), the strain cannot convert pyruvate to acetyl coenzyme A, the required precursor for acetate and ethanol production. Consequently, the strains could not produce an additional ATP required for their growth (Liu et al., 2012). Furthermore, in the conversion of xylose to succinate, there is not insufficient pyruvate and acetate generation to supply ATP for xylose utilization, which resulted in an ATP deficit for succinate production (Liu et al., 2012). The *E. coli* KJ122 strain lacking of PFL could not utilize xylose for their growth and succinate production probably due to insufficient ATP supply.

Considering succinate yield, the succinate yields by KJ122 with glucose as substrate were also slightly higher than those with xylose, 0.88 g/g glucose (metabolized) as compared with 0.81 g/g xylose (Table 5.2). More effective fermentation of both xylose and glucose is needed to allow the use of mixed sugars

from lignocellulose as a feedstock. The higher succinate yield obtained from hexose sugars fermentation when compared with pentose sugars could probably be explained by the fact that there are different in ATP yields and redox balances of these substrates. In particular, the conversion of xylose to PEP results in net consumption of ATP whereas the formation of PEP from glucose is ATP neutral (Andersson et al., 2007).

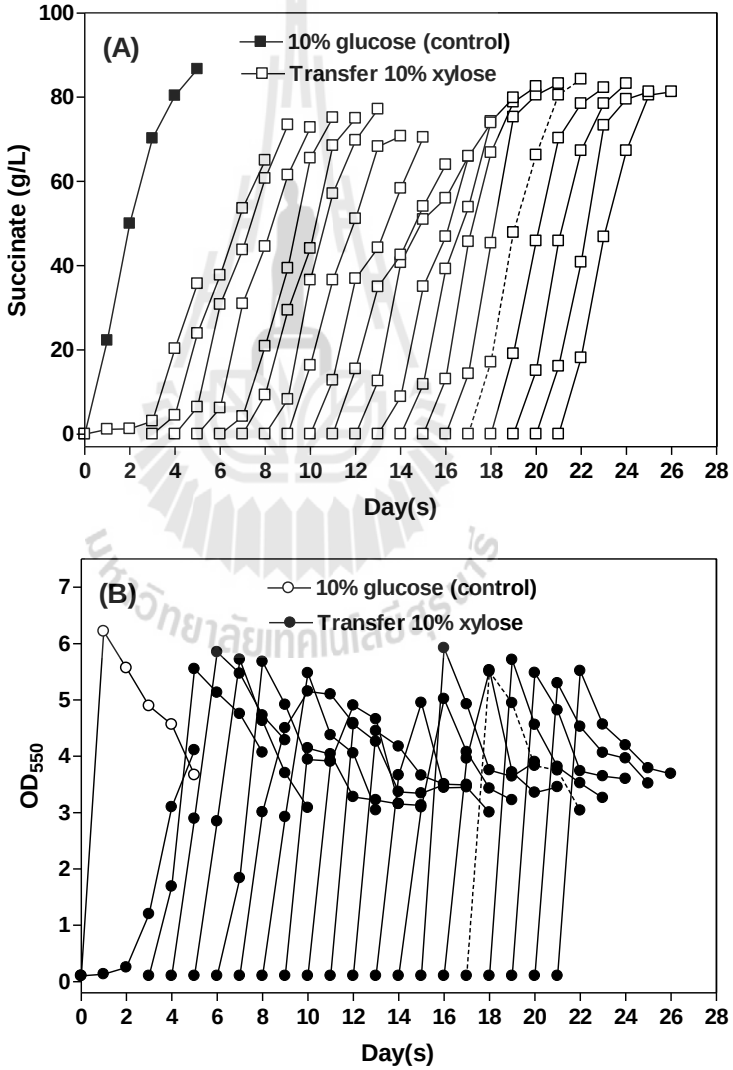

Figure 5.6 Metabolic evolution profile of *E. coli* KJ122 in xylose for succinate production. Dash lines indicate the source for isolation of AS1600a. (A) Succinate production; (B) Cell mass.

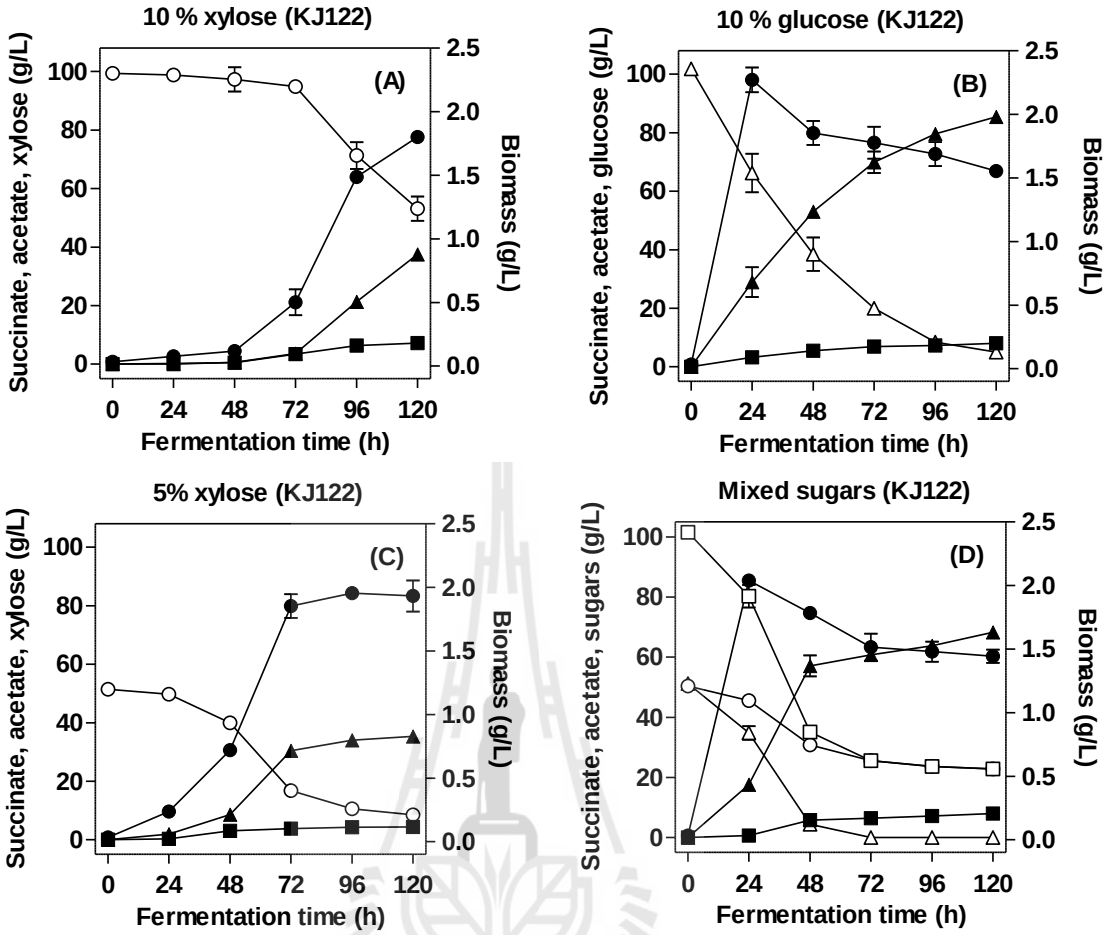

Figure 5.7 Time course of succinate production from glucose and xylose by *E. coli* KJ122 (parent) in AM1 mineral salts medium. (A) Fermentation of 10% xylose; (B) Fermentation of 10% glucose; (C) Fermentation of a xylose (5%); (D) Fermentation of a sugar mixture containing 5% xylose and 5% glucose. Symbols for all: total sugars (open square), xylose (open circle), glucose (open triangle), biomass (filled circle), succinate (filled triangle), acetate (filled square).

Table 5.2 Comparison of fermentation profiles of succinate production from glucose and xylose by *E. coli* KJ122 and its derivatives.

Strains	Carbon sources	Sugars residual (g/L)		Succinate (g/L)	Acetate (g/L)	Maximum biomass (g/L)	Succinate yield (g succinate/sugar consumed)[A]	Maximum productivity (g/L/h)[B]
		Glucose	Xylose					
KJ122	5% xylose	-	8.50±0.37	35.36±0.14^{l}	4.35±0.10^{h}	1.93±0.12^{dc}	0.82±0.01^{d}	0.42±0.00^{f}
KJ122	5% glucose	0.00±0.00	-	44.17±0.78^{j}	4.09±0.78^{h}	2.03±0.04^{cd}	0.88±0.01^{abc}	0.87±0.02^{d}
KJ122	10% glucose	5.02±0.24	-	85.46±1.78^{ab}	8.07±0.77^{cd}	2.27±0.10^{ab}	0.88±0.02^{abc}	1.21±0.21^{abc}
KJ122	10% xylose	-	53.15±1.10	37.49±1.72^{k}	7.15±0.78^{e}	1.80±0.03^{e}	0.81±0.06^{d}	0.31±0.01^{f}
KJ122	Mixed sugars[C]	0.00±0.00	22.81±2.13	68.28±1.71^{g}	7.94±0.35^{cd}	2.04±0.03^{cd}	0.87±0.00^{bc}	1.11±0.03^{abc}
KJ122	Hydrolysate[D]	0.00±0.00	17.42±0.68	66.45±0.94^{h}	5.52±0.43^{g}	NM	0.80±0.01^{d}	0.68±0.06^{e}
AS1600a	10% xylose	0.00±0.00	5.00±0.28	84.26±1.37^{b}	8.41±0.75^{ab}	2.14±0.10^{a}	0.88±0.00^{abc}	0.96±0.00^{d}
AS1600a	10% glucose	15.05±0.05	-	75.09±1.68^{d}	6.27±0.29^{f}	2.17±0.24^{bc}	0.86±0.01^{c}	0.91±0.05^{d}
AS1600a	Mixed sugars	3.24±0.36	5.03±0.57	84.23±0.46^{b}	7.90±0.93^{cd}	2.43±0.03^{a}	0.90±0.02^{a}	1.12±0.07^{abc}
AS1600a	Hydrolysate	13.53±0.84	1.21±0.45	72.66±0.59^{e}	8.33±0.31^{abc}	NM	0.87±0.01^{abc}	0.59±0.01^{e}
KJ122 Δ*galP* + pLOI5747	10% xylose	-	40.48±1.09	46.32±1.69^{i}	4.21±0.13^{h}	2.00±0.08^{cd}	0.75±0.01^{e}	0.39±0.01^{f}
KJ122 Δ*galP* + pLOI5747	10% glucose	6.31±0.96	-	86.88±0.93^{a}	8.42±0.37^{ab}	2.36±0.07^{a}	0.91±0.00^{a}	1.24±0.02^{ab}
KJ122 Δ*galP* + pLOI5747	Mixed sugars	0.99±0.25	21.16±1.64	70.20±1.58^{f}	9.70±0.23^{a}	1.88±0.06^{e}	0.87±0.00^{abc}	1.10±0.02^{c}
KJ122 Δ*galP* + pLOI5746	10% xylose	-	6.99±0.50	85.18±0.34^{ab}	8.83±0.03^{b}	2.15±0.13^{c}	0.90±0.00^{ab}	1.11±0.06^{bc}
KJ122 Δ*galP* + pLOI5746	10% glucose	12.39±0.57	-	78.90±0.95^{c}	7.73±0.13^{de}	2.38±0.12^{b}	0.88±0.01^{abc}	0.89±0.06^{d}
KJ122 Δ*galP* + pLOI5746	Mixed sugars	3.49±0.16	4.68±0.39	85.53±0.09^{ab}	8.78±0.21^{b}	2.17±0.09^{c}	0.90±0.00^{ab}	1.25±0.07^{a}

[A]The succinate yield was calculated as grams of succinate formed divided by grams of the sugars consumed.

[B]The maximum succinate productivity was calculated from succinate concentration in the medium divided by incubation time.

[C]Mixed sugars are comprised of 5% (w/v) xylose and 5% (w/v) glucose.

[D]Sugarcane bagasse hydrolysate contained 10% (w/v) of initial total sugars concentration. Arabinose and galactose in hydrolysate were completely consumed at the end of fermentation. pLOI5747 (native *galP*), pLOI5746 (mutant *galP*), NM (not measured).

5.3.2 Metabolic evolution for improvment of xylose utilization

Metabolic evolution has been previously used to improve biocatalysts performance for many fermentation products including ethanol (Olsson et al., 2007; Yomano et al., 2008), D-lactate (Utrilla et al., 2012; Zhou et al., 2003), and succinate (Jantama et al., 2008a). This method was applied to *E. coli* KJ122 to develop an improved biocatalyst for the fermentation of xylose to succinate (Figure 5.3, 5.6A and 5.6B). Poor growth of KJ122 in AM1 medium containing 10% (w/v) xylose was used as the basis for this selection process. The initial inoculum grew very poorly during the first 3 days of incubation. After 3 days, this culture was transferred to fresh medium, and then transferred at 24-h intervals thereafter. Succinate production and growth (16 serial transfers in 10% (w/v) xylose) exceeded that of KJ122 with 10% (w/v) glucose (Figures 5.6A, 5.6B, and 5.7B; Table 5.2). Colonies were isolated from solid medium and tested for succinate production in pH-controlled fermentations with 10% (w/v) xylose. All appeared similar and one was selected for further study, designated AS1600a (Figure 5.8A). This strain produced 84.26±1.37 g/L succinate from 10% (w/v) xylose with a yield of 0.88 g/g sugar metabolized) (Table 5.2). Succinate yield with AS1600a and xylose was equivalent to the parent with 10% (w/v) glucose (85.46±1.78 g/L). AS1600a exhibited a 3-fold improvement in volumetric productivity with 10% (w/v) xylose (0.96 g/L/h) as compared with KJ122 (0.31 g/L/h). Unlike KJ122, strain AS1600a grew on 10% xylose without a noticeable lag phase, closely resembling the fermentation pattern with KJ122 and 10% glucose. With strain AS1600a, xylose was fermented more effectively than glucose.

Differences were also observed during the fermentation of an equal mixture of glucose and xylose (10%, w/v total sugars). The mutant AS1600a co-

fermented both glucose and xylose to near completion without a noticeable lag period and at similar rates (Figure 5.8D). In contrast, the parent strain KJ122 fermented glucose to completion but only half of the xylose was fermented (Figure 5.8D and Table 5.2). With these two sugars, the combined rates of xylose and glucose fermentation to succinate (maximum productivities) for AS1600a and for KJ122 strains were similar to when either only 10% (w/v) xylose or only 10% (w/v) glucose was used, respectively. Bao et al. (2014) reported that after ten generations of culture transfer, an evolved stain *E. coli* BA408 showed a 5.6-folds improvement in succinate production in chemical define medium using sugars mixture of glucose and xylose when compared with that of the parental strain *E. coli* BA305, a *pflB*, *ppc*, and *ptsG* deletion overexpressing the ATP-forming PEPCK from *B. subtilis* 168. This finding suggested that the industrial *E. coli* biocatalyst capable of producing succinate from mixtures of hexose and pentose sugars derived from lignocellulosic materials could be developed through traditional biology techniques, metabolic evolution. Last but not least, the indentification of gene mutations after metabolic evolution is needed to be investigated in order to understand which gene helps the cell to grow in xylose, under ATP-limiting condition.

5.3.3 A single mutation in *galP* is sufficient to improve xylose metabolism in KJ122

Chromosomal DNA from the parent KJ122 and the mutant AS1600a strain was isolated, sequenced, and compared. Only 2 new mutations were found, G236D in *galP* (glycine to aspartate) and L287Q in a cryptic 4.5 kbp rhs-like gene (leucine to glutamine), which were absent from many *E. coli* strains. Each mutation

resulted from a single nucleotide change within the coding region. The native *galP* permease is important for glucose uptake by KJ122 strain (Zhang et al., 2009), due to a mutation in *ptsI* that blocks glucose uptake by the native phosphotransferase system. This *ptsI* mutation also conserved phosphoenolpyruvate for succinate production and increased ATP yield when coupled with pyruvate carboxykinase (up-regulated in KJ122).

The *galP** mutation in AS1600a strain was investigated as a possible cause for improved xylose metabolism. The coding region for *galP* was deleted from KJ122 (parent) by double homologous recombination to make KJ122Δ*galP*. This strain grew poorly in AM1 medium with either 10% (w/v) glucose or 10% (w/v) xylose and was maintained on LB glucose plates. Growth on AM1 with either glucose or xylose was restored by supplying GalP activity from plasmids. Strains KJ122Δ*galP*(pLOI5747; wild type *galP*) and KJ122Δ*galP*(pLOI5746; mutant *galP**) were compared with the AS1600a (mutant) during fermentation with either only 10% (w/v) xylose or only 10% (w/v) glucose was used, and with either a mixture of 5% (w/v) glucose or 5% (w/v) xylose (Figure 5.7 and Table 5.2). Strains KJ122Δ*galP*(pLOI5746; *galP**) and AS1600a were very similar. Both strains fermented 10% xylose to succinate (without any noticeable lag periods) as effectively as the parent KJ122 and the KJ122Δ*galP*(pLOI5747; wild type *galP*) strain which fermented 10% (w/v) glucose (Table 5.2). However, strains with the *galP** were less effective than the parent strain with 10% (w/v) glucose alone. The *galP*-deleted strain with the *galP** mutant plasmid (pLOI5746) and AS1600a co-fermented the mixture of glucose and xylose (5% each) while the parent KJ122 and KJ122Δ*galP* strain with pLOI5747 (wild type *galP*) used glucose preferentially. With these two strains,

almost half of the xylose remained after 120 h of fermentation. Expressing the *galP** mutation (G236D) in KJ122Δ*galP* fully duplicated the xylose utilization phenotype of mutant AS1600a, without the mutation in the cryptic rhs-like mutant gene.

Sugar transporters often utilize multiple sugars with differing affinities. GalP appears to be particularly very versatile proton symport, also transporting fructose, glucose, and lactose (Zhang et al., 2009; Zheng et al., 2010). McDonald et al. (1997) and Maiden et al. (1987) reported that the GalP from *E. coli* is homologus to the L-arabinose-H+ symporter (AraE) and the D-xylose-H+ symporters (XylE) of *E. coli*, with 64 and 33% indentity, respectively. Additionally, the sugar speficity of GalP is very similar to the glucose transporters from human erythrocytes (GLUT1) and rat adipocytes (GLUT4) (Macdonald et al., 1997). Expression of an unmutated *galP* was able to replace the native phosphotransferase system for glucose in *E. coli* KJ122 (Jantama et al., 2008b; Zhang et al., 2009; Hernández-Montalvo et al., 2003), restoring growth and succinate production. Overexpression of native *galP* from a high copy vector increased both glucose and xylose metabolism in *Enterobacter cloacea* engineered for butanediol production (Li et al., 2015).

5.3.4 Mutation in cryptic gene related to rhs C-terminal tip in *E. coli* W (ATCC9637)

A second point mutation was found in AS1600a, within a large reading frame (4.5 kbp) that is 90% identical to a cryptic gene in *E. coli* W (ATCC9637), a putative *rhs* C terminal tip (Archer et al., 2011). This gene is absent in most of *E. coli* strains, including K12 (MG1655). Little is known about the activity and function of this protein. Some *rhs*-like proteins have been associated with intercellular

competition and cell inactivation. Amplifying this gene by PCR and cloning have proven difficult due to the highly repeated regions of sequence and length. This mutation was not required for reconstruction of xylose utilization in a KJ122Δ*galP*. The *galP** mutation alone was sufficient to fully confer the improvements in xylose fermentation without the *rhs*-like mutant.

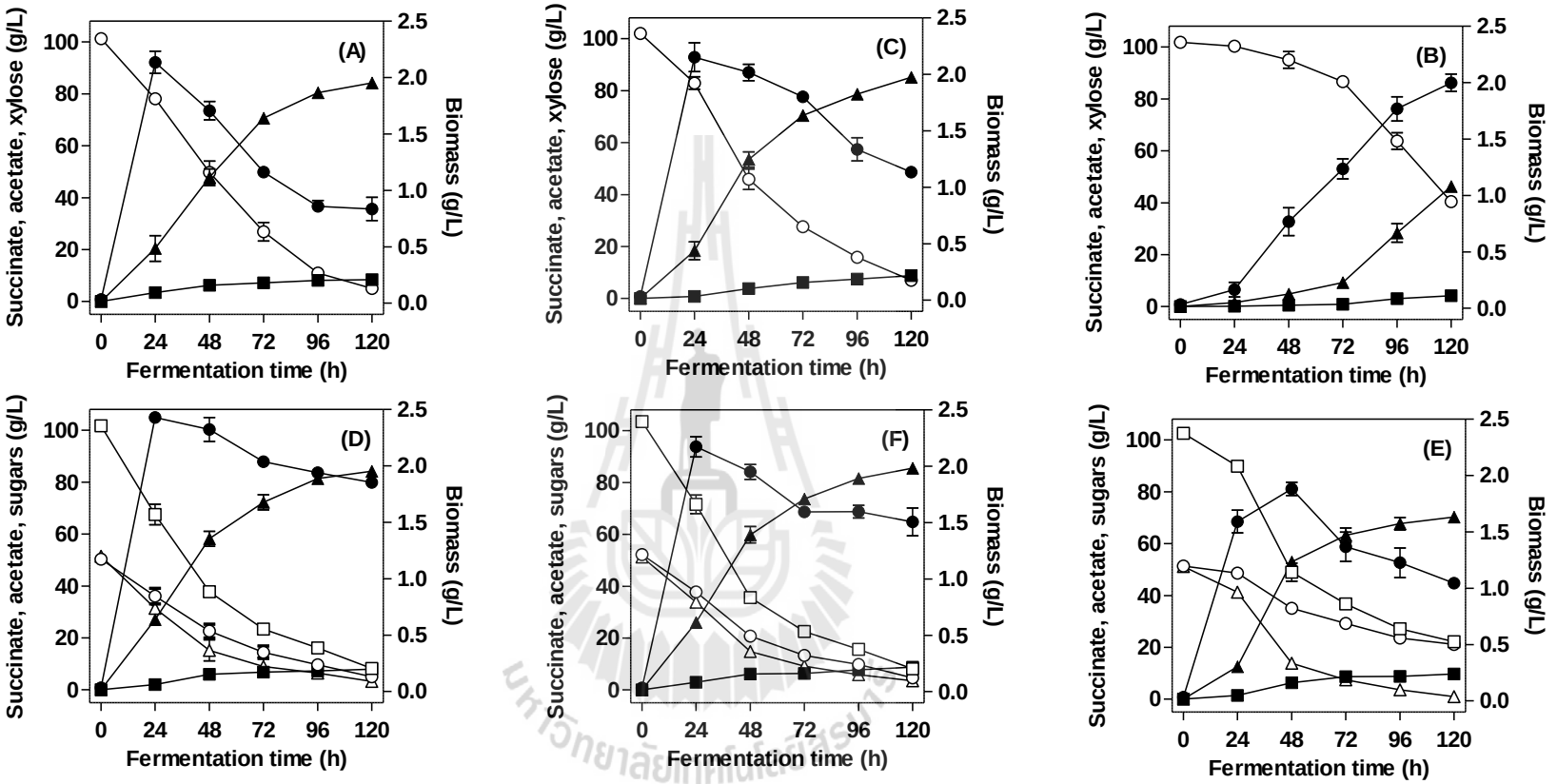

Figure 5.8 Effect of *galP** mutation on succinate production from glucose and xylose. A. AS1600a (improved mutant) with 10% xylose; B. KJ122Δ*galP*(pLOI5747 harboring wild type *galP*) with 10% xylose; C. KJ122Δ*galP*(pLOI5746 harboring *galP** mutation); D. AS1600a (improved mutant) with sugar mixture (5% xylose and 5% glucose); E. KJ122Δ*galP*(pLOI5747 harboring wild type *galP*) with sugar mixture (5% xylose and 5% glucose); F. KJ122Δ*galP*(pLOI5746 harboring *galP** mutation). Symbols for all: mixed sugars (open square), xylose (open circle), glucose (open triangle), biomass (filled circle), succinate (filled triangle), acetate (filled square).

5.3.5 Co-fermentation of sugars from lignocellulose hydrolysate derived from sugarcane bagasse

Mutant strain of AS1600a was compared with KJ122 (parent) strain using sugarcane bagasse as a substrate. Filtered sugarcane bagasse hydrolysate (55 g/L total sugar) was used to simulate hydrolyzed lignocellulose by adding 150 g/L glucose as a replacement for hydrolyzed cellulose fiber and diluting with an equal volume of water. The resulting broth contained mostly glucose and xylose with smaller amounts of arabinose and galactose, together with inhibitors from side reactions during dilute acid pretreatment. Concentrations of sugars (100 g/L) and inhibitors are equivalent to hydrolysate prepared from the slurry of 15% sugarcane bagasse (dry weight). The combination of treatments was used to mitigate toxicity in this broth based on prior studies with ethanologenic *E. coli* (Geddes et al., 2015). Furfural and other volatiles were removed by vacuum evaporation. At large scale, both may be useful as co-products. Other inhibitors were mitigated by the pH 9 treatment with ammonia (16 h incubation) and addition of 2 mM sodium metabisulfite. Phosphate used for dilute acid pretreatment and ammonia added for neutralization served as macronutrients. Trace metals and magnesium sulfate were added to complete the AM1 medium. All sugars were co-metabolized to differing extents. The parent KJ122 strain fermented small amounts of galactose and arabinose from sugarcane hydrolyslate to completion within 48 and 96 h of fermentation, respectively. The most abundant sugar glucose was fully metabolized after 144 h, but 80% of the xylose remained unfermented after 144 h (Figure 5.9A and 5.9C). In contrast, the AS1600a strain containing the *galP** mutation fermented galactose, arabinose and xylose to near completion (Figure 5.9B and 5.9D), but left 20% of the

glucose unused. With AS1600a, fermentations times were reduced by 24 h each for galactose and arabinose with much more reduction time in the case of xylose. Interestingly, the weights of unfermented sugars in hydrolysate (glucose with AS1600a and xylose with KJ122) were similar for both strains. The differences in arabinose and xylose utilization were surprising. Except for uptake, reversible xylose isomerization, and initial phosphorylation, remaining genes encoding metabolism are the same for both pentose sugars. Differences in rate of utilization must reside with these early steps. The change in sugar preference in AS1600a and KJ122Δ*galP*(pLOI5746) is presumed to be resulted from a change in GalP structure, consistent with uptake as the determining event. It also found that there was about 10% higher in succinate production from hydrolysate fermentation (72.66±0.59 g/L versus 66.45±0.94 g/L) by the AS1600a strain when compared with the KJ122 strain. However, the lag phase period of hydrolysate fermentation was longer than that of pure sugar mixture fermentation (Figure 5.8 and 5.9). It indicates that the improvement of simultaneous utilization of sugars mixture by the *E. coli* AS1600a dose not only increase xylose utilization but also enhance the succinate production from sugarcane bagasse hydrolysate.

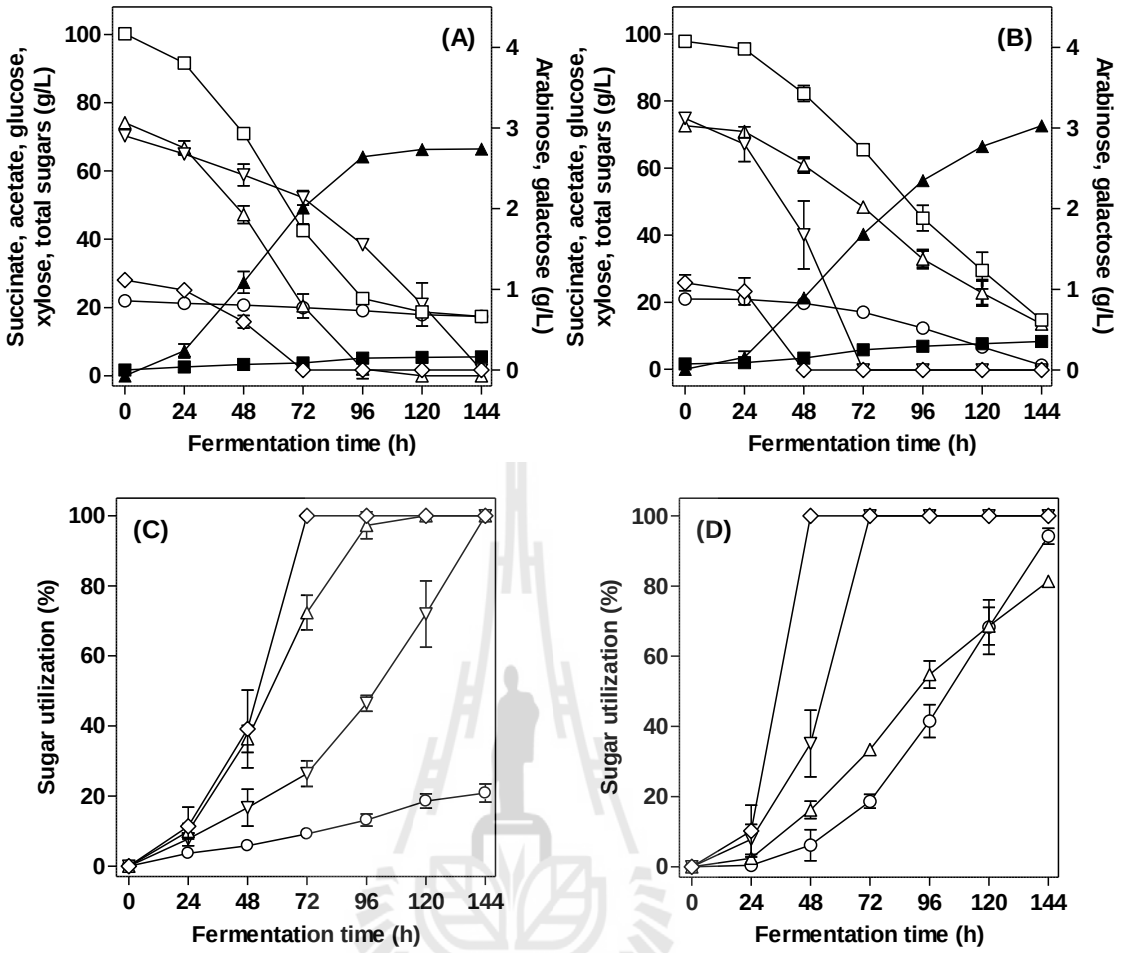

Figure 5.9 Time course of fermentation of mixed sugars from sugarcane bagasse hydrolysate by KJ122 (parent) and AS1600a (improved mutant). A. Succinate production by KJ122. B. Succinate production by AS1600a. C. Sugar utilization by KJ122. D. Sugar utilization by AS1600a. Symbols for all: total sugars (open square), xylose (open circle), glucose (open triangle), arabinose (open inverted triangle), galactose (open diamond), succinate (filled triangle), acetate (filled square).

5.3.6 Comparison of xylose fermentation by *E. coli* strains engineered for succinate production

Many mutants of *E. coli* have been constructed to be able to effectively ferment xylose alone, mixtures of sugars, and lignocellulose hydrolysates (corn stalk, sugarcane bagasse, etc). However, most of prior studies have been used complex medium supplemented with such as tryptone, yeast extract, corn steep liquor, or Luria broth (Table 5.3). Product yields have been rather good on a sugar basis in some cases, with product titers of over 50 g/L. However, these complex nutrients may be prohibitively expensive for most commercial processes. Others use multistep processes with a separate aerobic growth phase followed by a production phase (Andersson et al., 2007; Wang et al., 2011a; Liu et al., 2013a) or repetitive fermentations (Liang et al., 2013; cell recycle) which may reduce media costs. Our work using AM1 mineral salts medium (Martinez et al., 2007) in simple batch fermentations could achieve titers of over 70 g/L with pure xylose, an equal weight mixture of glucose and xylose, and from sugarcane bagasse hydrolysate with yield above 0.8 g/g sugar. The use of mineral salts medium offers two main advantages over complex medium supplementation: reduction in media cost and simpler purification.

Table 5.3 Comparison of stain development for succinate production from xylose, sugars mixture and hemicelluloses hydrolysate fermentation.

Strain designation	Media/mode of process	Carbon sources	Succinate (g/L)	Yield (g/g substrate)	References
E. coli AFP184, Δ*ldhA*, Δ*pflB* and Δ*ptsG*	Complex medium supplemented with 0.4 g/L corn steep liquor (50% solid), dual-phase fermentation	Xylose	25.0	0.50	Andersson et al., 2007
		Xylose/glucose mixtute	27.0	0.60	
E. coli SD121 Δ*ldhA*, Δ*pflB*, Δ*ptsG* and cyanoacterial ppc overexpression	Complex medium supplemented 20 g/L tryptone and 10 g/L yeast extract, two-stage culture	50 g/L glucose and 50 g/L xylose	58.6	0.59	Wang et al., 2011a
		Corn stalk hydrolysate (44 g/L initial sugars and maintain at 10 g/L during fermentation	57.8	0.87	
E. coli BA204, Δ*ldhA*, Δ*pflB*, Δ*ppc* and overexpression of ATP-forming (PEPCK)	Complex medium supplemented with LB, dual-phase fermentation	20 g/L xylose	9.58	0.87	Liu et al., 2012
		10 g/L xylose +10 g/L glucose	9.18	0.72	
		Corn stalk hydrolysate (20 g/L total sugars)	11.13	1.02	
E. coli BA305, Δ*ldhA*, Δ*pflB*, Δ*ppc* and overexpression of ATP-forming (PEPCK)	Complex medium, dual-phase fermentation.	2 g/L glucose + 13.45 g/L xylose + 2 g/L arabinose	18.88	1.10	Liu et al., 2013a
		Sugarcane bagasse hydrolysate (19.66 g/L total sugars)	19.20	0.96	
E. coli BA305, Δ*ldhA*, Δ*pflB*, Δ*ppc*, Δ*ptsG* and overexpression of ATP-forming (PEPCK)	Complex medium supplemented with LB, Simple batch fermentation	20 g/L xylose	5.2	0.72	Liu et al., 2013b
	Simple batch fermentation	9 g/L xylose + 9 g/L glucose	10.6	NR	
	Simple batch fermentation	Sugarcane bagasse hydrolysate	10.1	0.66	
	Feb-batch fermentation.	Sugarcane bagasse hydrolysate	39.3	0.97	

Table 5.3 (continued).

Strain designation	Media/mode of process	Carbon sources	Succinate (g/L)	Yield (g/g substrate)	References
E. coli BA305, Δ*ldhA*, Δ*pflB*, Δ*ppc* and overexpression of ATP-forming (PEPCK)	LB medium supplemented with chemically defined medium, repetitive fermentation.	Xylose	24.0	0.98	Liang et al., 2013
		Sugars mixture	29.5	0.95	
		Sugarcane bagasse hydrolysate	24.5	0.87	
E. coli DC115, Δ*ldhA*, Δ*pflB*, Δ*ptsG* selected by the atmospheric and room-temperature plasma mutation system combining with a 15th serials transfer in 1.5% xylose	LB medium supplemented with chemically defined medium, simple bath fermentation.	20 g/L xylose	12.1	0.67	Jiang et al., 2014
		3 g/L glucose, 2 g/L arabinose and 30 g/L xylose	27.7	0.79	
		Corn stalk hydrolysate (35 g/L total sugars)	21.1	0.76	
E. coli BA408, Δ*ldhA*, Δ*pflB*, Δ*ppc*, Δ*ptsG* and overexpression of ATP-forming (PEPCK) 10th serials transfer in 15 g/L sugars mixture	LB medium supplemented with chemically defined medium, simple bath fermentation.	24 g/L xylose + 6 g/L glucose	24.6	0.81	Bao et al., 2014
		Corn stalk hydrolysate (35 g/L total sugars)	23.1	0.85	
E. coli AS1600a , Δ*ldhA*, Δ*adhE*, Δ*ackA*, Δ(*focA-pflB*) ΔmgsA Δ*poxB* Δ*tdcDE* Δ*citF* Δ*aspC* Δ*sfcA* and 16th serials transfer in 10% xylose	A low salt medium (AM1, 4.2 g/L total salt), simple batch fermentation	100 g/L xylose	84.7	0.90	This study
		50 g/L glucose+ 50 g/L xylose	84.2	0.88	
		50% (v/v) sugarcane bagasse hydrolysate + 75 g/L glucose (100 g/L total sugars)	72.66	0.88	

4. Conclusion

E. coli strain KJ122 was designed for the fermentation of glucose to succinate (Jantama et al., 2008; Wang et al., 2013), However, it performed poorly with 10% (w/v) xylose in AM1 mineral salts medium. An improved strain was easily obtained by growth-based selection, designated AS1600a. This mutant strain could be able to also co-ferment a glucose-xylose mixture, and a mixture of 4 sugars in sugarcane bagasse hydrolysate. A single mutation in *galP* (G236D) was shown to be responsible for the improvement in pentose fermentation. This *galP** mutant gene may be useful for the improvement of sugar metabolism in other biocatalysts.

CHAPTER VI

IMPROVING FURFURAL TOLERANCE FOR SUCCINATE PRODUCTION FROM SUGARCANE BAGASSE HYDROLYSATE BY *ESCHERICHIA COLI* AS1600a

6.1 Introduction

Lignocellulosic biomasses are non-food feedstocks, abundant, renewable and cheap. It is considered as an attractive substrate for the production of bio-based economy like succinate, a commercial intermediate for bio-degradable plastics, surfactants, green solvents, and detergents (Zeikus et al., 1999). However, lignocellulosic materials are resistant to degradation, resulting in the need for pretreatment processes that allow access to the fermentable sugars (Keating et al., 2014). Dilute acid pretreatment is one of the most effective pretreatment methods characterized by a great advantage of producing pentose-rich syrups (*i.e.*, hydrolysate) that can be fermented by microorganisms without the use of hemicellulases or cellulases. During pretreatment, inhibitory compounds to microbial fermentation are generated (Keating et al., 2014). Among these, furfural has been shown to be a key inhibitory compound, acting synergistically with other inhibitors.

Furfural (2-furaldehyde) is a side product formed by the dehydration of pentose sugars and hexoses degraded to hydroxymethyl furfural (HMF) during dilute

acid pretreatment of lignocellulosic biomass (Zaldivar and Ingram, 1999). Furfural is more toxic than HMF (Rahman and Hadi, 1991). It damaged DNA by causing a single strand break and DNA mutagenesis, mainly in AT-rich region (Hadi et al., 1989). The reduction of furfural to furfuryl alcohol (less toxic compound) improved cell growth and fermentation of diluted acid hydrolysates of hemicelluloses (Zheng et al., 2013).

Many useful genes have been reported for furfural tolerance in ethanologenic *E. coli*. An expression of a native *fucO* gene, encoding an NADH-dependent, L-1,2-propanediol reductase, has been reported to increase furfural resistance in ethanologenic *E. coli* (Wang et al., 2011c). Zheng et al. (2013) applied the saturation mutagenesis combining with growth-based selection to isolate a mutated form of *fucO* by replacing the most abundant codon for leucine with the most abundant codon for phenylalanine. The mutant *fucO* (L7F), *fucO** gene increased FucO activity by more than 10-fold and doubled the rate of furfural metabolism during fermentation when compared with the wild-type *fucO*. Overexpression of *pntAB*, a cytoplasmic NADH/NADPH transhydrolgenase, has also been evaluated to increase furfural tolerance in ethanologenic *E. coli* (Miller et al., 2009). Wang et al. (2012) also found that plasmid expression of *ucpA*, a cryptic gene adjustment to sulfur assimilation operon, increased furan tolerance in ethanologenic strain LY180 and wild-type strain W. Deletion of *ucpA* shows decreasing of furfural tolerance, but the exact function of *ucpA* and its mechanism for conferring furfural tolerance is unknown. Zheng et al. (2012) demonstrated that the growth of *E. coli* in the presence of furfural was improved by overexpression of *thyA* (encoding for thymidylate synthase). More recently, plasmid-based expression of polyamine transporters, *puuP* and *potE* have

been evaluated to increase the metabolism of furfural and decreased the time required for xylose fermentation by ethanologenic strains (Geddes et al., 2014). However, increasing furfural tolerance using plasmid-based expression of furfural resistant genes in succinate-producing strain have not much been studied so far. The goal of this research was to improve a strain resistance to furfural, which is the most significant inhibitor present in sugarcane bagasse hydrolysates (Miller et al., 2009), by introducing the beneficial furfural resistant genes from ethanologenic *E. coli* strain including *pntAB*, *ucpA*, *fucO*, *fucO**, *thyA*, *puuP*, and *potE* into *E. coli* strain AS1600a. The AS1600a strain was originally developed to effectively ferment xylose and simultaneously utilize sugars mixture for succinate production (Sawisit et al., 2015).

6.2 Materials and methods

6.2.1 Strains and plasmids

Strains, plasmids, and primers used in this study are listed in Table 6.1. A Luria Bertani medium was used for plasmid constructions. After genetic manipulations, succinate biocatalysts were grown in AM1 mineral salts medium (Jantama at al., 2008b; Martinez et al., 2007). The *E. coli* strain AS1600a and derivatives were used to investigate furfural tolerance for growth and succinate production.

6.2.2 Construction of expression vector pLOI5883

The construction of a stable expression vector (pLOI5883) was previously described in section 5.2.3 by replacing the pBR322 *oriR* replicon in pTrc99a with the

RSF1010 replicon from pLOI707EH (Arfmann et al., 1992). The primer pairs RSF1010*rep* and pTrc99aΔ*oriR* were used to amplify the RSF1010 replicon and the pTrc99a backbone, omitting *oriR*. After amplification, both fragments were digested with *Spe*I and *cat*-ligated. The resulting plasmid, pLOI5883, was confirmed by Sanger sequencing (Interdisciplinary Center for Biotechnology Research, University of Florida).

6.2.3 Construction of furfural resistant plasmids

Genes encoding *potE*, *puuP*, *fucO*, *fucO**, *ucpA*, and *thyA*, were amplified (including ribosomal binding site and terminator region) from strain *E. coli* W (ATCC 9637) chromosomal DNA by using PCR. These fragments were cloned into *EcoR*I and *BammH*I sites of expression vector, pLOI5883 (Sawisit et al., 2015), to produce pLOI5420, pLOI5421, pLOI5422, pLOI5423, pLOI5424 and pLOI5425, respectively. A *pntAB* gene was aplified from *E. coli* C (ATCC 8739) chromosomal DNA and cloned into *Xba*I and *Hind*III sites of expression produce pLOI5426 using primers listed in Table 6.1. After ligation, plasmids were transformed into *E. coli* TOP10F'. Plasmids were purified using a QIAspin Spin Miniprep kit (Qiagen, Valencia, CA). Clones were verified by digestion with restriction enzymes, gel analysis of PCR products, and sequencing.

Table 6.1 Strains and plasmid used in this study.

Strains and plasmids	Relevant characteristics or sequence	Source or references
Strain		
E. coli KJ122	*E. coli* ATCC 8739 (Δ*ldhA*, Δ*adhE*, Δ*ackA*, Δ(*focA-pflB*) ΔmgsA, Δ*poxB*, Δ*tdcDE*, Δ*citF*, Δ*aspC*, Δ*sfcA*, *pck**, *ptsI**)	Jantama et al., 2008
E. coli AS1600a	Clone from 16th transfer of *E. coli* KJ122 in 10% xylose containing point mutations in *galP* (*galP**) and an *rhs*-like gene	This study
E. coli W (ATCC 9637)	Wild type	ATCC
E. coli C (ATCC 8739)	Wild type	ATCC
E. coli Top10F'	Host used for plasmid construction	Thermo Fisher
Plasmids		
pTrc99a	pTrc *bla oriR rrnB lacI*^{q}	Laboratory collection
pCR2.1-TOPO	*ori bla lacI*^{q} , vector for constructions	Thermo Fisher
pLOI707EH	Source of RSF1010 replicon	Arfman et al., 1992
pLOI5883	Expression vector, RSF1010 *rep* pTrc *bla rrnB lacI*^{q}	This study
pLOI5420	*potE* gene in *Eco*RI-*BamH*I digested pLOI5883	This study
pLOI5421	*puuP* gene in *Eco*RI-*BamH*I digested pLOI5883	This study
pLOI5422	*fucO* gene in *Eco*RI-*BamH*I digested pLOI5883	This study
pLOI5423	*fucO* (L7F), (*fucO**) gene in *Eco*RI-*BamH*I digested pLOI5883	This study
pLOI5424	*ucpA* gene in *Eco*RI-*BamH*I digested pLOI5883	This study
pLOI5425	*thyA* gene in *Eco*RI-*BamH*I digested pLOI5883	This study
pLOI5426	*pntAB* gene in *Xba*l-*Hind*III digested pLOI5883	This study
Primers		
RSF1010 *rep*	For-GGAGCAGAAGAGCATACATCTGG Rev-GGACTAGTCTGAAAGCGACCAGGTGCTCG	This study
pTrc99a Δ*oriR*	For-CGCTTACAGACAAGCTGTGACCG Rev-GGACTAGTGTCTGACGCTCAGTGGAACGAA	This study
potE cloning	For- CGGAATTCCTGTTGAAAGGGGAAAAATTATG Rev- CGGGATCCCTGTCTCCGGAAATGGTGAG	This study
puuP cloning	For-CGGAATTCTACGCAGGGGAGGCAGCAAT Rev-CGGGATCCGCTTCAGCAGCCATTTCATGTTG	This study
fucO cloning	For-CGGAATTCGTAAAGCAACAAGGAGAAGGATGATG Rev-CGGGATCCTTGCGGGGATCAGCAGTTCA	This study
ucpA cloning	For-CGGAATTCTTAACAAGGAGAGCATTAAAATGGGT Rev-CGGGATCCCTTTTGCCCATTGTTGCTCAA	This study
thyA cloning	For-CGGAATTCCAACACGTTTCCTGAGGAACCA Rev-CGGGATCCGTTGCGACTGCTGCCAGTATTG	This study
*fucO** cloning	For-CGGAATTCGTAAAGCAACAAGGAGAAGGATGATG Rev-CGGGATCCTTGCGGGGATCAGCAGTTCA	This study
pntAB cloning	For-AAGCAATCTAGAAGGCAAACCATCATCAATAA Rev-AAGCAAAAGCTTCCGTTCTGTTAAGCGATCTC	This study

Underlines indicate the recognition sites for cloning the genes into expression vector (pLOI5883). *Eco*RI: GAATTC, *BamH*I: GGATCC, *Xba*l: TCTAGA, *Hind*III: AAGCTT and *Spe*I: ACTAG

6.2.4 Preparation of sugarcane bagasse hydrolysate

Sugarcane bagasse hydrolysate was prepared at the University of Florida Biofuels Pilot Plant as described previously (Nieves et al., 2011). Briefly, sugarcane bagasse (Florida Crystals) was soaked for 4 h in a 14-fold excess of 0.5% phosphoric acid (w/w including moisture in bagasse). The dilute acid impregnated bagasse was steam-treated for 5 min at 190°C. Hemicellulose syrup (hydrolysate) was recovered using a screw press, discarding solids. After removal of fine particulates with a Whatman GF/D glass fiber filter, clarified hydrolysate was stored at 4°C (pH 3.0). The final composition of sugarcane bagasse hydrolysates used in this study was (g/L): xylose (44.18±0.31), glucose (4.88±0.16), arabinose (7.61±0.18), galactose (3.87±0.01), acetate (4.81±0.03), furfural (2.22±0.05), hydroxymethyl furfural (undetected), and total monomer sugars (56.67±0.64) (Nieves et al., 2011). For detoxification, the clarified hydrolysate was adjusted pH to 9.0 by addition of ammonium hydroxide (5N NH_4OH) and stored at room temperature for 16 h before inoculation (Geddes et al., 2015; Sawisit et al., 2015). For bisulfite addition, a freshly prepared solution was immediately added to culture before inoculation (1 mM final concentration of bisulfite is equal 0.5 mM sodium metabisulfite) (Nieves et al., 2011).

6.2.5 Tube assays for furfural and acetate tolerance

The minimum inhibitory concentration (MIC) was determined using a modified method described in Geddes et al. (2014) by measuring growth and succinate production after 48 h (37°C) using tube cultures (13 x 100 mm) containing 4 mL of AM1 medium. Furfural and isopropyl-β-D-thiogalactopyranoside (IPTG) were added as indicated. Cells were grown overnight on AM1 agar plates. Fresh colonies were

scraped, resuspended in AM1 medium containing 5% (w/v) sugars (glucose or xylose depending on experiment) and adjusted to an optical density at 550 nm of 1.0. Tube cultures were inoculated to an initial OD_{550nm} of 0.1 (0.33 mg DCW/mL/OD) and incubated in a water bath with 50 rpm shaking.

6.2.6 pH controlled batch fermentation

Fermentations were conducted in pH-controlled vessels (500 mL) with a 300 mL working volume (37°C, 150 rpm). The pH of the medium was maintained at 7.0 by automatic addition of a mixture of 6 N KOH and 3 M K_2CO_3 (1:4 ratio) (Jantama et al., 2008b). A low salt medium, AM1 (4.2 g/L total salt, Martinez et al., 2007) supplemented with 1 mM betaine, and 100 mM $KHCO_3$ was used as a fermentation medium. Seed cultures were grown overnight (16 h) in AM1 medium supplemented with 5% (w/v) sugars (glucose or xylose depending on experiment). Fermentations were initiated by inoculating to an initial OD_{550nm} of 0.1 and monitored for up to 120 h.

6.2.7 Fermentation products, furan and cell mass measurement

Fermentation broth was removed during fermentation for the measurement of cell mass, organic acids, furan and sugars. Cell mass was estimated from the optical density (OD) at 550 nm (0.33 mg of DCW/mL/OD) with a Bausch & Lomb Spectronic 70 spectrophotometer (Jantama et al., 2008b). Sugars, furans, and organic acids were analyzed by two high-performance liquid chromatography (HPLC) using Agilent Technologies 1200 series HPLC systems (Geddes et al., 2014). Sugars and furan were analyzed with a BioRad (Hercules, CA) Aminex HPX-87P ion

exclusion column (80ºC; nano-pure water as the mobile phase (0.6 mL/min) while organic acids were analyzed with a BioRad Aminex HPX-87H column (45ºC; 4 mM H_2SO_4 as the mobile phase, 0.4 mL/min).

6.2.8 Statistical analysis

Analysis of variance (ANOVA) was performed using SPSS software (SPSS 17.0 for Windows; SPSS Inc., Chicago, IL, USA). Triplicate determinations were conducted on each test and averages were reported. The differences among mean values were established using Duncan's multiple range tests at 95% significance level.

6.3 Results and discussion

6.3.1 Minimum inhibitory concentration (MIC) of furfural and acetate

The MIC of the most abundant inhibitory compounds residing in hemicellolose hydrolysate of sugarcane bagasse (furfural and acetate) was examined by measuring the growth of succinate-producing strains in tubes culture containing furfural and acetate. The results showed that furfural concentrations above 4 mM appear sufficient to prevent growth and metabolism of all succinate producing *E. coli* strains in the absence of furfural tolerance genes (Figure 6.1A and 6.1B) while all the strains were able to tolerate to the acetate concentration up to 165 mM (10 g/L) (Figure 6.1C and 6.1D). It indicated that furfural (<4 mM) was more potent inhibitor of fermentation than acetate (>165 mM). Furfural has been identified as one of the key inhibitors affecting fermentation of dilute acid hydrolysates (Almeida et al., 2009). It has been shown to cause strand breaks in DNA and cell membrane damage

(Hadi et al., 1989). Previous studies have shown that increasing concentrations of furfural has correlated with toxicity of diluted acid hydrolysates of hemicellulose (Geddes et al., 2014; Miller et al., 2009). In addition, acetate can be derived from structural components of the lignocellulose and/or decomposition of acetylxylan, a byproduct of hemicellulose degradation (Zaldivar and Ingram, 1999). Organic acids like acetate penetrate the cell membrane in an undissociated form and dissociate after entering the cytoplasm (Roe et al., 1998). This event collapses the cell's proton gradient resulting in a decrease of ATP, lowering the growth of microorganism. Figure 6.1A and 6.1B also revealed that the KJ122 strain exhibited a slightly higher furfural tolerance compared with AS1600a strain when using glucose as carbon source. However, the KJ122 strain could not grow and produce succinate when the medium was supplemented with xylose as a sole carbon source. In wild type *E. coli*, conversion of glucose to pyruvate yields 2 net ATPs per mole glucose, while metabolism of a xylose, to pyruvate only yields 0.67 net ATP per mole xylose due to the need for one (each) ATP for each mole xylose transport and xylulose phosphorylation (Hasona et al., 2004). During fermentative growth, the wild type *E. coli* produces equimolar amounts of acetate and ethanol from two pyruvates. These reactions generate one additional ATP from two pyruvates. Consequently, the conversion of xylose to acetate and ethanol increases the overall redox balance and the net ATP yield from 0.67 to 1.5 per mole xylose, which meets the requirement for xylose metabolism (Hasona et al., 2004). Nevertheless, Gonzalez et al. (2002) demonstrated that the strain lacking the lactate dehydrogenase (*ldhA*) and pyruvate dehydrogenase (*pflB*) could not grow in xylose due to failing to convert pyruvate to acetyl coenzyme A, the required precursor for acetate and ethanol production. It

indicated that the KJ122, an *ldhA* and *pflB* deletion strain (Jantama et al., 2008b), could not utilize xylose for their growth and succinate production probably due to insufficient ATP supply.

Figure 6.1C and 6.1D also revealed that the AS1600a strain exhibited the higher resistant to furfural and acetate when xylose was used as carbon source comparing with that of glucose, which was in accordance to the KJ122 strain. The probably reason could be due to a point mutation in galactose permease (GalP), replacement of glycine residue at position 236 with aspartate residue in the GalP protein. This mutation in GalP structure has been proved to be responsible for high xylose utilization for growth and production of succinate by the AS1600a strain (Sawisit et al., 2015), while the native GalP is important for glucose uptake in *E. coli* KJ122 strain (Zhang et al., 2009). Similarly, Utrilla et al. (2012) investigated that a point mutation in the *GatC* gene of galctitol PTS permease, which changed serine to leucine at position 184 (*gatC*S184L), altered the sugars metabolism of *E. coli* strain engineered for D-lactate production. The mutant *gatC*S184L contributes more to xylose transport than the native xylose transporter (*xylE*) when compared with the parental strain. In addition, a single amino acid change in *hSGLT*3 functioning as a sugar sensor *in vivo*, converts this sugar sensor into a sugar transporter similar to SGLT1 (intestinal glucose absortion) (Bianchi and Diez-Sampedro, 2010). Based on the above mentioned, it was implied that the replacement of glycine by aspartate (G236D) in the GalP caused the change in the GalP structure, which altered the affinity of sugar transporters. The mutant *galP* strain AS1600a might probably transports xylose and glucose through the mutant GalP transporter, but with different affinity.

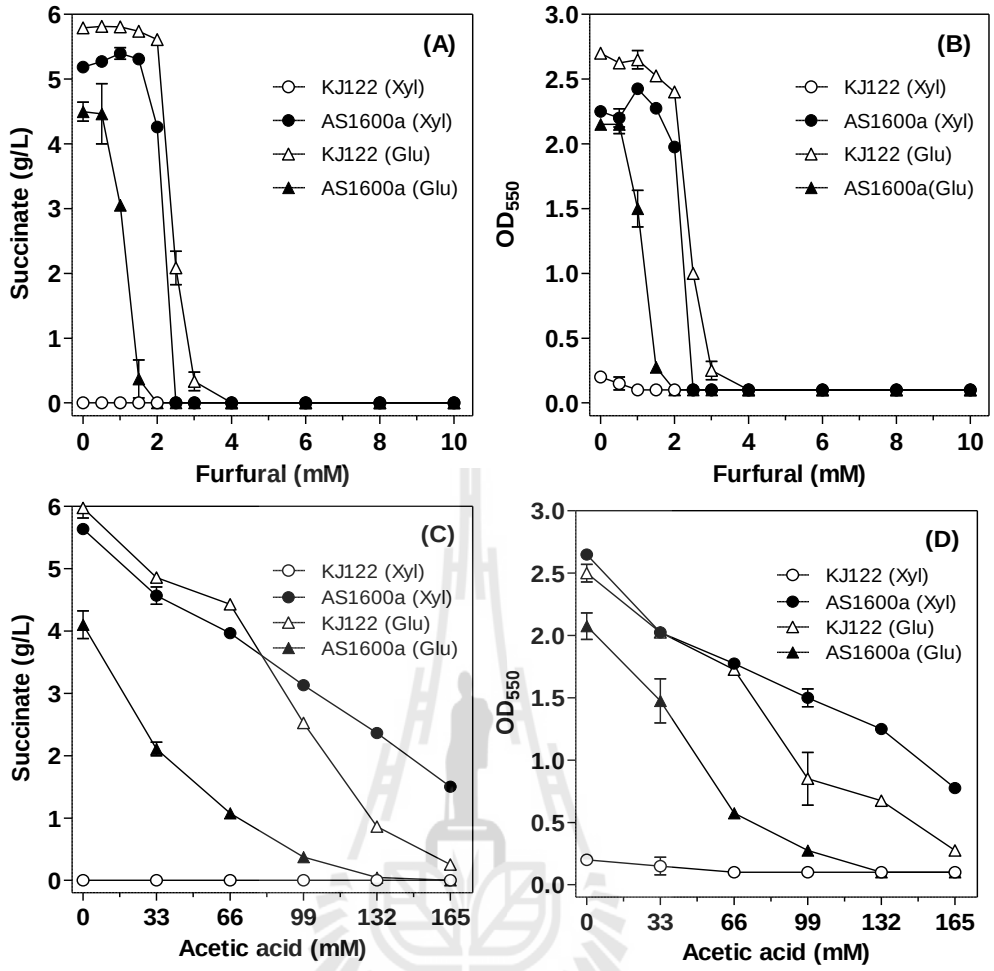

Figure 6.1 Furfural and acetate tolerance (MIC) by succinate-producing *E. coli*. A. Succinate production under furfural addition. B. Biomass formation under furfural addition. C. Succinate production under acetate addition. D. Biomass formation under acetate addition. Glu (glucose), Xyl (xylose).

6.3.2 Improving furfural tolerance using plasmid-based expression in tube culture

To reduce the cost of succinate production from sugarcane bagasse hydrolysate and to minimize the hydrolysate detoxification processes, the furfural resistant genes including *puuP*, *potE*, *thyA*, *fucO*, *fucO**, *ucpA*, and *pntAB* were

introduced into *E. coli* AS1600a. Furfural tolerance was examined in the tube culture using glucose and xylose as carbon sources supplementing with and without IPTG. The empty vector (EV) without furfural resistant genes was served as a control. The results revealed that all of the strains harboring the furfural resistant genes exhibited the higher succinate production along with the higher furfural tolerance when compared with the strain containing an empty vector. The strain harboring an empty vector appeared to be the most sensitive to furfural (Figure 6.2). The highest MIC to furfural at 8 mM was found in the strain harboring a *fucO** plasmid using glucose as carbon source and 10 µM IPTG induction. With *fucO** plasmid, the MIC of furfural increased from 4 to 8 mM when compared with that of the strain containing an empty vector (Figure 6.2C and 6.2D). In contrast, the strain harboring a *puuP* gene exhibited the highest furfural tolerance at 6 mM when xylose was used as a sole carbon source with 10 µM IPTG induction (Figure 6.3G and 6.3H). Increased expression of polyamines transporters (*puuP*, *potE*, *tABCD*, and *plaP*) and polyamine supplements have been reported to increase furfural tolerance in ethanologenic strain due to the binding of polyamine to negatively charge cellular constituents such as nucleic acids and phospholipids, providing protection from damage by furfural (Geddes et al., 2014). Interestingly, the higher furfural tolerance was found when using glucose as carbon source compared with xylose in which the MIC of furfural enhanced from 6 mM to 8 mM (Figure 6.3C and 6.3G). Miller et al. (2009) and Wang et al. (2011c) demonstrated that furfural tolerance could be increased by the replacement of xylose with glucose (increased NADPH production) or addition of complex nutrients like yeast extract or cystein (decreased biosynthesis demand of NADPH). Figure 6.3 also indicated that the addition of IPTG at concentration as 10 µM improved cell growth

and enhanced furfural tolerance. Similar beneficial effect of adding inducer (IPTG) for increasing expression of *fucO* (Wang et al., 2011c) and *fucO** (Zheng et al., 2013) has been found in ethnologenic *E. coli* strain. High level of *fucO* appears to be needed to increase furfural tolerance in *E. coli* (Wang et al., 2011c). Nevertheless, the negative effect of addition inducer has also been observed in several *E. coli* genes, including *pntAB* (Miller et al., 2009), trehalose biosynthetic genes (Purvis et al., 2005), and polyamine transporter genes (*potE* and *puuP*) (Geddes et al., 2014). Based on the result obtained in this study, the helpful genes for furfural tolerance from ethanologenic *E. coli* strain were also beneficially improved furfural tolerance in succinate-producing *E. coli* strain. The best furfural resistant genes exhibited the highest furfural tolerance in tube assay was found to be a *fucO** followed by *puuP*. These two genes were subsequently applied for further study.

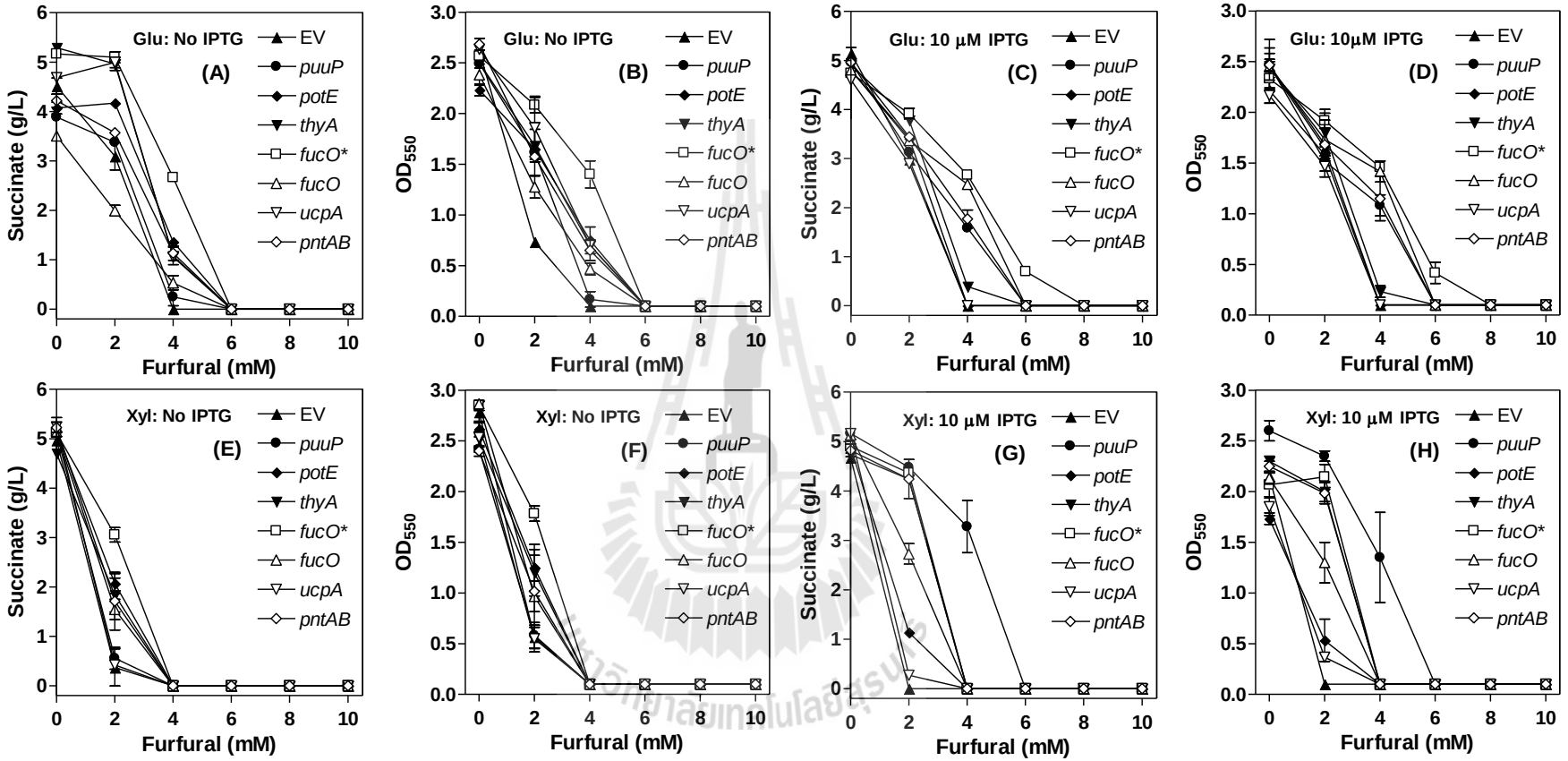

Figure 6.2 Effectiveness of furfural resistant genes in succinate production by *E. coli* AS1600a using glucose and xylose with and without IPTG. A. Succinate production from glucose without IPTG. B. Biomass production from glucose without IPTG. C. Succinate production from glucose with 10 µM IPTG. D. Biomass production from glucose with 10 µM IPTG. E. Succinate production from xylose without IPTG. F. Biomass production from glucose without IPTG. G. Succinate production from xylose with 10 µM IPTG. H. Biomass production from xylose with 10 µM IPTG.

6.3.3 Batch fermentation with furfural under pH-controlled vessel

6.3.3.1 Individual xylose fermentation

The xylose-evolved *E. coli* strain AS1600a harboring the *fucO** and *puuP* gene were applied for succinate production under a pH-controlled using 10% xylose as carbon source with and without IPTG induction. The strain containing an empty vector (without furfural resistant genes) was also carried out as a control. At 0 mM to 8 mM furfural addition, all strains started growing within the first 24 h with no lag phase. Furfural was completely metabolized within the first 24 h. Beyond 10 mM furfural addition, the delay in growth about 24 h and prolong furfural metabolism from 24 to 72 h of the strain harboring an empty vector was observed. Moreover, the furfural addition at 15 mM was completely inhibited the growth and succinate production of the strain harboring an empty vector. Furfural was partially metabolized during the initial 24 h of incubation. Thereafter the rate of furfural metabolism declined progressively. Even though, almost half of furfural was remained after 120 h fermentation. It was in contrast to what found in the strain harboring the *fucO** and *puuP*, which were able to grow and produce succinate when 20 mM furfural was added (Figure 6.3). Martinez et al. (2000) and Almeida et al. (2009) also found that the presence of furfural and furan derivatives prolonged the lag phase during the initial 24 h of fermentation in both bacteria and yeast. The cell growth was merely inhibited while furfural was being metabolized and resumed after complete reduction to furfuryl alcohol (less toxic than furfural) by NADPH-dependent enzyme such as propanediol oxidoreductase encoded by *fucO* (Miller et al., 2009) and pyridine nucleotide transhydrogenases encoded by *pntAB* (Wang et al., 2011c). Figure 6.4 indicates that addition of IPTG improves furfural metabolism and reduces the lag

period of growth in both strains (*fucO** and *puuP*). At the end of fermentation (20 mM furfural), succinate concentration of 70.21±0.93 g/L and 67.18± .13 g/L were obtained from the strain harboring the *fucO** and *puuP*, respectively. There were about 14.91% (*fucO**) and 23.82% (*puuP*) improved in succinate production when compared with that of the fermentation without IPTG induction. It suggested that addition of IPTG increased the expression of furfural resistant genes (*fucO** and *puuP* genes) and improved succinate production. The enzyme L-1,2-propanediol oxidoreductase encoded by *fucO* is an NADH-link, belongs to the iron-activated group III dehydrogenase family (Wang et al., 2011c). It catalyzes the interconversion between L-alactaldehyde and L-1,2-propanediol during the anaerobic dissimilation of fucose (Cocks et al., 1974). Expression of the native *fucO* from plasmids has been evaluated to enhance furfural tolerance in *E. coli*-based fermentation for ethanol and lactate production (Wang et al., 2011c). The furfural reductase activity in *fucO* offered an alternative route for furfural reduction to less toxic alcohol using NADH (abundant during fermentation) as the reductant and does not compete for NADPH biosynthesis (Wang et al., 2011c). Zheng et al. (2013) performed a mutagenesis of *fucO* gene by replacing the most abundant codon for leucine with the most abundant codon of phenylalanine resulted in a *fucO* (L7F), (*fucO**). The mutated *fucO** gene increased FucO activity more than 10-folds and exhibited a double rate of furfural metabolism during fermentation when compared with that of the wild type *fucO* (Zheng et al., 2013). This confirms that the overexpession of *fucO** exhibited the higher furfural tolerance than that of native *fucO*.

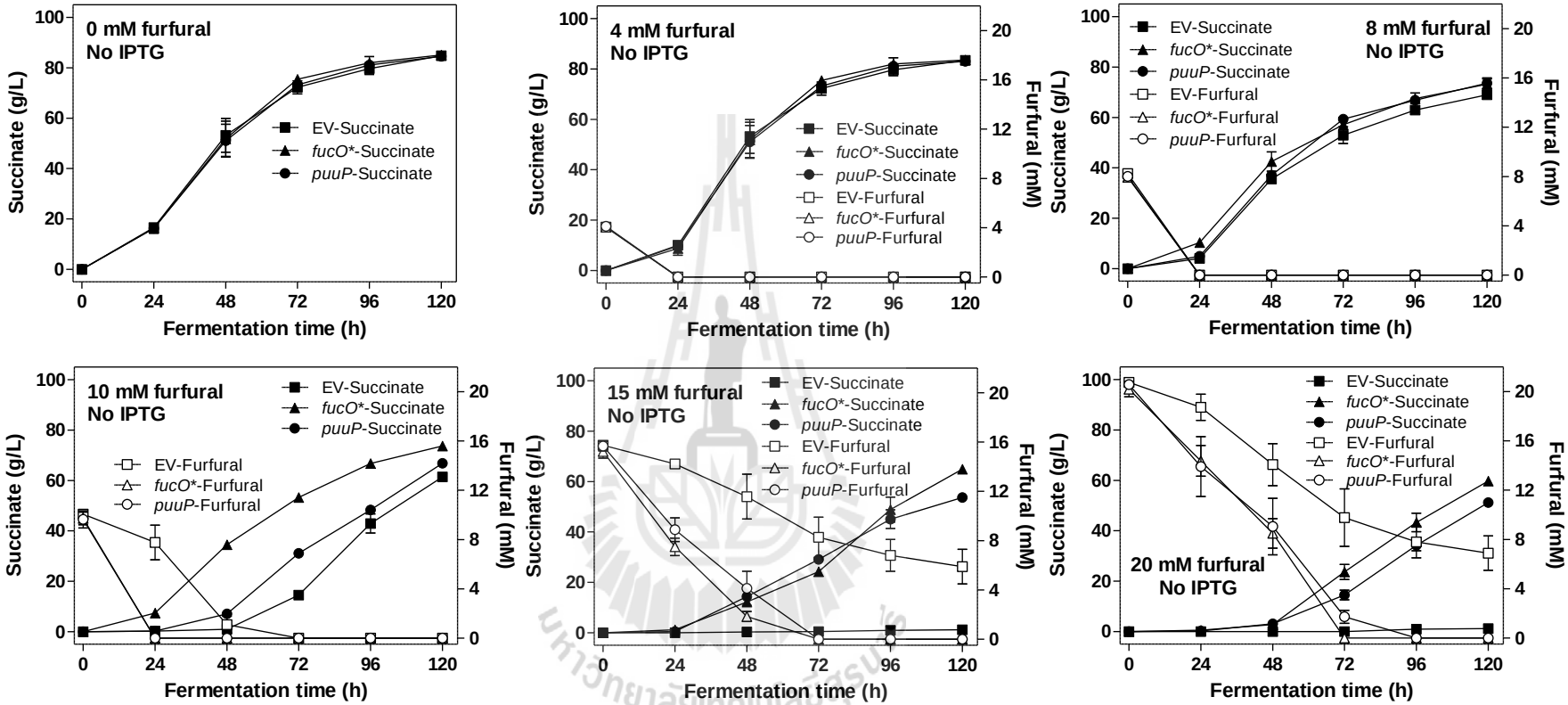

Figure 6.3 The effect of furfural concentrations on succinate production from 10% (w/v) xylose by *E. coli* AS1600a harboring the furfural resistant genes under controlled pH without IPTG induction. EV (empty vector).

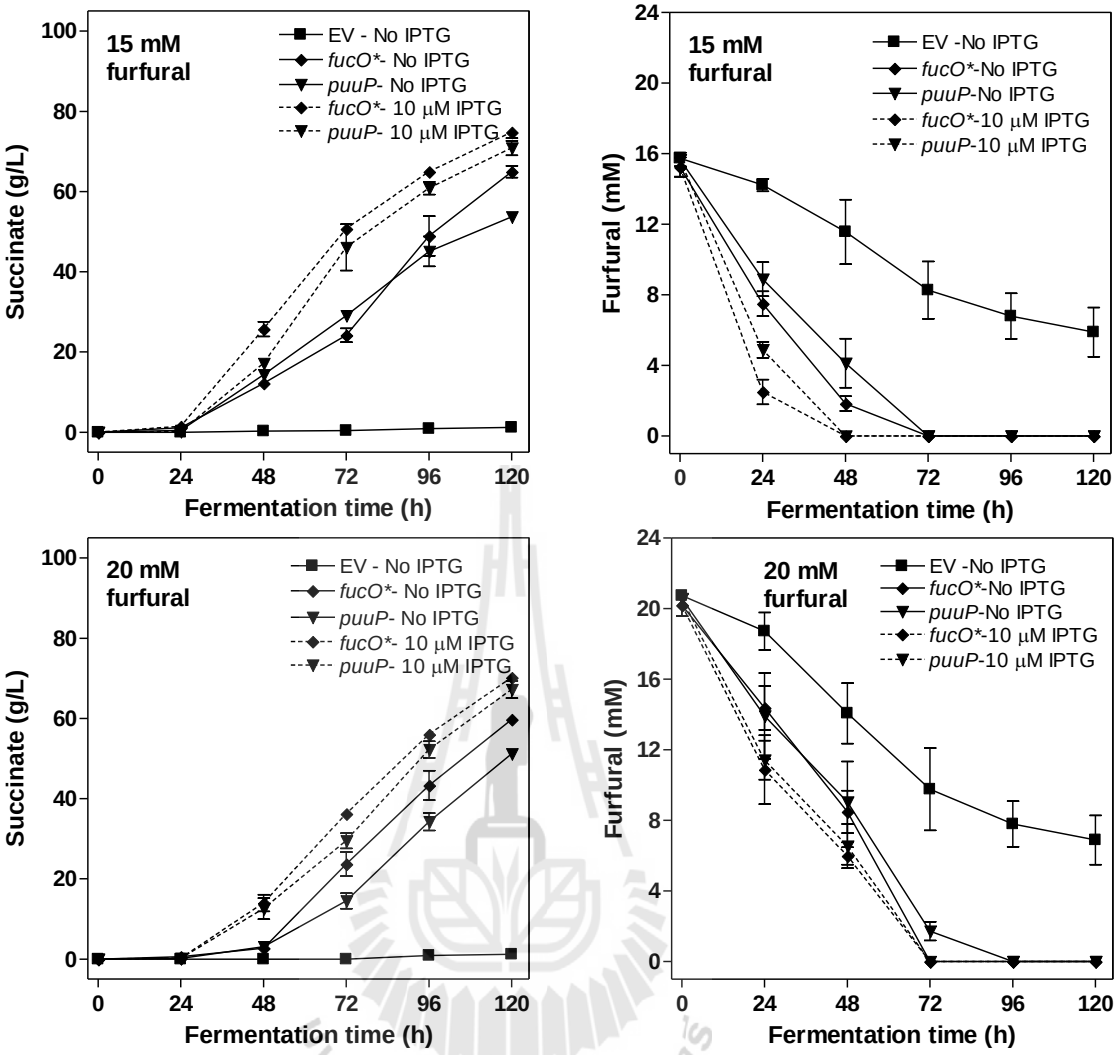

Figure 6.4 The effect of furfural concentration on succinate production from 10% (w/v) xylose by *E. coli* AS1600a harboring the furfural resistant genes under controlled pH with 10 μM IPTG induction. EV (empty vector).

6.3.3.2 Mixed sugars fermentation

The effect of furfural addition at 20 mM on succinate production by *E. coli* AS1600a harboring the *fucO** and *puuP* was also investigated under the pH-controlled using the mixture of 5% (w/v) glucose and 5% (w/v) xylose. The strains harboring *fucO** and *puuP* could consume sugars mixture of glucose and

xylose simultaneously throughout the fermentation without the catabolic repression. Unlike the strain harboring an empty vector, the *fucO** and *puuP* strains completely metabolized 20 mM furfural within 48 h, indicating that the expression of *fucO** and *puuP* increased the rate of furfural metabolism and permitted the fermentation of sugars mixture of 5% (w/v) glucose and 5% (w/v) xylose to succinate. Although furfural addition as 20 mM caused an initial lag of 24 h, the strains still required longer fermentation times than the condition in which no furfural was added (Figure 6.3). After 120 h incubation, the succinate production obtained from sugars mixture by the strain harboring a *fucO** (70.50±0.75 g/L) was slightly higher than that of the *puuP* strain (67.66±1.21 g/L). It is well known that the native *E. coli* usually consumes glucose first before consuming the other sugars during sugars mixture. This mechanism is known as a carbon catabolite repression (CCR) (Magasanik et al., 1961). As recognized, glucose uptake in *E. coli* with the aid of glucose-specific permease EIICB^{glc} encoded by *ptsG* in the phosphoenalpyruvate (PEP): carbohydrate phoshotransferase system (PTS). The PTS system catalyzes the uptake and concomitant phosphorylation of numerous carbohydrates, and plays a major role in *E. coli* CCR (Deutscher et al., 2006). Zhang et al. (2009) proposed that the *E. coli* KJ122, a parental strain of *E. coli* AS1600a, transported glucose to the cell with a non-phosphorylated form by a sugar-H+ symport mechanism via galactose permease (GalP), due to the inactivation of PEP-dependent PTS system by the combinations of loss of catabolite repression, gain of Mlc repression, presences of *ptsI* mutation, and regulation of RNaseE-dependent cleavage. This inactivation of native PTS system provides an energy mechanism to increase the pool size of PEP and to facilitate redox balance for succinate production by the KJ122 strain (Zhang et al., 2009). Beside, the

KJ122 strain could co-metabolize glucose and xylose during sugars mixture but stops using xylose when glucose was exhausted. During sugars mixture, the *E. coli* KJ122 might utilize ATP generated during glucose metabolism in an efficient consumption of xylose. Without glucose, ATP was not efficiently generated resulting in lower consumption of xylose. In contrast, the *E. coli* AS1600a could simultaneously utilize the sugars mixture of glucose and xylosse throughout the fermentation (Figure 6.5). The probable reason could be due to the mutation of galactose permease (GalP, Gly236 to Asp). The transport of xylose into the cell by the use of the mutant GalP (sugar-H^+ symporter) instead of a high-affinity ATP-independent transporter provided the benefit to the AS1600a strain, not only for generating the ATP to supply cell growth but also maintaining a redox balance for succinate production. Therefore, the AS1600a strain was able to consume xylose with high rate and enabled the efficient co-consumption of sugars mixture (Sawisit et al., 2015). Figure 6.5 also showes that the AS1600a strain containing the mutanted GalP consumed glucose and xylose at nearly similar rate during mixed sugars fermentation. It indicated that this GalP mutation dose not abolish glucose uptake, but cause a drastically increasing in the xylose affinity, and reduce the inhibition of xylose transporter by glucose, thus improve sugars mixture utilization. Furthermore, expressing the *galP** mutation gene in KJ122Δ*galP* fully resembled the xylose utilization phenotype of the mutant AS1600a and the AS1600a strain could ferment the sugars mixture without CCR (Sawisit et al., 2015). The changing in GalP structure was responsible for the high xylose consumption and co-uitilization of sugars phenotype in the adapted *E. coli* AS1600a strain. Interestingly, the addition of furfural dose not affecte the sugars

metabolism during sugars mixture fermentation but increased the lag phase of fermentation until it was fully reduced to the less toxic compound.

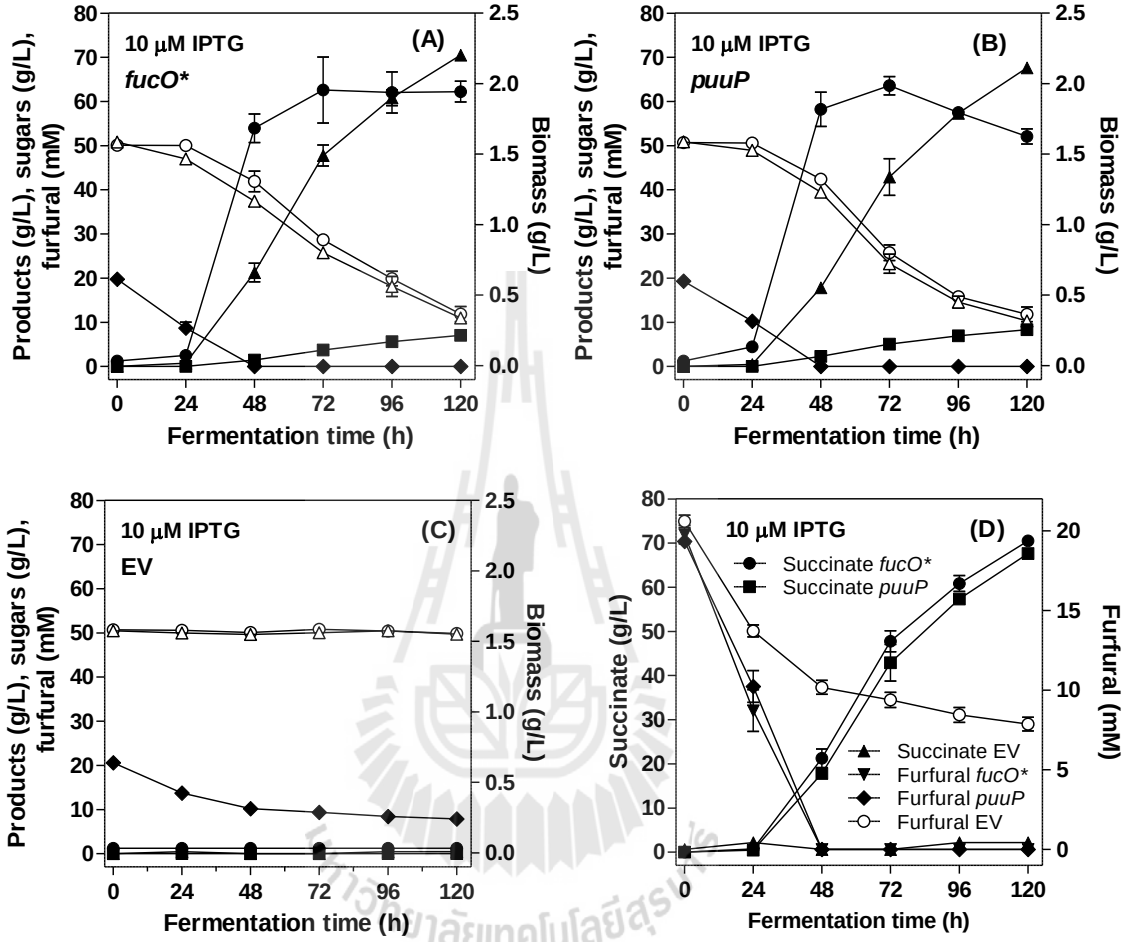

Figure 6.5 Time course of sugars mixture fermentation by *E. coli* AS1600a harboring the furfural resistant genes. A. Strain harboring a *fucO** plasmid. B. strain harboring *puuP* plasmid. C. Strain harboring an empty vector plasmid (EV). D. Comparison of succinate production and furfural metabolism. Cells were grown with a mixture of 5% (w/v) xylose and 5% (w/v) glucose in AM1 medium supplemented with 20 mM furfural and 10 μM IPTG. Symbols for all: xylose (open circle), glucose (open triangle), succinate (filled triangle), acetate (filled square), biomass (filled circle), furfural (filled diamond).

6.3.3.3 Sugarcane bagasse hydrolysate fermentation with furfural tolerance genes under pH-controlled vessel

The strain harboring *fucO** and *puuP* were also utilized for succinate production from sugarcane bagasse hydrolysate under the pH-controlled. The sugarcane bagasse hydrolysate containg 55 g/L total sugar was used to simulate the hydrolyzed lignocellulose by adding 150 g/L glucose as a replacement for hydrolyzed cellulose fiber and diluting with an equal volume of water (Sawisit et al., 2015). The resulting broth contained 100 g/L total sugars, mostly glucose and xylose with smaller amounts of arabinose and galactose, together with inhibitors from side reactions during dilute acid pretreatment. Concentrations of sugars (100 g/L) and inhibitors are equivalent to hydrolysate prepared from a slurry of 15% sugarcane bagasse (dry weight). Before inoculation, the hydrolysate inhibitors were mitigated by pH 9 treatment with ammonia (16 h incubation) and addition of 2 mM bisulfite. Figure 6.6 shows that furfural was completely metabolized within 48 h in strains harboring *fucO** and *puuP* while the depletion of furfural of the strain containing an empty vector (without fufural resistant genes) was observed at 72 h incubation. After furfural was fully metabolized, all strains started rapidly growth and produced succinate with similar phenomenon to what observed in the AS1600a strain without furfural resistant plasmids (Figure 6.3). After 144 h of incubation, the significant higher in succinate production was found in the strain harboring the *fucO** as compared with the *puuP* strain in which the succinate concentration of 72.76±0.98 g/L and 68.42±0.81 g/L were obtained, respectively. These were about 37% higher in succinate production (46.0 ±1.34 g/L) when compared with that of the empty vector (control), indicating that the furfural tolerance of *fuco** and *puuP* genes promotes the

metabolism of furfural and substantially reduce the time required to completely metabolized the hydrolysate. Nevertheless, the fermentation time required for succinate production from sugarcane bagasse hydrolysate was longer than of pure sugars fermentation even some detoxification processes were introduced. Moreover, the strain containing a furfural tolerance genes, *fucO** and *puuP* could not grow when using hydrolysate without pH 9.0 treatment as a carbon source (data not shown), indicating that other inhibitory compounds rather than furfural such as phenolic compounds retard the microbial growth. Keating et al. (2014) reported that the aromatic aldehydes derived from ammonia-pretreated lignocellulosic biomass, furfural and 5-hydroxymethylfurfural appeared to be reduced to its alcohol form (less toxic) by the ethanologen during fermentation, whereas phenolic acids and amide inhibitors were not metabolized. Phenolic compounds are known to partition into biological membranes altering the permeability and lipid/protein ratio, which therefore increase cell fluidity, leading to cell membrane disruption (Heipieper et al., 1994) Additionally, phenolic aldehydes has been reported to enhance the gerneration of ractive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), super oxides (O_2^-) and super hydroxyl (OH^-) that can interact with proteins/enzymes, which results in their denaturation (Ibraheem and Ndimba, 2013). With this regard, it suggested that further strain development for non-furfural hydrolysate resistance is needed to be performed in order to improve the efficiency of hydrolysate utilization, thus beneficial for improving industrial succinate production.

A few studies on succinate production using sugarcane bagasse hydrolysate by metabolically engineered *E. coli* have been investigated. Liu et al. (2013a) used sugarcane bagasse hydrolysate as the carbon source for succinate production by *E.*

coli BA204, a *pflB*, *ldhA*, and *ppc* deletion strain overexpressing the ATP-forming PEPCK from *B. subtilis* 168. During dual-phase fermentation, the BA204 strain produced a final succinate concentration of 18.88 g/L with a yield of 0.96 g/g total sugars. Liang et al. (2013) modified the *E. coli* BA204 strain by further deletion of *ptsG* gene resulted in a strain *E. coli* BA305. The *E. coli* BA305 strain could produce succinate concentration of 83 g/L with a yield of 0.87 g/g total sugars in 36 h under three repetitive fermentations (cell recycle) from sugarcane bagasse hydrolysate. In the same year, Liu et al. (2013b) applied fed-batch fermentation in *E. coli* BA305 to produce succinate from sugarcane bagasse hydrolysate. After 120 h fed-batch fermentation, the succinate concentration at 39.3 g/L with 11% higher of succinate yield comparing with the repetitive succinate production (cell recycle) of sugarcane bagasse hydrolysate by the BA305 strain was obtained. It is interesting to note that the succinate production from sugarcanes bagasse hydrolysate by *E. coli* AS1600a in this study was comparable to that obtained from previously published work. Meanwhile, succinate production by BA305 or BA204 strain could reach a high concentration of succinate yield but the need of a complex media and an air or carbon dioxide sparging are required in order to promote the good growth of microorganism, thereby increasing the cost of succinate production. In contrast to previous published works, the fermentation condition in this study differs in the use of inorganic salts medium rather than complex media, the lack of carbon dioxide gas providing and auxotrophic requirements, and the use of simple batch operation under anaerobic conditions. Growth and succinate production occur concurrently in a single step. This study indicated that succinate production from sugarcanes bagass hydrolysate by *E. coli* AS1600a would be expected to lower costs of succinate production due to a

simple use of carbon sources for both growth and succinate production. Importantly, the sugarcane bagasse hydrolysate showed a great potential usage of renewable biomass as a feedstock for an economical succinate production using *E. coli.*

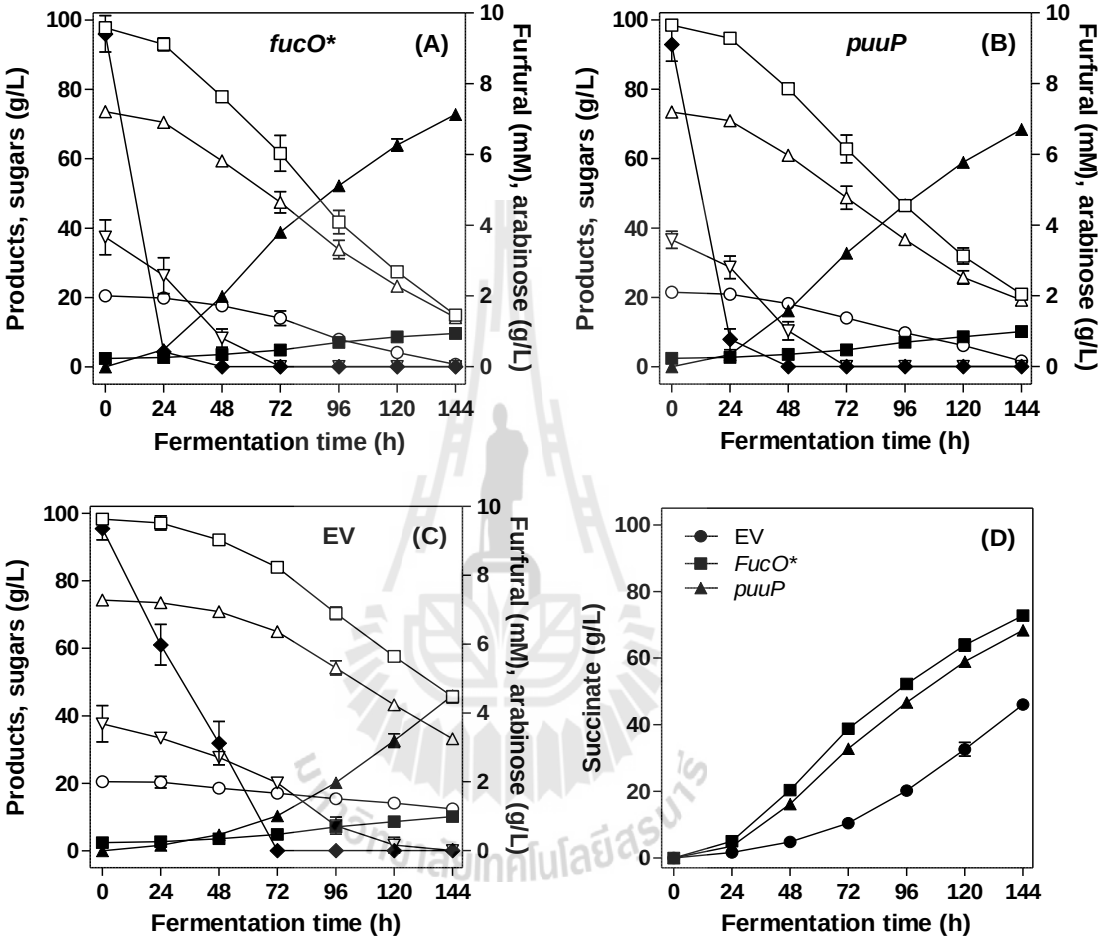

Figure 6.6 Fermentation of mixed sugars in sugarcane bagasse hydrolysate by *E. coli* AS1600a harboring the furfural resistant genes. A. Strain harboring a *fucO** plasmid. B. strain harboring *puuP* plasmid. C. Strain harboring an empty vector plasmid (EV). D. Comparison of succinate production and furfural metabolism. Symbols for all: total sugars (open square), xylose (open circle), glucose (open triangle), succinate (filled triangle), acetate (filled square), biomass (filled circle), furfural (filled diamond).

6.4 Conclusion

This study demonstrated that all of the effectiveness of furfural resistant genes from ethanologenic strain was also beneficial improved furfural tolerance in succinate producing *E. coli* strain AS1600a. In tube culture, the highest furfural tolerance was found to be a strain harboring the *fucO** plasmid followed by *puuP*. Under pH controlled and IPTG induction, the strain harboring the *fucO** and *puuP* could completely metabolize 20 mM furfural within 48 h and produced succinate with an impressive yield (up to 0.85 g/g) from 10% (w/v) xylose or sugars mixture of 5% (w/v) xylose and 5% (w/v) glucose. The strain harboring *fucO** gene produced about 37% higher in succinate production from sugarcane bagasse hydrolysate when compared with that of the control (empty vector). This study suggested that furfural resistant genes such as *fucO** and *puuP* may be useful for improving industrial succinate production from diluted acid of lignocellulosic biomasses.

CHAPTER VII

HYDROLYSATE RESISTANT DERIVATIVES OF *ESCHERICHIA COLI* AS1600a FOR CONVERSION OF SUGARCANE BAGASSE HYDROLYSATE INTO SUCCINATE

7.1 Introduction

Increased environmental concern and the depletion of mineral oil reserves stimulate the search for alternative energy sources and also for alternative biochemical processes. The key to success in the development of profitable industrial biochemical conversion technologies is the choice of target fermentations that can compete with the efficiency of the petrochemical industry (Beauprez et al., 2010). Since lignocellulosic materials are renewable natural, abundant availability and relatively inexpensive carbon source, the operating costs for producing industrial chemicals can be significantly reduced with the use of lignocellulosic materials as the source of carbon.

Sugarcane bagasse, a fibrous residue after the sugarcane stalks, is crushed to extract their juice. Per 1,000 kilograms of sugarcane provided 125 kilograms of bagasse, thus over 100 million tons of bagasses are produced annually throughout the world (Botha and Blottnitz, 2006). With this regard, sugarcane bagasse seems to be the most promising alternative feedstock for microbial production of bio-based

chemicals such as succinate, a commercial intermediate for biodegradable plastic, specialty chemical in food, agricultural, and pharmaceutical industries (McKinlay et al., 2007).

Sugarcane bagasse, like lignocellulosic materials in general, is resistant to degradation, resulting in the need for pretreatment processes that allow access to the fermentable sugars. Steam pretreatment of sugarcane bagasse with dilute mineral acids is an efficient approach to depolymerize hemicellulose into sugars (hydrolysate, primarily xylose) and to increase the access of cellulase enzymes (Geddes et al., 2011). The acid/steam pretreatment releases not only fermentable sugars but also causes the breakdown of lignin and dehydration of the sugars, producing the inhibitory compounds such as organic acids (acetic acid and formic acid), furan derivatives (furfural and 5-hydroxymethylfurfurral, HMF) and phenolic compounds (Keating et al., 2014). These compounds are known to inhibit microbial growth and to retard the fermentation (Miller et al., 2009).

A number of approaches have been made to remove toxic compounds from the sugarcane bagasse hydrolysate. For example, the use of vacuum evaporation (Chandel et al., 2013), addition of sodium meta-bisulfite (Nieves et al., 2011), high pH treatment with ammonium hydroxide (NH_4OH) (Geddes et al., 2013), and the combining of several treatments (vacuum evaporation, laccase, high pH, bisulfite, and microaeration) have been investigated. However, the detoxification process increases the process cost and causes the potential loss of fermentable sugars (Keating et al., 2014). Hence, it is need to develop biocatalysts that can tolerate the inhibitory compounds presenting in the hydrolysate.

A traditional strategy called metabolic evolution has been accomplished in combating the inhibitor effects of hydrolysate by maintaining continuous cultures to metabolically evolved cells in the presence of hydrolysate (Geddes et al., 2011). Previously, the improvement of furfural tolerance in succinate production *E. coli* strain AS1600a by expression of the native *fucO* (encoding for L-1,2-propanediol oxidoreductase), the mutant *fucO* (*fucO*(L7F), *fucO**), *pntAB* (nicotinamide nucleotide transhydrogenase), *ucpA* (cryptic gene), *thyA* (thymidylase syntase), *puuP* and *potE* (polyamine transporters), have been reported (Sawisit et al., 2015). However, the fermentation time requires for succinate production from the sugarcane bagasse hydrolysate by the strain harboring those furfural tolerance genes were still longer than that of pure sugars fermentation. Even though, furfural in hydrolysate was completely removed by vacuum evaporation. It is indicated that other inhibitory compounds in hydrolysate rather than furfural retard the microbial growth. The objective of this present study was to develop a metabolically evolved *E. coli* strain AS1600a capabling of using sugarcane bagasse hydrolysate without furfural for efficient production of succinate.

7.2 Materials and methods

7.2.1 Strains and plasmids

Strain, plasmids and primers used in this study are listed in Table 7.1. A hydrolysate resisitant strain *E. coli* AS2003, derivatives of *E. coli* AS1600a (Sawisit et al., 2015), was used as bacterial strain for succinate production throughout this study. For expression vector construction, the construction of the stable expression vector, (pLOI5883) and *fucO** (pLOI5425) plasmids were previously described

(Sawisit et al., 2015). The plasmids were purified using a QIAspin Spin Miniprep kit (Qiagen, Valencia, Ca). The purified plasmids were then transformed into *E. coli* AS2003 by heat shock at 42°C, 45 seconds. The colonies grown on ampicillin AM1 agar plate containing 10% (v/v) vacuum-treated sugarcane bagasse hydrolysate were selected for further study.

Table 7.1 Strains and plasmid used in this study.

Strains and plasmids	**Relevant characteristics or sequence**	**Source or references**
Strains		
E. coli KJ122	*E. coli* ATCC 8739 (Δ*ldhA*, Δ*adhE*, Δ*ackA*, Δ(*focA-pflB*) ΔmgsA, Δ*poxB*, Δ*tdcDE*, Δ*citF*, Δ*aspC*, Δ*sfcA*, *pck**, *ptsI**)	Jantama et al., 2008b
E. coli AS1600a	Clone from 16th transfer of *E. coli* KJ122 in 10% xylose containing point mutations in *galP* (*galP**) and an *rhs*-like gene	Sawisit et al., 2015
E, coli AS2003	Clone from 145th transfer of *E. coli* AS1600a in 10-80% vacuum bisulfite sugarcane bagasse hydrolysate	This study
E, coli AS020	The strain populations during serial transfer of *E. coli* AS1600a in 10-80% vacuum bisulfite sugarcane bagasse hydrolysate	This study
Plasmids		
pTrc99a	pTrc *bla oriR rrnB lacI^q*	Laboratory collection
pCR2.1-TOPO	*ori bla lacI^q* , vector for constructions	Thermo Fisher
pLOI707EH	Source of RSF1010 replicon	Arfman et al., 1992
pLOI5883	Expression vector, RSF1010 *rep* pTrc *bla rrnB lacI^q*	Sawisit et al., 2015
pLOI5423	*fucO* (L7F), (*fucO**) gene in *EcoR*I-*BamH*I digested pLOI5883	Sawisit et al., 2015
Primers		
RSF1010 *rep*	For-GGAGCAGAAGAGCATACATCTGG Rev-GGACTAGTCTGAAAGCGACCAGGTGCTCG	Sawisit et al., 2015
pTrc99a Δ*oriR*	For-CGCTTACAGACAAGCTGTGACCG Rev-GGACTAGTGTCTGACGCTCAGTGGAACGAA	Sawisit et al., 2015
*fucO** cloning	For-CGGAATTCGTAAAGCAACAAGGAGAAGGATGATG Rev-CGGGATCCTTGCGGGGATCAGCAGTTCA	Sawisit et al., 2015

Underlines indicate the recognition sites for cloning the genes into expression vector (pLOI5883). *Eco*RI: GAATTC, *BamH*I: GGATCC and *Spe*I: ACTAG.

7.2.2 Preparation of sugarcane bagasse hydrolysate

Sugarcane bagasse hydrolysate was prepared at the University of Florida Biofuels Pilot Plant as previously described (Nieves et al., 2011). Briefly, sugarcane bagasse (Florida Crystals) was soaked with phosphoric acid (0.5% of bagasse dry weight) for 4 h. The dilute acid impregnated bagasse was steam-treated for 5-7 min at 185 or 190°C. Hemicellulose syrup (hydrolysate) was recovered using a screw press, discarding solids. After removal of fine particulates with a Whatman GF/D glass fiber filter, clarified hydrolysate was stored at 4°C (pH 3.0).

7.2.3 Sugarcane bagasse hydrolysate detoxification

The sugarcane bagasse hydrolysate was pre-treated with 3 following steps; 1) vacuum treatment, 2) high pH treatment and 3) bisulfite addition. For vacuum treatment, the clarified hydrolysate was evaporated at 55°C under vacuum condition by using vacuum rotary evaporator (Buchi Rotavapor R110 evaporator, Flawil Switzerland) equipped with a Cole Palmer aspirator pump Model 7049-00 (Chicago, Illinois) (Geddes et al., 2015). Unless otherwise indicated, hydrolysate was evaporated to 50% by weight and restored to original weight by adding sterilized deionized water. For high pH treatment, the hydrolysate was adjusted pH to 9.0 by using ammonium hydroxide (5N NH_4OH) and stored at room temperature for 16 h before inoculation. Hydrolysate was adjusted pH to 6.3 with ammonium hydroxide at the day before fermentation and was used as a control (Geddes et al., 2015). For bisulfite addition, a freshly prepared solution was added to culture immediately before inoculation (1 mM final concentration of bisulfite is equal to 0.5 mM sodium metabisulfite) (Nieves et al., 2011).

7.2.4 Sugarcane bagasse hydrolysate toxicity

The hydrolysate toxicity was determined by measuring succinate production after 48 h (37°C) in tube cultures (13 x 100 mm) containing 4 mL of AM1 medium with various concentrations of hydrolysate (0-90%, v/v) as previously described (Geddes et al., 2014; 2015). Since inocula and media components represented up to 10% of the volume, the highest concentration of hydrolysate that could be examined was 90% (Figure 7.1). Xylose was added as needed to provide a monomer sugar concentration of approximately 50 g/L. The toxicity was estimated by comparing hydrolysate concentrations (treated and untreated control) that inhibited succinate production by 50% (IC_{50}) and by 100% (minimum inhibitory concentration, MIC). The inoculum was prepared by growing the cell overnight on AM1 xylose agar plates. Fresh colonies were scraped, re-suspended in AM1 medium and adjusted to OD_{550nm} 1.0. Tube cultures were inoculated to an initial OD_{550nm} of 0.1 (33.3 mg DCW/L) and incubated in a reciprocating water bath (50 rpm shaking, 37°C). All tube cultures and tests were prepared in triplicates and all experiments were repeated at least twice.

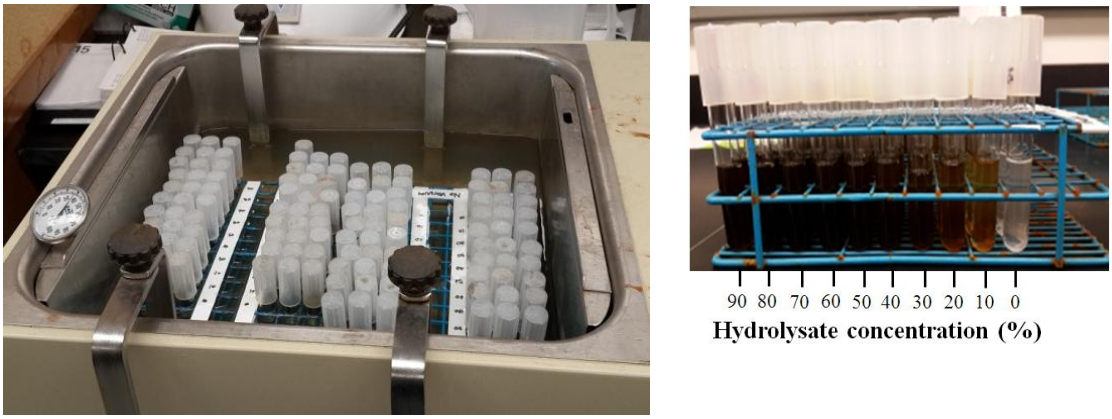

Figure 7.1 The experimental set up of the tube cultures (13 x 100 mm) containing 4 mL medium during incubating in the reciprocating water bath.

7.2.5 Fermentation and culture condition

Fermentations were carried out in 500 mL in-house-built small fermentation vessel with working volume of 300 mL as previously described (Sawisit et al., 2015). A low salts medium, AM1 (4.2 g/L total salts; Martinez et al., 2007) was used as a fermentation medium. This medium was supplemented with 100 mM $KHCO_3$ and 1 mM betaine. For seed culture preparation, one or two fresh colonies grown on AM1 agar supplemented with 5% (w/v) xylose or 10% (v/v) hydrolysate were inoculated in AM1 medium containing 5% xylose or 10% (w/v) hydrolysate depending on experiment, and grown for 16–18 h at 37ºC with 150 rpm shaking. Fermentations were inoculated with an initial OD_{550} of 0.1 (0.33 mg CDW/mL/OD). Anaerobiosis was rapidly achieved during growth with added bicarbonate serving to ensure an atmosphere of CO_2. During the fermentation process, the pH of the medium was maintained by automatic addition of a mixture of 6 M KOH and 3 M K_2CO_3 (1:4 ratio). IPTG was added as indicated in fermentation with the furfural resistant plasmids. No antibiotics were used for maintaing plasmids.

7.2.6 Metabolic evolution to select for hydrolysate-resistant strain

The hydrolysate resistant derivative of *E. coli* AS1600a was selected by metabolic evolution in 500 mL in-house-built small fermentation vessel with working volume of 300 mL. The AS1600a strain was repeated sub-culture in dilute acid sugarcane bagasse hydrolysate (initially 10% till 80%, v/v) pre-treated with vacuum and addition of 2 mM bisulfite. In order to keep the sugar concentration and achieve the appropriate dilution of hydrolysate, modified AM1 medium was prepared by diluting hydrolysate with AM1 medium containing 50 g/L xylose. The pH of hydrolysate was adjusted to 6.3 using 5 N NH_4OH solutions at the day prior to fermentation. Cultures (2%, v/v inoculum) were rapidly transferred when succinate concentration exceeded 5 g/L. The concentration of hydrolysate was increased in the selection medium when at least three successive transfers produced over 10 g/L succinate in 24 h (Geddes et al., 2011). The fermentation condition was operated as same as described in the xylose evolution experiment (Sawisit et al., 2015).

7.2.7 Isolation, sequencing, and comparison of chromosomal DNA

After 145 serial transfers, the hydrolysate resistant colonies were isolated from solid medium containing 10%, (v/v) vacuum-treated hydrolysate (Geddes et al., 2011). The succinate production was examined in pH-controlled fermentations with 60% (v/v) vacuum-treated hydrolysate and 2 mM bisulfite supplemented with 5% (w/v) xylose in AM1 medium. One of the best hydrolysate-resistant colonies exhibited a fast cell growth and produced the highest succinate from 60% (v/v) hydrolysate was assigned as *E. coli* AS2003. For genome sequencing, genomic DNA samples of *E. coli* AS1600a parental strain, and AS2003 strain were

purified according to the bacterial genomic DNA protocol from the DOE Joint Genome Institute (http://jgi.doe.gov). Next-generation sequencing was performed using Illumina paired-end technology (150 bp read length). Sequencing and bioinformatics was provided by the Tufts University Core Facility (Boston, MA). Sequences were aligned and compared (CLC Sequence Viewer, Qiagen, Valencia, CA) using *E. coli* ATCC 8739 (Accession number NC_010468.1, NCBI) as the template.

7.2.8 Analyses

Cell growth was estimated from the amount of succinate produced during hydrolysate fermentation due to the optical density measurement was obscured by color and color changes during fermentation, especially at higher hydrolysate concentrations (Geddes et al., 2014; 2015). Sugars, furans, and organic acids were analyzed by two high-performance liquid chromatography (HPLC) systems (Agilent Technologies 1200) as described previously (Geddes et al., 2014). Sugars and furan were analyzed using a BioRad (Hercules, CA) Aminex HPX-87P ion exclusion column (80ºC; nano-pure water as the mobile phase, 0.6 mL/min). Organic acids were analyzed using a BioRad Aminex HPX-87H column (45ºC; 4 mM H_2SO_4 as the mobile phase, 0.4 mL/min).

7.2.9 Statistical methods

Analysis of variance (ANOVA) was conducted using SPSS software (SPSS 17.0 for Windows; SPSS, Inc., Chicago, IL, USA). Results from at least 3 tests were reported as averages with standard deviations. Differences among mean values were established using Duncan's multiple range tests at 95% significance level.

7.3 Results and discussion

7.3.1 Sugarcane bagasse hydrolysate toxicity

Dilute acid sugarcane bagasse hydrolysate contained unwanted side products that retard fermentation. The detoxification processes are needed to be performed in order to minimize toxins in hydrolysate before fermentation. In this study, three detoxification methods were evaluated to reduce toxicity of dilute acid sugarcane bagasse hydrolysate. The result showed that the AS1600a strain could not grow and produce succinate once using hydrolysate without any pretreatments as a carbon source (pH 6.3 treatment, control) (Figure 7.2A). However, the strain exhibited a little growth at 10% (v/v) in non-detoxified hydrolysate when using pH 9.0 treatment (Figure 7.2B). The removal of volatile compounds with vacuum or addition of either bisulfile alone or the combination of those two approaches improved the IC_{50} and MIC levels. The combination of vacuum treatment and addition of bisulfile was more beneficial than those of either alone. With those combinations, the MIC of hydrolysate was greater improved from 10 to 50% (v/v) or 20 to 70% (v/v) in pH 6.3 and pH 9.0 treatments, respectively. Converti et al. (2000) reported that the improvement of xylitol production from hydrolysate after removal of acetic acid, furfural and other volatile compounds by vacuum was achieved. The concurrent reduction of toxicity and removal of furfural by vacuum evaporation suggests that furfural is an important inhibitor in sugarcane bagasse hydrolysate. The elimination of furfural from hydrolysate by vacuum evaporation improved cell growth and enhanced succinate production by the *E. coli* strains AS1600a. However, it was not only furfural removed by evaporation, but unquantified compounds of equal or greater importance may also be removed by evaporation. In addition, Nieves

et al. (2011) reported that the addition of reduced sulfur compound like sodium metabisulfite increased growth and fermentation of dilute acid sugarcane bagasse hydrolysate by ethanologenic *E. coli* LY180. The addition of sodium metabisufite at 0.5 mM sufficiently reduced hydrolysate toxicity and allowed an unclean hydrolysate (without solid-liquid separation) to be fermented by the strain (Nieves et al., 2011). Sodium metabisulfite is known to form bisulfite adducts with aldehydes, and to react with pyrimidine bases in DNA (Frommer et al., 1992). However, the mechanism of action for sodium metabisulfite in hydrolysate detoxification is not clearly understood. It does not suppress the toxicity of furfural in mineral salts medium (Nieves et al., 2011).

When individual testing, high pH treatment (pH 9.0) was more beneficial to improve the growth and succinate production of *E. coli* AS1600a when compared with those of pH 6.3 treatment (control) (Figure 7.2B). The higher MIC and IC_{50} values after high pH exposure indicated a reduction in hydrolysate toxicity. Martinez et al. (2000) demonstrated that using high pH treatment (calcium hydroxide over liming), helped to reduce levels of sugarcane bagasse hydrolysate toxicity by reduction of inhibitors both of furans (furfural and HMF), and to improve of the growth of ethanologenic *E. coli*. Though, this method caused a sugar loss about 10% (Chandel et al., 2013). Additionally, high pH treatments using sodium hydroxide and potassium hydroxide have also been showed to be effective in reducing the hydrolysate inhibitory compounds (Mohagheghi et al., 2006). Unlike those mentioned above, however, high pH treatment using ammonia caused very little sugar destruction less than 1% (data not showed). Based on this result, the best detoxification process showed the highest MIC and IC_{50} for succinate production by

E. coli AS1600a was found to be the combination of vacuum treatment and addition of bisulfite. Although, the MIC of hydrolysate only low as 40% (v/v) in pH 6.3 treatment and 70% (v/v) in pH 9.0 treatment were permitted the strain growth. Beyond these concentrations, the microbial growth was completely inhibited. This indicated that there were other inhibitory compounds rather than furfural retarding the growth of microbial fermentation. In addition, high pH treatment might not be suitable for succinate production in the industry application using a simultaneous saccharification and co-fermentation process (SScF) where hydrolysis of cellulose and fermentation takes place in a single reactor (Geddes et al., 2011; Nieves et al., 2011). Using high pH treatment, more acid is used to bring pH down to optimum pH of cellulase and hemicellulase (around 5.0-6.0). Therefore, in this study the metabolic evolution was further applied to improve cell growth and succinate production using vacuum and bisulfite-treated sugarcane bagasse hydrolysate adjusted pH to 6.3 before inoculation.

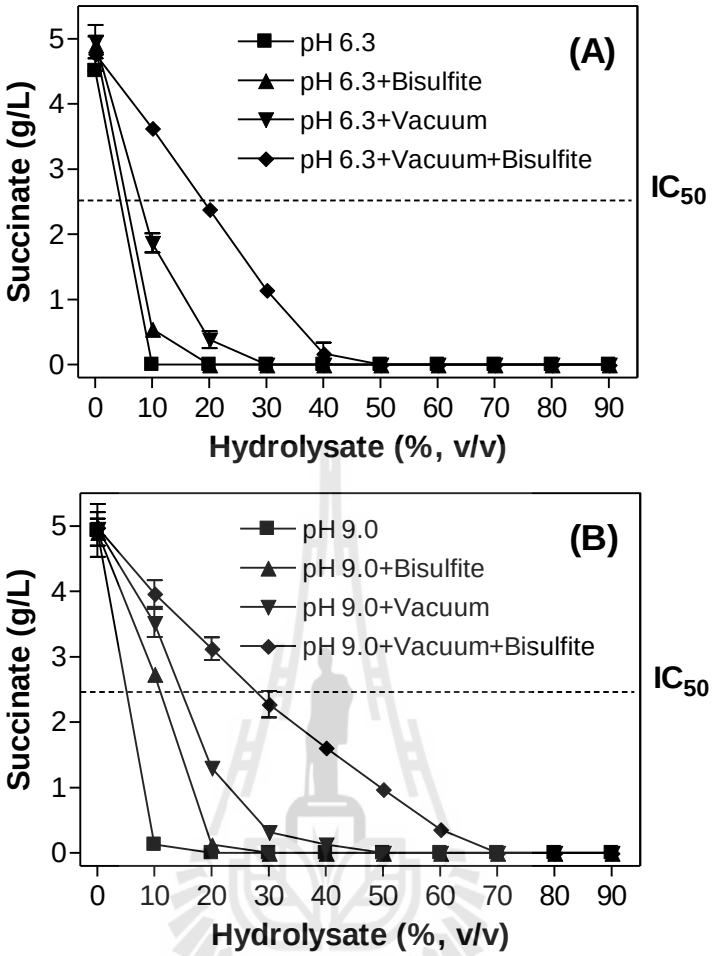

Figure 7.2 Minimum inhibitory concentration (MIC) and concentration of inhibitor that reduce succinate production by 50% (IC_{50}) for succinate production by *E. coli* AS1600a. A. pH 6.3 treatment, B. pH 9.0 treatment.

7.3.2 Development of hydrolysate-resistant strain

Genetics of *E. coli* strain AS1600a have been previously described (Jantama et al., 2008b; Sawisit et al., 2015). This strain could efficiently ferment sugars that are constituents of hydrolysate but it could not grow and produce succinate when hydrolysate used was used over 40% (v/v) even furfural was removed from hydrolysate and bisulfite was added (Figure 7.2A). It seems that other inhibitor

compounds rather than furfural such as phenolic compounds that inhibits the microbial growth. It is generally accepted that using higher hydrolysate concentration ratio up to 90% (v/v) for productions of succinate must enhance for industrial scale application to reduce operating costs. In this study, the metabolic evolution was further applied to improve the strain tolerance to hydrolysate regardless of furfural. The culture was serial transfered under the pH-controlled with initial transferring the culture at 10% (v/v) of vacuum bisulfite treated hydrolysate supplemented with 5% (w/v) xylose in AM1 medium. The components of sugarcane bagasse hydrolysate used during metabolic evolution were summarized in Table 7.2. As seen in Figure 7.3, an improvement of strain during serial transfers was observed. After 145 serial transfers, the strain could utilize 60 to 80% (v/v) vacuum bisulfite treated sugarcane bagasse hydrolysate with no lag phase. The strain could consume all sugars in hydrolysate and produce succinate with a yield up to 85% (based on total sugars consumed). With the use of a new batch or changing the higher concentration of hydrolysate according to Table 7.2, a delay in growth of the first transfers was noticed (Figure 7.3). The growth and succinate production were improved for sequential transfers. The probable reason could be that the higher hydrolysate concentration might contain more inhibitors, causing longer lag phase of cell growth when compared with that of the lower hydrolysate concentration. Once the cells adapted itself to hydrolysate inhibitors, the lag phase of cell growth was reduced. Additionally, during the 26 to 29th serial transfers, the small amount of side products such as lactate, ethanol and formate were detected. Genes, *ldhA*, *adhE* and *pflB* that responded for the production of lactate, formate and ethanol, respectively, were deleted from the parental strain before metabolic evolution (Jantama et al., 2008b).

The accumulation of those products might be caused by the contamination of other bacterial. This problem can be overcomed by restarting a new transfer with a clean stock to resume a transfer. Moreover, the higher inhibitory compounds found in hydrolysate that prepared by the METSO caused a longer lag phase of cell growth when compared with that of using hydrolysate from the steam gun (Table 7.2).

After 145th serial transfers, the hydrolysate-resistant colonies were isolated from solid medium and tested for succinate production in pH-controlled fermentations with 60% (v/v) vacuum bisulfite treated hydrolysate supplemented with 5% xylose (w/v) in AM1 medium. All appeared similarly and one was designated AS2003. The AS2003 strain was further examined for the MIC of hydrolysate compared with the parental strain, *E. coli* AS1600a (derivative of *E. coli* KJ122). Figure 7.4 indicated that the AS2003 strain was more resistant to the vacuum-treated hydrolysate when compared with that of the parental strain. The KJ122 strain could not ferment 0 to 20% (v/v) hydrolysate which contained mainly xylose. The strain started growing once using hydrolysate beyond 30% (v/v) due to more glucose was carried over from hydrolysate. The poor utilization of xylose by the KJ122 strain was previously described (Sawisit et al., 2015). The AS2003 strain grew on 70% (v/v) vacuum bisulfite treated hydrolysate (prepared by METSO, 185ºC, 7.5 min), and this condition completely inhibited the parental strain. There was about 20% (v/v) higher in hydrolysate concentration that strain was able to resist as compared with that of the parental strains (hydrolysate MIC; 80% versus 60%, v/v). Similarly, Geddes et al. (2011) applied a metabolic evolution of ethanologenic *E. coli* MM160 in diluted phosphoric acid hydrolysate of sugarcane bagasse. After 139 sequential transfers, the strain MM160 grew and fermented well in AM1 medium containing 60% (v/v)

phosphoric acid hydrolysate. The concentration completely inhibited the growth of the parent strain (*E. coli* LY180). Chen et al. (2010) used a metabolic evolution approach to improve a non-transgenic homoethanol *E. coli* SZ420 for xylose fermentation. Wang et al. (2011a) evaluated that during about 350 generations of adaptive evolution, the evolved mutant, *E. coli* SZ470, was able to grow anaerobically at 40 g/L ethanol, a two-fold improvement over the parent KC01 strain. This suggested that the metabolic evolution was very helpful approach for improving the strain tolerance to the conditions that was harmful to cells.

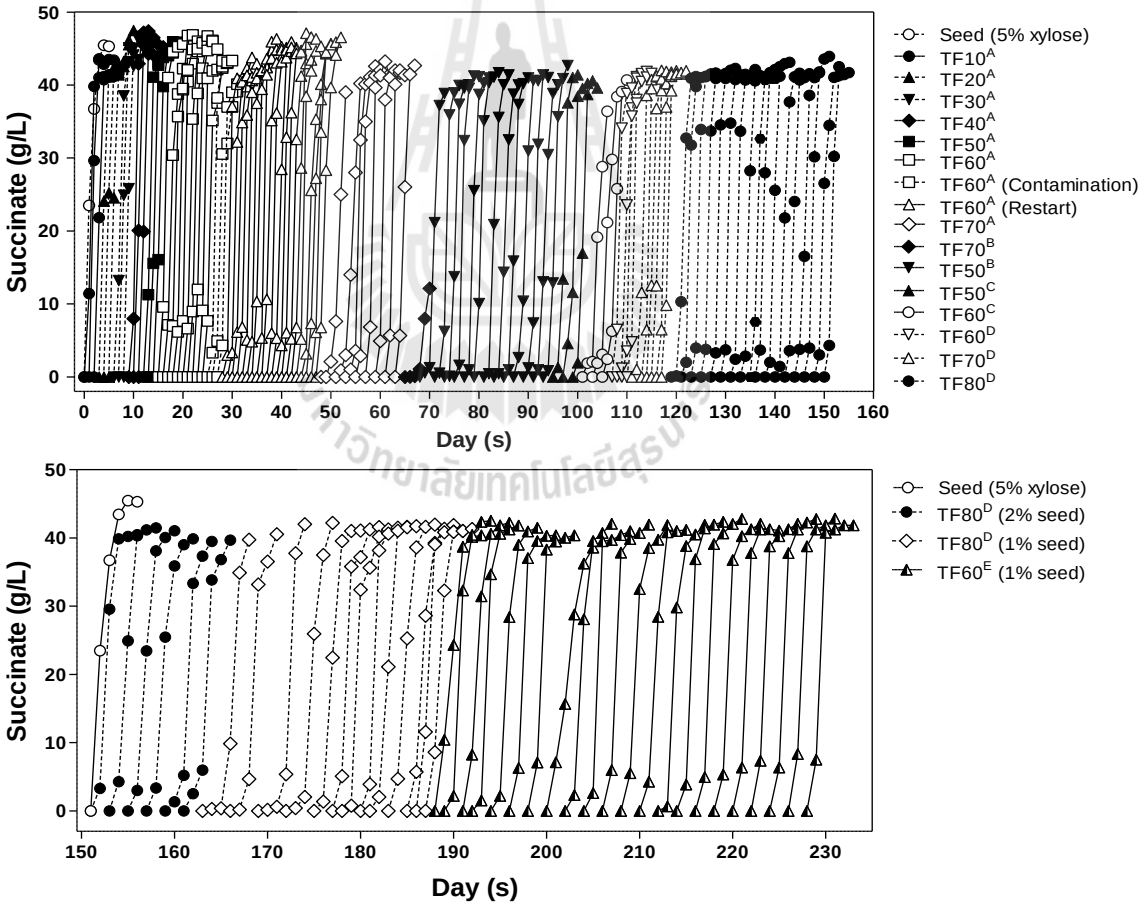

Figure 7.3 The metabolic evolution of *E. coli* AS1600a in diluted acid sugarcane bagasse hydrolysate under pH controlled vessel.
[A-E]The superscript represents the different batches of sugarcane bagasse hydrolysate and its compositions were summarized in Table 7.2.

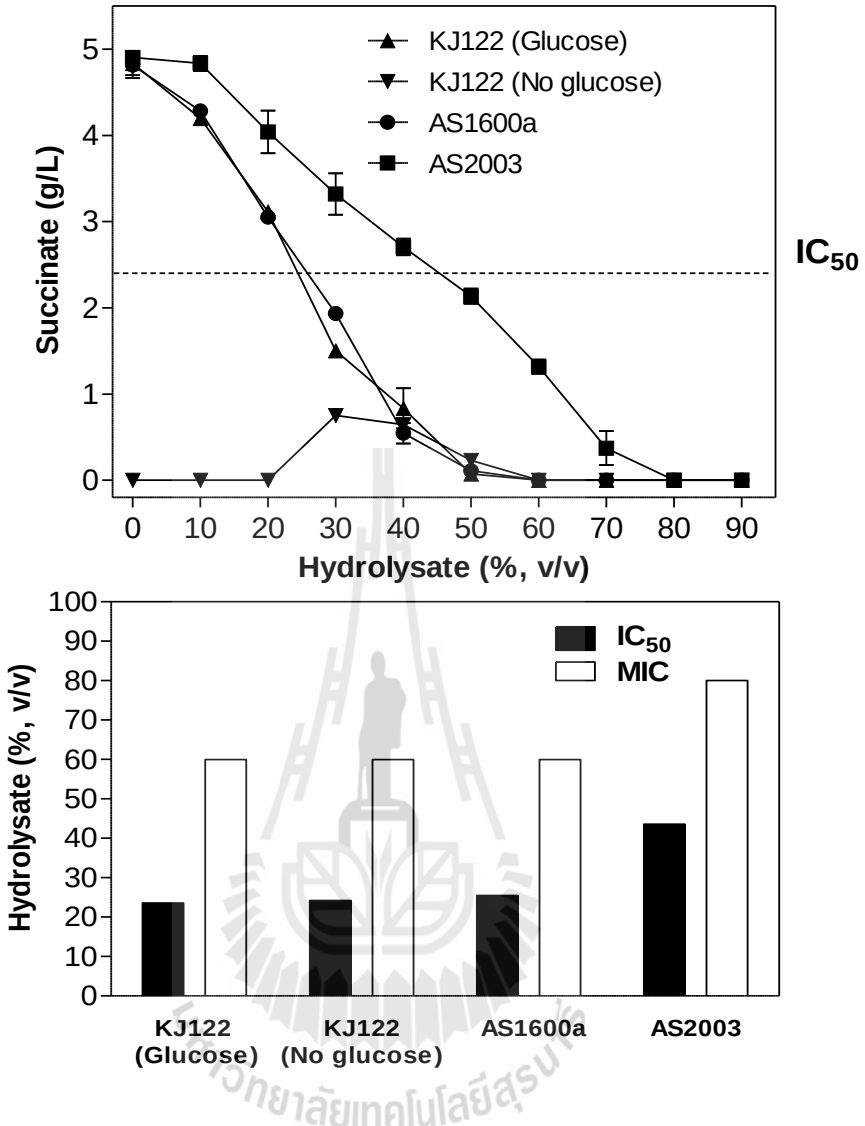

Figure 7.4 Comparison of the minimum inhibitory concentration (MIC) and the concentration of inhibitor that reduce succinate production by 50% (IC_{50}) of sugarcane bagasse hydrolysate for succinate production by succinate-producing *E. coli*.

Considering the hydrolysate component in Table 7.2, the total sugars content along with the amount of inhibitors forms (furfural + acetate) in sugarcane bagasse hydrolysate were varied and strongly depended on the type of pretreatments and

conditions used. The main sugar presenting in hydrolysate was found to be xylose which was in the range of 41.18±0.10 to 46.31±0.75 g/L, followed by arabinose (3.95±0.26 to 6.93±0.06 g/L), glucose (1.98±0.12 to 4.81±0.08 g/L), and galactose (2.24±0.29 to 3.31±0.10 g/L), respectively. The highest sugars content (58.65±0.30 g/L) and the highest inhibitors (furfural + acetate, 8.38±0.06 g/L) were obtained from steam pretreatment with the METSO (190ºC, 7.5 min). It indicated that using higher temperature and longer time processing during dilute acid/steam pretreatment of sugarcane bagasse produced more sugars but also generated more inhibitor compounds (Table 7.2). Kabel et al. (2007) reported that the level of inhibitor produced during acid/steam pretreatment was affected by the severity of conditions, and by the choice of acid. Additionally, the inhibitors generation during acid/steam pretreatment was increased concomitantly with time and temperature (Castro et al., 2014). It is interesting to note that the inhibitors content were reduced after vacuum evaporation. Furfural was completely removed (100%) while acetate was reduced about 40-60% after vacuum evaporation. Similarly, furfural (90%) and HMF (4%) were removed from wood hemicellulose hydrolysate using vacuum evaporation (Larsson et al., 1999). Furthermore, Chandel et al. (2013) confirmed that the evaporation under vacuum could remove volatile compounds like furfural, acetic acid and vanillin from hemicellulose hydrolysate. Geddes et al. (2015) also demonstrated that vacuum treatment with half the weight (50% evaporation) of sugarcane bagasse hydrolysate reduced furfural to undetectable amounts and also reduced acetate concentrations by 25%. It suggested that vacuum evaporation was beneficial for removing volatile toxic compounds in hydrolysate and provided a good growth for microbial fermentation.

Table 7.2 Comparison of sugarcane bagasse hydrolysate component obtained from different pretreatment methods.

Pretreatment methods		Hydrolysate components						
		Sugars (g/L)					Inhibitors (g/L)	
		Glucose	Xylose	Arabinose	Galactose	Total sugars	Furfural	Acetate
Steam gun[A]	Original	2.78±0.04[d]	45.84±0.17[ab]	4.24±0.01[b]	3.21±0.05[a]	56.08±0.20[bc]	1.74±0.16[b]	3.88±0.14[d]
(190ºC, 5 min)	Vacuum	2.85±0.08[d]	46.31±0.75[a]	4.25±0.23[b]	3.30±0.07[a]	56.72±0.83[b]	0.00±0.00[d]	2.10±0.10[h]
METSO[B]	Original	4.17±0.08[c]	45.04±0.24[b]	6.66±0.09[a]	2.77±0.06[b]	58.65±0.30[a]	2.15±0.01[a]	6.23±0.08[a]
(190ºC, 7.5 min)	Vacuum	4.21±0.06[c]	45.06±0.34[b]	6.77±0.11[a]	2.79±0.02[b]	58.82±0.38[a]	0.00±0.00[d]	3.37±0.02[e]
METSO[C]	Original	4.81±0.08[a]	41.18±0.10[d]	6.80±0.05[a]	2.24±0.29[c]	55.04±0.34[c]	1.98±0.18[a]	5.52±0.10[b]
(185ºC, 7.5 min)	Vacuum	4.80±0.05[a]	41.20±0.14[d]	6.82±0.06[a]	2.27±0.28[c]	55.08±0.35[c]	0.00±0.00[d]	2.99±0.12[f]
Steam gun[D]	Original	1.98±0.12[e]	42.92±1.17[c]	3.95±0.26[c]	2.46±0.16[c]	51.30±1.36[d]	0.84±0.12[c]	1.93±0.10[i]
(190ºC, 5 min)	Vacuum	2.06±0.04[e]	42.85±0.78[c]	3.95±0.36[c]	2.48±0.15[c]	51.34±0.86[d]	0.00±0.00[d]	1.05±0.08[j]
METSO[E]	Original	4.33±0.01[b]	43.36±0.12[c]	6.93±0.06[a]	2.15±0.03[d]	56.77±0.18[b]	2.09±0.27[a]	4.95±0.03[c]
(185ºC, 7.5 min)	Vacuum	4.32±0.02[b]	43.54±0.11[c]	6.75±0.11[a]	2.18±0.02[d]	56.80±0.10[b]	0.00±0.00[d]	2.65±0.09[g]

[(A-E)] The superscript represents the different batches of sugarcane bagasse hydrolysate that used during serial transfers of *E. coli* AS1600a.

[(a-j)] The values with different symbols in the same row are significantly different ($p<0.05$).

7.3.3 Batch fermentation of sugarcane bagasse hydrolysate under pH-controlled

Vacuum evaporation of hydrolysate is very effective at removal of volatile compounds particular furfural and enhances the hydrolysate fermentation. However, the construction of evaporation unit is cost associated (Geddes et al., 2015). To minimize the fermentation complexity and reduce the operating cost of hydrolysate fermentation, the best furfural tolerance gene, *fucO** obtained from previously study (Sawisit et al., 2015) was also introduced into *E. coli* AS2003. A non-detoxified sugarcane bagasse hydrolysate (pH 6.3 treatment) contained 56.77±0.18 g/L total sugars and 7.35±0.35 g/L total inhibitors (furfural and acetate) were used as carbon substrate. The fermentations were carried out under the pH controlled using sugarcane bagasse hydrolysate supplemented with 150 g/L glucose as a replacement for hydrolyzed cellulose fiber and 2 mM bisulfite in AM1 medium (100 g/L initial total sugars). The total sugars at 100 g/L contained mostly glucose and xylose with smaller amounts of arabinose and galactose, together with inhibitors from side reactions during diluted acid pretreatment. Concentrations of sugars (100 g/L) and inhibitors are equivalent to what obtained from 15% (w/w) biomass hydrolysis during liquefaction plus simultaneous sacharification and co-fermentation (L+SScF) (Geddes et al., 2011). In case of fermentation with the strains without furfural resistant genes, the hydrolysate used removed furfural and other volatiles by vacuum evaporation. The other hydrolysate inhibitors were mitigated by pH 9.0 treatment with ammonia hydroxide (16 h incubation) and addition of 2 mM bisulfite (Sawisit et al., 2015; Geddes et al., 2015). Figure 7.5 reveals that all strains could utilize all sugars containing in hydrolysate and produced succinate within the first 24

h without a lag period except the strain harboring an empty vector (without furfural resistant genes) exhibiting a lag phase about 24 h (Figure 7.5D). The strain harboring *fucO** fully metabolized the initial furfural concentration (10 mM) in hydrolysate without vacuum evaporation within 24 h (Figure 7.5C). Whereas, the strain harboring an empty vector completely metabolized furfural at 48 h (Figure 7.5F). At the end of fermentation (144 h), the succinate production obtaining from the hydrolysate (pH 6.3 treatment + bisulfite addition + vacuum treatment) and (pH 9.0 treatment + bisulfite addition + vacuum treatment) by the AS2003 strain (no furfural plasmids) were not significantly different resulted in 84.65±1.69 g/L and 85.64±0.28 g/L succinate concentrations, respectively. However, the significant higher of maximum productivity at 0.77±0.01 g/L/h was found in the pH 9.0 treatment (Table 7.3). Interestingly, the succinate production by the AS2003 strain harboring a *fucO** gene using the pH 6.3 treatment and addition of bisulfite was not significantly different when compared with the succinate production by the AS2003 (no furfural resistant genes) using the hydrolysate pretreated with pH 6.3 treatment + bisulfite addition + vacuum treatment. These were also significant higher from what observed in the AS2003 strain harboring an empty vector control (Table 7.3). An expression of a native *fucO* gene, encoding an NADH-dependent, L-1,2-propanediol reductase that is induced during fucose catabolism has been previously reported to increase furfural resistance in *E. coli* biocatalysts engineered for the production of lactate and ethanol (Wang et al., 2011a). The mechanism action of *fucO* gene in furfural tolerance is believed to catalyze the NADH-dependent reduction of furfural to the less toxic alcohol, furfuryl alcohol (Miller et al., 2009). Zheng et al. (2013) applied the saturation mutagenesis combined with growth-based selection to isolate a mutated

form of *fucO* by replacing the most abundant codon for leucine with the most abundant codon for phenylalanine. The mutant *fucO* (L7F), *fucO** gene increased FucO activity by more than 10-fold and caused the rate of furfural metabolism during fermentation for two-folds when compared with the wild type *fucO* gene. Based on the result obtained in this study, expressing a *fucO** gene in the AS2003 strain improved furfural tolerance, and shorted the fermentation time required for succinate production from non-detoxified sugarcane bagasse hydrolysate. Furthermore, it implied that the use of vacuum evaporation in this study could be omitted and the operating cost would be reduced.

Considering sugars metabolism as shown in Figure 7.6, the AS2003 strain was capable of co-utilizing sugars in sugarcane bagasse hydrolysate but the rate of sugars comsumption by the strain was different. At the end of fermentation, the strain completely consumed small amounts of galactose, arabinose and xylose in sugarcane bagasse hydrolysate, but the most abundant sugar glucose remained unfermented after 144 h. The fastest sugars utilization rate by the AS2003 strain in sugarcane bagasse hydrolysate fermentation was found to be galactose followed by arabinose. In most case, the rate of xylose utilization was rapidly increased once arabinose was fully metabolized (Figure 7.6). Similarly, Jiang et al. (2014) reported that glucose-xylose co-utilization during succinate production from corn stalk hydrolysate by a wild type *E. coli* was completely inhibited by the presence of arabinose. Kang et al. (1998) have observed that genes in xylose metabolism pathway were repressed when cells were grown in a mixture of arabinose and xylose. Genes in xylose metabolism pathway are repressed in the presence of arabinose due to the repression of AraC dependence resulting from the binding of arabinose-bound AraC at xylose promoters, which

consequently inhibit genes expression (Desai and Rao, 2009). On the other hand, the *ptsG E. coli mutant* FBRl4 grew on glucose at rate approximately equal to the parent strain and fermented arabinose and xylose simultaneously with glucose (Nichols et al., 2001). The AS2003 strain could simultaneously consume sugars mixture in sugarcane bagasse hydrolysate without carbon catabolic repression (CCR). The probable reason could be due to the AS1600a strain containing a *ptsI* mutation and regulating of RNaseE-dependent cleavage that blocks the glucose uptake by the native PTS system (Zang et al., 2009). The PTS system catalyzes the uptake and concomitant phosphorylation of numerous sugars playing a major role in *E. coli* CCR (Deutscher et al., 2006). The KJ122 strain transports glucose to the cell through the native galactose peramease (GalP), a non-phosportlate form by a sugar-H+ symporter (Zang et al., 2009). In addition, the AS1600a strain also contained a point mutation in the GalP (replacement of glycine residue at the position of 236 with aspatate in GalP protein (Sawisit et al., 2015). Furthermore, Sawisit et al. (2015) demonstrated that an expression of the *galP* mutation gene in KJ122Δ*galP* fully resembled the xylose utilization phenotype of the AS1600a strain. The AS1600a strain could ferment the mixture of glucose and xylose without CCR. This indicated that the changing in the GalP structure was responsible for the high xylose consumption and co-uitilization of sugars phenotype in the adapted *E. coli* AS1600a strain. As this regard, it implied that the strain AS2003 also contained the *ptsI* and *galP* mutations. It might probably transport both glucose and xylose through the mutatant GalP transporter, but with different affinity. Moreover, the transport of xylose into the cell by the use of the mutant GalP (sugar-H^+ symporter) instead of a high-affinity ATP-independent transporter provided the benefit to the AS2003a strain, not only for generating the

ATP to supply cell growth but also maintaining a redox balance for succinate production.

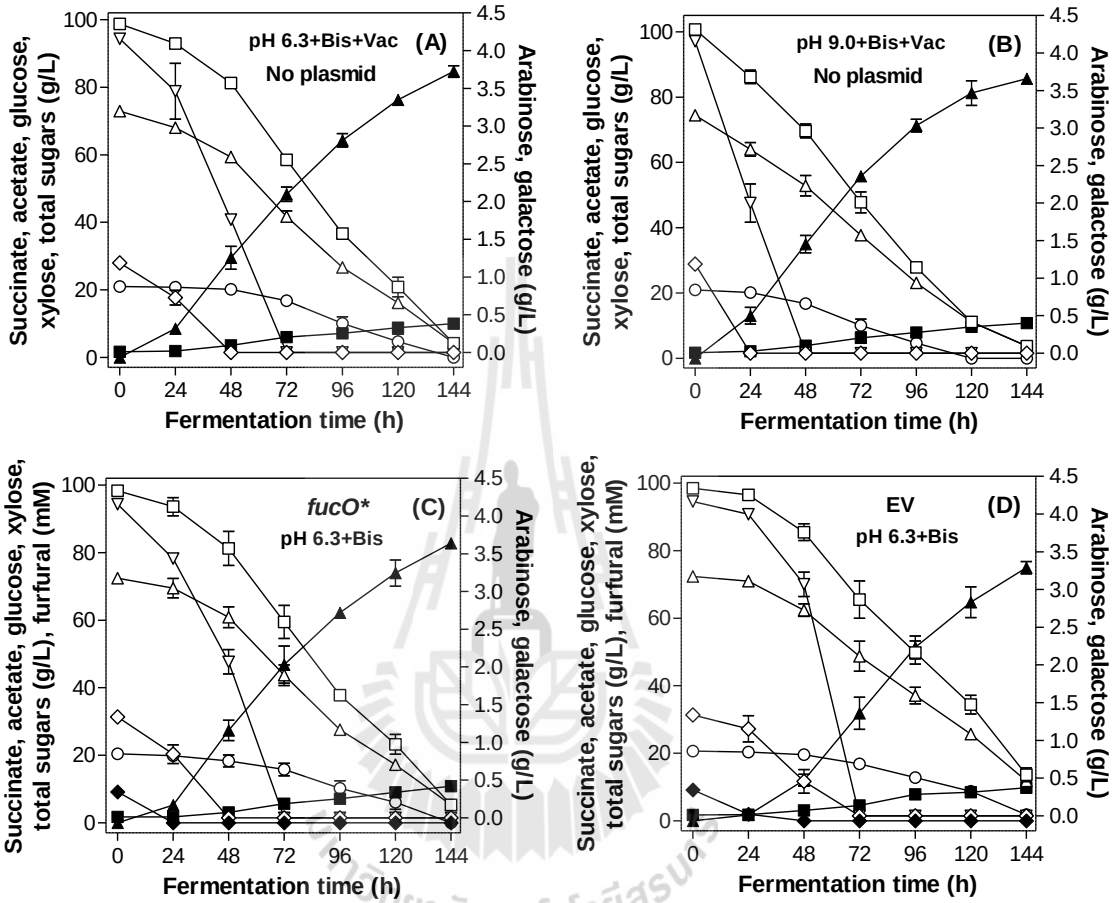

Figure 7.5 Fermentation profile of sugarcane bagasse hydrolysate by *E. coli* AS2003 and its derivatives. A. Succinate production by AS2003 (no plasmid) with pH 6.3 treatment. B. Succinate production by AS2003 (no plasmid) with pH 9.0 treatment. C. Succinate production by AS2003 (*fucO** plasmid) with pH 6.3 treatment. D. Succinate production by AS2003 (empty vector) with pH 6.3 treatment. Symbols for all: total sugars (open square), xylose (open circle), glucose (open triangle), arabinose (open inverted triangle), galactose (open diamond), succinate (filled triangle), acetate (filled square). EV (empty vector, pLOI5883), *fucO** (pLOI5423).

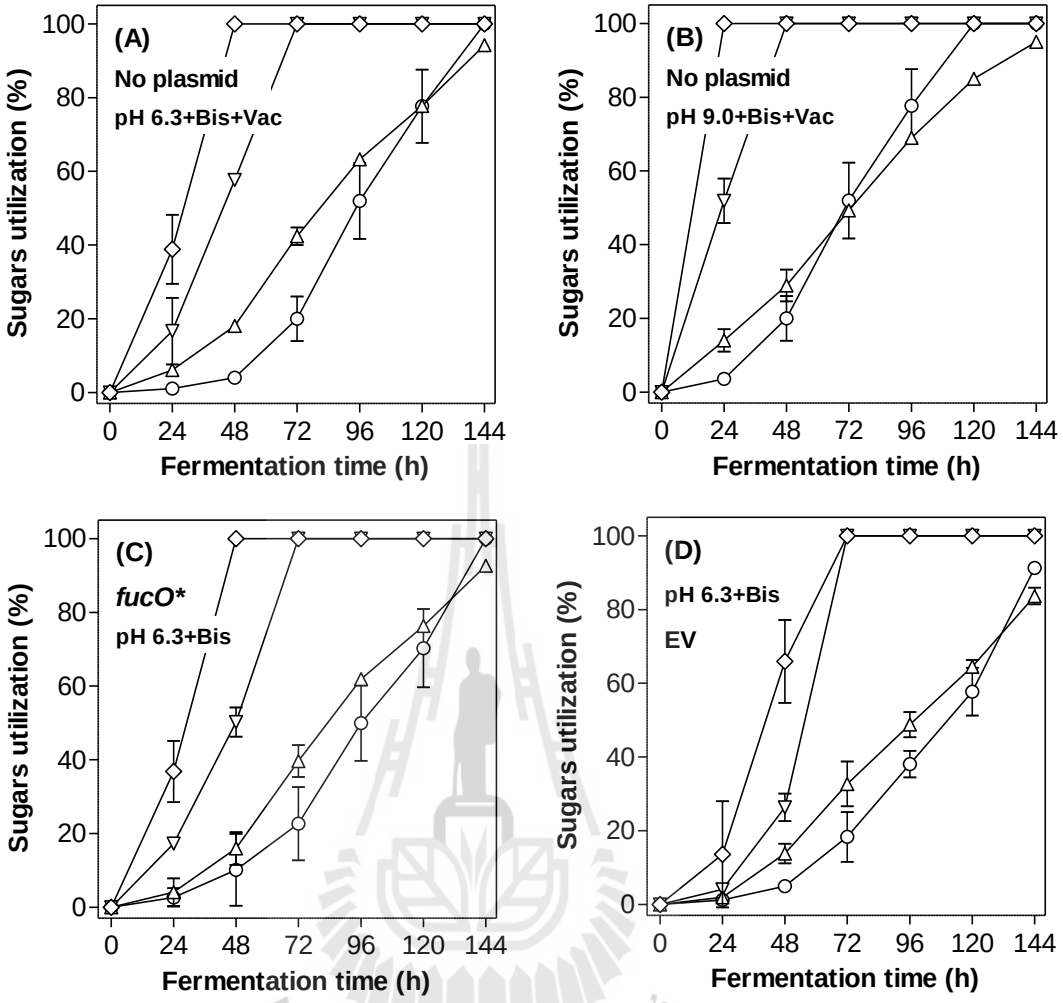

Figure 7.6 Sugars utilization during fermentation of sugarcane bagasse hydrolysate by *E. coli* AS2003 and its derivatives. A. Sugars utilization by AS2003 (no plasmid) with pH 6.3 treatment. B. Sugars utilization by AS2003 (no plasmid) with pH 9.0 treatment. C. Sugars utilization by AS2003 (*fucO** plasmid) with pH 6.3 treatment. D. Sugars utilization by AS2003 (empty vector) with pH 6.3 treatment. Symbols for all: xylose (open circle), glucose (open triangle), arabinose (open inverted triangle), galactose (open diamond). EV (empty vector), pLOI5883. *fucO**, pLOI5423.

According to Table 7.2, it is interesting to note that the succinate production obtained from the hydrolysate resistant *E. coli* strain AS2003 using the sugarcane bagasse hydrolysate pretreated by the combination of pH 9.0 treatment, addition of

bisulfite and vacuum treatment, was about 15% and 22% higher than that of the parental strain AS1600a before evolution and the KJ122 strain, respectively. This result suggested that the metabolic evolution provided an incremental benefit by increasing hydrolysate utilization and also promoted the succinate production up to 85 g/L. The metabolic evolution has successfully improved biocatalysts for many fermentation products including ethanol (Yomano et al., 2008), D-lactate (Utrilla et al., 2012), and succinate (Jantama et al., 2008b; Sawisit et al., 2015).

Table 7.3 Fermentation profile of succinate production from sugarcane bagasse hydrolysate by *E. coli* KJ122 and its derivatives with and without furfural resistant genes.

Strains	Pretreatment conditions	Sugars residual (g/L)		Succinate (g/L)	Acetate (g/L)	Succinate yield (g succinate/total sugars consumed)[A]	Maximum productivity (g/L/h)[B]
		Glucose	Xylose				
KJ122-No plasmid	pH 9.0+Bis+Vac	0.00±0.00^{d}	17.42±0.68^{a}	66.45±0.94^{d}	5.52±0.43^{c}	0.80±0.01^{b}	0.68±0.06^{b}
AS1600a-No plasmid	pH 9.0+Bis+Vac	13.53±0.84^{ab}	1.21±0.45^{bc}	72.66±0.59^{c}	8.33±0.31^{b}	0.87±0.01^{a}	0.59±0.01^{bc}
AS2003-No plasmid	pH 9.0+Bis+Vac	3.70±0.12^{c}	0.00±0.00^{c}	85.64±0.28^{a}	10.76±0.26^{a}	0.89±0.01^{a}	0.77±0.01^{a}
AS2003-No plasmid	pH 6.3+Bis+Vac	4.20±0.59^{c}	0.00±0.00^{c}	84.65±1.69^{ab}	9.98±0.13^{a}	0.88±0.01^{a}	0.67±0.30^{bc}
AS2003-EV	pH 6.3+Bis	11.83±1.63^{b}	1.80±0.38^{b}	74.94±1.80^{c}	9.79±0.14^{a}	0.88±0.01^{a}	0.54±0.04^{c}
AS2003-*fucO**	pH 6.3+Bis	5.33±0.49^{c}	0.00±0.00^{c}	82.77±0.98^{b}	10.79±1.55^{a}	0.89±0.01^{a}	0.65±0.08^{bc}

[A]The succinate yield was calculated as grams of succinate formed divided by grams of the total sugars consumed.

[B]The maximum succinate productivity was calculated from succinate concentration in the medium divided by the incubation time.

Arabinose and galactose in hydrolysate were completely consumed at the end of fermentation.

EV, empty vector (pLOI5885), *fucO** (pLOI5746), Bis (bisulfite), Vac (vucuum)

[(a-c)] The values with different symbols in the same row are significantly different ($p<0.05$).

7.3.4 Comparison of chromosomal DNA

The basis of genetic changes gained during the metabolic evolution could be examined by sequencing the chromosomal DNA of the organism and comparing the sequence data with that of the parental strain (Geddes et al., 2011). In this study, whole genome sequencing of the best three hydrolysate-resistant clones (AS2003, AS2004, AS2005) and the strain populations (AS020) obtained after 145th serial transfers in sugarcane bagasse hydrolysate was performed using AS1600a as the reference strain. The result showed that the strain populations and the best three hydrolysate-resistant clones contained the similar of 8 mutations which were existed >50% frequency when compared with that of the parental strain. Eight mutations were listed in Table 7.4. There were consisted of three tyrosine-aspartate (YD)-repeat, *rhs* genes mutation, one with single nucleotide variation (SNV) and one containing two mutations of a frame shift (fs) deletion and SNV mutation. The fourth mutation was a single nucleotide deletion in an *uspB* gene, universal stress (ethanol tolerance) protein B. The fifth mutation was a single nucleotide change in osmotic regulatory protein OmpR. The residue aspartate was changed to asparagine (Asp183 to Asn). The sixth mutation was the SNV mutation of cytidine synthetase (*pyrG*). A methionine residue was changed to isoleucine, Met84 to Ile. The seventh mutation was the SNV mutation in formaldehyde regulator of *frmRAB* operon (*frmR*). The alanine was changed to threonine (Ala44 to Thr). The last mutation was the SNV mutation in *lysR*-like putative transcriptional regulator with changing of asparagine to asparagine (Asp275 to Asn).

Because of mutations during metabolic evolution, the succinate production from sugarcane bagasse hydrolysate by the adapted AS2003 strain was

greatly improved when compared to that of the parental strain before evolution (85.46±1.69 versus 72.66±0.59 g/L) (Table 7.3). A single or combination of those mutations as mentioned above might be responsible for improving the AS2003 strain resistance to hydrolysate and enhanced succinate production. Further experiments need to be done in order to understand the mechanism of mutations that conferred a resistance to inhibitors in hydrolysate by *E. coli* AS2003 strain. This study suggested that the accessibility of whole genome sequences significantly enhanced our understanding of the physiology, genetics and evolutionary development of bacteria. Genome sequencing is expensive but is probably wise investment. Utrilla et al. (2012) has reported the use the genome sequencing to understand the new phenotype and the metabolic limitations of xylose conversion to D-lactate by *E. coli* JU15. The whole genome sequencing of the evolved strain identified a point mutation in the *gatC* gene, responsible for the high xylose consumption phenotype in the evolved strain. Sawisit et al. (2015) also applied the genome sequencing to search for the mutations that occurred after metabolic evolution of succinate producing *E. coli* AS1600 in xylose. The genome sequencing guided us to identify a point mutation in galactose permease (GalP), which was responsible for the capable of xylose utilization and simultaneously consumed sugars mixture by the evolved strain.

Table 7.4 The mutations in chromosomal DNA (>50% frequency) of the hydrolysate-resistant *E. coli* strain AS2003 compared with the parental strain, *E. coli* AS1600a.

Number of mutation	Reference position	Type	Length	Reference	Allele	Amino acid change	Genes/function
1.	253578	Deletion	1	A	-	Upstream of uspB	*uspB*/Universal stress (ethanol tolerance) protein B
2.	358889	SNV	1	G	A	YP_001723313.1:p.Asp183Asn	*ompR*/Osmotic regulatory protein
3.	1011291	SNV	1	G	A	YP_001723928.1:p.Met84Ile	*pyrG*/Cytidine synthetase, amination of UTP to CTP
4.	2416109	Deletion	1	G	-	YP_001725166.1:p.Pro1267fs	*rhs* family, YD repeat, cell wall or envelope
5.	2416111	SNV	1	G	T	YP_001725166.1:p.Pro1267Thr	*rhs* family, YD repeat, cell wall or envelope
6.	3371207	SNV	1	T	A	YP_001726030.1:p.Leu1387Gln	*rhs* family, YD repeat, cell wall or envelope
7.	3577387	SNV	1	G	A	YP_001726217.1:p.Ala44Thr	*frmR*- formaldehydeinduced neg regul of *frmRAB* operon
8.	3769668	SNV	1	G	A	YP_001726398.1:p.Asp275Asn	putative *lysR*-like regulator

Abbreviation; Fs (frame shift), SNV (single nucleotide variation).

Table 7.5 Comparison of stains development for succinate production from sugarcane bagasse hydrolysate fermentation.

Strain designation	Media/mode of process	Carbon sources	Succinate (g/L)	Yield (g/g substrate) [g/L/h]	References
E. coli BA305, Δ*ldhA*, Δ*pflB*, Δ*ppc* and overexpression of ATP-forming (PEPCK)	Complex medium, dual-phase fermentation.	Sugarcane bagasse hydrolysate (19.66 g/L total sugars)	19.20	0.96	Liu et al., 2013a
E. coli BA305, Δ*ldhA*, Δ*pflB*, Δ*ppc*, Δ*ptsG* and overexpression of ATP-forming (PEPCK)	Complex medium supplemented with LB, Simple batch fermentation	Sugarcane bagasse hydrolysate	10.1	0.66	Liu et al., 2013b
	Feb-batch fermentation.	Sugarcane bagasse hydrolysate	39.3	0.97	
E. coli BA305, Δ*ldhA*, Δ*pflB*, Δ*ppc* and overexpression of ATP-forming (PEPCK)	LB medium supplemented with chemically defined medium, repetitive fermentation.	Sugarcane bagasse hydrolysate	24.5	0.87	Liang et al., 2013
E. coli KJ122, Δ*ldhA*, Δ*adhE*, Δ*ackA*, Δ(*focA-pflB*) ΔmgsA Δ*poxB* Δ*tdcDE* Δ*citF* Δ*aspC* Δ*sfcA*, *pck*, ptsI**	A low salt medium (AM1, 4.2 g/L total salts), simple batch fermentation	50% (v/v) sugarcane bagasse hydrolysate + 75 g/L glucose (100 g/L total sugars)	66.45	0.80	This study
E. coli AS1600a , Δ*ldhA*, Δ*adhE*, Δ*ackA*, Δ(*focA-pflB*) ΔmgsA Δ*poxB* Δ*tdcDE* Δ*citF* Δ*aspC* Δ*sfcA*, *pck*, ptsI*, galP** and 16th serial transfers in 10% (v/v) xylose	A low salt medium (AM1, 4.2 g/L total salts), simple batch fermentation	50% (v/v) sugarcane bagasse hydrolysate + 75 g/L glucose (100 g/L total sugars)	72.66	0.88	This study
E. coli AS2003 , Δ*ldhA*, Δ*adhE*, Δ*ackA*, Δ(*focA-pflB*) ΔmgsA Δ*poxB* Δ*tdcDE* Δ*citF* Δ*aspC* Δ*sfcA pck*, ptsI*, galP** and 145th serial transfers in 10-80% (v/v) hydrolysate	A low salt medium (AM1, 4.2 g/L total salts), simple batch fermentation	50% (v/v) sugarcane bagasse hydrolysate + 75 g/L glucose (100 g/L total sugars)	85.64	0.89	This study

7.4 Conclusion

This study demonstrated that a metabolic evolution is such a helpful approach for improving the strain resistance to non furfural toxic compounds in sugarcane bagasse hydrolysate. After 145 serial transfers, the hydrolysate resistant *E. coli* strain AS2003 could ferment 60 to 80% (v/v) vacuum-treated hydrolysate into succinate with an impressive yield up to 85% without the lag phase, which was comparable to other published works with engineered *E. coli* strain (Table 7.5). With *fucO** gene, the strain AS2003-*fucO** was able to ferment non-detoxified hydrolysate and produced succinate at 82.77±0.98 g/L with a yield of 89% (based on total sugars consumed), which was not significant different to what obtained from vacuum-treated hydrolysate fermentation by the strain (84.65±1.69 g/L). This study indicated that high succinate yield derived from sugarcane bagasse hydrolysate demonstrated a great potential application of renewable biomasse as the feedstock for economical succinate production using metabolically evolved *E. coli*.

CAPTER VIII

GENERAL CONCLUSIONS

The work presented in this dissertation has been accomplished the three major objectives stated in the introduction: (1) demonstrate the feasibility of producing succinate from cassava pulp by metabolically engineered *E. coli* KJ122 using separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF), (2) develop a metabolically engineered *E. coli* KJ122 that was able to efficient convert xylose, sugar mixtures, and diluted acid hydrolysate of sugarcane bagasse to succinate using a metabolic evolution, (3) improve a succinate-producing strain that resist to inhibitor compounds in dilute acid hydrolysate of sugarcane bagasse, (3.1) enhancing a strain resistance to furfural by introducing furfural resistant genes from ethanologenic *E. coli* strain into succinate-producing strain and (3.2) developing a strain resistant to non-furfural hydrolysate inhibitors by applying a metabolic evolution.

Objective 1: The metabolically engineered *E. coli* KJ122 was capable of efficient utilization of cassava pulp to produce succinate with impressive titers up to 98 g/L, yields around 70 g/100 g dry pulp and productivities above 1.0 g/L/h, under simple anaerobic conditions in a low-cost medium using batch and fed-batch SSF. More importantly, the result obtained in this study was favorably compared with other published works. However, due to the high prices of commercial enzymes, a reduction in the amount of enzymes used for cassava pulp hydrolysis during SSF

process would improve the process economy. The strategies for decreasing enzymes loadings and for feeding substrate need to be further investigated in conjunction with improvements in succinate titer, yield, and productivity.

Objective 2: The *E. coli* KJ122 was unable to efficient convert xylose, sugar mixtures, and a dilute acid hydrolysate of sugarcane bagasse to succinate. The improved strain was easily obtained by growth-based selection (16^{th} serial transfers in 10% (w/v) xylose), designated AS1600a. This mutant co-fermented a glucose-xylose mixture and a mixture of 4 sugars in sugarcane bagasse hydrolysate for succinate production. A single mutation in *galP* (G236D) was shown to be responsible for the improvement in xylose and sugar mixtures fermentation. With this mutation, we have proposed a new mechanism of sugars metabolism by the mutant AS1600a. The AS1600a probably transports both glucose and xylose through the mutant GalP, but with different affinity. This *galP** mutant gene may be useful for the improvement of sugar metabolism in other biocatalysts.

Objective 3.1: The improved succinate-producing strain AS1600a resistance to furfural was obtained by expression the effectiveness of furfural resistant genes from ethanologenic *E. coli* strain into the succinate-producing strain. The best furfural resistant gene showing the highest MIC to furfural under glucose and xylose fermentation was found to be the *fucO** and *puuP*, respectively. Additionally, the strain harboring the *fucO** and *puuP* could entirely metabolize 20 mM furfural within 48 h and produced succinate with an impressive yield (up to 0.85 g/g) from 10% (w/v) xylose or sugars mixture of 5% (w/v) xylose and 5% (w/v) glucose. Furthermore, the AS1600a strain harboring *fucO** gene produced about 37% higher in succinate production from sugarcane bagasse hydrolysate when compared with that of

the control (without furfural resistant genes). However, the fermentation time required for succinate production from sugarcane bagasse hydrolysate by the strain harboring the furfural resistant genes was longer than that of pure sugars fermentation even some detoxification processes were introduced. This indicated that other inhibitory compounds rather than furfural that retards the microbial growth. Thus, further strain development for non-furfural hydrolysate resistance is needed to be investigated in order to improve the efficiency of hydrolysate utilization, thus beneficial for improving industrial succinate production.

Objective 3.2: The hydrolysate resistant *E. coli* strain AS2003 was attained after 145 serial transfers in vacuum bisulfite treated sugarcane bagasse hydrolysate. The AS2003 strain could ferment 60 to 80% (v/v) vacuum-treated hydrolysate into succinate with impressive yield up to 85% without noticeable lag phase, which was comparable to other published works with engineered *E. coli* strain. With *fucO** gene, the strain AS2003-*fucO** able to ferment non-detoxified hydrolysate and produced succinate at 82.77±0.98 g/L with a yield of 89% (based on total sugars consumed), which was not significantly difference to what obtained from vacuum-treated hydrolysate fermentation by the strain (84.65±1.69 g/L). This indicated that the use of vacuum evaporation removing volatile inhibitory compounds could be ignored and thus the operating cost of succinate production from hemicellulose hydrolysate would be reduced. The AS2003 was sequenced and found to contain 8 mutations. Therefore, the further experiments need to be carried out in order to understand the mechanism of mutations that conferred a resistance to inhibitors in hydrolysate by *E. coli* AS2003 strain.

In summary, the high succinate yield derived from cassava pulp and sugarcane bagasse hydrolysate demonstrated a great potential application of renewable biomasses as the feedstock for economical succinate production using metabolically evolved *E. coli*. Furfural resistant genes such as *fucO** and *puuP* may be useful for improving industrial succinate production from the other diluted acid of lignocellulosic biomasses. The accessibility of whole genome sequences significantly enhanced our understanding of the physiology, genetics and evolutionary development of bacteria.

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BIOGRAPHY

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