การหาสภาวะที่เหมาะสมและการเพิ่มขนาดกระบวนการผลิต 2,3 บิวเทนไดออล จากมอลโตเดกซ์ทรินด้วย *Klebsiella oxytoca* KMS005 ที่ดัดแปลงวิถีการสร้างและสลาย

นางสาวสิธา จัน

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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OPTIMIZATION AND SCALE-UP PRODUCTION PROCESS OF 2,3-BUTANEDIOL FROM MALTODEXTRIN BY METABOLICALLY ENGINEERED *KLEBSIELLA OXYTOCA* KMS005

Sitha Chan

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OPTIMIZATION AND SCALE-UP PRODUCTION PROCESS OF 2,3-BUTANEDIOL FROM MALTODEXTRIN BY METABOLICALLY ENGINEERED *KLEBSIELLA OXYTOCA* KMS005

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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สิธา จัน : การหาสภาวะที่เหมาะสมและการเพิ่มขนาดกระบวนการผลิต 2,3 บิวเทนไดออล จากมอลโตเดกซ์ทรินด้วย *Klebsiella oxytoca* KMS005 ที่ดัดแปลงวิถีการสร้างและสลาย (OPTIMIZATION AND SCALE-UP PRODUCTION PROCESS OF 2,3-BUTANEDIOL FROM MALTODEXTRIN BY METABOLICALLY ENGINEERED *KLEBSIELLA OXYTOCA* KMS005) อาจารย์ที่ปรึกษาที่มหาวิทยาลัยเทคโนโลยีสุรนารี : รองศาสตราจารย์ ดร.เขมวิทย์ จันต๊ะมา, อาจารย์ที่ปรึกษาที่ INSTITUT NATIONAL POLYTECHNIQUE DE TOULOUSE : PROF. DR. PATRICIA TAILLANDIER, 168 หน้า.

การหาวิธีการที่เหมาะสมด้วยการใช้วัตถุดิบราคาถูกและมีอยู่ล้นเหลือ ได้ถูกพิจารณาให้เป็นหนึ่งในปัจจัยที่มีผลต่อมูลค่าทางการค้าของการผลิต 2,3 บิวเทนไดออล การหาระดับที่เหมาะสม ของค่าความเป็นกรด-ด่าง อัตราการให้อากาศ ความเร็วในการกวน และความเข้มข้นของวัตถุดิบ (มอลโตเดกซ์ทริน) ได้ถูกนำมาหาสภาวะที่เหมาะสมในการหมัก 2,3 บิวเทนไดออล จากมอลโตเดกซ์ทรินด้วย *Klebsiella oxytoca* KMS005 ที่ดัดแปลงวิถีการสร้างและสลาย โดยใช้วิธีการแบบดั้งเดิมและวิธีการพื้นผิวตอบสนอง (RSM) แบบ Box-Behnken จากผลการทดลองพบว่าการใช้ค่าความเป็นกรด-ด่างที่ 6.0 อัตราการให้อากาศที่ 0.8 ปริมาตรอากาศต่อปริมาตรน้ำหมักต่อนาที การกวนที่ 400 รอบต่อนาที และใช้ความเข้มข้นของมอลโตเดกซ์ทรินที่ 150 กรัมต่อลิตร ตามลำดับเป็นสภาวะที่เหมาะสมที่สุด ส่วนผลจาก RSM ชี้ให้เห็นว่า ความเร็วในการกวนเป็นปัจจัยที่มีอิทธิพลมากที่สุดในการผลิต 2,3 บิวเทนไดออล เมื่ออัตราในการกวนและอัตราการให้อากาศเป็นอันตรกิริยาระหว่างกัน หรืออัตราการกวนและความเข้มข้นของวัตถุดิบเป็นอันตรกิริยาระหว่างกัน ทั้งนี้ความเร็ว ในการกวนเป็นปัจจัยที่มีความสำคัญอย่างยิ่งในการผลิต 2,3 บิวเทนไดออล และภายใต้สภาวะการหมักแบบกึ่งกะ ความเข้มข้นของ 2,3 บิวเทนไดออล ค่าผลผลิตและค่าอัตราการผลิต ในระยะเวลา 78 ชั่วโมงของการหมัก มีค่าเท่ากับ 88.1±0.2 กรัมต่อลิตร 0.412±0.001 กรัมของ 2,3 บิวเทนไดออลต่อกรัมของน้ำตาลที่เติมลงไปทั้งหมด และ 1.13±0.01 กรัมต่อลิตรต่อชั่วโมง ตามลำดับ นอกจากนั้นยังศึกษาอิทธิพลของสภาวะการหมักแบบให้อากาศเล็กน้อยต่อการเจริญของจุลินทรีย์และการผลิต 2,3 บิวเทนไดออลด้วย โดยการหมักแบบกะในถังปฏิกรณ์ชีวภาพได้ทำการทดสอบผลของอัตราการไหลอากาศ และอัตราการกวนผ่านการตรวจวัดค่า k_La ซึ่งพบว่าปริมาณออกซิเจนที่เหมาะสมสำหรับการเจริญของจุลินทรีย์และการผลิต 2,3 บิวเทนไดออล คือ 9.5 กรัม ซึ่งสอดคล้องกับค่า k_La ที่ 25.2 ต่อชั่วโมง ส่วนกระบวนการหมักแบบกึ่งกะ ได้ทำการทดสอบอัตราการเติมน้ำตาลกลูโคสที่แตกต่างกัน พบว่าการหมักแบบกะที่เริ่มเติมน้ำตาลกลูโคสด้วยอัตรา 2 กรัมต่อชั่วโมง ในช่วงสุดท้ายของการเจริญของจุลินทรีย์ ภายในเวลา 48 ชั่วโมง ตามด้วยการเติม

น้ำตาลกลูโคสในช่วงสุดท้ายของการหมักแบบกะที่ 40 ชั่วโมง ให้ผลเป็นที่น่าพอใจ ส่งผลให้ได้ 2,3 บิวเทนไดออล ความเข้มข้นสุดท้ายเท่ากับ 74.7 กรัมต่อลิตร ให้ค่าอัตราการผลิตเท่ากับ 0.64 กรัมต่อลิตรต่อชั่วโมง แต่ได้ผลผลิตพลอยได้เกิดขึ้นเล็กน้อย (ซักซิเนต อะซิเตท และเอทานอล ที่ความเข้มข้นรวมประมาณ 3 กรัมต่อลิตร)

ข้อมูลทั้งหมดที่ได้จากการศึกษาในถังปฏิกรณ์ชีวภาพขนาด 2 ลิตร ได้ถูกนำไปประยุกต์ใช้ในการผลิต 2,3 บิวเทนไดออล ในถังปฏิกรณ์ชีวภาพที่มีขนาดใหญ่ขึ้นเป็นลำดับจาก 10 ลิตร เป็น 90 และ 300 ลิตร โดยที่การทดลองหมักแบบกะจะควบคุมอัตราการให้อากาศไว้ที่ 0.8 ปริมาตรอากาศต่อปริมาตรน้ำหมักต่อนาที ซึ่งการใช้ถังปฏิกรณ์ชีวภาพขนาด 10 ลิตร ที่ควบคุมอัตราการกวนคงที่ที่ 295 รอบต่อนาที ได้ความเข้มข้นของ 2,3 บิวเทนไดออล และค่าผลผลิตเท่ากับ 53.8 กรัมต่อลิตร และ 0.40 กรัมของ 2,3 บิวเทนไดออลต่อกรัมของน้ำตาลที่เติมลงไปทั้งหมด ตามลำดับ ภายในระยะเวลา 48 ชั่วโมงของการหมัก ส่วนการหมักในถังปฏิกรณ์ชีวภาพขนาด 90 ลิตร ที่ควบคุมอัตราการกวนคงที่ที่ 130 รอบต่อนาที ได้ความเข้มข้นของ 2,3 บิวเทนไดออล เท่ากับ 52.53 กรัมต่อลิตร และได้ค่าผลผลิตที่ 0.43 กรัมของ 2,3 บิวเทนไดออลต่อกรัมของน้ำตาลที่ถูกใช้ไป ภายในเวลา 72 ชั่วโมงของการหมัก ทั้งนี้พบว่าช่วงเวลาที่เหมาะสมในการเตรียมกล้าเชื้อในระหว่างหมักด้วยถังปฏิกรณ์ชีวภาพขนาด 90 ลิตร คือบ่มเพาะกล้าเชื้อเป็นเวลา 12 ชั่วโมง ในช่วงระยะการเจริญ ให้ได้ค่าการดูดกลืนแสง (OD_{550}) ประมาณ 4 ก่อนที่จะใช้ถ่ายกล้าเชื้อลงในถังปฏิกรณ์ชีวภาพ สำหรับการหมักในถังปฏิกรณ์ชีวภาพระดับโรงงานต้นแบบขนาด 300 ลิตร ที่ควบคุมแรงกวนให้คงที่ที่ 70 รอบต่อนาที สามารถผลิต 2,3 บิวเทนไดออลได้ 45.02 กรัมต่อลิตร และให้ค่าผลผลิตเท่ากับ 0.43 กรัมของ 2,3 บิวเทนไดออลต่อกรัมของน้ำตาลที่ถูกใช้ไป หลังจากการหมักเป็นเวลา 72 ชั่วโมง

สาขาวิชาเทคโนโลยีชีวภาพ
ปีการศึกษา 2559

ลายมือชื่อนักศึกษา_____
ลายมือชื่ออาจารย์ที่ปรึกษา (at SUT)_____
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ลายมือชื่ออาจารย์ที่ปรึกษาร่วม (at INPT)_____

SITHA CHAN : OPTIMIZATION AND SCALE-UP PRODUCTION PROCESS OF 2,3-BUTANEDIOL FROM MALTODEXTRIN BY METABOLICALLY ENGINEERED *KLEBSIELLA OXYTOCA* KMS005. THESIS ADVISOR AT SURANAREE UNIVERSITY OF TECHNOLOGY : ASSOC. PROF. KAEMWICH JANTAMA, Ph.D., THESIS ADVISOR AT INSTITUT NATIONAL POLYTECHNIQUE DE TOULOUSE : PROF. PATRICIA TAILLANDIER, Ph.D., 168 PP.

OPTIMIZATION/SCALE-UP/MALTODEXTRIN/*KLEBSIELLA OXYTOCA* KMS005

An optimization process with a cheap and abundant substrate is considered one of the factors affecting the price of commercial 2,3-Butanediol (2,3-BD) production. The optimized levels of pH, aeration rate, agitation speed, and substrate concentration (maltodextrin) were optimized by a conventional method and Response Surface Methodology (RSM) with Box-Behnken design in which metabolically engineered *Klebsiella oxytoca* KMS005 utilized maltodextrin to produce 2,3-BD. Results revealed that pH, aeration rate, agitation speed, and maltodextrin concentration at levels of 6.0, 0.8 vvm, 400 rpm, and 150 g/L, respectively, were the optimal conditions. RSM indicated that the agitation speed was the most influential parameter when either agitation and aeration interaction or agitation and substrate concentration interaction played important roles for 2,3-BD production. Under interim fed-batch fermentation, 2,3-BD concentration, yield, and productivity were obtained at 88.1±0.2 g/L, 0.412±0.001 g/g sugar supplied, and 1.13±0.01 g/L/h, respectively, within 78 h. The influence of micro-aerobic conditions on microbial growth and 2,3-BD production was also studied. In batch bioreactors, air flow rate and agitation rate characterized through

k_La measurement were tested. The optimal amount of oxygen supply was evaluated at 9.5 g corresponding to a k_La of 25.2 h^{-1} for cell growth and 2,3-BD production. Then, a fed-batch process was investigated by different glucose feeding rate strategies. Fed-batch with a glucose feeding rate of 2 g/h starting at the end of the growth phase during 48 h, followed by a final batch phase of 40 h was found satisfactory. It resulted in a final 2,3-BD concentration of 74.7 g/L with a productivity of 0.64 g/L/h but few by-products formed (about 3 g/L including succinate, acetate and ethanol).

Validated information in the 2 L bioreactor was further applied in a larger scale production of 2,3-BD with series of bioreactors from 10, 90 and 300 L vessels. Batch experiments were conducted based on various agitation speeds with the fixed aeration rate at 0.8 vvm. As a result, 2,3-BD concentration, and yield were achieved at 53.8 g/L, and 0.40 g/g sugar supplied within 48 h, respectively, under the constant tip speed at 295 rpm using a 10 L vessel. Its concentration of 52.53 g/L and yield of 0.43 g/g sugar consumed within 72 h were attained under the condition of the constant tip speed at 130 rpm using a 90 L fermenter. An appropriate seed inoculum condition was found with an optical cell density (OD_{550}) around 4 at the log phase (12 h incubation) prior to transferring of the inoculum into the 90 L fermenter. Under the constant tip speed at 70 rpm, 2,3-BD concentration and yield were obtained at 45.02 g/L and 0.43 g/g sugar consumed in the pilot scale of 300 L bioreactor after 72 h incubation.

School of Biotechnology

Academic Year 2016

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LIST OF ABBREVIATIONS

°C	Degree Celsius
2,3-BD	2,3-butanediol
Ace	Acetate
ackA/*pt*a	Acetate kinase A/phosphotransacetylase gene
adhE	Alcohol dehydrogenase E gene
ADP	Adenosine diphosphate
ANOVA	Analysis of variance
ATP	Adenosine 5'-tri-phosphate
B. polymyxa	*Bacillus polymyxa*
cAMP	cyclic adenosine 3′, 5′-monophosphate
cAMP	Cyclic Adenosine monophosphate
CAP	Catabolite activator protein
CDW	Cell dry weight
CSL	Corn steep liquor
DE	Dextrose equivalent
DNA	Deoxyribonucleic acid
DO	Dissolved oxygen
E. aerogenes	*Enterobacter aerogenes*
E. cloacae	*Enterobacter cloacae*

LIST OF ABBREVIATIONS (Continued)

E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
Eth	Ethanol
FNR	Fumarate nitrate reductase
For	Formate
g/g CDW	Gram of product per gram of cell biomass
g/L	Gram (s) per liter
g/L/h	Gram (s) per liter per hour
h	Hour (s)
HPLC	High performance liquid chromatography
IPTG	Isopropyl-β-D-thiogalactoside
K. oxytoca	*Klebsiella oxytoca*
K. pneumonia	*Klebsiella pneumonia*
kg	Kilo gram (s)
k_La	Oxygen mass transfer coefficient
L	Liter (s)
Lac	Lactate
LB	Luria-Bertani
ldhA	Lactate dehydrogenase A
M	Molar

LIST OF ABBREVIATIONS (Continued)

MalT	Maltose transcriptional activators
min	minute
mM	Milli-molar
NAD^+	Nicotinamide adenine dinucleotide (Oxidized form)
NADH	Reduced form of Nicotinamide adenine dinucleotide
OD	Optical cell density
OTR	Oxygen transfer rate
OUR	Oxygen uptake rate
pflB	Pyruvate formate-lyase
poxB	Pyruvate oxidase
RNA	Ribonucleic acid
rpm	Revolutions per minutes
RQ	Respiratory quotient
RSM	Response surface methodology
S. cerevisiae	*Saccharomyces cerevisiae*
S. marcescens	*Serratia marcescens*
sp.	Species (singular)
spp.	Species (plural)
Suc	Succinate
U	Unit

LIST OF ABBREVIATIONS (Continued)

v/v	Volume per volume
vvm	Gas volume flow per unit of liquid volume per minute
w/v	Weight per volume
α-ALS	α-acetolactate synthase
μL	Microliter (s)
μM	Micro-molar (s)

CHAPTER I

INTRODUCTION

1.1 Background and scope of the study

Concerning with scarce crude oil reserve, gradually increasing its price, and environmental pollution, bio-refinery systems that integrate biomass conversion processes and equipment to produce fuels, power, and chemicals from annually renewable resources are at stage of worldwide development (Kamm and Kamm, 2004; Ragauskas et al., 2006). Current practice in industrial biotechnology demonstrates that the social (People), environmental (Planet) and economic (Profit) benefits of bio-based processes go hand in hand. In accordance with DMS company documented by Sijbesma, substantial reductions of 17-65% greenhouse gas emissions could be realized, and a more profound shift towards bio-based chemicals could potentially account for up to for chemical industry alone is estimated to be US $ 12.32 to 24.64 billion per annum by 2010 (http://www.sustentabilidad.uai.edu.ar/pdf/tec/industrial_white_biotech.pdf).

2,3-Butanediol (2,3-BD) is an example of a bulk chemical which can be produced by biotechnological routes. An interest in 2,3-BD started, when Harden and Walpole (1906) employed *Klebsiella pneumoniae* to produce this compound. An extensive application for 2,3-BD is in various fields such as fuel, chemical industry, food industry, softening agents, explosives, and plasticizers as well as pharmaceutical agents. Furthermore, dehydration of 2,3-BD can be used in the industrial solvents

such as methyl ethyl ketone (MEK) reviewed by Xiao-Jun et al. (2009). MEK, and 1,3-butadiene derived from 2,3-BD conversion were costly increased from US $ 1.67 to 2.32 and from US $ 2.20 to 3.99 per kg, respectively during the first-half of 2011 mainly due to prices of fossil raw materials (Cho et al., 2012).

To lower the overall price of fermentative 2,3-BD production, some strategies have been considered including microorganism, inexpensive substrate and medium, simple fermentation process, and purification. Recently, the derivative of *K. oxytoca* M5a1 named KMS005-73T have been engineered to enhance 2,3-BD production by Jantama et al. (2015). The strain utilized glucose and other sugar types as a sole carbon substrate supplemented in mineral salts medium to produce high yield of 2,3-BD under microaerobic conditions. Based on previous studies, biotechnological production of 2,3-BD from wastes and excessive biomass is a promising and attractive alternative for traditional chemical synthesis and for the development of viable processes for the generation of fuels or chemicals from sustainable resources (Yajun and James, 2009). Maltodextrin, a partial starch hydrolyzed product, is such an abundant carbon source feasible to be fermented to 2,3-BD because it is easily digestible, and being absorbed as rapidly as glucose. In addition to this abundant substrate, the cost effective medium such as simple mineral salt medium (AM1) has low price (0.424 $/L) possible to economical 2,3-BD production and is not composed of any complex nutrients beneficial to downstream process which costs around 60-70% of total production price (Song et al., 2006 and Xiao et al., 2011). The production of 2,3-BD by KMS005 fermentation from maltodextrin supplemented with AM1 medium would be further investigated extensively. Particularly, the development of a simple fermentation process under an optimal condition is vital to

obtain high concentration of the main product and minor amount of by-products in which 2,3-BD producing pathway is stimulated under micro-aerobic conditions and slightly acidic pH. Oxygen supply is the most important parameter since the 2,3-BD pathway participates in regulation of the NADH/NAD^+ ratio in order to maintain the redox balance in the cells. Previous studies applied oxygen controlled parameters such as respiratory quotient (RQ), oxygen mass transfer coefficient, etc. It is technically interesting to include optimization process as well as oxygen control strategy in laboratory scale prior to scaling up production process of 2,3-BD fermentation in pilot plant while the scale-up process is rarely reported in recently studies on 2,3-BD production. Hence, process designs of culture optimization and manufacture (specifically process scale-up) would be integrated in 2,3-fermentation process for further improvements of fermentation engineering and microbiology (Figure 1.1).

In this study, chemical and physical parameters affecting 2,3-BD production by KMS005 such as pH, aeration rate, agitation and substrate concentrations, would be optimized by conventional optimization and by response surface methodology (RSM). The result from this study would elucidate the best level for each parameter indicated to obtain high production yield, titer and productivity of 2,3-BD. Subsequently, the critical information from RSM would be used to search for the best condition for aeration supply under simply fed-batch strategy. Meanwhile, oxygen transfer was studied to understand the cell metabolism and to determine the suitable amount of oxygen consumption to micro-aerobic culture of KMS005. Coupling these methods, the useful information would be available for improving fermentation processes, and scaling up in pilot tanks to address and guide for further industrial needs.

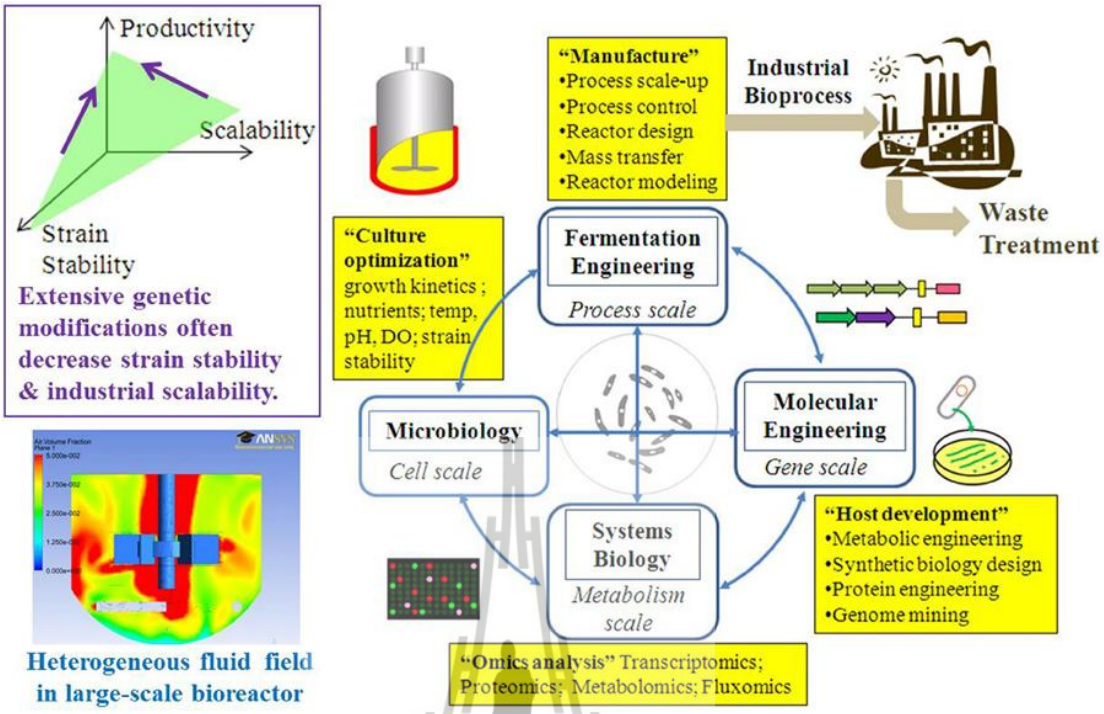

Figure 1.1 The design-engineering-analysis cycle for scale-up of bio-fuel fermentation (Hollinshead and Tang, 2014).

1.2 Objectives

To become more attractive and more promising in the 2,3-BD-production industry, the process optimization of 2,3-BD from metabolically engineered *K. oxytoca* KMS005 using an inexpensive carbon substrate would be studied. Maltodextrin was extensively employed as the model of sole carbon source. Hence, this project was focused on:

Objective 1:

1) To elucidate the parameters affecting the 2,3-BD production in fermentation process such as pH, aeration rate, agitation speed and substrate concentrations.

2) To optimize the positive or key parameters affecting 2,3-BD production obtained above to achieve the best optimum level for 2,3-BD by Response Surface Methodology (RSM) with Box-Behnken design.
3) To improve yield and productivity of 2,3-BD by applying a simple fed-batch strategy.

Objective 2:

1) To optimize oxygen supply by controlling oxygen transfer parameters such as k_La and RQ in batch and fed-batch modes using glucose as a sole carbon source.
2) To propose a robust fed-batch fermentation with constant feeding glucose based on growth rate and 2,3-BD production rate.

Objective 3:

1) To investigate the effect of agitation upon scaling up the production process of 2,3-BD in 10, 90 and 300 L fermenter vessels.
2) To investigate the scale-up fermentation process of 2,3-BD production from maltodextrin and to report achievements and obstacles found in pilot tanks.
3) To optimize inoculum age and density upon scaling up of 2,3-BD production.

CHAPTER II

LITERATURE REVIEW

2.1 Characterization of 2,3-BD and its applications

A reduction of natural resources has led to discoveries for renewable resources for sustainable development. Interestingly, microbial 2,3-BD had been known to have extensive applications in various fields such as fuel and chemical industry (Xiu and Zeng, 2008). An interest in 2,3-BD started in 1906, when *Klebsiella pneumoniae* was cultured to produce this compound.

2,3-BD is known as 2,3-butylene glycol, dimethylene glycol, or dimethylethylene glycol, mainly manufactured by microbial route and is acquired to be a considerable progress in the fermentation (Yajun and James, 2009). In physical property, 2,3-BD can form 3 isomeric structures, including D-(−)-, L-(+)- and meso-forms as shown in Figure 2.1 (Celińska and Grajek 2009). It is a colorless and odorless liquid chemical with a high boiling point and a low freezing point. Boiling points of the three stereoisomers are slightly different, ranging from 177 to 182 °C, the boiling points of meso-(181-182 °C), D-(179-180 °C) and racemic (177 °C). One of the applications in 2,3-BD is its conversion to 1,3-butadiene, which further can be used in synthetic rubber production. Due to its low freezing point at −60 °C, the chemical is also used as an antifreeze agent (Soltys et al., 2001). Furthermore, its derivatives have been found in manufacturing plastics and solvent production (Figure 2.2). For example, the product of 2,3-BD dehydrogenation converted as diacetyl,

served as a highly-valued flavoring agent in food products providing a buttery taste. Diacetyl was also a bacteriostatic food additive, since it inhibited growth of some microorganisms (Bartowsky and Henschke, 2004). Dehydration of 2,3-BD allowed the production of methyl ethyl ketone (MEK), which is an effective liquid fuel capable in a higher heat of combustion than that of ethanol.

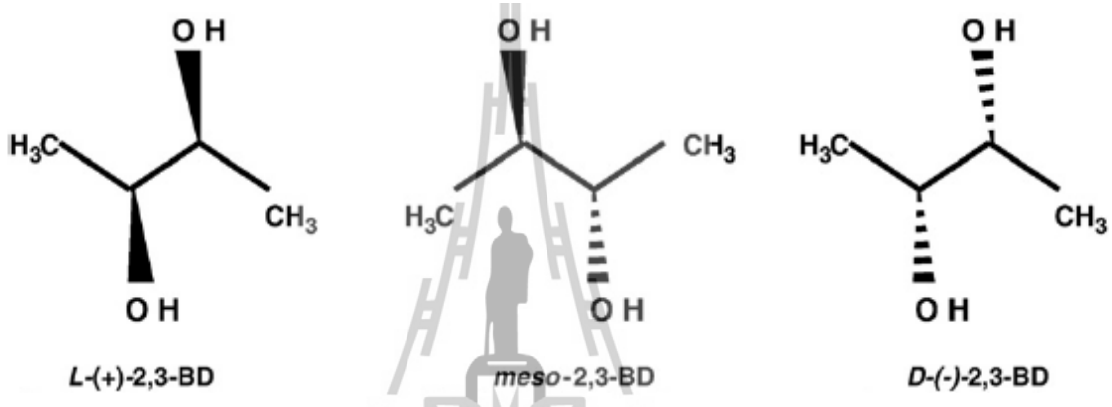

Figure 2.1 The stereoisomers of 2,3-butanediol (2,3-BD). (Celińska and Grajek, 2009).

Be noticed that 2,3-BD has its high octane number in which it can serve as an "octane booster" for gasoline. It can be used for synthesizing Polyurethane-melamides (PUMAs) by esterification with maleic acid. This chemical is useful in cardiovascular applications (Petrini et al., 1999). Other esterified products of 2,3-BD are used mainly in the pharmaceutical and cosmetics. Further potential applications of 2,3-BD are these productions of printing inks, perfumes, fumigants, spandex, moistening and softening agents, plasticizers such as cellulose nitrate, polyvinyl chloride, polyacrylates. In addition, 2,3-BD could also be ketalized with acetone to produce a

'tetramethyl' compound, which is a potential gasoline blending agent similar to the commonly used methyl tert-butyl ether (MTBE) (Voloch et al., 1985). In fact, it has been pointed out that synthesis of MTBE and other new alkyl ether blending agents from sources other than petroleum stocks is essential if they are to be of much real benefit in extending gasoline supply (Stinson, 1979).

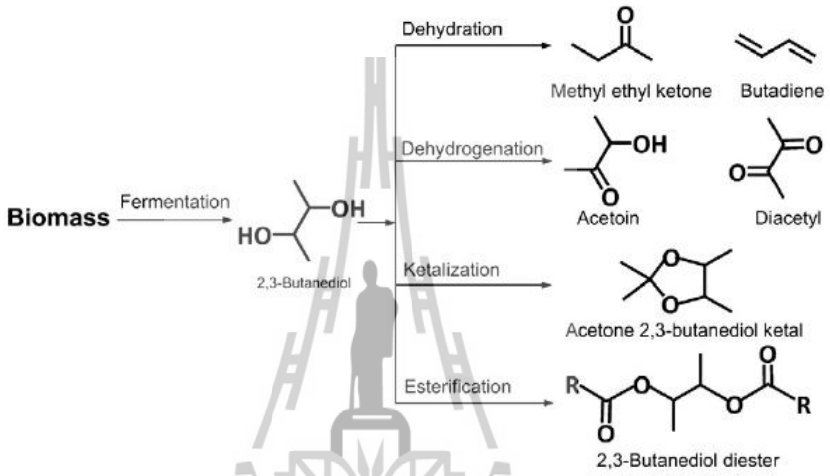

Figure 2.2 Derivatives of fermentative 2,3-BD. (Ji et al., 2011).

2.2 Microorganisms

2,3-BD is an intermediate produced in the metabolic pathway of several anaerobic and facultative microorganisms. Many bacterial species can ferment pyruvate to 2,3-BD. Experimental data infers the presence of the 2,3-BD pathway in the following taxonomic groups: *Aeromonas hydrophila*, *Bacillus subtilis*, *Brevibacillus brevis* S1, *Corynebacterium glutamicum*, *Enterobacter aerogenes*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Lactobacillus brevis*, *L. casei*, *L. helveticus*, *L. plantarum*, *Lactococcus lactis*, *Leuconostoc lactis*, *Leuconostoc*

mesenteroides subsp. *cremoris*, *Oenococcus oeni*, *Pediococcus pentosaceus*, *Raoultella terrigena*, *Serratia marcescens*, *Bacillus polymyxa*, *Klebsiella terrigena*, *Streptococcus faecalis*, *Enterobacter cloacae*, *Bacillus licheniformis*, *Bacillus amyloliquefaciens*, *Aerobacter indologenes*, *Rhizobacterium*, *Pseudomonas chlororaphis* O6 as well as the marine microalga, *Chlamydomonas perigranulata* (Hon-Nami 2006, Cho et al., 2008; Celińska and Grajek, 2009). However, *K. pneumoniae*, *K. oxytoca*, *B. polymyxa*, and *E. aerogenes* are considerable to be promising microorganisms for industrially fermentative 2,3-BD production. In addition, *K. pneumoniae* GT1 strain is able to convert glycerol almost completely to 2,3-BD under microaerobic conditions (Biebl et al., 1998). Both *K. oxytoca* and *B. polymyxa* can utilize pentoses as well as hexoses, which are of considerable practical importance since biomass materials have a ratio of pentose: glucose at 1:1.5 (Tsao et al., 1982). Cho et al. (2012) reported that *K. pneumonia* showed higher productivity than *K. oxytoca* did but it secreted large amounts of capsular polysaccharide, increasing pathogenicity and hindering the separation of cells from the fermentation broth during downstream processing. Different microorganisms produce different stereoisomers, as shown in Table 2.1. *Klebsiella sp.* and *Enterobacter sp.*, members of the *Enterobacteriaceae* family, produce L (+), D (-) and meso forms of 2,3-BD formation.

Table 2.1 2,3-BD stereoisomer produced by different species (based on Maddox, 1996); in parentheses: relative amount of the isomer is given.

Organism	L (+)-	D(-)-	meso-
K. oxytoca	+ (1)	-	+ (9)
E. aerogenes	+ (1)	-	+ (9)
Serratia marcescens	-	-	+
A. hydrophila	-	+	-
B. polymyxa	-	+	-
B. subtilis	-	+ (3)	+ (2)
B. amyloliquefaciens	-	+	+

2.3 Pathways of 2,3-BD metabolism in microorganisms

2,3-BD producing bacteria synthesize varying amounts of 2,3-BD as well as other products, including ethanol, lactic acid, succinic acid and formic acid during mixed acid fermentation. The bacteria in the family *Enterobacteriaceae* produce 2,3-BD in very high concentrations. There are several different pathways to produce 2,3-BD (Lee et al., 2004). Among them, the production of 2,3-BD in a mixed acid pathway exist acetoin as an intermediate prior to the formation of 2,3-BD (Jiayang et al., 2006). Remarkably, 2,3-BD synthesis is induced under acid supplementation, thus suggesting that 2,3-BD, is a neutral metabolite, and counteracts too high acidification. On the other hand, most studies on the influence of acid supplementation on 2,3-BD synthesis were performed with acetic acid (Nakashimada et al., 2000). The second function of the 2,3-BD pathway is the regeneration of the excess reducing power

associated with glycolysis. Thus, the 2,3-BD pathway, similar to other fermentative processes, participates in regulation of the NADH/NAD^+ ratio in the cell. Last but not least, the synthesis of 2,3-BD is regarded as a carbon and energy storing strategy (Xiao and Xu, 2007). 2,3-BD is reversely consumed in fermentation when glucose or other carbon source is depleted. A suitable harvesting time would be observed during each time course of fermentation to obtain a high product concentration.

Generally, 2,3-BD is produced from pyruvate in a mixed acid fermentation process via several intermediate compounds, including α-acetolactate, acetoin, and diacetyl (Figure 2.3). First, pyruvate from glycolysis can be converted either into lactate in a reaction which requires NADH (catalysed by L-/D-lactate dehydrogenase; LDH) or, after decarboxylation, into α-acetolactate (catalyzed by α-acetolactate synthase; α-ALS). α-acetolactate is mostly produced under low NADH availability. Further, α-acetolactate can be converted to acetoin by α-acetolactate decarboxylase (α-ALD), and this takes place under anaerobic conditions. If oxygen is present, α-acetolactate can undergo spontaneous decarboxylation producing diacetyl. Then, diacetyl reductase (DAR; also known as acetoin dehydrogenase) can convert diacetyl to acetoin. Ultimately, butanediol dehydrogenase (BDH; also known as acetoin reductase; AR) reduces acetoin to 2,3-BD. Under aerobic conditions, α-ALS is rapidly and irreversibly inactivated, thus ceasing 2,3-BD formation. However, in this case, pyruvate is broken down to acetyl-CoA by the action of the pyruvate dehydrogenase multienzyme complex, which is not synthesized in anaerobic environments and is generally inhibited by NADH (Gottshalk, 1986). The acetyl-CoA formed is then channeled mainly to the tricarboxylic acid (TCA) cycle. When culture conditions are not fully aerobic, lactate dehydrogenase (LDHA), pyruvate-formate lyase (PFLB),

and α-ALS act upon pyruvate to generate lactate, formate, and 2,3-BD. The formate generated can be further metabolized to carbon dioxide and hydrogen by the formate-hydrogen lyase (FDH) complex, which can be inhibited under aerobic conditions, and by the presence of nitrate in an anaerobic environment (Magee and Kosaric, 1987). The succinate originates from the flow of carbon through the phosphoenolpyruvate (PEP) branch point where PEP is carboxylated to oxaloacetate by PEP carboxylase (PPC), and then to succinate, under strict anaerobic conditions (Gottshalk, 1986).

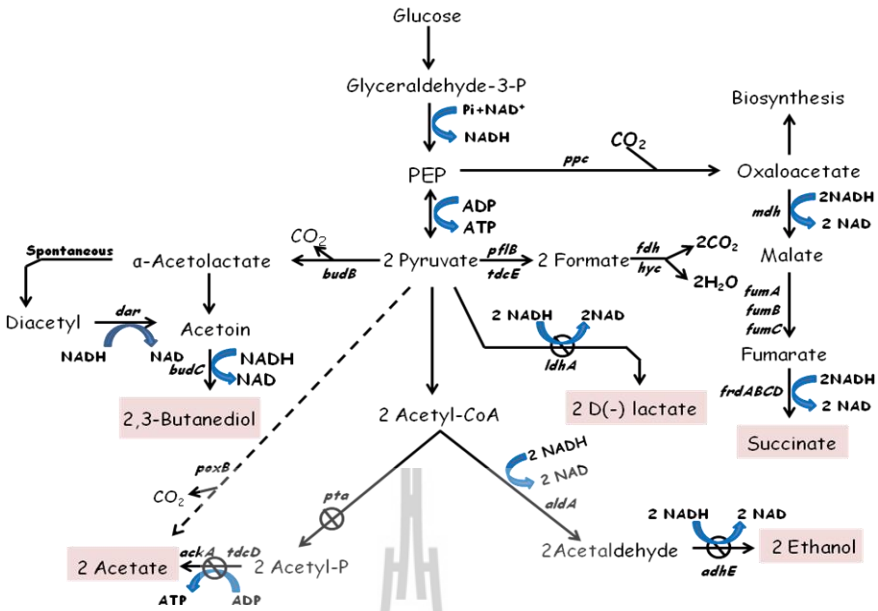

Figure 2.3 Fermentation pathway in *K. oxytoca* KMS005 under micro-aerobic conditions. Central metabolism indicating genes deleted in the engineered stain for 2,3-BD production. Solid arrows represent central fermentative pathways. Dashed arrow represents alternative acetate-producing pathway via pyruvate oxidase (*poxB*). Circled-crosses represent the gene deletions performed to obtain KMS005. Genes and enzymes: *ldhA*, lactate dehydrogenase; *pflB*, pyruvate formate-lyase; *pta*, phosphate acetyltransferase; *ackA*, acetate kinase; *tdcD*, propionate kinase; *tdcE*, threonine decarboxylaseE; *adhE*, alcohol dehydrogenase; *ppc*, phosphoenol-pyruvate carboxylase; *mdh*, malate dehydrogenase; *fumABC*, fumarase isozymes; *frdABCD*, fumarate reductase; *fdh*, formate dehydrogenase; *poxB*, pyruvate oxidase; *budA*, α-acetolactate decarboxylase; *budB*, α-acetolactate synthase; *budC*, butanediol dehydrogenase and *dar*, diacetyl reductase (modified from Jantama et al. (2015)).

2.4 *Klebsiella oxytoca* KMS005, derivative of wide type *K. oxytoca* M5a1: a newly potential microorganism for 2,3-BD production

K. oxytoca M5a1 has an ability to utilize such the broader range of carbon substrates. The bacterium exhibits fast growth, no requirements of complex nutrients medium, in addition to have available techniques for genetic manipulations. It would be a next target microorganism to alter the metabolic pathway for bio-based 2,3-BD production.

Physiology and morphology of the microorganism are known as Gram-negative, rod-shaped and enteric bacterium found in paper and pulp steams as well as around other sources of wood. This bacterium is in the phylum of Proteobacteria, the class of Gamma Proteobacteria, the order of *Enterobacteriales*, and the family of *Enterobacteriaceae* (Mahon et al., 2007). The microorganism is capable of growing at pH as low as 5.0 and temperature as warm as 35 °C in the minimal salts medium. Many genetic techniques can be applied to this microorganism in order to engineer or modify the metabolic pathway of this microorganism for chemical production in the industry. *K. oxytoca* M5a1 was developed by deleting competitive NADH consuming pathways resulting in a new potential strain, namely KMS005 (Jantama et al., 2015). The method was adapted from the gene deletion techniques performed in *E. coli* (Jantama et al., 2008). The strategy to enhance 2,3-BD production in KMS005 was done by a removal of *adhE* (alcohol dehydrogenase E), *ackA*/*pt*a (acetate kinase A/phosphotransacetylase), and *ldhA* (lactate dehydrogenase A) genes from its parental genome. KMS005 is expected to expense the carbon flux through pyruvate route rather than that through acetyl-coA production route in microaerobic fermentation. Furthermore, it is also expected to channel the pyruvate through α-acetolactic acid and

reduce subsequently to 2,3-BD. The potential strain grows on a wide variety of sugars including hexoses and pentoses, as well as on cellobiose and cellotriose without any requirements of expensive nutrients (Dien et al., 2003). Also this microorganism has an ability to utilize glycerol as a sole carbon substrate derived from biodiesel.

2.5 Operational parameters for production of 2,3-BD by microbial fermentation

The varieties of genetic approaches have been employed to engineer many bacterial strains for 2,3-BD production (Table 2.2). However, the production of 2,3-BD performed in rich nutrient media, and required antibiotics or Isopropyl β-D-1-thiogalactopyranoside (IPTG) for heterologous gene expression are concerned to increasing the total price for 2,3-BD fermentation process. Consequently, an engineered strains without these requirements for growth would be the potential microorganism gaining the benefit in economical 2,3-BD production. Considering advantages of KMS005 over capability to produce high 2,3-BD yield, less by-product formation, non pathogen and less exopolysaccharide production a research work on 2,3-BD production from agricultural product other than refine glucose would be studied. Meanwhile, physical conditions to suit and to improve the final product concentration would be observed in the optimization process.

Table 2.2 Comparison of 2,3-BD production from available carbon sources and microorganisms.

Organism	Substrate/Medium/Condition	2,3-BD (g/L) Yield [g/g]	Productivity g/L/h	References
B. licheniformis (Δ*gdh* and Δ*acoR*)	150 g/L glucose, supplemented with simple mineral medium, CSL, pH 6.0, 3 L/min and 350 rpm for the first 16 h, 1.5 L/min and 200 rpm for the rest, 70 h incubation, Fed batch, 37 °C.	98 [0.40]	0.94[a]	Qui et al., 2016
K. oxytoca KMS005	200 g/L glucose, supplemented with simple mineral salts medium AM1, pH 6.0, aeration 1.0 vvm, 96 h incubation, 37 °C, 300 rpm.	117 [0.49]	1.2[a]	Jantama et al., 2015
K. oxytoca NBRF4 (chemical mutation)	44 g/L glucose, supplemented with YP medium, pH 4.3, aeration 10% dissolved oxygen, batch, 18 h incubation, 38 °C, 200 rpm.	14.4 [0.32]	0.78	Han et al., 2013
K. pneumoniae KCTC2242 (Δ*wabG*)	70 g/L glucose, supplemented with minimal medium containing 5g/L yeast extract, trace elements solution, pH 5.5, batch, 72 h incubation, 37 °C without shaking.	22.44 [0.44]	0.31	Jung et al., 2013
K. oxytoca	90 g/L glucose, supplemented with a medium containing 5 g/L yeast extract, trace elements, pH 6.5, aeration 1.0 vvm, batch, 30 h incubation, 37 °C without shaking.	30 [0.33]	1.153	Park et al., 2013
K. pneumonia DSM 2026	72 g/L glucose, supplemented with minimal medium containing 5 g/L yeast extract, pH 6.5, aeration 3.0 L/min, batch, 10 h incubation, 37 °C, 150 rpm.	17.6 [0.27]	1.76	Cho et al., 2012
E. coli BL21 (DE3) over expression of *bud* genes	40g/L glucose supplemented with LB medium, 20 g/L diacetyl, 100 µg/mL ampicillin, 1mM IPTG, pH 7.4, batch, without aeration, 10 h incubation, 37 °C, 180 rpm.	16.1 [0.40]	2.70	Li et al., 2012
S. cerevisiae (Δ*adh1*Δ*adh3*Δ*adh5*Δ*gpd 2*Δ*ald6*) and overexpressed genes *B. subtilis alsS and E. aerogenes budA*	20 g/L glucose, supplemented with 6.7 g/L yeast nitrogen base without amino acid, 0.42 g/L Tween 80 and 0.01 g/L cholesterol, without pH control, batch, 85 h incubation, 30 °C, 250 rpm.	2.29 [0.113]	0.269	Ng et al., 2012

[a] 2,3-BD Yield = gram of 2,3-BD divided by gram substrate consumed (g/g) for fed-batch fermentation.

NA= Not available.

Table 2.2 (Continued).

Organism	Substrate/Medium/Condition	2,3-BD (g/L) Yield [g/g]	Productivity g/L/h	References
B. licheniformis (ΔldhA)	30 g/L glucose, supplemented with minimal medium, pH 5.0, aeration 0.5 L/min, batch fermentation, 12 h incubation, 50 °C, 250 rpm.	13.0 [0.45]	0.07	Wang et al., 2012
E. coli W3110 over-expressed *K. pneumonia budAC*	60 g/L glucose, supplemented with a minimal medium, 100 µg/ ml ampicillin, pH 6.8, aeration 1.5 L/min, batch, 48 h incubation, 30 °C, 200 rpm.	15.7 [0.25]	0.33	Lee et al., 2012
Enterobacter aerogenes EMY-01*(ΔldhA)*	90 g/L glucose, supplemented with a medium containing 5 g/L yeast extract, pH 6.0, aeration 1.0 vvm, batch, 12 h incubation, 37 °C, 150 rpm.	23.2 [0.30]	1.93	Jung et al., 2012
K. pneumoniae SGJSB04 over-expressed *budABC*	100 g/L glucose, supplemented with a minimal medium containing 5 g/L yeast extract, pH 5.5, aeration 0.75 vvm, fed-batch, 40 h incubation, 37 °C, 300 rpm.	101.53 [0.34]	2.54 [a]	Borim et al., 2012
K. pneumoniae CICC 10011	100 g/L glucose, supplemented with a minimal medium containing 0.5 g/L peptone, 1.5 g/L yeast extract, trace elements, pH 6.0, without aeration, batch, 6 h incubation, 37 °C, 120 rpm.	40.7 [0.44]	6.70	Liu et al., 2011
K. oxytoca ME-UD-3	220 g/L glucose, supplemented with a simple medium, pH 6.0, aeration 1.0 vvm, batch, 81 h incubation, 37 °C, 200 rpm.	86.2 [0.39]	1.06	Nie et al., 2011
B. amyloliquefaciens B10-127	200 g/L glucose, supplemented with a medium containing 4 g/L K_2HPO_4, 10 g/L corn steep liquor, 10 g/L soybean meal without pH, batch, 72 h incubation, 37 °C, 160 rpm.	74.3 [0.37]	0.74	Yang et al., 2011
C. acetobutylicum ATCC 824 over-expressed *budC*	60 g/L glucose, supplemented with MG medium containing 2.5 g/L yeast extract, 3.0 g/L *p*-aminobenzoic acid, 100 µg/mL ampicillin, 30 µg/mL chloramphenicol, 40 µg/mL erythromycin and 50 µg/mL IPTG, without pH, batch, 140 h incubation, 37 °C, without shaking.	2.4 [0.042]	0.017	Siemerink et al., 2011
K. oxytoca ME-UD-3 (Δ*ald*A)	200 g/L glucose, supplemented with a medium containing EDTA 0.05 M, pH 6.5, aeration 1.0 vvm, fed-batch, 60 h incubation, 37 °C, 200 rpm.	130 [0.48]	1.63 [a]	Ji et al., 2010a

[a] 2,3-BD Yield = gram of 2,3-BD divided by gram substrate consumed (g/g) for fed-batch fermentation.

NA= Not available.

Table 2.2 (Continued).

Organism	Substrate/Medium/Condition	2,3-BD (g/L) Yield [g/g]	Productivity g/L/h	References
K. oxytoca ME-XJ-8, pDK7 encoding a mutant CRP(in) from *E. coli* T25	Glucose–xylose (6% (w/w), 2:1, 1:1, 1:2), supplemented with LB medium containing 1 mM IPTG, pH 6.5, aeration 1.0 vvm, batch, 50 h incubation, 37 °C, 200 rpm.	23.9 [0.40]	0.70	Ji et al., 2010b
E. coli JM109 (Δ*ldhA*Δ*pta*Δ*adhE*Δ*poxB*) over-expressed of *alsSD B. subtilis*	60g/L glucose, supplemented with LB medium containing 100 μg/mL ampicillin, pH 6.8, aeration 1.5 L/min, batch, 36 h incubation, 37 °C, 200 rpm.	25.8 [0.43]	0.71	Li et al., 2010
Klebsiella pneumoniae SDM isolated from soil	Total glucose utilized 168 g/L, supplemented with medium, pH 7.0, air flow 1.5 vvm, fed-batch, 37 °C, 500 rpm.	150 [0.43]	4.21[a]	Ma et al., 2009
Enterobacter cloacae subsp. *dissolvens* SDM	188 g/L cassava powder hydrolysate, supplemented with corn steep liquor (10g/L) as nitrogen source, trace minerals, pH 6.0, batch (SSF), 24 h incubation, 30 °C, 100 rpm.	78.3 [0.42]	3.3	Wang et al., 2012
Syn. elongatus PCC7942	BG-11 medium, containing 50 mM $NaHCO_3$, 10 mg/L thiamine, and 10 mg/L gentamicin with constant light (55 $\mu E \cdot s^{-1} \cdot m^{-2}$), pH 7.5, 72 h incubation, 30 °C, 100 rpm.	2.38 [NA]	0.0098	Oliver et al., 2012
Clostidium ragsdalei, *C. autoethanogenum* and *C. ljungdalii*	0.5% (w/v) fructose for heterotrophic growth and steel mill waste gas containing 44% CO, 32% N_2, 22% CO_2, 2% H_2, supplemented with modified PETC medium for autotrophic growth. 30 °C for *C. ragsdalei*, 37 °C for *C. autoethanogenum* and *C.ljungdalii*, without pH, batch (strict anaerobe), 200 h incubation, without shaking.	0.13-0.18 [0.26-0.36]	NA	Köpke et al., 2011
K. pneumoniae subsp. pneumoniae G31	30 g/L glycerol, supplemented with a medium, pH fluctuation, aeration varied between 1.1-2.2 vvm, fed-batch, 150 h incubation, 37 °C, 200 rpm.	70.0 [0.39]	0.47 [a]	Petrov and Petrova, 2010

[a] 2,3-BD Yield = gram of 2,3-BD divided by gram substrate consumed (g/g) for fed-batch fermentation.

NA= Not available.

Table 2.2 (Continued).

Organism	Substrate/Medium/Condition	2,3-BD (g/L) Yield [g/g]	Productivity g/L/h	References
K. pneumonia SDM	70 g/L corncob molasses composed of 9% (w/v) glucose, 45% xylose, 14.6% arabinose, supplemented with a medium containing 8.27 g/L corn steep liquor powder, pH 6.0, aeration 1.0 vvm, batch, 27 h incubation, 37 °C, 500 rpm.	30.1 [0.42]	1.30	Wang et al., 2010
Serratia marcescens H30	90 g/L sucrose, supplemented with a medium containing 33.36 g/L yeast extract, pH 6.0, aeration at desired Respiratory Quotient (RQ), fed-batch, 45 h incubation, 30 °C, 200 rpm.	139.92 [0.47]	[3.49] [a]	Zhang et al., 2010
K. oxytoca ACCC 10370	Corncob hemicellulose hydrolysate (39.5 g/L xylose, 9.9 g/L glucose, 1.5 g/L arabinose, and 1.8 g/L acetate), supplemented with a medium containing 1.5 g/L yeast extract, pH 6.0, aeration 0.3 vvm, batch, 48 h incubation, 37 °C, 300 rpm.	23.5 [0.46]	0.49	Cheng et al., 2010
Klebsiella pneumoniae CICC 10011	Reducing sugars in initial hydrolysate of 300 g/L Jerusalem artichoke powder and 50g/L Jerusalem artichoke powder supplemented with salt nutrients medium, pH 6.0, fed-batch SSF fermentation, 0.15 l/min, 40 h incubation, 37 °C, 300 rpm.	84.0 [0.32]	2.29[a]	Sun et al., 2009

[a] 2,3-BD Yield = gram of 2,3-BD divided by gram substrate consumed (g/g) for fed-batch fermentation.

NA= Not available.

2.5.1 Oxygen supply

The most important variable in the 2,3-BD fermentation is oxygen supply. 2,3-BD is produced under low oxygen supply to maintain an internal redox balance with respect to the pyridine nucleotide pool (NAD) during glycolysis and biosynthesis (Converti et al., 2003). The ratio of oxygen demand and supply can control the proportions of metabolites produced (Figure 2.4). The NAD^+/NADH balance is maintained by relative production of acetoin: 2,3-BD (Blomqvist et al., 1993). It was found that under aerobic conditions α-acetolactate synthase (α-ALS) is rapidly and irreversibly inactivated, thus preventing 2,3-BD synthesis under high oxygen supply (Kosaric et al., 1992). Such facultative anaerobic microorganisms can obtain energy by two different pathways: respiration and fermentation. When the oxygen supply is limited, both pathways are simultaneously active. The yield of 2,3-BD depends on the relative activities of each pathway. The yield of 2,3-BD can be maximized by optimizing the oxygen supply, because this limits the respiration. In the absence of oxygen, usually ethanol is produced in approximately equimolar amounts with 2,3-BD (Voloch et al., 1985) along with formate, acetate, lactate, and acetoin. In addition, by lowering the availability of oxygen, cell mass is also lowered, and the 2,3-BD yield is therefore reduced. All three genes in 2,3-BD pathway (α-acetolactate synthase, α-acetolactate decarboxylase, and diacetyl (acetoin) reductase) are located in one operon. The operon is regulated at the transcriptional level, and the induction occurs in oxygen-limited conditions (Magee and Kosaric, 1987; Mayer et al., 1995).

According to previous studies summarized in Table 2.2, the optimum range of aeration rate were supplied from 0.3-2.2 vvm for cell growth and 2,3-BD production. Since the oxygen requirement in production of cell biomass and the product formation

are different, further published works have attempted to establish proper oxygen supply strategies to ensure efficient 2,3-BD production. Those strategies such as OTR (oxygen transfer rate), k_La (volumetric oxygen transfer coefficient), OUR (oxygen uptake rate) and RQ (respiratory quotient) guided oxygen supply control strategies were successfully applied in 2,3-BD fermentation and proved to be effective. Beronio and Tsao (1993) maintained the OTR constantly at oxygen limitation level resulting in 2,3-BD productivity increased by 18%. In a fermentative (R,R)-2,3-BD production process using *P. polymyxa*, the best results of 44 g/L (R,R)-2,3-BD with a productivity of 0.79 g/(L h) were obtained by using this programmed k_La change method (Fages et al., 1986). The technique was conducted based on a programmed variation of k_La process, which k_La was set at three different levels: 40 h^{-1} (0–19 h); 21 h^{-1} (19–41 h) and 8 h^{-1} (41–55 h) to control the flow of substrate being oxidized through the aerobic respiratory and the anaerobic fermentative pathways. In another continuous culture study, optimum oxygen uptake rates (OURs) were determined and were found to vary with dilution rate (Zeng et al., 1990). By altering the speed of the impeller with constant aeration, different OURs were obtained. As the dilution rate increased, the yield and product concentration decreased. Product formation was not dependent on growth rate. Even at low or no growth rates, higher specific productivity could be expected. At low OURs, the cells used fermentation for growth and maintenance and continued to convert substrate to 2,3-BD. Another approach, the control of 2,3-BD fermentation process by the respiratory quotient (RQ) was performed. Zhang et al. (2010) developed a suitable control strategy which combined RQ control with the constant residual sucrose concentration fed-batch for 2,3-BD production by *Serratia marcescens*. The RQ value was separately set at 1.0-1.5 for

the cell growth and 1.8–2.0 for 2,3-BD production, respectively, based on the stoichiometric calculation of sucrose metabolism under anaerobic or micro-aerobic conditions. However, the parameters mentioned above (OTR, k_La, OUR and RQ) are not technically to control, thus restricting the application of those strategies (Ji et al., 2011). In contrast, some published works applied the strategies (OTR, k_La and RQ) as control parameter for oxygen supply in scale-up process of 2,3-BD fermentation (Byun et al., 1994; Zeng et al., 1994).

Interestingly, newly simple oxygen supply method based on agitation speed control was developed to realize efficient 2,3-BD fermentation. The processes of 2,3-BD fermentation by *K. oxytoca* were compared at different oxygen supply conditions, which was accomplished by changing agitation speed. Subsequently, a two-stage agitation speed control strategy, aimed at achieving high concentration, high yield, and high productivity of 2,3-BD, was designed based on the analysis of kinetic parameters during batch processes controlled by a single agitation speed (Ji et al., 2009). Hence, in this study, an optimized aeration rate will be investigated by optimization process before conducting a modified aeration supply for efficient 2,3-BD production using different aeration rate which will be contrasted to the one from Ji et al. (2009). This would be benefit for reducing the cost of power on stirrer and for oxygen supply.

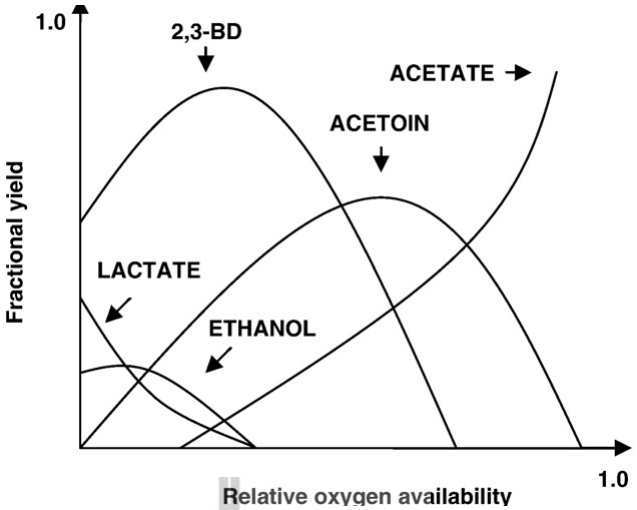

Figure 2.4 The effect of relative oxygen availability on fractional product yields in *B. polymyxa* (De Mas et al., 1988).

2.5.2 pH

For 2,3-BD biosynthesis from pyruvate, three key enzymes are involved, i.e. α-acetolactate synthase (ALS), α-acetolactate decarboxylase (ALDC), and 2,3-BD dehydrogenase (BDH; acetoin reductase). Alkaline conditions favour formation of organic acids with a simultaneous decrease in the 2,3-BD yield. In contrast, under acidic conditions, organic acid synthesis is reduced (over 10-fold) and 2,3-BD synthesis is increased (3–7-fold). The enzyme ALS has an optimum selectivity under slightly acidic conditions (pH 6). The enzyme conducts a two-step reaction: in the initial stage, pyruvate is complexed with thiamine pyrophosphate (TPP) to form acetyl-TPP. This compound is subsequently condensed with a second molecule of pyruvate to yield α-acetolactate (Magee and Kosaric, 1987). However, the optimum pH for 2,3-BD production strongly depends on the microorganism and substrate used (Garg and Jain, 1995). Some microorganisms have evolved defensive

strategies, like switching the metabolism to production of less toxic compounds, such as alcohols or glycols. 2,3-BD pathway induction is caused by accumulation of acidic products in the medium rather than by altering the internal pH. The pH gradient at cell membrane causes accumulation of acetate, which would induce the enzymes involved in 2,3-BD synthesis. Therefore, lowering the culture pH causes an increase in the pH gradient concomitantly, with 2,3-BD production before the external pH becomes too high and the culture is inactivated. It was noticed that pH range of 5.5-7.4 was controlled in fermentation (Table 2.2). 2,3-BD production by *K. oxytoca* is in the pH range from 5 to 6 and by *E. aerogenes* in the pH 6 (Voloch et al., 1985; Converti et al., 2003). Stormer (1977) found that pH above 6 causes a sharp decrease in the activity of ALS in the 2,3-BD pathway of *K. pneumoniae*. Perego et al. (2000) determined pH 6 as the optimum for the production of 2,3-BD by *E. aerogenes*. Experimentally pH was tested in the range from 4.0 to 8.0. The conversion yield was nearly constant within a narrow range of pH 5.0-6.5, and it sharply decreases either at lower or at higher pH values. The strongest effect was observed under acidic conditions, which stems from the fact that the lower pH value usually inhibits the biomass growth as well as the bioprocess itself.

2.5.3 Substrate concentration and maltodextrin

Klebsiella species, *K. pneumonia* and *K. oxytoca*, have ability in the best production of 2,3-BD by fermentation from a wide range of substrates, including pentoses (xylose and arabinose), hexoses (glucose, mannose, and galactose), and disaccharides (sucrose, lactose, cellobiose). As far as bulk chemical production is concerned, the major cost in most biomass conversion processes appears to be the

substrate cost and the price of the final product is mostly affected by the raw material cost. Availability of an inexpensive carbohydrate raw material is essential for developing an economical fermentation process for the production of 2,3-BD. The most frequently applied initial sugar concentrations were in the range of 5–10%. A high sugar concentration (exceeding 50 g/L) was reported to inhibit 2,3-BD formation and sugar utilization (Yu and Saddler, 1983). Accordingly, at low sugar concentrations (4 to 6%), the fermentation of sucrose in sugar beet molasses proceeded rapidly to completion in 24 h (McCall and Georgi, 1954). On the other hand, at higher sucrose concentrations (up to 17%) fermentations were incomplete and less efficient. *K. pneumoniae* is able to utilize sugars in concentrations of 5 to 15% in complex fermentation media containing various types of raw materials. However, when *K. pneumoniae* was grown on chemically defined medium containing 1–2% D-glucose or D-xylose, solvent production at higher sugar concentration was less efficient. Furthermore, when supplemented with 0.5% acetate, D-glucose at a concentration of 4% was completely utilized within 24 h, resulting in a 2,3-BD yield of 19.6 g/L. Similarly, D-xylose at a concentration of 4% was completely utilized over 48 h, and a 2,3-BD yield of 22 g/L was obtained. However, Nie et al. (2011) reported that initial 220 g/L glucose was used to ferment 2,3-BD at yield and concentration of 0.39 g/g, and 86.2 g/L under batch condition, respectively.

As listed in Table 2.2, recently several research have still focused on 2,3-BD production from refined glucose as substrate with less concerning on the price of the substrate when the best obtainable condition from laboratory studies are applied in industrial scale. On the other hand, many studies have been attempted to the fermentation of sugars from wood hydrolysates, especially those derived from the

hemicellulose fraction. However, due to the lack of cellulases and hemicellulases, *K. oxytoca* requires pretreated substrate. Unfortunately, some of the pretreatment procedures release toxic derivatives into the culture medium (Frazer and McCaskey, 1991) and even produce low yield and concentration of 2,3-BD, for example, using corncob molasses and corncob hemicelluloses hydrolysate as substrates resulted in the 2,3-BD titers at 30.1 and 23.5 g/L, respectively (Cheng et al., 2010; Wang et al., 2010). So far, one promising alternative carbon source is derived from non-cellulosic substrate including starch hydrolysate, sucrose, and wastes from food industries rich in glucose and compatible with fermentation by microorganisms. They found that waste starch hydrolysate was the most promising raw material, ensuring the highest product yield and volumetric productivity, which was nearly twice as estimated for synthetic glucose solutions (Ji et al., 2011). However, food industrial wastes including waste starch hydrolysate, sugarcane molasses, whey permeate, etc, might have met hurdle in purification step, which is the primary barrier to commercialization of microbial 2,3-BD production. Therefore, the rational study would concentrate on the use of partial hydrolyzed starch, or maltodextrin which is abundant, pure and rich in efficient catabolism of α (1-4)-linked glucose polymers for potential fermentation and lowering cost of downstream process.

2.5.4 Model of operation (Batch, and fed batch)

The effects of reactor operation mode on 2,3-BD formation is one of critical factors in the establishment of an optimal process design. For efficient 2,3-BD production, a series of reactor operation modes were adopted previously, including batch and fed-batch, continuous culture, cell recycle, and immobilized cell systems.

The three latter modes (continuous culture, cell recycle, and immobilizes cell) is neglected in the literature review because they would not be employed in this study due to complicated, non practical and high sophisticated techniques to set up the systems in industry.

While a concentrated product stream is desirable in any fermentation scheme, it is essential in fermentative 2,3-BD production due to the difficulties encountered in 2,3-BD recovery (Xiu and Zeng, 2008). A minimum 2,3-BD concentration of approximately 80 g/L must be acquired for economically feasible recovery (Magee and Kosaric, 1987). This has led to the belief that successful production of this compound on a commercial scale requires a batch mode of operation. However, some disadvantages in the batch process exist, such as relatively low productivity and high substrate inhibition. These problems could be avoided by supplying additional sugar at a slow rate during cultivation. High product concentrations could be obtained using a fed-batch mode with the capability of producing a high final 2,3-BD concentration while minimizing the effects of initial substrate inhibition (Ramachandran et al., 1990). Ma et al. (2009) compared different fed-batch strategies for 2,3-BD production using *K. pneumoniae*, including pulse, constant feed rate, constant residual glucose concentration, and exponential fed-batch. The results showed that the maximum 2,3-BD concentration of 150 g/L could be obtained by the constant residual glucose concentration from 20 to 30 g/L in feeding strategy. However, the feeding occasion is not so easy to determine due to the lack of glucose online analysis equipment, and this system is not sufficiently robust to control glucose concentrations consistently at the desired levels. Also, the feeding amount of glucose is according to the glucose consumption before interval, and thus cannot respond to the real information. In some

cases, this would cause the glucose not to be timely fed and 2,3-BD would be reused as the carbon source for cell growth. To overcome this problematic feeding, Nie et al. (2011) designed a proper feeding strategy which could resolve the problem and keep the glucose concentration at a relative low level by feeding glucose and sodium hydroxide mixture solution at the same time due to declining the culture pH according to the synthesis of 2,3-BD as a mixed acid fermentation process.

2.6 Pathway of maltodextrin uptake

The maltose system is responsible for the uptake and efficient catabolism of α (1-4)-linked glucose polymers (maltodextrins) up to 7 to 8 glucose units. There are three phenomena in maltodextrin metabolism including transcriptional controlled system, maltodextrin transport system and enzyme system for maltose degradation summarized (Figure 2.5).

2.6.1 Positive transcriptional activator

The MalT proteins, which is the activator at all *mal* promoters (Richet and Raiband, 1897), and which binds MalT boxes, have provided an important alternative view to the *lac* operon about how transcription is activated. Although the precise mechanism of how MalT interacts with and stimulates RNA polymerase is still unknown, there is considerable information about the structural requirements for MalT binding to DNA as well as its ability to form a nucleoprotein complex with the catabolite activator protein (CAP).

The expression of *malT* is not autoregulated by MalT but is subjected to catabolite repression and therefore requires the presence of the cAMP/CAP complex (Chapon, 1982). According to the current view, the mechanism of catabolite

repression is based mainly on the regulation of intracellular cAMP levels. This, in turn, is a function of adenylate cyclase, controlled by the enzyme IIAGlc of the phosphoenolpyruvate (PEP)-dependent sugar phosphotransferase system (PTS) in its phosphorylated (activating) and dephosphorylated (inactivating) states (Postma et al., 1996). Indeed, mutants lacking adenylate cyclase or CAP are unable to grow on maltose. Expression of *malT* is controlled by a repressor called Mlc. In mutants lacking Mlc, the expression of *malT* is increased by a factor of 2 to 3 when grown in glycerol. On the other hand, overexpression of Mlc from a multicopy plasmid strongly reduces *malT* expression (Decker, 1997). By DNA footprint analysis, it was shown that Mlc binds to *malT* DNA at a position 1 to 23 bp from the start of the *malT* transcript.

2.6.2 Maltose/maltodextrin transport system

The maltose/maltodextrin transport system is a member of the family of multi-component and periplasmic binding protein-dependent ABC high-affinity transport systems of Gram-negative enteric bacteria (Davidson et al., 1992; Shuman and Panagiotidis, 1993). The substrate recognition site of the system is determined primarily by the soluble binding protein with a high affinity for maltose and maltodextrins (K_D around 1 mM) (Kellerman and Szmelcman, 1974; Szmelcman et al., 1976) that is located in the periplasm in high concentration. This maltose binding protein (MalE protein or MBP) consists of two nearly symmetrical lobes between which the binding site is formed (Quiocho et al., 1997; Spurlino et al., 1991).

2.6.3 Enzyme for maltodextrin system

Incoming maltose and maltodextrins of up to seven glucose moieties are metabolized to glucose and glucose-1-phosphate by the combined action of three cytoplasmic enzymes, amylomaltase (MalQ), maltodextrin phosphorylase (MalP), and maltodextrin glucosidase (MalZ) (Figure 2.5). Since maltodextrins larger than six glucose moieties are not very well transported by the ABC transporter, they are reduced in size by a periplasmic amylase, the MalS protein. MalS cleaves maltodextrins except maltose. Its preferred product released from larger dextrins is maltohexaose. The function of the enzyme is most probably the degradation of longer dextrins that enter the periplasm to shorter dextrins that can be transported by the binding protein-dependent maltose/maltodextrin transport system (Freundlieb et al., 1988). Even though MBP, the recognition site of the transport system, binds all maltodextrins from maltose to amylose, only dextrins up to the size of maltohexaose can be transported across the membrane (Ferenci, 1980a; Ferenci et al., 1980b).

2.6.3.1 Amylomaltase Amylomaltase (Wiesmeyer and Chon, 1960), encoded by *malQ*, is a dextrinyl transferase that can transfer maltosyl and longer dextrinyl residues onto glucose, maltose, and longer maltodextrins. The smallest substrate which amylomaltase recognizes is maltotriose (Palmer et al., 1976). Acting on maltotriose, it releases glucose from the reducing end, forms a maltosyl-enzyme complex, and transfers the maltosyl residue onto the nonreducing end of an acceptor, being as glucose, maltose, or any larger maltodextrin. It is important to note that maltose is not a substrate of amylomaltase but only an acceptor in the transfer reaction catalyzed by the enzyme. Thus, it follows that for maltose degradation, the cell has to be able to internally produce small amounts of maltodextrins as primers with the minimum size of maltotriose. Amylomaltase is essential for maltose degradation.

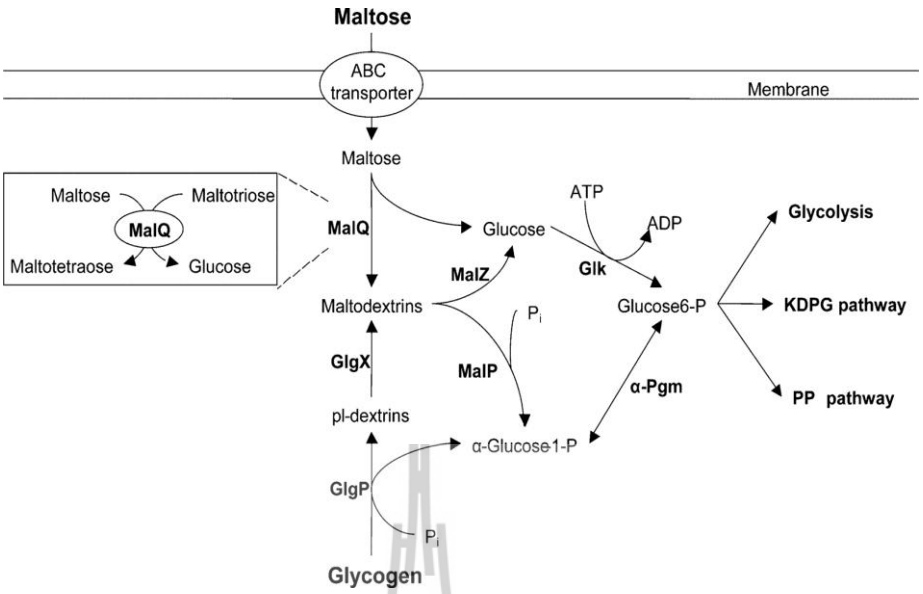

Figure 2.5 Maltose degradation by the maltose enzymes. The enzymes amylomaltase (*malQ*), maltodextrin phosphorylase (*malP*), and maltodextrin glucosidase (*malZ*) are indicated by their genes. After transport of maltose by the binding protein-dependent ABC transporter, a maltosyl, maltotriosyl, or maltotetraosyl (and so on) residue is transferred from maltotriose, maltotetraose, or maltopentaose (and so on) onto the incoming maltose releasing glucose in the process. Maltopentaose and longer maltodextrins are recognized by the maltodextrin phosphorylase, forming glucose-1-phosphate and a maltodextrin that is smaller by one glucosyl residue. Maltodextrin glucosidase recognizes maltotriose and longer maltodextrins (up to maltoheptaose), releasing glucose consecutively from the reducing end of the maltodextrin (Boos and Shuman, 1998).

2.6.3.2 Maltodextrin Phosphorylase Maltodextrin phosphorylase, encoded by *malP*, forms glucose-1-phosphate by sequential phosphorolysis of the nonreducing end glucose moieties of larger dextrins. As discussed above, amylomaltase does not split the glycosidic bond of maltose, but the net products of the action of MalP on maltose are glucose and maltodextrins. Since glucose will be removed in vivo by glucokinase to form glucose-6-phosphate, maltodextrins would accumulate.

Maltodextrin phosphorylase (Becker et al., 1994; Palm et al., 1985) recognizes maltopentaose and longer linear maltodextrins and forms α-glucose-1-phosphate by phosphorolysis from the nonreducing end of the maltodextrin. Obviously, it is important that maltodextrin phosphorylase does not attack maltotetraose and maltotriose, since dextrins of a minimum size are required for full activity of amylomaltase.

2.6.3.3 Maltodextrin glucosidase The cloning and sequencing of the *malZ* gene and the isolation and biochemical characterization of the encoded protein revealed an enzyme that hydrolyzed maltoheptaose and smaller maltodextrins to glucose and maltose. The smallest substrate is maltotriose; maltose is not a substrate. In contrast to other glucosidases, the MalZ enzyme preferentially removes glucose (and to some extent maltose) consecutively from the reducing end of the maltodextrin chain (Tapio et al., 1991).

2.6.3.4 Role of glucokinase and phosphoglucomutase in maltose/ maltodextrin metabolism The final products of the combined action of amylomaltase, maltodextrin phosphorylase, and maltodextrin glucosidase are glucose and α-glucose-1-phosphate. Therefore, to funnel these end products of the specific maltose enzymes

into general metabolism, the cells rely on glucokinase (encoded by *glk*) for the phosphorylation of glucose to glucose-6-phosphate and on phosphoglucomutase (encoded by *pgm*) for the transformation of a glucose-1-phosphate to glucose-6-phosphate, which enters into glycolysis (Buhr et al., 1992).

2.7 Inoculum effect

Appropriate inoculum size and inoculum age have a great impact on the production of enzymes and product formation. Commonly, inoculum amount are as follows: 1-5 % for bacteria, 5-10 % for yeast and 7- 15 % for mold. When the inoculation amount is too low, the activity of the enzymes is not high and the production cycle is extended. When the inoculum amount is high, microorganisms immediately enter the exponential phase, and production phase is concomitantly proceeded. However, excessive inoculation is not necessary because seed culture is time consuming and too much metabolic waste affects normal fermentation. Inoculum age is the physical age when culture in seed pots can begin to be planed to the production tank. Generally, it is better to inoculate at a late logarithmic growth phase. Too young an inoculum age will make offspring culture growth slow, extending the fermentation period but too old an inoculum age will cause the strain to decline prematurely leading to a decline in production capacity (Chen, 2014).

2.8 Response surface methodology

Response surface methodology (RSM) is a collection of statistical and mathematical techniques useful for developing, improvement, and optimizing processes in the industrial world in where several input variables potentially influence

some performance measure or quality of the product or process. By careful design of **experiments,** the objective is to optimize a **response** (output variable) which is influenced by several **independent variables** (input variables). An experiment is a series of tests, called **runs**, in which changes are made in the input variables in order to identify the reasons for changes in the output response. In general, suppose that a researcher wants to find the levels of temperature (X_1) and pH (X_2) that maximize the concentration of 2,3-BD (Y). The concentration of 2,3-BD is a function of the levels of temperature and pH, as follows: $Y = f(X_1, X_2) + e$ (where e represents the noise or error observed in the response Y, when X_1, X_2 are known as independent variables). The surface represented by $f(X_1, X_2)$ is called a *response surface*. Although the precise form of the response function f is always unknown, experience has shown that it can usually be approximated by suitable linear or quadratic functions of the independent variables. A linear regression relationship,

$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + ...+ \beta_n X_n + e$ is the simplest and is known as the first model or equation. The second order model is the quadratic regression relationship

$$Y = \beta_0 + \beta_1 X_1 + ...+ \beta_n X_n + \beta_{11} X_1^2 + ...+ \beta_{nn} X_n^2 + \beta_{12} X_1 X_2 + ...+ \beta_{n-1,n} X_{n-1} X_n + e$$

β_0 = the intercept (grand mean), and its estimate is donated by b_0

β_i = the linear effect of X_i and its estimate is donated by b_i (i = 1,…,n)

β_{ii} = the quadratic effect of X_i and its estimate is donated by b_{ii} (i = 1,…,n)

β_{ij} = the interaction effect of X_i and X_j and its estimate is donated by b_{ij} (i < j, i = 1,… n-1 and j = 1,…n).

The true response function is seldom known, and hence must be estimated. Consequently, the contribution to the error variation is not only due to the experimental errors alone but also to the inadequacy or the so-called **lack of fit** of the estimated model prior to examining the characteristic of the fitted surface to decide what action is appropriate. Fitting response model must include the quadratic and interaction (second order model) since the first order model is often inadequate and provide a poor description of geometric shape of the response surface.

The response can be represented graphically, either in the three-dimensional space or as contour plots that help visualize the shape of the response surface. Contours are curves of constant response drawn in the X_i, X_j plane keeping all other variables fixed. Each contour corresponds to a particular height of the response surface, as shown in Figure 2.6.

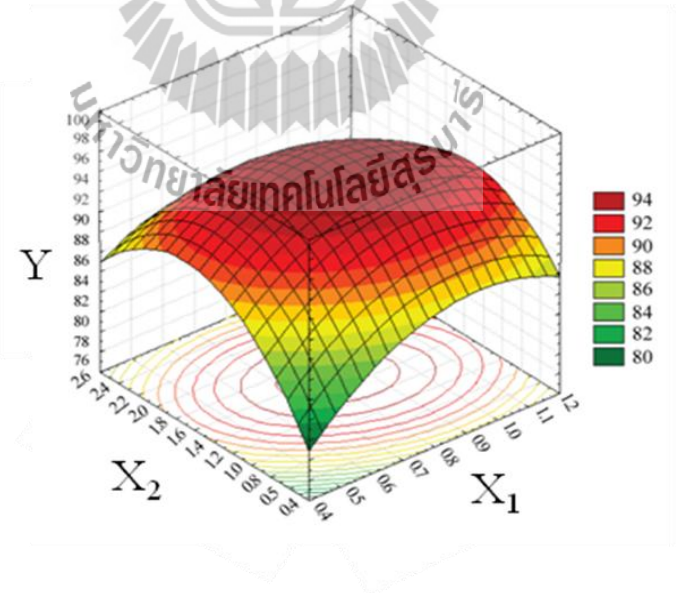

Figure 2.6 Three-dimensional response surface and the corresponding contour plot for 2,3-BD concentration. X_1 is temperature (°C) and X_2 is pH.

An important aspect of RSM is the **design of experiments** (Box and Draper, 1987), usually abbreviated as DoE. The objective of DoE is the selection of the points where the response should be evaluated. In statistics, Box–Behnken designs are experimental designs for response surface methodology, (Box et al., 1978). It is an efficient three-level design for fitting second order response surface. The behavior of the system is described by the following quadratic equation:

$$Y = b_0 + b_1 X_1 + ... + b_n X_n + b_{11} X_1^2 + ... + b_{nn} X_n^2 + b_{12} X_1 X_2 + ... + b_{n-1,n} X_{n-1} X_n$$

where Y is the predicted response; b_0 is the intercept; b_i will be the linear effect; b_{ii} is be the squared effect; and b_{ij} is the interaction effect. X_i and X_j is the coded independent variables.

Therefore, Box-Behnken design is alternative and useful to search for the fitted response surface of second order term resulting graphs called response surface plots and contour map as shown in Figure 2.6. Based on statistically mathematical model, optimization of few parameters at once using RSM rather than “one-at-a-time” appears to be the most reasonable approach since fermentation process is a multivariable system (Oh et al., 2008). RSM can be adopted to derive a statistical model for the individual and interactive effects of such parameters as medium composition, pH, cultivation time, or temperature.

To become more feasible, the production of 2,3-BD should be performed in a mineral salt medium by any microorganisms that can utilize broader range of carbon substrates such as sucrose, cellobiose, cellotriose, and glycerol or even pure hydrolyzed starch of maltodextrin which is readily abundant in Thailand.

2.9 Scale-up process

Bioreactor design and scale-up are important in the development of bioprocess. Once the conditions are optimized in laboratory unit, then, the selecting a proper reactor type and scaling it up to large production size are face with. Widely applications of bioreactor type are known such as stirred tank reactors (STR) and tower reactor (bubble column and air lift) detailed information by (Schugerl, 1990; Deckwer et al., 1974; Chisti and Moo, 1993). In most of the cases, scale up of bioreactors may cause serious problems. The problems associated with the strong decency of the transport parameters (mixing, shear, k_La, etc.) on the dimensions of the reactors. The transport characteristics of bioreactors included hydrodynamic, heat and mass transfer properties. Since the influence of reactor hydrodynamics on growth and metabolism of organisms are rarely predictable, the wildly used scale-up strategies in today's practice of biotechnology are still rules of thumbs, trial and error (Schmidt, 2005). The criterion of constant k_La or constant OTR has been successfully used in scale up some aerobic and even oxygen-limited culture. It is not certain if this criterion can also be used for scale-up of oxygen sensitive culture with the product formation like 2,3-fermentation. The work conducted by Byan et al. (1994) opposed that the OTR is not a suitable parameter to control micro-aerobic conditions, particularly at large scale unit due to the strong influence of reactor hydrodynamics on the distribution and utilization of oxygen in the whole reactors. Once the global OTR of a reactor is controlled, the local oxygen availability may vary, as OTR is not uniformly distributed over the whole tank volume. A controlled and optimum oxygen supply is required for achieving high product concentration and productivity. A too

low oxygen supply rate often leads to the formation of toxic by-products when oversupply of oxygen suppresses the main product formation.

Commonly, there are three aspects affecting by scaling up in fermentation process. First, biological factors was impacted by scale such as the number of generations associated with the inoculum development and production phases, mutation probability, contamination vulnerability, pellet formation and selection pressure (Sweere et al., 1987; Jem, 1989; Ettler, 1990; Enfors et al., 2011). Second, chemical factors triggered by scale include (a) pH control agents (i.e., type and concentration of acid and/or base), medium quality (i.e., purity of components) and water quality (Jem, 1989); (b) carbohydrate (e.g., oil), nitrogen (e.g., ammonia), phosphorus and product concentrations (Young, 1979); (c) redox potential and foam formation due to surface tension changes (Votruba and Sobotka, 1992). Last but not least, physical factors was affected by scale including tank configuration, aeration, agitation, back-pressure (and hydrostatic pressure), medium sterilization, temperature control/heat transfer and removal, and mixing (Votruba and Sobotka, 1992; Jem, 1989). Liden (2002) and Schmidt (2005) concluded that any approach and model are always approximate and a compromise but their relationships are not entirely reflected the highly complex intonations of the physical conditions covering the fermentation scale-up. Successful scale up in most cases would not be the outcome of a conclusive and straight-lined experimental strategy, but rather is the outcome of an independent optimization on each scale which highly depends on the experience, skill and the intuition of the experimentalist.

2.10 Previous studies of 2,3-BD production by fermentation

A number of species of the genera *Klebsiella, Bacillus, Serratia* and *Pseudomonas* have the ability to produce butanediol, but only two organisms, *K. pneumoniae and B. polymyxa*, have been shown to be potentially useful (Long and Patrick, 1963). The yield of 2,3-BD by *B. polymyxa* with all substrates is uniformly lower than that by *K. pneumoniae*. *K. pneumoniae* is widely distributed in nature, it is stable under a wide range of environmental conditions and produces double the amount of diol with much higher yields than does *B. polymyxa* (Garg and Jain 1995). *B. polymyxa* is one of the few *Bacillus* species that is capable of anaerobic growth and has been of historic fermentation interest for its ability to produce only the levo-isomer of 2,3-BD (Hespell, 1996). Unfortunately, few genetic techniques are available to engineer this microorganism to produce homo-2,3-BD under anaerobic conditions via gene deletions. This microorganism also requires rich nutrient sources such as whey based medium and yeast extract for optimal growth. A bacterium that has fast growth, no requirements of special and expensive sources of nutrients, and has available techniques for genetic manipulations, would be an ideal microorganism for 2,3-BD production. *K. pneumoniae* mainly produces meso-BD and is often used for the production of 2,3-BD because of its more complete fermentation, broad substrate spectrum and cultural adaptability (Garg and Jain, 1995). *K. pneumoniae* grows rapidly in a simple medium and it is able to metabolize a wide variety of sugars. If genetic improvement of this organism becomes desirable, its similarity to *E. coli* could prove to be a fortuitous advantage. Perhaps the most important attribute of *K. pneumonia* related to biomass conversion is its ability to convert all of the major sugars present in hemicellulose and cellulose hydrolysates into 2,3-butanediol. Yu and

Saddler (1983) obtained a 2,3-BD concentration of 113 g/L using fed-batch operation with *K. pneumoniae* but the productivity was relatively low (0.94 g/L/h). Although 2,3-BD production has been improved, the concentration and productivity are not high enough for economical industrial production. Therefore, it is essential to further improve 2,3-BD production by selecting high-yield strains or systematically optimizing the fermentation conditions. However, several strategies have been widely employed to enhance 2,3-BD production, such as introducing super productive strains, optimizing fermentation, operating conditions, and building mathematical models (Garg and Jain, 1995; Lee and Maddox, 1986). In 2009, Ma and coworkers have already published the enhancement of 2,3-BD production carried out by *K. pneumoniae* SDM. Corn steep liquor powder and $(NH_4)_2HPO_4$ were identified as the most significant factors to produce 2,3-BD by the two-level Plackett–Burman design. Fed-batch fermentations were conducted using different feeding strategies in a 5-L bioreactor resulting remarkable 2,3-BD yield and productivity were obtained. *K. pneumoniae* SDM has no requirements of the heterologous gene expression under anaerobic conditions; however, it has some limitations. For example, the bacterium utilizes a limited source of carbon substrates, only glucose. Furthermore, Zhang et al. (2010) employed RSM to find the optimized conditions for 2,3-BD from sucrose using *S. marcescens* H30. The concentration of yeast extract and sodium acetate at 33.4 g/L and 4.0 g/L, respectively, were found to produce 2,3-BD at 139.9 g/L with the diol (Acetoin + 2,3-BD), productivity of 3.5 g/L/h and the yield of 94.7% at 42 h. Wang et al. (2012) employed RSM with Central composite design (CCD) to optimize concentrations of cassava, glucoamylase and CSL. Under an optimal condition, 78.3 g/L of 2,3-BD was produced after 24 h in simultaneous saccharification and

fermentation (SSF), with a yield of 0.42 g/g cassava powder and a specific productivity of 3.3 g/L/h. The requirement of yeast extract or CSL was not the target for economic bio-based chemical production. So far, there were a few reports on oxygen control parameters coupling with scale-up process for micro-aerobic fermentation of 2,3-BD. Zeng et al. (1994) achieved to use respiratory quotient (RQ) as the control parameter for optimum production of 2,3-BD in both laboratory and pilot scales up to 1500 L resulting in 55 g/L of 2,3-BD concentration from glucose. Yang et al. (2012) scaled up the process to 30 L bioreactor by maintaining agitation and aeration rates as same as smaller tank conditions using *B. amyloliquefaciens* B10-127. The final 2,3-BD and yield were obtained at 61.4 g/L and 0.38 g/L from glucose. Recently, Priya et al. (2016) claimed that dual stage pH and agitation control regime were considered to enhance accumulation of 2,3-BD in a 150 L bioreactor. In the initial 10 h, the pH value was 7.5 and the agitation rate used was 200 rpm favoring bacterial growth, while these values were subsequently changed to a pH value of 6.5 and an agitation rate of 150 rpm. Using *E. cloacae* as biocatalyst, 2,3-BD concentration, total productivity and a yield are at 85 g/L, 1.73 g/L/h and 0.48 g of 2,3-BD per g of glucose, respectively.

CHAPTER III

GENERAL PROCEDURES

3.1 Microorganism and medium

Metabolically engineered *K. oxytoca* KMS005 was employed to produce 2,3-BD as a main product along with minor concentration of by-products such as succinate, acetate and ethanol (Jantama et al., 2015) throughout this study. The seed culture was maintained on LB agar composed of (g/L): peptone, 10; yeast extract, 5; NaCl, 5; agar, 20 at 4 °C. Chloramphenicol at concentration of 20 µg/ mL was also added into to the LB agar as necessary. Stock culture of strain KMS005 was kept in the medium supplemented with glycerol at the ratio of 3:1. A mineral salts medium (AM1) was adapted from Martinez et al. (2007) and used as a basic medium for preparing inocular and fermentation (Table 3.1).

3.2 Culture conditions

KMS005 inocula were prepared by inoculating a single loop of seed into LB broth at working volume of 100 mL (250 mL Erlenmeyer flasks). Inocula were incubated at 37 °C, 250 rpm for 16 h. Fermentation medium in the production tank was included AM1 medium and substrate as maltodextrin to grow KMS005 which was inoculated at OD_{550} of 0.1 (0.033 g/L dry cell weight). As shown in Figure 3.1, the fermentation was carried out in a 2 L bioreactor with 1 L of working volume at 37 °C, 250 rpm and aeration rate of 0.5 volume of air per volume of liquid per minute (vvm).

A stirrer was equipped with two Rushton turbines (impellers). The pH of the fermentation medium was maintained after being dropped to 6.0 by automatic addition of 3.0 M KOH using a program-controlled peristaltic pump. Variable parameters such as sugar concentration, pH, aeration rate, agitation speed, and tank scale were varied depending on experiment setup. The total incubation time was 48 h and all experiments were performed in duplicate.

Table 3.1 Composition of AM1 medium supplemented with 1 mM betaine (excluding carbon source).

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

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

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

3.3 Analytical methods

Two milliliters of culture broth were sampling every 6 h to measure the concentration of biomass, organic acids, and sugars. Cell mass was estimated from the optimal density at 550 nm (0.033 g/L of dry cell weight of OD_{550} is 0.1) with a spectrophotometer Spekol®1500. Organic acids and sugars were determined by using high performance liquid chromatography, HPLC, (Agilent, 2009) equipped with UV and refractive index detectors by a Bio-Rad Aminex HPX-87H ion exclusion column. Sulfuric acid was used as mobile phase at concentration of 4 mM with 0.4 mL/min. The culture was withdrawn and centrifuged at maximum speed 13,500 rpm for 4 min to separate cells and supernatant. The supernatant was filtered through 0.2 µm filter membrane before injecting to HPLC. Ten micro-liters of injection volume were automatically analyzed.

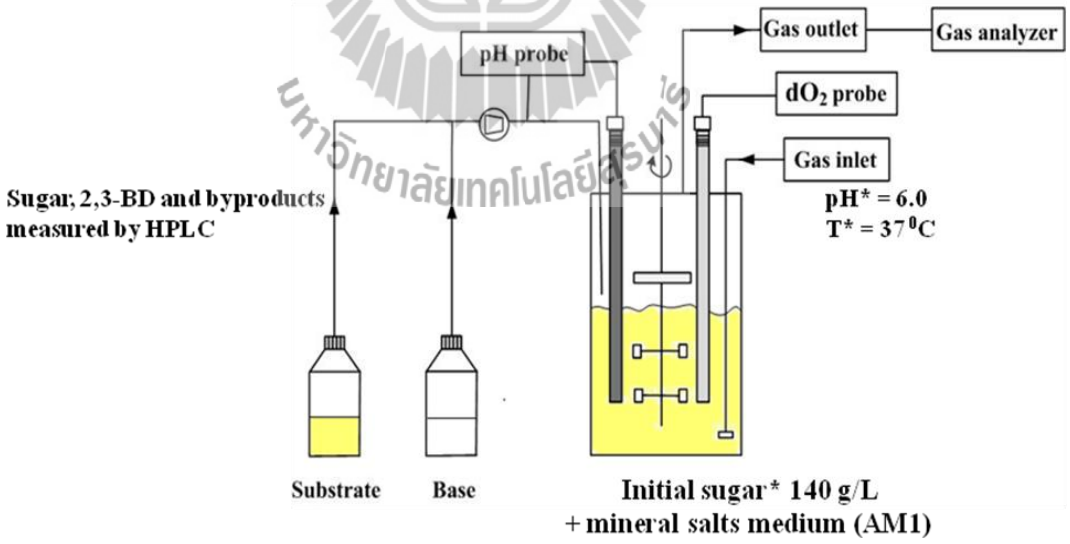

Figure 3.1 Schemetic diagram set up for 2,3-BD production (adapted from Nie et al. (2011)).

CHAPTER IV

PROCESS OPTIMIZATION ON MICRO-AERATION SUPPLY FOR HIGH PRODUCTION YIELD OF 2,3-BD FROM MALTODEXTRIN BY METABOLICALLY ENGINEERED *KLEBSIELLA OXYTOCA* KMS005

4.1 Introduction

Bio-refinery systems that integrate biomass conversion processes and are equipped to produce fuel, power, and bio-based chemicals from renewable resources are the focus of worldwide development due to concerns about scarce crude oil reserves, gradual increases in price, and environmental pollution (Kamm and Kamm, 2004; Ragauskas et al., 2006). 2,3-Butanediol (2,3-BD) is an example of bulk chemicals produced by fermentation that has raised much interest. An extensive application for 2,3-BD occurs in various fields, such as fuel, chemical industry, food industry, softening agents, explosives, plasticizers, and pharmaceutical agents. Furthermore, dehydration of 2,3-BD can be used in industrial solvents, such as methyl ethyl ketone (Ji et al., 2009). There are several microorganisms, including *Klebsiella pneumoniae, K. oxytoca, Bacillus polymyxa, Serratia marcescens,* capable of naturally producing 2,3-BD (Maddox, 1996). Among these species, *Klebsiella* spp. were comprehensively studied for fermentative 2,3-BD production (Yu and Saddler, 1983; Voloch et al., 1985; Blomqvist et al., 1993; Lee et al., 2009; Ma et al., 2009). For

example, Yu and Saddle (1983) obtained very high concentration of 2,3-BD at the concentration of 113 g/L using a fed-batch operation by *K. pneumonia*. Ma et al. (2009) also utilized *K. pneumonia* SDM isolated from soil for 2,3-BD production with very high productivity from glucose. However, *K. oxytoca* has an advantage in the purification step over *K. pneumoniae* owing to less formation of capsular polysaccharide during 2,3-BD production (Cho et al., 2012). Therefore, this might make *K. pneumoniae* unsuitable for 2,3-BD production on a large scale. The conventional optimization method and response surface methodology (RSM) were employed for the fermentative production of 2,3-BD. These strategies were mostly applied in flask experiments prior to bio-reactors leading to medium optimization. However, only a few studies reported on process optimization of physiological parameters affecting 2,3-BD, such as pH, aeration rate, and agitation speed (Jiang et al., 2012). Some attempted to establish proper oxygen supply strategies to ensure efficient 2,3-BD production. Strategies to control parameters, including oxygen transfer rate (OTR), volumetric oxygen transfer coefficient (k_La), oxygen uptake rate (OUR), and respiratory quotient (RQ), were applied for 2,3-BD fermentation. The application of those strategies is restricted as these parameters are not easily controlled (Ji et al., 2009). Most previously published works reported the use of complex and rich nutrients for the promotion of microbial growth and improvement of 2,3-BD production (Voloch et al., 1985; Blomqvist et al., 1993; Ma et al., 2009; FAOSTAT, 2011; Ge et al., 2011; Ji et al., 2011; Nie et al., 2011; Jiang et al., 2012; Sangproo et al., 2012; Lee et al., 2013; Park et al., 2013). It is expected that high production costs related to medium preparation, downstream processing, and waste disposal have not substantially met criteria for process economy (Song and Lee,

2006). Robust microorganisms, the use of inexpensive substrates and media, and simple downstream processes were taken into account to ensure the economic feasibility of 2,3-BD production (Zeng and Sabra, 2011). In addition, the development of a simple fermentative production process utilizing cheap agricultural substrates is vital to obtain high concentrations and yields of 2,3-BD. Maltodextrin, a product of partially hydrolyzed corn or cassava starch, is a cheap and abundant carbon source that can be fermented to 2,3-BD due to its highly pure substrate, accessible digestion, and rapid absorption. There is only one publication of the use of maltodextrin for ethanol production (Formanek et al., 1997). Therefore, the use of a low-cost medium without supplementation of any complexes, rich, and expensive nutrients with a cheap substrate such as maltodextrin is an attractive consideration for the economic production of 2,3-BD. In this study, optimized parameters affecting 2,3-BD production were investigated with a recently published *K. oxytoca* KMS005 (Δ*adhE*Δ*ack-pta*Δ*ldhA*) strain using maltodextrin as the sole carbon source supplemented in a mineral salts medium. The KMS005 strain was previously reported to produce the highest yield ever published of 2,3-BD at the level of 0.49 g/g glucose with less by-products formation in AM1 medium (only 4 g/L of salts) containing glucose without addition of rich and expensive nutrients (Jantama et al., 2015). By the use of conventional optimization coupled with RSM, the optimized levels of four parameters (pH, aeration rate, agitation speed, and maltodextrin concentration) were precisely indicated. The key parameters are necessary to 2,3-BD fermentation pathway which is stimulated under micro-aerobic cultivation and slightly acidic pH.

Meanwhile, statistical analysis led to an understanding of the interaction effects between the parameters. A single stage fermentation was established using

maltodextrin to produce 2,3-BD with a high production yield. The optimized conditions obtained were also validated in batch and interim fed-batch fermentation. The concentration of 2,3-BD was obtained at the level of 88.1 g/L with the yield of 0.41 g/g maltodextrin consumed within 78 h. Interestingly, the low cost of fermentative 2,3-BD production calculated at $2.04/kg and the total production cost ranged from $5.5 to $7/kg based on the results in our study may be effective in competition with chemical-based 2,3-BD production.

4.2 Materials and methods

4.2.1 Microorganism and media

Metabolically engineered *K.oxytoca* KMS005 (Δ*adhE*-Δ*ack-pta*Δ*ldhA*) which was previously produced by Jantama et al. (2015) was used in this study. Maltodextrin was purchased from Nakhon Ratchasima, Thailand. The percentage of solid content in maltodextrin is approximately 84% (w/w) and its dextrose equivalent (DE) is 6.4. A simple mineral salts medium (4 g/L of salts), AM1 (Martinez et al., 2007) was used as a fermentation medium throughout this study. As shown in Table 3.1, AM1 medium is composed of inorganic nitrogen source, $MgSO_4$, betaine-KCL as an osmotic protectant, and trace minerals. Luria-Bertani (LB) agar was used for maintaining bacterial cultures.

4.2.2 Culture conditions

For seed culture preparation, KMS005 was cultured on a LB agar. The plate was incubated at 37 °C for 24 h. A full single loop of fresh colonies was inoculated into 250 mL Erlenmeyer flasks containing 60 mL LB medium. The inoculum was incubated at 37 °C and 200 rpm for 16 h. The seed culture was

inoculated in AM1 medium supplemented with maltodextrin at the concentration equivalent to 0.033 g/L dry cell weight (OD_{550} = 0.1). Fermentation experiments were carried out at 37 °C in a 2 L bio-reactor with a working volume of 1 L. The fermentation broth was supplied with sterile air at desired flow rates and controlled at desired agitation speeds. The pH of the medium was constantly maintained by the automatic addition of 3.0 M KOH. The percentage of dissolved oxygen was also measured by dissolved oxygen (DO) probe.

4.2.3 Process optimization of 2,3-BD production

The levels of the parameters affecting 2,3-BD such as pH, aeration rate, agitation speed, and maltodextrin concentration, were initially screened using a 'one variable at a time' strategy prior to subjection to RSM. The parameters were: pH (5.0, 5.5, 6.0, 6.5 and 7.0); aeration rates (0.1, 0.5, 0.8, 1.0 and 1.2 vvm); agitation rates (200, 300, 400 and 500 rpm); maltodextrin concentrations (5, 10, 15, 20 and 25% (w/v)). An agitator was equipped with two Rushton turbines. Experiments were performed in duplicate. Data were analyzed by the SPSS program (version 15.0). Comparisons between means were carried out using a Duncan's new multiple range test at p-value < 0.05.

RSM with Box-Behnken design was employed to determine optimal levels of aeration rate, agitation speed, and maltodextrin concentration for 2,3-BD production by the KMS005 strain. Design expert version 8.0 (licensed) was used to program the Box-Behnken design to maximize the response of 2,3-BD production. All fermentation experiments were carried out in 2 L bio-reactor in which pH was controlled at 6.0 and temperature was at 37°C.

4.2.4 Batch and fed-batch fermentations

Optimized conditions obtained from RSM were validated under batch conditions prior to being employed under fed-batch conditions. During an interim-feeding fermentation, maltodextrin solution at the stock concentration of 80% (w/v) was added to the bio-reactor thus maintaining sugar in the broth at concentrations of 45-60 g/L when the residual sugar concentrations were in the range of 30-35 g/L.

4.2.5 Analytical methods

Two milliliters of culture broth were withdrawn every 6 h to measure the concentrations of cell mass, organic acids, and sugars. Cell mass was estimated from the optimal density at 550 nm (0.033 g/L of dry cell weight of OD_{550} is 0.1) with a spectrophotometer Spekol® 1500. Sugars, 2,3-BD, and other by-products were determined by high performance liquid chromatography (HPLC) (Agilent, 2009) equipped with UV and refractive index detectors by a Bio-Rad Aminex HPX-87H ion exclusion column. Sulfuric acid was used as a mobile phase at the concentration of 4 mM. Residual maltodextrin left in the broth was fully hydrolyzed to glucose. A Glucoamylase (Siam Victory Chemicals Co., Ltd.) at 500 U was added to the broth. The reaction was incubated for 2 hours at 65 °C and 200 rpm prior to filtration. Ten micro liters of filtered broth were injected for analysis by HPLC. A percentage of dissolved oxygen (DO) in fermentation was measured by dissolved oxygen probe. The probe was mounted in the medium prior to calibration by de-gassing with oxygen-free nitrogen gas. After inoculation, decreasing values of DO as a function of time were recorded.

4.3 Results and discussion

4.3.1 Effects of pH

The effects of pH in the range of 5.0 to 7.0 on 2,3-BD production by the KMS005 strain were initially investigated with fixed aeration rate and agitation speed of 0.5 vvm and 200 rpm respectively, and maltodextrin at the concentration of 100 g/L (equivalent to 93 g/L glucose). As shown in Table 4.1, 2,3-BD production at the concentration of 31.8±0.3 g/L was maximized with a yield of 0.34±0.01 g/g maltodextrin supplied at pH 6.0. However, there was no significant difference in 2,3-BD production at pH 6.5 in terms of concentration, productivity and yield compared with the results at pH 6.0. Detectable levels of acetate, lactate, and formate were not observed at pH 6.0. It may be implied that acetate acted as an inducer and may be directed and utilized for 2,3-BD production through the activity of α-acetolactate synthase (Biebl et al., 1998). Noticeably, an increase in pH over 6.5 resulted in lowering 2,3-BD production in terms of concentration and yield. In addition, the highest levels of by-products, mainly succinate and acetate, were accumulated at pH 7.0. This observation was in agreement with Lee et al. (2013) whose study stated that an increased pH led to higher proportions of fermentative metabolites such as acetate, suucinate, and ethanol, resulting in a decreased level of 2,3-BD in *K. pneumoniae*. This may be due to an inactivation of α-acetolactate synthase activity at higher levels of pH than 6.0. Stormer (1968) also found that a pH above 6.0 caused a sharp decrease in the activity of α-acetolactate synthase in the 2,3-BD producing pathway in *K. pneumoniae*, thus diverting the carbon flow to other fermentative pathways. Our findings also demonstrated that lower pH values than 6.0 caused significant reductions in substrate utilization and biomass formation, thus detrimentally affecting

2,3-BD production. This may be explained by inefficient maltodextrin transport at low pH conditions. Pajatsch et al. (1998) demonstrated that *Klebsiella* species was able to deliver linear maltodextrins (maltose up to maltoheptaose) through actions of a binding protein-dependent ABC transporter. Pedersen and Carafoli (1987) revealed that the prevailing reaction for ATP synthesis via proton motive force (PMF) by ATP synthase was not thermodynamically favored at pH lower than 6.9. Our results clearly showed that maltodextrin was greatly accumulated in the fermentation broth at lower pH values and even uncontrolled pH conditions (Table 4.1). These results may suggest that the sufficiently high acid concentration caused a collapse of the pH gradient across the cell membrane at pH values less than 6.0, resulting in an impairment of ATP production via PMF. Inefficient ATP production may cause the activity of the ABC transporter for maltodextrin to be ineffective at low pH values. In addition, the KMS005 strain was tested for 2,3-BD production under a non-controlled pH experiment (Table 4.1). This resulted in the lowest production of 2,3-BD (11.6±1.5 g/L) when pH in the medium was changed from 7.2 to 4.8. This finding confirmed that maltodextrin was not efficiently utilized and not consumed by the strain when pH was gradually lowered in an uncontrolled pH experiment, indicating the highest level of residual sugar accumulating in the fermentation broth. This result was not in agreement with the result observed by Biebl et al. (1998) who claimed that 2,3-BD concentration and yield were impressively obtained when the pH of the fermentation broth of *K. pneumoniae* was not controlled. The pH values of the broth changed continuously from 7.0 to 5.5. Therefore, our findings that contrasted with the work of Biebl et al. (1998) may be caused by differences of micro-organism strains, bio-reactor configurations, and operational conditions.

Table 4.1 Fermentation profile for 2,3-BD production by *K. oxytoca* KMS005 at various pH values in AM1 medium containing 100g/L maltodextrin.

pH	Residual sugar (g/L)	Max CDW (g/L)	2,3-BD (g/L)	Gross yield (g/g)[a]	Productivity (g/L/h)[a]	By-products (g/L)				
						Suc[d]	Lac	Eth	For	Ace
Uncontrolled	67.1±2.2	1.0±0.1	11.6±1.5$^{c,\beta}$	0.12±0.01$^{\beta}$	0.16±0.01	ND[b]	ND	ND	ND	1.5±0.1
5.0	60.1±4.3	2.4±0.1	15.1±1.8$^{\gamma}$	0.16±0.01$^{\gamma}$	0.21±0.02	ND	ND	ND	ND	1.4±0.3
5.5	33.0±2.2	2.9±0.1	23.2±1.6$^{\theta}$	0.25±0.01$^{\theta}$	0.32±0.02	1.8±0.3	ND	ND	ND	1.1±0.1
6.0	5.3±1.2	3.9±0.1	31.8±0.3$^{\pi}$	0.34±0.01$^{\pi}$	0.44±0.01	2.2±0.1	ND	1.9±0.1	ND	ND
6.5	1.0±0.3	4.2±0.1	31.3±1.2$^{\pi}$	0.33±0.01$^{\pi}$	0.43±0.02	3.4±0.1	ND	2.2±0.1	ND	1.7±0.1
7.0	ND	3.5±0.4	26.2±0.6$^{\Upsilon}$	0.28±0.01$^{\Upsilon}$	0.36±0.04	8.6±0.4	ND	ND	ND	10.7±0.9

[a] Gross yield was calculated as product concentration divided by initial total sugar concentration equivalent to 93 g/L of sugar content at 72 h incubation. Productivity was calculated at 72 h.

[b] ND = not detected.

[c] All data represent the averages of two fermentations with standard deviations. Values bearing different Greek symbol are significantly different ($P < 0.05$).

[d] Suc: Succinate, Lac: Lactate, Eth: Ethanol, For: Formate, and Ace: Acetate

4.3.2 Effects of aeration rates

The effects of aeration rates ranging from 0.1 to 1.2 vvm on 2,3-BD production were also investigated to find a suitable level of micro-aerobic conditions to ensure an activation of enzymes involved in 2,3-BD production, such as α-acetolactate synthase and 2,3-BD dehydrogenase (Lee et al., 2013). Experiments were performed at fixed parameters of 200 rpm agitation speed, pH 6.0, and 10 % (w/v) maltodextrin. As shown in Table 4.2, higher 2,3-BD concentrations ranging from 32 to 34 g/L and their improved productivities ranging from 0.67 to 0.72 g/L/h were observed as aeration rates varied from 1.0 to 1.2 vvm. The results revealed insignificant differences in 2,3-BD concentrations and yields ($p<0.05$) between both aeration rates. Thus, the lower aeration rate at 1.0 vvm was sufficient enough to provide preferable conditions for 2,3-BD production by the KMS005 strain. Stanbury and Whitaker (1995) suggested that the aeration rate should be in the range of 0.5-1.0 vvm. An aeration rate in this range is maintained in large scale operations for low energy consumption and industrially operational viability. It should be noted that the aeration rate had an equivalent effect on 2,3-BD production and cell growth compared to those of pH (Tables 4.1 and 4.2). The majority of enzymes involving in 2,3-BD producing pathway is activated under micro-aerobic conditions, and irreversibly inactivated by oxygen under fully aerobic conditions. This was clearly confirmed by our results (Table 4.2). The concentrations and production yields of 2,3-BD were significantly increased when aeration rates were increased to 1.2 vvm. Voloch et al. (1985) suggested that oxygen was less soluble in water than those of carbon substrates was. Thus, the oxygen demand of an industrial fermentation process for 2,3-BD production is normally satisfied by aerating the fermentation broth. Then, the production yield of

2,3-BD can be maximized by adjusting the suitable oxygen supply to limit the respiration. Our results revealed that the 2,3-BD production yields and concentrations improved when a suitable aeration rate was provided. Generally, ethanol is usually produced in approximately equimolar amount with 2,3-BD along with formate, acetate, lactate, and acetoin by *K. oxytoca* in the absence of oxygen. However, the KMS005 strain was metabolically engineered to delete *adhE* and *ldhA* genes encoding enzymes involved in NADH reoxidation pathways. Two pathways in central metabolism for succinate and 2,3-BD productions are responsible for NADH reoxidation in the KMS005 strain. No lactate was observed but certain amount of ethanol was detected (Table 4.2). It was likely that other isoenzymes, such as acetaldehyde dehydrogenase 2 (encoded by *mhpF*), ethanol dehydrogenase P (encoded by *adhP*), acetaldehyde dehydrogenase (encoded by *ald*), and 1,3-propanediol oxidoreductase (encoded by *yqhD-1*), were suspected of taking responsibility for ethanol formation under micro-aerobic conditions (Blomqvist et al., 1993; Zhuge et al., 2010). These genes are usually activated by FNR (fumarate-nitrate reduction regulatory) protein. A high level of active FNR protein appeared to be present during micro-aerobic respiration (Levanon et al., 2005). Therefore, it is suspected that FNR activity is correlated with the high redox potential ($NADH/NAD^+$ ratio) obtained under micro-aerobic growth performed in our study. Succinate was still detected under micro-aerobic conditions (0.8-1.2 vvm) but KMS005 seemed not to effectively produce 2,3-BD and succinate under non-aerated conditions (Table 4.2) due to impaired growth. Usually, succinate-producing pathway via phosphoenol pyruvate (PEP) carboxylase (PPC: encoded by *ppc*) and malate dehydrogenase (MDH: encoded by *mdh*) activities are responsible for NADH re-oxidation under

anaerobic conditions (Gottschalk, 1986; Reed et al., 2003). The results showed that the KMS005 strain is more preferably dissimilated PEP fluxes through 2,3-BD production pathway to conserve free energy and to reduce the ratio of NADH to NAD^+ respectively. This did not prevent the production of succinate. This situation may be explained by the fact that PEP is also essential to supply precursor metabolites, including amino acids for biomass formation. Then, the requirement of intracellular PEP in KMS005 was still maintained with a small extent of carbon flux to oxaloacetate (OAA). Upon OAA availability, KMS005 strain may produce succinate from OAA via MDH activity to partially maintain the redox balance ($NADH/NAD^+$ ratio). Recently, Jantama et al. (2015) revealed that the KMS005 strain exhibited a low level of MDH activity during micro-aerobic conditions. The *mdh* expression was kept at very low levels during micro-aerobic conditions by the activity of ArcA (encoded by *arcA*) protein (Levanon et al., 2005). In addition, it was also demonstrated that the MDH activity was highest based on an availability of OAA under micro-aerobic conditions (Park et al., 1995). On the other hand, succinate accumulation may be involved with an activity of putative FNR protein. In 2,3-BD pathway, three genes encoding for α-acetolactate synthase, α-acetolactate decarboxylase, and acetoin reductase are known to be clustered in one operon called *budABC*. In the operon, FNR binding site appeared at position -6 to facilitate transcription and regulation of the gene cluster during the anaerobic process (Blomqvist et al., 1993; Mayer et al., 1995). At low aeration rate (0.8 vvm), FNR may down-regulate the expression of *budABC* genes causing the highest production of succinate. By increasing aeration, more oxygen was able to activate α-acetolactate synthase while FNR was down-regulated resulting in a low expression of genes

involving succinate producing pathway, including fumarate dehydrogenase (FDH: encoded by *fdh*). FDH activity functionally converts fumarate to succinate under both anaerobic and micro-aerobic conditions. Hence, the more aeration increased, the more succinate decreased. It may also be concluded that high 2,3-BD production along with the production of succinate and ethanol showed that the ratio of NADH/NAD^+ was well-balanced to prevent a retarded glycolytic flux caused by the low efficiency of NADH re-oxidation.

Table 4.2 Fermentation profile for 2,3-BD production by *K. oxytoca* KMS005 at various aeration rates in AM1 medium containing 100 g/L maltodextrin.

Aeration rate (vvm)	Residual sugar (g/L)	Max CDW (g/L)	2,3-BD (g/L)	Gross yield (g/g)[a]	Productivity (g/L/h)[a]	By-products (g/L)		
						Suc	Eth	Ace
No air	84.0±3.0	0.6±0.1	1.6±0.1$^{c,\beta}$	0.03±0.01$^{\beta}$	0.02±0.01	0.8±0.1	0.9±0.1	2.1±0.1
0.1	78.6±1.8	2.2±0.2	6.2±0.3$^{\gamma}$	0.06±0.01$^{\gamma}$	0.12±0.01	0.6±0.1	ND[b]	1.0±0.1
0.8	12.7±1.9	4.7±0.2	27.9±0.1$^{\alpha}$	0.30±0.01$^{\alpha}$	0.58±0.01	3.2±0.1	2.2±0.2	0.6±0.1
1.0	4.5±2.5	4.4±0.2	32.1±1.4$^{¥}$	0.34±0.01$^{¥}$	0.67±0.03	2.2±0.2	1.4±0.3	1.1±0.9
1.2	7.9±1.6	4.7±0.1	34.6±2.2$^{¥}$	0.35±0.02$^{¥}$	0.72±0.05	1.5±0.2	1.3±0.1	0.2±0.1

[a] Gross yield was calculated as product concentration divided by initial total sugar concentration (10% (w/v) at 48 h incubation. Productivity was calculated at 48 h.

[b] ND = not detected.

[c] All data represent the averages of two fermentations with standard deviations. Values bearing different Greek symbol are significantly different ($P < 0.05$).

4.3.3 Effects of agitation speeds

The effect of agitation speeds in the range of 200 to 500 rpm on 2,3-BD production were investigated with fixed aeration rate of 1.0 vvm, pH 6.0, and maltodextrin concentration of 10% (w/v). Our results revealed that the agitation speed at 400 rpm provided a faster cell growth (Figure 4.1) accompanied with the high 2,3-BD concentration of 35 g/L. The agitation speed at 400 rpm led to faster cell growth but provided a comparable 2,3-BD production than that obtained at the agitation speed of 300 rpm (Fig 4.1). There was no significant differences in 2,3-BD concentrations and yields between agitation rates of 300 and 400 rpm at 48 h incubation (Table 4.3). Our findings were supported by Cho et al. (2015) who stated that the agitation speed of 400 rpm was the best condition to drive both cell growth and 2,3-BD production using *K. oxytoca* species from glucose.

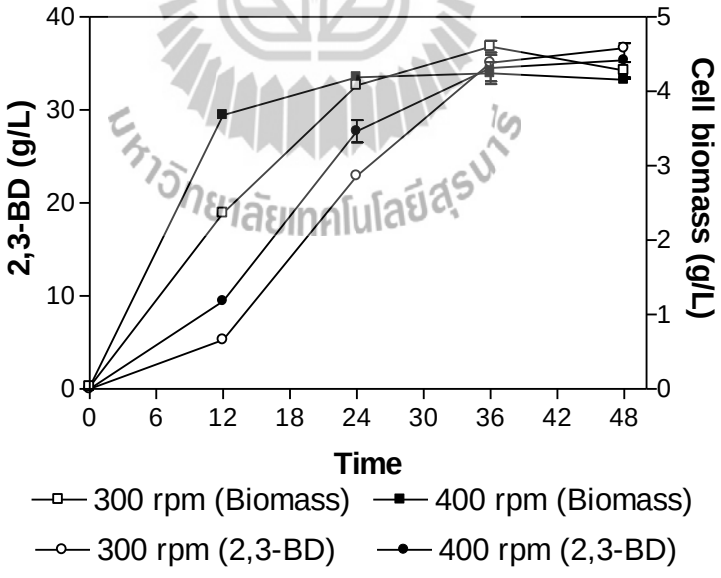

Figure 4.1 Comparison of cell biomass and 2,3-BD production between agitation speeds 300 and 400 rpm. Aeration rate, pH, and maltodextrin concentration were fixed at 1.0 vvm, 6.0, and 10% (w/v) respectively.

Table 4.3 Fermentation profile for 2,3-BD production by *K. oxytoca* KMS005 at various agitation speeds in AM1 medium containing 100 g/L maltodextrin.

Agitation speed (rpm)	Residual sugar (g/L)	Max CDW (g/L)	2,3-BD (g/L)	Gross yield (g/g)[a]	Productivity (g/L/h)[a]	By-products (g/L)		
						Suc	Eth	Ace
200	4.5±2.5	4.4±0.2	32.1±1.4[b, β]	0.34±0.01[β]	0.67±0.03	2.2±0.2	1.4±0.3	1.1±0.9
300	1.6±0.1	4.8±0.1	36.6±0.6[π]	0.39±0.01[π]	0.76±0.01	2.2±0.4	0.8±0.1	1.2±0.2
400	1.0±0.1	4.4±0.1	35.4±2.6[π]	0.38±0.02[π]	0.73±0.05	1.4±0.1	<0.1	0.9±0.1
500	31.3±3.1	4.4±0.2	23.7±0.1[¥]	0.25±0.01[¥]	0.49±0.01	<0.1	<0.1	2.6±0.6

[a] Gross yield was calculated as product concentration divided by initial total sugar concentration (10% (w/v) at 48 h incubation. Productivity was calculated at 48 h.

[b] All data represent the averages of two fermentations with standard deviations. Values bearing different Greek symbol are significantly different ($P < 0.05$).

Banks (1976) demonstrated that degree of agitation had a profound effect on oxygen-transfer efficiency in agitated fermenters. According to our results, a lower agitation speed at 200 rpm did not only cause a decrease in 2,3-BD production in terms of yield and concentration but also accumulation of high levels of succinate, ethanol, and acetate (Table 4.3). This indicated that the conditions at agitation rates of 200 to 300 rpm favored succinate and ethanol-producing pathways (Zhuge et al., 2010) in some magnitude due to lower oxygen availability. In addition, a higher agitation speed of 500 rpm had an obvious negative effect on 2,3-BD production by the KMS005 strain. The 2,3-BD concentration and yield dramatically decreased by about 32% and 34% respectively compared with the results obtained at the agitation speed of 400 rpm. The combination between the agitation speed of 500 rpm and the aeration rate of 1.0 vvm in our study may have affected the culture environment of the broth in fermenter. It was likely that micro-aerobic conditions may be altered to partially aerobic conditions by the increase in oxygen solubility in the fermentation broth. This led the decreasing in 2,3-BD production due to an irreversible inactivation of α-acetolactate synthase by its exposure to a greater level of soluble oxygen when the agitation speed of 500 rpm was applied. However, our findings were contrasted with those reported by Ma et al. (2009) who applied the agitation speed of 500 rpm and the aeration rate of 1.5 vvm to produce a high level of 2,3-BD from glucose within 38 h under fed-batch fermentation by *K. pneumoniae*. Surprisingly, a very high concentration of maltodextrin still accumulated in the broth at 500 rpm agitation compared with those accumulated at lower agitation speeds, even though the incubation time was prolonged up to 48 h (31.3±3.1 g/L). It was likely that the KMS005 strain did not efficiently utilize maltodextrin under partially aerobic

conditions reflected by high agitation rates. The explanation of this is that when culture conditions are not fully aerobic, generally combined activities of lactate dehydrogenase (*ldhA*) and α-acetolactate synthase (*budB*) play synergistic roles to dissipate pyruvate to generate lactate and 2,3-BD, thus preventing an accumulation of pyruvate and an inhibition of glycolysis caused by an imbalanced NADH/NAD^+ ratio in the wild type strain. However, the KMS005 strain was metabolically engineered to delete *ldhA*, *adhE*, and *ackA* genes to maximize carbon flux through a 2,3-BD producing pathway (Jantama et al., 2015). Therefore, the strain only possesses a 2,3-BD producing pathway and succinate-production pathway to maintain the redox balance (NADH/NAD^+ ratio) under fermentative conditions in a central metabolism pathway. The KMS005 strain was not able to utilize a succinate-producing pathway to maintain a redox balance of NADH/NAD^+ due to non restrictive anaerobic conditions and absence of an external carbon dioxide source under our fermentation conditions at 500 rpm agitation speed. This was confirmed by no detectable concentration of succinate in the broth. Also, at the same condition (500 rpm agitation speed), genes coding for 2,3-BD formation (*budABC* operon) may be down-regulated at the transcription level by FNR activity (Blomqvist et al., 1993), and α-acetolactate synthase enzyme may be also partially inactivated (Gottschalk, 1986) due to excess oxygen above the threshold favoring 2,3-BD production. This was observed by the decrease in concentration of 2,3-BD (Table 4.3). The high ratio of NADH/NAD^+ and accumulated pyruvate usually inhibit glycerol-3-phosphate dehydrogenase enzyme thus resulting in glycolysis inhibition (Gottschalk, 1986). Therefore, the KMS005 strain stopped consuming maltodextrin to lower a glycolytic flux and to avoid a detrimental effect caused by a redox imbalance (high ratio of NADH/NAD^+) within

the cells. Considering the carbon balance, very low amounts of by-products were detected in the fermentation broth (Table 4.3) and there was no observation of pyruvate in the broth. It is possible that the KMS005 strain may dissipate some of the pyruvate pool to acetyl-CoA and formate besides succinate and ethanol. Pyruvate-formate lyase (encoded by *pflB*) (Gottschalk, 1986) and α-ketobutyrate/pyruvate formate-lyase (encoded by *tdcE*) (Reed et al., 2003) can assimilate pyruvate to formate and acetyl-CoA under non-fully aerobic conditions. Lots of bubbles were observed during cell growth but no formate was observed in the fermentation broth of KMS005 under our fermentation conditions. This phenomenon suggested that the strain may channel formate to produce CO_2 and H_2 via formate hydrogen-lyase (encoded by *fdh* and *hyc*) to support growth. Thauer et al. (1977) revealed that the formate hydrogen-lyase reaction produced the electro gradient that was able to generate ATP by proton motive force. Axley et al. (1990) also demonstrated that formate served as a growth substrate in many microorganisms when carbon sources provided in the broth were limited. This effect was more pronounced where maltodextrin consumption was stalled due to the inhibition of glycolysis in the fermentation at 500 rpm agitation speed. Therefore, formate may be further consumed to compensate for the capability deficit in energy production due to low flux through acetate kinase (encoded by *ackA*) or low glycolytic flux in KMS005. In addition, a few amounts of acetyl-CoA were converted to acetate via a propionate kinase (TdcD) encoded by *tdcD*, which is suspected to compensate for acetate kinase activity in the KMS005 strain (Sangproo et al., 2012).

4.3.4 Effects of substrate concentrations

Maltodextrin concentrations were varied from 5 to 25% (w/v) at the conditions of pH 6.0, aeration rate 1.0 vvm, and agitation speed 400 rpm. As shown in Table 4.4, maltodextrin concentration at 200 g/L provided the highest concentration and yield of 2,3-BD within 72 h of fermentation. Improvement in terms of 2,3-BD concentration (74.7±0.2 g/L), productivity (1.04±0.16 g/L/ h), and yield (0.40±0.01 g/g maltodextrin supplied) was achieved under this condition. However, employing the initial maltodextrin concentration at 250 g/L was found to affect substrate utilization. It was likely that the substrate inhibition would be considered. The comparable 2,3-BD concentration was obtained at the level of 73.4±2.4 g/L. But a dramatic reduction in gross yield (0.31±0.01 g/g maltodextrin supplied) of about 22.5% was observed compared with that of the fermentation condition with the initial concentration of 200 g/L maltodextrin. Also, there was more sugar left over (78.3±2.4 g/L) after 72 h incubation at the initial concentration of 250 g/L maltodextrin. Our findings are similar to the results of Wang et al. (2012) who found that a significant increase of 2,3-BD concentration with an increase of cassava powder as substrate from 100 to 200 g/L in *Enterobacter cloacae.* However, when the cassava powder concentration was greater than 200 g/L, the residual sugar level sharply increased and the growth of the strain stalled. The production yield of 2,3-BD was also reduced. This may be explained by the fact that the osmotic pressure contributed from the high substrate concentrations resulted in a slower proliferation of microbial cells. The higher concentration of substrate also affected pH, viscosity and the activity of the fermentation medium. In addition, long exposure to high substrate concentration may also cause catabolic repression of microbial strains (Thomas et al., 1992). In addition,

Wang et al. (2012) revealed that when the initial glucose concentration was over its optimum point, not only *K. pneumoniae* SDM did stop the utilization of glucose substrate but the cell biomass was also significantly stalled. It had a detrimental effect on 2,3-BD production reflected by a lowering in 2,3-BD concentration compared with that obtained at the condition with optimal substrate concentration.

Table 4.4 Fermentation profile for 2,3-BD production by *K. oxytoca* KMS005 at various maltodextrin concentrations in AM1medium.

Maltodextrin concentration (g/L)	Residual sugar (g/L)	Max CDW (g/L)	2,3-BD (g/L)	Gross yield (g/g)[a]	Productivity (g/L/h)[a]	By-products (g/L)		
						Suc	Eth	Ace
50	ND	2.8±0.1	15.1±0.3$^{b,\alpha}$	0.32±0.02$^{\alpha}$	0.21±0.03	1.4±0.5	1.0±0.2	2.3±0.1
100	1.6±0.1	4.8±0.1	36.6±0.6$^{\beta}$	0.39±0.01$^{\beta}$	0.73±0.05	2.2±0.4	0.8±0.1	1.2±0.2
150	16.6±4.1	4.8±0.3	50.4±2.4$^{\pi}$	0.36±0.02$^{\pi}$	1.05±0.05	1.0±0.1	0.6±0.1	0.9±0.0
200	30.7±4.5	4.9±0.0	74.7±0.2$^{¥}$	0.40±0.01$^{\beta}$	1.04±0.16	0.9±0.2	0.5±0.1	0.8±0.1
250	78.3±2.4	4.6±0.0	73.4±2.4$^{¥}$	0.31±0.01$^{\alpha}$	1.02±0.03	1.1±0.4	0.6±0.1	0.7±0.3

[a] Yield was calculated as product concentration divided by initial total sugar concentration at 48 h for substrate concentration of 50, 100 and 150 g/L and at 72 h for 200 and 250 g/L. Productivity was calculated at 48 h for substrate concentration of 50, 100 and 150 g/L and at 72h for 200 and 250 g/L.

[b] All data represent the averages of two fermentations with standard deviations. Values bearing different Greek symbol are significantly different ($P < 0.05$).

Noticeably, the KMS005 strain still exhibited a robustness in 2,3-BD production in AM1 medium containing high maltodextrin concentration. The strain was able to produce up to 90 g/L of 2,3-BD from 250 g/L of maltodextrin within 120 h incubation under batch conditions (data not shown). It seemed that the strain did not stop utilizing but gradually consumed maltodextrin, thus delaying 2,3-BD production at a high substrate concentration. *E. coli* and *Klebsiella* spp. can transport linear maltodextrins via a binding protein-dependent ABC transporter consisting of maltoporin (LamB), the maltodextrin-binding protein (MBP or MalE), cytoplasmatic membrane proteins (MalF and MalG), and ATP-binding protein (MalK). The linear maltodextrins are further degraded intracellularly into glucose and glucose-1-phosphate by the enzymes amylomaltase (MalQ), maltodextrin phosphorylase (MalP), and maltodextrin glucosidase (MalZ). Among these proteins, MBP is essential for the transport of maltodextrins. MBP can bind linear maltodextrins, cyclic maltodextrins, and various maltodextrin analogues although only linear maltodextrins up to maltoheptaose are substrates for transport (Ferenci et al., 1986). Maltodextrin is a polymer of glucose and is a mixture of oligosaccharide chains with different numbers of glucose sub-units connected in chains of variable length linked with α-1,4-glycosidic bonds. In our study, maltodextrin with a dextrose equivalent (DE) value of 6.4 was used. It contained only 6.4% of the reducing power of dextrose or glucose that had a DE of 100. The average degree of polymerization (DP) in maltodextrin was approximately 19 glucose sub-units (DE*DP = 120) (Kearsley and Dziedzic, 1995). Therefore, the transport of maltodextrin substrate used in this study was still dependent on the cleavage of the α-1,4-glycosidic linkages of high DP-maltodextrin to be low DP-maltodextrins (up to 7 glucose sub-units). In *Klebsiella* strains, the

disproportionation activity of the extracellular α-cyclodextrin-glucanotransferases (CGTases) is required to degrade maltodextrin (Bender, 1990). . Therefore, the higher substrate concentration allowed a longer time to break down high-DP maltodextrins. Thus, the longest chain of high-DP maltodextrins may be the last one utilized and gradually consumed by the KMS005 strain resulting in delaying 2,3-BD production.

4.3.5 Response Surface Methodology

Response Surface Methodology (RSM) was applied to minimize the distance of parameters to their optimum points. Box Behnken design was used to investigate the optimal levels of agitation speed, aeration rate, and substrate concentration for 2,3-BD production by the KMS005 strain from maltodextrin. In RSM, upper and lower levels of significant parameters were defined based on previous results from the conventional optimization. The level of each parameter and the design matrix are shown in Table 4.5. However, pH was not among the selectable parameters for RSM strategy. Table 4.5 represents all 13 experiment runs. By applying multiple regression analysis to the experimental data, the following second-order polynomial equation was obtained:

$$Y = -0.265 + 0.587X_1 + 0.00613X_2 - 0.00741X_3 - 0.00310X_1X_2 + 0.00189X_1X_3 + 1.03*10^5 X_2X_3 + 0.0647X_1^2 - 6.60*10^6 X_2^2 + 2.45*10^6 X_3^2,$$

where Y is the predictable gross yield of 2,3-BD, X_1 is the aeration rate, X_2 is the agitation speed, and X_3 is the maltodextrin concentration. From a model analysis (Table 4.6), the Model F-value of 23.61 implied the significance of the model. Values of Prob > F less than 0.0001 indicated the model terms are significant. In this case X_2, X_3, X_1X_2, X_2X_3, and X_2^2 were significant model terms ($P < 0.0001$). P-value above

0.0001 suggested that X_1, X_1X_3, X_1^2, and X_3^2 were not significant. Hence, the short model of quadratic equation was obtained:

$$Y = -0.265 + 0.00613X_2 - 0.00741X_3 - 0.00310X_1X_2 + 1.03*10^5 X_2X_3 - 6.60*10^6 X_2^2$$

Table 4.5 Experimental design of RSM strategy using three independent variables.

Runs	Aeration rate (vvm)	Agitation speed (rpm)	Maltodextrin concentration (g/L)	2,3-BD Gross Yield (g/g)[a] at 72 h		Biomass (g/L)
				Predicted	Observed	
1	0.8	350	150	0.427±0.025	0.438±0.020	4.4±0.2
2	1.0	250	250	0.149±0.025	0.203±0.020	3.2±0.3
3	1.2	350	150	0.393±0.025	0.430±0.010	4.6±0.1
4	1.0	350	200	0.354±0.025	0.355±0.016	4.2±0.3
5	1.2	350	250	0.337±0.025	0.348±0.014	4.5±0.1
6	1.0	250	150	0.345±0.025	0.364±0.006	4.1±0.1
7	1.0	450	250	0.347±0.025	0.341±0.002	4.6±0.1
8	1.2	450	200	0.279±0.025	0.263±0.004	4.8±0.3
9	0.8	350	250	0.296±0.025	0.277±0.008	3.9±0.3
10	0.8	250	200	0.180±0.025	0.216±0.001	3.4±0.2
11	1.0	450	150	0.337±0.025	0.306±0.005	4.9±0.1
12	1.2	250	200	0.307±0.025	0.302±0.011	3.8±0.3
13	0.8	450	200	0.399±0.025	0.431±0.001	4.9±0.2

[a] Gross yield is calculated as gram of 2,3-BD divided by gram of total sugar supply.

The fitness of the model was checked by the coefficient of correlation R^2, which was calculated to be 0.9341 showing that 93.41% of variability in the response could be explained by the model. An R^2 value higher than 0.9 was considered to have a very high correlation. The value of the adjusted determination coefficient (Adj R^2 =89.45%) was also satisfied to advocate for a high significance of the model. The

predicted R^2 of 0.8084 was in reasonable agreement with the Adj R^2 of 0.8945. The Adeq Precision measures the signal to noise ratio that should be greater than 4 (Khuri, 1998). Our ratio of 16.228 indicated an adequate and desirable signal. Therefore, this model can be used for navigating the design space.

Table 4.6 ANOVA summary.

ANOVA for Response Surface Quadratic Model

Analysis of variance table [Partial sum of squares - Type III]

Source	Sum of Squares	Df	Mean Square	F Value	p-value Prob > F	
Block	1.66E-06	1	1.66E-06			
Model	0.133641	9	0.014849	23.61415818	< 0.0001	significant
X_1-Aeration	0.00016	1	0.00016	0.253662887	0.6218	
X_2-Agitation	0.018301	1	0.018301	29.1043915	< 0.0001	significant
X_3-Substrate	0.03458	1	0.03458	54.99161255	< 0.0001	significant
X_1X_2	0.030785	1	0.030785	48.95692557	< 0.0001	significant
X_1X_3	0.002496	1	0.002496	3.969613577	0.0649	
X_2X_3	0.018489	1	0.018489	29.40303056	< 0.0001	significant
X_1^2	3.06E-05	1	3.06E-05	0.048640668	0.8284	
X_2^2	0.019856	1	0.019856	31.57723914	< 0.0001	significant
X_3^2	0.000131	1	0.000131	0.207642375	0.6551	
Residual	0.009432	15	0.000629			
Core Total	0.143075	25				

Std. Dev=0.025, C.V. %=7.55, R^2=0.9341, Adj R^2=0.8945, Pred R^2=0.8084, Adeq Precision=16.228.

The regression model is also reasonable to analyze the trends in the responses. Furthermore, the effects of aeration rate, agitation speed, and maltodextrin concentration on 2,3-BD production were also evaluated by the 3D response surface (Figure 4.2 a-c). The red surfaces in 3D structures corresponded with maximum responses of 2,3-BD concentration. Based on the model and the 3D plots, the optimal levels of aeration rate, agitation speed, and maltodextrin concentration were 0.8 vvm, 400 rpm and 15% (w/v), respectively. The predicted maximum 2,3-BD yield was calculated at the level of 0.45±0.025 g/g maltodextrin supplied. From the model, all three significant parameters provided an interaction effect on each other. Agitation speed had a great significant effect on the model. It also had interaction effects on the other two parameters, aeration rate and substrate concentration, to drive flow and mixing as well as optimum micro-aerobic conditions for 2,3-BD production yield in the KMS005 strain. Aeration rate and substrate concentration had no significance on their main effects or on their interaction with each other. Both of them had significance on the model by means of their interactions with agitation speed. This may imply that the difference in flow pattern of mixing caused by different agitation speeds and aeration rates reflected cell growth and 2,3-BD production yield by the KMS005 strain. Lee et al. (2013) claimed that aeration rate (3.5 vvm) and CLS concentration (45 mL/L) did not significantly affect the proportion of 2,3-BD. This supported our findings that no interaction effect between aeration rates (X_1) and substrate concentration (X_3) was found. Fortunately, our results found that the optimized aeration rate at 0.8 vvm fell in the range of 0.5-1.5 vvm. The rate is expected to be easily maintained in scaling up (Stanbury et al., 1995). The optimized substrate level at 15% (w/v) was estimated to be equivalent to glucose concentration

of 140 g/L. The concentration was appropriate to fermentation under batch condition, as suggested by Yu and Saddler (1983) who stated that final 2,3-BD concentrations were found to be highest for cultures grown on the initial glucose concentration of 150 g/L, particularly when the inoculum was first acclimatized to high sugar levels.

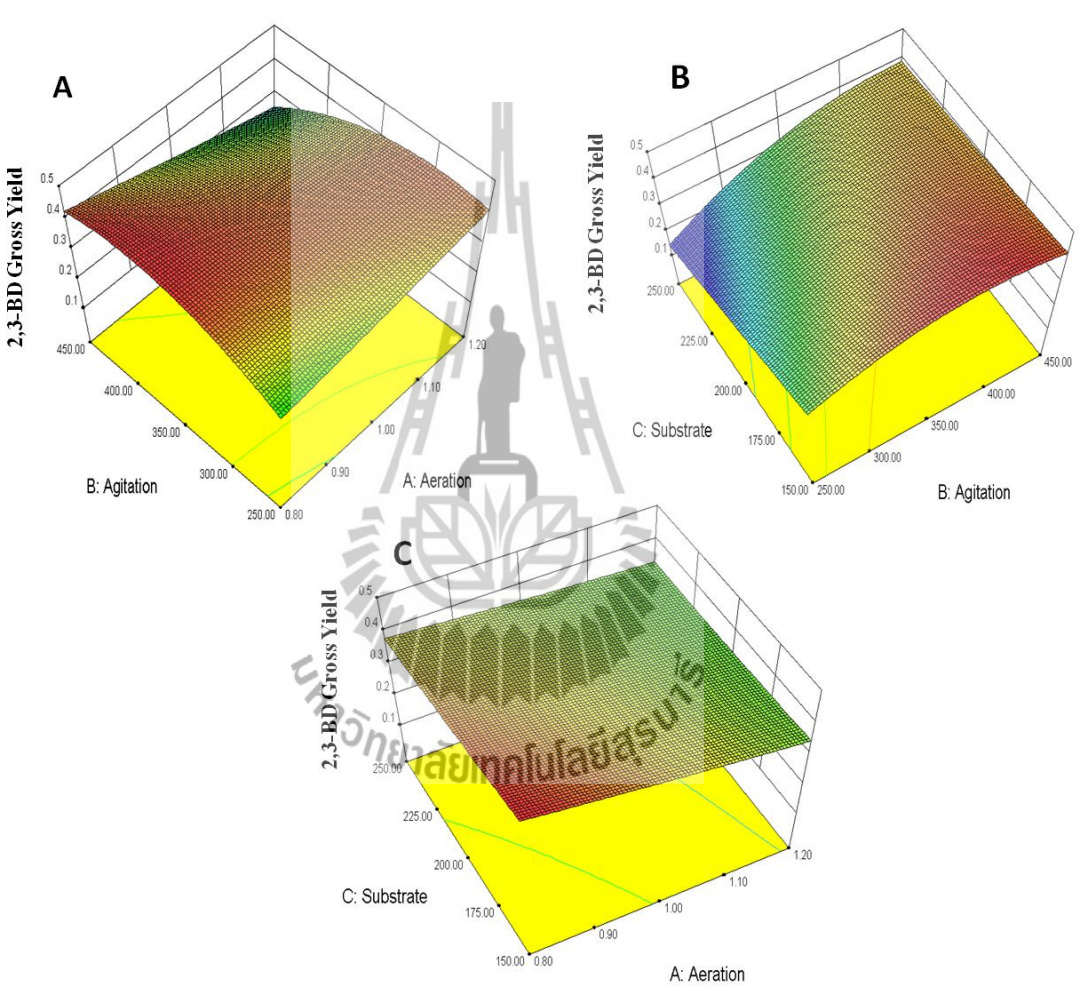

Figure 4.2 3D structures of optimum points of aeration rate, agitation speed, and substrate concentration: (A) the plot between aeration rate and agitation speed, (B) the plot between agitation speed and substrate concentration, (C) the plot between aeration rate and substrate concentration.

4.3.6 Validation of the mathematic model obtained from RSM strategy

To confirm the availability of the mathematic equation for predicting maximum 2,3-BD production yield, the experiment was carried out in batch fermentation in a 2 L fermenter to validate the model. Optimum 2,3-BD conditions were observed by the model at the agitation speed of 400 rpm, and aeration rate of 0.8 vvm with 150 g/L of maltodextrin. At 60 h incubation, 2,3-BD production yield at 0.433±0.002 g/g maltodextrin supplied was obtained (Figure 4.3). The validated yield of 2,3-BD obtained from the condition using optimized parameters was statistically acceptable and satisfactory compared with that of the predicted yield at 0.450±0.025 g 2,3-BD produced/g maltodextrin supplied calculated by the model. Interestingly, the optimum condition was able to achieve the yield of 0.433 g 2,3-BD produced/g maltodextrin supplied at as shorter incubation time (60 h) than that of other conditions (obtained at 72 h) (Table 4.5). Also, 2,3-BD production yield from optimized conditions was elucidated to be the best among other conditions, especially those from run 1, 3, and 13 (Figure 4.4).

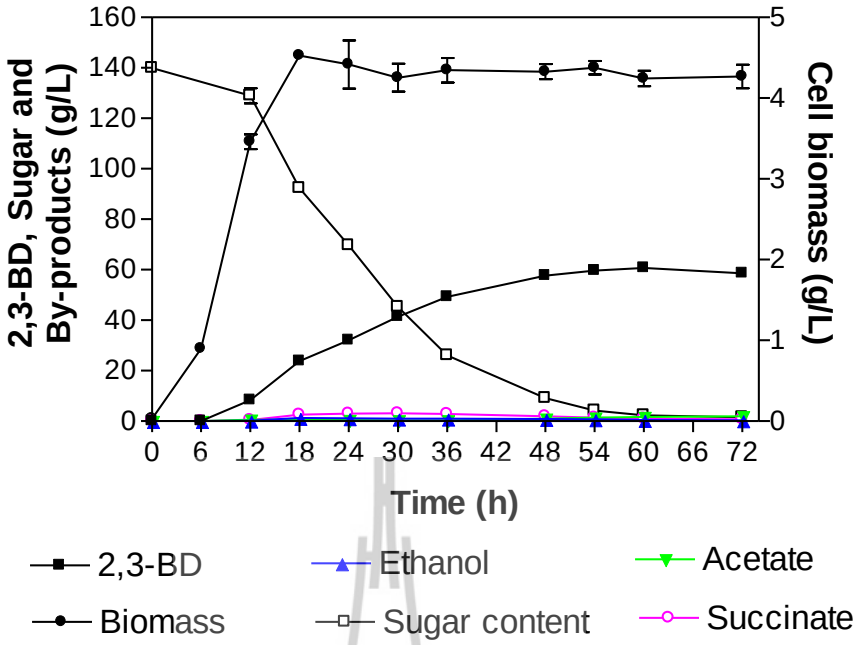

Figure 4.3 Batch fermentation profile for 2,3-BD production from maltodextrin performed under optimized agitation speed, aeration rate, and maltodextrin concentration.

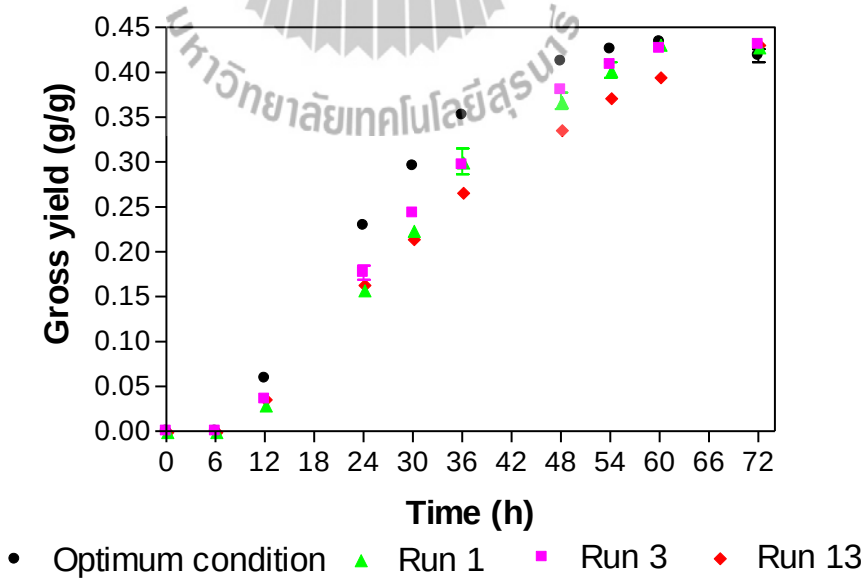

Figure 4.4 Gross yield comparisons obtained during fermentation.

Compared to the conventional method, RSM strategy provided advantages for fermentative 2,3-BD optimization process. The aeration rate was reduced from 1.0 to 0.8 vvm thus resulting in lowering the energy consumption for 2,3-BD production. Second, the production of 2,3-BD by the KMS005 strain from 150 g/L maltodextrin (48 h) in terms of concentration (50.4±2.4 versus 57.7±0.5 g/L), yield (0.360±0.017 versus 0.412±0.003 g 2,3-BD produced/g maltodextrin supplied) and productivity (1.05±0.05 versus 1.20±0.01 g/L/h) was significantly improved (Table 4.4 and Figure 4.3).

4.3.7 Fed-batch fermentation

The optimum condition in RSM was applied in the interim fed-batch fermentation. At 78 h incubation, 2,3-BD concentration, gross yield, and productivity were achieved at 88.1±0.2 g/L, 0.412±0.001 g 2,3-BD produced/g maltodextrin supplied, and 1.13±0.01 g/L/h, respectively. The by-products including succinate, ethanol, and acetate were at concentrations of 0.3±0.1, 0.5±0.1, and 0.8±0.2 g/L respectively (Figure 4.5). The concentrations of by-products under the fed-batch experiment were less than those obtained under batch fermentation. Considering dissolved oxygen, cells consumed oxygen until 48 h before the dissolved oxygen increased gradually until the end of fermentation. Since 2,3-BD became a mixed growth-associated product by the KMS005 strain, its incremental production of 2,3-BD concomitantly occurred in the log phase addition to the stationary phase. In wild type strains, all enzymes in 2,3-BD pathway are activated in the late log and stationary phases under oxygen limitation, and induced by acetate at low pH (Célinska and Grajek, 2009). Recently, Wong et al. (2014) stated that 2,3-BD production by

Klebsiella Zmd30 was also found to be growth-associated provoking lower productivity in fed-batch than that obtained in batch mode. This supported our findings which slightly decrease by 1.13 g/L/h of fed batch productivity was obtained.

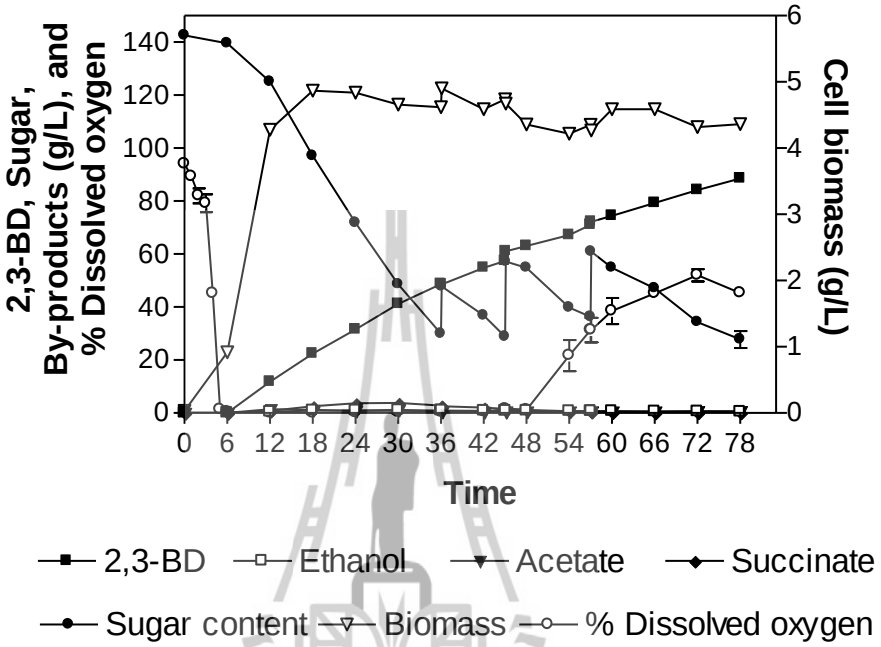

Figure 4.5 Fed-batch fermentation profile for 2,3-BD production from maltodextrin by KMS005 under optimum conditions.

Our study was the first to report the use of maltodextrin as a cheap carbon substrate for high production yield of 2,3-BD. Even though the product concentration and productivity of 2,3-BD in this study was not the highest level ever published (Table 4.7) due to the use of mineral salts medium that contained the least nutrients essential for bacterial growth. However, most previously published works on 2,3-BD production were performed in media composed of complex, rich, and expensive nutrients. Antibiotics were also supplied to maintain heterologous gene expression for activating 2,3-BD producing pathway (Banks, 1979; Song and Lee, 2006; Ma et al.,

2009). These led to high production of 2,3-BD in terms of titers and productivities, but they contributed to an increase in production costs including nutrients and chemical prices, and required additional steps of product recovery. However, simple mineral salts medium with less nitrogen sources and trace minerals, and transparently-pure maltodextrin used in our study are expected to reduce some obstacles in product recovery. Consequently, costs related to medium preparation and waste disposal also decreased. In addition, bio-based 2,3-BD production appears economically attractive because the estimated production cost was about $2.04/kg 2,3-BD produced, based on the results in our study. The cost included AM1 medium at $0.43/kg and maltodextrin at $0.69/kg. As the market price of glucose is about $1.0-1.50/kg, the fermentation cost for 2,3-BD production from maltodextrin would be cheaper than that derived from glucose. In addition, it is likely that the price of maltodextrin derived from cassava starch was cheap when no enzyme utilization in hydrolysis and pre-treatment step of the raw material were required in our process. Unlike maltodextrin, the price of cassava starch is sold in the market at $0.40/kg, much cheaper than maltodextrin (Jantama et al., 2015). However, high investment cost on enzymatic hydrolysis is required for saccharification steps. The prices of amyloglucosidase and α-amylase at costs of $7.57/kg and $3.50/kg (Jantama et al., 2015), respectively would contribute to higher production cost of 2,3-BD if cassava starch was utilized. Thailand is known as one of the world's leading countries for production and export of cassava starch (about 2 million tons annually). The use of cassava starch in the form of maltodextrin for 2,3-BD production is not concerned to be competitive for human consumption (FAOSTAT, 2011; Marcuschamer et al., 2011). Our total production cost of 2,3-BD was estimated in the range of $5.5 to $7/kg.

Compared with 2,3-BD production derived from chemical synthesis, the selling price in the market ranged from $9.12 to $19.77/kg (Ge et al., 2011). Therefore, our 2,3-BD selling price ($5.5 to $7/kg) is a solid benefit and makes it possible and alternative to bring the model development into industrial production scale. Further improvement of 2,3-BD production in terms of concentration and productivity is yet possible by means of the KMS005 strain development. The cost for the recovery of 2,3-BD from a model medium on a production scale of 500 tons per year by using single distillation, reverse osmosis followed by distillation and combination of distillation and extraction were estimated to be 0.73, 0.69 and 1.09 DM/kg 2,3-BD respectively (Sridhar, 1989).

Table 4.7 Comparison of 2,3-BD production by *Klebsiella* species from glucose-based medium and microorganisms.

Organism	Substrate/Medium/Condition	2,3-BD (g/L) Yield [g/g]	Productivity g/L/h	References
K. oxytoca KMS005	150 g/L maltodextrin (140 g/L of glucose), supplemented with AM1 mineral salt medium, pH6.0, 0.8 vvm, Fed batch, 78 h incubation, 37 °C, 400 rpm.	88.1 [0.412]	1.13 [a]	This study
B. licheniformis (Δ*gdh* and Δ*acoR*)	150 g/L glucose, supplemented with simple mineral medium, CSL, pH 6.0, 3 L/min and 350 rpm for the first 16 h, 1.5 L/min and 200 rpm for the rest, 70 h incubation, Fed batch, 37 °C.	98 [0.40]	0.94[a]	Qui et al., 2016
K. oxytoca M1/pUC18CM-budC	80-100 g/L glucose, supplemented with define medium, 5 g/L yeast extract 10 g/L casamino acid, 25 μg/ mL chloramphenicol, pH6.0, 1.0 vvm, Fed batch, 97 h incubation time, 30 °C, 400 rpm.	142.5 [0.42]	1.47 [a]	Cho et al., 2015
K. oxytoca NBRF4 (chemical mutation)	44 g/L glucose, supplemented with YP medium, pH 4.3, aeration 10% dissolved oxygen, batch, 18 h incubation, 38 °C, 200 rpm.	14.4 [0.32]	0.78	Han et al., 2013

[a] Productivity calculated under fed-batch.

Table 4.7 (Continued).

Organism	Substrate/Medium/Condition	2,3-BD (g/L) Yield [g/g]	Productivity g/L/h	References
K. oxytoca	90 g/L glucose, supplemented with a medium containing 5 g/L yeast extract, trace elements, pH 6.5, 1.0 vvm, batch, 30 h incubation, 37 °C without shaking.	30.0 [0.33]	1.15	Park et al., 2013
K. oxytoca ACCC 10370	Corncob hemicellulose hydrolysate (39.5 g/L xylose, 9.9 g/L glucose, 1.5 g/L arabinose, and 1.8 g/L acetate), supplemented with a medium containing 1.5 g/L yeast extract, pH 6, 0.3 vvm, batch, 48 h incubation, 37 °C, 300 rpm.	23.5 [0.46]	0.49	Cheng et al., 2010
K. oxytoca ME-UD-3 (Δ*ald*A)	200 g/L glucose, supplemented with a medium containing EDTA 0.05 M, pH 6.5, 1.0 vvm, fed-batch, 60 h incubation, 37 °C, 200 rpm.	130.0 [0.48]	1.63 [a]	Ji et al., 2010
K. oxytoca ME-UD-3	220 g/L glucose, supplemented with a medium, pH 6.0, aeration 1.0 vvm, batch, 81 h incubation, 37 °C, 200 rpm.	86.2 [0.39]	1.06	Nie et al., 2011
K. pneumoniae SDM isolated from soil	Total glucose utilized 168 g/L, supplemented with medium, pH 7.0, 1.5 vvm, fed-batch, 48 h incubation time, 37 °C, 500 rpm.	150.0 [0.43]	4.21[a]	Ma et al., 2009

[a] Productivity calculated under fed-batch.

4.4 Conclusion

To the best of our knowledge, this is the first study on 2,3-BD production from maltodextrin, an abundant and pure substrate derived from cassava. Fermentative operational parameters including pH, aeration rate, agitation speed, and substrate concentration were optimized. Strain *K. oxytoca* KMS005 is able to efficiently hydrolyze maltodextrin and produce 2,3-BD at the concentration and yield of 88.1 g/L and 0.412 g/g maltodextrin supplied, respectively, along with minor amounts of by-products within 78 h under optimum conditions in fed-batch operation. Meanwhile, the fermentation process was performed with regard to the reduction of total production cost by lowering the cost in fermentation and purification steps by employing simple mineral salts medium without additional complex nutrients, utilization of pure and abundant substrate derived from cassava rather than directly refined glucose, eliminating the enzymatic hydrolysis step, and taking advantages of using bio-catalyst downstream processing as less by-products formation. However, further study is required to improve titer, yield, and productivity to meet the commercial requirements of 2,3-BD production in the near future.

CHAPTER V

DEVELOPMENT OF MICRO-AEROBIC FED-BATCH FERMENTATION FOR 2,3-BD PRODUCTION BY METABOLICALLY ENGINEERED *KLEBSIELLA OXYTOCA* KMS005

5.1 Introduction

2,3-BD can be produced by anaerobic and facultative aerobic microorganisms. Oxygen supply is the most important parameter since the 2,3-BD pathway participates in regulation of the NADH/NAD^+ ratio in order to maintain the redox balance in the cells. Under not fully aerobic cultivation, lactate dehydrogenase, pyruvate-format lyase and α-acetolactate synthase acts upon pyruvate to generate lactate, formate and 2,3-BD. Besides, succinate, ethanol and acetate also present in the pathway (Figure 2.3).

The 2,3-BD is generally produced under microaerobic metabolism under slightly acidic pH (Celińska and Grajek, 2009). Several researches in micro-aerobic fermentation of 2,3-BD production have been performed to optimize oxygen supply by means of various methods including the oxygen mass transfer coefficient (k_La), the respiratory quotient (RQ), or the oxygen uptake rate (OUR). Among studies, oxygen supply was constant or varied during fermentation. Fages et al. (1986) attempted to use k_La to optimize the 2,3-BD production. High (R,R)-2,3-BD concentration of 44 g/L was obtained with a productivity of 0.79 g/L/h by programming k_La variation.

A two stage air supply strategy by varying agitation speeds and aeration rates was reported in previous works (Lee et al., 2013; Cho et al., 2015). For example, a two-stage agitation speed strategy was performed to supply different oxygen conditions for cell growth and 2,3-BD production based on the analysis of kinetic parameters controlled by a single agitation speed (Ji et al., 2009). RQ was also assigned to determine optimal oxygen supply. Zeng et al. (1994) controlled the process to obtain high 2,3-BD concentration around 96 g/L by employing an optimum RQ range between 4 and 4.5. Furthermore, Zhang at al. (2010) provided two stage oxygen supply based on changing RQ value for cell growth and 2,3-BD production phases from 1-1.5 to 1.8-2.0. In this work, to optimize oxygen supply, k_La was chosen since it depends on bioreactor's configuration, media composition, concentration and cell morphology (Tuffile and Pinho, 1970; Montes et al., 1999) as well as making scale-up possible.

In order to obtain a high final 2,3-BD concentration, high amount of sugar should be consumed, otherwise high initial concentration of sugar could be inhibitory. In previous studies, fed batch strategy was successfully superior compared with batch and continuous cultivations. It improved the final product concentration avoiding the effect of substrate inhibition by maintaining and supplying additional sugar at a relatively low level (Lee et al., 1999; Ezeji et al., 2004). Among fed batch strategies including pulse, constant feed rate, constant sugar residue concentration, and exponential fed-batch, the constant sugar residue concentration strategy was shown to be preferential for 2,3-BD production by *K. pneumoniae* (Nie et al., 2011). However, constant fed rate strategy is commonly considered as the simplest one because it does not require computer coupled with peristaltic pump, substrate sensors or other sophisticated equipments for detection. In our case, this last strategy was chosen.

The aim of this study was to improve the 2,3-BD production as well as to lower residual substrate at the end of fermentation by a metabolically engineered strain of *K. oxytoca* KMS005. The final objective is to use this strain to produce low cost 2,3-BD from maltodextrin derived from the hydrolysis of cassava in a minimal medium. Oxygen supply for optimum 2,3-BD production under microaerobic conditions was first optimized by varying k_La value under both constant and two stage microaeration. Then, several fed-batch fermentations were carried out in order to investigate optimal feeding strategy (initial substrate concentration, feeding starting, feeding rate).

5.2 Materials and methods

5.2.1 Microorganism

Metabolically engineered *K. oxytoca*, KMS005 was kindly provided by Jantama et al. (2015). The mutant designated, KMS005 was developed by deleting NADH competing pathways such as removals of *adhE* (alcohol dehydrogenase E), *ackA*/*pt*a (acetate kinase A/phosphotransacetylase), and *ldhA* (lactate dehydrogenase A). Luria-Bertani (LB) agar composed (per liter) of 5 g yeast extract, 5 g NaCl, 10 g peptone, and 20 g agar was used to store the strain at 4 °C.

5.2.2 Culture method

For seed preparation, KMS005 was cultured on a Luria-Bertani (LB) agar. The plate was incubated at 37 °C for 24 h. A full single loop of the freshly seed was inoculated into 250 mL Erlenmeyer flasks containing 60 mL LB medium. The inoculum was incubated at 37 °C, 250 rpm for 16-18 h. Culture media for fermentations was composed of a simple mineral salts medium, AM1 adapted from

Martinez et al. (2007) in which KCl was excluded and glucose concentration was estimated from our previous study in 2,3-BD production from maltodextrin.

5.2.3 Batch fermentations

Fermentations were carried out in 2 L bioreactors (BIOFLO 110, New Brunswick Scientific) containing 1 L of AM1 medium supplemented with 14% (w/v) glucose and seed with the inoculum LB broth at OD_{550} (optical density at 550 nm) of 0.1. The temperature was regulated at 37 °C. The pH of medium fermentation was maintained after being dropped to 6.0 by automatically addition of 3.0 M KOH. The inlet gas flow rate was set-up at 0.8 or 1 vvm with air flow meter. Agitation with two Rushton turbines was controlled at 250 or 400 rpm. The reactors were equipped with an automatic gas analyzer (SERVOMEX 4100) for on-line measurement of CO_2 and O_2 concentrations in the exit gas. Dissolved oxygen (DO) in fermentation was measured by dissolved oxygen probe (Ingold). Once a day, samples were taken for determination of cell dry weight, glucose, 2,3-BD, succinate, acetate and ethanol concentrations.

5.2.4 Fed-batch fermentations

A batch phase was first carried out in the same way as described above except for the initial sugar concentration which varied among the experiment (90, 120 or 140 g/L). Then, the fermenter was fed with a glucose stock solution (800 g/L of glucose in diluted AM1 medium (1:4 ratio)) at constant flow rate depending on the targeted glucose rate supply. The starting and duration of the feeding phase were varied with the experiments. Feeding rate was estimated based on sugar consumption rate in an optimum condition batch and was varied (1.25, 2.0, or 2.5 mL/h). The feeding rate at 2 g/h is equivalent to 2.5 mL/h in the study.

5.2.5 Analytical methods

5.2.5.1 Cells dry weight The optical density (OD) at 550 nm of 1 mL sample was measured by a spectrophotometer Spekol®1500. Cell mass was estimated from a linear equation derived from a plot of OD vs cell dry weight (CDW) as shown in Figure 5.1.

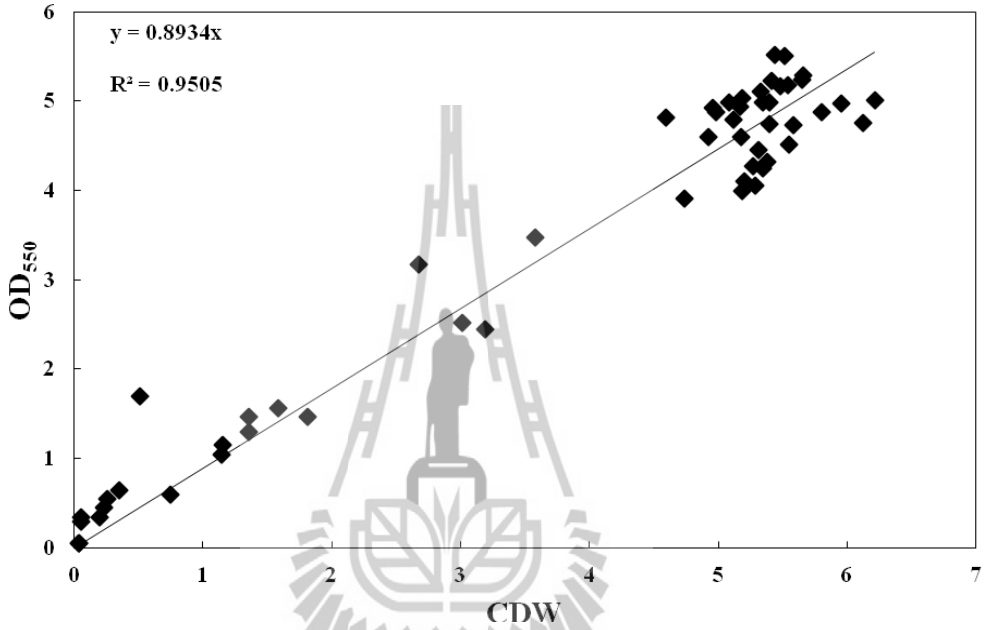

Figure 5.1 Interpolation of optical cell density at 550 nm and cells dry weight.

5.2.5.2 Glucose, 2,3-BD, acetate, succinate and ethanol concentrations Three mL of culture broth was centrifuged at 13,500 rpm for 4 min to separate cells and supernatant. The supernatant was filtrated through 0.2 μm filter membrane. Twenty five micro-liters of injection volume were automatically analyzed by HPLC (Thermo Scientific, France) equipped with a column, Rezex ROA organic acid H^+ (8%), 250*4.6 mm phase–reverse column (Phenomenex, France) thermostated at 30 °C and associated with a Refractive Index detector in series with a UV detector. The elution was performed at 170 μL/ min with an aqueous solution of sulphuric acid 10 mM.

5.2.5.3 Determination of volumetric oxygen mass transfer coefficient (k_La) The k_La values were estimated by a dynamic gassing-out method (Nielsen and Villadsen, 1994) by monitoring the increase in dissolved oxygen concentration in the reactor containing 1.0 L working volume of AM1 medium with 14% (w/v) glucose monohydrate without microorganism. Different couples of gas flow rate (between 0.8 and 1 vvm) and stirring speed (between 250 and 400 rpm) were tested.

5.2.5.4 Calculation of respiratory quotient (RQ) Gas balancing method was used to calculate OUR (oxygen uptake rate) and CPR (CO_2 production rate) from O_2 and CO_2 concentration in the outlet gas, under the hypothesis of well-mixed reactor and steady-state conditions (Garcia-Ochoa and Gomez, 2010). O_2 and CO_2 concentration in the inlet gas were supposed to be constant and equal to 21% and 0% respectively. Then, the respiratory quotient (RQ) was calculated as:

$$RQ = CPR / OUR \ (mol.mol^{-1})$$

5.2.5.5 Calculation of total consumed oxygen during batch fermentation Mass balance for the dissolved oxygen (DO) can be written as:

$$dC_L/dt = k_La \ (C_L^* - C_L) - OUR$$

where C_L is the concentration of dissolved oxygen in the liquid phase and C_L^* is the concentration of dissolved oxygen at saturation ($C_L^* = 8.10^{-3}$ g/L). Since C_L is constant and close to zero during fermentation, it can be written:

$$k_La \ (C_L^*) = OUR$$

So, the total amount of consumed oxygen was estimated as follow:

$$\text{Total } O_2 \text{ consumed (g)} = k_La \ .C_L^*. \ t. \ V$$

where V is the volume of the liquid phase and t the duration of batch experiment.

5.2.5.6 Carbon balances Quantities (Q in g) of glucose consumed and carbonated molecules produced (2,3-BD, acetate, succinate, ethanol, CO_2 and biomass) were calculated between the beginning and the end of fermentations. For biomass, calculation was done by supposing the average formula of *K. oxytaca* cell was eqivalent to $CH_{1.73}O_{0.43}N_{0.24}$ (Bommarius and Riebel, 2004). So, the percentage of carbon recovery was calculated as:

$$\%C \text{ recovery} = \text{sum } [n_i (Q_i / M_i)] / [6 (Q_{glucose} / M_{glucose})]$$

where index i referred to the products, n_i is equal to the number of carbon mole by mole of compound i, Q_i is the mass of compound i produced during fermentation, and M_i is the molecular weight of compound i. $Q_{glucose}$ is the quantity of glucose consumed during fermentation and $M_{glucose}$ is the molecular weight of glucose.

5.3 Results and discussion

5.3.1 Oxygen supply in batch fermentation

Oxygen transfer rate (OTR) is considered as the most important operating parameter for 2,3-BD production. Higher OTR mainly leads to high growth of cells, whereas limitation in oxygen increases 2,3-BD yield, although decreasing the overall production rate is due to a lower cell density. At extremely low oxygen level, equal molar amounts of 2,3-BD and ethanol are formed (Syu, 2001). Hence conditions for high 2,3-BD concentration and productivity are not the same as those for high yield. With these considerations, several oxygen supply strategies were tested in batch conditions. For the different conditions of aeration rate and stirring rate tested, the k_La values were measured (Table 5.1). The values of $k_La = 36.1\ h^{-1}$ was obtained at

400 rpm and 1.0 vvm; k_La = 25.2 h^{-1} was obtained at 400 rpm and 0.8 vvm and the value of k_La = 19.6 h^{-1} was obtained at 250 rpm and 0.8 vvm.

First two runs with one step aeration were carried out with low (B1L) and high (B1H) OTR (Table 5.1). For both experiments (Figure 5.2a and b), cell growth phase occurred until 24h. At the beginning of the experiments, the oxygen transfer was not a limiting factor as DO concentration was not equal to zero and specific growth rate was maximal. After 8 h, oxygen transfer became limiting and specific growth rate became linear meanwhile 2,3-BD production started (a mixed growth associated production). During the stationary phase (from 24 to 52 h), the 2,3-BD production was no more growth associated and productivity was lower than during cell growth phase (1.3 g/L/h during growth phase for both B1L and B1H against 1.0 g/L/h for B1L and 0.9 g/L/h for B1H during stationary phase). No significant difference could be observed in cell biomass production but there was significant in the final 2,3-BD concentration with the productivity between the two aeration strategies. For the stationary phase, the lower productivity observed for B1H could be explained by formation of more by-products and lower sugar consumed (Table 5.1). The RQ profiles were quite similar for both aeration conditions: they reached a maximal value during growth phase then started to decrease during oxygen limitation phase. However, for the higher aeration the RQ value was kept constant around 3.5 until the end of the growth phase. For both aerations the stationary phase was associated with a value of RQ between 1.0 and 2.0. It suggested that the difference in RQ value might depended on whether it is associated to growth (greater than 3 because of additional CO_2 production) or not (between 1.0 and 2.0).

Table 5.1 Comparison of the performance of batch fermentation for 2,3-BD production with different aeration strategies.

	k_La		Cell DW[a] (g/L)	2,3-BD Concentration (g/L)	Yield	By products[c]	Productivity At 52 h	Sugar[d] consumed	Carbon balance
					g/g[b]	g/L	g/L/h	(%)	%
	Growth phase	Stationary phase							
	0 - 24 h	24 - 52 h							
One-step aeration (B1)									
B1L (Low aeration)	25.2 h^{-1}	25.2 h^{-1}	4.9±0.1$^{\mu}$	49.4±3.0$^{e,\mu}$	0.37±0.01	2.4±0.7$^{\mu}$	0.95±0.06$^{\mu}$	93	96
B1H (High aeration)	36.1 h^{-1}	36.1 h^{-1}	4.6±0.2$^{\mu}$	45.1±2.0^{p}	0.37±0.01	3.2±0.2$^{\mu}$	0.86±0.04^{p}	85	93
Two-step aeration (B2)									
B2L (Low aeration)	25.2 h^{-1}	19.6 h^{-1}	5.1±0.3$^{\mu}$	41.0±5.5$^{\alpha}$	0.37±0.01	7.3±0.5^{p}	0.78±0.10$^{\alpha}$	87	99
B2H (High aeration)	36.1 h^{-1}	25.2 h^{-1}	5.0±0.4$^{\mu}$	50.2±0.1$^{\mu}$	0.37±0.01	4.7±0.3$^{\alpha}$	0.96±0.01$^{\mu}$	97	95

[a] At the end of growth phase

[b] Yield is calculated as gram of 2,3-BD produced divided by gram of glucose consumed

[c] By products = ethanol + succinic acid + acetic acid

[d] Initial sugar concentration: 140 g/L

[e] All data represent the averages of two fermentations with standard deviations. Values bearing different Greek symbol are significantly different ($P < 0.05$).

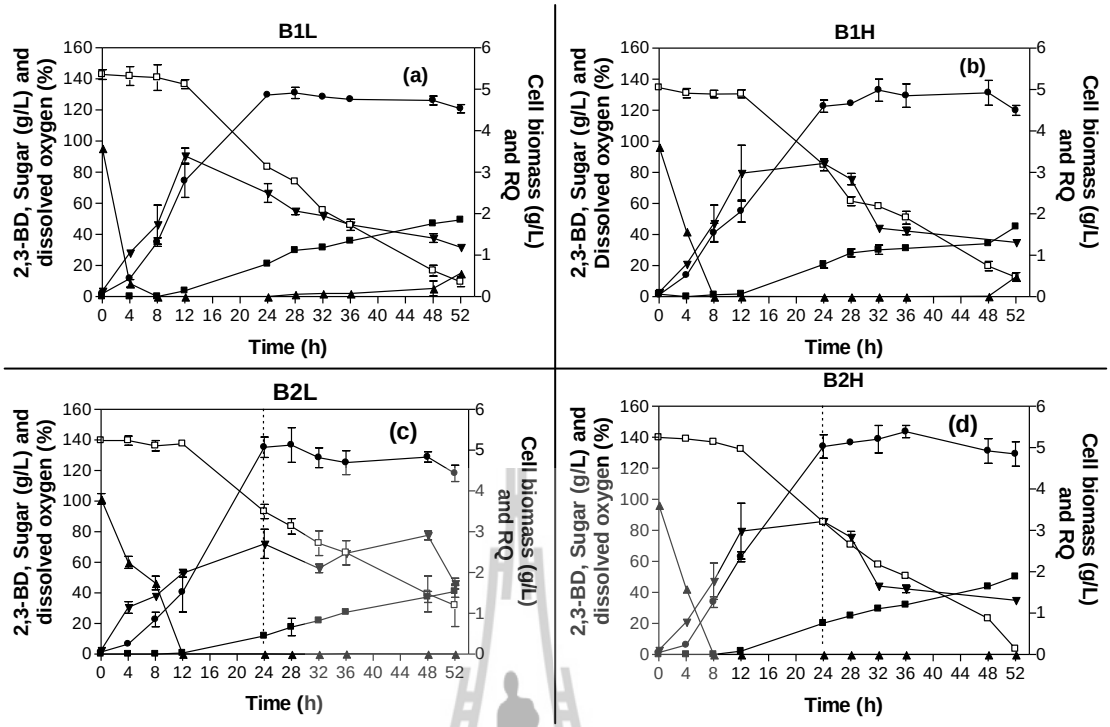

Figure 5.2 Time course of 2,3-BD production by *K. oxytoca* KMS005 during batch fermentation with different aeration strategies: one step aeration with (a) low aeration and (b) high aeration, and 2 stage aeration with (c) low aeration and (d) high aeration. The symbols represents: 2,3-BD (■), dissolved oxygen (▲), RQ (▼), cell biomass (●), sugar consumed (□) and oxygen supply change (…..).

Two other batches were carried out by decreasing the OTR during the stationary phase: B2L with lower aeration and B2H with higher aeration (Table 5.1). Low aeration resulted in a decreased 2,3-BD concentration compared with the one stage aeration and for the high aeration rate. These results showed that an optimal oxygen supply did not exit for the mixed growth-associated 2,3-BD production. In both cases the accumulation of by-products was increased during two phase's aeration batches. To compare the formation of these by-products for the 4 experiments, Figure

5.3 shows variations in final concentrations of 2,3-BD and by-products (acetate and succinate) as a function of total consumed O_2 during the experiment. According to these results, B1L appeared to be the optimal experiment by minimizing by-products formation for a high 2,3-BD concentration. For this experiment, OUR was equal to 6.3 mmol/L/h which was corresponded to a total oxygen consumption of 9.6 g. The optimum k_La at 25.2 h^{-1} obtained under 400 rpm and 0.8 vvm in our case was similar to the k_La at 26.7 h^{-1} obtained under 300 rpm and 1.0 vvm condition for cell growth by Ji et al. (2009). Our findings were also supported by previous reports on the effect of aeration change strategy for improvement of 2,3-BD production (Fages et al., 1986; Ji et al., 2009; Zhang et al., 2010). As shown in Table 5.1, B2H resulted in the high 2,3-BD concentration at 50.2 g/L, however, low initial aeration condition in B2L triggered a lower production of 2,3-BD by 41 g/L. Interestingly, the highest 2,3-BD concentration around 50.2 g/L was achieved under batch mode when total transferred oxygen supplied was from 9.5 to 11.7 g (Figure 5.3).

The RQ could reach the range varied from 3.5 to 4.5 at 12 h during the growth phase and between 1.0 and 2.0 during the stationary phase (Figure 5.2). Zeng et al. (1994) reported that optimum 2,3-BD production was observed at an RQ between 4.0 to 4.5 which was in a well agreement with the optimum RQ at 4.0 derived stoichiometrically from the bioreactions involved. However, Zhang et al. (2010) claimed that the RQ was separately controlled at 1.0-1.5 for cell growth and 1.8 to 2.0 for 2,3-BD production based on the stoichiometric calculation of sucrose metabolism under anaerobic or micro-aerobic condition. Zhang et al. (2010) used *S. marcescens* H30 to ferment sucrose to 2,3-BD in complex nutrient medium supplemented with peptone and yeast extract when our study employed *K. oxytoca* KMS005 to ferment

glucose to 2,3-BD in simple mineral salts medium. In addition, carbon balance confirmed an accuracy for the carbon distribution in the pathway of the strain KMS005 by converting 6 carbons of glucose to 2,3-BD, cell biomass, by-products and carbon dioxide (Table 5.1).

Our finding confirms that the strain KMS005 preferred an oxygen supply at the optimal level to drive 2,3-BD pathway and to maintain proper regulation of NAD^+/NADH inside the cells (Converti et al., 2003). The proper regulation was clarified by the low by-product concentrations obtained under appropriate microaerobic condition. The 2,3-BD metabolism by the strain KMS005 become partially growth associated product similar to what Wong et al. (2014) found. The 2,3-BD was produced at different rates during growth and stationary phases resulting in different productivities. These observations would be explained and come up with understanding of enzymatic pathways via the mixed acid fermentation in wide type and genetically engineered strain KMS005. Strains of *Klebsiella* spp. are such a group of microorganisms growing and obtaining energy by respiratory and fermentation. When under excessive oxygen supply, the only products are CO_2 and biomass. However, if employed under anaerobic conditions, 2,3-BD and other pathways serve as products during fermantation. Since the strain KMS005 was developed by gene deletions of alcohol dehydrogenase, acetate kinase-phosphotransacetylase, and lactate dehydrogenase, there are only small amounts of by-products produced such as succinic acid, acetic acid and trace of ethanol (Jantama et al., 2015). But when the transferred oxygen amount was increased over 9.5 g, acetate formation was increased in both B1H and B2H. Syu (2001) also reported that further increase in O_2 availability causes higher production of acetic acid. Furthermore, Jantama et al. (2015) claimed

that the production of acetic acid were still detected even though, acetate kinase-phosphotransacetylase genes had been successfully deleted in the strain KMS005. This result might postulate the activation of other acetate-producing pathways instead. Abdel-Hamid et al. (2001) claimed that pyruvate oxidase (encoded by *poxB*) was necessary for cell survival during the stationary phase under micro-aerobic conditions (Figure 5.1). Therefore, it implies that POXB compensated PTA-ACKA activities and over oxygen demand stimulated this pathway of acetic acid production in the KMS005 strain.

Unlike acetate, succinate was sharply increased when transferred oxygen amount was lowered to 8.5 g but it kept minor constant when the amount of total oxygen consumption was increased from 9.5 to 13.7 g. It seems that the more aeration increased, the more succinate decreased. The first possibility is that minor succinate detection in KMS005 was resulted from considerably low and constant activity of malate dehydrogenase (MDH), responsible for succinate production from oxaloacetate in TCA cycle under micro-aerobic conditions (Lenvanon et al., 2005a; 2005b). The cycle provides precursors of certain amino acids as well as the reducing agent NADH for cell growth. The second possibility is likely to involve with an activity of putative FNR protein to regulate transcription level of functional genes in 2,3-BD producing pathway. FNR binding site appeared at position-6 was found in an operon, known as *budABC*. The operon exists a cluster of genes encoding for α-acetolactate synthase, α-acetolactate decarboxylase, and acetoin reductase. FNR also induces the genes involved in succinate production anaerobically (Guest et al., 1996). In the absence or minor amount of oxygen (lower than 8.5 g), FNR might down-regulate the expression of *budABC* genes resulting in the highest production of succinate. By increasing

transferred oxygen amount until optimum micro-aeration (9.5 g), more oxygen stimulates α-acetolactate synthase while FNR was down-regulated resulting in low gene expression for succinate producing pathway. However, aerobically supply (more than 11.7 g oxygen amount) simulates acetate producing pathway and declines 2,3-BD production even if succinate route is less active. NAD^+/NADH balance and energy conservation derived from NADH oxidation in KMS005 might be catalyzed by NADH dehydrogenase I (NdhI). The enzyme is found during microaerobic fermentation, in the late log and stationary phase of cell growth and during carbon limitation for essential fumarate respiration (Tran et al., 1997). Albeit, NdhI participated in other fermentative pathways such as succinate and ethanol, a huge amount of NADH reoxidation was expected through 2,3-BD producing pathway. Hence, it was transparent to demonstrate the important role of the optimum micro-aeration to maximize 2,3-BD, to minimize by-products and to regulate NADH/NAD^+ ratio.

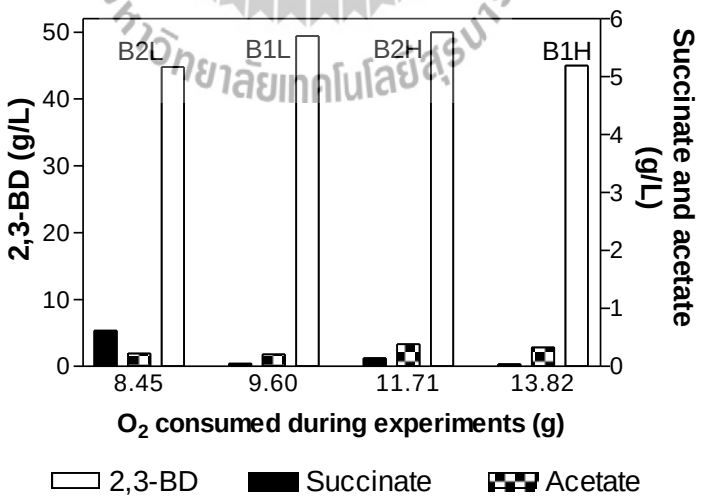

Figure 5.3 Effect of oxygen consumption on final concentrations of 2,3-BD, succinate and acetate during batch fermentation.

5.3.2 Sugar supply: fed-batch fermentation

A series of fed-batch experiments was carried out with the objective to increase the final 2,3-BD concentration in comparison to B1L batch experiment, by extending the stationary phase beyond 52 h. During all these experiments, aeration was constant (k_La = 25.2 h^{-1}) and sugar feeding rate, feeding time and initial sugar concentration varied (Table 5.2).

First experiment (FB-S140-2.5=Fed batch at initial substrate concentration of 140 g/L and feeding rate at 2.5 mL/h) was similar to batch B1L except that after 48 h, sugar started to be fed to the culture at a flow rate of 2 g/h (2.5 mL/h of stock solution of 800 g/L of glucose) which corresponded to the sugar consumption rate observed in batch B1L during stationary phase (calculated between 24 and 48 h). As can be seen from Figure 5.4a, the production of 2,3-BD was similar to Batch B1L until 48 h. After sugar feeding, the 2,3-BD production rate was maintained at 0.8 g/L/h until 76 h meanwhile sugar and biomass concentration were quite constant. After 76 h, a decrease was observed in 2,3-BD production rate (0.4 g/L/h). At the same time sugar concentration increased as well as dissolved O_2 concentration (data not shown), indicating a decrease in the biomass formation. Consequently, the global productivity was lower than that observed in batch experiment whereas final concentration and yield of 2,3-BD were higher (Table 5.2).

In the second fed-batch FB-S140-2.5b, sugar feeding began earlier (at 36 h) and the flow rate was decreased when sugar concentration increased (Table 5.2). This resulted in a higher production of 2,3-BD during the first part of feeding (until 75 h) and so a higher final concentration (77 g/L) comparing with FB-S140-2.5. However the global productivity was still lower because of a decreasing activity of the cells

after 75 h. By calculating the global yield at time 75 h, when final concentration was similar than that obtained in FB-S140-2.5 (65 g/L), the productivity was 0.88 g/L/h. Besides, residual sugar concentrations in these two fed-batch were very high (Table 5.2) and could decrease the efficiency of the down-stream separation step. For the third fed-batch (FB-S140-1.25), sugar feeding rate was decreased to 1.25 mL/h to avoid substrate accumulation in the reactor and started at 36 h.

Table 5.2 Comparison of the performance of fed-batch fermentation for 2,3-BD production with different sugar feeding strategies.

	Max CDW (g/L)	Initial sugar (g/L)	Feeding rate (mL/h)	Yield (g/g)[a]	2,3-BD concentration (g/L)	Productivity (g/L/h)	Residual sugar (g/L)	By-products[b] (g/L)
FB-S140-2.5	4.5	140	2.5 (48-120 h)	0.45	65.3	0.56	67.3	3.5
FB-S140-2.5b	5.3±0.2	140	2.5 (36-75 h) 2.0 (75-100 h) 1.25 (100-120 h)	0.48±0.04	77.0±0.3	0.54±0.01	77.1±12.4	4.0±0.3
FB-S140-1.25	4.7	140	1.25 (36-120 h)	0.39	60.4	0.51	17.9	3.3
FB-S90-2.5	6.1	90	2.5 (24-76 h)[c]	0.38	66.6	0.65	4.9	4.0
FB-S120-2.5	5.2	120	2.5 (20-68 h)[d]	0.5	74.7	0.64	32.4	5.2

[a] Yield is calculated as gram of 2,3-BD produced divided by gram of glucose consumed

[b] By-products = ethanol + succinic acid + acetic acid

[c] Fermentation time from 76-100 h without sugar feeding

[d] Fermentation time from 68-116 h without sugar feeding

Between 36 and 56 h, production rate was lower than that observed in FB-S140-2.5b during the same period (0.66 g/L/h for FB-S140-1.25 and 0.9 g/L/h for FB-S140-2.5b). After 56 h, sugar concentration was constant at 20 g/L (Figure 5.4a) and production rate decreased to 0.25 g/L/h at 120 h. This suggested that production rate could be increased at higher sugar concentration. It could be concluded from these 3 fed-batch results that a good yield and 2,3-BD concentration could be obtained but with a high residual sugar concentration. The low productivity was due to the decrease of production rate with time.

The two last fed batches (FB-S90-2.5 and FB-S120-2.5) were performed with the aim to decrease residual sugars. Two strategies were proposed to obtain different sugar concentrations during the feeding phase (about 20 g/L for FB-S90-2.5 and 50 g/L for FB-S120-2.5). The feeding phases were shortened (52 h for FB-S90-2.5 and 48 h for FB-S120-2.5) and started at the end of the growth phase. Then a second batch phase was performed at the end of feeding to exhaust residual sugar. 2,3-BD and sugar concentrations versus time are presented on Figure 5.5. During the feeding phase, 2,3-BD production rate was 1.0 g/L/h for residual sugar concentration of 50 g/L (FB-S120-2.5) and 0.72 g/L/h for residual sugar concentration of 20 g/L (FB-S90-2.5). Then production rate decreased to 0.69 g/L/h for FB-S120-2.5 and 0.4 g/L/h FB-S90-2.5 during last batch phase. For FB-S90-2.5, sugar was exhausted (< 5 g/L) after 116 h whereas residual sugar concentration of 32 g/L was found in FB-S120-2.5. So FB-S120-2.5 gave the best result in term of yield, 2,3-BD concentration and productivity.

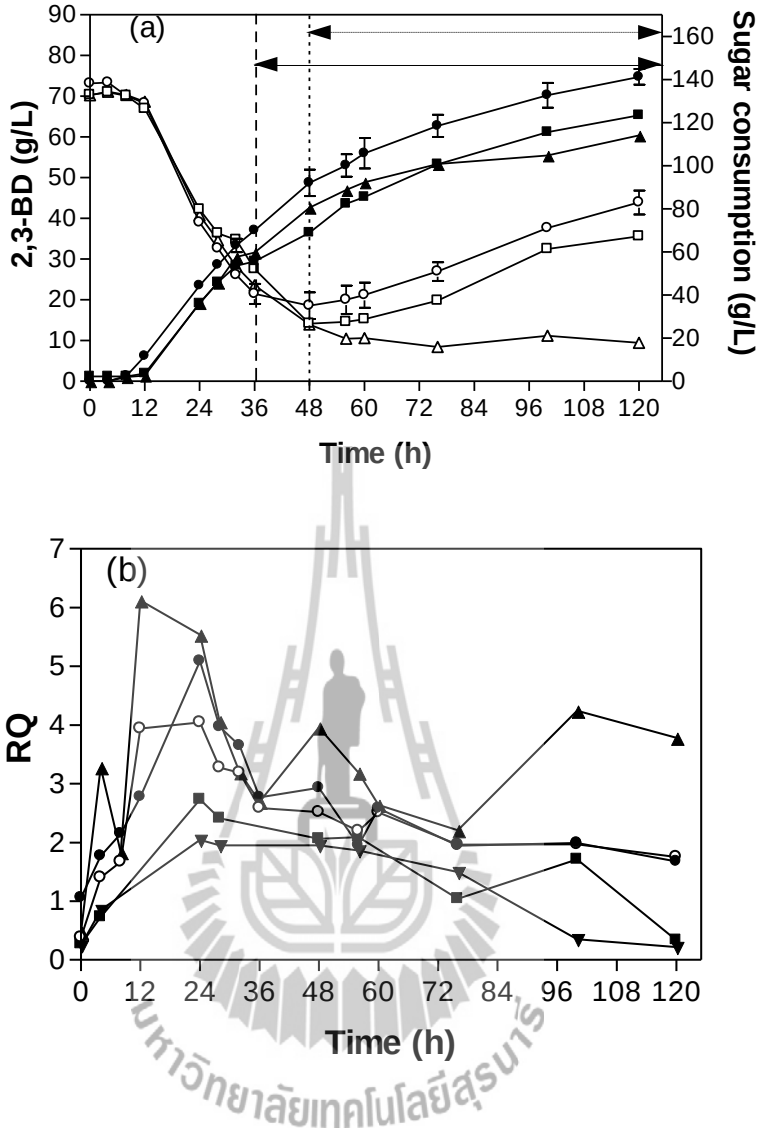

Figure 5.4 Fermentation profile of (a): 2,3-BD and sugar concentrations, and (b): RQ during fed-batch fermentation with initial sugar concentration of 140 g/L. The symbols represent (a): 2,3-BD for FB-S140-2.5 (■), FB-S140-2.5b (●), and FB-S140-1.25 (▲); sugar consumption for FB-S140-2.5 (□), FB-S140-2.5b (○), and FB-S140-1.25 (Δ); feeding time for FB-S140-1.25 and 2.5 b (◄——►); feeding times for FB-S140-2.5 (◄·····►). The symbols represent (b): FB-S140-2.5 (○), FB-S140-2.5b (▲), FB-S140-1.25 (●), FB-S90-2.5 (▼), FB-S120-2.5 (■).

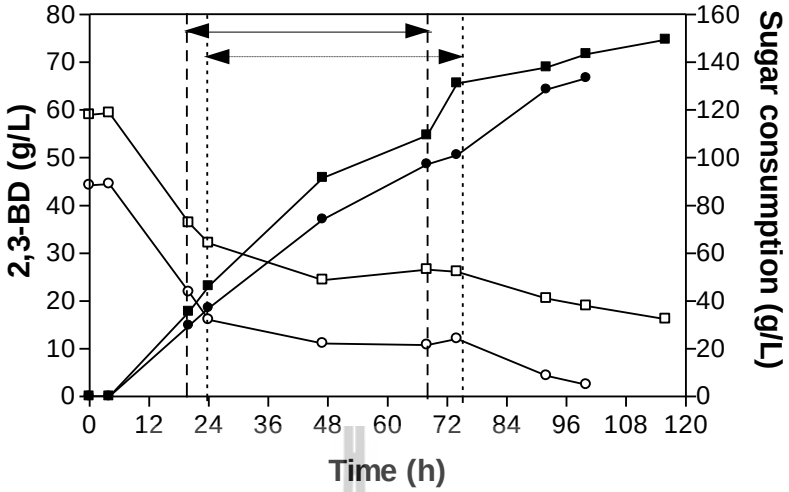

Figure 5.5 Fermentation profile of 2,3-BD and sugar concentrations during fed-batch fermentation with varied initial sugar concentration and feeding time. The symbols represent as 2,3-BD for FB-S90-2.5 (●), and FB-S120-2.5 (■); sugar consumption for FB-S90-2.5 (○) and FB-S120-2.5 (□); feeding period for FB-S90-2.5 (◄·····►), and FB-S120-2.5 (◄——►).

These results might be linked to the modification of the 2,3-BD metabolism in the modified strain changed to the mixed growth associated production (Wong et al., 2014). Maximum biomass at 6.1 g/L was obtained in FB-S90g/L, the highest cell concentration among all fed-batches. Garge and Jain (1995) reported that the most commonly used initial sugar concentrations were in the range of 5 to 10%. The range could be changed depending on type of substrates, inhibitory compounds present and media (Célinska et al., 2009). Remarkably, slightly increasing in acetate and higher biomass concentration were also detected in FB-S90g/L and FB-S120g/L (Table 5.2) suggesting that feeding substrate-medium in exponential phase might cause more activation of pyruvate oxidase (PoxB) for good growth under microaerobic condition

(Abdel-Hamid et al., 2001; Jantama et al., 2015). The greater 2,3-BD production rate when the sugar concentration in the medium was maintained at 50 g/L (1 g/L/h) rather than 20 g/L (0.72 g/L/h) could be due to a shift of the metabolic ways from growth to 2,3-BD production regulated by the sugar level. Our 2,3-BD concentration was lower than other previous reports (Ji et al., 2010; Cho et al., 2015), however, it was higher than some using *K. oxytoca* to produce 2,3-BD (Han et al., 2013; Jung et al., 2013). Under fedbatch mode, the concentration of 2,3-BD was increased with more accumulation of by-products (Table 5.2). This circumstance was found in the research of Cho et al. (2015) in which 15.5 g/L of by-products (acetate, ethanol and succinate) were produced with 142.5 g/L of 2,3-BD. Nevertheless, the previous works are conducted with supplementation of EDTA, yeast extract and casamino acid in media which increased the fermentation cost and more steps in purification process. Unlike the expensive complex nutrients, simple mineral salts medium with less nitrogen sources and trace metals used in our study are expected to reduce some obstacles in product recovery (Song and Lee, 2006). Consequently, costs related to medium preparation and waste disposal would be decreased. Also, the study might guide for the further improvement of simply feeding strategy for 2,3-BD production.

5.4 Conclusion

2,3-BD production under microaerobic condition was studied and optimized based on oxygen mass transfer coefficient (k_La) and respiratory quotient (RQ) to maximize our product and minimize byproduct formations. Employing constant optimum k_La 25.2 h^{-1} and reaching RQ value around 3.5-4.0 during growth phase and between 1.0 and 2.0 during stationary phase, high 2,3-BD concentration around 50

g/L with a productivity of 0.95 g/h/L was achieved along with low byproducts formation. Under constant feeding rate of 2 g/h, fed batch enhanced 2,3-BD production to reach 74.7 g/L starting the feeding at the end of growth phase and maintaining a sugar concentration in the medium around 50 g/L during the feeding period.

CHAPTER VI

SCALE UP PRODUCTION PROCESS FOR 2,3-BD FROM MALTODEXTRIN BY METABOLICALLY ENGINEERED *KLEBSIELLA OXYTOCA* KMS005

6.1 Introduction

Manufacturing of bulk precursors derived from bio-based chemicals was broadening the knowledge from laboratorial to industrial scale. Biotechnological 2,3-BD production have been developed in the past decades. Proper understanding of the 2,3-BD pathways and genetic approaches had been studied and well known (Magee and Kosaric, 1987; Caspi, 2008). Along with the improvement of molecular genetic engineering, fermentation process including optimization of medium components and of physical parameters (pH, temperature, and oxygen supply) were studied recently (Ma et al., 2009; Wong et al., 2014; Cho et al., 2015). Although the microbiological aspects of oxygen on the microaerobic culture of 2,3-BD pathway are broadly studied, a few studies on the reactor design and scale up of the process were reported. Zeng et al. (1994) achieved to use respiratory quotient (RQ) as the control parameter for optimizing production of 2,3-BD in both laboratory and pilot scales up to 1,500 L. Yang et al. (2012) scaled up the process to 30 L bioreactor by maintaining agitation speed and aeration rate as same as smaller tank conditions. The catalog scale of bioreactors was classified as laboratory ranging from shake flasks to 30 L, pilot plant

ranging from 100 to 19,000 L and industrial scale from 10,000 to 500,000 L (Junker, 2004; Papagianni, 2011). To simplify and commercialize the 2,3-BD fermentation process, our previous work employed abundant substrate as maltodextrin and low cost simple mineral salts medium as AM1 under an optimum condition programmed by response surface methodology (RSM). The optimum condition included agitation speed, aeration rate and maltodextrin concentration at 400 rpm, 0.8 vvm and 150 g/L substrate concentration, respectively, using 2 L stirred bioreactors. It is beneficial to scaling up the process since the fermentation cost-effectiveness was estimated at 2.04$/kg of 2,3-BD in laboratory scale. Common problems related to scaling-up were caused by reduced mixing quality and enhanced stress exposure (Schmidt, 2005). The changed geometric and physical conditions in larger scales lead to be a less favorable mixing behavior and to be impaired physiological reaction conditions thus decreasing specific yields and increasing unwanted side products as well as product quality. A controlled and optimum oxygen supply is critical for biological 2,3-BD process favorable microaerobic condition. This reflects the importance of parameter for oxygen supply's strategy appropriated to micro-aeration.

In this study, optimum agitation speed at 400 rpm obtained from 2 L bioreactor was scaled up based on impeller diameter of larger scales of 10, 90 and 300 L. Effect of changed configurations between laboratory and pilot scale was investigated while effect of inoculum size was studied to improve seed quality in pilot scale. Production of 2,3-BD in scale up process by *K. oxytoca* species using maltodextrin a feedstock derived from abundant and cheap cassava, has been the first ever study.

6.2 Materials and methods

6.2.1 Microorganism and media

Metabolically-engineered *K. oxytoca* KMS005 (Δ*adhE*-Δ*ack-pta*Δ*ldhA*) was previously constructed (Jantama et al., 2015). Maltodextrin was purchased from Nakhon Ratchasima, Thailand. The percentage of solid content in maltodextrin is approximately 84% (w/w) and its dextrose equivalent (DE) is a simple mineral salts medium (4 g/L of salts), AM1 (Martinez et al., 2007) was used as a fermentative medium throughout this study. Luria-Bertani (LB) agar was used for maintaining bacterial cultures.

6.2.2 Seed preparation

For seed preparation, KMS005 was cultured on a Luria-Bertani (LB) agar. The plate was incubated at 37 °C for 24 h. A full single loop of fresh colonies was inoculated into 250 ml Erlenmeyer flasks containing 60 mL LB medium. The inoculum was incubated at 37 °C and 200 rpm for 16 h. To study the effect of inoculum, agitation speed and incubation time were varied to investigate the cell stage and cell density for seed quality.

6.2.3 Scale up process by agitation speed variation

Scale up process was performed based on fermentation configuration including scales, tank diameters, and impeller diameters (Table 6.1). All scales were conducted with 50 % working volume. The seed culture was inoculated in AM1 medium supplemented with maltodextrin at the concentration equivalent to 0.033 g/L dry cell weight (OD_{550}= 0.1). Fermentation experiments were carried out at 37 °C in a 2 L scale bio-reactor with a working volume of 1 L. The fermentation broth was supplied with sterile air at 0.8 vvm and controlled at 400 rpm. The pH of the medium

was constantly maintained at 6.0 by the automatic addition of 10 M KOH. The percentage of dissolved oxygen was also measured by dissolved oxygen (DO) probe.

The 2 L stirred tank reactor had stirrers with two six flat-blade Rushton turbine impellers when 10, 90 and 300 L reactors had that with three six flat-blade turbines. Physical scale up parameter is agitation rate to be optimized and to obtain appropriate oxygen supply, heat transfer and mixing behavior. Various agitation speeds were calculated based on scaling up criterion such as impeller Reynolds number (R_{ei}), constant tip speed (N_iD_i), constant volumetric power input $\left(\frac{p}{v}\right)$ and constant stirred speed (N_i). An optimum agitation speed in a smaller scale was selected for further scale up process. Each equation of physical scale-up of agitation speed was mentioned as

The constant Reynolds number (R_{ei}) as $\frac{\rho_l D^2{}_{i,1} N_{i,1}}{\mu_l} = \frac{\rho_l D^2{}_{i,2} N_{i,2}}{\mu_l}$

where $R_{ei,1}$ is the constant Reynolds number in smaller scale and can be found from the graph (see in Appendix); ρ_l is density of the medium; μ_l is dynamic viscosity; $D_{i,1}$ impeller diameter in small scale; $D_{i,2}$ is impeller diameter in large scale; $N_{i,1}$ is stirrer speed in small scale; $N_{i,2}$ is stirrer speed in large scale.

The constant tip speed (N_iD_i) as $N_{i,1}D_{i,1} = N_{i,2}D_{i,2}$

where $D_{i,1}$ is impeller diameter in small scale; $D_{i,2}$ is impeller diameter in large scale; $N_{i,1}$ is stirrer speed in small scale; $N_{i,2}$ is stirrer speed in large scale.

The constant volumetric power input $\left(\frac{p}{v}\right)$ as $\left(\frac{p}{v}\right)_1 = \left(\frac{p}{v}\right)_2$ or $N^3{}_{i,1}D^2{}_{i,1} = N^3{}_{i,2}D^2{}_{i,2}$

where $\left(\frac{p}{v}\right)_1$ is volumetric power input is small scale; $\left(\frac{p}{v}\right)_2$ is volumetric power input in large scale; $D_{i,1}$ is impeller diameter in small scale; $D_{i,2}$ is impeller diameter in large scale; $N_{i,1}$ is stirrer speed in small scale; $N_{i,2}$ is stirrer speed in large scale.

The constant stirred speed (N_i) of larger fermenter is kept at constant rate of smaller tanks.

The scale up equations were used to optimize agitation speeds in 10 and 90 L tanks when the optimized agitation speed in 2 L tank was obtained from RSM and one in 300 L tank was from optimum condition selected from 90 L tank (Table 6.2). Laboratory tanks were designed by SARTORIUS BIOSTAT model 338, Germany (Figure 6.1). Pilot tanks were belonged to B.E.MARUBISHI model MPF-U_3, Japan (Figure 6.2).

Table 6.1 Configuration of fermenters in laboratorial and pilot scales.

Scale (L)	Tank inner diameter (mm)	Impeller diameter (mm)	Impeller distance[a] (mm)	Speed range (rpm)	Air flow (L/min)[b]	Motor power (kw)
2	128	51	25.5	200-500	0.8	0.09
10	190	69	34.5	218-400	4	0.09
90	400	160	80	55-295	36	2.2
300	650	296	148	70	120	3.7

[a] Distance between the air sparger to the impeller.

[b] Air flow was calculated based on an optimum aeration rate at 0.8 vvm.

Table 6.2 Various agitation speeds based on scaling up criterion.

Scales (L)	Agitation speed (rpm)
2	400(optimum)
10	Constant N_i = 400
	Constant $\left(\frac{p}{v}\right)$=326
	Constant N_iD_i=295 (optimum)
	Constant R_{ei}=218
90	Constant N_i=295
	Constant $\left(\frac{p}{v}\right)$=170
	Constant tip N_iD_i=130 (optimum)
	Constant R_{ei}=55
300	Constant N_iD_i=70

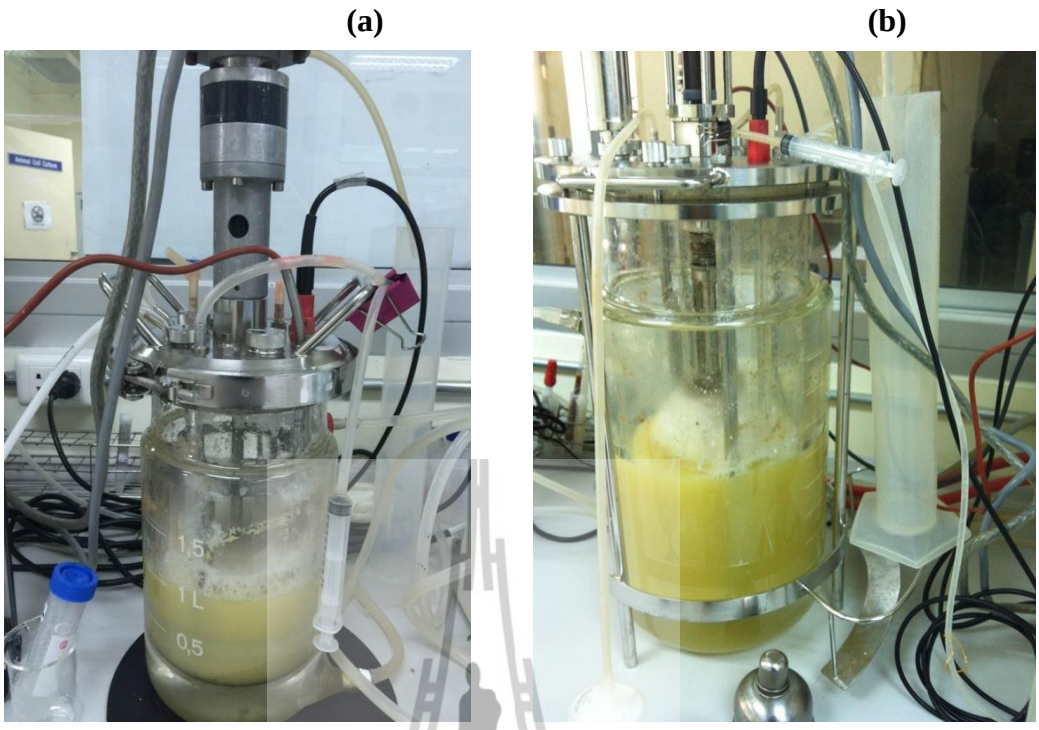

Figure 6.1 Laboratory scale of (a) 2 L and (b) 10 L tanks.

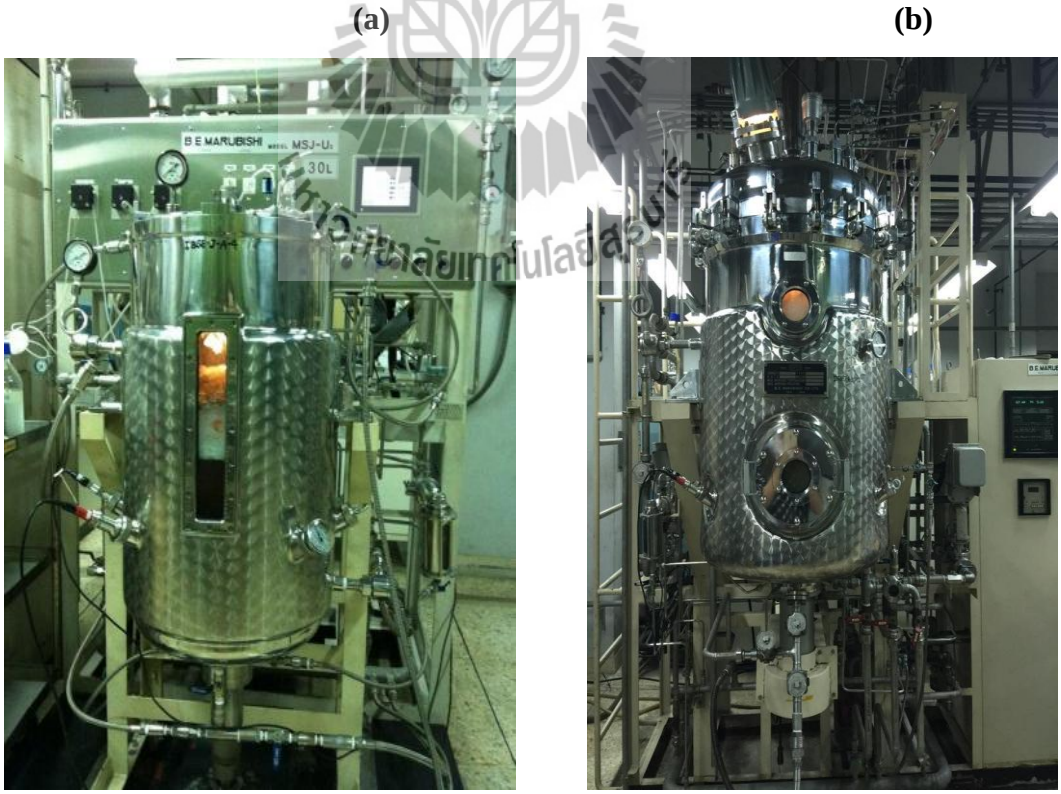

Figure 6.2 Pilot scales of (a) 90 L and (b) 300 L fermenter tanks.

6.3 Results and discussion

6.3.1 Effect of various agitation speeds in 10 L fermenter

Agitation speed was used as a parameter to control dissolved oxygen concentration throughout which aeration was fixed at 0.8 vvm in every scale. Under 10 L scale, agitation speed at 295 rpm (N_iD_i) resulted in the highest 2,3-BD concentration, yield and productivity at 53.8 g/L, 0.40 g/g of maltodextrin consumed and 1.12 g/L/h (Figure 6.3). There were similarities of fermentation profiles in 2 L (400 rpm) and 10 L (295 rpm) scales such as cell growth, 2,3-BD concentration, sugar consumption, dissolved oxygen concentration and by-product formations (Figure 6.4 and Table 6.3). It was noticed that the highest and lowest agitation speeds at 400 and 218 rpm resulted in lower 2,3-BD production whereas agitation rates at 295 and 326 rpm resulted in higher 2,3-BD production. Hence, agitation speed, 295 rpm, was selected as the optimum parameter for further scale-up process in pilot tank due to slightly higher 2,3-BD production and lower energy consumption compared with agitation speed at 326 rpm. Our current results demonstrated the effect of bioreactor configuration, especially impeller diameter on mixing behavior and oxygen transfer in biological 2,3-BD pathway. The trends for oxygen depletion during the growth phase and fermentation time spent in both tanks prior to oxygen limiting were similar and reproducible. Moreover, by-product concentrations were not different between 2 L (400 rpm) and 10 L tanks (295 rpm), thus implying that micro aerobic conditions using constant tip speed at 295 rpm, and aeration rate at 0.8 vvm was optimum for 2,3-BD production. The high succinate (3.2 g/L) and acetate (2.4 g/L) production in applied agitation rate at 218 rpm and 400 rpm, respectively, would clarify the effect of different agitation speeds previously mentioned and explained in Chapter IV. Yang et

al. (2012) revealed that similar amount of 2,3-BD was obtained in lab-scale using 5, 10 and 30 L vessels. There were a slightly reduction on the main product at a 30 L tank. It was understood and explained that pH and temperature were difficult to maintain constantly in an entire area of reactor tank (Oh et al., 2009). Our results demonstrated that there was no obstacle to scale-up in laboratory unit. Hence, further experiment was carried out to scale up 2,3-BD production process to pilot scale from 90 to 300 L bioreactors.

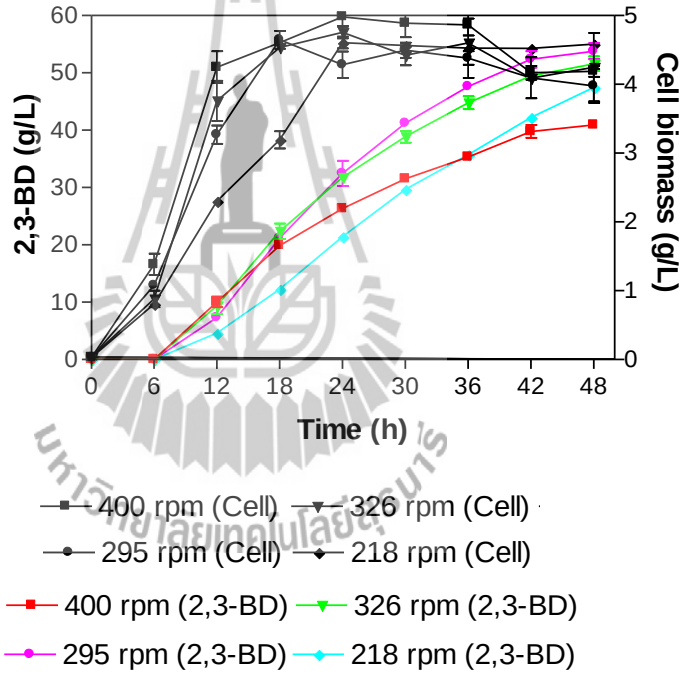

Figure 6.3 Comparison of four agitation rates in 10 L tank.

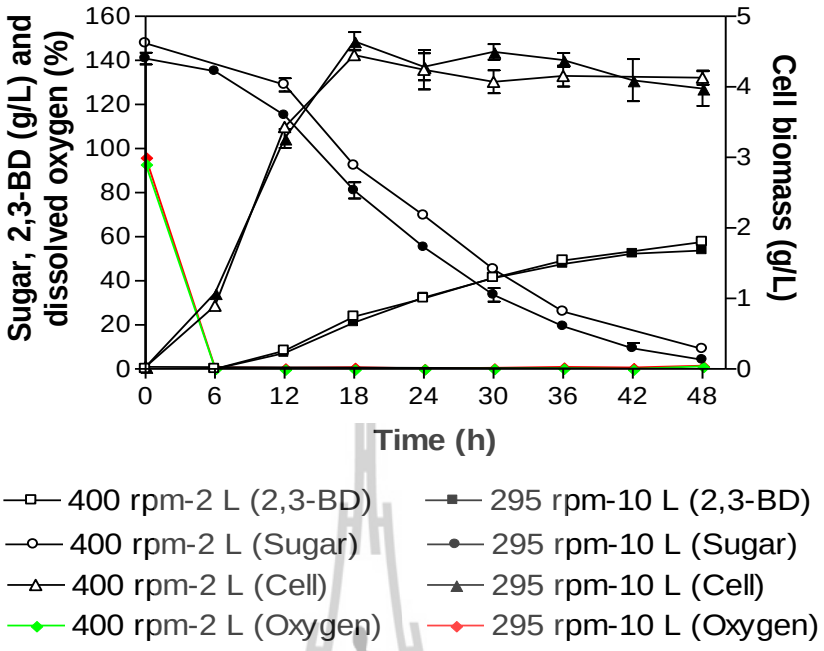

Figure 6.4 Comparisons between optimum conditions in 2 L and 10 L bioreactors.

Table 6.3 Summary of fermentation profile for 2,3-BD production *by K. oxytoca* KMS005 in laboratory unit.

(Scale) Agitation	Residual sugars (g/L)[a]	Max CDW (g/L)	2,3-BD concentration (g/L)	Gross Yield (g/g)	Conversion Yield (g/g)	Average Productivity (g/L/h)[b]	Byproducts (g/L)		
							Succinate	Ethanol	Acetate
(2 L)									
400 rpm	9.09±0.90	4.52±0.13$^{c,\mu}$	57.71±0.51$^{\varepsilon}$	0.41±0.01$^{\alpha}$	0.44±0.01$^{\alpha}$	1.20±0.01$^{\varepsilon}$	1.87±0.34$^{\mu}$	0.88±0.07$^{\mu}$	0.95±0.22$^{\mu}$
(10 L)									
218 rpm	20.48±1.89	4.60±0.12$^{\mu}$	47.47±0.92$^{\mu}$	0.34±0.01$^{\rho}$	0.37±0.01$^{\mu}$	0.98±0.01$^{\mu}$	3.19±2.08$^{\alpha}$	1.60±0.23$^{\varepsilon}$	0.41±0.11$^{\mu}$
295 rpm	4.43±1.75	4.64±0.18$^{\mu}$	53.78±1.95$^{\alpha}$	0.39±0.01$^{\alpha}$	0.40±0.01$^{\varepsilon}$	1.12±0.04$^{\alpha}$	1.98±0.22$^{\mu}$	0.72±0.06$^{\mu}$	1.07±0.52$^{\mu}$
326 rpm	8.86±0.41	4.75±0.12$^{\mu}$	51.58±1.79$^{\alpha}$	0.37±0.01$^{\alpha}$	0.39±0.02$^{\varepsilon}$	1.07±0.03$^{\alpha}$	0.59±0.28$^{\mu}$	0.43±0.20$^{\mu}$	0.90±0.32$^{\mu}$
400 rpm	28.05±2.35	4.98±0.19$^{\varepsilon}$	40.90±0.76$^{\rho}$	0.29±0.01$^{\varepsilon}$	0.33±0.01$^{\rho}$	0.85±0.01$^{\rho}$	0.19±0.01$^{\mu}$	0.47±0.19$^{\mu}$	2.36±0.82$^{\varepsilon}$

[a]Aeration and maltodextrin concentration was fixed at 0.8 vvm at 15% (w/v), respectively.

[b] Productivity is calculated at 48 h.

[c] All data represent the averages of two fermentations with standard deviations. Values bearing different Greek symbol are significantly different ($P < 0.05$).

6.3.2 Effect of various agitation speeds in 90 L fermenter

Scale-up process in pilot plant was investigated in 90 L tank under four various conditions which were the same as in 10 L tank. Inoculum preparation such as cell age, stage, and density was observed to be similar to the inoculum preparation between laboratory and pilot scale. Seed inoculation was cultured at 200 rpm for 16 h for four agitation speeds tested in 90 L (Figure 6.5 and 6.6). The result showed that different cell density of the seed inoculum was obtained when using different incubator shakers for seed preparation of 2 L and 90 L fermenters. It was likely that the incubator shaker for seed preparation of 90 L fermenter provided higher cell density than one for seed preparation of 2 L fermenter. The constant tip speed N_iD_i at 130 rpm was still kept to be the best condition of 2,3-BD production and its dissolved oxygen trend was similar to those observed in smaller scales (Figure 6.6). However there were obstacles in pilot plant such as low 2,3-BD production, high sugar residue, and prolonged cell growth. In comparison to the laboratory scale, the concentration of 2,3-BD was 2 folds decreased while sugar concentration was slowly consumed and left over at final fermentation. Growth curves at the constant tip speed, N_iD_i (130 rpm) and Reynolds number, R_{ei} (55 rpm) were likely prolonged during lag phase period while higher agitation speeds of constant volumetric power input, $\frac{P}{V}$ (170 rpm) and stirred speed N_i (295 rpm) could enhance cell growth but not 2,3-BD production.

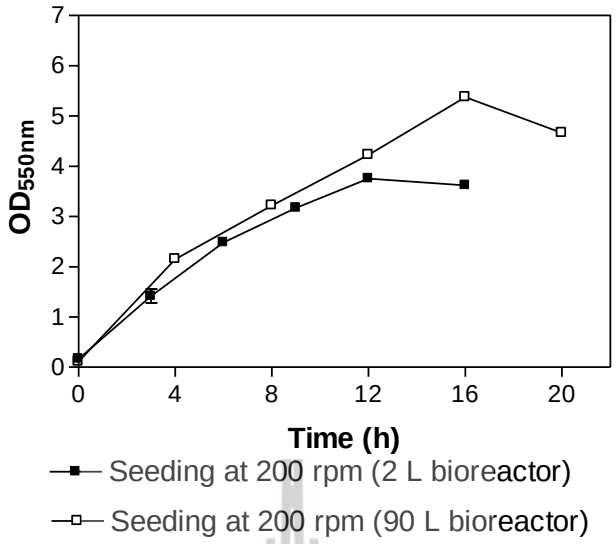

Figure 6.5 Growth curve of seed preparation in 2 L and 90 L scales.

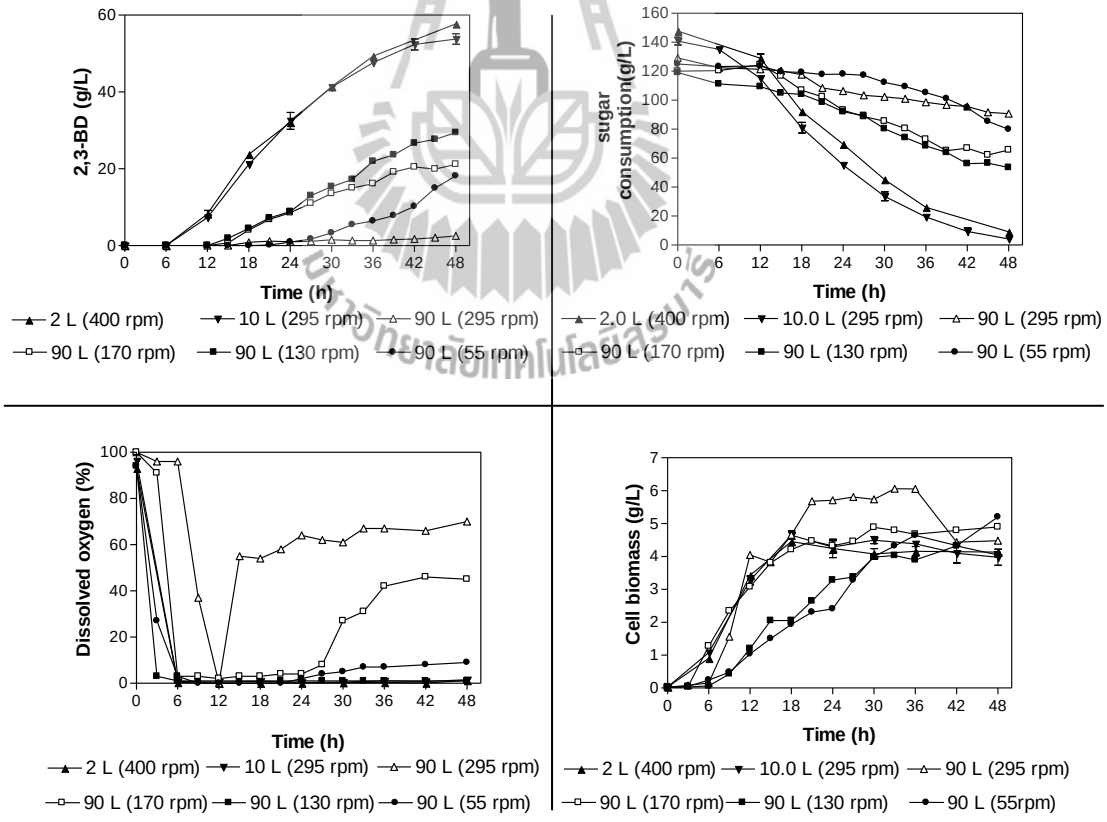

Figure 6.6 Fermentation profiles and time course of four agitation speeds in a 90 L bioreactor. Seed was prepared at 200 rpm for 16 h incubation time.

Furthermore, inoculum growth curve in 90 L tank was changed to be similar to the one in 2 L tank when using agitation speed at 150 rpm. As shown in Figure 6.7, both growth curves were similar from 0 to 12 h but not at 16 h. Incubation time of the inoculum for 90 L tank was assigned at 12 and 16 h. Total fermentation time was extended to 72 h in the production tank (Figure 6.8). The results showed that 12 h incubation time of the inoculum at cell density (OD_{550}) around 4 had a positive effect on 2,3-BD production in 90 L production tank. Interestingly, 2,3-BD concentration at 52.5 g/L was attained in 90 L. However, when the total fermentation time was prolonged up to 72 h, sugar consumption was slowly consumed (Figure 6.9). Strain KMS005 was prolonged in growth phase, it resulted in longer duration of 2,3-BD production phase in pilot scale. Remarkably, maximum cell biomass was 6.01 g/L in 90 L scale, which was much higher than 4.64 g/L in 10 L (Figure 6.10). To confirm that the constant tip speed, 130 rpm, was the best condition in 90 L tank, different oxygen supply was performed using inoculum preparation for 12 h. Figure 6.11 shows that oxygen supply by the constant tip speed 130 rpm, 0.8 vvm resulted in highest 2,3-BD production when oxygen supply by higher or lower agitation speed or aeration rate did not improve 2,3-BD production.

Usually, inoculum size was reported ranging from 1-5% and the age was in early stationary phase for bacteria (Sasikala et al., 1995). In our current study, concentration of inoculum was measured as optical cell density (OD) at 550 nm. The initial cell concentration to cultivate in the production tank was fixed at OD_{550} of 0.1, equivalent to inoculum size of 2.5% (v/v) and cell concentration around of 0.033 g/L when pre-culture reached OD_{550} around 4. Hence, only cell age and stage would be determined and observed their effects in pilot scale. The inoculum preparation by

agitating at 150 rpm for 12 h fell into the logarithmic phase which most of the cells are active to biomass production and could enhance 2,3-BD production. This could postulate that seed inoculum preparation in log phase had positive effect on 2,3-BD production rather than that in late log phase. Our finding was supported by Basak and Das (2007) revealing that an inoculum of purple non-sulfur (PNS) bacteria during exponential phase was the most appropriate for higher yield of hydrogen production. Even though 52.3 g/L of 2,3-BD concentration were achieved, there are three main problems observed in fermentation process such as longer fermentation time, high maximum cell biomass, and the elevation of side product concentrations such as succinate and acetate in 90 L tank (Table 6.4). Probably, large location inside geometry of the fermenter and low initial cell concentration might cause the problems in scale up. Dada et al. (2012) suggested that inoculum concentration at 1.5 g/L was the best for production of acetone, butanol and ethanol. Initial cell concentration of inoculum used in our study at 0.033 g/L might be too low for proper cell propagation. On the other hand, Junker (2004) reviewed that inoculum development and production phase as well as selection pressure are biological factors affected by scale. Selection pressure known as hydraulic pressure, or back pressure was occurred due to fluid flow behavior and mixing in larger scale which often influenced oxygen transfer rate (OTR). Imagine that KMS005 took more time for self-adaptation in the environment of larger size undertaken physiology effects such as tank configuration, aeration rate, agitation speed, back pressure medium sterilization, temperature control/ heat transfer, and removal, and mixing (Trilli, 1986; Jem, 1989; Votruba and Sobotka, 1992). Furthermore Okada and Iwamatu (1997) reported their investigation on large scale fermentation of milbemycine production that scale-up deteriorated production

syndrome was in terms of culture morphology change which caused packed cell volume and viscosity, carbon substrate uptake changes and productivity reduction. Often, the previous research on large scale formation found that the biomass production is declined by the scale up of aerobic process. Around 20% of maximum cell density was dropped when scaling up from 0.003-9 m^3 for recombinant protein production by *E. coli* (Bylund et al., 1998). Similarly, Zeng et al. (1994) showed that 18 g/L of maximum cell concentration in bench-scale was achieved within 22 h compared with 10 g/L of that in pilot scale within 26 h for 2,3-BD production by *E. aerogenes*. In contrast, maximum cell biomass concentration was highly obtained in pilot scale even though lag phase was prolonged and maximum cell biomass was obtained late at 33 h in our study. It is certain that cell physiology was altered accompanying with longer time for cell adaption in large volume. However, after lag phase for cell adaption, KMS005 could grow without discrimination on the zone of oxygen limitation. The strain KMS005 was derived from KMS004 which has parental strain *Klebsiella oxytoca* M5a1 by some gene deletions techniques in the central metabolic pathway (Jantama et al., 2015). The strain KMS004 was able to produce lactic acid under anaerobic conditions (Sangproo et al., 2012). However, the increase in maximum cell biomass is still not understandable and explained in the pilot plant.

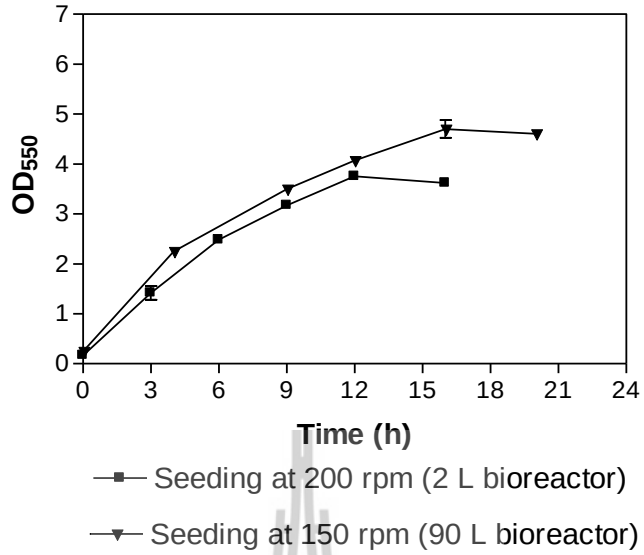

Figure 6.7 Growth curve in seed inoculum preparation in 2 L and 90 L scales.

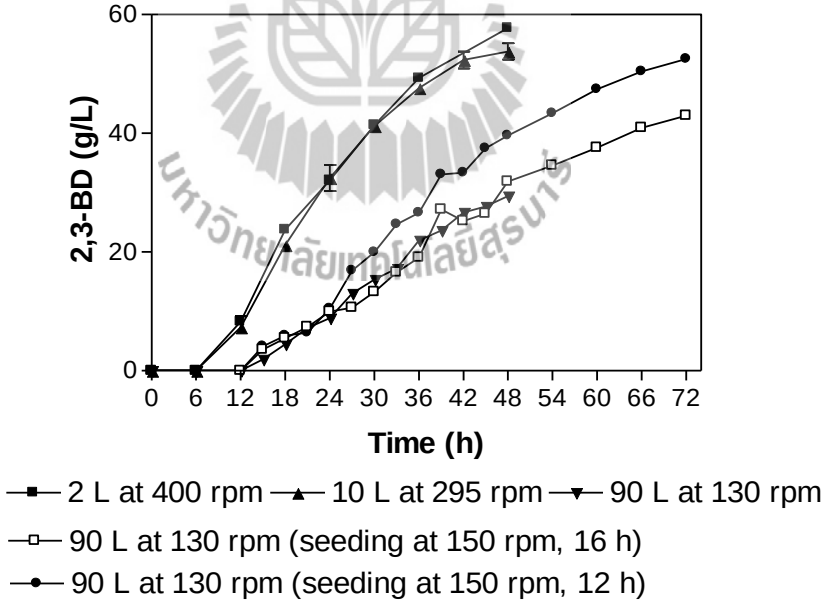

Figure 6.8 Effect of inoculum on 2,3-BD production profiles in 2, 10 and 90 L vessels.

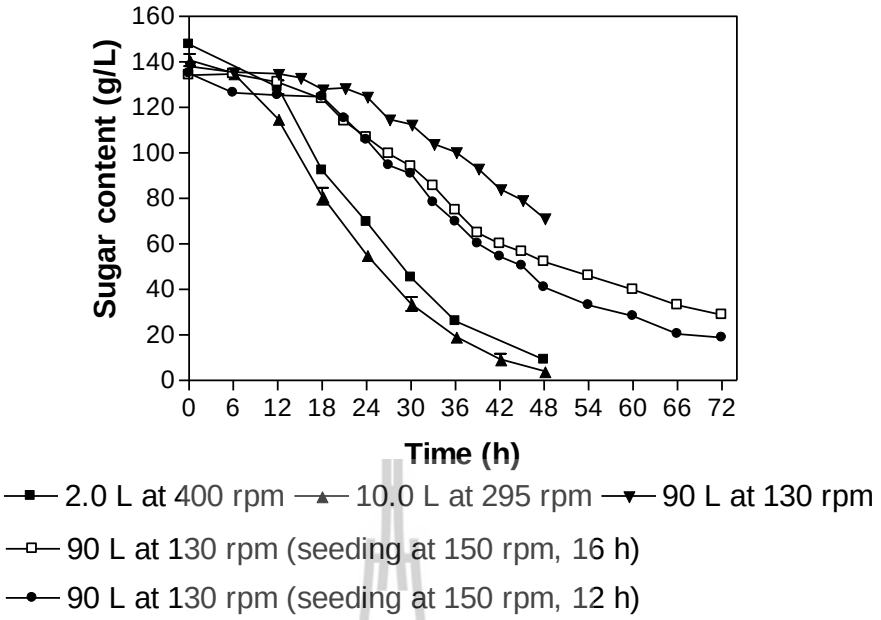

Figure 6.9 Effect of inoculum on sugar consumption profiles in 2, 10 and 90 L tanks.

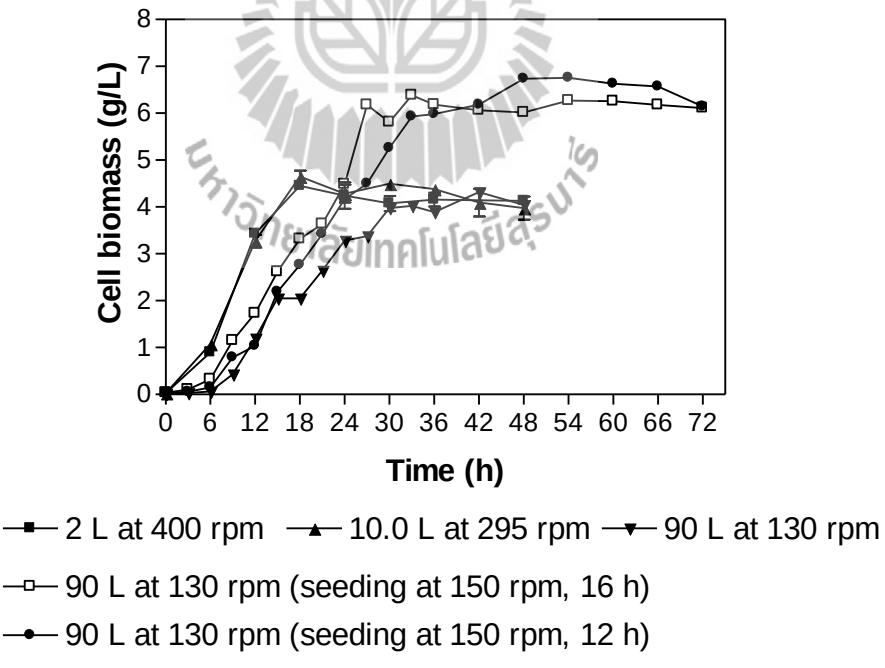

Figure 6.10 Effect of inoculum on cell biomass profiles in 2, 10 and 90 L bioreactors.

Figure 6.11 demonstrates that 2,3-BD concentration was not improved when either higher or lower oxygen supplies through agitation higher than 130 rpm were provided. Oxygen transfer coefficient (k_La) at 29.52 h^{-1} using agitation speed at 130 rpm and aeration rate 0.8 vvm were likely to be suitable for micro-aeration of 2,3-BD pathway. The k_La value estimation in the pilot scale was not different to what Ji et al. (2009) reported at 26.7 h^{-1} for growth phase of *K. oxytoca* ME-UD-3 in a 3 L stirred fermenter. The k_La value is currently one of the most applied physical scale-up variable since it includes the relevant parameters influencing oxygen supply like agitation and aeration employed for reactor scale up (Yawalkar, 2002). Wong et al. (2002) successfully scaled up *E. coli* fermentation from 5 to 200 L by keeping constant k_La and aeration rate (vvm) under variation of power input, and working volume. However, Byan et al. (1994) and Zheng et al. (1994) argued that the criterion of constant k_La or constant OTR is not applicable for the scale-up of 2,3-BD metabolic activity due to the strong influences of reactor hydrodynamics. So far, the concentration of 2,3-BD in scale-up process was not exceeded 60 g/L under batch condition (Byun et al., 1994; Zheng et al., 1994; Yang et al., 2012). Zeng et al. (1994) used respiratory quotient (RQ) from 4 to 4.5 as a control parameter for optimum oxygen supply in 1,500 L stirred tank. Around 55 g/L of 2,3-BD concentration was obtained in scale-up process by *E. aerogenes* within 32 h. However, slow glucose consumption and 2,3-BD production rate were observed in pilot tanks. According to Zeng et al. (1994), starting at initial glucose concentration around 175 g/L in laboratory and pilot reactors, glucose concentration was almost consumed at 19 h in laboratory but at 30 h in pilot plants. Laboratory scale could perform fermentation of 2,3-BD to reach 55 g/L but pilot plant reactor did as less as at 35 g/L at 19 h. In

comparison to our current finding, the longer fermentation time 72 h was required in 90 L vessel to obtain similar amount of 2,3-BD concentration at 53 g/L at 48 h in 10 L tank. This hurdle cannot be solved by increasing stirred speed and energy inputs. The mixing time inevitably increased in larger scale because homogenization deteriorated due to the larger volume to be stirred (Schmidt, 2005; Nienow, 1998).

Last but not least, the concentration of succinate and acetate were increased in 90 L tank. The phenomenon was known as stress-induced metabolic caused by misincorperation of amino acid into native proteins (Fenton et al., 1997). The stress response triggered in the change of glycolysis, citric acid cycle, and pathways involved in anaerobic and mixed acid fermentation. As shown in Figure 6.12, acetate and succinate were increased to 3 and 5.55 g/L, respectively, under the constant tip speed, 130 rpm of 90 L scale. Acetate accumulation can be explained by overflow-metabolism which is an important physiological characteristic of *E.coli*, a common industrial microorganism under aerobic cultivation (Bylund et al., 1998; Enfors et al., 2001). Bylund et al. (2000) stated that oxygen limitation following glucose overflow was the critical parameter for byproduct formation where formate was the dominating factor and not acetic acid. Our current result reflected that metabolic flow caused unwanted byproducts increasingly due to higher succinate and acetate concentrations in micro-aerobic culture for 2,3-BD fermentation scale-up. Taking into account the effect of different oxygen supply on by-product formations in 90 L plant, acetate is likely to be less undertaken by oxygen limitation zone and metabolic flow compared with the change of succinate concentration. The presence of acetate was almost kept constant from 18-72 h for constant tip speed 130 rpm with 0.8 and with 0.8/0.4 vvm,

but it was slightly reduced for constant $\left(\frac{p}{v}\right)$, 170 rpm agitation speed. Take succinate in turn, the more oxygen was supplied, the less succinate was produced. Obviously, two circumstances were occurred in which succinate concentration was jumped up and dropped sharply and it was consumed during fermentation process runs by constant N_iD_i at 130 rpm and constant $\left(\frac{p}{v}\right)$ at 170 rpm. The first behavior might be resulted from oxygen limitation zone and dissolved CO_2 accumulation. Dissolved CO_2 is higher in the larger vessel than the smaller vessel due to the added head pressure (Jem, 1989). Hence, low mixing referred to low agitation speed along with high dissolved carbon dioxide presence would spread more oxygen limitation zone and stimulate high succinate concentration. In opposition, high agitation speed enhanced more oxygen supply resulting in low succinate due to more partially aerobic zones. The second observation related to succinate re-uptake was demonstrated in large and small scales (Figure 6.12). In the large scale tanks, there often led to a local high consumption of oxygen thus forming zones of high glucose and low oxygen concentration which favor accumulation of acetate, formate, lactate, succinate and ethanol. These metabolite products are easily re-consumed in well mixed and aerated part of the bioreactor (Enfors, 2004). This would support our postulation that constant $\left(\frac{p}{v}\right)$ at 170 rpm changed micro-aerobic conditions to partially aerobic conditions in which succinate was well consumed.

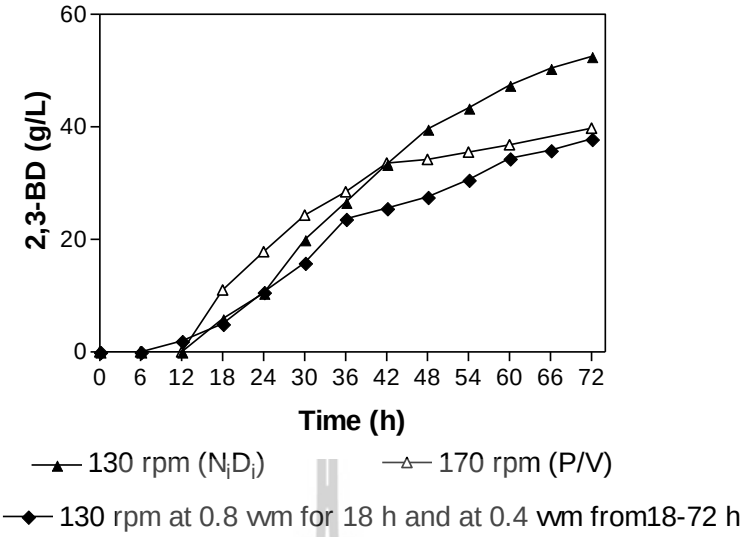

Figure 6.11 Comparison of different oxygen supply in 90 L scale. Seed was cultured at 150 rpm for 12 h incubation time.

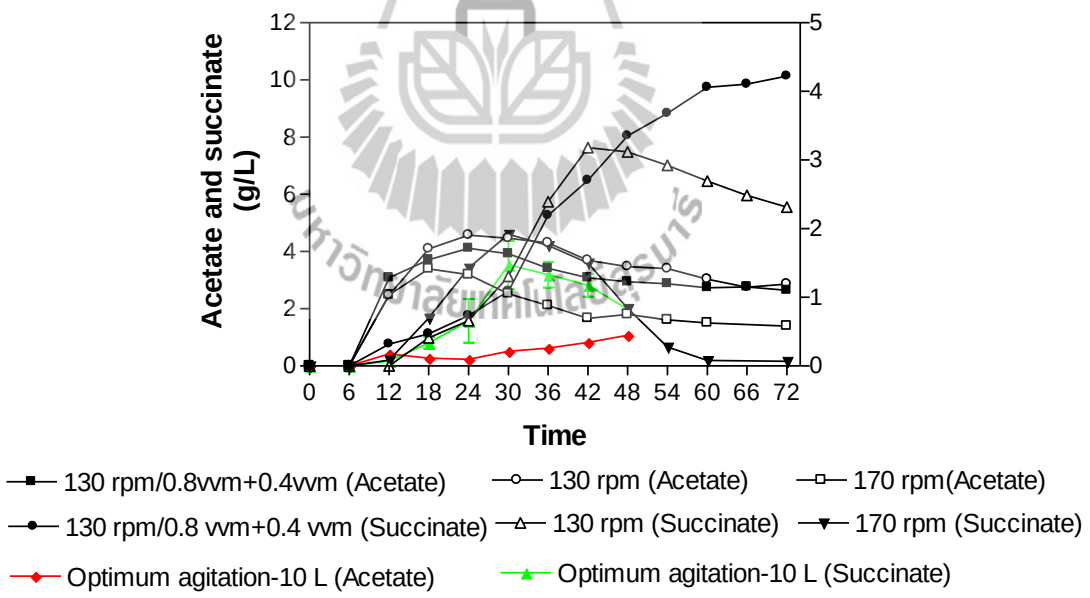

Figure 6.12 By-product concentration under oxygen supply in 90 L reactor. Acetate and succinate concentrations in 10 L scale were as control. Condition of 130 rpm/0.8 vvm+0.4 vvm represented agitation rate at 130 rpm with 0.8 vvm from 0-24 h and with 0.4 vvm from 24-72 h.

6.3.3 2,3-BD production in 300 L tank

Production of 2,3-BD was finally scaled-up to 300 L tank. Inoculum was fixed by incubating at 37 °C 150 rpm for 12 h. As resulted, cell biomass was gradually increased to 7.4 g/L and 2,3-BD production was produced at 45.02 g/L within 72 h (Table 6.4). The same phenomena between 90 and 300 L tanks were found such as high concentration of maximum cell biomass, long lag phase and prolonged fermentation time by slowly consuming maltodextrin and low producing 2,3-BD (Figure 6.13-6.15). For by-product comparison in both pilot scales, acetate did not increased but succinate did (Table 6.4). Succinate was increased from 5.55 g/L to 12.28 g/L when the fermenter scale was increased from 90 L to 300 L. This would imply that succinate is a dominating factor by metabolic flow rather than acetate. In addition, the increase in succinate production transparently demonstrated that partially anaerobic conditions were spread in larger tank geometry.

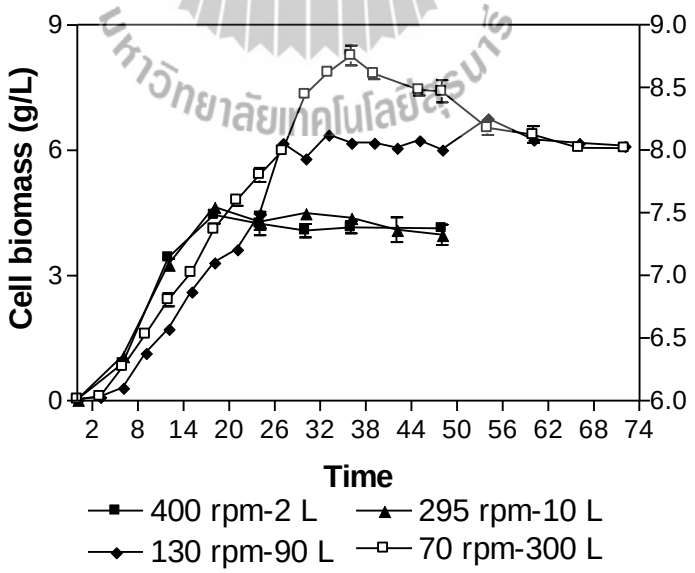

Figure 6.13 Comparison of biomass production in laboratory and pilot scales.

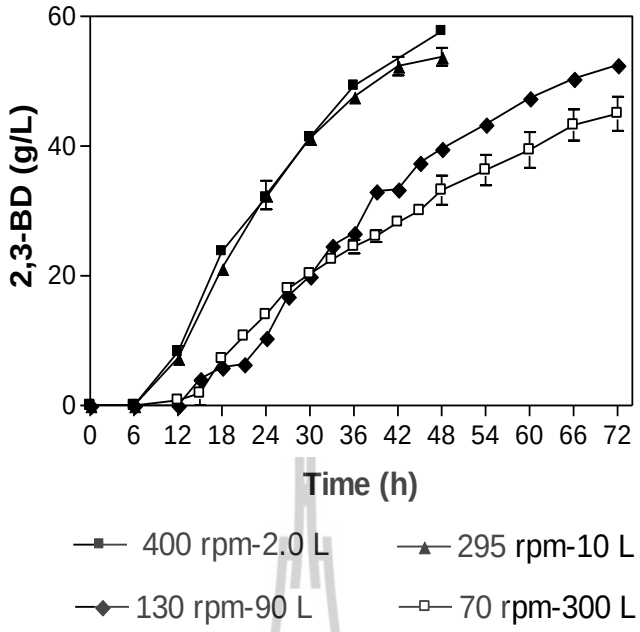

Figure 6.14 Comparison of 2,3-BD production in laboratory and pilot scales.

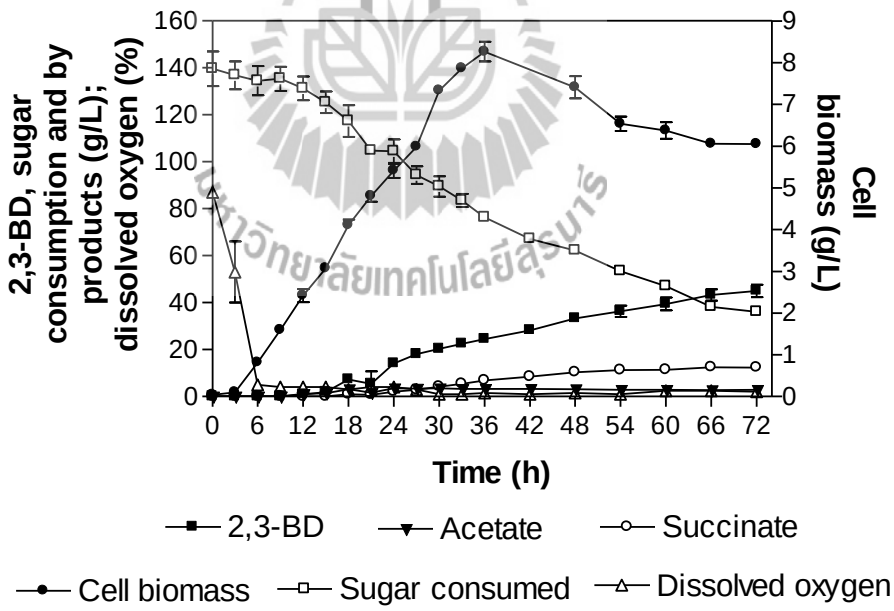

Figure 6.15 Fermentation profile of 2,3-BD production in 300 L scale under constant tip speed at 70 rpm.

Table 6.4 Summary of fermentation profile in 2, 10, 90 and 300 L bioreactors.

Scale		Residual Sugars (g/L)	Maximum CDW (g/L)	2,3-BD (g/L)	Conversion Yield[a] (g/g)	Average Productivity (g/L/h)	Specific Productivity (g/gCDW/h)	K_La (h^{-1})	Byproducts (g/L)				
									Suc	Lac	For	Eth	Ace
2L (400 rpm), 48 h		9.1±0.9	4.4±0.1	57.7±0.5	0.44±0.01	1.20±0.01	0.27	-	1.9±0.3	-	-	0.9±0.1	0.9±0.2
10L (295 rpm), 48 h		4.4±1.7	4.6±0.2	53.8±1.9	0.40±0.01	1.12±0.04	0.24	-	2.0±0.2	-	-	0.7±0.1	1.1±0.5
90 L (295 rpm)	48 h	90.7	6.1	2.5	0.06	0.05	0.01	46.44	<0.1	-	-	-	3.0
	72 h	83.5	6.1	5.2	0.11	0.07	0.01	46.44	<0.1	0.2	<0.1	-	3.6
90 L (170 rpm)		57.6	4.9	20.9	0.33	0.43	0.09	45.72	0.9	<0.1	-	0.5	2.7
90 L (130 rpm)		53.6	4.4	29.4	0.44	0.61	0.14	29.52	4.3	-	-	0.5	2.1
90 L-seeding at 200 rpm, 16 h (130 rpm)	48 h	46.1±0.1	5.4±0.4	36.2±2.5	0.41±0.02	0.75±0.05	0.14	29.52	8.3±1.2	-	-	1.7±0.1	2.4±0.1
	72 h	20.4±1.1	5.4±0.4	47.0±0.4	0.40±0.01	0.65±0.01	0.12	29.52	7.4±0.9	0.2±0.2	-	1.5±0.1	2.2±0.4
90 L (55 rpm)	48 h	79.9	4.8	18.2	0.40	0.38	0.08	18.01	3.9	0.7	0.5	2.6	3.5
	72 h	49.6	4.8	33.2	0.44	0.46	0.10	18.01	7.3	0.3	0.2	1.4	3.7
90 L-seeding at 150 rpm, 12 h (130 rpm)	48 h	41.0	6.0	39.9	0.40	0.82	0.14	29.52	7.5	0.7	-	2.5	3.4
	72 h	18.9	6.0	52.5	0.43	0.72	0.12	29.52	5.5	-	-	2.0	3.0
300 L (70 rpm)	48 h	62.2±1.2	7.4±0.4	33.2±3.2	0.43±0.01	0.69±0.06	0.10	-	10.4±1.2	-	-	-	3.2±0.2
	72 h	36±1.3	7.4±0.4	45.0±3.7	0.43±0.01	0.62±0.05	0.08	-	12.3±0.1	-	-	-	2.9±0.3

[a] Conversion yield was calculated as gram of 2,3-BD divided by gram of sugar consumed.

6.4 Conclusion

2,3-BD production was scaled up to 300 L tank using KMS005 strain as biocatalyst and maltodextrin as substrate. Around 45 g/L of 2,3-BD concentration was obtained after 72 h. Even though, slight decrease in 2,3-BD concentration and dramatically decrease in productivity were obtained, KMS005 showed its ability for cell growth and product formation in larger scale. Especially, it would explain that the negative effect resulting in low productivity and higher by-product (succinate) were triggered by physical parameters such as tank geometry and mixing, other than cell biology (cell-lost ability). Our study would be a guidance for further improvements relevant to industrial 2,3-BD production.

CHAPTER VII

GENERAL CONCLUSION AND RECOMMENDATIONS

In this dissertation, the research works have been reported in the production of 2,3-BD from maltodextrin using metabolically engineered *Klebsiella oxytoca* KMS005 as biocatalyst and simple mineral salts medium as basic and inexpensive medium. There are three parts comprised in novelty of the study including optimization process for 2,3-BD production, determination of optimum oxygen supply based on oxygen control parameters, and scale-up process in pilot plant.

First, pH, agitation speed, aeration rate and substrate concentration were optimized using conventional optimization. The optimum range obtained from one variable at time of optimization process was used as criteria to set up for further Response surface methodology (RSM) which allows all of the parameters to run at time. Consequently agitation speed, aeration rate and substrate concentration were selected as variables and their exact optimum points were found at 400 rpm, 0.8 vvm and 15% (w/v), respectively. Fermentative 2,3-BD production in terms of concentration, yield and productivity was improved using RSM as a statistical tool for optimization process. Under fed batch mode and optimum condition obtained from RSM, KMS005 could produce 2,3-BD at 88.1 g/L with the yield of 0.412 g/g of maltodextrin supplied after 78 h using 2 L bioreactor. The program indicated that agitation speed was the main effect and had interaction to aeration rate and substrate

concentration. The validated information recruited maltodextrin as alternative renewable source and push the further step in scale-up process of 2,3-BD production when estimated cost of 2,3-BD in our study could compete with fossil based 2,3-BD production.

Second, another strategy to enhance production was investigated evaluating the effect of oxygen concentration using glucose and simple mineral salts medium. There are some common oxygen parameters used such as oxygen mass transfer coefficient (k_La), and respiratory quotient (RQ). Both of the parameters assisted to evaluate the optimum oxygen supply to induce 2,3-BD producing pathway. Results demonstrated that optimum k_La 25.2 h^{-1} and RQ value from 3.5 to 4.0 for growth phase and between 1 and 2 for stationary phase were the best oxygen supply condition for high 2,3-BD concentration around 50 g/L with a productivity of 0.95 g/L/h and low byproduct formations. In addition, constant feeding rate-fed batch was attempted after optimum batch condition was found. Around 74.7 g/L of 2,3-BD concentration was achieved by constant feeding rate at 2 g/h, concomitantly starting the feed at the end of log phase and keep a sugar constant in the medium around 50 g/L.

Third, the several efforts in scaling up production of 2,3-BD from laboratorial to pilot scales were investigated. RSM had analyzed that agitation is a critical parameter to improve 2,3-BD concentration, this would support for the scale-up criterion based on various agitation speeds derived from mathematical equations involved with impeller diameters of each scale. As scaled-up from 2 to 10 L tanks, there was no certain obstacle to face with, however, there was sharply reduction in productivity and slightly decrease in 2,3-BD concentration in 90 L and 300 L fermenters. As resulted, around 45 g/L of 2,3-BD concentration was produced within

72 h using 300 L vessel. The increase in byproducts, especially succinate, would be taken into account for any changes of environmental condition inside the larger tanks. One of possibilities is related to oxygen distribution and poor mixing which are sensitive to prolonged cell growth and succinate accumulation.

Therefore the outcome of the studies would contribute to the current biotechnology of 2,3-BD process including

1. Introducing the optimization process, oxygen control strategy and simple fed batch as well as scale-up process to innovate 2,3-BD production;
2. Systematically investigating the effect of operating conditions, strain performance and different scale-up approaches on the extent of 2,3-BD production;
3. Guidance in the fermentation process design with the advance consideration of downstream processing, and waste deposal;
4. Encouragement and support the commercial bio-based 2,3-BD production according to the cost effectiveness of 2,3-BD production in the study which is competitive with fossil-based 2,3-BD process;
5. Reporting the achievements and the obstacles found in the fermentation process to overcome the target of industrial 2,3-BD production in the future.

Last but not least, the future studies are necessary to be pursued:

1. Optimization on seed inoculum for pilot scale will be proposed to shorten long lag phase occurred in larger fermentation;
2. The RQ method will be proposed as the oxygen control parameter for scale-up process;

3. Different types of bioreactor in pilot plants will be proposed to find out which is the most suitable geometry for 2,3-BD production;
4. Further improvement in strain KMS005 will be proposed via genetic engineering techniques to eliminate succinate and push carbon flux through to 2,3-BD metabolic pathway.

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APPENDICES

APPENDIX A

AGITATION SPEED CALCULATION IN SCALE-UP

Case I: **Constant** $\left(\frac{p}{v}\right)$

For turbulent region $\left(\frac{p}{v}\right) \propto N^3{}_i D^2{}_i$,

$\left(\frac{p}{v}\right)_1 = \left(\frac{p}{v}\right)_2$, $N^3{}_{i,1} D^2{}_{i,1} = N^3{}_{i,2} D^2{}_{i,2}$

$(6.66)^3\ (51*10^{-3})^2 = N^3{}_{i,2}\,(69*10^{-3})^2 \Rightarrow N^3_{i,2} = \frac{295.4*2{,}601*10^{-6}}{4{,}761*10^{-6}}$

$N_{i,2} = \sqrt{161.38} = 5.44\ \text{s}^{-1} = 326.6\ \text{rpm}$

$\left(\frac{N_{i,2}}{N_{i,1}}\right) = \left(\frac{5.44}{6.66}\right) = 0.8168$

Turbulent $P \propto N^3{}_{i,} D^5{}_i$

$$\frac{P_2}{P_1} = \frac{N^3{}_{i,2} D^5{}_{i,2}}{N^3{}_{i,1} D^5{}_{i,1}} = (0.8168)^3 * \left(\frac{69*10^{-3}}{51*10^{-3}}\right)^5 = 2.47$$

Determine P_1 from graph Figure 1 which $P_{(no)1} = 6.75$

$P_{(no)1} = \frac{P_1}{\rho_l N^3{}_{i,1} D^5{}_{i,1}} \quad \Rightarrow \quad P_1 = P_{(no)1} * \rho_l N^3{}_{i,1} D^3{}_{i,1}$

$P_1 = 6.75*(1000\ \text{kg/m}^3)(6.66\ \text{s}^{-1})^3(51*10^{-3}\text{m})^5 \;=\; 0.687\ \text{W}$

$$R_{ei,2} = \frac{\rho_l D^2{}_{i,2} N_{i,2}}{\mu_l} = \frac{(1000\ \text{kg/m}^3)(5.44\ \text{s}^{-1})(69*10^{-3}\text{m})^2}{1.002*0.001\ \text{kg.m/s}} = 25848.14 > 10^4$$

Determine $P_{(no)2}$ from Figure A.1 → $P_{(no)2} = 6.75$

$$P_{(no)1} = \frac{P_1}{\rho_l N^3{}_{i,1} D^5{}_{i,1}} \quad \Rightarrow \quad P_2 = P_{(no)2} * \rho_l N^3{}_{i,2} D^3{}_{i,2}$$

$$P_2 = 6.75*(1000\ \text{kg/m}^3)(5.44\ \text{s}^{-1})^3(69*10^{-3}\text{m})^5 \ = \ 1.7\ \text{W}$$

Scale up 2,3-BD process

Stirred tank 2 L	Stirred tank 10 L
Impeller D_{ia}, D_i = 51 mm	69 mm
Tank ID = 128 mm	190 mm
Air flow rate 0.1-1.2 vvm	0.1-1.2 vvm
Agitation speed 20-600 rpm	20-600 rpm
400 rpm, 0.8 vvm	
working volume at 1 L	Condition in 2 L to be scaled up
6 blades 3 turbine	

$$\frac{D_{i,2}}{D_{i,1}} = \frac{69\ \text{mm}}{51\ \text{mm}} = 1.35 \quad \Rightarrow \quad V \propto D^3{}_i, \quad \rightarrow \quad \frac{V_2}{V_1} = (1.35)^3 = 2.47$$

$$R_{ei} = \frac{\rho_l D^2{}_i N_i}{\mu_l}$$ Assume $\rho_l = \rho_{H_2O}$, $\mu_l = \mu_{H_2O}$

Viscosity SI unit Pa.S, Pa.S = 1 kg/m.s

CP of H_2O at 20 °C, $\mu_l = 1.002$ CP

1P = 1 g/cm.s = 1 Pa.S

10 P = 1 kg/m.s = 1 m Pa.S

$N_{i,1}$ = 400 rpm = 400 min^{-1} = 400/60 = 6.66 A^{-1}

$$R_{ei,1} = \frac{\rho_l D^2_{i,1} N_{i,1}}{\mu_l} = \frac{(1000 \text{ kg/m}^3)(6.66 \text{ s}^{-1})(51*10^{-3}\text{m})^2}{1.002*0.001 \text{ kg/m.s}} = 17,288.08 > 10^4 \rightarrow \text{Turbulent flow}$$

For turbulent region, power input is independent of R_{ei}

$$P \propto N_i^3 D_i^5$$

$$\frac{P}{V} \propto N_i^3 D_i^2$$

$$\frac{R_{ei,2}}{R_{ei,1}} = \frac{25848.14}{17288.08} = 1.49$$

$$N_{i,1}D_{i,1} = (6.66 \text{ s}^{-1})*(51*10^{-3}\text{m}) = 0.34 \text{ m/s}$$

$$N_{i,2}D_{i,2} = (5.44 \text{ s}^{-1})*(69*10^{-3}\text{m}) = 0.37 \text{ m/s}$$

$$\frac{N_{i,2}D_{i,2}}{N_{i,1}D_{i,1}} = \frac{0.37}{0.34} = 1.11$$

$$N_a = \frac{Fg}{N_i D_i^3}$$

In 2 L fermenter, V_R =1 L (50%), 0.8 vvm = 0.8 L/min = $\frac{0.8*10^{-3}\text{m}^3}{60 \text{ s}} = 1.33*10^{-5}$ m^3/s

$$Na_1 = \frac{1.33*10^{-5}\text{m}^3/\text{s}}{(6.66 \text{ s}^{-1})(51*10^{-3}\text{m})^3} = 0.015 = 1.5*10^{-2}$$

From Figure A.2, read P_a/P = 0.9

$$\frac{Pa_1}{P_1} = 0.9 \quad \Rightarrow \quad Pa_1 = 0.9*0.69 \text{ W} = 0.62 \text{ W}$$

In 10 L fermenter, V_R = 5 L (50 %), 0.8 vvm = 4 L/min = $\frac{4*10^{-3}\text{m}^3}{60\text{ s}} = 6.66*10^{-5}\text{m}^3/\text{s}$

$$Na_2 = \frac{6.66*10^{-5}\text{m}^3/\text{s}}{(5.44\text{ s}^{-1})(69*10^{-3}\text{m})^3} = 0.0372 = 3.72*10^{-2}$$

From Figure A.2, read P_a/P = 0.78

$$\frac{Pa_2}{P_2} = 0.78 \quad \Rightarrow \quad Pa_2 = 0.78*1.7\text{ W} = 1.33\text{ W}$$

$$\frac{Pa_2}{Pa_2} = \frac{1.33}{0.62} = 2.14$$

$$F_l \propto N_i D_i^3$$

$$\frac{F_{l,2}}{F_{l,1}} = \frac{N_{i,2}D_{i,2}^3}{N_{i,1}D_{i,1}^3} = \frac{(6.66\text{ s}^{-1})(51*10^{-3}\text{m})^3}{(5.44\text{ s}^{-1})(69*10^{-3}\text{m})^3} = 0.49$$

$$\frac{F_l}{V} \propto N_i \quad \rightarrow \quad \frac{N_{i,2}}{N_{i,1}} = \frac{5.44}{6.66} = 0.81$$

Case II: The tip speed constant N_iD_i

$$N_{i,1}D_{i,1} = N_{i,2}D_{i,2} \quad \Rightarrow \quad (6.66\text{ s}^{-1})(51*10^{-3}\text{m}) = N_{i,2}(69*10^{-3}\text{m})$$

$$N_{i,2} = 4.92\text{ s}^{-1} = 295.35\text{ rpm}$$

$$\frac{N_{i,2}}{N_{i,1}} = \frac{4.92}{6.66} = 0.74$$

$$R_{ei,2} = \frac{\rho_l D^2_{i,2} N_{i,2}}{\mu_l} = \frac{(1000\text{ kg/m}^3)(4.92\text{ s}^{-1})(69*10^{-3}\text{m})^2}{1.002*0.001\text{ kg/m.s}} = 23386.86$$

$$\frac{R_{ei,2}}{R_{ei,1}} = \frac{23386.86}{17288.08} = 1.35$$

$$\frac{(F_l/V)_2}{(F_l/V)_1} = \frac{N_{i,2}}{N_{i,1}} = 0.74$$

$$\frac{F_{l,2}}{F_{l,1}} = \frac{N_{i,2}D^3{}_{i,2}}{N_{i,1}D^3{}_{i,1}} = (0.74)(1.35)^3 = 1.83$$

$$\frac{(P/V)_2}{(P/V)_1} = \frac{N^3{}_{i,2}D^2{}_{i,2}}{N^3{}_{i,1}D^2{}_{i,1}} = (0.74)^3(1.35)^2 = 0.74$$

$$\frac{P_2}{P_1} = \frac{N^3{}_{i,2}D^5{}_{i,2}}{N^3{}_{i,1}D^5{}_{i,1}} = (0.74)^3(1.35)^5 = 1.83$$

$$P_2 = (1.83)(0.69\ \text{W}) = 1.26\ \text{W}$$

Case III: The constant R_{ei}

$$R_{ei,1} = R_{ei,2} = \frac{\rho_l D^2{}_{i,2}\ N_{i,2}}{\mu_l}$$

$$17288.08 = \frac{(1000\ \text{kg/m}^3)(N_{i,2})(69*10^{-3}\text{m})^2}{1.002*0.001\ \text{kg/m.s}}$$

$$N_{i,2} = 3.64\ \text{s}^{-1} = 218.30\ \text{rpm}$$

$$\frac{N_{i,2}}{N_{i,1}} = \frac{3.64}{6.66} = 0.54$$

$$\frac{F_{l,2}}{F_{l,1}} = \frac{N_{i,2}\ D^3{}_{i,2}}{N_{i,1}\ D^3{}_{i,1}} = (0.54)(1.35)^3 = 1.35$$

$$\frac{(P/V)_2}{(P/V)_1} = \frac{N^3{}_{i,2}D^2{}_{i,2}}{N^3{}_{i,1}D^2{}_{i,1}} = (0.54)^3(1.35)^2 = 0.30$$

$$\frac{P_2}{P_1} = \frac{N^3{}_{i,2} D^5{}_{i,2}}{N^3{}_{i,1} D^5{}_{i,1}} = (0.54)^3 (1.35)^5 = 0.73$$

$$P_2 = (0.73)(0.69\ \text{W}) = 0.51\ \text{W}$$

In 10 L fermenter

$$N_{a2} = \frac{Fg}{N_{i,2} D_{i,2}^3} = \frac{6.66 * 10^{-5} \text{m}^3/\text{s}}{(3.64\ \text{s}^{-1})(69 * 10^{-3}\text{m})^3} = 0.05 = 5.57 * 10^{-2}$$

Case IV: The constant stirred speed N_i

$$R_{ei,2} = \frac{\rho_l D_{i,2}^2 N_{i,2}}{\mu_l} = \frac{(1000\ \text{kg/m}^3)(6.66\ \text{s}^{-1})(69 * 10^{-3}\text{m})^2}{1.002 * 0.001\ \text{kg/m.s}} = 31,644.97$$

$$\frac{R_{ei,2}}{R_{ei,1}} = \frac{31,644.97}{17288.08} = 1.83$$

$N_{i,1} D_{i,1} = (6.66\ \text{s}^{-1})(51 * 10^{-3}\text{m}) = 0.34$

$N_{i,2} D_{i,2} = (6.66\ \text{s}^{-1})(69 * 10^{-3}\text{m}) = 0.46$

$$\frac{N_{i,2} D_{i,2}}{N_{i,1} D_{i,1}} = 1.35$$

$$\frac{F_{l,2}}{F_{l,1}} = \frac{N_{i,2}\ D^3{}_{i,2}}{N_{i,1}\ D^3{}_{i,1}} = (1.35)^3 = 2.48$$

Turbulent $\frac{(P/V)_2}{(P/V)_1} = \frac{N^3{}_{i,2} D^2{}_{i,2}}{N^3{}_{i,1} D^2{}_{i,1}} = (1.35)^2 = 1.83$

$$\frac{P_2}{P_1} = \frac{N^3{}_{i,2} D^5{}_{i,2}}{N^3{}_{i,1} D^5{}_{i,1}} = (1.35)^5 = 4.55$$

$$P_2 = (4.55)(0.69\ \text{W}) = 3.121\ \text{W}$$

In 10 L fermenter

$$N_{a2} = \frac{Fg}{N_{i,2}D_{i,2}^3} = \frac{6.66*10^{-5}\,\mathrm{m^3/s}}{(6.66\ \mathrm{s^{-1}})(69*10^{-3}\,\mathrm{m})^3} = 0.034 = 3.04*10^{-2}$$

From Figure A.2, read $P_a/P = 0.8$

$$\frac{Pa_2}{P_1} = 0.8 \quad \Rightarrow \quad Pa_2 = 0.8*3.12\ \mathrm{W} = 2.50\ \mathrm{W}$$

$$\frac{Pa_2}{Pa_1} = \frac{2.50}{0.61} = 4.04$$

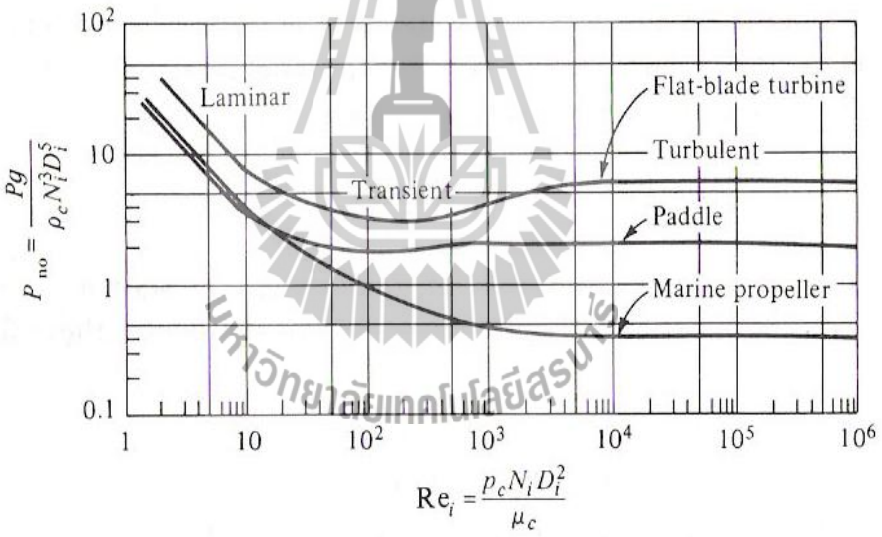

Figure A.1 The correlation between power number (P_{no}) and Reynolds number (R_{ei}).

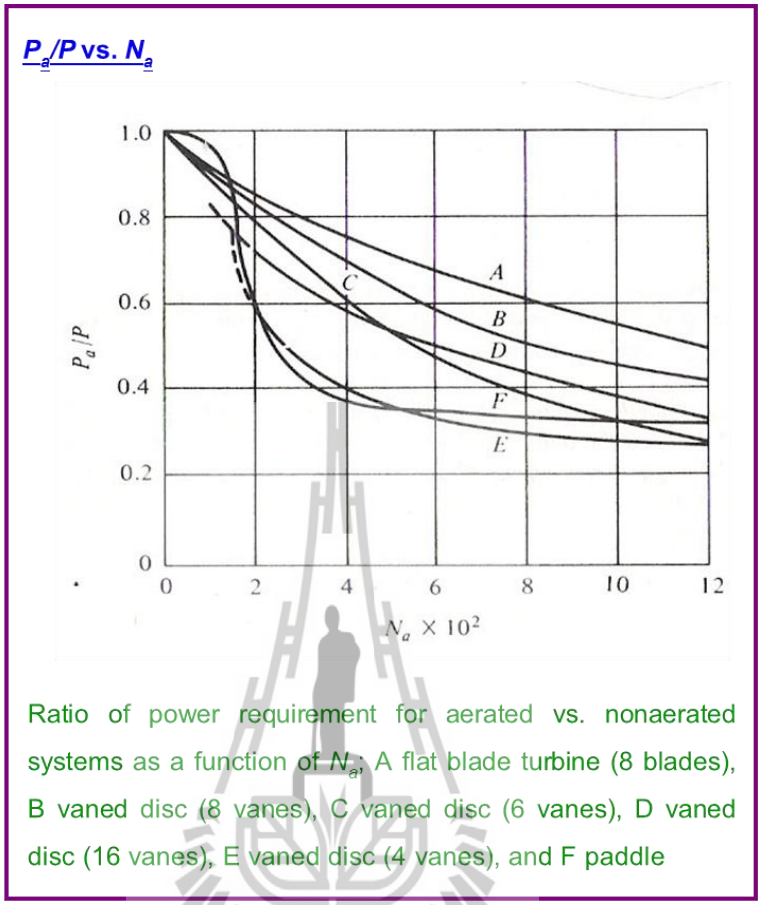

Figure A.2 The correlation between ratio of power requirement in aerated (P_a/P) vs non aerated vessels (N_a).

APPENDIX B

PUBLICATION

Chan, S., Jantama, S. S., Kanchanatawee, S., Jantama, K. (2016): Process optimization on micro-aeration supply for high production yield of 2,3-butanediol from maltodextrin by metabolically-engineered *Klebsiella oxytoca*. **PLOS ONE** DOI:10.1371/journal.pone.0161503.

ABSTRACT

An optimization process with a cheap and abundant substrate is considered one of the factors affecting the price of the production of economical 2,3-Butanediol (2,3-BD). A combination of the conventional method and response surface methodology (RSM) was applied in this study. The optimized levels of pH, aeration rate, agitation speed, and substrate concentration (maltodextrin) were investigated to determine the cost-effectiveness of fermentative 2,3-BD production by metabolically-engineered Klebsiella oxytoca KMS005. Results revealed that pH, aeration rate, agitation speed, and maltodextrin concentration at levels of 6.0, 0.8 vvm, 400 rpm, and 150 g/L respectively were the optimal conditions. RSM also indicated that the agitation speed was the most influential parameter when either agitation and aeration interaction or agitation and substrate concentration interaction played important roles for 2,3-BD production by the strain from maltodextrin. Under interim fed-batch

fermentation, 2,3-BD concentration, yield, and productivity were obtained at 88.1±0.2 g/L, 0.412 ±0.001 g/g of maltodextrin supplied, and 1.13±0.01 g/L/h respectively within 78 h.

Keywords: Metabolic engineering, Optimization, Response surface methodology, *Klebsiella*, Maltodextrin

BIOGRAPHY

Miss Chan Sitha was born on June 8th, 1986 in Battambong province, Cambodia. In 2008 she accomplished her Bachelor Degree (Biochemistry) in Royal University of Phnom Penh, CAMBODIA. In 2011, she graduated master degree of Science (Biotechnology) in Suranaree University of Technology, THAILAND. She pursued her PhD program in the same year. In 2014, she had awarded an international joint PhD program between SUT and The Institut National Polytechnique of Toulouse, FRANCE. She performed a part of her research in Laboratoire de Génie Chimique, INPT, for 8 months. In 2015, she had experience to practice pilot scale of bioreactors in Institute of Biotechnology and Genetic Engineering, Chulalongkorn University for almost a year. Besides she had a chance to participate an International conference 10th European congress of chemical engineering 3rd European congress of Applied Biotechnology and 5th European Process Intensification Conference, Nice, FRANCE from September 27- October 1st, 2015 (poster presentation). She was selected to be a participant in The 2016 Southeast Asia International Joint-research and Training Program for Green Energy Technologies, Taipei, TAIWAN from July 8-16, 2016. Her PhD thesis was entitled optimization and scale up production process of 2,3-butanediol from Maltodextrin by metabolically engineered *Klebsiella oxytoca* KMS005. Apart of her results had been published as Process optimization on micro-aeration supply for high production yield of 2,3-butanediol from maltodextrin by metabolically-Engineered *Klebsiella oxytoca*.