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BIOSYNTHESIS OF FOUR CARBON ALCOHOLS FROM SUSTAINABLE FEEDSTOCK

XIN FENGXUE

NATIONAL UNIVERSITY OF SINGAPORE

2015

BIOSYNTHESIS OF FOUR CARBON ALCOHOLS FROM SUSTAINABLE FEEDSTOCK

XIN FENGXUE

(B.Sc. Ludong Univ., P.R.China
M.Sc. Jiangxi Agr Univ., P.R. China)

A THESIS SUBMITTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF CIVIL AND ENVIRONMENTAL ENGINEERING

NATIONAL UNIVERSITY OF SINGAPORE

2015

DECLARATION

I hereby declare that this thesis is my original work and it has been written by me in its entirety. I have duly acknowledged all the sources of information which have been used in the thesis. This thesis has also not been submitted for any degree in any university previously.

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XIN FENGXUE

18 August 2015

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TABLE OF CONTENTS

Declaration i

Acknowledgements ii

Table of Contents iii

Summary x

List of Tables xiii

List of Figures xiv

List of Abbreviations xvii

Chapter 1 Introduction 1

1.1 Research background 2

1.1.1 Importantce of butanol and problem statement 2

1.1.1.1 Importance of butanol in industry 2

1.1.1.2 Challenges in ABE fermentation 5

1.1.1.2.1 High cost of traditional feedstock and lignocelluloses-degrading enzymes 5

1.1.1.2.2 Butanol toxicity in ABE fermentation 8

1.1.1.2.3 By-products in ABE fermentation 9

1.1.2 Importantce of 2,3-butanediol and problem statement 11

1.2 Research objectives 13

1.3 Organization of thesis 15

Chapter 2 Literature review 17

2.1 Microbial production of butanol 18

2.1.1 Chatacteristics of ABE fermentation 18

2.1.1.1 History of ABE fermentation 18

2.1.1.2 Microorganisms 19

2.1.1.3 Metabolic pathway in ABE fermentation 21

2.1.1.4 pH and acid crash 26

2.1.2 Advances to improve clostridial-based butanol production 28

2.1.2.1 Feedstock selection 28

2.1.2.1.1 Lignocellulosic biomass 28

2.1.2.1.2 Genetic engineered plants 31

2.1.2.2 Strain development 32

2.1.2.3 Development of novel fermentation processes 38

2.1.2.3.1 Co-production of riboflavin (VB2) 38

2.1.2.3.2 Conversion of butanol to more economically valuable product 39

2.2 Microbial production of 2,3-butanediol 41

2.2.1 Microorganisms 41

2.2.2 Metabolic pathway 43

2.2.3 Operating conditions 45

2.2.3.1 pH 45

2.2.3.2 Substrate concentration ... 46

2.2.4 Strategies for efficient and economical 2,3-butanediol production ... 47

Chapter 3 Simultaneous fermentation of glucose and xylose to butanol by *Clostridium* sp. strain BOH3 ... 51

3.1 Abstract ... 52

3.2 Introduction ... 53

3.3 Materials and methods ... 55

3.3.1 Growth medium and culture conditions ... 55

3.3.2 Preparation of horticultural waste hydrolysates ... 56

3.3.3 Expression of genes related to xylose utilization as measured by quantitative real time PCR ... 57

3.3.4 Xylose isomerase and xylulose kinase assays ... 58

3.3.5 Analytical methods ... 59

3.4 Results ... 60

3.4.1 Efficient co-production of butanol and riboflavin from xylose by strain BOH3 .. 60

3.4.2 Simultaneous utilization of glucose and xylose by strain BOH3 ... 65

3.4.3 Fermentation of lignocellulosic hydrolysate by strain BOH3 ... 71

3.4.4 Expression of xylose isomerase and xylulokinase in strain BOH3 ... 73

3.5 Discussion ... 76

3.6 Conclusion ... 79

Chapter 4 Characterization of a thermostable xylanase from a newly isolated *Kluyvera* species and its application for biobutanol production 80

4.1 Abstract 81

4.2 Introduction 82

4.3 Materials and methods 84

4.3.1 Isolation and culture growth 84

4.3.2 Coculture of *Kluyvera* sp. strain OM3 and *Clostridium* sp. strain BOH3 85

4.3.3 16S rRNA gene sequencing and phylogenetic analysis 86

4.3.4 Enzyme activity and hydrolysis assays 87

4.3.5 Enzymatic properties of xylanase 88

4.3.6 Gas chromatography analysis of metabolic products 89

4.4 Results and discussion 89

4.4.1 Cultivation of a xylanase-producing culture OM3 89

4.4.2 Biochemical characterization of the xylanase from culture OM3 92

4.4.3 Hydrolysis of untreated lignocellulosic biomass 97

4.4.4 Butanol production by xylanase from *Kluyvera* sp. strain OM3 plus *Clostridium* sp. strain BOH3 and co-culturing of *Kluyvera* sp. strain OM3 plus *Clostridium* sp. strain BOH3 directly from xylan 100

4.5 Conclusion 105

Chapter 5 Integration of biobutanol and lipase-catalyzed butyl-butyrate production in a single ractor 107

5.1 Abstract 108

5.2 Introduction 109

5.3 Materials and methods 111

5.3.1 Growth medium and cultural conditions 111

5.3.2 Shake bottle fermentation 111

5.3.3 Fed-batch fermentation with sodium butyric feeding 112

5.3.4 Determination of partition coefficient 112

5.3.5 Lipase assay 113

5.3.6 Analytical methods 113

5.4 Results and discussion 114

5.4.1 Butyl-butyrate production with induction of lipases by *Clostridium* sp. strain BOH3 115

5.4.2 Butyl-butyrate production catalyzed by commercial lipases by *Clostridium* sp. strain BOH3 118

5.4.3 High butyl-butyrate production by *Clostridium* sp. strain BOH3 integrated with in-situ extraction 123

5.4.4 Productivity and cost comparison using different strategies by *Clostridium* sp. strain BOH3 127

5.5 Conclusion 129

Chapter 6 Production of 2,3-butanediol from sucrose by a Klebsiella species 130

6.1 Abstract 131

6.2 Introduction 132

6.3 Materials and methods 133

6.3.1 Isolation, characterization and phylogenetic analysis of strain XMR21 133

6.3.2 Optimization of fermentation conditions 134

6.3.3 Batch and fed-batch fermentation 136

6.3.4 Mutation and screening of mutants 136

6.3.5 Analytical methods 136

6.4 Results and discussion 137

6.4.1 Strain isolation and characterization 137

6.4.2 Optimization of fermentation conditions for enhanced 2,3-butanediol production 140

6.4.3 Effect of sucrose concentration 144

6.4.4 2,3-Butanediol production by strain XMR21 under fed-batch fermentation 145

6.4.5 Enhanced 2,3-butanediol production by mutating strain XMR21 146

6.5 Conclusion 150

Chapter 7 Conclusions and recommendations 151

7.1 Conclusions 152

7.2 Recommendations 156

References 158

Bibliography 172

Appendix: List of publications and awards.. 173

SUMMARY

Butanol and 2,3-butanediol, four-carbon primary alcohols, are not only important intermediate chemicals, but also considered as promising next-generation liquid fuels. However, the high cost of traditional feedstocks and low solvent production restrict the development of industrial acetone-butanol-ethanol (ABE) fermentation. A lignocellulosic biomass-based route and high butanol-producing microbes have been regarded as thorough solutions for sustainable development of ABE fermentation. However, the unefficient utilization of pentose containing in the lignocellulosic hydrolysates by solventogenic *Clostridium* limites the development of ABE fermentation through biological routes. Therefore, microbial isolates possessing capabilities of high butanol production and efficient pentose utilization or novel fermentation strategies to fulfill direct butanol production from lignocelluloses are needed. For 2,3-butanediol bioproduction, novel strains capable of producing high concentrations of 2,3-butanediol without any by-products using low-cost carbon sources are also highly desirable to achieve the industrial scale up. So one of the main goals of this dissertation is to isolate and characterize novel microbial isolates able to simultaneously ferment glucose and xylose, which are the main components in lignocellulosic hydrolysate to butanol and produc high concentrations of 2,3-butanediol without by-products using low-cost carbon sources. The other objective is to design novel co-culturing microbial systems to achive direct butanol production from hemicellulose or new fermentation strategies to convert toxic butanol to non-toxic and more value-added butyl-butyrate.

This dissertation firstly demonstrated fermentation of glucose and xylose by the newly reported *Clostridium* sp. strain BOH3 (Chapter 3). Compared to existing bacteria,

strain BOH3 not only shows the most efficient utilization of xylose, but also eliminates the carbon catabolic repression no matter in pure glucose/xylose mixtures or horticultural waste hydrolysate. This high xylose-utilization capability of strain BOH3 is attributed to its high xylose-isomerase (0.97 U/mg protein) and xylulokinase (1.16 U/mg protein) activities. Interestingly, strain BOH3 was also found to co-produce riboflavin during the fermentation process. Hence, *Clostridium* sp. strain BOH3 is an attractive candidate for application in efficiently converting lignocellulosic hydrolysates to biofuels and other value-added products, such as riboflavin.

This thesis further reported isolation of a new hemicellulose-degradaing *Kluyvera* sp. strain OM3 and characterization of its cellulase-free (5.12 IU/mL) and thermostable (70 ° C and pH 8.0) xylanase (Chapter 4). Moreover, a co-culturing system consisting of *Kluyvera* sp. strain OM3 and *Clostridium* sp. strain BOH3 was designed, which could directly convert birchwood xylan to 1.2 g/L butanol. To the best of our knowledge, this represents the first report on the production, characterization of a xylanase from genus *Kluyvera* and its application for butanol production directly from hemicelluloses.

In Chapter 5, the capability of biosyntheisis of butyl-butyrate via ABE fermentation using *Clostridium* sp. strain BOH3 was investigated. The strategy of integration of ABE fermentation by using strain BOH3, lipase-catalyzed esterification and *in situ* extraction using kerosene led to final 22.4 g/L butyl-butyrate obtained in the solvent phase (kerosene). This concentration is 4.5 - fold higher than that in a previously reported system. The strategy above could be used for production of butyl-butyrate in the future.

In Chapter 6, a wild-type *Klebsiella* sp. strain XRM21 was isolated, which is capable of producing only 2,3-butanediol and ethanol as its end metabolic products from sucrose. And further process optimization and strain improvement via chemical mutagenesis resulted in 119.4 g/L and 22.5 g/L of 2,3-butanediol and ethanol under optimized fed-batch fermentation process with a total productivity of 2.18 g/L/h, which is comparable to the reported highest 2,3-butanediol concentration produced by previous strains. Thus, the mutant *Klebiesella* sp. strain XMR21 can serve as a potential industrial strain when using sucrose-based material as the substrate, such as sugar cane juice or molasses wastes, etc.

In summary, the unique capabilities in efficient xylose utilization and simultaneous cofermentation of glucose and xylose by the newly isolated *Clostridium* sp. strain BOH3, the sequential co-culture system consisting of hemicellulose-degrading strain OM3 and solventogenic strain BOH3 and process integratation for bioconvertion of toxic butanol to non-toxic and more valued-added product - butyl-butyrate pave the way for cost-efficient butanol and butyl-butyrate fermentation in industrial scale. And lastly, strain improvement and process optimization provide a feasible and economical strategy to produce industrially important 2,3-butanediol from low-cost sucrose and make the bioprocesses of 2,3-butanediol economically applicable.

LIST OF TABLES

Table 1.1 Comparison of properties of different fuels.

Table 2.1 Batch fermentation performance of various biomass hydrolysates by solventogenic clostridia.

Table 2.2 Various mutagenesis attempts in butanol producing microorganisms.

Table 2.3 Metabolic engineering of *C. acetobutylicum* for butanol production.

Table 2.4 Comparison of 2,3-butanediol production by different microbes from different substrates.

Table 3.1 Xylose consumption and total solvent (acetone, butanol, and ethanol) production by different *Clostridium* species fed with 60 g/L xylose.

Table 3.2 Specific enzymatic activities (U/mg protein) of xylose isomerase and xylulokinase associated with various glucose/xylose ratios in *Clostridium* sp. strain BOH3 and *Clostridium* sp. strain G117.

Table 4.1 Comparison of xylanase activities among mesophilic anaerobic bacteria.

Table 5.1 Partition coefficients of solutes in hexadecane/aqueous system.

Table 5.2 Comparison of the butyl-butyrate productivities at different stages of process development and associated overhead costs (cost of extractant not included).

Table 6.1 RSM experimental design for 2,3-butanediol production and corresponding results.

Table 6.2 Comparison of 2,3-butanediol production by different *Klebsiella* strains and substrates under fed-batch fermentation conditons.

LIST OF FIGURES

Fig. 2.1 Hexoses and pentoses metabolism in *C. acetobutylicum.*

Fig. 2.2 Acetone-butanol ethanol fermentation pathway in *C. acetobutylicum*: (A) and their relation to the life cycle stages (B). (A) The red letters show the enzymes involved in the fermentative pathways: Ldh, lactate dehydrogenase; Pdc, pyruvate decarboxylase; Hyd, hydrogenase; Pfor, pyruvate:ferredoxin oxidoreductase; Fd, ferredoxin; PTA, phosphotransacetylase; AK, acetate kinase; AdhE, aldehyde/alcohol dehyrogenase; CtfAB, acetoacetyl-CoA:acyl-CoA transferase; Adc, acetoacetate decarboxylase; Thl, thiolase; Hbd, 3-hydroxybutyryl-CoA dehydrogenase; Crt, crotonase, Bcd, butyryl-CoA dehydrogenase; Ptb, phosphotransbutyrylase, BK, butyrate kinase. (B) Acidogenesis and solventogenesis referring to the respective growth stages.

Fig. 2.3 2,3-Butanediol fermentationpathway (modified, based on Maddox, 1996; Magee and Kosaric, 1987). 1, Embden–Meyerhof and pentose phosphate pathway enzymes; 2, pyruvate kinase; 3, pyruvate– formate lyase; 4, acetaldehyde dehydrogenase; 5, etha nol dehydrogenase; 6, phospho-t ransacetylase; 7, acetate kinase; 8, α-acetolacta te synthase; 9, α -acetolactate decarboxylase; 10. acetoin reducta se (2,3-butanediol dehydrogenase); 11, lactate dehydrogenase; 12, phosphoenolpyruvate decarboxylase; 13, malate dehyd rogenase; 14, fumarase; 15, succ inate dehydrogenase; 16, formate-hydrogen lyase complex; 17, pyruvate dehydrogenase multi-enzyme complex; and 18, citroyl synthetase.

Fig. 2.4 Scope of the research work.

Fig. 3.1 Growth and fermentation profiles of *Clostridium* sp. strain BOH3 in reduced mineral medium containing 60 g/L of xylose (A and B) and 60 g/L of glucose in batch bottles (C and D).

Fig. 3.2 Growth and fermentation profiles of *Clostridium* sp. strain BOH3 in reduced mineral medium containing a mixture of 30 g/L of glucose and 30 g/L of xylose (A and B), 40 g/L of glucose and 20 g/L of xylose (C and D), and 20 g/L g of glucose and 40 g/L of xylose (E and F).

Fig. 3.3 Sugar-consumption profiles. (A) xylose was added into glucose-containing medium, and (B) glucose was added into xylose-containing medium, after 24 hrs of cultivation.

Fig. 3.4 Fermentation of (A) cellulosic hydrolysate (containing 39.8 g/L glucose and 20.8 g/L xylose) from horticultural waste and (B) hemicellulosic hydrolysate

(containing 5.9 g/L glucose and 58.3 g/L xylose) from horticultural waste by *Clostridium* sp. strain BOH3.

Fig. 3.5 Comparison of relative xylose isomerase transcription levels between *Clostridium* sp. strain BOH3 and *Clostridium* sp. strain G117in a medium containing 60 g/L xylose as a carbon source.
Xylose isomerase mRNA-cDNA copy numbers were first normalized against the mRNA-cDNA copy numbers of housekeeping genes (*fabZ* for strain BOH3, *pepT* for strain G117) at each time point. The fold of transcript increment is then calculated by normalized xylose isomerase copy numbers at each time point / normalized xylose isomerase copy numbers at the time point of 8 hrs. Values are averages of duplicate determinations.

Fig. 4.1 Phylogenetic tree of strain *Kluyvera* sp. strain OM3 using the neighbor-joining method (MEGA 4.0) based on 16S rRNA gene sequences.

Fig. 4.2 (A) Optimal pH and (B) pH stability of the partially purified xylanase from *Kluyvera* sp. srain OM3. The influence of pH on xylanase activity was determined at 70 °C using 0.05 M of buffer (citrate (pH 3.0-5.0), phosphate (6.0-7.0), and glycine/NaOH (pH 8.0–12.0)). The remaining activity was measured after incubation for 10 min at 70 °C over various pH ranges. (C) Optimal temperature and (D) thermostability of the partially purified xylanase from *Kluyvera* sp. strain OM3. The reaction was determined by using 0.05M glycine/NaOH buffer at pH 8.0.

Fig. 4.3 Hydrolysis of different lignocellulosic wastes (WC: wood chips; SD: sawdust; PO: palm oil fiber; CB: waste carton box; NP: waste newspaper) by the partially purified xylanase from *Kluyvera* sp. strain OM3 after 72 hrs of incubation at 60 °C.

Fig. 4.4 Profiles of reducing sugars and fermentation product formation by partially purified xylanase and *Clostridium* sp. strain BOH3 amended with 4% (w/w) birchwood xylan. Arrow means inoculation point of *Clostridium* sp. strain BOH3.

Fig. 4.5 (A) Enzyme activities and (B) product concentrations by culture *Kluyvera* sp. strain OM3 when amended with 4% (w/w) birchwood xylan at 35 °C. (C) Time-dependent enzyme activities and (D) product concentrations in the co-culture system consisting of culture *Kluyvera* sp. strain OM3 and *Clostridium*

sp. strain BOH3 amended with 4% (w/w) birchwood xylan. Arrow means the inoculation point of *Clostridium* sp. strain BOH3 at 35 °C.

Fig. 5.1 Butyl-butyrate production and lipase activity with addition of different inducers. Bio-OSR, olive oil or glyceryl tributyrate was added to the culture medium in aratio of 1:5 (V/V). Samples were tested after 122 hrs of fermentation by *Clostridium* sp. strain BOH3.

Fig. 5.2 Schematic diagram of an esterification in the mini-emulsion fromed using Bio-OSR.

Fig. 5.3 Batch fermentation results of *Clostridium* sp. strain BOH3 with addition of commercial lipase at different time points. A: 24 hrs; B: 48 hrs; C, 72 hrs. D: Fed-batch fermentation result of *Clostridium* sp. strain BOH3 with addition of sodium butyric. Solid arrows mean supplement of lipase. Dashed arrows mean supplement of sodium butyric.

Fig. 5.4 A. Butyl-butyrate and butanol (in solvent and aquatic phases) production with addition of different extractants using *Clostridium* sp. strain BOH3under 60 g/L xylose. B. Fermentation profiles of *Clostridium* sp. strain BOH3 in reduced mineral medium containing 70 g/L xylose with addition kerosene. Commercial lipase was added at time of 24 hrs. 30 mM sodium butyric was fed twice at 48 and 72 hrs. Samples were tested after 122 hrs of fermentation. pH was controlled at 5.0-5.2.

Fig. 6.1 Phylogenetic tree of *Klebsiella* sp. strain XMR21 using the neighbor-joining method (MEGA 4.0) based on 16S rRNA gene sequences.

Fig. 6.2 Production of 2,3-butanediol and ethanol via fermenting 60 g/L glycerol, xylose, glucose and sucrose by *Klebsiella* sp. strain XMR21. Data shown are after 48 hrs of fermentation.

Fig. 6.3 Three dimensional response surface and contour plot showing the effects of pH values and concentration of sodium acetate (mM) on 2,3-butanediol production (g/L).

Fig. 6.4 Production of 2,3-butanediol at various amount of sucrose initial concentrations (50-300 g/L) by *Klebsiella* sp. strain XMR21. Data shown are after 48 hrs of fermentation under the optimized conditions.

Fig. 6.5 Production of 2,3-butanediol and ethanol under fed-batch fermentation in a 3.0-L bioreactor by *Klebsiella* sp. strain XMR21.

Fig. 6.6 (A) 2,3-Butanediol production by different mutants of strain XMR21 from fermenting 150 g/L of sucrose after 48 hrs. (B) Production of 2,3-butanediol and ethanol under fed-batch fermentation in a 3.0-L bioreactor by the mutant *Klebsiella* sp. strain XMR21.

LIST OF ABBREVIATIONS

ABE	Acetone-butanol-ethanol
BB	Butyl-butyrate
EMP	Embden-Meyerhof-Parnas pathway
PPP	Pentose Phosphate pathway
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
PCR	Polymerase chain reaction
GC-FID	Gas chromatography-flame ionization detector
HPLC	High-performance liquid chromatography
16S rRNA	16S Ribosomal RNA
TAE buffer	Tris-acetate-EDTA buffer
dNTP	Deoxyribonucleotide triphosphate
BSA	Bovine serum albumin
Ldh	Lactate dehydrogenase
Pdc	Pyruvate decarboxylase;
Hyd	Hydrogenase
Pfor	Pyruvate:ferredoxin oxidoreductase

Fd	Ferredoxin
PTA	Phosphotransacetylase
AK	Acetate kinase
AdhE	Aldehyde/alcohol dehyrogenase
CtfAB	Acetoacetyl-CoA:acyl-CoA transferase
Adc	Acetoacetate decarboxylase
Thl	Thiolase
Hbd	3-hydroxybutyryl-CoA dehydrogenase;
Crt	Crotonase
Bcd	Butyryl-CoA dehydrogenase
Ptb	Phosphotransbutyrylase
BK	Butyrate kinase

Chapter I

Introduction

1.1 Research background

Over the last 150 years, the synthesis of inexpensive chemicals from fossilized forms of carbon (e.g. oil, coal, natural gas) has dramatically altered society through their broad applications, ranging from cosmetics to plastics. However, the depletion of these fossilized reserves coupled with their negative environmental impacts, has inherently limited current chemical approaches towards producing these chemicals. Instead, biorefinery systems that integrate biomass conversion, microbial fermentation and process optimization to produce fuels, power, and chemicals from annually renewable resources are at the stage of worldwide development (Ranjan and Moholkar, 2012; Demirbas, 2007). Many fuels and chemicals that could only be produced by chemical processes in the past now have the potential to be generated biologically using renewable resources (Danner and Braun, 1999; Hatti Kaul et al., 2007). Microbial production of four-carbon alcohols (butanol and 2,3-butanediol) are such good examples, which have shown wide application in the industry and are also listed in the top 30 industrial organic chemicals used in the USA (Kamm and Kamm, 2004; Ragauskas et al., 2006, McCoy et al., 2007). However, bio-production of butanol and 2,3-butanediol is still not economically competitive compared to petrochemical-derived production because of their drawbacks. Hence, the importance of these compounds and the challenges impeding the development of the corresponding microbial production platforms are discussed in the following subsections.

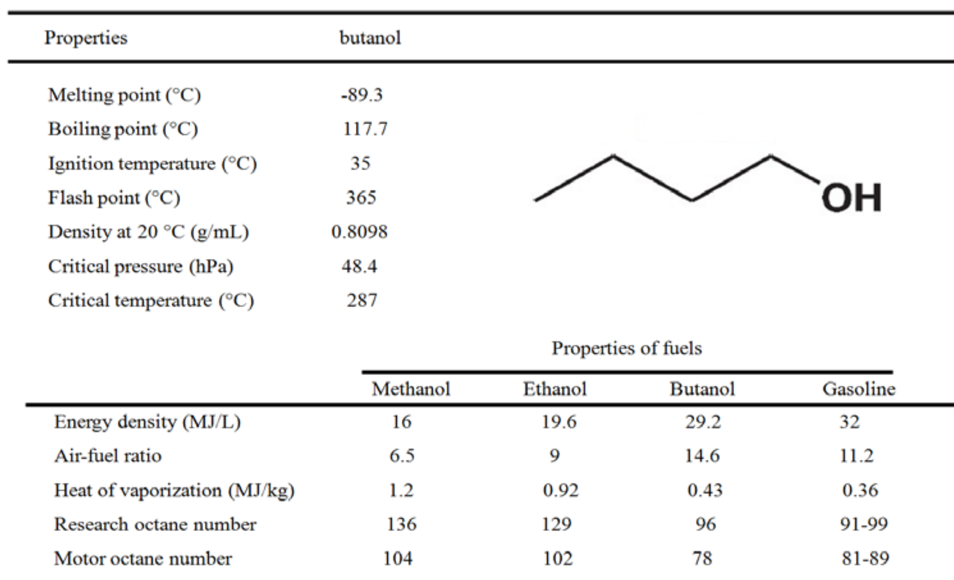
1.1.1 Importance of butanol and problem statement

1.1.1.1 Importance of butanol in industry

Butanol (C_4H_8O), a four carbon primary alcohol, is not only an important intermediate chemical, but is also considered as a promising next generation liquid fuel (Yang Gu, 2011). Table 1.1 lists and compares some properties of butanol with other liquid fuels. The distinct merits of butanol over other biofuels are as follows:

1) It has sufficiently similar characteristics to gasoline and can be used as a direct replacement of gasoline or as fuel additive without the requirement for engine modification and/or substitution.
2) Butanol is easy to transport because of its low vapor pressure. It is also less corrosive than ethanol, so that it can extend the service life of the pipeline through which it is transported.
3) The heat of vaporization of butanol was 0.43MJ/kg, which is slightly higher than gasoline (0.36MJ/kg) and much lower than ethanol (0.92MJ/kg). Therefore, an engine running on butanol-blended gasoline should not have the cold weather issues as ethanol or methanol blended gasoline.
4) It is not hygroscopic, which allowed blending of gasoline at the refinery, well ahead of storage and distribution.
5) Low solubility of butanol resulting in less possibility of groundwater contamination.
6) The butanol derivatives of dibutyl ether and butyl-butyrate have the potential for diesel and avation fuel (Alasfour, 1997; Dürre, 2007; Ranjan and Moholkar, 2012).

Table 1.1 Comparison of properties of different fuels (Dürre, 2007).



Properties	butanol
Melting point (°C)	-89.3
Boiling point (°C)	117.7
Ignition temperature (°C)	35
Flash point (°C)	365
Density at 20 °C (g/mL)	0.8098
Critical pressure (hPa)	48.4
Critical temperature (°C)	287

	Properties of fuels			
	Methanol	Ethanol	Butanol	Gasoline
Energy density (MJ/L)	16	19.6	29.2	32
Air-fuel ratio	6.5	9	14.6	11.2
Heat of vaporization (MJ/kg)	1.2	0.92	0.43	0.36
Research octane number	136	129	96	91-99
Motor octane number	104	102	78	81-89

In addition to a potential biofuel, butanol is an important bulk chemical precursor. Half of the butanol production was used in the form of butyl acrylate and methacrylate esters used in latex surface coating, enamels and lacquers (Kirschner, 2006). Butyl glycol ether, butyl acetate and plasticizers are all important butanol derivatives. Recently, butyl-butyrate ($C_8H_{16}O_2$) attracts great attention as it is not only used as additives for the food industry, but also found to be a good aviation fuel additive (Phodri et al., 2013). It shows good compatiblility with petrol and has similar properties to the recently published alternative fuel substitutes ethyl acetate, ethyl propionate and ethyl butyrate (Phodri et al., 2013). Unlike these fuels, butyl butyrate is compatible with kerosene with a melting point below -47°C and a flash point above 38 °C, as well as remaining misciblewith kerosene at low temperatures (Phodri et al., 2013). Butyl butyrate is thus a promising additive for the aviation sector.

Furthermore, butanol is an excellent diluent for brake fluid formulations and solvent for the manufacturing of antibiotics, vitamins and hormones (Lee et al., 2008). The annual production of butanol has been estimated to be 10-12 billion pounds, which accounts for 7-8.4 billion dollar market with a projected market expansion of 3% per year (Donaldson et al., 2007 and Kirschner, 2006).

1.1.1.2 Challenges in ABE fermentation

As an important solvent and transport fuel additive, butanol can be produced by microbial acetone-butanol-ethanol (ABE) fermentation. The best known strains for butanol fermentation are the solventogenic *Clostridium acetobutylicum* and *C. beijerinckii*. Attempts to generate a superior microbial producer of butanol and develop a more economical bio-process for butanol production have been made. However, to date, butanol bio-production is still not economically competitive compared to petrochemical-derived production because of its major drawbacks, such as, high cost of the feedstocks and lignocelluloses-degrading enzymes, low butanol concentration in the fermentation broth and the co-production of low-value by-products (acetone and ethanol). Here we analyze the main challenges in microbial ABE fermentation.

1.1.1.2.1 High cost of traditional feedstock and lignocelluloses-degrading enzymes

Butanol production by ABE fermentation is one of the oldest fermentation processes employed for commercial production of a chemical to benefit mankind (Lee et al., 2008). Traditional microbial ABE fermentation uses starchy feed-stocks (such as

corn) or molasses as preferred substrates. However, utilization of such substrate is impeded by its high cost. For instance, production of each ton of ABE solvents required 4.0~4.5 tons of corn, 13~25 tons of steam, 20~30 tons of water and 700~1000 kWh of electricity in China (Yang et al., 2011). Corn prices had reached around 322 $/ton (Jilin, corn belt in China) by the first half of 2011, which means that the feedstock cost represent over 70% of total production cost (Gu et al., 2011). Other feedstocks, such as cassava and molasses, are unsatisfactory for meeting the demands of fermentative ABE production because their basic price has also been rapidly pushed up in recent years (Gu et al., 2011). Therefore, searching for other low-cost renewable resources has become a fundamental requirement for sustainable development of ABE production.

In this regard, lignocellulose being the most abundant renewable resource is recognized as having great potential as the substrate for ABE fermentation, provided that the hemicellulose and cellulose components can be enzymatically hydrolyzed and efficiently utilized by solventogenic *Clostridium* species (Annous and Blaschek, 1991; Demirbas, 2007; Ezeji et al., 2007). However, the inefficiency of the bacteria in the utilization of pentoses in the lignocellulosic hydrolysate and cost of lignocellulose-degrading enzymes limited the scale up of ABE fermentation. As known, pentose constitutes 20 to 60% of the sugars in the hydrolysate of lignocellulose (Zaldivar et al., 2001). So far, the biofuel-generating microbes in common use are either unable to utilize pentoses at all (e.g., *Saccharomyces cerevisiae*) or consume hexoses first and then pentoses with a low yield. Good examples are the solventogenic *Clostridium* sp. (e.g., *C. acetobutylicum*, *C. beijerinckii*, and *C. pasteurianum*), which are among the few microorganisms able to ferment both pentose and hexose sugars (Compere and Griffith,

1979). However, the solvents, including butanol, are still produced mainly from glucose, while small amounts are produced from xylose, as observed in cultures of *C. acetobutylicum* ATCC 824 and *C. beijerinckii* NCIMB 8052 when fermenting a mixture of glucose and xylose. Unless glucose and xylose are both utilized efficiently, converting lignocellulosic biomass into biologically based products is unfavorable from an economic viewpoint, as yields would be limited. So, novel strains with efficient pentose utilization are needed urgently to make utilization of lignocellulosic hydrolysate more applicable.

Another issue in bioconversion of lignocellulose to biobutanol is the costs of lignocellulose-degrading enzymes. Although solventogenic *Clostridium* sp. utilizes a broad range of monosaccharides, disaccharides, starches, and other substrates, such as inulin, pectin, and whey, it can not directly utilize cellulose and hemicellulose for butanol production. With the catalysis of lignocellulose-degrading enzymes, such as cellulase and xylanase etc., the solventogenic *Clostridium* sp. could ferment the component sugars (hexoses and pentoses) in the lignocellulosic hydrolysate to biobutanol (Ezeji et al., 2007). According to recent techno-economic evaluations, lignocellulose-degrading enzyme (10–20 %) is the third contributor to the overall costs of producing biofuel from lignobiomass followed by the raw material (30–40 %) and the capital investment (30–45 %). Producing biobutanol directly from cellulose or hemicellulose, known as consolidated bioprocessing, is believed to reduce costs substantially compared to a process in which cellulose/hemicellulose degradation and fermentation to butanol are accomplished in separate steps. Consolidated bioprocessing utilizes micro-organisms to perform biomass hydrolysis and the fermentation of the sugars into biofuel within a single process. Althought different attemps, such as metabolic reconstruction of strains with capability of

both butanol production and cellulose/hemicellulose degradation and co-culturing bacterial systems have been made to achieve direct butanol production; however, only lower amounts of butanol or only acids were produced. For example, Higashide et al., have successfully constructed a *C. cellulolyticum* strain for isobutanol synthesis directly from cellulose by exploiting the host's natural cellulolytic activity and the amino acid biosynthesis pathway and diverting its 2-keto acid intermediates toward alcohol synthesis. Finally, only 0.66 g/L isobutanol could be produced directly from cellulose (Higashide et al., 2011). On the other hand, only fatty acids were generated from cellulose by coculturing cellulolytic *C. cellulolyticum* H10 with *C. acetobutylicum*, suggesting that the fermentation process had stopped at the acid-forming phase prior to the solvent-forming phase for *C. acetobutylicum* (Petitdemange et al., 1983). Similar results were also reported by using *C. thermocellum* and *C. acetobutylicum* (Yu et al., 1985). The failure of solventogenesis to occur was attributed to the slow reducing sugars releasing rate as a direct result of the rate-limiting enzymatic hydrolysis of the lignocellulosic substrates. To overcome this barrier, more efficient lignocellulose degrading cultures are needed to apply to this co-culturing system to achieve direct butanol production from celulose/hemicellulose.

1.1.1.2.2 Butanol toxicity in ABE fermentation

The tolerance of microbes to their products is always one of the issues widely focused on biological production of chemicals and biofuels. Butanol is a lipophilic solvent, which could change the membrane structures and interfere with normal functions by partitioning into the cytoplasmic membranes (Liu and Qureshi, 2009). Membrane fluidity increases by 20-30% in response to 1% butanol exposure was observed, which

would result in the disruption of membrane associated functions such as various transport processes, glucose uptake and membrane-bound ATPase activity. Most of the butanol-producing bacteria could not tolerate more than 20 g/L butanol (Lin and Blaschek, 1983). Cell growth would be reduced by 50% in the presence of 7-13 g/L of butanol. While the addition of acetone and ethanol up to 40 g/L reduced growth by 50%. Butanol is the only solvent that can reach the toxic level during the ABE fermentation process (Jones and Woods, 1986). This low butanol concentration directly increases the recovery cost. According to economic analyses, if the final butanol titer can reach 19 g/L in the fermentation broth, the product recovery cost would be reduced by half. Attempts have been made to improve the butanol concentration up to 17.6 g/L by genetically manipulating the wild-type *Clostridium* species (Harris et al., 2001). Nevertheless, genetically modified bacteria are usually unstable due to plasmid excision (Heap et al., 2007), leading to the deterioration of butanol-producing capability within batches of experiments. Considering this, conversion of lipophilic butanol to non-toxic and more economically valuable product, such as long-chain ketones and short-chain esters provides another alternative strategy. However, the rational strategies are still needed to be rationally designed to further improve the final ketones or esters production.

1.1.1.2.3 By-products in ABE fermentation

In the typical ABE fermentation pathway, acetone, butanol and ethanol are normally produced in a ratio of 3:6:1. Acetone and ethanol are still two non-ignorable products, normally representing 30% and 10%, respectively (Jones and Woods, 1986). Actually, the existence of acetone and ethanol impacts the economics of ABE fermentation: they are much less profitable than butanol and, to separate these three

products, a continuous distillation method utilizing a series of distillers has to be used. Therefore, the engineering strategies for improving butanol selectivity, namely converting ABE fermentation into a butanol-only fermentation, or isolation of novel strains with non by-products formation have attracted general interest. This alternation of solventogenic clostridia will increase butanol yield and simplify the recovery process.

However, it should be noted that owing to our relatively poor understanding of complex metabolic pathways of solventogenic clostridia, great challenges still remain in realizing a real single-butanol process by metabolic engineering, such as, how to block branch pathways without causing undesired phenotypes (e.g., acids assimilation, deficient growth rate), how to direct the flow specifically to butanol, how to offer enough reducing force to support butanol formation, etc. The most recent advancement was disruption of acetoacetate decarboxylase gene (adc) in the hyperbutanol-producing industrial strain *C. acetobutylicum* EA 2018 using TargeTron technology. The butanol ratio increased from 70% to 80%, with acetone production reduced to approximately 0.21 g/L in the adc-disrupted mutant (2018adc). However, due to the deficiency of reducing force, additional reducing equivalents, such as artificial electron acceptor methy viologen had to be added, although the final butanol concentration still remained at 12-13 g/L (Jiang et al., 2009). By far, the most efficient approach to solve this barrier is the isolation of novel wild-type microbes with non by-products (acetone or ethanol) production. For instance, a new butanol-acetone-producing *Clostridium* sp. strain G117 was isolated in our lab, which could produce 13.5 g/L butanol from 60 g/L glucose with a butanol/acetone ratio of 2.14 (Chua et al., 2013). The elimination of ethanol in the fermentation broth could decrease the downstream separation cost. The lack of ethanol-forming dehydrogenase is

responsible for its non-ethanol production. It is know that acetone does not qualify as fuel and should be separated from the final products which results in net low yield of solvents. Recently, a butanol-ethanol producing *C. sporogenes* BE01 was isolated (Gottumukkala et al., 2013). The culture is not considered as an efficient producer of butanol in comparison with commercial strains like *C. acetobutylicum*, because it only produced 5.52 g/L butanol. However, the ability of the culture to grow and produce butanol-ethanol only in rice straw hydrolysate indicates the potential to be used for biobutanol production. Considering this, further work is still needed to improve the final butanol production and isolate or metabolically construct only butanol-producing microbes.

1.1.2 Importance of 2,3-butanediol and problem statement

2,3-butanediol ($C_4H_{10}O_2$) is also known as 2,3-butylene glycol, dimethylene glycol, dimethylethylene glycol and the IUPAC name is butane-2,3-diol. One of 2,3-butanediol's possible applications is its conversion to 1,3-butadiene, which can be further used in synthetic rubber production. Due to its low freezing point of -60 °C, 2,3-butanediol is used as an antifreeze agent (Soltys et al., 2001). Furthermore, this compound or its derivatives have shown wide applications in plastics and solvent production. The product of 2,3-butanediol dehydrogenation, diacetyl, can serve as a highly-valued flavoring agent in food products, giving a buttery taste (Bartowsky and Henschke, 2004). Diacetyl is also a bacteriostatic food additive, since it inhibits growth of some micro-organisms. Dehydration of 2,3-butanediol gives methyl ethyl ketone (MEK;butan-2-one), which is an effective fuel additive having a higher heat of

combustion than ethanol. MEK can also be used as the solvent for resins and lacquers. The heating value of 2,3-butanediol (27 198 J g^{-1}) compares favorably with other liquid fuels e.g. methanol (22 081 J g^{-1}) and ethanol (29 05 5 J g^{-1}) (Flickinger, 1980). Equimolar mixture of ethanol and 2,3-butanediol can provide a combined heating value of 27 660 J g^{-1}, so 2,3-butanediol can be used as the fuel additive (Yu and Saddler, 1982b). 2,3-butanediol due to its high octane number can serve as an "octane booster " for petrol. Polyurethane-mel-amides (PUMAs), synthesized by esterification of 2,3-butanediol with maleic acid, were found to be useful in cardiovascular applications (Petrini et al.,1999). Other products of 2,3-butanediol esterification are used mainly in the pharmaceutical and cosmetical branch. Further potential applications of 2,3-butanediol are: production of printing inks, perfumes, fumigants, spandex, moistening and softening agents, plasticizers (e.g. cellulose nitrate, polyvinyl chloride, polyacrylates) and carrier for pharmaceuticals (Garg and Jain, 1995).

Due to the wide application of 2,3-butanediol, the world butanediol consumption increased by nearly 6% to reach 1.76 million tones in 2011. Asia accounted for over half (55%) of the overall butanediol consumption volume. It was followed by North America and Europe with shares of 25% and 19.5%, respectively. The global butanediol production is forecast to increase by about 6% annually through 2017, exceeding the 2.49 million tonnes mark by the end of the forecast period. Asia is primed to maintain its leadership position in the overall butanediol market in the oncoming years (http://mcgroup.co.uk/news/20140516/ butanediol-bdo-production -volume-249-mln-tonnes.html).

So far, absolutely unbeatable in efficient production of 2,3-butanediol are *Klebsiella pneumoniae*. However, its by-products, like formic acid, lactic acid and acetic acid etc will affect the final yield of 2,3-butanediol. Hence, novel strains with only 2,3-butanediol or other value-added product as final metabolic products are needed urgently. As far as bulk chemicals production is concerned, the major cost in most bioconversion processes appears to be the substrate cost and the price of the final product is mostly affected by the raw material cost (Ji et al., 2011). Thus, availability of an inexpensive carbohydrate raw material is essential for developing an economical fermentation process for the production of 2,3-butanediol.

1.2 Research objectives

This thesis attempts to address the bioprocessing challenges of the widely used four carbon alcohols - butanol and 2,3-butanediol. The specific aims with regards to butanol production are specified below, while that for 2,3- butanediol production is provided in the subsequent section.

1) To make ABE fermentation process more economically applicable, novel wild-type microbes capable of cofermenting glucose and xylose in lignocellulosic hydrolysate to butanol (up to 15 g/L) and other value-added product, such as riboflavin will be cultivated and characterized. The mechanism of efficient utilization of xylose and elimination of xylose

repression will be evaluated by analysis of the expression levels of xylose-utilizating enzymes.

2) To achieve direct butanol production from hemicellulose, novel bacterium capable of efficiently degrading lignocellulose will be cultivated first, and then a co-culturing bacterial system containing lignocelluloses-degrading and solventogenic strains will be set up. The high reducing sugars releasing rate in this system will result in higher butanol production than previous studies.

3) To solve the problems of butanol toxicity in ABE fermentation, strategies for bioconversion of butanol to more value added product - butyl-butyrate will be developed. Through integration of ABE fermentation by *Clostridium* sp. strain BOH3, lipase-catalyzed esterification and *in situ* extraction, higher butyl-butyrate production will be obtained. This short-chain ester – butyl-butyrate could be applied in different setors, such as food, health and fuel etc.

To make microbial production of 2,3-butanediol more economically competitive, novel strains with little by-products and efficient utilization of cheap carbon-source, sucrose are needed. The specific scope of the study is:

4) To isolate novel 2,3-butanediol producing strains capable of utilizing low cost sugar - sucrose without by-products production. Strain improvement and process optimization using statistical methods will be both adopted to further improve the final 2,3-butanediol production and yield.

1.3 Organization of thesis

The thesis is subdivided into the following chapters, each defining a specific area of study that contributed to meeting the overall objective. Each chapter will contain individual introduction, materials and methods, results and discussion section specific to the area of study.

- **Chapter 2: Literature review**

  This chapter provides a comprehensive review of the development and advances of ABE fermentation and 2,3-butanediol production.

- **Chapter 3: Simultaneous fermentation of glucose and xylose to butanol by *Clostridium* sp. strain BOH3**

  This chapter demonstrates efficient xylose utilization and elimination of carbon catabolic repression in *Clostridium* sp. strain BOH3 and co-production of butanol and riboflavin from glucose and xylose mixtures.

- **Chapter 4: Characterization of a thermostable xylanase from a newly isolated *Kluyvera* species and its application for biobutanol prodcution**

  This chapter illustrates characterization of a novel cellulase-free thermostable xylanase from a newly isolated *Kluyvera* sp. strain OM3 and set up of a co-culturing system for direct butanol production from hemicelluloses.

- **Chapter 5: Integration of biobutanol and lipase-catalyzed butyl-butyrate production in a single reactor**

This chapter exhibits different strategies to achieve high butyl-butyrate production via ABE fermentation by *Clostridium* sp. strain BOH3, lipase-catalyzed estefication and *in situ* extraction.

- **Chapter 6: Production of 2,3-butanediol from sucrose by a *Klebsiella* species**

  This chapter demonstrates high 2,3-butanediol and ethanol production by a novel *Klebiella* species from sucrose via process optimization and strain improvment.

- **Chapter 7: Conclusion and recommendations**

  The overall conclusion and recommendations for future studies are presented.

Chapter 2

Literature Review

2.1 Microbial production of butanol

Early industrial production of butanol was based on fermentation of the bacterium *C. acetobutylicum* which ferments carbohydrates and produces mainly butanol and acetone (Jones and Woods, 1986). However, increasing demand for butanol and the dramatic growth of the petrochemical industry saw biological processes replaced by more efficient chemical processes. In recent years, high crude oil price and increasing concerns over global warming have renewed interest in biotechnological production of butanol, not only as a chemical but also as an alternative fuel. Reflecting this, a number of companies are developing bio-butanol processes. BP and DuPont, for example, recently announced a joint effort to develop a fermentative butanol process. BP has also created a subsidiary, BP Biofuels, to commercialize butanol by fermentation. Many venture companies have also been established aiming to commercialization of biobutanol production (Lee et al., 2008). The successful industrial-level butanolfermentation in these companies can provide guidelines to our current effort to produce butanol in large-scale.

This review focused on characteristics of ABE fermentation, factors which may influence ABE fermentation and advances in ABE fermentaion that were (or potentially could be) applied to the process performed at industrial-scale.

2.1.1 Characteristics of ABE fermentation

2.1.1.1 History of ABE fermentation

The clostridial ABE fermentation represents one of the oldest industrial fermentation processes known, ranking second in scale only to ethanol fermentation by

yeast. ABE can be traced back to 1862, when the famous French scientist Louis Pasteur reported this alcohol to be a fermentation product of "Vibrion butyrique", which was produced by a mixed culture presumably containing clostridia of the *C. butyricum* or *C. acetobutylicum* type (Jones and Woods, 1986). As a pioneer in biobutanol production study, Pasteur paved the way for fermentative ABE to prosper during the early 20th century and became the second largest industrial fermentation process ever performed (after ethanol). In 1945, approximately two thirds of the world's butanol supply stemmed from fermentation. Unfortunately, the biological process lost its competitiveness by 1960s due to the increase of substrate costs and low crude oil prices, rendering chemical butanol production more attractive except in Russia and in South Africa. Until the late 1980s to early 1990s, the biological butanol production refineries continued to operate in South Africa and Russia, where the substrate and labor costs were low (Zverlov et al., 2006). From then until 2005, butanol was only considered as a bulk chemical feedstock. However, the ABE fermentation process revived in 2007 as BP and DuPont has announced that they were restarting the industrial biobutanol production for biofuel supply in the United Kingdom. Meanwhile, China and Brazil were also reopening their fermentation plants. From then on, the prospect of biobutanol production has really been brightened (Dürre, 2007; Lee et al., 2008).

2.1.1.2 Microorganisms

There are a number of species of *Clostridium* capable of producing a significant amount of sovlents during the later stages of batch fermentation under the appropriate conditions. Clostridia are rod-shaped, spore-forming Gram positive bacteria and typically strict anaerobes. The strain that has been used most extensively in producing

butanol is what we now commonly know as *C. acetobutylicum* (Lee et al., 2008). Other strains, including *C.beijerinckii, C. saccharobutylicum*, and *C. saccharoperbutylacetonicum* that also produce solvents are also identified. They are classified mainly based on the research results of phylogeny, genomic study and fermentation performance (Johnson et al., 1997; Keis et al., 1995; Shaheen et al., 2000). Solventogenic clostridia can utilize a large variety of substrates from monosaccharides including many pentoses and hexoses to polysaccharides (Jones and Woods, 1986). Complex nitrogen sources such as yeast extract are generally required for good growth and solvent production, but otherwise the nutrient requirements for the growth of Clostridia are rather simple. Besides, Clostridia require high redox potential to produce butanol (and ethanol) and the supply of additional reducing power results in increased butanol formation (Keis et al., 1995).

The choice of strains for use in industrial fermentation depends on the nature of the raw material used, the ratio of end products required, the need for additional nutrients, and phage resistance (Jones and Woods, 1986). In the majority of cases, to make ABE fermentation more economically applicable strains exhibiting the desired characteristics, such as simultaneous utilization of glucose and xylose in the lignocellulosic hydrolysate, high butanol production and co-production of other value added products are favourable. Meanwhile, with the delevopment of metabolic engineering tools and isolation strategies, more attempts have been made to improve strains by means of genetic manipulation and isolation of novel wild-type microbes with desired characteristics, such as non by-products formation etc.

2.1.1.3 Metabolic pathways in ABE fermentation

Fermentation process inherently relies on the level of metabolic activities of the microorganism. Fortunately, the metabolic pathways of *Clostridium* species (*C. acetobutylicum* and *C. beijerinckii*) are alike. Major end products from metabolic pathway activities are solvents (acetone, butanol, and ethanol), acids (acetic acid and butyric acid) and gases (carbon dioxide and hydrogen) (Lee et al., 2008). At the beginning of the metabolism of *Clostridium*, depending on the type of carbon source used, pathways that lead to the formation of pyruvate are different. When hexoses (glucose, fructose, and galactose) are used, they are catabolized through the Embden-Meyerhof-Parnas (EMP) pathway, whereas when pentoses (xylose and arabinose) are used, it follows the pentose phosphate pathway to finally produce pyruvate (Jones &Woods, 1986) (Fig. 2.1). After that, pyruvate is converted to acetyl-CoA by pyruvate ferredoxin oxidoreductase (Pfor) before it branches into different pathways thereafter (Dürre, 2007; Lee et al., 2008).

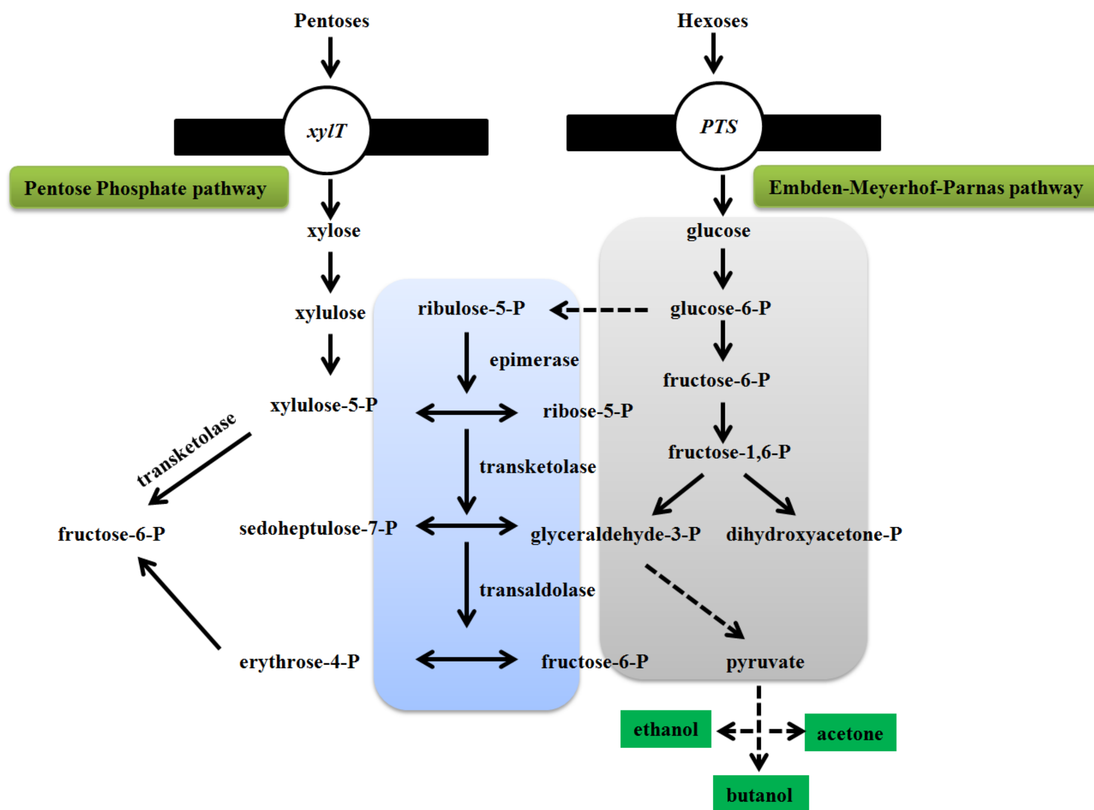
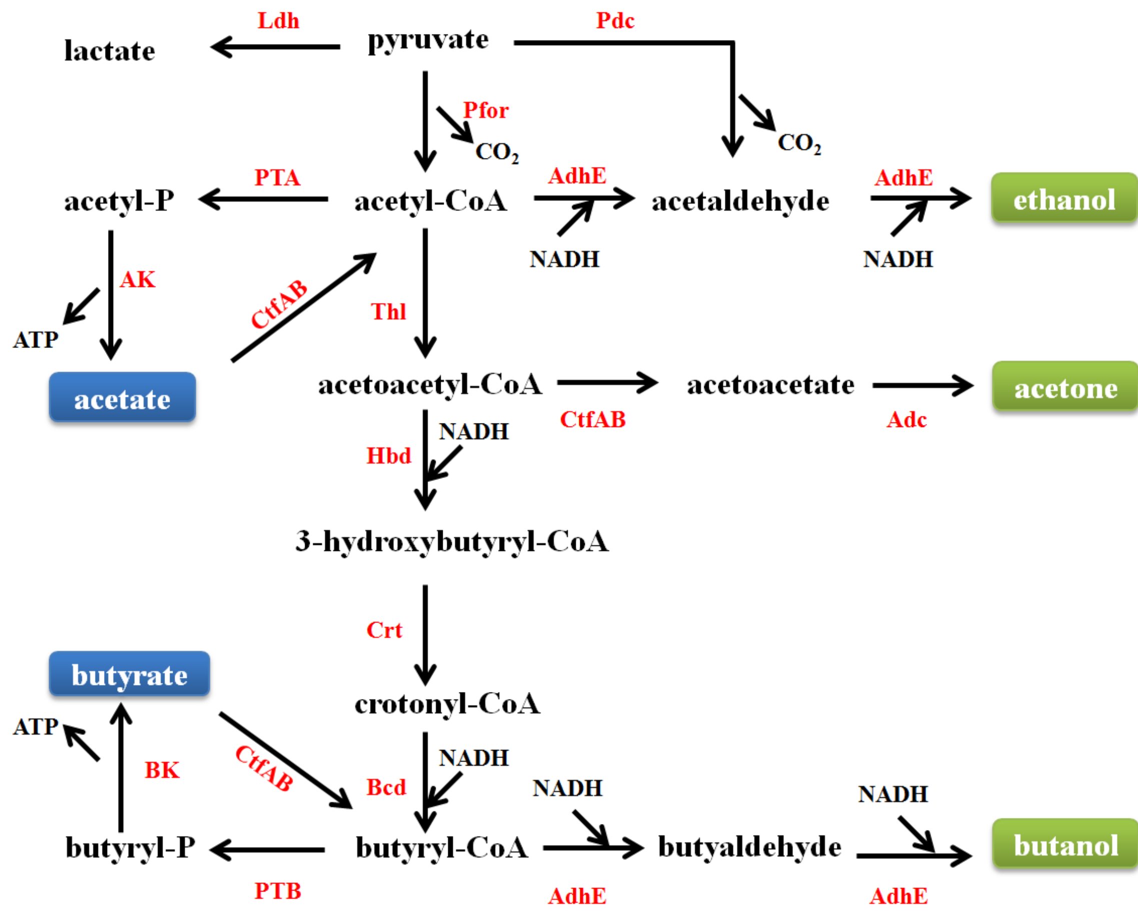


Fig. 2.1 Hexoses and pentoses metabolism in solventogenic *C. acetobutylicum*.

The carbon flow from acetyl-CoA through the main branches of the pathway leading to the formation of acids and solvents is shown in Fig. 2.2. These branch points arise from three key intermediates, acetyl-CoA, acetoacetyl-CoA, and butyryl-CoA and lead to the first phase of fermentation – acidogenesis phase (Fig. 2.2A). During this phase, acetate and butyrate are produced from acetyl-CoA and butyryl-CoA by means of two analogous steps which result in the production of the corresponding acyl-phosphate, followed by the generation of ATP. As ATP produced is used to support rapid growth of *Clostridium* in this phase, so bacteria grow exponentially in the acidogenesis phase along the formation of acids (mostly acetate and butyrate), leading to rapid decrease of pH to ~ 4.5 (Lee et al., 2008).

A



B

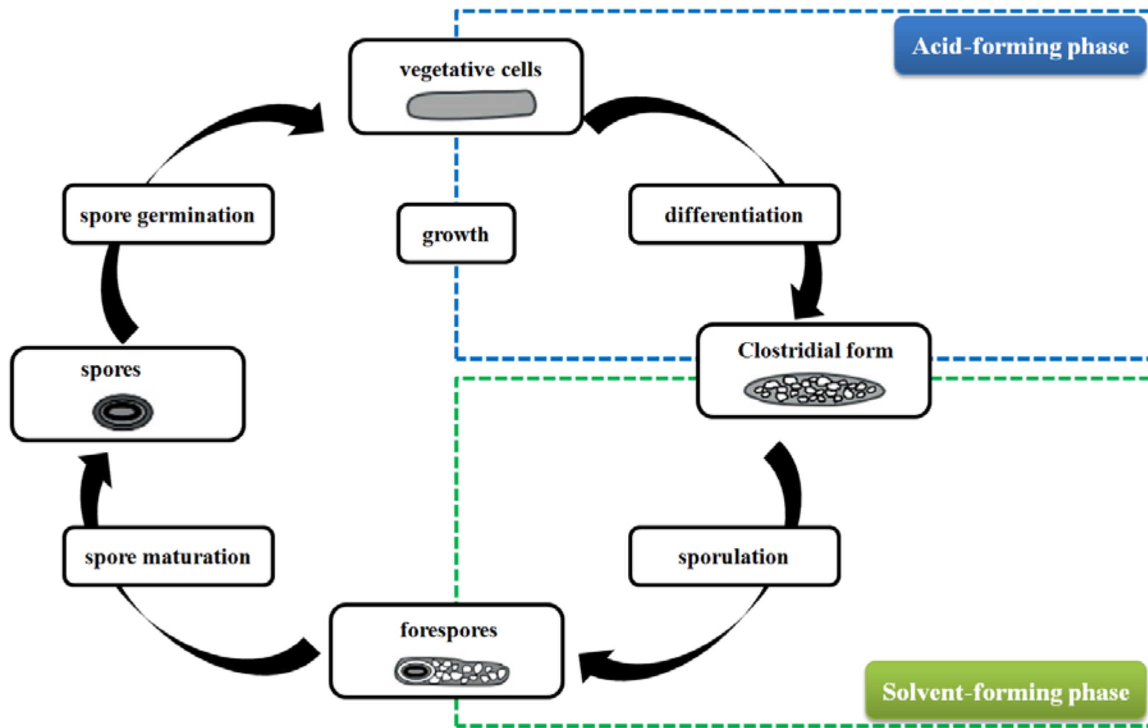


Fig. 2.2 Acetone-butanol-ethanol fermentation pathway in *C. acetobutylicum*: (A) and their relation to the life cycle stages: (B). (A) The red letters show the enzymes involved in the fermentative pathways: Ldh, lactate dehydrogenase; Pdc, pyruvate decarboxylase; Hyd, hydrogenase; Pfor, pyruvate:ferredoxin oxidoreductase; Fd, ferredoxin; PTA, phosphotransacetylase; AK, acetate kinase; AdhE, aldehyde/alcohol dehyrogenase; CtfAB, acetoacetyl-CoA:acyl-CoA transferase; Adc, acetoacetate decarboxylase; Thl, thiolase; Hbd, 3-hydroxybutyryl-CoA dehydrogenase; Crt, crotonase, Bcd, butyryl-CoA dehydrogenase; Ptb, phosphotransbutyrylase, BK, butyratekinase. (B) Acidogenesis and solventogenesis referring to the respective growth stages.

The second phase is the solventogenic phase during which acetyl-CoA and butyryl-CoA function as the key intermediates for ethanol and butanol production. These pathways produce acetylaldehyde and butyraldehyde, respectively, as intermediates, and the pathway requires the function of two sets of dehydrogenases to accomplish the necessary reductions to produce ethanol and butanol. Early workers observed that once the shift to solvent production had taken place, the acid end products produced during the initial fermentation phase were reassimilated. The uptake of acetate and butyrate, however, only occurred when sugars were metabolized concurrently (Andersch et al., 1983). It has been suggested that the uptake of acids (normally accompanied by an increase in pH) which occurs during solvent production functions as a detoxification process initiated in response to the accumulation of acid end products, which result in conditions unfavorable for growth (Andersch et al., 1983).

There should also be noticed that addition of exogenous acids, such as acetate and butyrate could improve solvent production and eliminate strain degeneration (Lee et al., 2008). The addition of sodium acetate to chemically defined MP2 medium was found to increase and stabilize solvent production and also increase glucose utilization by *C. beijerinckii* NCIMB 8052. RNA and enzyme analyses indicated that coenzyme A (CoA) transferase was highly expressed and had higher activity in *C. beijerinckii* NCIMB 8052 grown in MP2 medium containing added sodium acetate than in the microorganismgrown without sodium acetate (Chen and Blaschek, 1999). In addition to CoA transferase, *C. beijerinckii* NCIMB 8052 grown in MP2 medium containing added acetate demonstrated higher acetate kinase- and butyrate kinase-specific activity than when the culture was grown in MP2 medium containing no added acetate (Chen and Blaschek, 1999). Based

on this, exogenous acetate should be optimized and added to the medium to further improve butanol production and stabilize the strains.

2.1.1.4 pH and acid crash

The influence of pH has been recognized as a key factor in determining the outcome of ABE fermentation, and many of the early reports relating to the industrial production of solvents noted that the initiation of solvent production occurred only after the medium pH had decreased to around 4.5 to 5.0 (Andersch et al., 1983; Jones and Woods, 1986). Generally, cultures at high pH produce mainly acids, whereas in cultures maintained at a low pH solvent production usually predominates. However, the pH range over which solvent formation may occur appears to vary quite widely depending on the particular strain and the culture conditions used. If *Clostridium* is growing at or close to its maximum growth rate or the metabolic rate is very high, excess acids will be accumulated and prevent the switch from acidogenesis to solventogenesis occurring. Under this condition, if the medium pH is uncontrolled, solvents are generally not produced. This phenomenon is known as “acid crash” (Dürre, 2007; Lee et al., 2008). Jones and Woods explained the occurrence of "acid crash" by stating that "acetate and butyrate in their undissociated form, they are able to partition into the cell membrane and behave as uncouplers which allow protons to enter the cell from the medium" (Jones & Woods, 1986). When the concentration of undissociated acids reaches a sufficiently high value, collapse of the pH gradient across the membrane occurs and causes a rapid decrease in the NTP/NDP ratio, which eventually ceases all the metabolic activities in the cell.

There are four techniques available to prevent "acid crash" in batch fermentation which have the common goal to minimize the acid production rate in the cultures and hence the concentration of undissociated acids. Firstly and probably the most direct way is to control the pH of the cultures to be above certain value that promises solvent production (e.g. pH 5.0). At this pH value, majority of the acids will be in dissociated form and are less harmful to the growth of cells. Secondly, enhance the buffering capacity by the addition of strong buffer agents (such as, 2-(N-morpholino) ethanesulfonic acid (MES)). Highly buffered medium was found to increase the stationary phase cell density, carbohydrate utilization, and the final butanol concentration (Lee et al., 2008). This is a simple method for achieving a high concentration of the less toxic butyrate ion before inhibitory levels of undissociated butyric acid were achieved. Thirdly, the fermentation can be carried out under a lower temperature (e.g. 28°C) to control the metabolic rate of the cells and the acid production rate. However, the shortcoming of this technique is that the productivity would be too low. Lastly, instead of maintaining a constant lower temperature throughout the experiment, it is possible to perform the fermentation under a lower temperature first and then increase the temperature once the solventogenesis phase has taken place. Although it prevents acid crash and at the same time maintains the productivity, however, the cell growth rate will be lower because the cell growth mainly occurs during the acid phase. On a practical basis, finding the optimical pH of the strain, controlling the medium pH at the optimal level and adding desired levels of buffering agents are more efficient and economically feasible for the batch fermentation process.

2.1.2 Advances to improve clostridia-based butanol production

Cost of fermentaion substrates and lignocelluloses-degrading enzymes, butanol toxicity and by-products are three of the most critical problems in ABE fermentation (Chapter I). Substrate and enzymes costs impact the economics of butanol production; butanol toxicity limits the utilization of carbon substrate resulting in low final solvent concentration and productivity; the existence of by-products also impacts the economics of ABE fermentation. To solve these barriers, the advances based on the feedstock selection, strain improvement and novel processes were developed.

2.1.2.1 Feedstock selection

At present, sugar- and starch-based feedstocks are used for butanol production; however these food supplies or main ingredients of animal feed to produce protein product for human being are not sustainable at large scales for fuel use. Therefore, alternative feedstocks that are abundantly available and not food-related need to be developed.

2.1.2.1.1 Lignocellulosic biomass

A lignocellulosic biomass-based route is one of the thorough solutions for sustainable development of ABE fermentation. Because solventogenic clostridia are not able to efficiently hydrolyze lignocellulose, the current route mainly consists of feedstock pretreatment, detoxification, cellulase/xylanase-based saccharification, fermentation and product recovery. Recently, numerous *Clostridium* strains have been evaluated in batch fermentations of various biomass hydrolysates (Table 2.1). These solvent values are comparable to the level achieved in corn- or molasses-based fermentation. The

enzymatic hydrolysate of pretreated corn stover, without following detoxification of the inhibitors, was also suitable for butanol production by *Clostridium* strains, reaching a solvent titer of >14 g/L and a yield of 0.35 g/g fermentable sugars.

Table 2.1 Batch fermentation performance of various biomass hydrolysates by solventogenic clostridia.

Strains	Feedstocks	Total solvents (g/L)	Productivity (g/L/h)	References
C. beijerinckii P260	Wheat straw	25	0.59	Qureshi et al., 2007
C. beijerinckii BA101	Corn fiber	9.3	0.11	Qureshi et al., 2008
C. beijerinckii P260	Wheat straw	13.1	0.14	Qureshi et al., 2008
C. beijerinckii P260	Wheat straw	22.1	0.55	Qureshi et al., 2008
C. beijerinckii ATCC 55025	Wheat bran	11.8	0.16	Liu et al., 2011

It should be noted that, considering the great differences (e.g., different inhibitors and pentoses) occurring in various hydrolysates, we can not expect a strain to efficiently ferment all kinds of hydrolysates. Therefore, developing or isolating ideal clostridial strains for utilizing biomass hydrolysates must be based on the specific pretreatment and hydrolysis process. Pentoses are the basic building blocks of lignocellulosic hydrolysate, among which xylose is normally the monosaccharide unit present in largest amount (Jones and Woods, 1986). As discussed in Chapter I, although the model solventogenic *C. acetobutylicum* is capable of utilizing xylose, its xylose utilization is inherently inferior to that of glucose, thus resulting in a low solvent titer and productivity when using xylose as the sole carbon source (Compere and Griffith, 1979; Gottachalk 1986).

This low utilization of xylose leads to carbon catabolic repression (CCR) when using mixtures of glucose and xylose, such as lignocellulosic hydrolysate (Gu et al., 2009). Due to the greater availability of genetic information and tools in recent years, there has been some progress in improving pentose utilization and eliminating CCR of solventogenic clostridia (Xiao et al., 2013). In *Bacillus subtilis*, a model Gram-positive bacterium, it has been found that a pleiotropic protein, catabolite control protein A (CcpA), is the key regulator for exerting CCR (Kim et al., 1995). However, it will not be known whether the "same" CcpA regulator also exist in *Clostridium* strains until the publication of genome sequencing results for *C. acetobutylicum ATCC* 824. Bioinformatics analysis based on the genome information of the elements of catabolite repression was first performed by Tangney et al. (Tangney et al., 2003) and a putative ccpA gene was then proposed in *C. acetobutylicum*. The experimental validation was recently provided by Ren et al. (Ren et al., 2010) and, interestingly, the knockout of ccpA gene realized partial elimination of glucose repression on xylose metabolism, indicating that a CcpA-mediated CCR does exist in *C. acetobutylicum*. Recently, *Xiao* et al. have metabolically constructed *C. acetobutylicum ATCC* 824 by strengthening xylose transporter (*XylT*), inactivation of phosphoenolpyruvate (PES) dependent phosphotransferase system (PTS), and overexpression of xylose-utilization enzymes (xylose isomerase and xylulokinase), which nearly consumed all xylose (20 g/L) in the presence of glucose (40 g/L) and improved butanol production from 7.85 g/L to 9.11 g/L. This represents the highest butanol production in utilization of glucose and xylose mixtures; however, bottleneck still existed because of its low butanol tolerance in xylose

(Xiao et al., 2011). Hence, novel strains with more efficient xylose utilization are still needed urgently.

Lignocellulosic hydrolysate provides a more cost-effective substrate for scaling up of ABE fermentation, however, the cost of lignocellulose-hydrolyzing enzymes still takes a high percent of 10–20 % during the process (Fahimeh and Radhakrishnan, 2013). Consolidated bioprocessing, in which cellulosic biomass is fermented to desired products in one step without adding externally produced enzymes provides a potential way to eliminate the costs associated with the cellulase production stage. Clostridial co-culture containing cellulolytic and solventogenic species is a potential consolidated bioprocessing approach for producing biochemicals and biofuels from cellulosic biomass. It has been demonstrated that the rate of cellulose utilization in the co-culture of *C. acetobutylicum* and *C.cellulolyticum* is improved compared to the mono-culture of *C. cellulolyticum*, however, the butanol production in this process is still too low (0.2 g.L) because of the inefficient degradation of cellulose (Fahimeh and Radhakrishnan, 2013). Nevertheless, this paves a way for direct convertion of lignocelluloses to butanol. More efficient cellulose/hemicelluloses-degrading strains could be applied to this process to further improve the yield of butanol.

2.1.2.1.2 Genetic engineered plants

Lignocellulosic biomass, particularly residues from agriculture and forest industry that are abundantly available at low cost, and dedicated energy crops that can grow well in marginal land not suitable for grain production are sustainable feedstocks (Heaton et al., 2008). However, like ethanol production from lignocellulosic biomass, these

feedstocks also present challenges for butanol production due to their recalcitrance to degradation and release sugars for fermentations (Himmel et al., 2007).

Plant genetic engineering cannot be employed to grain crops to render the recalcitrant issue of their residues for biofuel production due to the risk in compromising grain output. In contrast, when grain crops are modified for high yield, their residues become more recalcitrant to degradation. However, energy crops such as perennial C4 grasses not only capture solar energy more efficiently than annual C3 grain crops, but also require less inputs and energy consumption (Heaton et al., 2008). Most importantly, energy crops can be genetically modified to improve biomass yield, and in the meantime make them less recalcitrant for degradation (Taylor et al., 2008). For example, the US Department of Agriculture (USDA) sponsored the project: engineering switchgrass to express cell wall -degrading enzymes, which could be induced during processing by elevated temperature not experienced under field growth conditions. Moreover, key enzymes for lignin biosynthesis can also be modified to make the lignin carbohydrate complex less recalcitrant (Saathoff et al., 2011).

2.1.2.2 Strain development

The prime challenge of industrialization of ABE fermentation lies on low butanol tolerance capability of the organism as it destroys the cell membrane. To overcome this challenge, strain improvement programs and isolation of novel wild-type strains were applied to obtain the high butanol producing strains. Chemical mutagenesis is the most traditional and simple method to improve the performance of industrial strains. Mutagenesis was executed randomly to metamorphose the DNA sequence of genes

responsible for ABE production. N-methyl-N-nitro-N-nitroso-guanidine, ethyl methane sulphonate and UV exposure were recognized as effective to improve strain performance (Table 2.2). Recently, Mao et al. have generated a butanol-tolerant mutant strain *C. acebotutylicum* RH8 via chemical mutagenesis and genome shuffling, which showed tolerance to 18 g/L butanol and 15.3 g/L butanol production (increased 23%, as compared to wild strain). Comparative proteomic analysis of Rh8 strain revealed that some proteins involved in ATP synthesis, solvent formation, and protein folding were differently expressed compared with the wild type, indicating that the butanol tolerance is a complex global response (Bao et al., 2014; Mao et al., 2010). In another way, with the development of screening strategies, more novel wild-type strains were isolated and characterized. Cheng et al. have isolated a solvent-producing bacterial microflora, which could produce 17.5 g/L butanol with addition of butyric acid (Cheng et al., 2012). In our laboratory, a unique *Clostridium* sp. strain G117 was obtained, which is capable of producing dominant butanol from glucose. 13.50 g/L of butanol was produced when culture G117 was fed with 60 g/L glucose, which is ~20% higher than previously reported butanol production by wild-type *C. acetobutylicum* ATCC 824 under similar conditions (Chua et al., 2012). Chemical mutagenesis on these wild-type strains could further improve the butanol production.

Table 2.2 Various mutagenesis attempts in butanol producing microorganisms.

Parent microorganism	Mutagens	Mutants	Remarks	References
C. acetobutylicum ATCC 824	Butanol	SA-1	Enhanced butanol tolerance (121% higher than the parentorganism)	Lin and Blaschek, 1983
C. acetobutylicum PTCC-23	UV exposure, N-methyl-N-nitro-N-nitrosoguanidine and ethyl methane sulphonate	MEMS-7	Yield enhanced by 20%	Syed et al., 2008
C. acetobutylicum ATCC 824	N-methyl-N'-nitro-N-nitrosoguanidine together with selectiveenrichment on the glucoseanalog 2-deoxyglucose	BA101, BA-105	Amylolytic activity increased by 82% (BA-101), 25% (BA-105), and increase to nearly 2% final concentration of butanol	Annous and Blaschek, 1991; Formanek et al., 1997
C. acetobutylicum DSM 1731	Chemical mutagenesis and genome shuffling	Rh8	Enhanced butanol tolerance(18 g/L) and production (15.3 g/L,increased 23% than the parent organism)	Mao et al., 2010

Over the last few years, with the improved genetic accessibility available, various metabolic engineering strategies have been tried in solventogenic *Clostridium* species (Table 2.3). Many endeavors that involve in either altering the metabolic regulatory system of *C. acetobutylicum* or overexpressing solvent-producing genes in *C. acetobutylicum* have been made to enhance solvent production. However, these approaches did not improve butanol production significantly. Sillers et al. only improved butanol to 13.2 g/L compared to 13.0 g/L of wild strain by construction of the promoters for enhanced *adhE*, *thl* overexpression and ctfB downregulation (Sillers et al., 2008a). As *C. acetobutylicum* is known to be an obligate anaerobe, so its energy production is rather inefficient. However, protein synthesis is rather energy intensive, so overexpression of related enzymes probably disrupts the biosynthesis of other metabolites that indirectly favor the production of butanol (Oh and Liao, 2000). Therefore, improving the activity of enzyme products by protein engineering may be an alternative method for enhancing solventproduction. Rational design and directed evolution (laboratory evolution) are the approaches generally used in protein engineering. Glieder et al. (Glieder et al., 2002) obtained a highly active alkane hydroxylase by directed evolution. The evolved enzyme exhibited a turnover rate that was 20 times higher than the wild type for the selective oxidation of hydrocarbons of small to medium chain length. Therefore, it is feasible to apply this technique to improve the butanol production.

Table 2.3 Metabolic engineering of *C. acetobutylicum* for butanol production.

Strain	Strategy	Engineered strain/control	References
C.acetobutylicum ATCC 824	Overexpression of adc, ctfA, ctfB	Butanol: 13.2/9.6 g/L Acetone: 8.6/4.5 g/L	Mermelstein et al., 1993
C.acetobutylicum ATCC 824	SolR-inactivated, plasmid-encoded copy of theaad gene	Butanol: 17.6/11.7 g/L Acetone: 8.2/4.9 g/L Ethanol: 2.2/0.7 g/L	Harris et al., 2001
C.acetobutylicum ATCC 824	SpoOA overexpression	Butanol: 10.2/9.2 g/L Acetone:	Thormann et al., 2002
C.acetobutylicum ATCC 824	Promoters for enhanced adhE, thl overexpression and ctfB downregulation	Butanol: 13.2/13.0 g/L Acetone: 3.5/6.3 g/L Ethanol: 14.0/0.9 g/L	Sillers et al. (2008a, 2008b)

By-product is another issue for ABE fermentation. Development of a high butanol ratio or butanol only solventogenic clostridia is an attractive idea, which would significantly improve the economics of current ABE fermentation. To realize this purpose, metabolic engineers mainly focus on eliminating by-product production, primarily acetone, in solventogenic clostridia. The mutant strain *C. acetobutylicum* 2-BrBu1, developed by Janati-Idrissi et al. (Janati-Idrissi et al., 1987) through traditional chemical mutagenesis, no longer produced acetone; however, an undesired decrease of butanol titer and ratio also occurred. In 2003, an antisense RNA strategy was used in *C. acetobutylicum* ATCC824 to down-regulate the expression of *adc* gene, encoding acetoacetate decarboxylase, but no changes were observed in acetone formation (Tummala et al., 2003). The same method was applied to down-regulate *ctfAB* gene,

encoding coenzyme A (CoA) transferase, and, although acetone production was successfully repressed, the production of butanol was also significantly reduced (Tummala et al., 2003). To overcome this problem, the *adhE* gene was re-introduced into the *ctfAB* - repressed *C. acetobutylicum*, which resulted in an increased ethanol titer, but there was no improvement in butanol formation and butanol ratio instead decreased (Tummala et al., 2003). Real progress was achieved with M5 or DG1 (mutant strains without megaplasmid pSOL1). Through expressing the *aad* gene in the M5 strain, the butanol ratio was increased to over 90% despite the unsatisfactory solvent titer, which only reached half the level seen with the wild-type strain (Nair and Papoutsakis, 1994; Cornillot et al., 1997). A better result was obtained by expressing both *adhE1* and *ctfAB* in the M5 strain, and the metabolically engineered M5 was able to produce 154 mM (11.4 g/L) butanol with a butanol ratio of 0.84 (Lee et al., 2009). A novel advance involved in disrupting the *adc* gene in *C. acetobutylicum* EA 2018, a strain that has been licensed to several plants in China (Green, 2011), resulting in a dramatic decrease of acetone production (Jiang et al., 2009). Through the subsequent pH-controlled fermentation and by adding methyl viologen (anelectron acceptor), the butanol ratio of EA 2018 was increased to over 82%, and butanol concentration reached 12-13 g/L, a value comparable to the level of the wild-typestrain. It is clear that, owing to our relatively poor understanding of complex metabolic pathways of solventogenic clostridia, great challenges still remainin realizing a real single-butanol process by metabolically engineering, such as, how to block branchpathways without causing undesired phenotypes (e.g., acids assimilation, deficient growth rate), how to direct the flow specifically to butanol, how to offer enough reducing force to support butanol formation,

etc. Compared to the genetical modification of wild-type strains, sucessfuel isolation of novel ones provides another perspective. *Clostridium* sp. strain G117 isolated by our laboratory has shown high butanol production (13.6 g/L) with non-ethanol production (Chua et al., 2013) and the whole genome sequence has also been available (Wu et al., 2012). Meanwhile, a butanol-ethanol producing *C. sporogenes* BE01 was also isolated (Gottumukkala et al., 2013). However, there are still no only-butanol producing strains reported. Modification of these bacteria will show great potential for only butanol production in the further studies.

2.1.2.3 Development of novel fermentation processes

2.1.2.3.1 Co-production of riboflavin (VB2)

In addition to butanol production, certain biofuel-generating microbes, such as *Clostridium*, can produce other value-added products (e.g., riboflavin, also known as vitamin B2), which adds to the economical value of the butanol fermentation process. Actually, in addition to producing butanol, *Clostridium* species are also one of the commercial microorganisms used to produce riboflavin on an industrial scale, although they only generate a very small amount of riboflavin (<50 mg/L) (Xin et al., 2014). Riboflavin, a yellow, water-soluble vitamin, is biosynthesized solely by plants and numerous microorganisms but not by vertebrates. Thus, this vitamin has to be supplied by foods and dietary supplements. Currently, industrial production has shifted completely from chemical synthesis to microbial fermentation, which has become one of the major fermentation products in the biotechnology industry, with an annual market demand of several million kilograms (Stahmann et al., 1979). Therefore, the

coproduction of butanol and riboflavin would make the fermentation process more economically feasible (Qureshi and Blaschek, 2001). By over-expression of riboflavin operon *ribGBAH* and purine pathway gene of *purF* in *C. acetobutylicum* and bufferting the culture pH, *C. acetobutylicum* ATCC 824 (pJpGN) could accumulate more than 70 mg/L riboflavin while producing 14.1 g/L butanol (Cai et al., 2011). The riboflavin production was shown to exert no effect on solvent production at these levels, which supports the rationale of co-production of a high-value product in improving the economics of the ABE fermentation process. When better riboflavin-producing strains and lignocellullosic substrates are used, it can be expected that higher amount of riboflavin will be produced and more economic value will be obtained during ABE fermentation.

2.1.2.3.2 Conversion of butanol to more economically valuable product

The recovery of butanol from fermentation broth is energy-intensive since typical butanol concentrations in the fermentation broth are below 15 g/L. To prevent butanol inhibition and high downstream processing costs, bioconversion of ABE to more value-added product, such as long chain ketones or alcohols or short-chain esters provies another perspective. Anbarasan et al. have proposed a chemical route to convert ABE fermentation products from a variety of renewable carbohydrate sources into hydrocarbons which can be used for petrol, jet fuel and diesel (Anbarasan et al., 2011). Because solventogenic fermentation products have lower carbon numbers that are appropriate for these fuels, coupling chemistry can be used to produce molecules that are larger than these natural fermentation products. This process provides a means to

selectively produce petrol, jet and diesel blend stocks at yields near their theoretical maxima (Anbarasan et al., 2011). Most recently, Sreekumar et al. described a catalytic strategy to upgrade all three of the solvents produced during ABE fermentation to long-chain ketones, which after hydrodeoxygenation produce the types of hydrocarbons that are also components of gasoline, jet and diesel fuel (Sreekumar et al., 2015).

Another strategy to overcome butanol toxicity is to convert butanol to non-toxic and more valuable short chain esters. Short-chain esters are a class of compounds that can be derived by the condensation of an alcohol with a carboxylic acid (Phodri et al., 2013). These esters are additives for the food industry as flavoring compounds naturally present in pineapple, mango, and banana. During these esters, butyl butyrate is found also to be a good aviation fuel additive (Phodri et al., 2013). Currently, these esters are mainly synthesized via environmentally costly chemical routes by the use of a proper inorganic catalyst at 200-250 °C. However, the increasing demand of these esters has adversely affected market prices, the environment, and national energy security. To tackle this problem, recent research has focused on exploiting microbial conversion routes to produce these chemicals and fuels from renewable and sustainable biomass feedstocks. Van den Berg et al. have set up a proof-of-principle experiment for the one-pot bio-ester production from glucose using *C. acetobutylicum*, which led to 5 g/L in the hexadecane phase (Van den Berg et al., 2012). So far, this is the first report on butyl-butyrate production via ABE fermentation process. However, the product yield is still low because of the limited butanol production. Better solventogenic strains, more rational process strategies could further improve the final butyl-butyrate production.

These advances based on process development will overcome the butanol inhibiton and enable a route for the economical conversion of biomass into liquid transportation fuels.

2.2 Microbial production of 2,3-butanediol

Similar to butanol, 2,3-butanediol is another widely used four carbon alcohol. This review provides information on the biotechnological production of 2,3-butanediol, which gives an alternative to conventional chemistry routes. Hence, the review deals to some extent with the idea of bio-based chemical industry, which should have a significant impact on production of bulk chemicals within the next few years. It is focused on employed microorganisms, factors which may influence the process, and types of biomass or waste substrates that were (or potentially could be) applied to the process performed on industrial-scale.

2.2.1 Microorganisms

A number of microorganism species are able to accumulate 2,3-butanediol, but only a few do so in what might be considered significant quantities. Species which are noted for this ability include those belonging to the genera *Klebsiella*, *Enterobacter*, *Bacillus* and *Serratia*, which are considered of industrial importance in the production of 2,3- butanediol (Ji et al., 2011) (Table 2.4). The wide distribution among bacteria of the ability to generate this compound is evident from the classification of key 2,3-butanediol producers.

Table 2.4 Comparison of 2,3-butanediol production by different microbes from different substrates.

Strains	Substrates	Methods	2,3-BDO production			Reference
			Con. (g/L)	Pro. (g/L/h)	Yield (g/g)	
Klebsiella pneumoniae G31	Glycerol	Fed-batch	70.0	0.47	0.39	Petrov and Petrova, 2010
Klebsiella pneumoniae CICC 10011	*Jerusalem artichoke* tuber	Fed-batch	84.0	2.29	0.29	Petrov and Petrova, 2010
Klebsiella pneumoniae SDM	Corncob molasses	Fed-batch	78.9	1.30	0.47	Wang et al., 2010
Klebsiella pneumoniae	Sucrose	Fed-batch	70.0	2.00	0.40	Berbert-Molina et al., 2001
Klebsiella pneumoniae DSM2026	Glucose	Fed-batch	50.9	0.72	0.29	Cho et al., 2012
Klebsiella oxytoca ATCC43863	Glucose	Fed-batch	34.1	0.48	0.17	Cho et al., 2012
Bacillus amyloliquefaciens B10-127	Glucose	Fed-batch	61.4	1.71	0.38	Yang et al., 2011
Bacillus polymyxa	Glucose	Fed-batch	40.5	-	0.81	Mas et al., 1987
Enterobacter cloacae subsp. *dissolvens SDM*	Cassava powder	Fed-batch	93.9	2.0	0.42	Wang et al.,2012
Enterobacter aerogenes DSM 30053	Glucose	Fed-batch	110.0	5.4	0.47	Zeng et al.,1991
Enterobacter aerogenes NRRL B-23289	Arabinose	Batch	20.0	50.63	0.40	Saha and Bothast, 1999

Until now, *K. pneumonia* and *K. oxytoca* have been unbeatable in the efficient production of 2,3-butanediol. *E. aerogenes* and *S. marcescens* are also considered promising microorganisms for this application. In light of this, it seems to be desirable to consider the advantages or disadvantages of *Klebsiella* sp. in some what greater detail. *Klebsiella* sp. typically produces at least twice the amount of 2,3-butanediol obtainable from other genera. Another advantage of 2,3-butanediol production using *Klebsiella* sp.

is that the species are easy to cultivate. They grow rapidly in a simple medium and metabolize all of the major sugars present in hemicellulose and cellulose hydrolysates into 2,3-butanediol (Chandel et al., 2010; Kosaric et al.,1992). However, the pathogenicity of opportunistic infection caused by the encapsulated *Klebsiella* species is generally thought to be an obstacle hindering the large-scale 2,3-butanediol production using this method.

2.2.2 Metabolic pathway

2,3-butanediol is produced from pyruvate in a mixed acid fermentation pocess via several intermediate compounds, including α-acetolacte, acetoin (acetylmethyl-carbinol), and diacetyl (Bryn et al.,1973; Nicholson, 2008; Stormer, 1968b; Ji et al., 2011). 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 (catalysed byα-acetolactate synthase; α-ALS). α-Acetolactate is mostly produced under low NADH availability. Furthermore, 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. Finally, butanediol dehydrogenase (BDH; also knownas acetoin reductase; AR) reduces acetoin to 2,3-butanediol. It was reported that acetate at low pH (i.e. acetic acid) is an effective inducer of all the three enzymes playing a role in the formation of 2,3-butanediol from pyruvate (Bryn et al.,1973; Stormer, 1968b). All enzymes and compounds involved in 2,3-butanediol pathway are normally produced during the late log and stationary phases of fermentation,

when oxygen-limiting conditions exist (Mallonee and Speckman, 1988). Apart from 2,3-butanediol, other end-products are also synthesized, i.e. ethanol, acetate, lactate, formate, and succinate, depending on microorganism and applied conditions (Fig. 2.4).

Similar to butanol fermentation, some exogenous additives, such as acetic acid, which is a by-product involved in 2,3-butanediol fermentation, can also act as an inducer for the three enzymes in the conversion of pyruvate to 2,3-butanediol. It was shown that acetic acid added at concentration of less than 1.0% could enhance 2,3-butanediol production by *K. pneumoniae* by two- to three- fold (Yu and Saddler, 1982a). However, this effect varies with culture pH. At pH of 5.5, less than 1.0 g/L of acetic acid inhibited the product formation, whereas at a culture pH of 6.7, it took roughly ten times more acetic acid for the same effect to be observed. This inhibitory effect is due to the high percentage of the un-dissociated form of acetic acid under low pH conditions because un-dissociated organic acids are normally more toxic to bacteria than their dissociated forms. However, it has been shown that ionized acetate can induce ALS formation, and could thus enhance the efficiency of pyruvate conversion to 2,3-butanediol (Ji et al., 2011). Based on this observation, Nakashimada et al. developed a fed-batch culture process by feeding glucose and acetate at a ratio of 0.35 mol acetate per mol glucose at pH 6.8 for production of 2,3-butanediol by using *P. polymyxa*, resulting in enhancement of 2,3-butanediol production without a reduction in optical purity (Nakashimada et al., 2000).

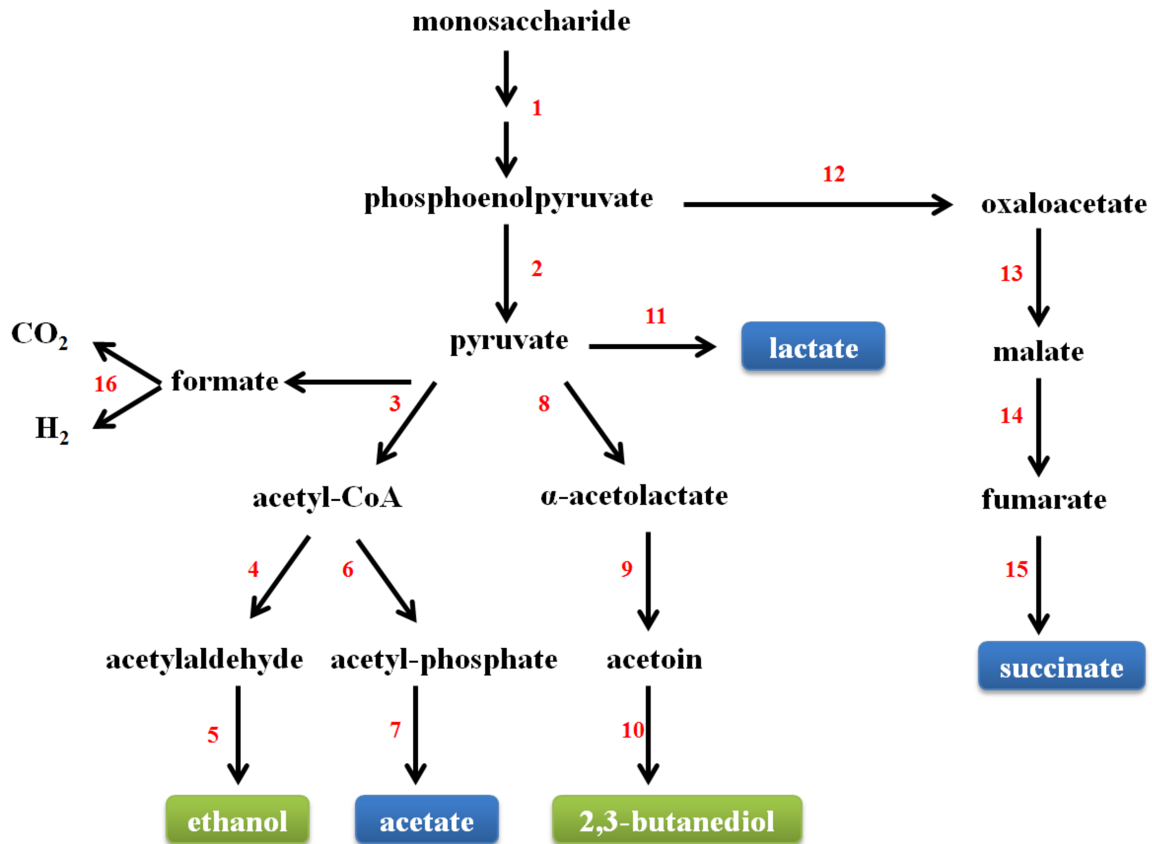


Fig. 2.3 2,3-Butanediol fermentaion pathway (Ji et al., 2011). 1, Embden–Meyerhof and pentose phosphate pathway enzymes; 2, pyruvate kinase; 3, pyruvate– formate lyase; 4, acetaldehyde dehydrogenase; 5, ethanol dehydrogenase; 6, phospho-transacetylase; 7, acetate kinase; 8, α-acetolactate synthase; 9, α -acetolactate decarboxylase; 10. acetoin reductase (2,3-butanediol dehydrogenase); 11, lactate dehydrogenase; 12, phosphoenolpyruvate decarboxylase; 13, malate dehydrogenase; 14, fumarase; 15, succinate dehydrogenase; 16, formate-hydrogenlyase complex; 17, pyruvate dehydrogenase multi-enzyme complex; and 18, citroyl synthetase.

2.2.3 Operating conditions

2.2.3.1 pH

One of the major factors which has influence on 2,3-butanediol production is the pH. According to Garg and Jain, alkaline conditions favour formation of organic acids, with a simultaneous decrease in the 2,3-butanediol yield (Garg and Jain, 1995). Contrary to this, under acidic conditions, organic acid synthesis is reduced (over 10 folds) and diol

synthesis is increased (3-7 folds). However, the optimum pH for diol production strongly depends on the microorganism and substrate used.

Most anaerobic fermentation processes are coupled with formation of organic acids. Thus, in the course of fermentation, the culture acidifies, and the concentration of toxic undissociated form of the acids increases. Growth and substrate turnover gradually cease and the culture is finally inactivated by its own products. Some microorganisms have evolved defensive strategies, like switching the metabolism to production of less toxic compounds, such as alcohols or glycols (Garg and Jain, 1995). Maddox explained the phenomenon that 2,3-butanediol pathway induction is caused by accumulation of acidic products in the medium rather than by altering the internal pH (Maddox, 1996). The resulting transmembrane pH gradient causes accumulation of acetate, which would induce the enzymes involved in 2,3-butanediol synthesis. Hence, lowering the culture pH causes an increase in the pH gradient, and 2,3-butanediol production occurs before the external pH becomes too high and the culture is inactivated. At neutral pH, *Klebsiella* sp. synthesizes acetic acid and ethanol, but below pH 6, 2,3-butanediol and ethanol are produced.

2.2.3.2 Substrate concentration

Generally, initial sugar concentrations of up to 200 g/L can be fermented, and even a large increase in concentration usually leads to an improvement in 2,3-butanediol yield. However, it was observed that at sugar concentration above 20 g/L, a decrease in specific growth rate occurs, which is probably a result of a fall in water activity. The 2,3-butanediol productivity is much less influenced and maximum values occur at an initial concentration of approximate 100 g/L. However, it should be noted that, since the

reduction of water activity is a function of molarity, lactose (disaccharide) is less inhibitory than glucose (monosaccharide) (Celińska and Grajek, 2011). Results obtained with *E. aerogenes* with regard to the glucose concentration showed almost no influence of the initial glucose concentration (in a range from 9 to 72 g/L) on the molar production of 2,3-butanediol (Converti and Perego, 2002). In other studies (carried out with *B. polymyxa*), the growth inhibition occurred at substrate concentrations exceeding 150 g/L (De Mas et al., 1988). The influence of xylose concentration on 2,3-butanediol appears to be species specific, since contrary data have been reported (Garg and Jain, 1995). In general, it seems that the influence of the substrate type and concentration on 2,3-butanediol production is species-dependent.

2.2.4 Strategies for efficient and economical 2,3-butanediol production

2,3-Butanediol is a very useful bulk chemical owing to a variety of its applications. It can be produced by biotechnological routes from waste biomass, which makes it an extremely attractive alternative to traditional production. The utilization of waste materials from renewable sources makes the process economically feasible. Still, the bio-based synthesis needs to compete with less expensive chemical routes and in order to “win the battle” it needs improvement.

First of all, a high-throughput screening for novel and safe microorganisms able to produce 2,3-butanediol with small amount of by products should be carried out. Isolated microorganisms could be subjected to a random mutagenization and directed evolution in vitro protocol. Such an approach could potentially result in isolation of a safe “super-producer” of 2,3-butanediol. An advantage of this approach is the possibility

of introduction of beneficial modifications into the microorganism's genome without previous knowledge of their importance in 2,3-butanediol synthesis. Furthermore, this allows taking advantage of less known and poorly described microorganisms. Alternatively, genetic engineering (precisely: metabolic engineering) of known and described microorganisms able or not to produce 2,3-butanediol could be applied. At least few directions of genetic modifications should be taken into account: improvement of 2,3-butanediol productivity and yield, elimination of by products synthesis, ability to utilize "difficult" substrate, most preferably without pretreatment, and resistance to environmental stresses that the microorganism will inevitably encounter during an industrial-scale bioprocess. Identification of new targets for directed mutagenization seems to be crucial. Successfully isolated or constructed microorganism would be employed in a fermentation process. In order to choose the most optimal conditions for a selected microorganism it is necessary to test at least few variants of fermentation. In general, fed-batch fermentation allows omitting the problem of high substrate concentration. In batch and fed-batch processes, higher concentration of the final product can be obtained, however the volumetric productivity is usually low (Table 2.4). On the other hand, in continuous cultures the productivity is higher, but the product concentration is rather low. Cell recycling usually gives a few-fold increase in the process productivity. What is more, drawn off cell-free culture liquid is easier in down-stream processing than unfiltered mash. Another attractive method worth considering for increasing productivity of the process is cell immobilization on a porous carrier. Such an approach, if precisely optimized, can result in a stable and efficient bioprocess. Fine and thorough optimization of the process parameters is a necessity. Optimization of few parameters at once rather

than“one-at-a-time” appears to be the most reasonable approach since fermentation process is a multivariable system. Mathematical and statistical modeling, like response surface methodology (RSM) serves the purpose perfectly (Ji et al., 2011). 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. As known, separation of the final product is the most expensive stage of the production. Therefore, development of new separation methods is a way to lower the overall cost of the process. The hybrid approaches (coupling few separation methods) appear to be very promising in this area.

Biotechnological production of 2,3-butanediol on an industrial-scale is still in its early stage but with strong prospects of growth. In order to achieve economically feasible, efficient and safe bioprocess of bio-based 2,3- butanediol still much has to be done. However, growing awareness of shortage of fossil fuels, benefits that stem from a bio-based processes and the emergence of “green chemistry” prompt us to undertake further efforts in this area. The potential that lies in strain improvement and intensified fermentation methods coupled with mathematical modeling in optimization procedures will stimulate rapid development of the process.

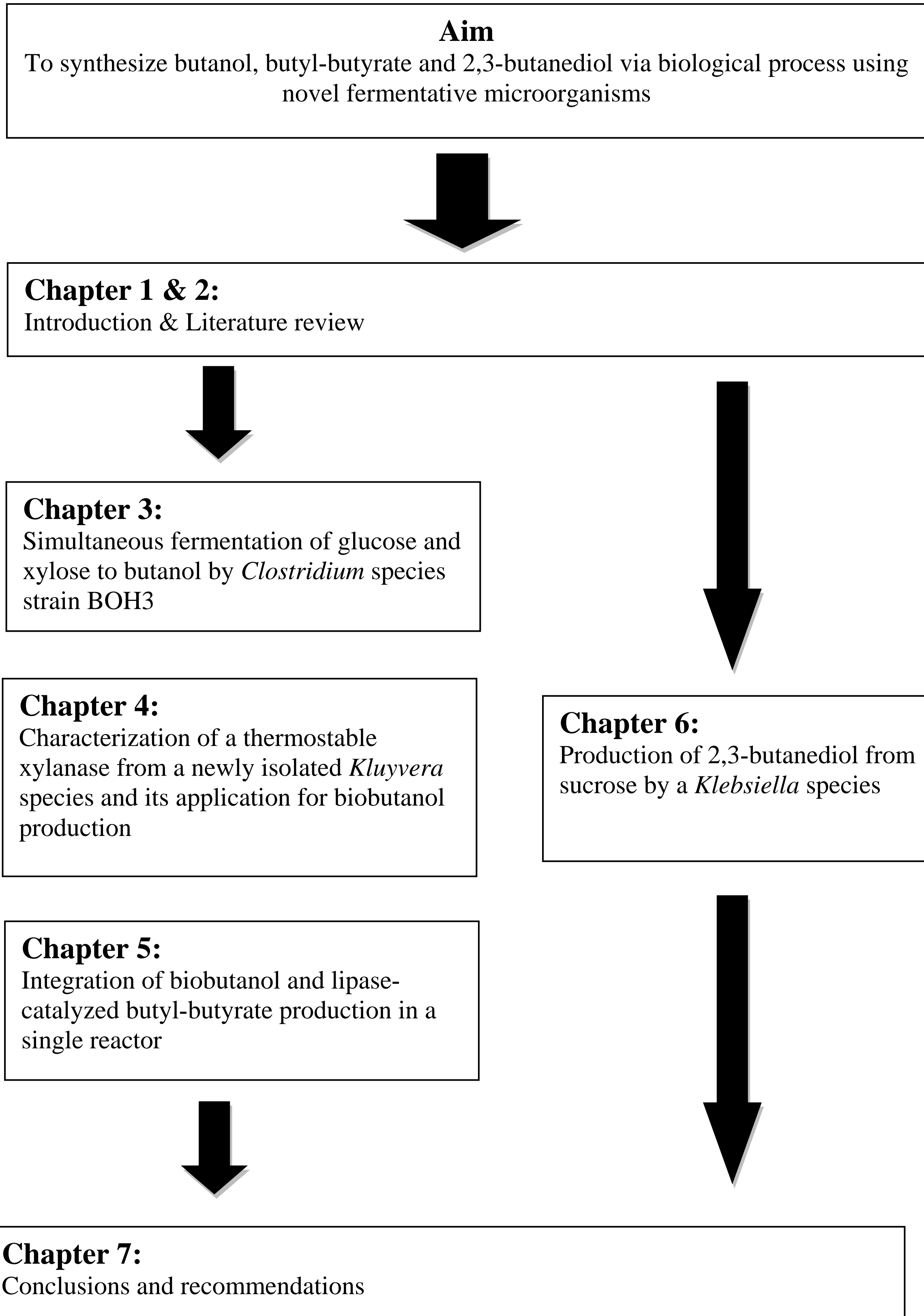


Fig. 2.4 Scope of the research work.

Chapter 3

Simultaneous fermentation of glucose and xylose to butanol by *Clostridium* species strain BOH3

3.1 Abstract

The production of ABE through biological processes has a long history. Among the fermentation products, butanol is not only an important bulk industrial chemical but also a high-quality transportation fuel. To address an economic bottleneck, namely, the excessively high feedstock cost in ABE bioproduction, traditional cereal substrates (e.g., maize and wheat) are gradually being abandoned, whereas lignocellulose, the most abundant renewable biomass, is arousing worldwide interest. Cellulose and hemicellulose constitute the major components in sustainable feedstocks, which could be used as substrates for biofuel generation. However, following hydrolysis to monomer sugars, the solventogenic *Clostridium* will preferentially consume glucose due to transcriptional repression of xylose utilization genes. This is one of the major barriers in making the best of lignocellulosic hydrolysates to produce butanol. In comparison with studies on existing bacteria, this study demonstrates that a newly reported *Clostridium* sp. strain BOH3 is capable of fermenting 60 g/L of xylose to 14.9 g/L butanol, which is similar to the 14.5 g/L butanol produced from 60 g/L of glucose. More importantly, strain BOH3 consumes glucose and xylose simultaneously, which is shown by its capability for generating 11.7 g/L butanol from a horticultural waste cellulosic hydrolysate containing 39.8 g/L glucose and 20.5 g/L xylose; as well as producing 11.9 g/L butanol from another horticultural waste hemicellulosic hydrolysate containing 58.3 g/L xylose and 5.9 g/L glucose. The high xylose utilization capability in strain BOH3 is attributed to its high xylose-isomerase (0.97 U/mg protein) and xylulokinase activities (1.16U/mg protein) when compared with low-xylose utilizing solventogenic strains, such as *Clostridium* sp. strain G117. Interestingly, strain BOH3 was also found to produce

riboflavin, 110.5 mg/L from xylose and 76.8 mg/L from glucose during the fermentation process. In summary, *Clostridium* sp. strain BOH3 is an attractive candidate for application in efficiently converting lignocellulosic hydrolysates to biofuels and other value-added products such as riboflavin.

3.2 Introduction

Conversion of lignocellulosic biomass to biofuels (e.g., butanol) is usually limited by the inefficiency of the bacteria in the utilization of pentoses, which constitute 20-60% of the sugars in the hydrolysate of lignocellulose (Zaldivar et al., 2001). So far, the biofuel generating microbes in common use are either unable to utilize pentoses at all (e.g., *Saccharomyces cerevisiae*) or consume hexoses first and then pentoses with a low yield. A good example are the solventogenic *Clostridium* species (e.g., *C. acetobutylicum*, *C. beijerinckii*, and *C. pasteurianum*), which are one of the few microorganisms able to ferment both pentose and hexose sugars (Compere and Griff, 1979). However, the solvents including butanol are still produced mainly from glucose, while small amounts are produced from xylose as observed in cultures *C. acetobutylicum* ATCC 824 and *C. beijerinckii* NCIMB 8052 when fermenting a mixture of glucose and xylose (Gu et al., 2009; Hu et al., 2011; Xiao et al., 2012). Gu et al. have shown that *C. acetobutylicum*ATCC 824 utilized 86% of glucose within 40 hrs, whereas only 6% of xylose was consumed even after elongated incubation time (Gu et al., 2009). This sequential utilization extends fermentation time and results in incomplete substrate consumption (Mitchell, 1998; Vinuselvi et al., 2012). The phenomenon of preferring

glucose over xylose is referred as carbon catabolite repression, in which microorganisms preferentially utilize a rapidly metabolizable carbon source and inhibit the expression of some genes and enzyme activities related to the catabolism of non-preferred carbon resources (Mitchell, 1998; Vinuselvi et al., 2012). Unless glucose and xylose are both utilized efficiently, converting lignocellulosic biomass into bio-based products is unfavorable from an economic viewpoint (Joshua et al., 2011; Kim et al., 2010; Nichols et al., 2001) as yields would be limited. Hence, in order to fully utilize the sugars in the hydrolysates of lignocellulosic biomass, microbes capable of fermenting both glucose and xylose with a high butanol yield are highly desirable to improve the efficiency of biofuel production systems.

In addition to butanol production, certain biofuel-generating microbes such as *Clostridium* can produce other value-added products (e.g., riboflavin, also known as vitamin B2), which adds to the value of the butanol fermentation process. Actually, in addition to producing butanol, *Clostridium* species are also one of the commercial microorganisms used to produce riboflavin on an industrial scale, although they only generate a very small amount of riboflavin (<50 mg/L) (Hickey, 1945; Perlman, 1979; Pridham, 1946). Riboflavin - a yellow water soluble vitamin - is solely biosynthesized by plants and numerous microorganisms, but not by vertebrates. Thus, this vitamin has to be supplied by foods and dietary supplements. Currently, industrial production has shifted completely from chemical synthesis to microbial fermentation (Vandamme, 1992), which has become one of the major fermentation products in biotechnology industry with an annual market demand of several million kilograms (Stahmann et al., 1979). Therefore,

the co-production of butanol and riboflavin would make the fermentation process more economically feasible (Qureshi and Blaschek, 2001).

The aim of this study is to explore the wild-type *Clostridium* sp. strain BOH3's capability to utilize the five-carbon sugar, xylose. A further study was then conducted on both butanol and riboflavin production from fermenting a mixture of glucose and xylose and hydrolysates from horticultural wastes. Lastly, the activities of xylose-utilizing enzymes (xylose isomerase and xylulose kinase) in strain BOH3 were evaluated to determine the underlying mechanism of co-fermentation of glucose and xylose.

3.3 Materials and methods

3.3.1 Growth medium and culture conditions

Clostridium sp. strain BOH3 and *Clostridium* sp. strain G117 were used in this study (Bramono et al., 2011; Wu et al., 2012). Batch cultures were grown at 35°C in defined mineral salts medium containing (per liter of distilled water): KH_2PO_4, 0.75 g; K_2HPO_4, 0.75 g; CH_3COONH_4, 2 g; yeast extract, 5 g. In addition, 1 mL of trace element solution (Widdel and Hansen, 1992), 1 mL of Na_2SeO_3-Na_2WO_4 solution (Brysch et al., 1987) and 10 mg of resazurin were added to 1 L of the medium. After the medium was boiled and cooled down to room temperature under N_2, reductants Na_2S, L-cysteine, and $_{DL}$-dithiothretol were added to a final concentration of 0.2, 0.2, and 0.5 mM, respectively (He et al., 2003). Subsequently, 20 mM 2-(N-Morpholino) ethanesulfonic acid (MES) was added to the medium to adjust its initial pH to 6.0. Then the medium was dispended to serum bottles, which were sealed with butyl stoppers, autoclaved for 20 min, and cooled down to room temperature. Glucose, xylose, or a mixture of glucose/xylose was amended to the above medium before inoculation.

Cultures for inoculation were grown in 50 mL mineral salts medium amended with glucose, xylose or a glucose/xylose mixtureat 35°C for ~20 hrs (late-exponential phase) unless otherwise stated. Inocula of 5 mL were added to 45 mL of the reduced mineral salts medium in 160 mL serum bottles, which were incubated in a shaker at a rotary rate of 150 rpm at 35 °C. The pH was adjusted to 5.0 using 2M NaOH after 24 hrs. Experiments were carried out in duplicates.

3.3.2 Preparation of horticultural waste hydrolysates

Horticultural wastes collected from a horticultural waste treatment plant (ecoWise Solution Pte Ltd) were used as raw materials, which were dried and milled to a small size (200–500 μm). Pretreatment of the horticultural waste was carried out by using the organosolv method as described by Zhang et al. (Zhang et al., 2012) and Geng et al. (Geng et al., 2012). Initially, the organic solvent ethanol was used to dissolve hemicellulose and lignin to the solvent phase. The ethanol was then removed by vacuum evaporation and the hemicellulosic fraction carrying mainly xylose was further detoxified using activated charcoal powder and concentrated to obtain a final sugar mixture containing 5.9 g/L of glucose and 58.3 g/L of xylose (Zhang et al., 2012). The solid residues from the pretreatment step contained mainly cellulose, which was hydrolyzed by enzymes of Celluclast 1.5 L and Novozym 188 to glucose and designated as cellulosic hydrolysate (Geng et al., 2012). Supplemental xylose (16.9 g/L) was added to the cellulosic hydrolysate to attain a 2:1 ratio of glucose (39.8 g/L) to xylose (20.8 g/L). Prior to filter sterilization, the pH values of the detoxified hemicellulosic hydrolysate (containing 5.9 g/L of glucose and 58.3 g/L of xylose) and cellulosic hydrolysate (containing 39.8 g/L of glucose and 20.8 g/L of xylose) were adjusted to 6.0 by titrating

with 10M NaOH solution. This was followed by supplementing KH_2PO_4, K_2HPO_4, CH_3COONH_4, yeast extract, trace element solution, Na_2SeO_3-Na_2WO_4 solution and reductants as described in the above section.

3.3.3 Expression of genes related to xylose utilization as measured by quantitative real-time PCR

Total RNA from cell pellets of *Clostridium* sp. strain BOH3 and G117 was extracted by using a modified Trizol methodand RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the supplier's instructions. The remaining trace DNA was removed using RNase-free DNase Kit (Qiagen, Hilden, Germany). cDNA was synthesized in one step using iScript One-Step RT-PCR kit (BioRad Lab-oratories, Munich, Germany). A quantitative real-time PCR (qPCR) (ABI 7500 Fast real-time PCR system; ABI, Foster City, CA) assay was performed by using QuantiTest SYBR Green (QIAGEN, Gmbh, Germany) to quantify the transcription levels of the xylose isomerase gene. The xylose isomerasegene-specific primers used for qPCR were designed based on the xylose isomerase gene sequence of culture BOH3 and G117, with the following forward primers (strain BOH3:5'-ATGTTGCAGTTACAGAGGGAGA-3'; strain G117:5'-CTGTTTTACTAATCCAAGGTATGTTCA-3) and reverse primers (strain BOH3:5'-TTTCATCTTGGCTTACCTTGTC-3'; G117:5'-ATCTAGCTCTTTTGTTATTTCAATTGC-3'). Primers for the housekeeping genes ((3R)-hydroxymyristoyl-ACP dehydratase (*fabZ*) for strain BOH3 (Nolling et al., 2001); peptidase T (*pepT*) for strain G117 (Wu et al., 2012)) are as follows: strain BOH3-forward-5'-AAATAGAACCAGGGAAAAGAGCA-3'; strain BOH3-reverse-5'-GCAACACCACCAAGTTGAGC-3';strain G117-forward-5'-

TGATGGAGGCGAGGAAGGTG-3'; strain G117-reverse-5'-CATTGTATTCTTTGCAGACCCTGG-3'. The following real-time PCR program was used: 10 min at 95°C, 45 cycles 10 s at 95°C and 30 s at 55°C, and followed by a melting point analysis (45–95°C) after the final PCR step to verify the specificity of amplified products. The real-time PCR data were analyzed with iQ5 software, relative quantification was done by using the "delta–delta CT method" (Pfaffl, 2001), and was normalized with the abundance of housekeeping genes.

3.3.4 Xylose isomerase and xylulose kinase assays

Cell extracts obtained from the batch cultures were used for enzymatic activity assays. The chemicals used in the enzymatic activity assessment were obtained from Sigma-Aldrich (St. Louis, MO). Cultures at the exponential phase were harvested by centrifugation at 10,000 ×g at 4°C for 10 min. Cell pellets were washed once with extraction buffer (100 mM Tris-HCl [pH 7.5], 20 mM KCl, 20 mM $MgCl_2$, 5mM $MnSO_4$, 0.1 mM EDTA, and 2 mM $_{DL}$-dithiothreitol). The resulting cell pellets were sonicated using an ultrasonic homogenizer in an ice water bath for 2-min, followed by 2-min cooling intervals, which were repeated three times. Cell debris was removed by ultra centrifugation (12,000×g, 4°C, 30 min). The cell lysates were subsequently used for enzyme assays.

The activity of xylose isomerase of the whole-cell lysate was carried out in a 1-mL reaction volume containing 70 mM xylose, 20 mM $MgCl_2$, 5 mM $MnSO_4$ and 2 mM $_{DL}$-dithiothreitol in 100 mM Tris buffer (pH 7.5) (Shamanna and Sanderson, 1979). The reaction mixture was incubated for 30 min at 30°C, and then 0.5 M $HClO_4$ were added to stop the reaction. The xylulose produced was quantified by using the cysteine-carbazole-

sulfuric acid assay by measuring the absorbance at 540 nm (Dische and Borenfreund, 1951). The enzyme activity of xylulose kinase was determined by the reduction of xylulose in the reaction mixture as described previously with minor modifications (Shamanna and Sanderson, 1979). The reaction mixture contained the following: 50 mM Tris/HCl buffer (pH 7.5), 2.0 mM $MgCl_2$, 2.0 mM ATP, 0.2 mM phosphoenolpyruvate, 0.2 mM NADH, 10 U pyruvate kinase (E.C. 2.7.1.40), 10 U lactate dehydrogenase (E.C. 1.1.1.27) and 8.5 mM xylulose. Consumption of NADH was measured by a spectrophotometer at 340 nm. One unit of enzyme activity was defined as the amount of enzyme which generated 1 μmol of product per minute. The protein amount in the cell extracts was detected by using the Coomassie Protein Assay Reagent (Braford, 1976).

3.3.5 Analytical methods

The biomass in the fermentation broth was determined on a UV–visible spectrophotometer set at a wavelength of 600nm (Lambda-25, Perkin-Elmer, USA). Riboflavin was initially identified and quantified by using a high-performance liquid chromatography (HPLC) and was later quantified at the wavelength of 444 nmon a Beckman DU-800 spectrophotometer based on a modified protocol from Sauer (Sauer et al., 1996). Briefly, the fermentation broth was diluted with 0.1 M phosphate buffer and heated at 80°C for 10 min before centrifugation. The supernatant was then assayed. Similarly, for sugar and biosolvent analysis, culture broths were centrifuged at 10,000×g for 10 min at 4°C and the supernatant fluids were stored at -20°C until further analysis. The concentrations of glucose and xylose were measured using the YSI Life Sciences multiparameter biochemistry analyzer 7100 MBS. Glucose and xylose sensors are configured in the same electrode chamber allowing simultaneous measurement of these

sugars in one-minute. Biosolvents (i.e., acetone, ethanol and butanol) were measured by a gas chromatography (GC, model 7890A; Agilent Technologies, U.S.A.) on a Durabond (DB)-WAXetr column (30 m × 0.25 mm × 0.25 μm; J&W, U.S.A.) equipped with a flame ionization detector (FID). The oven temperature was initially held at 60°C for 2 min, increased at 15°C/min to 230°C, and held for 1.7 min. Helium was used as the carrier gas, with a column flow rate of 1.5mL/min. Five-point standard curves were established by running standard solutions containing acetone, butanol, and ethanol. The yields of butanol and total solvents here are defined as the mass of products (g) / the mass of substrate (g).

3.4 Results

3.4.1 Efficient co-production of butanol and VB2 from xylose by strain BOH3

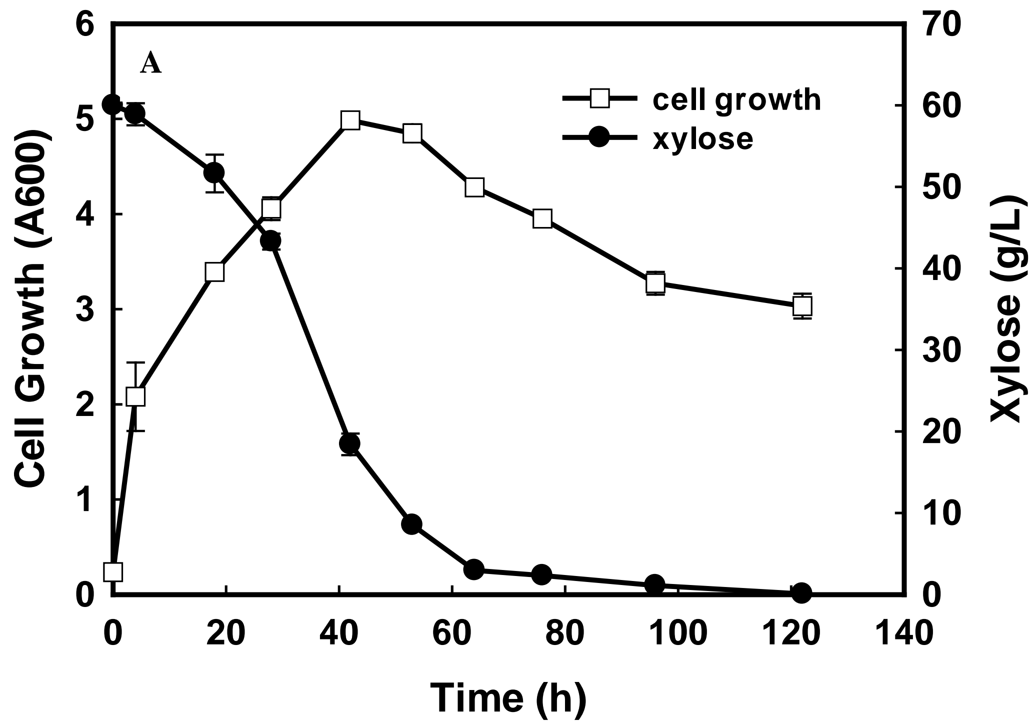
In contrast to the two most well-known butanol producing species, *C. acetobutylicum* ATCC 824 and *C. beijerinckii* NCIMB 8052, the newly discovered *Clostridium* sp. strain BOH3 could produce slightly higher amount of butanol (7.4 vs. 7.0 g/L) from xylose than from glucose (30 g/L each) (Bramono et al., 2011). The above observation inspired further investigation on the strain BOH3's capability to utilize xylose at a higher concentration. When 60 g/L of xylose was fed as the sole carbon source to culture BOH3, cells multiplied rapidly (optical density at a wavelength of 600 nm reached 4.9) during the first 28 hrs with only small amounts of butanol production (3.4 g/L). After that, acetone, butanol and ethanol concentration increased rapidly to a highest value of 5.3, 14.9 and1.2 g/L within 62 hrs, respectively (Fig. 3.1A and 3.1B).

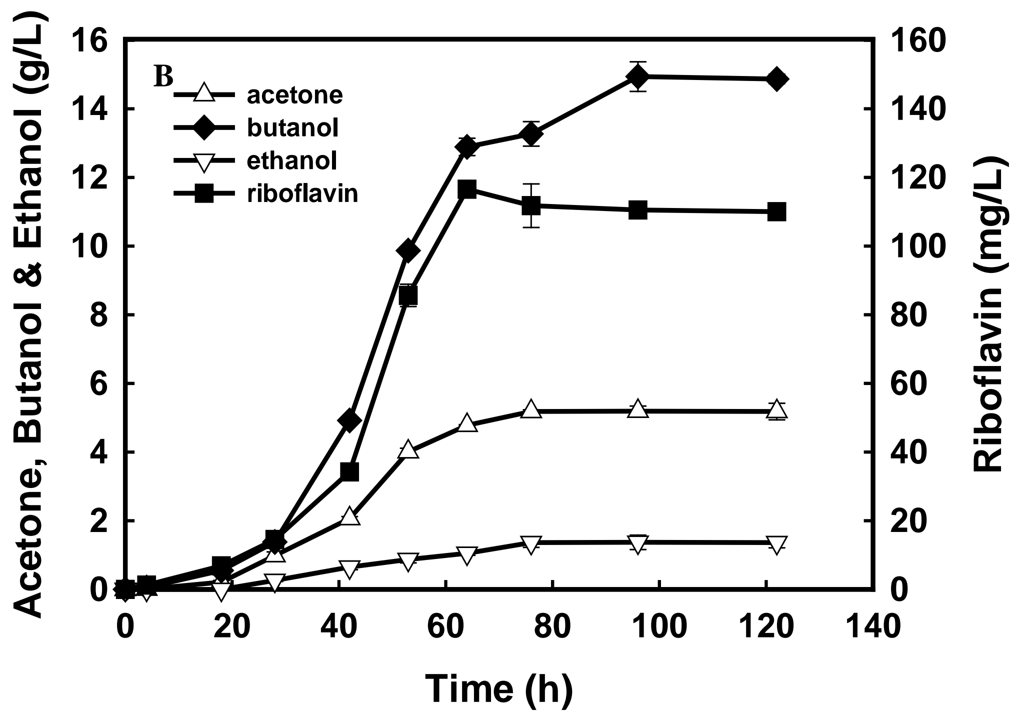
This corresponds to a yield of 0.25 g/g for butanol and 0.36 g/g for total solvents of acetone, butanol and ethanol (ABE). Interestingly, riboflavin also appeared during the fermentation process and reached a concentration of 110.5 mg/L, which is much higher compared to previous studies using either wild-type (50 mg/L) or gene-modified *Clostridium* strains (70 mg/L) (Bacher et al., 2001; Cai and Bennett, 2011). In comparison, when glucose (60 g/L) was used as the sole carbon source, the substrate-consumption curves almost overlapped with that of xylose (Fig.3.1A and 3.1C). Strain BOH3 exhibited a similar sugar-consumption rate of 0.83 g/L/h and generated slightly lower amounts of riboflavin (76.8 mg/L), butanol (14.5 g/L) and ABE (20.1 g/L) than those using xylose as a sole substrate (Fig. 3.1C and 3.1D). Currently, many works have focused on improvement of butanol production from xylose. *C. acetobutylicum* ATCC 824-TAL could produce 5.1 g/L butanol from xylose via transformed with *E. coli* transaldolase (*TAL*) gene (Gu et al., 2009). Most recently, Xiao et al., have improved butanol production to 11.6 g/L from 60 g/L xylose via gene-modifying *C. beijerinckii* NCIMB 8052 (Xiao et al., 2011). Strain BOH3 thus represents the first wild-type solventogenic *Clostridium* that could ferment xylose to butanol (14.9 g/L) more efficiently than previous reported wild-type (5.8 g/L) or gene-modified (11.6 g/L) *Clostridium* strains under similar operational conditions (Table. 3.1).

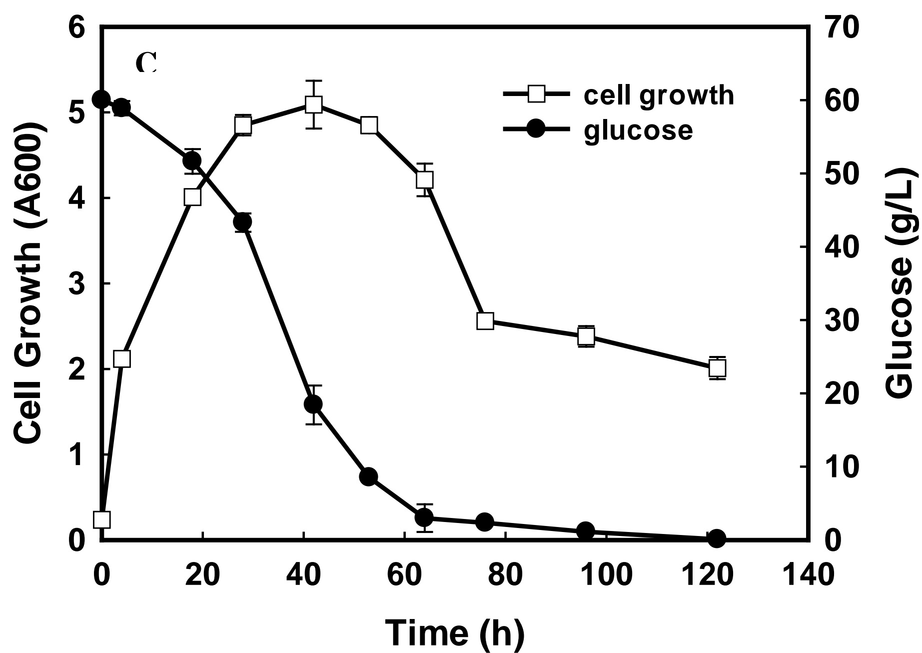
Table 3.1 Xylose consumption and total solvent (acetone, butanol, and ethanol) production by different *Clostridium* species fed with 60 g/L xylose.

Strain	Gene types	Initial xylose (g/L)	Residue xylose (g/L)	Solvent titer (g/L)		Reference
				Butanol	ABE	
C. acetobutylicum ATCC824	Wild strain	60	36.7±0.2	4.2±0.1	6.7±0.2	Gu et al., 2009
*C. acetobutylicum*824-TAL[a]	Gene modified	60	32.5±0.3	5.1±0.2	8.1±0.2	Gu et al., 2009
C. acetobutylicum ATCC824	Wild strain	60	35.7±0.5	4.3±0.3	6.8±0.1	Hu et al., 2011
C. acetobutylicum EA2018[b]	Mutant	60	23.6±1.2	8.5±0.1	11.6±0.1	Hu et al., 2011
C. beijerinckii NCIMB8052	Wild strain	60	27.3±0.9	5.8±0.2	7.9±0.7	Xiao et al., 2012
C. beijerinckii 8052xylR-xylT$_{ptb}$[c]	Gene modified	60	4.2±0.1	11.6±0.1	15.9±0.3	Xiao et al., 2012
Clostridium sp. strain G117	Wild strain	60	24.6±0.6	5.2±0.1	7.2±0.1	This study
Clostridium sp. strain BOH3	Wild strain	60	0	14.9±0.2	21.4±0.2	This study

[a]*C. acetobutylicum* 824-TAL: Transformant from *C. acetobutylicum* ATCC 824 bearing the *E. coli* transaldolase (*TAL*) gene (Gu et al., 2009).
[b]*C. acetobutylicum* EA2018: Mutant obtained from *C. acetobutylicum* ATCC 824 after several rounds of mutagenesis using NTG (*N*-methyl-*N'*-nitrosoguanidine) (Hu et al., 2011).
[c]*C. beijerinckii* 8052xylR-xylT$_{ptb}$: Engineered strain from *C. beijerinckii* NCIMB 8052 (putative xylose repressor gene (*xylR*) inactivation plus xylose proton-symporter gene (*xylT*) overexpression driven by *ptb* promoter) (Xiao et al., 2012).
All of the fermentation was carried out with 60 g/L of initial xylose at 35-37°C.


A
cell growth
xylose
Cell Growth (A600)
Xylose (g/L)
Time (h)
0
1
2
3
4
5
6
0
10
20
30
40
50
60
70
0
20
40
60
80
100
120
140


B
acetone
butanol
ethanol
riboflavin
Acetone, Butanol & Ethanol (g/L)
Riboflavin (mg/L)
Time (h)
0
2
4
6
8
10
12
14
16
0
20
40
60
80
100
120
140
160
0
20
40
60
80
100
120
140



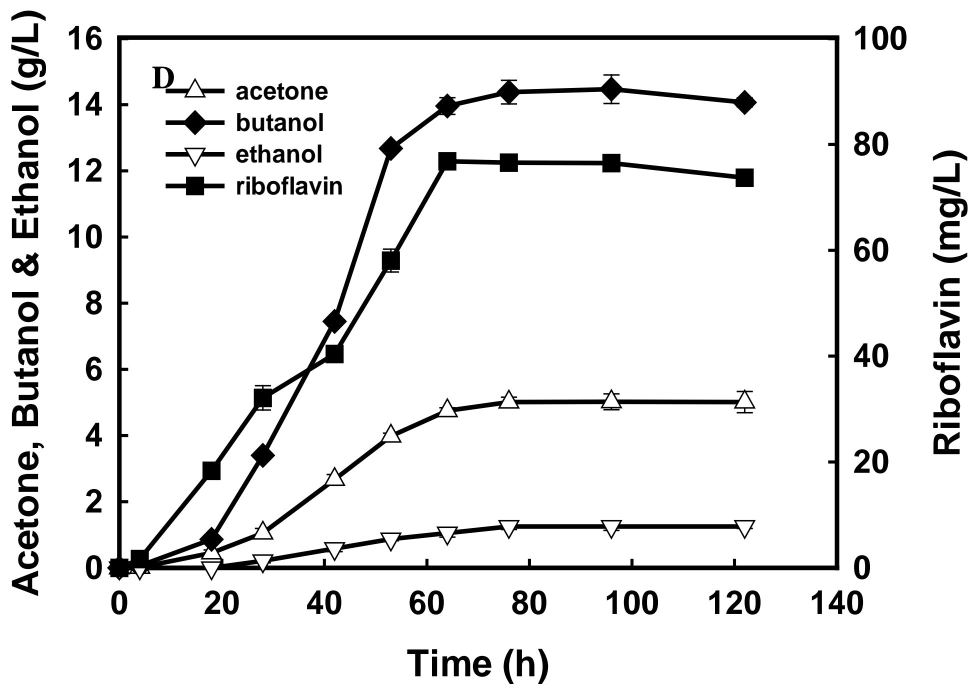
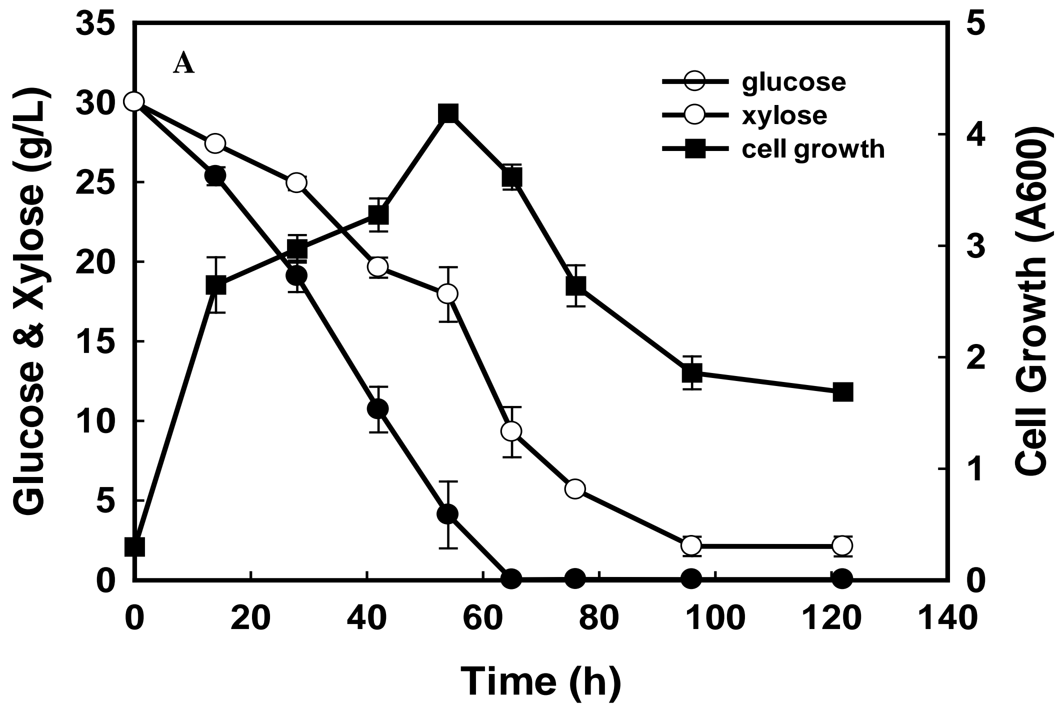
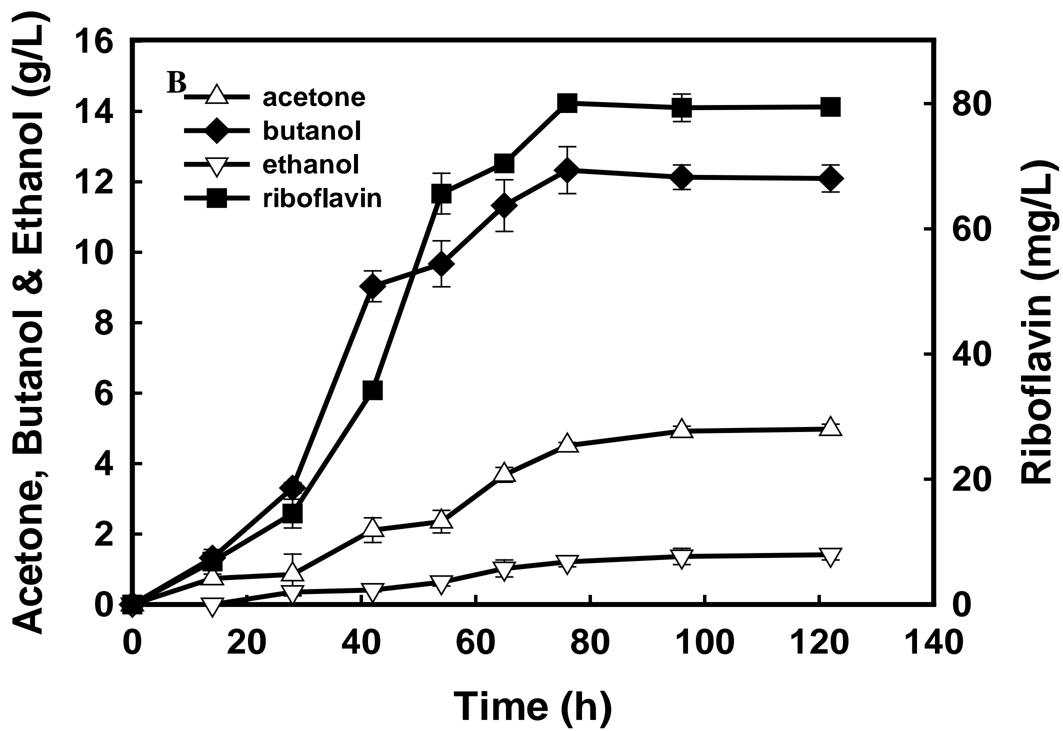


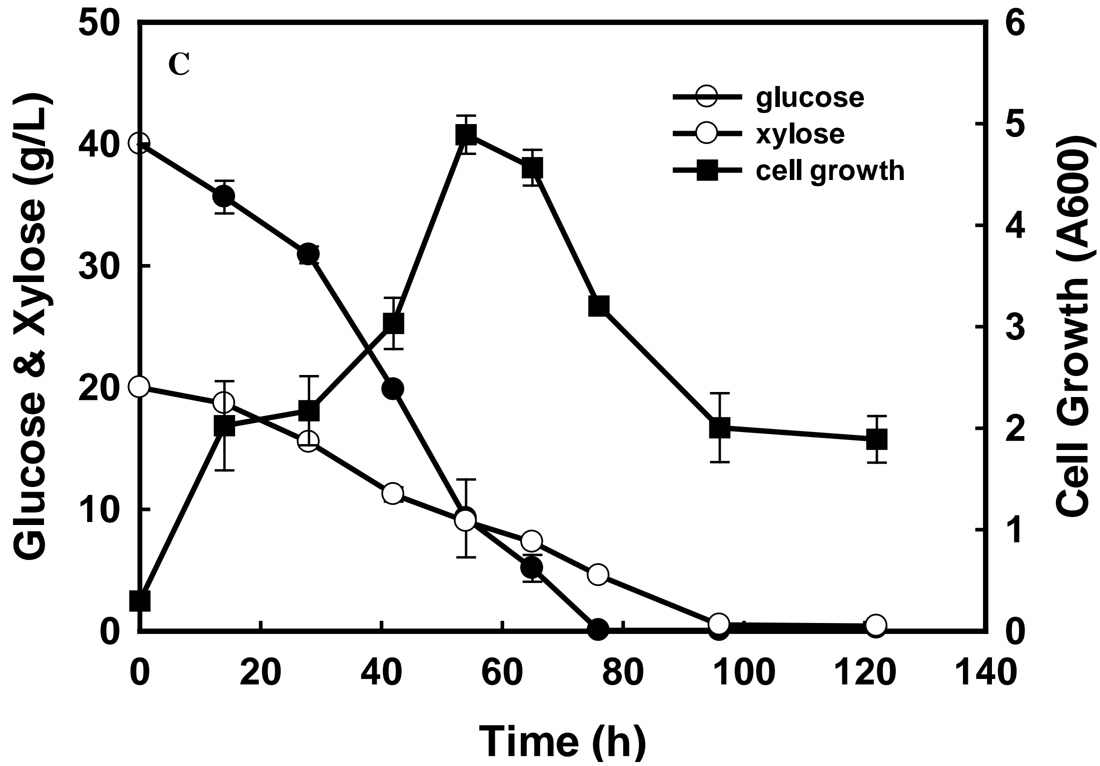
Fig. 3.1 Growth and metabolite profiles in batch fermentation of *Clostridium* sp. strain BOH3 in reduced mineral medium containing 60 g of xylose/liter (A and B) and 60 g of glucose/liter (C and D).

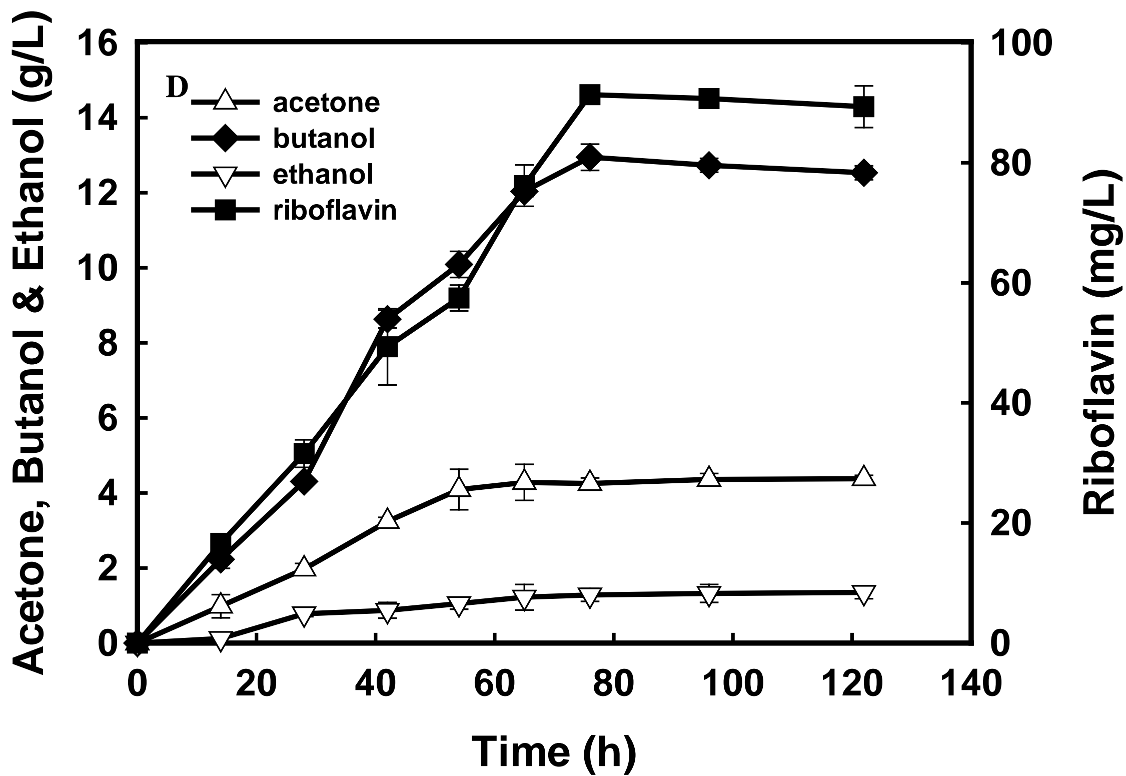
3.4.2 Simultaneous utilization of glucose and xylose by strain BOH3

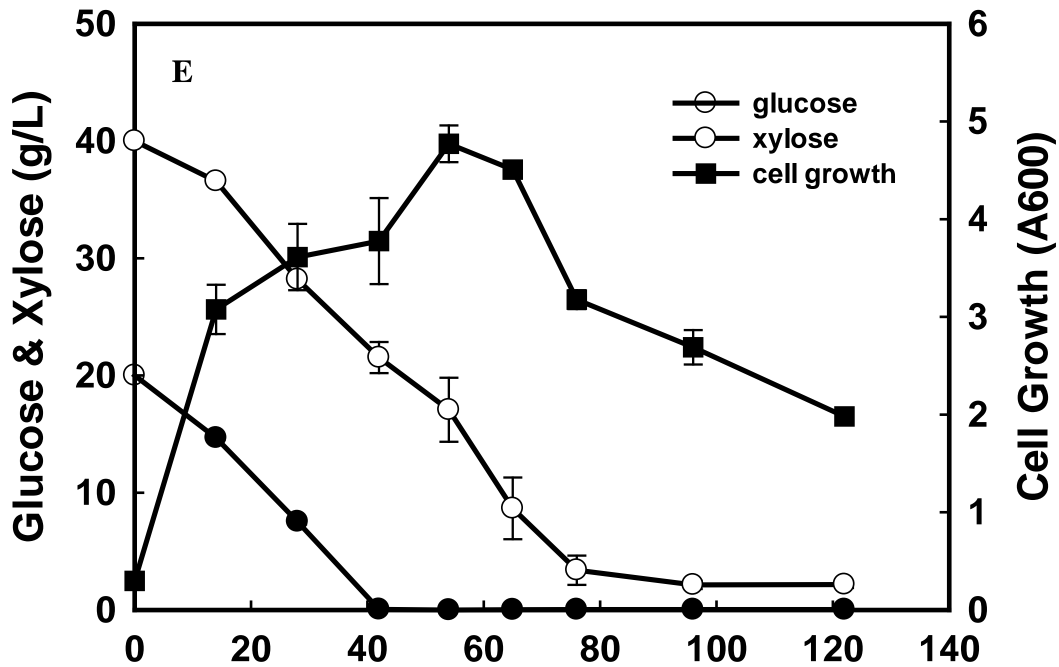
Glucose and xylose are the two major components in the hydrolysates obtained after pretreatment of lignocelluloses and enzymatic saccharification. As the ratio of glucose and xylose varies widely among different lignocellulosic biomass types, strain BOH3 was fed with a mixture of glucose and xylose at different ratios (~ 2:1, 1:1 and 1:2) (Fig. 3.2). In any ratio of glucose/xylose mixture, strain BOH3 rapidly and simultaneously fermented both glucose and xylose to produce butanol, acetone, ethanol, and riboflavin (Fig. 3.2). In contrast, previous studies exhibited typical sequential utilization, consuming glucose first and then followed by xylose (Chua et al., 2013;Gu et al., 2009; Qunine et al., 1985;Wu et al., 2012; Xiao et al., 2011; Xiao et al., 2012). For instance, when providing a mixture of glucose and xylose (30 g/L each), strain G117 consumed all the glucose in the first 48 hrs, and xylose utilization was initiated only after the glucose was completely consumed (after 48 hrs), resulting in a total of 6.5 g/L butanol after 100 hrs of fermentation. In comparison, strain BOH3 consumed all the glucose and most of the xylose in the glucose/xylose mixtures after 72 hrs incubation (Fig. 3.2A, C and E). The more xylose in the glucose/xylose mixture, the slightly higher amounts of butanol and riboflavin were produced (Fig. 3.2B, D and F). For example, when fed with glucose and xylose in a ratio of 1:2, strain BOH3 produced the highest butanol (13.0 g/L) and riboflavin (91.4 mg/L) among the above three substrate ratios, which is consistent with the results in Fig. 3.1 showing higher butanol and riboflavin production after consuming the same amount of xylose as glucose.


A
glucose
xylose
cell growth
Glucose & Xylose (g/L)
Cell Growth (A600)
Time (h)
35
30
25
20
15
10
5
0
5
4
3
2
1
0
0
20
40
60
80
100
120
140


B
acetone
butanol
ethanol
riboflavin
Acetone, Butanol & Ethanol (g/L)
Riboflavin (mg/L)
Time (h)
16
14
12
10
8
6
4
2
0
80
60
40
20
0
0
20
40
60
80
100
120
140


C
glucose
xylose
cell growth
Glucose & Xylose (g/L)
Cell Growth (A600)
Time (h)


D
acetone
butanol
ethanol
riboflavin
Acetone, Butanol & Ethanol (g/L)
Riboflavin (mg/L)
Time (h)



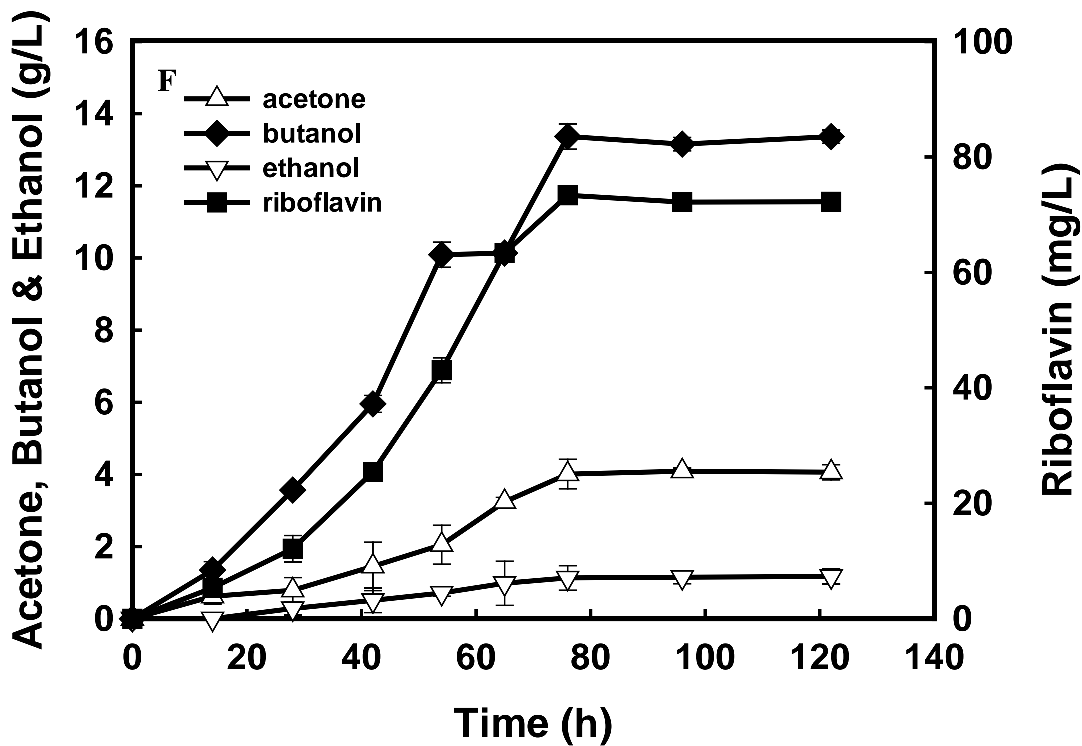
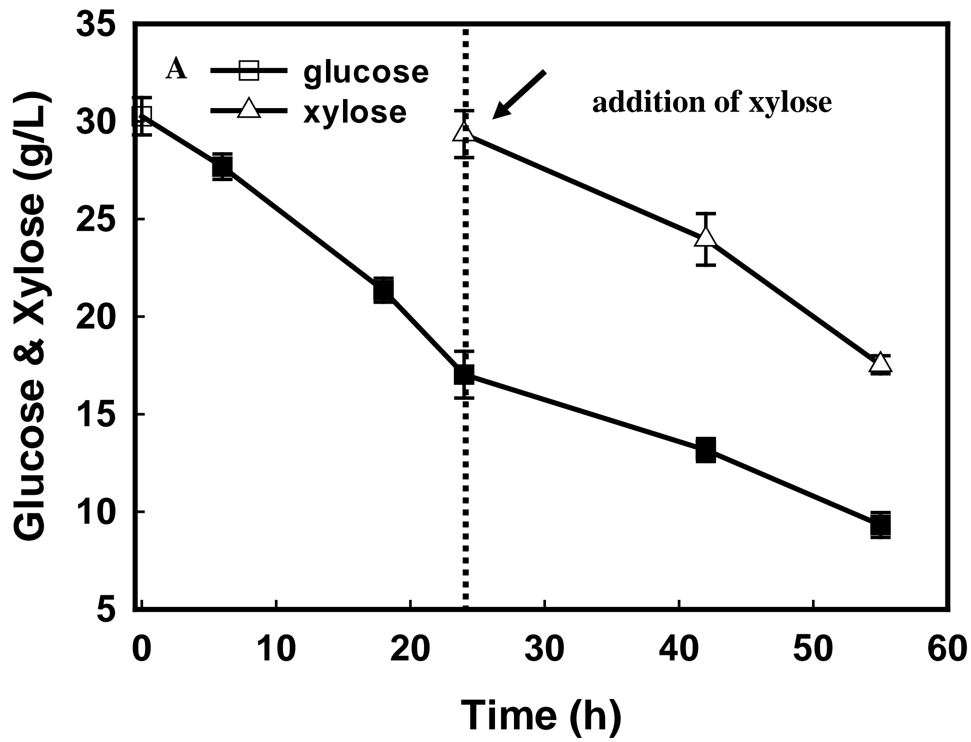


Fig. 3.2 Growth and metabolite profiles in batch fermentations of *Clostridium* sp. strain BOH3 in reduced minenal medium containing a mixtures of 30 g of glucose/liter and 30 gof xylose/liter (A and B), 40 g of glucose/liter and 20 g of xylose/liter (C and D) and 20 g of glucose/liter and 40 g of xylose/liter (E and F).

To further investigate the variation between glucose and xylose utilization, the culture initiated with glucose or xylose as the sole carbon source was supplemented with xylose or glucose after 24 hrs of incubation (Fig. 3.3). For the initial 24 hrs cultivation on 30 g/L glucose, the average glucose-consumption rate was 0.54 g/L/h. Xylose was then introduced to a final concentration of 30 g/L at the time of hr 24 (while 17g/L of glucose remained). During the next 20 hrs (the period from 24 to 44 hrs), xylose was consumed at an average rate of 0.33 g/L/h, whereas the glucose consumption rate decreased to 0.23 g/L/h (Fig. 3.3A). However, the total sugar consumption rate was 0.56 g/L/h, which was nearly equal to the rate before xylose supplementation. Similar results were obtained when glucose was introduced after 24 hrs to the culture initiated with xylose (Fig. 3.3B); glucose was consumed at 0.33 g/L/h and the xylose-consumption rate reduced from 0.57 g/L/h to 0.22 g/L/h. The total sugar-consumption rate of 0.55 g/L/h was very close to the rate before glucose supplementation. Therefore, when the medium was supplemented with a second sugar, the sugar-consumption capacity was redistributed between the two sugars.



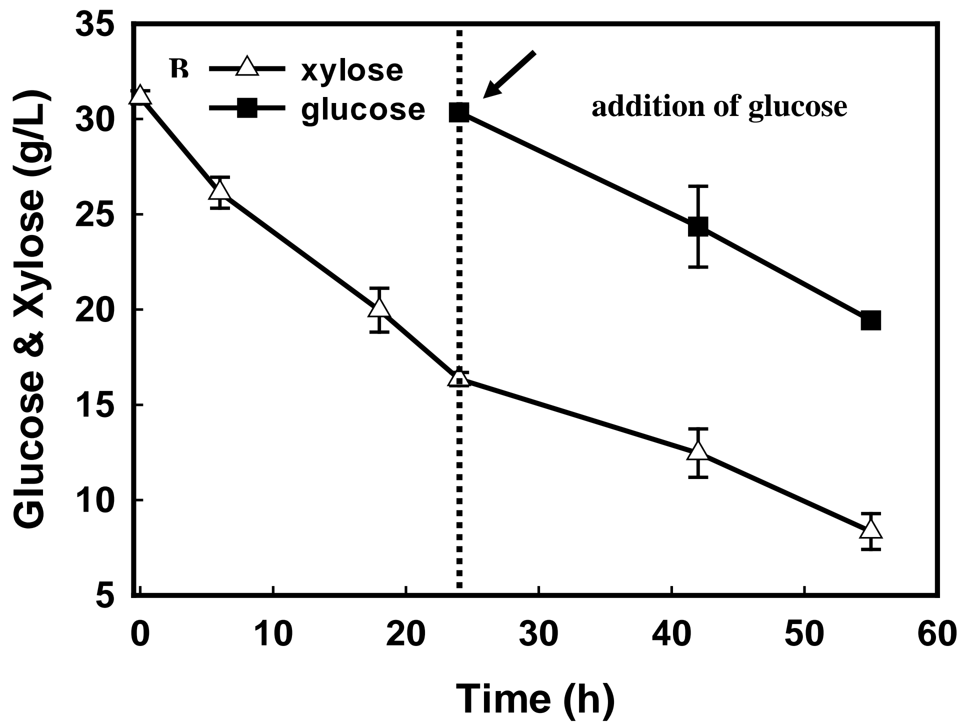


Fig. 3.3 Sugar-consumption profiles for the sugar supplementation experiments. (A) xylose added into glucose-containing medium and (B) glucose was added into xylose-containing medium, after 24 hrs of cultivation.

3.4.3 Fermentation of lignocellulosic hydrolysate by strain BOH3

To further test the strain BOH3's capability to ferment both glucose and xylose in the hydrolysate of lignocellulosic biomass, horticultural waste was chosen for the subsequent experiment. After pretreatment, enzymatic hydrolysis and supplementation of xylose (Geng et al., 2012), the horticultural cellulosic hydrolysate contained 39.8 g/L glucose and 20.5 g/L xylose in a ratio of ~ 2:1. Strain BOH3 fermented both glucose and xylose in the hydrolysate simultaneously after an adaption phase of 28 hrs (Fig. 3.4A). With a glucose/xylose ratio of 2:1, strain BOH3 consumes glucose and xylose at the same rate irrespective of whether the sugars came from hydrolysate or pure chemicals (Fig. 3.3A versus Fig. 3.2C). However, strain BOH3 produced 11.2 g/L butanol from the hydrolysate medium and with a slightly lower yield of 0.19 g/g when compared with the glucose/ xylose mixture medium (12.4 g/L butanol). Riboflavin was also detected at a concentration of 51.2 mg/L in the hydrolysate spiked medium (Fig. 3.4A), which is lower than in the glucose/ xylose mixture medium (91.4 mg/L) (Fig. 3.2F). On the other hand, the hemicellulosic hydrolysate obtained from the horticultural waste was further concentrated and detoxified resulting in a content mainly of xylose (58.3 g/L) and small amount of glucose (5.9 g/L) (Fig. 3.4B) (Zhang et al., 2012). After 28 hrs lag, strain BOH3 rapidly converted all of the xylose into 65.3 mg/L riboflavin and 11.9 g/L butanol with a final yield of 0.19 g/g, which is similar to fermenting the mixture of glucose/xylose (Fig. 3.2). Therefore, strain BOH3 is able to simultaneously ferment both glucose/xylose in hydrolysates of lignocellulosic biomass.

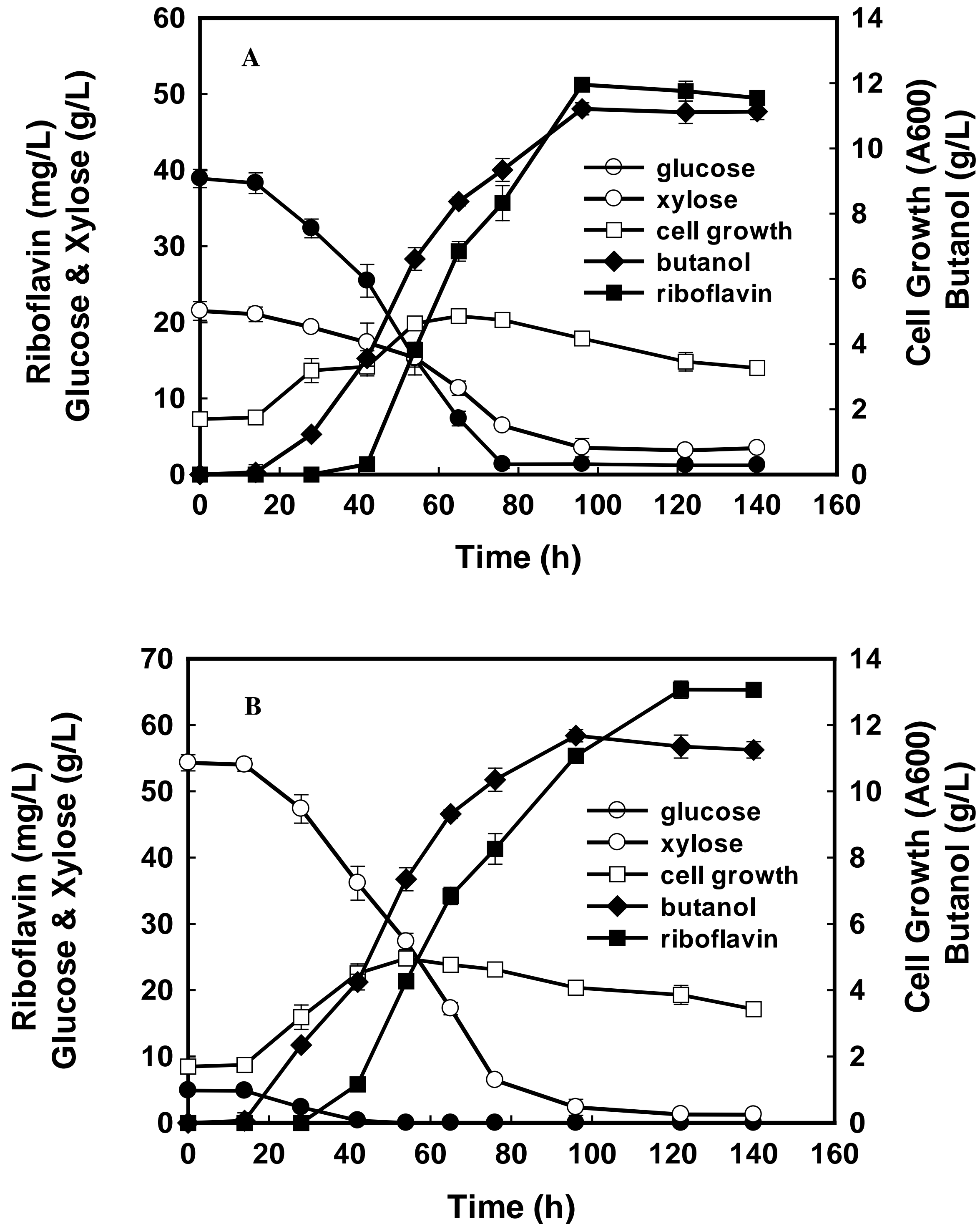


Fig. 3.4 Fermentation of (A) cellulosic hydrolysate (containing 39.8 g/L glucose and 20.8 g/L xylose) from horticultural waste and (B) hemicellulosic hydrolysate (containing 5.9 g/L glucose and 58.3 g/L xylose) from horticultural waste by *Clostridium* sp. strain BOH3.

3.4.4 Expression of xylose isomerase and xylulokinase in strain BOH3

Xylose isomerase (EC 5.3.1.5) and xylulokinase (EC 2.7.1.17) are two key enzymes in xylose metabolism (Aristidou and Penttila, 2000; Jeffries, 1983). To elucidate whether the high xylose utilization is related to a high degree of expression of xylose isomerase in culture BOH3, gene expression analysis was conducted on strain BOH3, and compared with the low xylose-utilizing solventogenic *Clostridium* sp. strain G117 (producing 5.2 g/L butanol from 60 g/L xylose) (Chua et al., 2013; Wu et al., 2012). During the acidogenic phase (0-20 hrs), the expression level of xylose isomerase in strain BOH3 increased up to 165 fold, which is 9 times higher than strain G117 (Fig. 3.5). The enhanced transcription of gene xylose isomerase in strain BOH3 supports its higher xylose utilization capability than other solventogenic strains, such as strain G117.

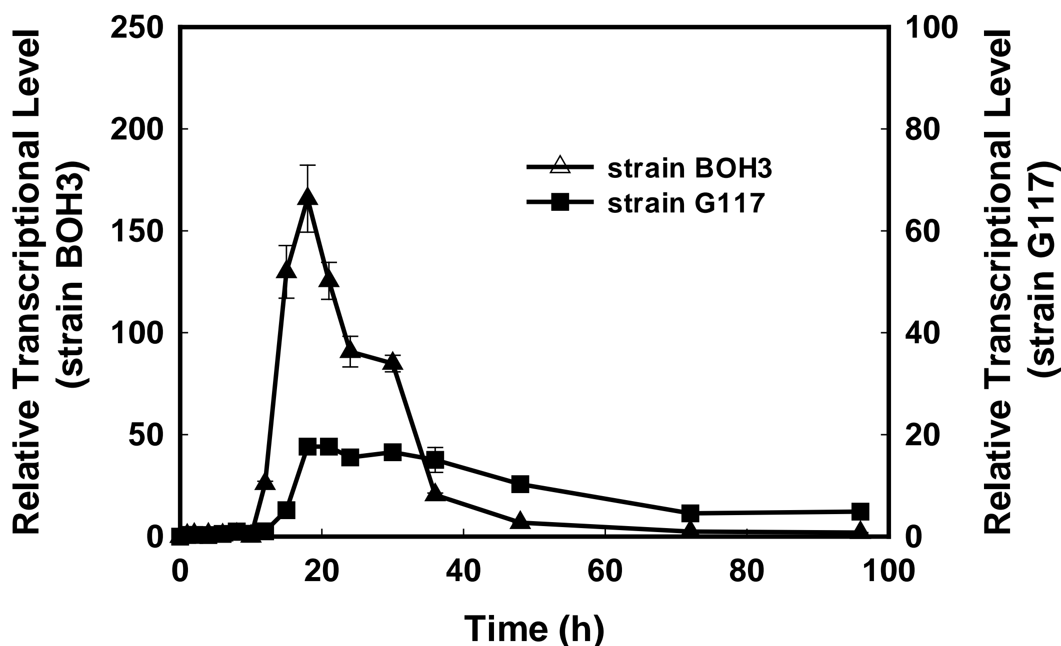


Fig. 3.5 Comparison of relative xylose isomerase transcription levels between *Clostridium* sp. strain BOH3 and *Clostridium* sp. strain G117 in a medium containing 60 g/L xylose as a carbon source.

Xylose isomerase mRNA-cDNA copy numbers were first normalized against the mRNA-cDNA copy numbers of housekeeping genes (*fabZ* for strain BOH3, *pepT* for strain G117) at each time point. The fold of transcript increment is then calculated by normalized xylose isomerase copy numbers at each time point / normalized xylose isomerase copy numbers at the time point of 8 hrs. Values are averages of duplicate determinations.

In previous studies, xylose isomerase and xylulose kinase are only induced by the presence of xylose, and their expressions are inhibited by glucose, a phenomenon of which is referred as carbon catabolite repression (Grimmler et al., 2010). To further elucidate the underlying reason for the BOH3 strain's efficient utilization of xylose and simultaneous utilization of glucose and xylose, activities of xylose isomerase and xylulose kinase were measured in the cell-free extract obtained at the cultures' exponential growth phase (36 hrs) with various amount of glucose and xylose (i.e., glucose, glucose and xylose mixture at different ratios, and xylose) (Table 3.2). As

expected, nearly no xylose isomerase and xylulose kinase activities were observed in the samples fed solely with glucose. For culture BOH3, the increments of enzymatic activities of xylose isomerase (0.25-0.66 U/mg protein) and xylulose kinase (0.46-0.84 U/mg protein) are consistent with the increased amount of xylose in the glucose/xylose mixtures (from 2:1 to 1:2), and the highest activities of xylose isomerase (0.97 U/mg protein) and xylulose kinase (1.16 U/mg protein) were reached when xylose was the sole carbon source (Table 3.2). For culture G117, the highest activities of xylose isomerase (0.42 U/mg protein) and xylulose kinase (0.76 U/mg protein) were reached when xylose was the sole carbon source. However, the activities of xylose isomerase and xylulose kinase were severely inhibited when glucose was present in the medium. For example, the xylose isomerase and xylulose kinase activities for strain BOH3 are 0.43 and 0.63 U/mg protein in a glucose and xylose mixture of 30g/L:30g/L, while the activities for strain G117 are only 0.09 and 0.12 U/mg protein, respectively. When fed with 60 g/L xylose only, the xylose isomerase and xylulose kinase activities for culture BOH3 increased to 0.97 and 1.16 U/mg protein, whereas these were only 0.42 and 0.76 U/mg protein for strain G117. The increments of xylose isomerase and xylulose kinase activities for strain BOH3 are proportional to the xylose ratio, while for strain G117, it is strongly inhibited by glucose. These findings suggest that expressions of xylose isomerase and xylulose kinase genes in strain BOH3 are not repressed in the presence of glucose.

Table 3.2 Specific enzymatic activities (U/mg protein) of xylose isomerase and xylulokinase associated with various glucose/xylose ratios in *Clostridium* sp. strain BOH3 and *Clostridium* sp. strain G117.

Substrate	Xylose isomerase activity[a]		Xylulokinase activity[a]	
	Strain BOH3	Strain G117	Strain BOH3	Strain G117
Glucose (60g/L)	ND[b]	ND	ND	ND
Glucose:Xylose (40g/L:20g/L)	0.25±0.12	0.06±0.01	0.46±0.05	0.09±0.02
Glucose:Xylose (30g/L:30g/L)	0.43±0.01	0.09±0.02	0.63±0.11	0.12±0.04
Glucose:Xylose (20g/L:40g/L)	0.66±0.11	0.10±0.05	0.84±0.06	0.16±0.06

[a]The data are calculated from triplicates, and data are represented as the average ±standard deviation. The samples were taken at a time point of 36 hrs.

[b]ND: not detected(detection limit is <0.01).

3.5 Discussion

In this study, the newly reported *Clostridium* sp. strain BOH3 shows efficient xylose utilization and co-fermentation of glucose and xylose simultaneously, which are supported by (i) generating higher amount of butanol (14.9 versus 14.5 g/L) and riboflavin (110.5 mg/L versus 76.8 mg/L) from xylose (60 g/L) than glucose (60 g/L); (ii) full utilization of glucose and xylose in glucose/xylose mixtures and hydrolysates from horticultural wastes; (iii) higher expression of the xylose isomerase gene and higher enzymatic activities of xylose isomerase and xylulose kinase responsible for xylose utilization. Moreover, compared to previously reportedwild and mutant strains in batch fermentations fed with 60 g/L xylose, strain BOH3 showed the highest xylose utilization (100%) and butanol production (14.9 g/L) (Table 3.1). Therefore, among all of the

reported solventogenic microorganisms, simultaneous utilization of glucose and xylose enables *Clostridium* sp. strain BOH3 to produce the highest amount of butanol (12.4 g/L) from glucose and xylose mixtures under similar growth conditions (Gu et al., 2009;Xiao et al., 2012; Xiao et al., 2013).

During the fermentation process, strain BOH3 co-produces riboflavin, which adds to the economical value of the process. Usually, amajor concern for a co-production process is whether thesecond product affects the yield of the major product. For strain BOH3, high butanol production (14.9 g/L) still occurred accompanying 110.5 mg/L riboflavin generation, which is close to its saturationamount (120 mg/L) in the medium (Perlman, 1979; Vandamme, 1992). Therefore, resultsin this studysupport the rationale of co-production of another value-added product in the ABE fermentation process so as to make it more economically feasible. However, butanol and riboflavin produced from the hydrolysates of horticultural wastes are still lower than pure monosaccharides mixture. It is known that furfural and hydroxymethylfurfural present in lignocellulosic hydrolysates can be converted to their alcohol or acid derivatives by microorganisms through $NAD(P)H/NAD(P)^+$-dependent redox reactions (Almeida et al., 2009; Zhang et al., 2012). These processes may break the cofactor balance in sugar metabolism of *Clostridium* to affect butanol formation (Almeida et al., 2009). In addition, other components (e.g. vanillin) in the hydrolysate may also affect thesugar-transport system (Almeida et al., 2009; Zhang et al., 2012).

For solventogenic *Clostridium*, xylose is usually transported into cytoplasm initially by a xylose transporter and then is further converted into xylulose-5-phosphate by xylose isomerase and xylulose kinase (Jeffries, 1983; Xiao et al., 2011; Xiao et al.,

2012). Strengthening this xylose transport system in *C. acetobutylicum* ATCC 824 and *C.beijerinckii* NCIMB 8052 have been proven to partially improve xylose uptake, however, they still show lower xylose utilization compared to glucose and carbon catabolite repression which still exists in *C. acetobutylicum* ATCC 824 (Xiao et al., 2011; Xiao et al., 2012). For example, xylose was still left at 13 g/L after glucose was fully utilized in a glucose and xylose mixture (40 g/L:20 g/L) when using gene modified *C. acetobutylicum* ATCC 824 (overexpression of *XylT* and inactivation of phosphoenolpyruvate (PES) dependent phosphotransferase system (PTS)) (Xiao et al., 2011). Only by further overexpression of xylose isomerase and xylulose kinase, *C. acetobutylicum* ATCC 824 nearly consumed all xylose (20 g/L) in the presence of glucose (40 g/L) and improved butanol production from 7.9 g/L to 9.1 g/L, although the bottleneck still existed because of its low butanol tolerance in xylose (Xiao et al., 2011). In contrast, strain BOH3 shows a higher transcription level of xylose isomerase and enzymatic activities of xylose isomerase and xylulose kinase when using xylose alone, compared to the low-xylose utilizing strain such as G117 (Table 3.2). Most importantly, the activities of xylose isomerase and xylulose kinase in strain BOH3 are not inhibited even in the presence of glucose. In contrast, their enzymatic activities in strain G117 were strongly inhibited by glucose (Table 3.2). As is known, the inhibition of expression of genes encoding for enzymes responsible for catabolism of carbon source over the preferred ones is the main cause of carbon catabolic repression. Moreover, the higher butanol tolerance (15.2 g/L) of strain BOH3 (Bramono et al., 2011) than previously reported wild-type microbes (e.g., 9.0 g/L for *C. acetobutylicum* ATCC 824 and 7.0 g/L *Clostridium* sp. strain G117) could efficiently alleviate the butanol inhibitory effect on

the energy-requiring xylose transport system located in the cell membrane (Bowles and Ellefson, 1985; Qunine et al., 1985; Volleherbst-Schneck et al., 1984). Therefore, the high expression of xylose degradation enzymes (xylose isomerase and xylulose kinase) in the presence of glucose contributes to its efficient xylose consumption and relief from carbon catabolite repression.

3.6 Conclusion

This study further explored the efficient utilization of xylose and elimination of carbon catabolic repression by *Clostridium* sp. strain BOH3. Compared with previous reported wild-type or gene-modified *Clostridium* strains, strain BOH3 could completely utilize 60 g/L xylose and produced the highest of 14.9 g/L butanol under similar operational conditions. Meanwhile, strain BOH3 shows unique capabilities in simultaneously consuming glucose and xylose to produce butanol at yields up to 14.9 g/L with riboflavin as a co-product. Strain BOH3 is therefore a promising candidate in transforming lignocellulosic biomass into biofuel due to its efficient usage of both xylose and glucose.

Chapter 4

Characterization of a thermostable xylanase from a newly isolated *Kluyvera* species and its application for biobutanol production

4.1 Abstract

Kluyvera species strain OM3 isolated from spent mushroom substrate produced high level of cellulase-free xylanase (5.12 U/mL). This xylanase showed maximum activities at 70 °C and pH 8.0, which could retain 100% and 71% activity after 1 hr incubation at 60 °C and 70 °C, and maintain stability over a wide range of pHs (5.0-9.0), indicating its thermal and pH stability. Moreover, the xylanase could hydrolyze untreated lignocellulosics (e.g., palm oil fiber) to reducing sugars with a yield of 27.1 - 46.9 mg/g. A co-culture consisting of *Kluyvera* sp. strain OM3 and *Clostridium* sp. strain BOH3 could directly convert birchwood xylan to 1.2 g/L butanol, which is comparable to the amount of butanol (1.7 g/L) generated via separate hydrolysis by the xylanase and fermentation by *Clostridium* sp. strain BOH3. This is the first report on the production, characterization of a xylanase from genus *Kluyvera* and its application for butanol production directly from hemicelluloses.

4.2 Introduction

Hemicellulose is the most abundant heteropolymer and second most abundant renewable biomass in nature after cellulose, which accounts for 25-35% of lignocellulosic biomass (Saha, 2003). Xylan constitutes a major portion of hemicelluloses, so hydrolysis of xylan becomes an important step towards proper utilization of the abundantly available lignocellulosic materials (Kumar et al., 2008; Saha, 2003). Chemical hydrolysis of xylan has been practiced extensively in industries, though faster, this process is accompanied with the formation of inhibitors (e.g., furfural, hydroxymethylfurfural, or 4-hydroxybenzaldehyde), which can exert a significant negative effect on the subsequent fermentation reaction (Beg et al., 2001). Enzymatic hydrolysis of xylan to xylose provides a viable alternative to chemical hydrolysis since it does not produce any inhibitors and thus is an environment friendly process (Bajpai, 1997).

The hemicellulose hydrolyzing enzymes, i.e., xylanases, have several different industrial applications, including in biodegradation of lignocellulose in animal feed, foods, textiles, and biopulping in the paper and pulp industry (Bajpai, 2007; Collins et al., 2005). Nowadays, increasing interest has been shown in using cellulase-free xylanases in the paper and pulp industry to improve pulp properties via removal of hemicelluloses and lignin residues but without disturbing the required structural properties of cellulose micro fibrils (Baijpai, 1997). Furthermore, the incoming pulp for enzymatic bleaching is usually hot and alkaline, thus the use of thermostable alkaline xylanases is very attractive from economical and technical point of view (Collins et al., 2005). However, most of the xylanases from fungi or bacteria show optimal activities at neutral (in particular for

bacterial xylanases) or slightly acidic (in particular for fungal xylanases) pH values and at mesophilic temperatures (approximately between 40°C and 55°C) (Collins et al., 2005; Wang et al., 2012). Relatively few microorganisms produce xylanases that are optimally active either at elevated temperature or alkaline conditions (Subramaniyan and Prema, 2002). However, thermostable enzymes show many advantages, such as increased flexibility of process concepts, reduction of enzyme amount due to higher specific activity and elongated hydrolysis time (Zhang et al., 2011). As a result, new xylanases possessing the above properties are desired.

The importance of xylanases is not limited to the paper and pulp industry, thus there are other industries with equal importance of applicability. Potential applications of xylanases also include hydrolysis of lignicellulosic biomass to reducing sugars, which can be fermented for biofuel (e.g., ethanol, butanol) generation. Especially, compared with aerobic fungi and bacteria, few studies have been conducted on hydrolytic enzymes from anaerobic bacteria (Bajpai, 2007; Beg et al., 2001). However, the anaerobic process shows advantages in development of unique biotechnological processes, such as direct bioconversion of lignocellulosic biomass into biofuel (bioethanol or biobutanol) by using xylanase or lignocelluloses hydrolyzing microbes and biofuel-producing bacteria (Kumar et al., 2008). The primary advantage of using a co-culture system is that it eliminates the costly enzymatic hydrolysis step and thus has a potential for cost-effective consolidated bioprocessing. Previous studies demonstrated butanol production from crystalline cellulose by co-culturing cellulose-degrading and biobutanol-producing bacteria under anaerobic conditions (Petitdemange et al., 1983; Yu et al., 1985). However, few studies

have reported fermentation directly from hemicellulose to biofuel (Kumar et al., 2008; Yu et al., 1985).

The aim of this study is to cultivate an anaerobic bacterium capable of producing effective cellulase-free xylanase that can tolerate high temperature and pH and to characterize this enzyme to uncover its potential application. Most importantly, the generated xylanase or the cultivated culture is applied to hydrolysis of hemicellulose to reducing sugars for following biobutanol production.

4.3 Materials and methods

4.3.1 Isolation and culture growth

A three-year old spent mushroom substrate (SMS) collected from Mycofarm Pte Ltd., Singapore was used as inoculum for screening xylanase-producing bacteria. Reduced mineral salts medium was used for isolation and cultivation. The procedure to prepare the medium is briefed described as follows: the serum bottles were spiked with a medium containing 1 g/L of NaCl, 0.5 g/L of $MgCl_2 \cdot 6H_2O$, 0.2 g/L of KH_2PO_4, 0.3 g/L of NH_4Cl, 0.3 g/L of KCl, 0.015 g/L of $CaCl_2 \cdot H_2O$, 0.2 g/L of $MgSO_7 \cdot H_2O$, and amended with 10 g/L xylan or other carbon source such as, palm oil fiber, sawdust and wood chips. In addition, 1 mL of trace element solution (Widdel et al., 1992), 1 mL of Na_2SeO_3-Na_2WO_4 solution (Brysch et al., 1987), and 10 mg of resazurin were added per liter of medium. After the medium was boiled and cooled down to room temperature (20-25°C) under N_2, reductants Na_2S, L-cysteine, and $_{DL}$-dithiothretol were added to a final concentration of 0.2, 0.2, and 0.5 mM, respectively (He et al., 2003). Subsequently, $NaHCO_3$ (30 mM) was added to the medium and the pH was adjusted to 7.0. Then, 50-

mL liquid medium was dispensed into 160-mL bottles, which were sealed with butyl stoppers, autoclaved for 20 min, and cooled down to room temperature (20-25°C). Lastly, a vitamin solution (Wolin et al., 1963) was added to each bottle. After five transfers in birchwood xylan-amended medium, the enrichment culture (0.1mL; successively diluted to 10^{-5} times) was repeatedly streaked on agar plates poured with mineral salts medium containing birchwood xylan as the sole carbon source. After incubation for six days, Congo red staining was used to indicate xylanase activities of the colonies. The xylanase activity of each colony was determined by measuring the zone of clearance on the agar plates. Through such processes, a pure bacterial culture, designated OM3, was obtained. All the enrichment, isolation and cultivation were performed in an anaerobic chamber filled with mixed gas (90% N_2, 5% CO_2 and 5% H_2) and operated at 25°C.

Culture growth and xylanase production were assessed by feeding the culture with various carbon sources at 40 °C and pH 7.0. Either birchwood xylan, beechwood xylan, sawdust, wood chips or palm oil fiber was used as a carbon source in the liquid medium. After 6 days of culture growth, enzyme activity was assessed. To investigate the influence of temperature, cultures were assayed at different temperatures ranging from 25 °C to 50 °C. Culture growth and enzyme production were also evaluated at various initial pHs from 5.0 to 11.0 at 40 °C. All of the fermentation was carried out in duplicates.

4.3.2 Coculture of *Kluyvera* sp. strain OM3 and *Clostridium* sp. strain BOH3

The coculture experiments were conducted in butyl rubber sealed-serum bottles. The medium used for fermentation was the same as the above mineral salts medium and amended with 4% (w/v) birchwood xylan plus 0.5% (w/v) yeast extract. To determine the role of xylanolytic *Kluyvera* sp. strain OM3 in hemicellulose hydrolysis step, a co-culture was established stepwise by initiating a mono-culture of strain OM3 with 10% inoculation (v/v) and followed by the addition of the solvent-producing *Clostridium* sp. strain BOH3 (Bramono et al., 2011) when soluble reducing sugars had accumulated to a stable concentration after 72 hrs incubation. Prior to adding strain BOH3, it was pre-grown to exponential phase and was collected after 24 hrs by centrifugation, washed, and suspended in the same mineral salts medium without any carbon source. A 10% inoculum (v/v) of culture BOH3 was then added to bottles containing 50 mL medium. All cultures were analyzed at regular intervals for enzyme activities, reducing sugars, biosolvents, and volatile fatty acids.

4.3.3 16S rRNA gene sequencing and phylogenetic analysis

The genomic DNA of cell pellets from isolate OM3 was extracted and purified with DNeasy Tissue Kit (Qiagen GmbH, Germany) according to the manufacturer's instructions with minor modifications. With the genomic DNA of OM3 as a template, PCR amplification of the 16S rRNA genes was performed with a pair of universal bacterial primer 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1392R (5'-ACGGGCGGTGTGT-3') (Maniatis et al., 1982). After cleaning the PCR products with a PCR purification kit (Qiagen GmbH, Germany), the 16S rRNA genes were sequenced on a ABI DNA Sequencer. After Basic Local Alignment Search Tool (BLAST), the 16S

rRNA gene sequences of the isolate and other closely related strains were aligned using CLUSTAL X software (Thompson et al., 1997). Phylogenetic trees were constructed using the neighbor-joining method (Saitou and Nei, 1987) implemented in the MEGA program (Kumar et al., 2004). The topologies of the tree were evaluated by bootstrap analysis, based on 1,000 replicates. The nucleotide sequence of culture OM3 was deposited in the GenBank under an accession number of HQ860790.

4.3.4 Enzyme activity and hydrolysis assays

Total extracellular soluble protein content, an indicator of the enzyme released, was measured by the Lowry protein assay using bovine serum albumin as the standard (Lowry et al., 1951). Xylanase activity was measured according to Bailey et al. (Bailey et al., 1992) when serial enzyme dilutions were amended with 1% (w/v) birchwood xylan, 0.05 M glycine/NaOH buffer (pH 8.0). This mixture was incubated at 70 °C for 10 min. Endoglucanase (CMCase) and exoglucanase (Avicelase) activities were measured according to IUPAC recommendations (Ghose, 1987) when enzyme solution was amended with 1% (w/v) carboxymethyl cellulose or microcrystalline cellulose (Avicel) in 0.05 M sodium acetate buffer (pH 5.0). Both of the reaction mixtures were incubated at 50 °C for 1 hr. Reducing sugars released from xylanase, CMCase and Avicelase activities assays were measured by using the 3,5-dinitrosalicylic acid (DNS) method (Miller, 1959). One international unit (U) of each enzyme was defined as the enzymatic activity required for the release of 1 μmol of glucose or xylose equivalents per unit volume and minute of reaction. Enzymatic hydrolysis was carried out at a substrate

loading of 2.5% (w/v) with 10 U xylanase/g substrate at 60 °C for 48 hrs. Reaction without any addition of xylanase was used as control.

4.3.5 Enzymatic properties of xylanase

To obtain xylanase, solid ammonium sulfate was added to the culture supernatant with constant stirring at 4 °C to achieve an initial 30% saturation. After centrifugation at 9000 rpm for 15 min, the precipitates were discarded and the supernatant was subsequently adjusted to 70% saturation with the addition of appropriate amounts of ammonium sulfate and the precipitates were dissolved in 20 mL 0.05 M glycine NaOH buffer (pH 8.0). The enzyme solution was subjected to dialysis for 24 hrs at 4 °C against 0.05 M glycine NaOH buffer (pH 8.0). Enzyme activity and protein estimation were carried out from the dialyzed sample.

The influence of pH on xylanase activity was determined at 70 °C using 0.05 M of buffer (citrate (pH 3.0-5.0), phosphate (6.0-7.0), and glycine/NaOH (pH 8.0–12.0)). The pH stability of xylanase was assessed at pH ranging 4.0-12.0 at 60 °C after 30 min incubation. To determine the thermostability of xylanase activity, the xylanase was incubated at different temperatures (60 °C - 80 °C) in the absence of substrate. After incubating for 1 hr, the residual xylanase activity was determined at 70 °C for 10 min (Bailey et al., 1992). Additionally, the effects of various reagents (0.01 M) on xylanase activity were determined by pre-incubating the enzyme with individual reagents in 0.05 M glycine/NaOH (pH 8.0) at 30 °C for 30 min. Activities were then measured at 70 °C for 10 min in the presence of the metal ions or reagents. The activity assayed in the absence of reagents was recorded as 100%. Finally, the kinetic constants K_m and V_{max}

were determined following the method of Lineweaver and Burk (Bajpai, 1997; Gupta et al., 2000).

4.3.6 Gas chromatography analysis of metabolic products

Volatile fatty acids (i.e., acetic and butyric acids) and biosolvents (i.e., ethanol and butanol) were measured by a gas chromatography (GC, model 7890A; Agilent Technologies, U.S.A.) on a Durabond (DB)-WAXetr column (30 m × 0.25 mm × 0.25 μm; J&W, U.S.A.) equipped with a flame ionization detector (FID). The oven temperature was initially held at 60 °C for 2 min, increased at 15 °C/min to 230 °C, and held for 1.7 min. Helium was used as the carrier gas, with a column flow of 1.5 mL/min. Five-point standard curves were obtained by running standard solutions containing acetone, butanol, ethanol, acetic acid, and butyric acid.

4.4 Results and discussion

4.4.1 Cultivation of a xylanase-producing culture OM3.

A wild-type anaerobic xylanase-producing bacterium was isolated on agar plates with inocula from a spent mushroom substrate and birchwood xylan as the only carbon source at 35 °C. This isolate was picked according to its highest xylanase activities determined by the size of orange digestion halos formed on birchwood xylan plates using Congo red staining. Phylogenetic analysis of the 16S rRNA genes showed that this isolate shares 99% sequence identity with the 16S rRNA gene of *Kluyvera georgiana* (Fig. 4.1), thus it is designated as *Kluyvera* sp. strain OM3.

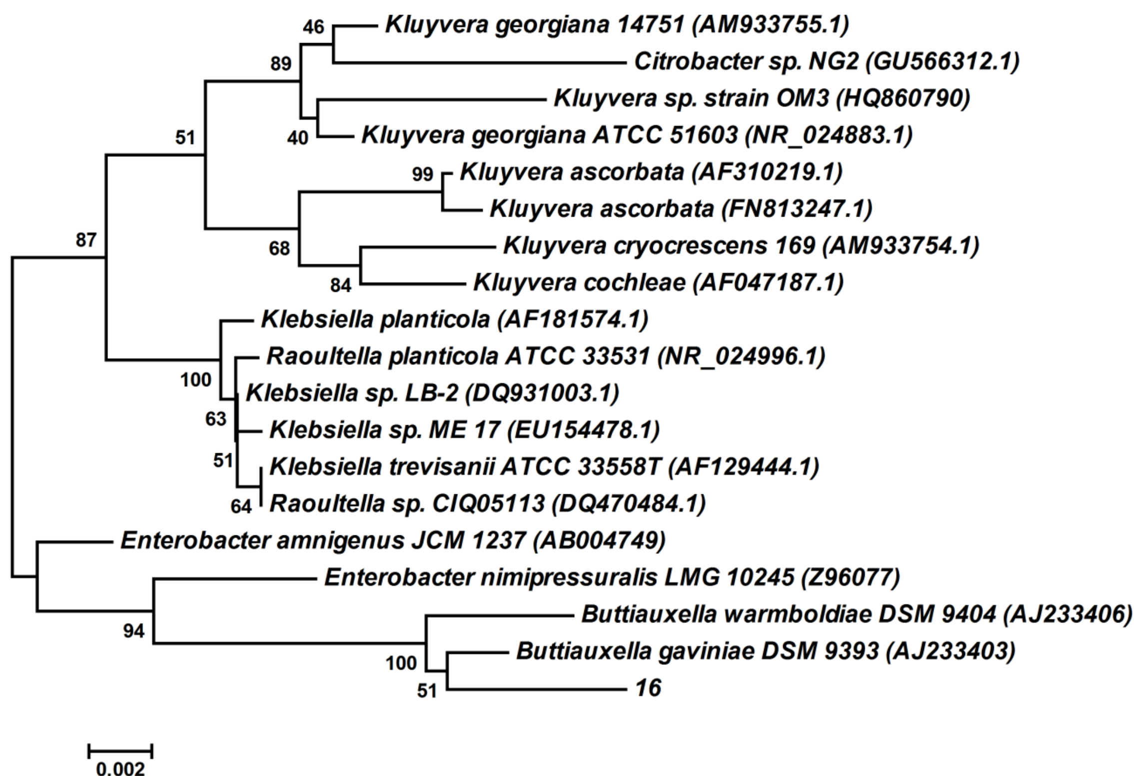


Fig. 4.1 Phylogenetic tree of strain *Kluyvera* sp. strain OM3 using the neighbor-joining method (MEGA 4.0) based on 16S rRNA sequences.

Strain OM3 can produce high-level of xylanase (5.12 U/mL) with a production of 0.88 mg/mL when the growth medium was supplemented with birchwood xylan at a pH and temperature of 8.0 and 40 °C, which is higher comparing with previous reported anaerobic bacteria such as *Ruminococcus flavefaciens* (0.07 U/mL), *Butyrivibrio fibrisolvens* (0.33 U/mL), *Clostridium* sp. (0.048-4.2 U/mL) (Marichamy and Mattiasson, 2005; Murty and Chandra, 1991) (Table. 4.1). Moreover, no cellulase activities could be detected, which is a positive attribute in terms of utilizing for pulp paper bleaching since cellulase could break down cellulose fibers. Xylanase activity and soluble protein concentration decreased when OM3 was grown at above 40 °C, indicating mesophilic nature of the bacterium. The optimum pH for the production of xylanase by OM3 was in

the range of pH 8.0 (5.12 U/mL) to 9.0 (4.86 U/mL). Most bacterial species are unable to grow at alkaline pH, and the common xylanase from fungal species favors acidic pH (Subramaniyan and Prema, 1998). There are only few reports of xylanase production from insoluble polysaccharides under alkaline conditions by anaerobic bacteria, such as *Tepidimicrobium xylanilyticum* BT14 at pH 9.0 (Phitsuwan et al., 2010). Noteworthy, besides hemicellulose, strain OM3 can also utilize hemicellulose-containing lignocelluloses, such as palm oil fiber, wood chips, and sawdust with a xylanase activity reaching 0.54, 0.43 and 0.42 U/mL and a production of 0.58, 0.43 and 0.46 mg/mL, respectively. Since large scale xylanase production from purified hemicellulose is uneconomical, agricultural residues offer a cost-effective alternative for xylanase production due to their abundance and low cost.

Table4.1 Comparison of xylanase activities among mesophilic anaerobic bacteria.

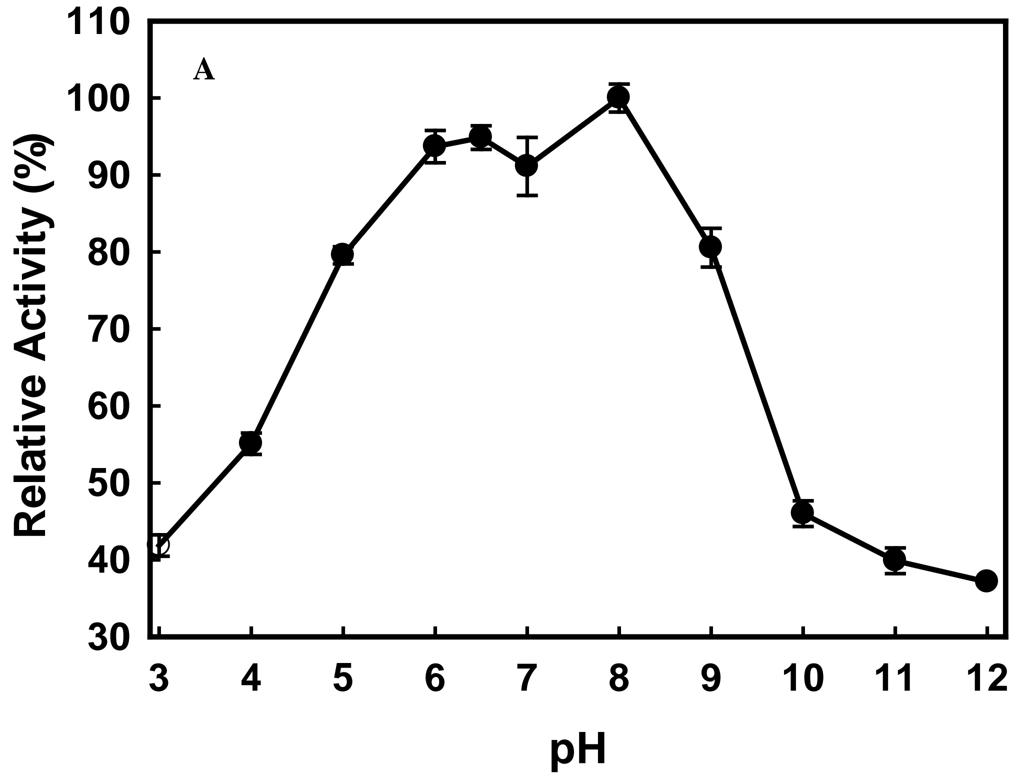
Organism	Growth substrate	T (°C)	pH	Xylanase activity (U/mL)	References
Clostridium acetobutylicum	OX	35^a	5.7^a	0.048	Murty and Chandra, 1991
Butyrivibrio fibrisolvens	LX	37^a	6.5^a	0.33	Murty and Chandra, 1991
Bacteroides ovatus B4-11	Hemicellulose	37^a	6.8^a	0.0052	Murty and Chandra, 1991
Ruminococcus flavefaciens	Cellobiose	45^b	6.4^b	0.07	Murty and Chandra, 1991
Clostridium SAIV	LX	50^b	$5.5\text{-}7.0^b$	0.52	Marichamy and Mattiasson, 2005
Clostridium acetobutylicum LU-1	OX	50^b	5.0^b	3.30	Marichamy and Mattiasson, 2005
*Clostridium butylicum*LU-1	OX	50^b	5.0^b	1.50	Marichamy and Mattiasson, 2005
Clostridium beijerinckii LU-1	OX	60^b	5.0^b	3.10	Marichamy and Mattiasson, 2005
*Clostridium bifermentans*LU-1	OX	70^b	4.0^b	4.20	Marichamy and Mattiasson, 2005
Kluyvera sp. OM3	BX	70^b	8.0^b	5.12	Present study

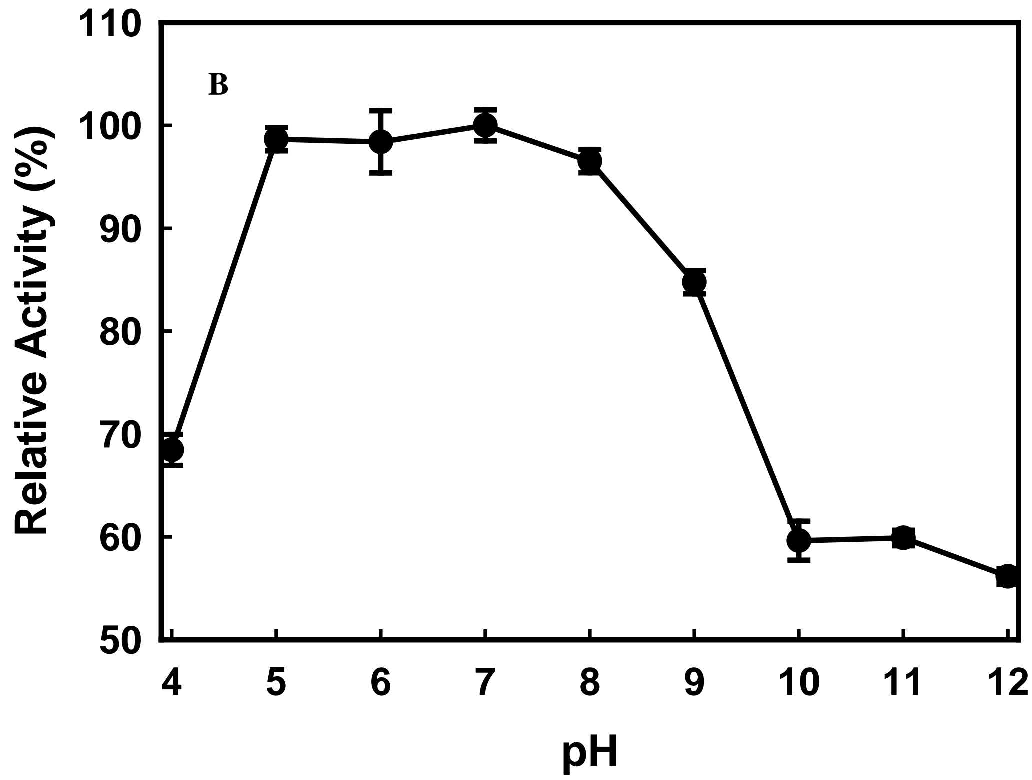
[a]: reaction temperature and pH; [b]: optimum reaction temperature and pH. OX: oat spelt xylan; LX: larchwood xylan; BX: birchwood xylan; FPC: filter paper cellulose.

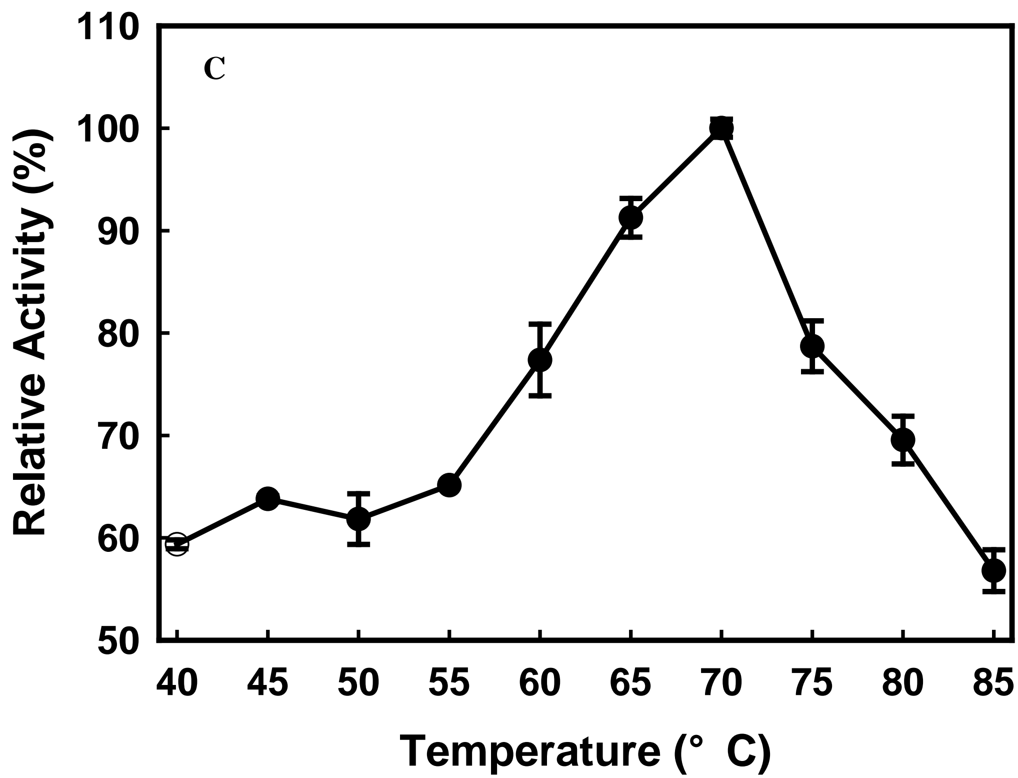
4.4.2 Biochemical characterization of the xylanase from culture OM3

During industrial application, whole cells are usually used as the source of enzymes but the efficiency can be improved by using purified enzymes or by excluding certain other unwanted enzymes (Bajpai, 1997). The crude xylanase from culture OM3 can be partially purified 2.6-fold with a yield of 67.2% by ammonium sulphate fractionation (30-70% saturation) and followed by dialysis with glycine NaOH buffer (pH 8.0). After applying the precipitated xylanase to agar plates containing birchwood xylan, the appearance of more clear zones also suggests higher hemicellulose hydrolysis activity. This xylanase was active between pH 5.0-9.0 and retained more than 80% of its activity while exhibiting an optimum pH ranging from 5.0- 8.0 by retaining almost 100% activity at 60°C for 0.5 hr. The enzyme activity beyond this range dropped dramatically, for example, only 54% and 46% activity were retained at pH 4.0 and 10.0, respectively (Fig. 4.2A and 4.2B). The optimum temperature for hydrolysis reactions was 70 °C (with a reaction time of 10 min) with 77% and 70% of the maximum activity at 50 °C and 80 °C, respectively. The enzyme activity kept increasing with an increase in temperature from 40 °C to 70 °C but further increase in temperature adversely affected the enzyme activity (Fig. 4.2C). Since utilization of enzymes in industrial applications often encounters its thermal inactivation, thermostability studies were carried out by pre-incubating the enzyme up to 1 hr in a temperature range of 60, 70 and 80 °C. The xylanase from culture OM3 could retain 100% activity at 60 °C after 1 hr of incubation while still exhibited nearly 71% and 49% of its maximum activity after incubation at 70 °C and 80 °C, respectively (Fig. 4.2D). The utilization of thermostable xylanase in

industry could improve the technical and economic feasibility of industrial processes (Bajpai, 1997).




B
Relative Activity (%)
110
100
90
80
70
60
50
4
5
6
7
8
9
10
11
12
pH


C
Relative Activity (%)
110
100
90
80
70
60
50
40
45
50
55
60
65
70
75
80
85
Temperature (° C)

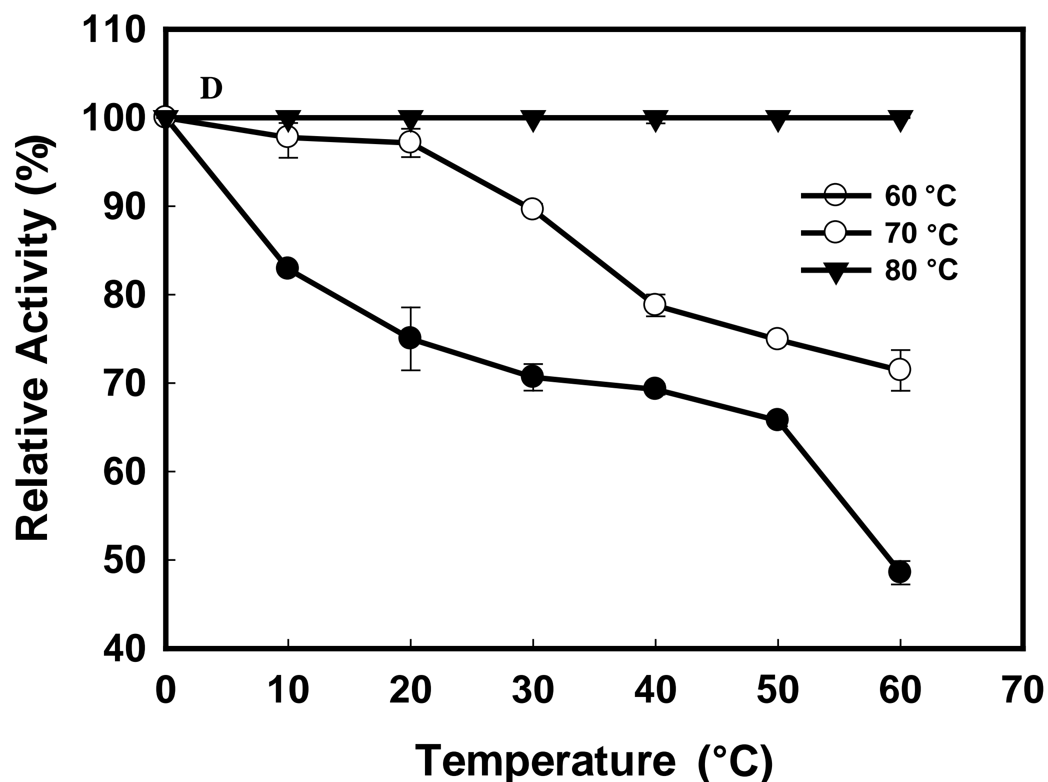


Fig. 4.2 (A) Optimal pH and (B) pH stability of the partially purified xylanase from *Kluyvera* sp. strain OM3. The influence of pH on xylanase activity was determined at 70 °C using 0.05 M of buffer (citrate (pH 3.0-5.0), phosphate (6.0-7.0), and glycine/NaOH (pH 8.0–12.0)). The remaining activity was measured after incubation for 10 min at 70 °C over various pH ranges. (C) Optimal temperature and (D) thermostability of the partially purified xylanase from *Kluyvera* sp. OM3. The reaction was determined by using 0.05M glycine/NaOH buffer at pH 8.0.

Usually, thermophilic aerobic bacteria possess the capability of producing thermostable xylanase, so do to several mesophilic aerobic bacteria, e.g, *Bacillus* spp. (Beg et al., 2001; Collins et.al., 2005). Till now, there are very few reports of xylanases from mesophilic anaerobic bacteria optimally active under thermo- and alkaline-conditions (Bajpai, 1997; Beg et al., 2001; Collins et al., 2005). For example, the xylanase produced by *Clostridium bifermentans* LU-1 grown at 37 °C showed an optimum pH of 4.0 and an optimum temperature of 70 °C but only 5 min half life

(Marichamy and Mattiasson, 2005). It is still unclear why some mesophiles have evolved thermostable enzymes, but it is possible that these genes in mesophilic bacteria were acquired via horizontal gene transfer from organisms that flourished under high temperature conditions (Beg et al., 2001; Collins et al., 2005; Kulkarni et al., 1999). Indeed, the spent mushroom substrate could reach high temperature during the process of composition and there is such possibility that the thermostable xylanase genes were acquired via horizontal transferation from other thermophilic bacteria. Currently, the major application of xylanases is in the pulp and paper industry with high temperatures (55–70 °C) and an alkaline pH of the pulp substrate, which requires thermo-alkaliphilic enzymes for efficient biobleaching (Beg et al., 2001; Collins et al., 2005). In this aspect, the xylanase from culture OM3 has a great potential on its application to pulp and paper industry. On the other hand, thermo-alkaliphilic xylanases could also find its wide usage in hydrolysis of hemicellulose from biomass to reducing sugars due to better solubility of hemicellulose under thermo- and alkaline- conditions.

Further characterization was conducted on the effect of potential inhibitors or activators on the precipitated xylanase. Compared with enzyme activity without any addition of reagents, the enzyme was strongly inhibited by 0.01 M Cu^{2+} and Ag^{+} with 34.0% and 47.4% of remaining activity, respectively. The activity of the enzyme was slightly inhibited in the presence of Fe^{3+} (65.8%), Ni^{2+} (70.6%), ethylenediaminetetraacetic acid (EDTA; 75.3%), sodium dodecyl sulfate (SDS; 87.2%), Zn^{2+} (94.5%), and Na^{+} (98.6%). Cu^{2+} is known to catalyze autooxidation of cysteines, which leads to the formation of intra molecular and inter molecular disulfide bridges or to the formation of sulfenic acid (Vieille and Zeikus, 2001). The inhibition by Ag^{+} may be

due to its interaction with sulfhydryl groups (Beg et al., 2001), suggesting that there is an important cysteine residue in or close to the active site of the enzyme, while similar results were also reported previously (Beg et al., 2001; Collins et al., 2005; Kulkarni et al., 1999). Metal ions can be involved in enzyme catalysis in a variety of ways (Palmer, 2001). This was further confirmed by the observed increase in xylanase activity in the presence of Co^{2+} (118.6%), Fe^{2+} (110.9%), Mg^{2+} (112.6%), and Mn^{2+} (115.2%). The xylanase activity can also be stimulated in the presence of the protein disulfide reducing reagents, L-Cys and Dithiothreitol (DTT) (125.9% and 122.5% relative activity), confirming the presence of reduced disulfide bonds of cysteine in the enzyme of strain OM3 (Kulkarni et al., 1999).

The precipitated xylanase showed a strong specificity towards birchwood and beechwood xylan, and exhibited a much higher activity with birchwood xylan. No activity was detected against carboxymethyl cellulose and Avicel, confirming that it is a cellulase-free xylanase. The Michaelis–Menten constant K_m and V_{max} values of the precipitated xylanase towards birchwood xylan were 4.59 mg/mL, and 187µmol/mg/min, respectively, which were obtained from Lineweaver-Burke plot of specific activities at 70 °C with different substrate concentrations. The xylanase from culture OM3 exhibited comparable K_m and V_{max} values to the xylanases isolated from other bacteria such as *Bacillus* sp. (Dey et al., 1992) and *Staphylococcus* sp. (Gupta et al., 2000).

4.4.3 Hydrolysis of untreated lignocellulosic biomass

To investigate the saccharification feasibility by the xylanase of culture OM3, various untreated lignocellulosic biomass, i.e. wood chips, sawdust, palm oil fiber, waste

carton box and waste newspaper, were tested with the precipitated xylanase under conditions of 60 °C and pH 8.0 since the xylanase could maintain up to 86% maximum activity after 48 hrs (Fig. 4.3). Among the lignocellulosics, waste newspaper showed the best yield (46.9 mg of reducing sugars per gram substrate), followed by waste carton box (44.1 mg/g), palm oil fiber (34.1 mg/g), wood chips (31.7 mg/g), and sawdust (27.1 mg/g) when using 10 U xylanase precipitation/g of substrate. In previous study, some other agro-industrial wastes were also used to test the xylanase saccharification abilities from other microorganisms, such as soybean hull, soybean fiber, and rice straw by purified xylanase from *B. circulans* witha yield of 29.5, 21.5 and 14 mg/g, respectively. Previous reaction mixtures contained 1 mL of purified enzyme solution and 50 mg dry weight (Heck et al., 2006). Also, a maximum yield of reducing sugars of 14 mg/g from wheat bran and 5.5 mg/g from the industrial pulp were obtained by purified xylanase from *Burkholderia* sp DMAX with 30 U partially purified xylanase/g of substrate (Sarayu et al., 2008). Compared to those data, xylanase produced by OM3 could more efficiently hydrolyze lignocelluloses than previous reported xylanases.

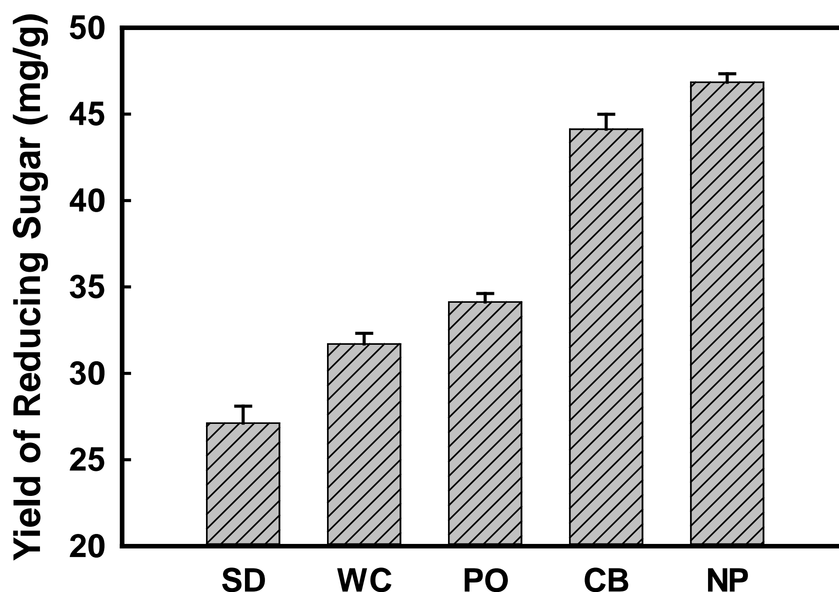


Fig. 4.3 Hydrolysis of different lignocellulosic wastes (WC: wood chips; SD: sawdust; PO: palm oil fiber; CB: waste carton box; NP: waste newspaper) by the partially purified xylanase from *Kluyvera* sp. OM3 after 72 hrs of incubation at 60 °C.

The factors affecting enzymatic degradation of lignocellulosic materials are lignin contents, since inhibition in both enzyme production and activity have been reported for lignin-rich materials (Xin and Geng, 2010). Lignin, which is a phenolic macromolecule, is resistant to enzyme attack and degradation, thus its content and distribution are recognized as the most important factors in determining cell wall recalcitrance to hydrolysis (Shi et al., 2011). Sawdust and wood chips both have higher lignin contents (36.6% and 34.9%), newspaper has the lowest lignin content (11.2%) (Xin and Geng, 2010), which is also reflected by the generated reducing sugar amounts as shown in this study. Various strategies of pretreatment (e.g., dilute acid pretreatment, steam explosion and organosolv pretreatment) can be adopted to disrupt the lignin structure, leading to biomass swelling, internal surface area increase, and improved accessibility of hydrolytic enzymes to cellulose and hemicellulose fibers (Kumar et al., 2008). The pretreated

biomass becomes more bioavailable than the raw biomass and makes the lignocellulosics more suitable for enzyme saccharification (Zhang et al., 2011).

4.4.4 Butanol production by xylanase from *Kluyvera* sp. strain OM3 plus *Clostridium* sp. strain BOH3 and co-culturing of *Kluyvera* sp. strain OM3 plus *Clostridium* sp. strain BOH3 directly from xylan

To evaluate the potential application of the xylanase from strain OM3 in biofuel application, a separate hydrolysis and fermentation process was carried out using 40 g/L of birchwood xylan with 4 U/mL xylanse at 60 °C. During the enzymatic saccharification of birchwood xylan, a regular increase of reducing sugars was observed till 24 hrs of incubation, which remained almost constant (9.5 g/L), thereafter, *Clostridium* sp. strain BOH3 was inoculated to initiate fermentation (Fig. 4.4). Fermentation was essentially completed after 72 hrs with 1.7 g/L butanol produced, which demonstrates the feasibility of using the xylanase and butanol-generating microbes for biofuel production from hemicellulose. The butanol production can be improved by addition of more enzymes in order to hydrolyze birchwood xylan more efficiently for higher reducing sugar production. However, a disadvantage is the expense of enzymes during the production and purification process, which covers a big part of biofuel cost.

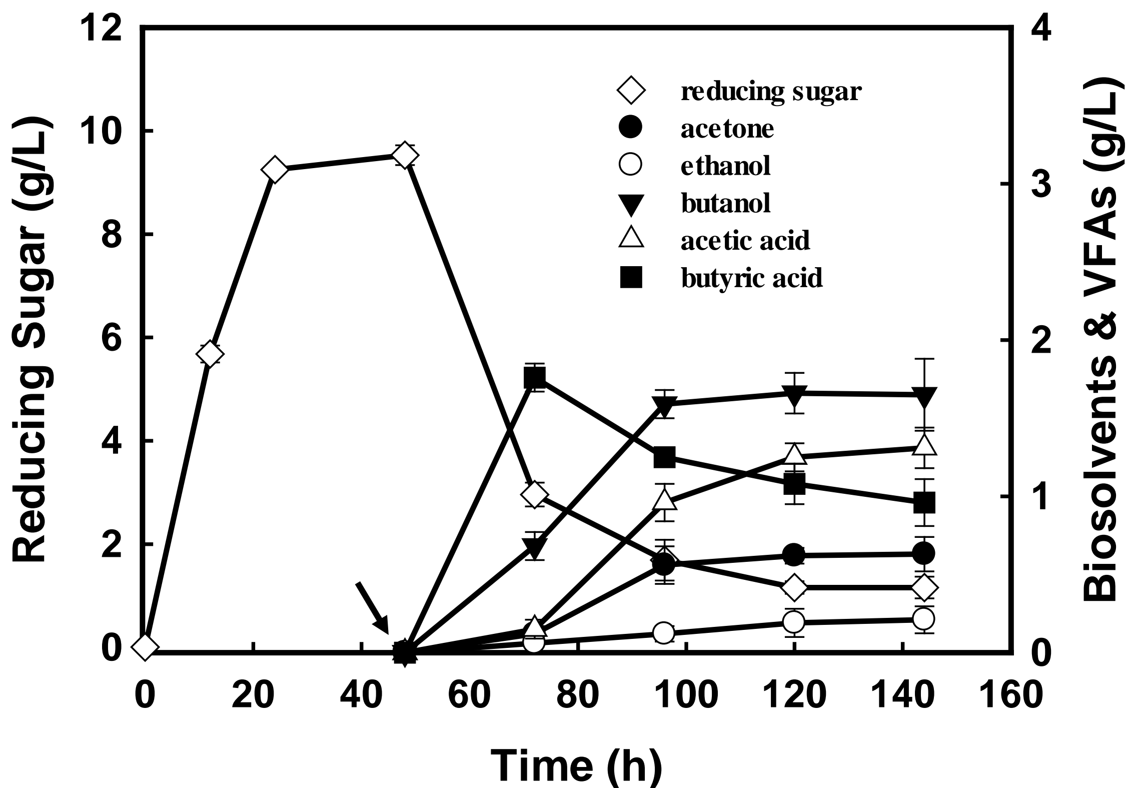
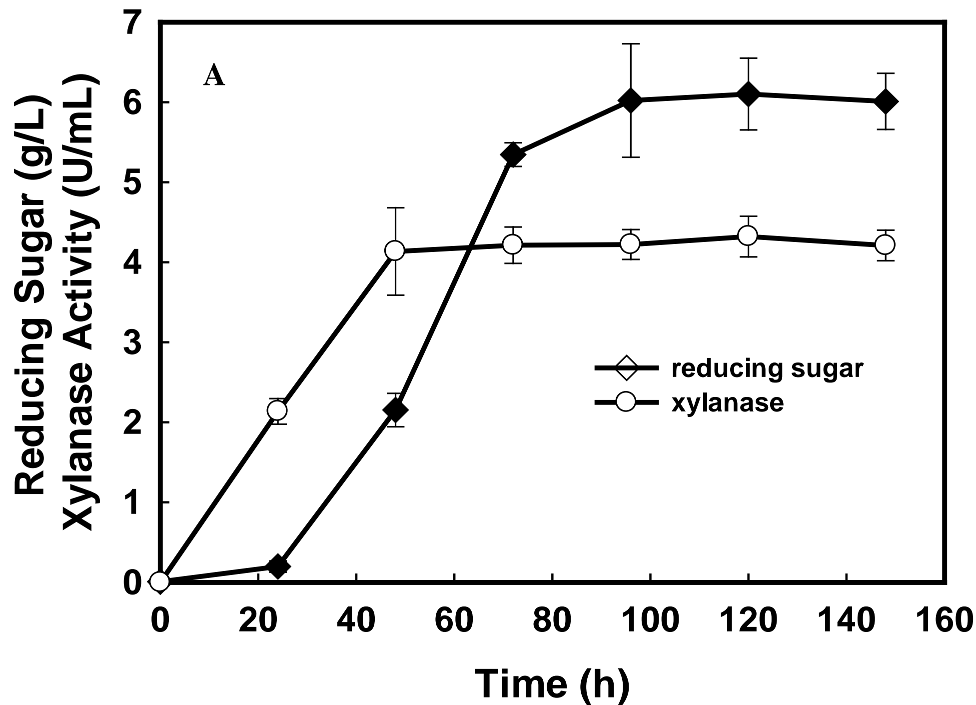


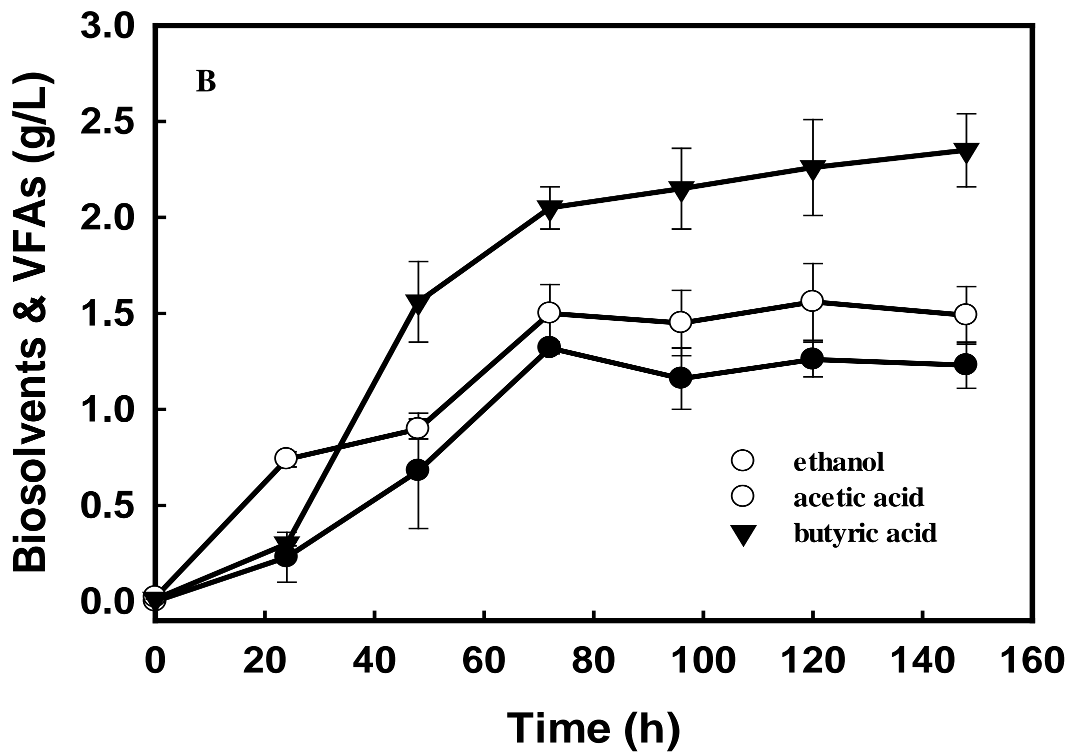
Fig. 4.4 Profiles of reducing sugars and fermentation product formation by xylanase of culture OM3 and*Clostridium* sp. strain BOH3 amended with 4% (w/v) birchwood xylan. Arrow means inoculation point of *Clostridium* sp. strain BOH3.

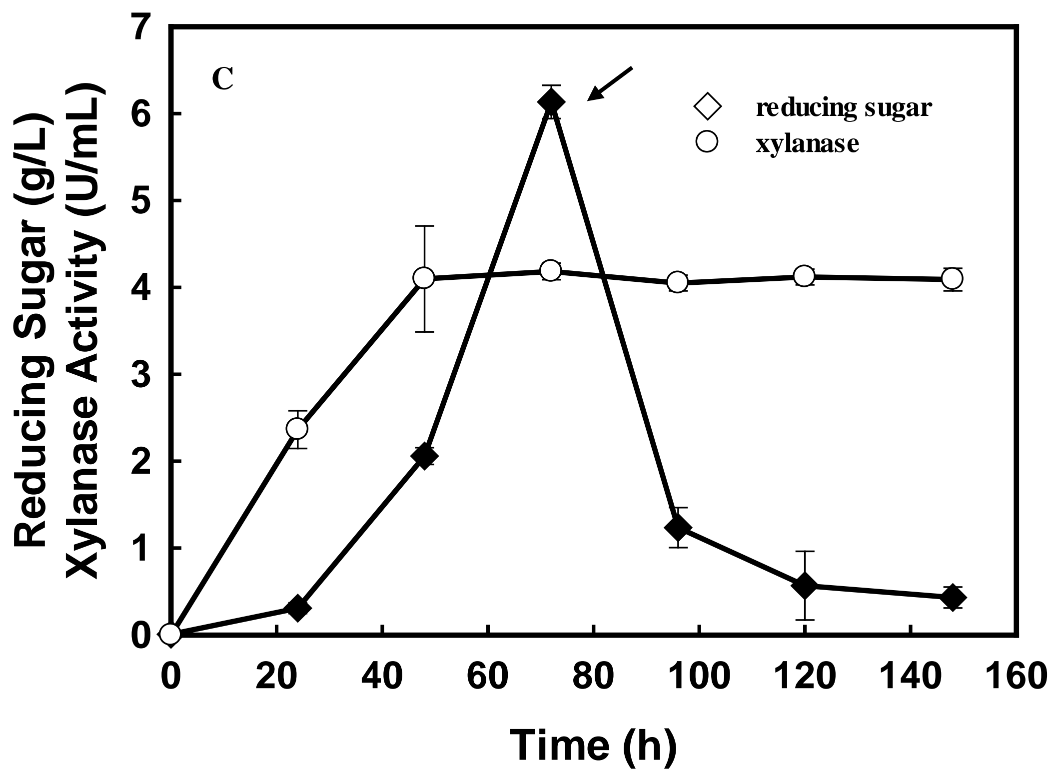
To overcome the above disadvantages, a one step process by co-culturing *Kluyvera* sp. strain OM3 and *Clostridium* sp. strain BOH3 was then performed for butanol production from birchwood xylan. Initially, *Kluyvera* sp. strain OM3 was grown on birchwood xylan, and extracellular xylanase was released into the culture medium (Fig. 4.5A). The xylanase could readily hydrolyze birchwood xylan to reducing sugars in the culture medium (Fig. 4.5A) and low levels of ethanol (1.2 g/L), acetic acid (1.5 g/L) and butyric acid (2.4 g/L) were formed with 144 hrs (Fig. 4.5B). Subsequently, *Clostridim* sp. strain BOH3 was added to *Kluyvera* sp. strain OM3 culture medium after 72 hrs, a compromised time for the co-culture system. Xylanase activity was relatively

stable in the culture medium during the incubation time of 144 hrs, indicating that active enzymes were present for continuous hydrolysis of birchwood xylan to fermentable reducing sugars (Fig. 4.5C). Most importantly, the addition of *Clostridim* sp. strain BOH3 to the *Kluyvera* sp. strain OM3 led to the rapid utilization of the reducing sugars accumulated in the medium (Fig. 4.5C) with the formation of additional fermentation products (0.5 g/L acetone, 1.9 g/L ethanol, 1.2 g/L butanol, 3.2 g/L butyric acid, and 3.3 g/L acetic acid) (Fig. 4.5D). The total fermentation products obtained in the co-culture were almost twice those obtained in the corresponding monoculture of strain OM3 grown under identical conditions. To our knowledge, this is the first demonstration of butanol production directly from hemicellulose. Previous studies have demonstrated fatty acids generation from cellulose by coculturing cellulolytic *C. cellulolyticum* H10 with *C. acetobutylicum* (Petitdemange et al., 1983). This co-culture system produced mainly acetic and butyric acids rather than ethanol and butanol, suggesting that the fermentation process had stopped at the acid-forming phase prior to the solvent-forming phase for *C. acetobutylicum* (Petitdemange et al., 1983). Similar results were also reported by using *C. thermocellum* and *C. acetobutylicum* (Yu et al., 1985). The failure of solventogenesis to occur was attributed to the slow reducing sugars releasing rate as a direct result of the rate-limiting enzymatic hydrolysis of the lignocellulosic substrates. Petitdemange et al. have also demonstrated that low reducing sugar concentrations in the fermentation medium resulted in only acid formation rather than solvent formation (Petitdemange et al., 1983). In addition, the amount of butyric acid is another important factor for solvent formation because the intracellular and/or extracellular levels of butyric acid are implicated in the induction of solventogenesis in *C. acetobutylicum* (Bramono et al.,

2011). Butyric acid must be added to the medium containing cellulose for butanol production when using *C. thermocellum* and *C. acetobutylicum* co-culture system (Yu et al., 1985). The initial addition of butyric acid to the medium containing reducing sugars (e.g., xylose) resulted in a 26.8% increase of butanol when using *Clostridium* sp. strain BOH3 (Bramono et al., 2011). Therefore, in the coculture system of this study, the high initial reducing sugar concentration (6.1g/L), constantly stable xylanase activity (4U/mL), and high initial butyric acid concentration (2.1g/L) before the inoculation of *Clostridium* sp. strain BOH3 contribute to the onset of butanol formation. Results in this study demonstrated the potential of converting lignocellulosic substrates directly to fuels and chemicals by using this co-culture system, consisting of culture OM3 - an efficient strain for reducing sugars release from hemicelluloses and culture BOH3 - a strain for butanol production.




B
Biosolvents & VFAs (g/L)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
ethanol
acetic acid
butyric acid
0
20
40
60
80
100
120
140
160
Time (h)


C
Reducing Sugar (g/L)
Xylanase Activity (U/mL)
7
6
5
4
3
2
1
0
reducing sugar
xylanase
0
20
40
60
80
100
120
140
160
Time (h)

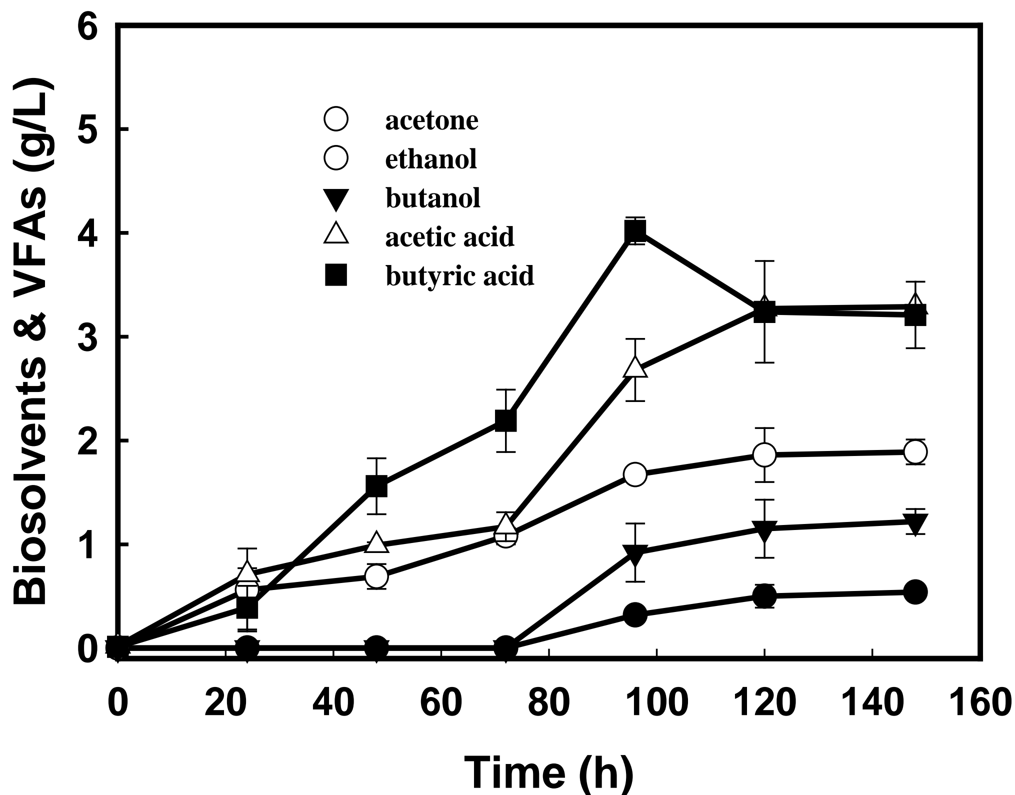


Fig. 4.5 (A) Enzyme activities and (B) product concentrations by culture *Kluyvera* sp. strain OM3 when amended with 4% (w/v) birchwood xylan at 35 °C. (C) Time-dependent enzyme activities and (D) product concentrations in the co-culture system consisting of culture *Kluyvera* sp. strain OM3 and *Clostridium* sp. strain BOH3 amended with 4% (w/v) birchwood xylan. Arrow means the inoculation point of *Clostridium* sp. strain BOH3 at 35 °C.

4.5 Conclusion

The cellulase-free and thermo-alkali-stable xylanase produced by the newly isolated anaerobic bacterium - *Kluyvera* sp. strain OM3 - is one of the rare xylanases because of its stability at extreme process conditions prevailing in the paper industry. The xylanase was able to release reducing sugars from natural lignocellulosic biomass, which could be further fermented to biofuel by the solventogenic *Clostridium* sp. strain BOH3. Moreover, a sequential co-culture consisting of strain OM3 and strain BOH3 demonstrated 1.2 g/L butanol production directly from hemicellulose for the first time.

In all, this study sheds lights on efficient hemicellulose hydrolysis and conversion of lignocellulosic materials to biofuels.

Chapter 5

Integration of biobutanol and lipase-catalyzed butyl-butyrate production in a single reactor

5.1 Abstract

Product inhibition in acetone-butanol-ethanol (ABE) fermentation can be reduced by conversion of lipophilic butanol to non-toxic and more economically valuable product, such as butyl-butyrate. In this study, lipases expression by *Clostridium* sp. strain BOH3 could be efficiently induced by oil materials. By further increasing the interface area between water and organic extractant in the form of oil emulsion, a maximum of 6.31 g/L of butyl-butyrate with indigenous lipase activity of 0.2 U/mL was obtained without the usage of lipases. In order to further improve the butyl-butyrate production, we integrated ABE fermentation process by *Clostridium* sp. strain BOH3 and commercial lipase-catalyzed (2.3 U/mL) esterification. By simultaneous *in situ* extraction using aviation fuel additives, such as kerosene, 22.35 g/L butyl-butyrate was produced in the solvent phase (25 mL solvent: 50 mL medium) from fed-batch reactor bottles spiked with 70 g/L xylose and 30 mM butyric acid. The principle may be extended to a wide range of esters, especially to longer chain ones, meanwhile the different strategies adopted in this study could be applied in different butyl-butyrate production sectors, like fuel, health and food.

5.2 Introduction

Esters derived from short-chain carboxylic acids and alcohols (such as butyric acid and butanol) are a large group of flavor and fragrance compounds, which are widely used in food, beverage, cosmetic, perfumes, solvents and pharmaceutical industries (Horton and Bennett, 2006; Park et al., 2009). Among them, butyl butyrate has shown great potential for being an aviation fuel constituent, which possesses good compatibility with kerosene with a melting point below -47°C and a flash point above 38 °C, as well as remaining miscible with kerosene at low temperature. Meanwhile, it also shows high octane rating of 97.3, higher than the minimum of 95 set out in the European Standard (EN) 228 (Rhodri et al., 2013). Thus butyl butyrate is a promising addition for the aviation sector.

Currently, most of the esters (R_1COOR_2) are produced through the Fisher esterification of an organic acid R_1COOH and an alcohol R_2OH catalyzed by an inorganic catalyst (e.g. acid) at high temperature (200-250 °C). However, the hazardous conditions caused by corrosive acid/base at high temperature and increasing scarcity of fossil fuels - source of alcohol and carboxylic acid have limited large quantities production of these esters. Since esters are commonly found in living species, such as plants and microbes, the concept of a natural ester made by lipase and natural substrate components from renewable and sustainable feedstocks under mild reaction conditions is more attractive than those from chemical routes (Horton and Bennett, 2006; Stergiou et al., 2013). As shown in literatures, lipases (triacylglycerol lipases E.C. 3.1.1.3) display catalytic activity towards a large variety of alcohols and acids in ester biosynthesis (Stergiou et al., 2013). Ester synthesis by lipases can be performed at room temperature

and atmospheric pressure, providing an energy-saving procedure under neutral pH in reaction vessels operated either batchwise or continuously. Enzyme-catalyzed esterification is also highly specific, which means less side products and waste (Rhodri et al., 2013). Therefore, the use of lipases to carry out esterification alleviates the necessity of a wide variety of complex post-reaction separation processes and thus leads to lower overall operation costs.

The feasibility of enzymatic production of esters such as butyl butyrate by transgenic *C. acetobutylicum* and *Escherichia coli* has been demonstrated, however, the production is still relatively low (0.29 g/L) (Horton and Bennett, 2006). So new symmetrical strategies or novel microbial biocatalysts that can efficiently convert substrate components into targeted esters are needed urgently. Solventogenic *Clostridium* sp. naturally synthesizes acids and alcohols by two distinct characteristic phases in their catabolic pathway, being the acidogenic and solventogenic phases (Lee et al., 2008; Jones et al., 1986). The main metabolic products in these two phases (acids: butyric acid; alcohols: butanol) are the natural substrates which can be catalyzed by lipases for ester synthesis. Thus, the integration of ABE fermentation process and lipase-catalyzed butyl-butyrate production will not only alleviate butanol toxicity to the cells, but also be able to convert butanol to a more value-added fuel constituent.

The aim of the present study was to design two efficient strategies for butyl-butyrate production. First, butyl-butyrate production using indigenous lipases from *Clostridium* sp. strain BOH3 was explored. Then, the fed-batch fermentation study integrated with ABE fermentation, commercial lipases catalyzed esterification and simultaneous *in situ* extraction of butyl-butyrate using aviation fuel additive solvents was

conducted to obtain high butyl-butyrate production. The obtained butyl-butyrate could be applied in different sectors, like food and fuel

5.3 Materials and methods

5.3.1 Growth medium and culture conditions

Bio-OSR was purchased from Alpha Biofuels & Keppel Offshore & Marine Technology Centre, Singapore. All the other chemicals were purchased from Sigma-Aldrich and had a purity of > 99%. *Clostridium* sp. strain BOH3 was used in this study (Bramono et al., 2011; Xin and He, 2013; Xin et al., 2014). Batch cultures were grown at 35°C in defined mineral salts medium containing (per liter of distilled water): KH_2PO_4, 0.75 g; K_2HPO_4, 0.75 g; CH_3COONH_4, 2 g; yeast extract, 5 g. In addition, 1 mL of trace element solution (Widdel and Hansen, 1992), 1 mL of Na_2SeO_3-Na_2WO_4 solution (Brysch et al., 1987) and 10 mg of resazurin were added to 1 L of the medium. After the medium was boiled and cooled down to room temperature under N_2, reductants Na_2S, L-cysteine, and $_{DL}$-dithiothretol were added to a final concentration of 0.2, 0.2, and 0.5 mM, respectively (He et al., 2003). Subsequently, 20 mM 2-(N-Morpholino) ethanesulfonic acid (MES) and 30 mM sodium butyric were added to the medium and the initial pH was adjusted to 6.2. Then the medium was dispended to serum bottles, which were sealed with butyl stoppers, autoclaved for 20 min, and cooled down to room temperature. 60 g/L xylose was amended to the above medium before inoculation.

5.3.2 Shake bottle fermentation

Cultures for inoculation were grown in 50 mL mineral salts medium amended with xylose at 35°C for ~20 hrs (late-exponential phase) unless otherwise stated. Inocula

of 5 mL were added to 45 mL of the reduced mineral salts medium in 160 mL serum bottles. Furthermore, 0.2 g lipase from *Candida rugosa* and 25 mL O_2 free extractant (hexadecane, kerosene, or paraffil oil) or 10 mL oil-based materials (olive oil, glyceryl tributyrate or Bio-OSR) were added to the serum bottles, which were incubated in a shaker at a rotary rate of 150 rpm at 35 °C. The pH was adjusted to 5.2 using 2M NaOH after 24 hrs. Experiments were carried out in duplicates.

5.3.3 Fed-batch fermentation with sodium butyric feeding

Prior to inoculation, 25 mL (O_2 free) extractants (hexadecane, kerosene, paraffin oil) were added to 50 mL fermentation broth. After 48 hrs of cultivation, the sodium butyric was almost completely consumed in the fermentation broth. Therefore, 3M of concentrated sodium butyric solution was added to the fermentationbroth to raise the sodium butyric concentration to 30 mM at time points of 48 and 72 hrs. The purpose of feeding sodium butyric here was to evaluate the performance of sodium butyric addition on the enhancement of butyl-butyrate production in the fed-batch operation mode.

5.3.4 Determination of partition coefficient

The partition coefficient of the expected fermentation products was determined as follows: a stock solution was prepared by dissolving 2.0 g butanol, 2.0 g butyl-butyrate, and 0.5 g butyric acid in 100-ml fermentation medium broth. Fifty milligrams of extractants (hexadecane, kerosene, paraffin oil) was mixed with the stock solution in a conical flask, which was then sealed with a rubber stopper and incubated at 30 °C for 24 hrs in a constant temperature and humidity chamber. The concentrations of butanol, butyric acid and butyl-butyrate in the solvent and medium broth were then determined. The partition coefficient was calculated by the following formula: $K = C_E/C_B$, where C_E is

the concentration of the analyte in the solvent and $C_B$is the concentration of the analyst in the medium broth.

5.3.5 Lipase assay

The activity of free lipase was determined spectrophotometrically using p-nitrophenyl palmitate (p-NPP) as the substrate according to the method of *Nawani et al.* with some modifications (Nawani et al., 2006). The reaction mixture containing 0.3 mL of 0.05M phosphate buffer (pH 8.0), 0.1 mL of 0.8 mM p-NPP and 0.1 mL of lipase was incubated at 37 °C for 10 min. The reaction was then terminated by adding 1 mL ethanol. A control without addition of the sample was run simultaneously, which contained the same contents but the reaction was terminated prior to addition of the enzyme. Absorbance of the resulting yellow colored product was measured at 410 nm in a spectrophotometer. One International Unit (IU) of lipase activity was defined as the amount of enzyme catalyzing the release of 1 μmol of p-nitrophenol per min from p-NPP under the standard assay conditions.

5.3.6 Analytical methods

The biomass in the fermentation broth was determined on a UV - visible spectrophotometer set at a wavelength of 600nm (Lambda-25, Perkin-Elmer, USA). Riboflavin was initially identified and quantified by using a high-performance liquid chromatography (HPLC) and was later quantified at the wavelength of 444 nm on a Beckman DU-800 spectrophotometer based on a modified protocol from Sauer (Sauer et al., 1996). Briefly, the fermentation broth was diluted with 0.1 M phosphate buffer and heated at 80°C for 10 min before centrifugation. The supernatant was then assayed. Similarly, for metabolic product analysis, culture broths were centrifuged at 10,000×g for

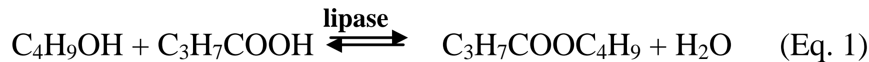
10 min at 4°C and the supernatant fluids were stored at -20°C until further analysis. Butyl-butyrate, butanol and butyric acid were measured by a gas chromatography (GC, model 7890A; Agilent Technologies, U.S.A.) equipped with a Durabond (DB)-WAXetr column (30 m × 0.25 mm × 0.25 μm; J&W, U.S.A.) and a flame ionization detector (FID). The oven temperature was initially held at 60°C for 2 min, increased at 15°C/min to 230°C, and held for 1.7 min. Helium was used as the carrier gas, with a column flow rate of 1.5 mL/min.

5.4 Results and discussion

Solventogenic *Clostridium* sp. strain BOH3 has shown tremendous potential over reported ABE producing strains so far, such as its high butanol production, efficient utilization of xylose and simultaneous fermentation of glucose and xylose (Bramono et al., 2011; Xin and He, 2013; Xin et al., 2014). Moreover, bioprocessing strategies using strain BOH3 are also advantaged by its capability of co-production of other value-added products, such as riboflavin (VB2), thereby showing great potential towards designing cost-effective processes. As shown previously, strain BOH3 produces both butyric acid and butanol as its main products in the biphasic fermentation processes and butyric acids produced during the acidogenesis phase would be reutilized by solventogenic strain BOH3 to synthesize butanol (Bramono et al., 2011; Xin et al., 2014). In this work, the bioconversion of butyric acid and butanol within the reactant site to more valuable product - butyl butyrate, using a lipase-catalyzed esterification process is exploited (Eq. 1). Accordingly, we presented two different strategies: i) butyl-butyrate production with catalysis of the indigenous lipase from solventogenic strain BOH3; and followed by ii)

high butyl-butyrate production from xylose using solventogenic strain BOH3 with commercial lipases.

5.4.1 Butyl-butyrate production with induction of lipases by *Clostridium* sp. strain BOH3



$$C_4H_9OH + C_3H_7COOH \xrightleftharpoons{\text{lipase}} C_3H_7COOC_4H_9 + H_2O \quad \text{(Eq. 1)}$$

During the last stage of ABE fermentation process by *Clostridium* sp. strain BOH3, a sweet smell occurred coupling with high butanol production (18.7 g/L) (Li et al., 2014). Further characterization using GC-FID showed that this pineapple-scented biofuel is butyl-butyrate with low production (0.15 g/L). Butyl-butyrate can be biosynthesized via the above equations (Eq. 1) with the catalysis of lipases. This interesting phenomenon indicated that the indigenous lipases could be expressed from strain BOH3 for butyl-butyrate synthesis. It is known that microbial lipases can be found in all living organisms and are mostly inducible extracellular enzymes, synthesized within the cell and exported to its external surface or environment (Stergiou et al., 2013). In the presence of lipids such as oil, triacyglycerol or any other fatty acid, lipases can be efficiently induced and expressed (Stergiou et al., 2013). For instance, 0.21 U/mL lipase could be efficiently expressed in *Aspergillus niger* when using live oil as the inducer, which can be used for the synthesis of biodiesel (Xiao et al., 2010). It would thus be interesting to investigate whether such enzyme(s) could be more efficiently indigenously induced within strain BOH3 and subsequently utilized for higher butyl-butyrate production. When the inducers, like olive oil or glyceryl tributyrate were added to the culture medium in the ratio of 1:5 (V/V), lipases could be efficiently induced and expressed by strain BOH3 with enzymatic activities of 0.2-0.3 U/mL (Fig. 5.1). More

importantly, the expression of lipases within strain BOH3 could lead to 1.21 g/L and 1.73 g/L of butyl-butyrate production after 6 days without addition of commercial lipases.

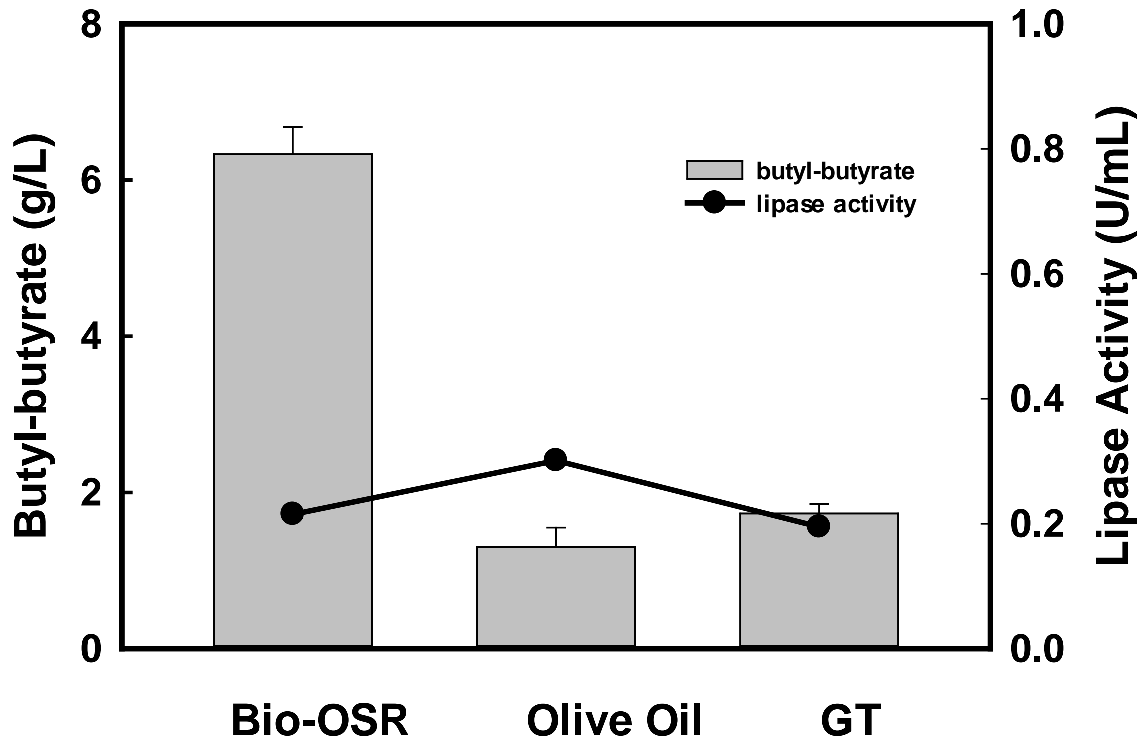


Fig. 5.1 Butyl-butyrate production and lipase activity with addition of different inducers. Bio-OSR, olive oil or glyceryl tributyrate was added to the culture medium in aratio of 1:5 (V/V). Samples were tested after 122 hrs of fermentation by *Clostridium* sp. strain BOH3.

As shown in Eq.1, ester and water are generated; the accumulation of water or ester in the reaction medium has significant influence on the chemical equilibrium (de Barros et al., 2009). For successful high-yield esterification reaction, the simultaneous extraction or removal of the end-products (butyl-butyrate or water) could possibly shift the equilibrium towards the butyl-butyrate production. It was therefore hypothesized that

addition of an oil based extractant can be advantageously used to induce *in situ* lipase production as well as simultaneously extract the produced butyl-butyrate. To test this hypothesis, another oil-based material called Bio-OSR (a plant based oil remover), which can easily form oil emulsion in the fermentation medium when kept under shear (130 rpm), was tested for its capability to synthesize lipase and accordingly lead to butyl-butyrate production using strain BOH3 (Fig. 5.2). Interestingly, similar to olive oil and glyceryltributyrate, the addition of oil-based Bio-OSR could efficiently induce the indigenous expression of lipase within strain BOH3, as 0.2 U/mL of lipase activity could be tested in the fermentation broth (Fig. 5.1). Meanwhile, the indigenous synthesis of lipase gave the highest butyl-butyrate production (6.31 g/L) compared to other two inducers after 6 days of fermentation. It is known that lipases have been characterized by their catalytic action preferably at the interface between water and insoluble substrates or organic solvent (e.g., oil-based material) containing the reactant (de Barros et al., 2009). Considering the fact that oil-water interfacial area gets enormously magnified in the case of emulsions, the emulsifying property of emulsion was considered to be highly advantageous, particularly because lipases are known to catalyze their reactions best at the oil-water interfaces (Fig. 5.2). Accordingly, the highest butyl-butyrate production of 6.31 g/L could be observed when adding Bio-OSR, compared to olive oil and glyceryl tributyrate which could not form emulsions (Fig. 5.1). Although genetic modification of *C. acetobutylicum* by overexpressing lipase genes has been carried out for biosynthesis of short-chain ester, however, only lower amount of butyl-butyrate (0.29 g/L) was produced (Horton and Bennett, 2006). Hence, the strategy via integration of induction of lipases

with *in-situ* extraction of butyl-butyrate shows more advantag over sole strain improvement.

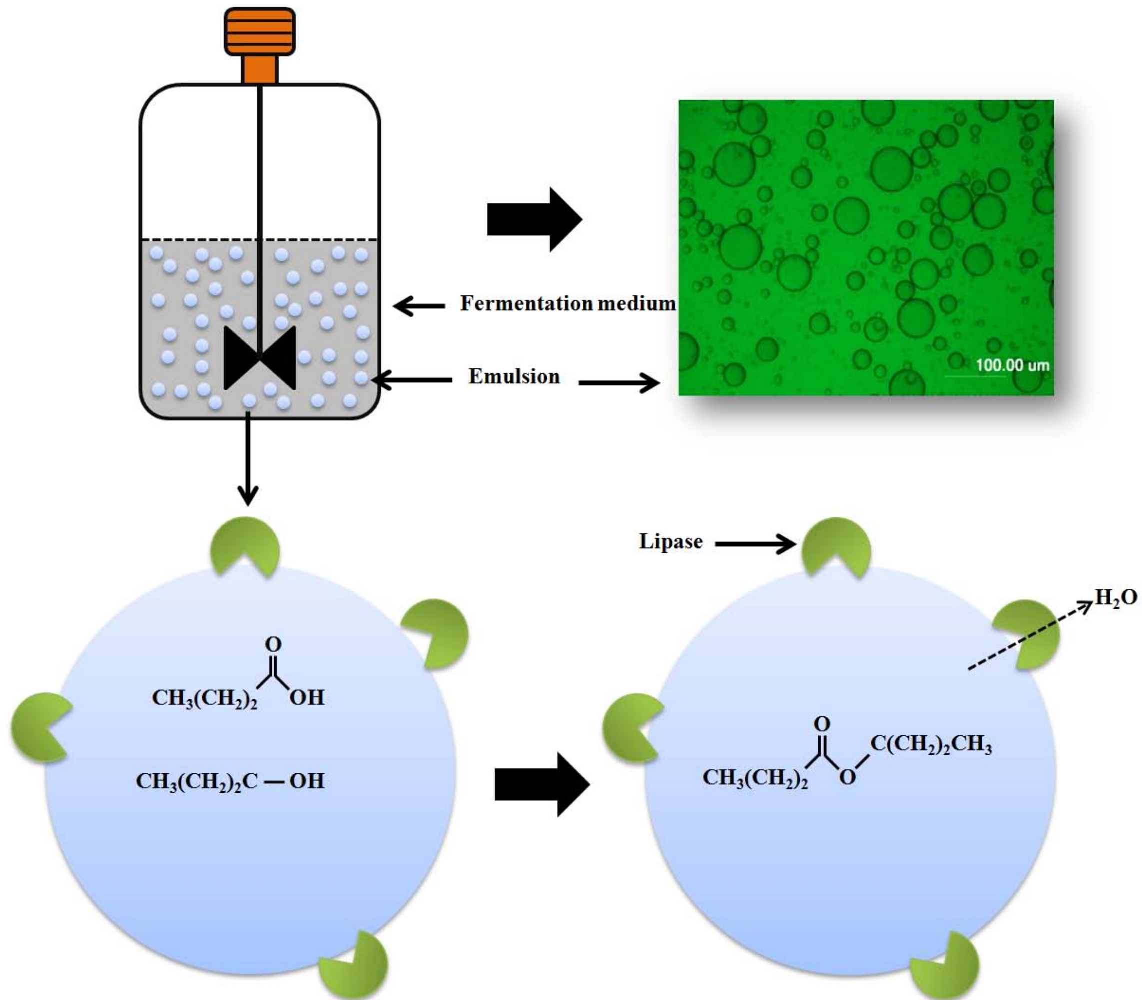


Fig. 5.2 Schematic diagram of an esterification in the mini-emulsion fromed using formed using Bio-OSR.

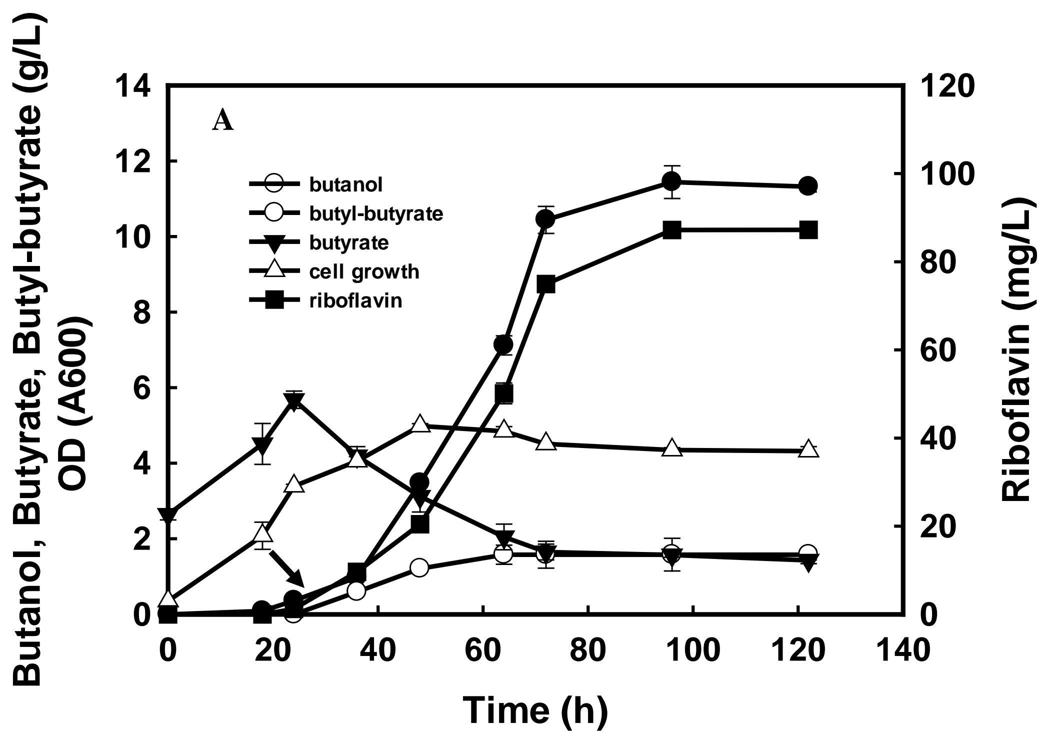
5.4.2 Butyl-butyrate production catalyzed by commercial lipases by *Clostridium* sp. strain BOH3

From Eq. 1, it can be calculated that 3.24 g/L butanol was utilized for production of 6.31 g/L of butyl-butyrate with indigenous lipase activity of 0.2 U/mL. The low lipase

activity would be the limiting factor in this low conversion yield. To further improve the final butyl-butyrate production, commercial lipases (2.3 U/mL, 0.2 g) were added into the fermentation broth (50 mL) at different fermentation phases, namely, acidogenic phase, early solventogenic phase, and: late solventogenic phase. As shown in Fig. 5.3, the lipase-addition timing shows obvious effects on butyl-butyrate production. The maximum of 1.56 g/L butyl-butyrate could be synthesized when lipases were added at the acidogenic phase (24 hrs) (Fig. 5.3A). Conversely, only small amounts of butyl-butyrate (0.98 and 0.23 g/L) were produced when lipases were added at the early (48 hrs) and late solventogenic (72 hrs) phases (Fig. 5.3B and 5.3C). Meanwhile, higher butyl-butyrate production correlated with higher butyric acid concentration in the fermentation medium, for instance, accompanying 1.56 g/L butyl-butyrate, 1.12 g/L butyric acid was also produced when lipases were added at the time of 24 hrs, however, only 0.23 g/L butyl-butyrate and 0.35 g/L butyric acid were present when lipases were added at the time of 72 hrs. It also should be noted that riboflavin production was affected with the co-production of butyl-butyrate and showed correlation with butanol production. For example, 86 mg/L riboflavin was co-produced with 1.56 g/L butyl-butyrate and 11.85 g/L butanol, however, when the maximum of 111 mg/L riboflavin was synthesized, only 0.23 g/L butyl-butyrate and 13.35 g/L butanol were generated. In any case, the results obtained here indicated the feasibility of butyl-butyrate production integrated with ABE fermentation and laid the foundation for further improvement of butyl-butyrate production using strain BOH3.

Being one of the essential substrates for biosynthesis of butyl-butyrate (Eq. 1), the lower amount of butyric acid could be another limiting factor for butyl-butyrate

production, as the typical ratio of alcohol and acid for esterification ratio is around 1:1 (Gupta et al., 2004; Stergiou et al., 2013). So to further enhance the final butyl-butyrate production, the fed-batch fermentation with supplement of 30 mM butyric acid at early and solventogenic (48 and 72 hrs) phases has been carried out in the following study (Fig. 5.3D). As shown in Fig. 5.3D, the supplemented butyric acid could be efficiently utilized and led to the final 4.10 g/L of butyl-butyrate. Interestingly, the final riboflavin production reached up to 115 mg/L, which correlated with the highest butanol production of 17.82 g/L. So in the following studies, 30 mM sodium butyric was fed twice at time of 48 and 72 hrs.


A
butanol
butyl-butyrate
butyrate
cell growth
riboflavin
Butanol, Butyrate, Butyl-butyrate (g/L)
OD (A600)
Riboflavin (mg/L)
Time (h)
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120
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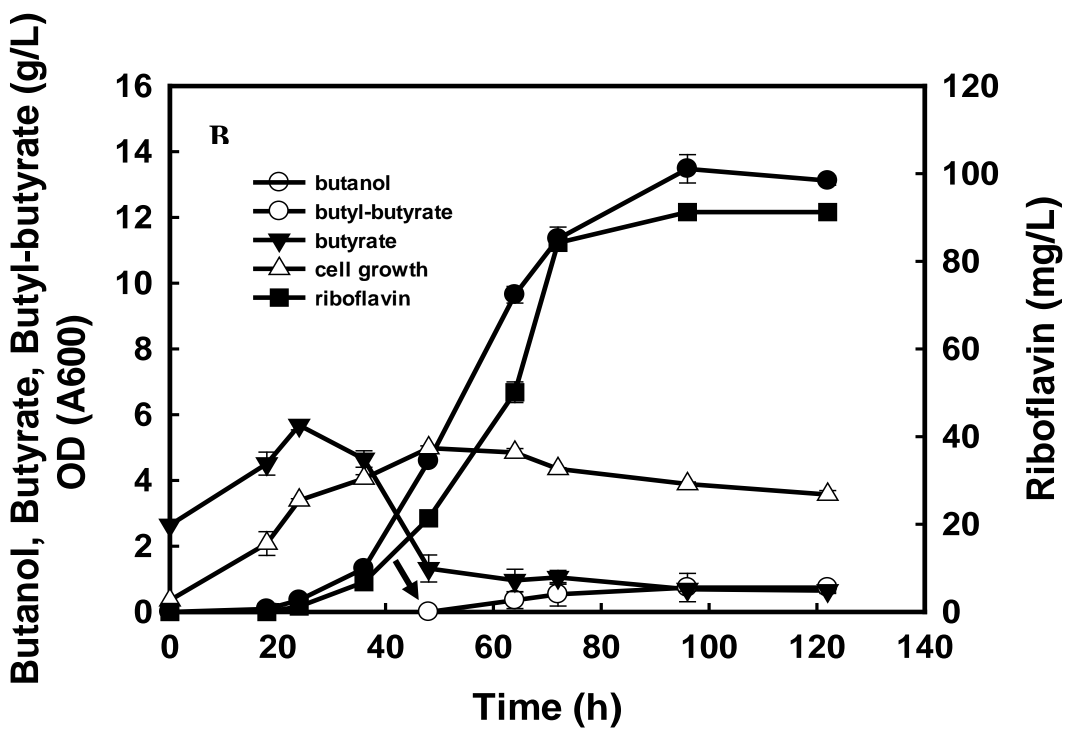
B
butanol
butyl-butyrate
butyrate
cell growth
riboflavin
Butanol, Butyrate, Butyl-butyrate (g/L)
OD (A600)
Riboflavin (mg/L)
Time (h)
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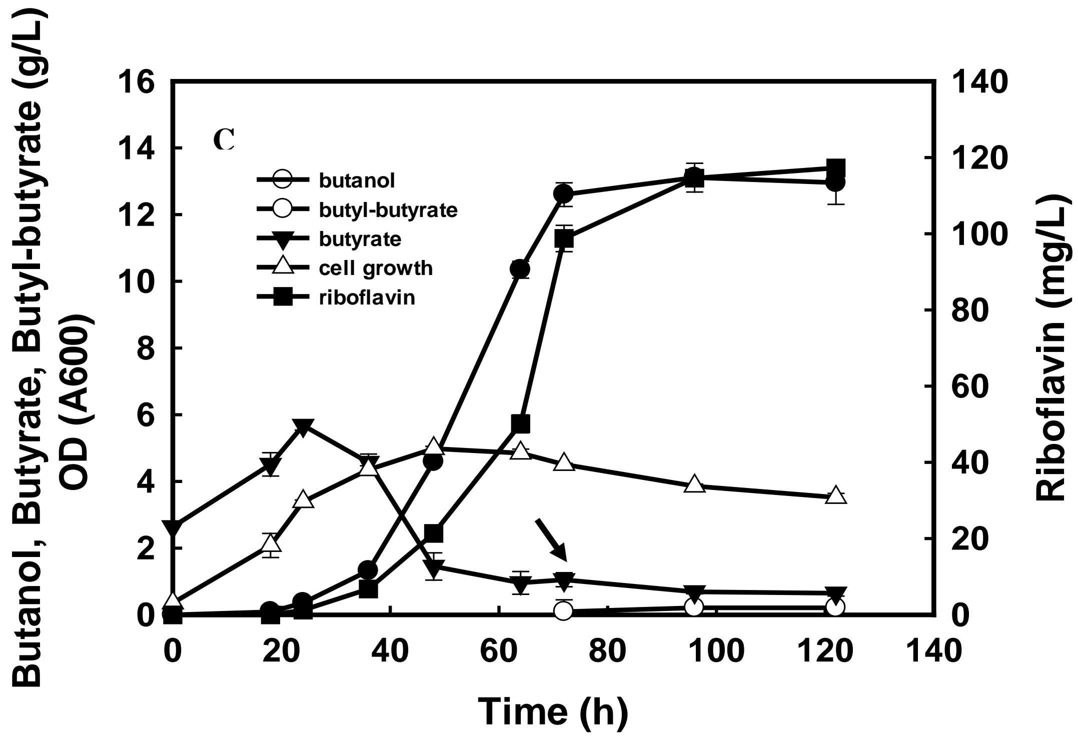

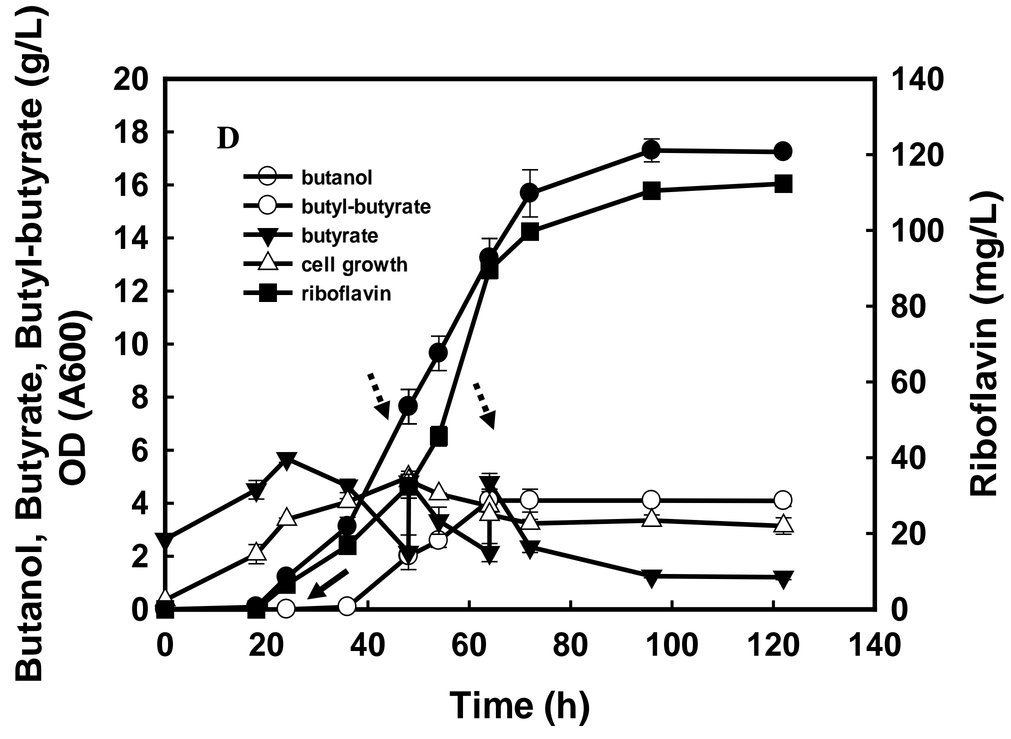

Fig.5.3 Batch fermentation results of *Clostridium* sp. strain BOH3 with addition of commercial lipase at different time points. A: 24 hrs; B: 48 hrs; C, 72 hrs. D: Fed-batch fermentation result of *Clostridium* sp. strain BOH3 with addition of sodium butyric. Solid arrows mean supplement of lipase. Dashed arrows mean supplement of sodium butyric.

5.4.3 High butyl-butyrate production by *Clostridium* sp. strain BOH3 integrated with in-situ extraction

Based on the results obtained so far, the following conclusions could be deduced: i) The esterification reaction could occur with addition of commercial or indigenous lipases induced by oil based materials (Fig. 5.1 and 5.3) using strain BOH3 and ii) the esterification reaction could be enhanced by the addition of a biocompatible extractant (Fig. 5.2). The high lipase activity and *in-situ* extraction of butyl-butyrate using suitable extractant are two essential factors for high butyl-butyrate production. Hence, in order to further pull the reaction to the synthesis side, we considered the integration of ABE fermentation, commercial lipase-catalyzed esterification and ester extraction using a suitable *in-situ* ester extractant. Notably, an ideal extractant should not only be compatible with the bacterial growth, but also possess high partition coefficient for butyl-butyrate. Although hexadecane shows a high partition coefficient of 340 for butyl-butyrate, it would necessitate adoption of subsequent cost-intensive downstream separation steps (Van den Bert et al., 2013). It was therefore considered advantageous to adopt an extractant that could be directly used as an enriched fuel without the need for any separation process.

Table 5.1 Partition coefficients of solutes in hexadecane/aqueous system.

Product	Partition coefficient		
	Hexdecane	**Kerosene**	**Paraffin Oil**
Butanol	0.21	0.17	0.16
Butyric acid	0.13	0.09	0.08
Butyl-butyrate	340	356	348

Being commercial aviation fuels, kerosene and paraffin oil also show high partition coefficient for butyl-butyrate and lower partition coefficient for butanol and butyric acid, which could be good optional extractants for this integrated process (Table. 5.1). Hence, 25 ml of kerosene or paraffin oil was adopted as the extractant (for 50 ml of fermentation broth) to perform the fed-batch fermentation under similar conditions as in Fig. 4A. After fed with 30 mM butyric acid twice (48 hrs and 72 hrs) and 122 hrs of fermentation, the control experiment (hexadecane as the extractant) for the one-pot bio-ester production finally led to 8.20 g/L butyl-butyrate using strain BOH3, which is 1.6 fold higher than previous work (Van den Bert et al., 2013), indicating the feasibility and advantage of this strategy (Fig. 5.4A). Under similar conditions, addition of paraffin oil led to slightly lower amount of butyl-butyrate (7.16 g/L) compared to hexadecane (8.20 g/L) (Fig. 5.4A). Interestingly, kerosene gave the highest butyl-butyrate production of 19.68 g/L from 60 g/L xylose in the solvent phase. To further explore the maximum potential for butyl-butyrate production using this strategy, higher concentration of substrate - xylose (70 g/L) was fed into this system (Fig. 5.4B). After 122 hrs of fermentation under similar conditions, a maximum of 22.35 g/L of butyl-butyrate

occurred in the solvent phase, which also represents the highest butyl-butyrate production via biological pathway (van den Berg et al., 2012). The kerosene enriched with butyl-butyrate can be directly used as aviation fuels to eliminate the further separation process. This is superior to similar systems, where hexadecane was adopted as the extractant (van den Berg et al., 2013). Further increment of xylose concentration did not obviously improve the butyl-butyrate production. Meanwhile, the maximum of 121 mg/L riboflavin was still co-produced when using kerosene as the extractant (Fig. 5.4B). The riboflavin production was shown to exert no effect on butyl-butyrate production at these levels, which supports the rationale of co-production of a high-value product in improving the economics of the biosynthesis of butyl-butyrate. It also should be noted that 8.15 g/L butanol still occurred in the aquatic phase when kerosene was adopted as the extractant. The overall converted butanol production in both solvent (used as the intermediate for butyl-butyrate production) and aquatic phases equals to 19.63 g/L from 70 g/L xylose with the yield of 0.28 g/g. This high conversion yield efficiently solves the problems of product inhibition caused by butanol toxicity.

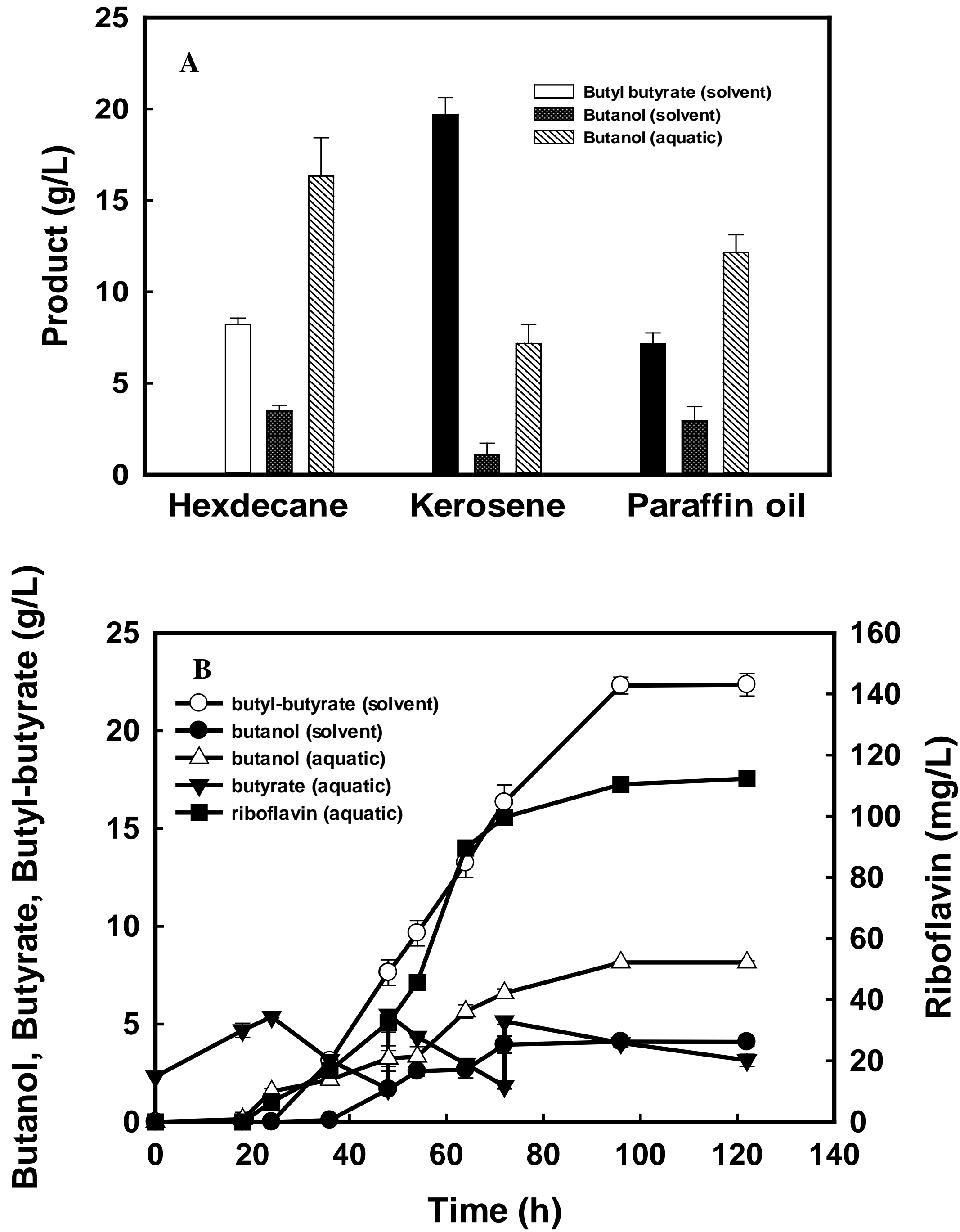

Fig. 5.4 A. Butyl-butyrate and butanol (in solvent and aquatic phases) production with addition of different extractants using *Clostridium* sp. strain BOH3 under 60 g/L xylose. B. Fermentation profiles of *Clostridium* sp. strain BOH3 in reduced mineral medium containing 70 g/L xylose with addition of kerosene. Commercial lipase was added at time of 24 hrs. 30 mM sodium butyric was fed twice at 48 and 72 hrs. Samples were tested after 122 hrs of fermentation. pH was controlled at 5.0-5.2.

5.4.4 Productivity and cost comparison using different strategies by *Clostridium* sp. strain BOH3

Butyl-butyrate shows diverse usages in fuel, food and health sectors. Therefore, different strategies using strain BOH3 were designed in this study to fulfill its goals in these sectors. The butyl-butyrate productivities and associated feedstock costs (i.e. raw material costs) using different strategies were compared in Table 5.2. The cost of lipases is a main contributor in this bio-process (5.75 $/kg). Although immobilization of lipases can make the bio-production of butyl-butyrate more acceptable, the efficiency of its catalysis after several reuse is still an issue. Thus, it would be advantageous if the fermenting microorganism would be capable of producing *in situ* lipases (Gupta et al., 2004). Fortunately, lipases within strain BOH3 could be efficiently induced and expressed using oil-based materials, like olive oil, Bio-OSR et al. Meanwhile, the formation of emulsions using these oil-based materials in the medium also pulls the esterification process to the synthesis of butyl-butyrate (Fig. 5.2). Therefore, these oil-based materials will play roles of both inducer and enhancer. In process 1, *in situ* induction of lipase using Bio-OSR achieves the comparative butyl-butyrate productivity (0.052 g/L/h), but at much lower prices compared to other processes (Table 5.2). On the other hand, addition of the aviation fuel, like kerosene as the extractant (Process 4) would not only double the butyl-butyrate productivity (0.092 g/L/h Vs 0.034 g/L/h) compared to the control (Process 2), but also spare the separation cost as the enriched fuel can be directly used as an aviation fuel. Considering this, we can design the butyl-butyrate and jet fuel plants in the same site in the future, thus the costs of transporting jet fuel will be saved. More importantly, the increment of butyl-butyrate productivity would not

increase the feedstock costs. Additionally, its co-production of riboflavin can further improve the overall process economics. However, one of the issues in this process is still the usage of lipases. Therefore, to improve this process, future work will focus on *in situ* surface-displayed lipases of strain BOH3. It is to be noted that the price of the medium was not considered in the calculations for Table 5.2. Strain BOH3 has shown efficient utilization of the lignocellulosic waste hydrolysate (Xin et al., 2014), indicating further cost reduction instead of using pure mono-carbon source, e.g., glucose, xylose et al.

Table 5.2 Comparison of the butyl-butyrate productivity at different stages of process development and associated feedstock costs (cost of extractant not included)

Process Design*	Butyric Acid	Lipase	Bio-OSR	Overhead Cost**	BB Con. (g/L)	BB Pro. (g/L/h)
1	30 mM	-	5 mL	0.28 $	6.31	0.052
2	30 mM	0.2 g	-	5.75	1.56	0.013
3	90 mM	0.2 g	-	6.01	4.1	0.034
4	90 mM	0.2 g	-	6.01	11.18	0.092

Process 1: Batch fermentation with addition of Bio-OSR and butyric acid (0, 48, 72 hrs).
Process 2: Batch fermentation with addition of lipases (24 hrs) and butyric acid (0 hr);
Process 3: Fed batch fermentation with addition of lipases (24 hrs) and butyric acid (0, 48, 72 hrs);
Process 4: Fed batch fermentation with addition of lipases (24 hrs), butyric acid (0, 48, 72 hrs) and extractant (kerosene);
* All fermentations were run for 122 hrs. Butyl-butyrate concentration is equivalent to concentration produced in the medium.
**Feedstock Cost implies costs incurred due to the addition of the additives within the media (lipases, 5.75 $/kg; butyric acid, 8$/kg; Bio-OSR, 1.7 $/L) (http://www.alibaba.com).
BB conc.: butyl-butyrate concentration; BB Pro.: butyl-butyrate productivity.

5.5 Conclusion

Different strategies for butyl-butyrate production by using *Clostridium* sp. strain BOH3 were demonstrated and compared in this study. In the absence of additional lipases, strain BOH3 could only produced 6.3 g/L of butyl-butyrate in culture medium amended with Bio-OSR. On the other hand, 22.35 g/L butyl-butyrate was achieved through fed-batch fermentation integrated with ABE fermentation, lipase-catalyzed esterification and simultaneous *in situ* extraction of butyl-butyrate using aviation fuel additives with addition of 7.9 g/L butyric acid. This concentration is 4.5 – fold higher of that in a previsouly reported system. These strategies above could be used in different sectors for butyl-butyrate application.

Chapter 6

Production of 2,3-butanediol from sucrose by a *Klebsiella* species

6.1 Abstract

Chemical 2,3-butanediol is an important platform compound possessing diverse industrial applications. So far, it is mainly produced by using petrochemical feedstock which is associated with high cost and adverse environmental impacts. Hence, finding alternative routes (e.g., via fermentation using renewable carbon sources) to produce 2,3-butanediol are urgently needed. In this study, we report a wild-type *Klebsiella* sp. strain XRM21, which is capable of producing 2,3-butanediol from a wide variety of carbon sources including glucose, sucrose, xylose and glycerol. Among them, fermentation of sucrose leads to the highest production of 2,3-butanediol. To maximize the production of 2,3-butanediol, fermentation conditions were first optimized for strain XMR21 by using response surface methodology (RSM) in batch reactors. Subsequently, a fed-batch fermentation strategy was designed based on the optimized parameters, where 91.2 g/L of 2,3-butanediol could be produced from substrate sucrose dosing in 100 g/L for 3 times. Moreover, random mutagenesis of strain XMR21 resulted in a highly productive mutant strain, capable of producing 119.4 g/L and 22.5 g/L of 2,3-butanediol and ethanol under optimized fed-batch fermentation process within 65 h with a total productivity of 2.18 g/L/h, which is comparable to the reported highest 2,3-butanediol concentration produced by previous strains . This study provides a potential strategy to produce industrially important 2,3-butanediol from low-cost sucrose

6.2 Introduction

The chemical 2,3-butanediol has extensive usage in diverse industries including rubber, printing, cosmetic, pharmaceutical and others (Celińska and Grajek, 2009; Ji et al. 2011; Zeng and Sabra 2011). It can also be used as a fuel additive due to its relatively high heating value (27.2 kJ g^{-1}), which compares favorably with other liquid fuels such as methanol (22.1 kJ g^{-1}) and ethanol (29.1 kJ g-1) (Celińska and Grajek, 2009). Despite the increasing global market demand for 2,3-butanediol (~74.4 kilo tons by 2018), production of 2,3-butanediol via petrochemical routes is impeded by the shortage of fossil fuel supplies, fluctuating petroleum prices and associated environmental pollution (Celińska and Grajek ,2009; Ji et al., 2011). Thus, production of bio-based 2,3-butanediol from renewable feedstock seems attractive, since it will provide a sustainable supply for 2,3-butanediol with low production cost (Celińska and Grajek, 2009; Zeng and Sabra, 2011).

Bio-production of 2,3-butanediol has been achieved through microaerobic fermentation by various bacteria such as *Klebseilla pneumonia* and *K. oxytoca* (Celińska and Grajek, 2009; Ji et al., 2011; Zeng and Sabra, 2011). However, co-production of unwanted organic acids (e.g. acetate, lactate, formate and succinate) always occurs, which adversely impacts product recovery and process economics (Ji et al., 2011; Zeng and Sabra, 2011). To address these issues, strain modifications, substrate selection and process optimization are needed to improve the yield of 2,3-butanediol and its purity (Celińska and Grajek, 2009). Novel strains capable of producing high concentrations of 2,3-butanediol using low-cost carbon sources without any by-products are highly desirable.

In this study, we aimed to cultivate a wild-type *Klebsiella* strain which can produce high levels of 2,3-butanediol from sustainable substrate - sucrose. To further optimize the production of 2,3-butanediol, a statistical design of experimental strategy known as response surface methodology (RSM) was used to evaluate the interaction between different parameters, including pH, inducers and nitrogen source. Further yield improvement was conducted through random mutation of this wild-type strain.

6.3 Materials and Methods

6.3.1 Isolation, characterization and phylogenetic analysis of strain XMR21

Soil samples collected from the rain forest (Bukit Batok Nature Park) in Singapore were used as inoculum for screening 2, 3-butanediol-producing bacteria. Mineral salts medium was used for screening, which contained 0.75 g/L of K_2HPO_4, 0.75 g/L of KH_2PO_4, 5.0 g/L of yeast extract, 1 mL of trace element solution, 10 mL salt solution and 1 mL of Na_2SeO_3-Na_2WO_4 solution to 1 liter solution (Xin and He, 2013). Under N_2 flow, reductants Na_2S, L-cysteine, and $_{DL}$-dithiothretol were added to a final concentration of 0.2, 0.2, and 0.5 mM, respectively (Xin and He, 2013). Subsequently, 20 mM (2-N-Morpholino ethanesulfonic acid) (MES) and 10 g of birchwood xylan (sole carbon source) were added and the pH was adjusted to 6.5. The medium was autoclaved for 20 min and cooled down to room temperature. Then, approximately 2.5 g of soil was added to the autoclaved 160-mL bottles and 50-mL liquid medium was dispensed into the bottles under N_2 flow, which were sealed with butyl stoppers and incubated at 35 °C. The enriched culture (0.1mL; successively diluted to 10^{-5} times) was repeatedly streaked on agar plates poured with the reduced mineral salts medium containing 10 g/L birchwood

xylan and the plates were incubated for 24 h in the anaerobic chamber filled with mixed gas (90% N_2, 5% CO_2 and 5% H_2) at room temperature (Xin and He, 2013). Based on the colony type and size, representative colonies were picked from the agar plates and inoculated into 15 mL of mineral salts medium. Carbon substrates (glucose, sucrose, xylose or glycerol) were filter-sterilized (0.22 μm) and added into the medium to a final concentration of 60 g/L for further fermentation studies. Then, the culture bottles were incubated at 35°C and shaking at 150 rpm. All of the fermentation was carried out in duplicates.

Genomic DNA of the cultures was extracted and purified with DNeasy tissue kit (Qiagen, Germany) according to manufacturer's instructions. The genomic DNA was used as a template for PCR amplification of the 16S rRNA gene with a pair of universal bacterial primer 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1392R (5'-ACGGGCGGTGTGT-3') (Xin and He, 2013). The obtained PCR products were purified with a PCR purification kit (Qiagen, Germany) and sequenced using an ABI DNA sequencer (Applied Biosystems, USA). Basic local alignment search tool (BLAST) analysis of the obtained 16S rRNA gene sequence was conducted by using CLUSTAL X software (Aiyar, 1999). Phylogenetic tree for the 16S rRNA gene sequence was constructed by using Mega4 software (Tamura et al., 2007). The nucleotide sequence of strain XMR21 was deposited in GenBank under an accession number of KM241871. The strain XMR21 is deposited in American Type Culture Collection (ATCC) with an accession number of PTA-121320.

6.3.2 Optimization of fermentation conditions

All batch fermentation studies were carried out in 160 mL serum bottles containing 50 mL of mineral salts medium (10.0 g of K_2HPO_4, 2.0 g of KH_2PO_4, 6.6 g of $(NH_4)_2SO_4$, 5.0 g of yeast extract, 1.0 mL of trace element solution, 1 mL of Na_2SeO_3-Na_2WO_4 solution and 10 mL of salt solution (pH = 6.0) in 1 L)) (Xin and He, 2013). The medium-filled serum bottles were sealed with rubber septa and aluminum caps before being autoclaved. The bacteria will utilize the oxygen in the medium for the biomass growth, and after the consumption of oxygen, fermentation will be onset. Sucrose stock solution was first filter-sterilized and then was added into the medium as the sole carbon source. Inoculation was done by adding 2% of seed culture (strain XMR 21) to the medium. The bottles were incubated at 35 °C and 150 rpm in a shaking incubator for 48 hrs. Detailed optimization studies regarding the production of 2,3-butanediol was carried out by using response surface methodology (RSM) (Bezerra et al., 2008; Rajagopalan and Krishnan, 2008; Basu et al., 2011; Basu and Leong 2012; Basu et al., 2013). Three factors (pH, sodium acetate, and ammonium sulfate) were chosen as independent variables, while concentration of 2,3-butanediol was the dependent variable. An experimental strategy based on central composite design (CCD) was obtained using Design Expert version 7.0. (Stat-Ease, Minneapolis, USA) (Merrill, 1994). The values of the response variable obtained were fitted to a second-order polynomial equation:

$$Y_i = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij} x_i x_j \quad \text{Eq.1}$$

where Y_i is the predicted response, x_i, x_j are independent variables which influence the dependent variable Y; β_0 is the offset term; β_i is the ith linear coefficient; β_{ii} is the ith quadratic coefficient and β_{ij} is the ij^{th} interaction coefficient. Statistical analysis of the

model was performed by using analysis of variance (ANOVA) in a statistical software package (Merrill, 1994).

6.3.3 Batch and fed-batch fermentation

Batch fermentation in serum bottles was carried out by feeding different amount of sucrose (50-300 g/L) and the culture bottles were incubated for 48 hrs. Subsequently, the optimized condition obtained from batch fermentation was applied to fed-batch fermentation in a 3.0-L bioreactor (BIOSTAT® B plus, Sartorius, Germany) equipped with a pH probe. The bioreactor was filled with 1.5 L of culture medium and was operated at 35°C with an agitation rate of 150 rpm. Fermentation was carried out for at least 65 hrs and the sucrose concentration was determined by using phenol sulfuric acid method (Mecozzi, 2005). When the sucrose level dropped to approximately 20 g/L, fresh sucrose stock solution (1000 g/L) was added to the fermentation broth to reach a final sucrose concentration of 100 g/L.

6.3.4 Mutation and screening of mutants

Random mutagenesis was conducted on active wild-type strain XMR21 by using ethyl methyl sulfonate according to the method described by Jiang et al. with minor modifications (Jiang et al., 2011). Fifty microliters of ethyl methyl sulfonate was added to 10 ml of XMR21 cell suspension. After reaching 84% lethal rate based on cell density, the cells were washed by using sterile saline, spread on agar selection plates containing 140 g/L of 2,3-butanediol, and incubated at 35°C for 3 days. Colonies were selected for inoculation in the fermentation medium (50 ml) as mentioned earlier.

6.3.5 Analytical methods

Dry cell weight (DCW) was determined by heating the cell suspension at 105°C until the weight became constant. Cultures of 2 ml were centrifuged at 12,000 rpm for 10 min at 4°C and the supernatant fluids were stored at -20°C until further analysis. Fermentation products including acetic acid, 2,3-butanediol and ethanol were measured by a gas chromatography (GC, model 7890A; Agilent Technologies, U.S.A.) equipped with a Durabond (DB)-WAXetr column (30m × 0.25mm × 0.25µm; J&W, U.S.A.) and a flame ionization detector (FID). The oven temperature was held at 60 °C for 2 min, increased at 15°C/min to 230°C, and held for 1.7 min. Helium was used as carrier gas with a flow rate of 1.5 mL/min. Five-point standard curves were obtained by running standard solutions containing 2,3-butanediol, ethanol, and acetic acid with concentrations ranging from 2.5 to 15.0 g/L.

6.4 Results and Discussion

6.4.1 Strain isolation and characterization

In order to screen bacteria that can convert xylan to value-added biochemicals, soil samples from rain forest were used as inocula to setup microcosms. After enrichment in liquid medium and isolation on agar plates with xylan as the sole carbon source, fourteen well-grown colonies were picked for further batch fermentation using glucose as the substrate. Only one colony was capable of producing the high value chemical 2,3-butanediol. Phylogenetic analysis based on its 16S rRNA gene sequence demonstrated that this isolate showed 99% identity with *K. pneumonia* (Fig. 6.1), and hence it is designated as *Klebsiella* sp. strain XMR 21.

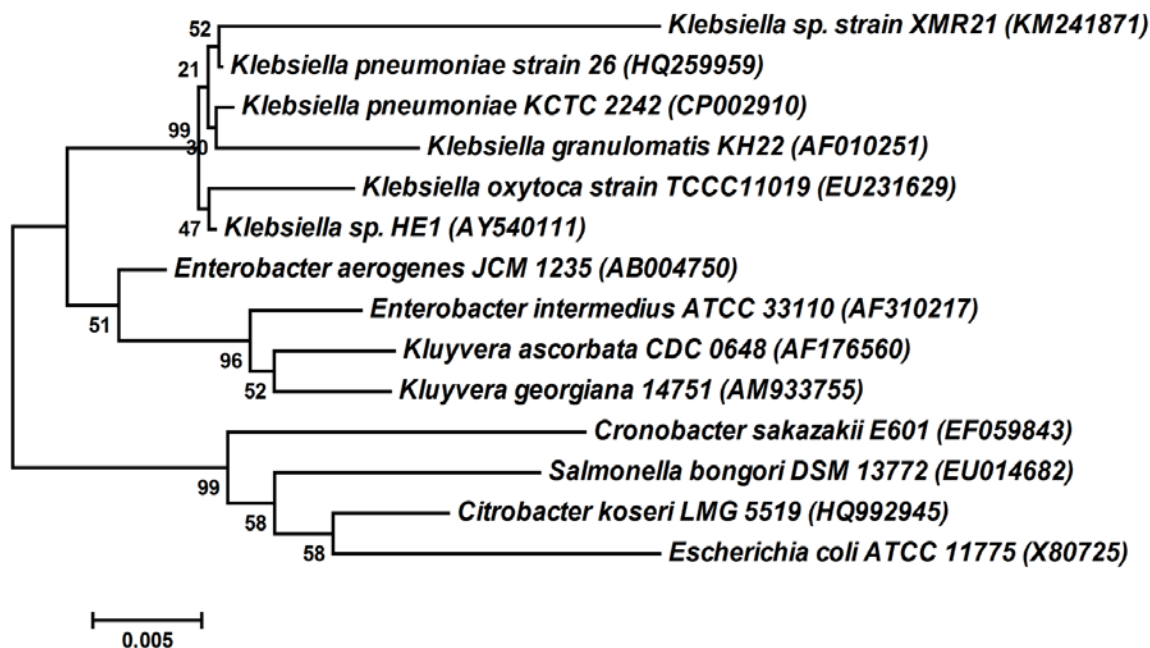

Fig. 6.1 Phylogenetic tree of *Klebsiella* sp. strain XMR21 using the neighbor-joining method (MEGA 4.0) based on 16S rRNA gene sequences.

Strain XMR 21 is capable of producing 2,3-butanediol from four different carbon sources including glycerol, xylose, glucose and sucrose (60 g/L), which are the main components in lignocellulosic materials, industrial waste and food waste (Ji et al., 2011). As shown in Fig. 6.2, although strain XMR21 could produce 2,3-butanediol and ethanol from all four carbon sources, it produced the highest amount of 2,3-butanediol (14.5 g/L) when using sucrose as the substrate. Even though corn-based glucose is the most common carbon source for fermentation (Ji et al., 2011), sucrose from sugarcane would be preferable substrate for 2,3-butanediol production Firstly, it is a cheap substrate as it can be directly fermented (as cane juice), whereas glucose has to be converted from starch by milling and enzymatic hydrolysis; secondly, sugarcane sucrose-based bioprocesses are more environmentally friendly and sustainable than glucose-based

bioprocesses (Ji et al., 2011; Zeng and Sabra, 2011; Syu, 2001). In addition, sucrose is highly abundant and readily available. Therefore, production of 2,3-butanediol from sucrose provides a more economically feasible approach compared with glucose as the substrate. In addition to 2,3-butanediol, a decent amount of ethanol (3.0-6.0 g/L) was also produced during the fermentation process by strain XMR21. Most importantly, production of fatty acids (such as acetate acid) and other byproducts is negligible, which simplifies the following separation processes.

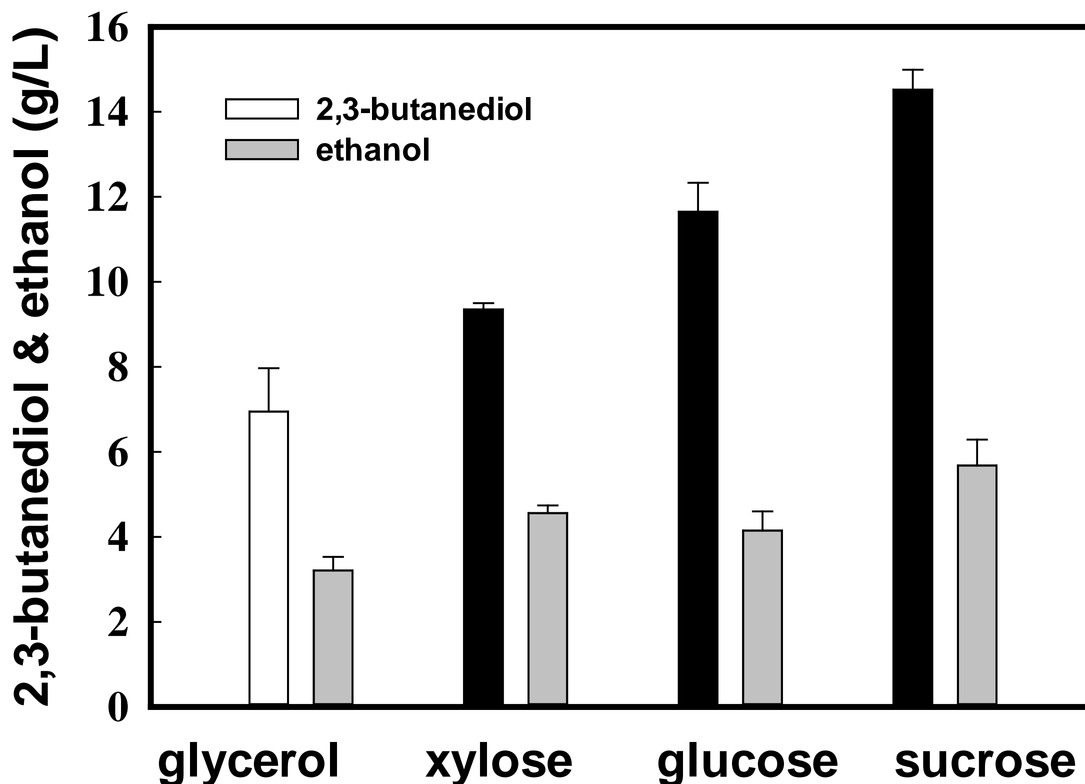

Fig. 6.2 Production of 2, 3-butanediol and ethanol via fermenting 60 g/L glycerol, xylose, glucose and sucrose by *Klebsiella* sp. strain XMR21. Data shown are after 48 hrs of fermentation.

6.4.2 Optimization of fermentation condition conditions for enhanced 2,3-butanediol production

Strain XMR 21 shows capability of producing 2,3-butanediol from a broad spectrum of carbon sources, however, the yields were low (0.24 g/g from sucrose). Accordingly, further improvement in 2,3-butanediol production was carried out by optimizing fermentation conditions using a statistical experimental design methodology. For the response surface analysis, 20 experiments were conducted according to the RSM design, with the results shown in Table 6.1, where Y is the 2,3-butanediol production (g/L), X_1 denotes pH, X_2 denotes sodium acetate level (mM) and X_3 denotes ammonium sulfate level (g/L). According to the response values obtained from the experimental results, a second order regression equation was generated for the response surface, as follows: $Y = 39.31 + 10.98X_1 + 1.75X_2 - 8.27X_1^2 - 1.79X_2^2$ (Eq. 1, [$R^2 = 0.9535$; $R^2(Adj) = 0.9411$; $R^2(Pred) = 0.8587$])

Table 6.1 RSM experimental design for 2,3-butanediol production and corresponding results.

Run	X_1	X_2	X_3	Y
1	0	0	0	39.0
2	0	0	0	42.3
3	-1	1	-1	16.4
4	0	0	0	41.4
5	-1	1	1	19.4
6	-1	-1	-1	13.3
7	0	1.682	0	39.8
8	1.682	0	0	38.5
9	0	0	0	39.0
10	0	0	0	42.3
11	1	1	1	40.5
12	0	0	0	39.1
13	1	1	-1	39.6
14	1	-1	-1	35.6
15	0	0	-1.682	36.9
16	0	0	1.682	40.0
17	1	-1	1	34.54
18	-1.682	0	0	0
19	-1	-1	1	16.0
20	0	-1.682	0	35.4

Note: Y is the 2,3-butanediol production (g/L), X_1 denotes pH, X_2 denotes sodium acetate level (mM) and X_3 denotes ammonium sulfate level (g/L).

The regression model (Eq. 1) fits the experimental data quite well, with a high R^2 value of 0.9535. The three-dimensional response surfaces as obtained from Eq. 2 are shown in Fig. 6.3. The highest point on the contour profiles in Fig. 6.3 indicates the optimal parameter values for the highest 2,3-butanediol production. The optimal 2,3-butanediol production value predicted from the response surface model was 45.1 g/L, when the pH was 6.0-6.4 and sodium acetate was 75-104 mM, respectively. However, neither the ammonium acetate level (X_3) nor its corresponding interaction terms (X_1X_3/ X_2X_3) played any important roles in the 2,3-butanediol production. This implied that the added yeast extract (5 g/L) provided sufficient nitrogen source to support bacterial growth and target metabolite production.

The fact that the optimal solvent production was found under acidic conditions aligns well with previous studies reporting alkaline conditions favoring organic acid production instead of 2,3-butanediol. Acidic conditions on the other hand are known to reduce organic acid synthesis (over 10 fold) and increase 2,3-butanediol concentration by 3-7 fold (Celińska and Grajek, 2009). However, the ultimate 2,3-butanediol production yields would be highly dependent on the microorganism and the substrate(s) (Ji et al., 2011). Presence of exogenous inducers like acetates is also known to trigger the biosynthesis of three key enzymes (α-acetolactate synthase,α-acetolactate decarboxylase, and acetoin reductase) essential for the conversion of pyruvate to 2,3-butanediol (Celińska and Grajek, 2009; Ji et al., 2011). Correspondingly, Fig. 6.3 demonstrates this interactive effect of pH and acetate levels on the 2,3-butanediol production. Therefore, RSM can be an effective tool to determine the optimized medium composition to be used

during the subsequent fed-batch experiments for 2,3-butanediol production. The following validation experiment shows that 42.6 g/L of 2,3-butanediol can be produced from 100 g/L of sucrose under the optimized conditions (Fig. 6.4), which is consistent with data from the predicted model (Eq. 2).

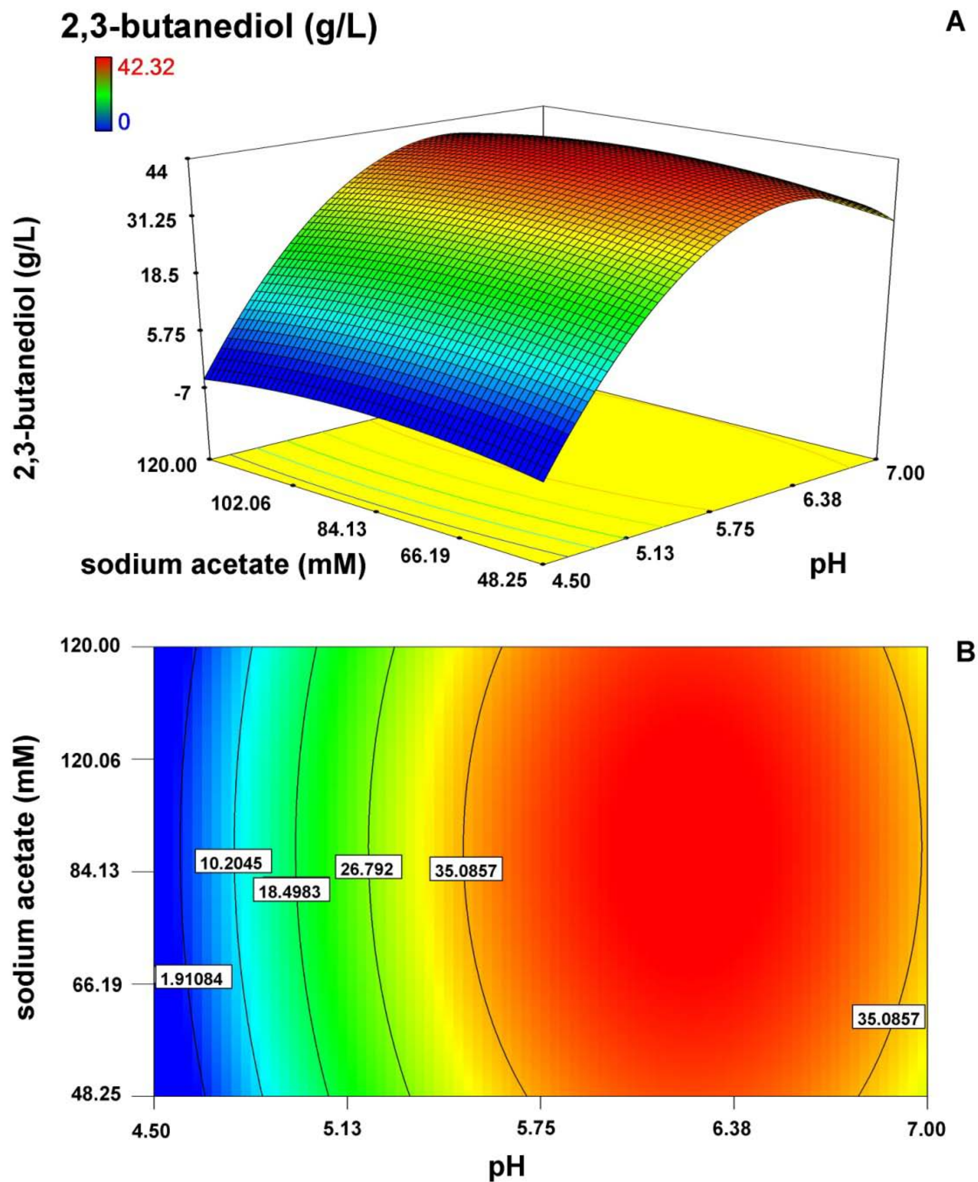

Fig. 6.3 Three dimensional response surface and contour plot showing the effect of pH values and concentration of sodium acetate (mM) on 2,3-butanediol production (g/L).

6.4.3 Effect of sucrose concentration

After the optimized conditions for 2,3-butanediol production were determined, various initial sucrose concentrations between 50 and 300 g/L were further investigated on its effect on 2,3-butanediol production in a batch system. Results showed that the amount of 2,3-butanediol increased with ascending sucrose concentration up to 200 g/L. Meanwhile, a maximum dry cell weight (DCW) reached 11.6 g/L at 100 g/L sucrose, however, further increment of the sucrose concentration led to the decrease of the DCW. Noticeably, the productivity and yield declined when the initial sucrose concentration was higher than 100 g/L (Fig. 6.4). This decreased yield and productivity could be attributed to the low cell biomass caused by the high osmotic pressure at high concentrations of sucrose (Ji et al., 2011). For instance, the minimum DCW of 5.1 g/L was obtained when using the highest concentration of sucrose of 300 g/L, which correlated with the lowest 2,3-butanediol yield (0.14 g/g) and productivity (0.29 g/L/h). Interestingly, during the entire process, the ethanol concentration was relatively stable, ranging from 15.2 to 18.1 g/L and low amount of acetate acid (< 0.5 g/L) was produced. Hence, a maximal sucrose concentration of 100 g/L was applied in the subsequent fed-batch fermentation studies in order to further improve the productivity and yield of 2,3-butanediol.

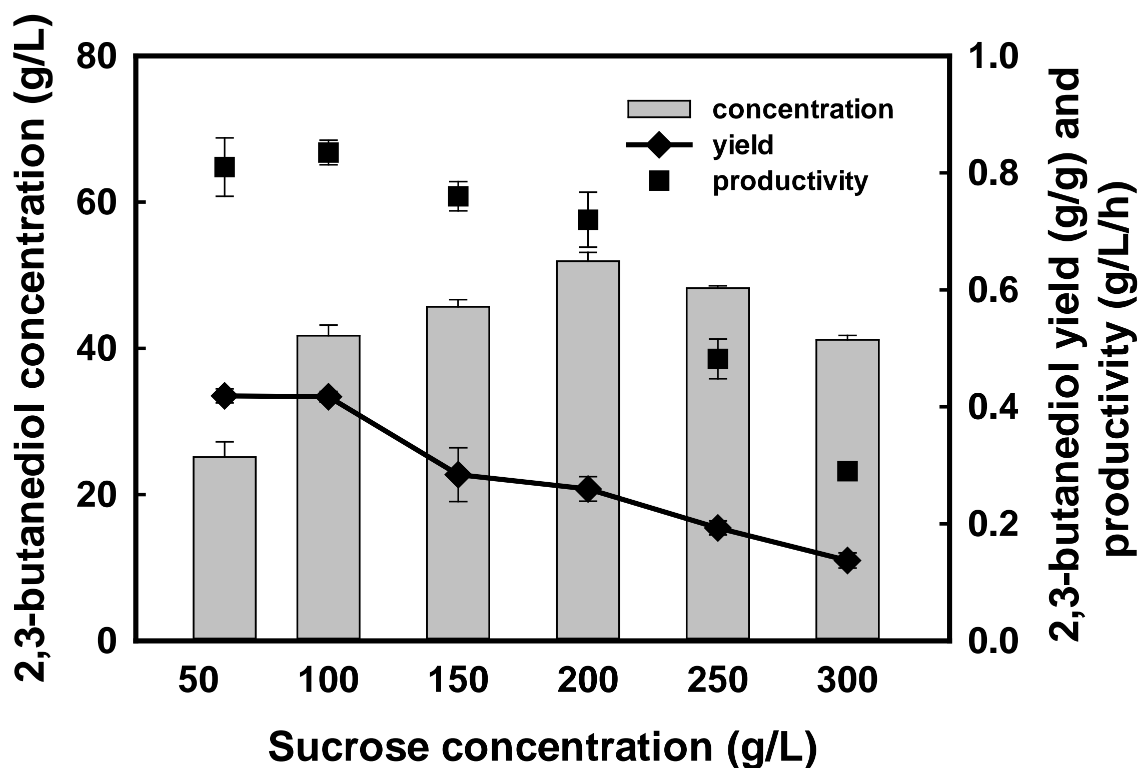

Fig. 6.4 Production of 2,3-butanediol at various amount of sucrose initial concentrations (50-300 g/L) by *Klebsiella* sp. strain XMR21. Data shown are after 48 hrs of fermentation under the optimized conditions.

6.4.4 2,3- Butanediol production by strain XMR21 under fed-batch fermentation

Subsequently, a fed-batch fermentation process was carried out for enhancement of 2,3-butanediol production. In the fed-batch fermentation process, sucrose was fed into the reactor three times to reach a concentration of 100 g/L after consumption of previous dosage, resulting in a nominal concentration of 300 g/L in total. In this fed-batch system, a maximum DCW of 13.1 g/L could be obtained at 24 hrs. The final 2,3-butanediol concentration through the fed-batch strategy reached 91.2 g/L (Fig. 6.5) which corresponded to a 2.4-fold increment compared to the batch fermentation conducted as

shown in Fig. 6.4. Meanwhile, ethanol in the fed-batch system accumulated to 20.1 g/L and less than 0.5 g/L acetate acid was detected. Thus, the overall 2,3-butanediol and ethanol is equivalent to a total solvent yield of ~0.37 g/g sucrose. This yield is about 80% of the theoretical yield (Ji et al., 2011; Zeng and Sabra, 2011). Hence, the fed-batch fermentation process surpasses 80 g/L of 2,3-butanediol, which is the minimal level needed for economic product recovery (Celińska and Grajek, 2009).

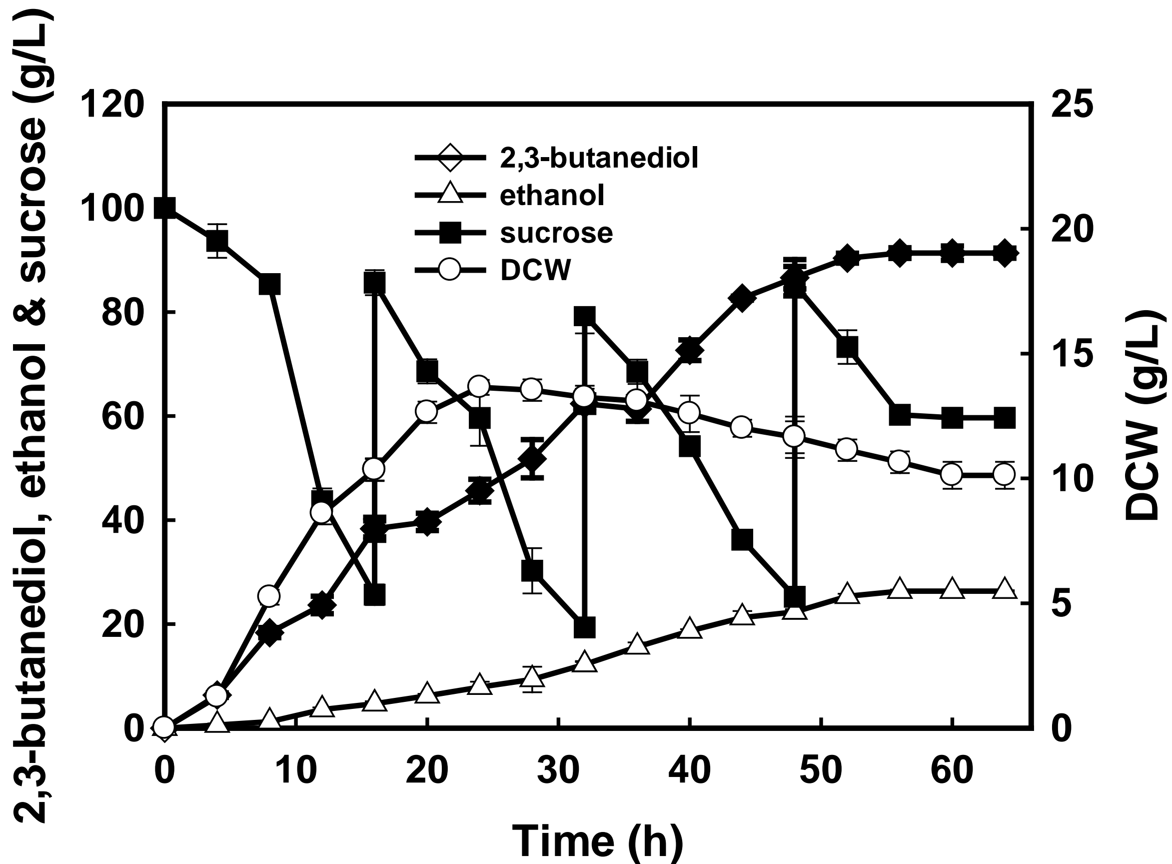

Fig.6.5 Production of 2,3-butanediol and ethanol under fed-batch fermentation in a 3.0-L bioreactor by *Klebsiella* sp. strain XMR21.

6.4.5 Enhanced 2,3-butanediol production by mutating strain XMR21

To further enhance the production of 2,3-butanediol, strain XMR21 was mutated via chemical mutagenesis in the presence of 0.5% (v/v) ethyl methyl sulfonate. After that, the treated cells were spread on agar plates amended with 80 to 180 g/L of 2,3-butanediol.

This screening procedure allowed us to select only those mutants which could tolerate higher levels of 2,3-butanediol compared to the wild-type strain. Cell growth was only observed at plates amended with less than 140 g/L of 2,3-butanediol, while no growth appeared on the agar plates containing more than 140 g/L of 2,3-butanediol. Among the 18 colonies appeared on the agar plates amended with 140 g/L of 2,3-butanediol, only one showed decent amount of 2,3-butanediol production (62.1 g/L) and yield (0.41 g/g) from fermenting 150 g/L of sucrose with still negligible by-products (Fig. 6.6A). Meanwhile, the mutated strain could produce the highest amount of ethanol (12.5 g/L) among all the colonies. Furthermore, the 2,3-butanediol production capacity of this mutant was quite stable as determined by five continuous subculture experiments conducted at 35 °C in a shaking bottles. On average, 63.7 ± 0.6 g/L of 2,3-butanediol was produced from 150 g/L of sucrose, which is 1.5-fold higher than that of the wild type strain (42.1 g/L).

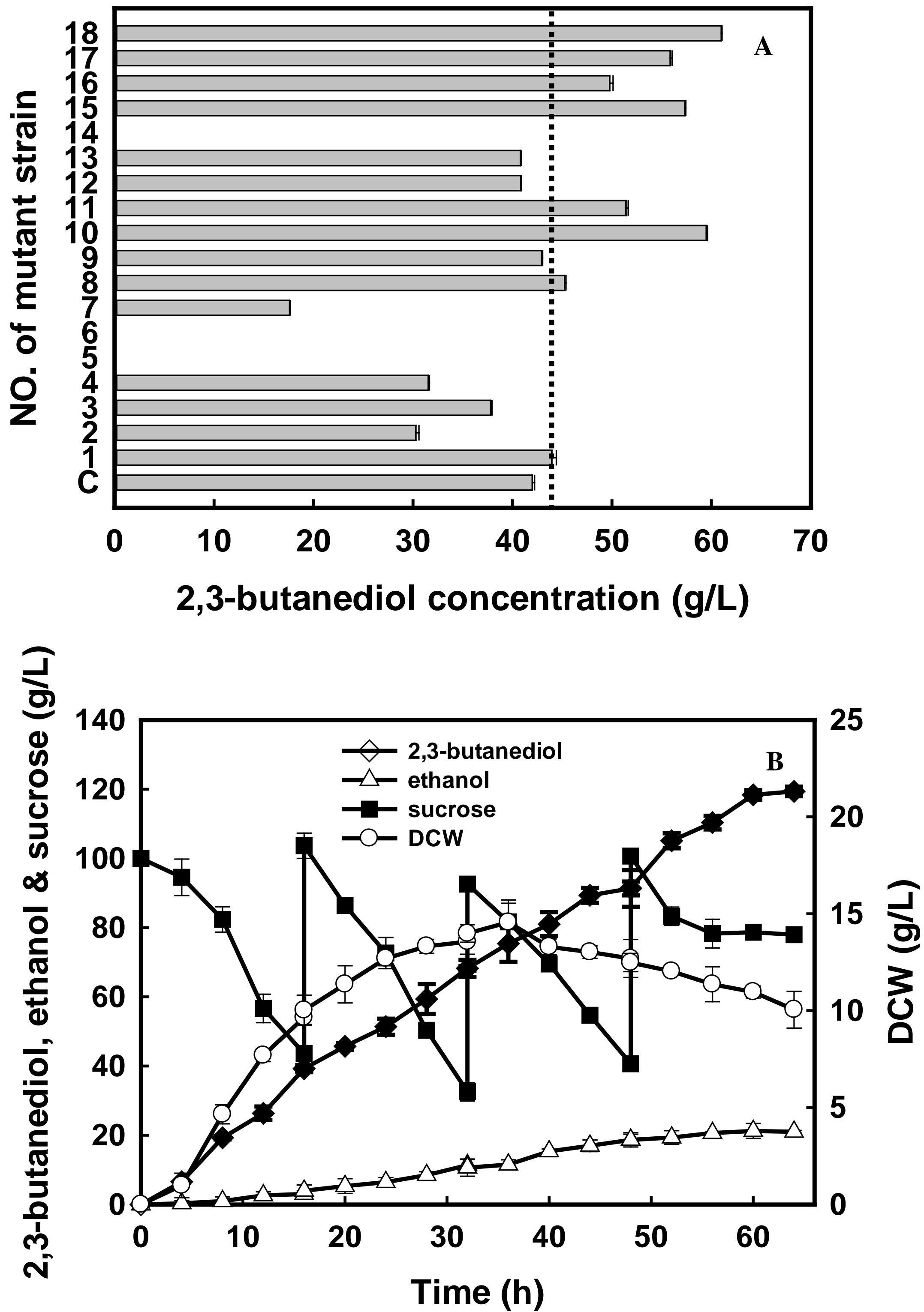

Fig. 6.6 (A) 2,3-Butanediol production by different mutants of strain XMR21 from fermenting 150 g/L of sucrose after 48 hrs. (B) Production of 2,3-butanediol and ethanol under fed-batch fermentation in a 3.0-L bioreactor by the mutant *Klebsiella* sp. strain XMR21.

Subsequently, this mutant strain was transferred to a fed-batch fermentation reactor by dosing 100 g/L sucrose for three times under optimized conditions (Fig. 6.4). As shown in Fig. 6.6B, the maximum DCW was as high as 15.0 g/L at 35 hrs, which was 1.1-fold higher than that of the wild type strain at similar fermentation conditions. The production of 2,3-butanediol and ethanol increased sharply with the rapid growth of the mutant strain, indicating that the production of 2,3-butanediol is associated with the cell growth. After 65 hrs of fermentation, the 2,3-butanediol concentration reached up to 119.4 g/L, which is 1.3-fold higher than that (91.2 g/L) of the wild strain and 1.7-fold higher than that (70 g/L) of other *Klebsiella* strain when fermenting sucrose (Berbert-Molina et al., 2001). This correlated with the corresponding 1.83 g/L/h and 0.40 g/g of 2,3-butanediol productivity and yield (Petrov and Petrova, 2009; Wang et al., 2010; Sun et al., 2009; Berbert-Molina et al., 2001; Qureshi and Cheryan, 1989; Cheng et al., 2010). Meanwhile, the final ethanol concentration through this fed-batch strategy reached 22.5 g/L, which will further add to the economic value of the process. In all, the final solvent (2,3-butanediol and ethanol) accumulated to 141.9 g/L with a final productivity and yield of 2.18 g/L/h and 0.47 g/g, which is higher than those in previous studies (Petrov and Petrova, 2009; Wang et al., 2010; Sun et al., 2009; Berbert-Molina et al., 2001; Qureshi and Cheryan, 1989; Cheng et al., 2010) and only 8.1 g/L less than that of the so far reported highest 2,3-butanediol production, where glucose was used as the substrate (Ma et al., 2009). In all, the data here represented the highest 2,3-butanediol bioproduction using sucrose as the sole carbon source. Thus, the mutant *Klebiesella* sp. strain XMR21 can serve as a potential industrial strain when using sucrose-based material as the substrate, such as sugar cane juice or molasses wastes, etc.

Table 6.2 Comparison of 2,3-butanediol production by different *Klebsiella* strains and substrates under fed-batch fermentation conditions.

Strains	Substrate	2,3-Butanediolproduction			References
		Con. (g/L)	Yield (g/g)	Pro. (g/L/h)	
K. pneumoniae SDM	glucose	150	0.4	3.95	Ma et al., 2009
K. pneumoniae G31	glycerol	70	0.39	0.47	Petrov and Petrova, 2009
K. pneumoniae SDM	corncob molasses	78.9	0.47	1.3	Wang et al., 2010
K. pneumoniae CICC 10011	Jerusalem artichoke tube	84	0.29	2.29	Sun et al., 2009
K. pneumoniae	sucrose	70	0.4	2	Berbert-Molina et al., 2001
K. oxytoca NRRL B-199	glucose	85.5	0.46	2.98	Qureshi and Cheryan, 1989
K. oxytoca ATCC 10370	corncob hydrolysate	35.7	0.50	0.59	Cheng et al., 2010
Klebsiella sp. strain XM18*	sucrose	119.4	0.40	1.83	This study

Con. : Concentration; Pro. : Productivity; *: Mutant strain.
All of the fermentation was carried out at 35-37°C.

6.5 Conclusion

A wild-type *Klebiesella* sp. strain XMR21 was obtained to ferment sucrose to 2,3-butanediol and ethanol without by-products. To maximize the production of 2,3-butanediol, fermentation conditions were first optimized for strain XMR21 by using response surface methodology (RSM) in batch reactors and then chemical mutagenesis was carried out to further improve the performance of strain XMR21. After optimization and mutagenesis of strain XMR21, 119.4 g/L and 22.5 g/L of 2,3-butanediol and ethanol can be produced from 300 g/L of sucrose within 65 hrs. This study provides a feasible and economical strategy to produce industrially important 2,3-butanediol from low-cost sucrose.

Chapter 7

Conclusions and recommendations

7.1 Conclusions

The work of this thesis work originated from the novel cultures and bio-processes designs involved in butanol, butyl-butyrate and 2,3-butanediol production from lignocellulosic biomass. In brief,

- One wild-type solventogenic *Clostridium* isolate, namly strain BOH3 was characterized with respect to its efficient utilization of xylose and co-fermentation of glucose and xylose;
- One hemicellulose-degrading *Kluyvera* isolate, namly strain OM3 was obtained and its cellulase-free xylanase was further characterized, meanwhile, a new co-culture system was set up using strains BOH3 and OM3 for direct butanol production from hemicelluloses;
- One bio-process was designed and evaluated with respect to butyl-butyrate production integrated with ABE fermentation by strain BOH3, lipase-catalyzed esterification and in situ extraction;
- One 2,3-butanediol-producing *Klebsiella*. sp strain XMR21was isolated and its high 2,3-butaneidol production was achieved via process optimization and strain improvement.

The key conclusions made in this study are listed below.

A lignocellulosic biomass-based route is one of the thorough solutions for sustainable development of ABE fermentation. However, this process is usually limited by the inefficiency of the solventogenic *Clostridium* spp. in the utilization of pentoses (mainly xylose) which constitute 20 to 60% of the sugars in the hydrolysate of lignocelluloses. Our newly isolated wild-type *Clostridium* sp. strain BOH3 efficiently

overcomes this obstacle and is a promising candidate in transforming lignocellulosic biomass into biofuel:

1. The wild-type *Clostridium* sp. strain BOH3 shows the most efficient xylose utilization (100% utilization of 60 g/L xylose) and highest butanol production (14.9 g/L from 60 g/L xylose) compared to other reported wild-type or gene modified strains. Meanwhile, its' co-production of riboflavin (110.5 mg/L from 60 g/L xylose versus 76.8 mg/L from 60 g/L glucose) will add to the economic value of the ABE process.

2. *Clostridium* sp. strain BOH3 eliminates carbon catabolic repression, which was clarified by its simultaneous consumption of glucose and xyloseto butanol, regardless of whether it was in pure glucose and xylose mixtures or horticultural waste hydrolysates at high yields with riboflavin as a co-product.

3. The higher expression of the xylose isomerase gene and higher enzymatic activities of xylose isomerase and xylulose kinase in strain BOH3 are responsible for efficient xylose utilization.

Direct butanol production from lignobiomass without the usage of commercial cellulase/xylanase could further reduce the economical cost of ABE fermentation. Hence, novel hemicellulose-degrading strain and co-culturing systems were isolated and designed, respectively:

4. The newly isolated anaerobic *Kluyvera* sp. strain OM3 could produce high cellulase-free and thermo-alkali-stable xylanase, which is one of the rare

xylanases because of its stability at extreme process conditions prevailing in the paper industry.

5. The co-culture system consisting of strain OM3 and strain BOH3 demonstrates 1.2 g/L butanol production directly from hemicellulose for the first time, which sheds light on efficient hemicelluloses hydrolysis and conversion of lignocellulosic materials to biofuels.

Butanol toxicity is one of the main obstacles in ABE fermentation. Compared with butanol, butyl-butyrate is a more value added product, which could be used in health, food and fule sectors. Microbial conversion of butanol to butyl-butyrate shows great advantage of mild reaction conditions, high catalytic efficiency and inherent selectivity compared with chemical synthesis. Two main strategies using strain BOH3 were designed to fulfill demands of butyl-butyrate in different sectors.

6. 19.7 g/L butyl-butyrate could be obtained by the integrationof ABE fermentation using strain BOH3, commericial lipase-catalyzed esterfication and *in situ* extraction using kerosene. This represents the highest butyl-butyrate production using microbial conversion method. The enriched aviation fuel can be directly applied in fuel sector.

7. Oil-based material, Bio-OSR could play bi-functional roles as both inducor and extractant in butyl-butyrate production process. 6.3 g/L butyl-butyrate could be obtained without any usage of commercial lipase and extractant. By further separation, the butyl-butyrate could be used in food, health sector.

With the deleption of oil reserves, bio-refinery systems that integrate strain improvement and process optimization to produce fuels and bulk-chemicals from renewable resources are at the stage of worldwide development. Similar to butanol, 2,3-butanediol is also a widely used four carbon bulk chemical and fuel. High production of 2,3-butanediol using our isolated *Klebsiella* sp. strain XRM21 after process optimization were finally achieved.

8. The newly isolated wild-type *Klebsiella* sp. strain XRM21 is capable of producing 2,3-butanediol with negligible by-products from a wide variety of carbon sources including glucose, sucrose, xylose and glycerol. Among them, sucrose leads to the highest concentration of 2,3-butanediol during fermentation.

9. Further process optimization using statistical experimental design methodology led to 91.2 g/L of 2,3-butanediol, which exceeds the minimal level needed for economic product recovery (80 g/L).

10. Moreover, the productive mutant stain XMR21 obtained by random mutagenesis resulted in 119.4 g/L and 22.5 g/L of 2,3-butanediol and ethanol under optimized fed-batch fermentation process with a total productivity of 2.18 g/L/h, which is comparable to the highest production.

In conclusion, this work has fulfilled its objectives and provides new insights into biosynthesis of butanol, butyl-butyrate and 2,3-butanediol using novel wild-type strains isolated from Singapore's tropical environment. These studies serve as a solid knowledge-base for further work to apply the novel strains and technologies to industry sectors.

7.2 Recommendations

In Chapter 3, the pure culture *Clostridium* sp. strain BOH3 characterized in this study provides scientific basis for understanding the simultaneous production of butanol using glucose and xylose mixtures. One of the contributing factors is the higher butanol tolerance (15.2 g/L) of strain BOH3 than previously reported wild-type microbes (e.g., 9.0 g/L for *C. acetobutylicum* ATCC 824 and 7.0 g/L for *Clostridium* sp. strain G117), which could efficiently alleviate the butanol inhibitory effect on the energy-requiring xylose transport system located in the cell membrane (Bowles and Ellefson, 1985; Ounine et al., 1985; Vollherbst-Schneck et al., 1984). Therefore, more information about the mechanism of its high butanol tolerance is needed in the further study. The proteomic study analysis that targets both the cell membrane and intracellular proteins can be used to identify the bacteria related to xylose transportation.

In Chapter 4, the pure culture *Kluyvera* sp. strain OM3 obtained and co-culturing system set up in this work sheds light on efficient hemicelluloses hydrolysis and conversion of lignocellulosic materials to biobutanol. Althought direct butanol production could be achieved from hemicelluloses, the low production (1.2 g/L) will limit its further application. As known, the level and availability of reducing cofactors could be a limiting step in reducing cofactor-dependent production systems (Berríos-Rivera et al., 2002 and Knepper et al., 2008), especially the solventogenic *Clostridium* sp. involving NADH- and NADPH-dependent butanol dehydrogenases. Consequently, a lack of or inefficient regenerations of the cofactor NADH or NADPH would shutdown the butanol dehydrogenase reaction. Accordingly, reducing cofactor manipulations could

potentially become a powerful tool for improvement of co-culturing process yield and productivity.

In Chapter 5, the proof-of-principle experiment set up in this study for the one-pot bio-ester production from xylose using *Clostridium* sp. strain BOH3 may be extended to a wide range of esters, especially to longer chain ones. However, the overall economics of this process could still be improved by metabolically reconstructing strain BOH3 with *in situ* surface-displayed lipase. Meanwhile, the real lignocellulosic hydrolysate, like horticultural waste hydrolysate could be used as the carbon source to replace the expensive pure glucose or xylose.

In Chapter 6, high 2,3-butanediol production was achieved via strain improvement and process optimization. Further study will choose sugar cane juice to replace sucrose (the main component of sugar cane juice) as the carbon source to further reduce the cost of this process. As a potential mutant strain for 2,3-butanediol production, strain XMR21 will be further identified and characterized with its wild-type.

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Bibiography

Mr. Fengxue Xin is currently the PhD candidate in Department of Civil & Environmental Engineering, National University of Singapore (NUS). He finished his master research in State Key Laboratory of Microbial Technology, National Glycoengineering Reseasrch Center, Shandong University, China in 2008. Before joining NUS, he had been working as a Research Assistant in School of Life Science and Chemical Technology, Ngee Ann Polytechnic, Singapore between the year of 2008-2010. His research mainly focuses on biodegradation of lignobiomass and butanol fermentation using new isolated strains.

Appendix: List of publications and awards

International journal publications:

1. **Fengxue Xin**, Jianzhong He*. Characterization of a thermostable xylanase from a newly isolated *Kluyvera* species and its application for biobutanol production. ***Bioresource Technology***. 2013, 135, 309-315.
2. **Fengxue Xin**, Yi-Rui Wu, Jianzhong He*. Simultaneous fermentation of glucose and xylose to butanol by *Clostridium* species strain BOH3. ***Applied Environmental Microbiology***. 2014, 180(15), 4771-4778.
3. **Fengxue Xin**, Anindya Basu, Michelle Cheung Weng, Kun-Lin Yang and Jianzhong He*. Production of 2,3-butanediol from sucrose by a *Klebsiella* species. ***Bioenergy Research***. 11 Jul 2015. DOI 10.1007/s12155-015-9653-7.
4. **Fengxue Xin**, Anindya Basuand, Kun-Lin Yang Jianzhong He*.Strategies for production of butanol and butyl-butyrate through lipase-catalyzed esterification. ***Bioresource Technology***. 2015, 202, 214-219.
5. Anindya Basu, **Fengxue Xin,** Lim Tech Kwang, Lin Qinsong and Jianzhong He*. Proteomics studies reveal efficient xylose utilization mechanisms for solventogenic *Clostridium* sp. strain BOH3. In preparation.
6. Yi-Rui Wu, **Fengxue Xin,** Jianzhong He*. Heterologous expression, characterization and application of a new β-xylosidase identified in solventogenic *Clostridium* sp. strain BOH3. ***Bioresource Technology.*** Under review.

Conference presentations:

1. **Fengxue Xin** and Jianzhong He*. **Poster**: Production and partical characterization of a novel thermostable xylanase by newly isolated *Kluyvera Georgiana* OM3. JGI (DOE Joint Genome Institute, US Department of Energy, Office of Science) User Meeting. 21.3-24.3.2011, Walnut Creek, California,

United States.

2. **Fengxue Xin** and Jianzhong He*. **Poster**: 241st American Chemical Society National Meeting & Exposition. 27.3-31.3.2011, Anaheim, California, United States.
3. **Fengxue Xin,** Eko Bramono Sandhi**,** and Jianzhong He*. **Invited Speaker**: A new butanol producing *Clostridium* species with high level of hemicellulosic and cellulosic hydrolyzing activity. The 14th Asia Pacific Confederation of Chemical Engineering Congress. 21.2-24.2.2012, Singapore.
4. **Fengxue Xin** and Jianzhong He*. **Poster**: Direct conversion of hemicelluloses to butanol by a solventogenic *Clostridium* species. The Australian Society of Microbiology 2012 Annual Scientific Meeting. 1.7-4.7.2012, Brisbane, Australia.
5. **Fengxue Xin,** Eko Bramono Sandhi, Yirui Wuand Jianzhong He*. **Invited Speaker**: Investigation of biobutanol generation and overexpression of a β–xylosidase gene from *Clostridium* sp. BOH3. 2012 International Symposium on Advanced Biological Engineering. 25.10-29.10.2012, Guilin, China.
6. **Fengxue Xin,** Eko Bramono Sandhi and Jianzhong He*. **Speaker and Poster**: Investigation of biobutanol generation from xylose by a unique *Clostridium* sp. BOH3. Bioenergy Biorefinery Conference-Southeast Asia. 26.3-28.3.2013, Singapore.
7. **Fengxue Xin,** Yirui Wu; Eko Bramono Sandhi and Jianzhong He*. **Speaker**: *Clostridium* sp. BOH3 that simultaneously utilizes glucose and xylose to produce biofuels. 15th Asian Chemical Conogress. 19.8-23.8.2013, Singapore.
8. **Fengxue Xin** and Jianzhong He*. **Poster**: Integration of acetone-butanol-ethanol (ABE) fermentation process and lipase-catalyzed butyl-butyrate prodution. V International Conference on Environmental, Industrial and Applied Microbiology. 2.10-4.10.2013, Madrid, Spain.
9. **Fengxue Xin**, Anindya Basu, Michelle Cheung Weng, Kun-Lin Yang, and Jianzhong He*. **Poster:** Production of 2,3-butanediol from sucrose by a *Klebsiella* speices. HT micro HOT TOPICS IN MICROBIOLOGY. 23.4-26.4.2015, High Tatras, Slovakia.

Awards

2014 Chinese Government Award for Outstanding Self-financed Students