BIOPRODUCTION OF 3-HYDROXYPROPIONALDEHYDE AND 3-METHYLCATECHOL BY ORGANIC SOLVENT-TOLERANT BACTERIA

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ในปัจจุบันการผลิตสารเคมีด้วยวิธีทางชีวภาพมีความสำคัญและมีข้อได้เปรียบเหนือกว่าการผลิตด้วยวิธีทางเคมี แต่อย่างไรก็ตาม ความเป็นพิษของสารตั้งต้น และ/หรือสารไฮโดรคาร์บอนผลิตภัณฑ์ อาจส่งผลกระทบต่อการอยู่รอดและการทำงานของตัวเร่งทางชีวภาพ แบคทีเรียที่ทนตัวทำละลายอินทรีย์ (organic solvent-tolerant; OST) เป็นแบคทีเรียที่เจริญได้ในภาวะที่มีตัวทำละลายอินทรีย์ความเข้มข้นสูงได้เนื่องจากมีกลไกการทนทานภาวะดังกล่าว ดังนั้น ในงานวิจัยนี้ได้ศึกษาเพื่อประยุกต์ใช้ OST แบคทีเรีย และกระบวนการผลิตทางชีวภาพที่เหมาะสมในการผลิตสารไฮโดรคาร์บอนอุตสาหกรรมประเภทสารเคมีที่ไม่ชอบน้ำและชอบน้ำด้วยวิธีทางชีวภาพ ประการแรก สำหรับการผลิต 3-เมทิลแคทิคอล (3MC) ซึ่งเป็นสารเคมีที่ไม่ชอบน้ำนั้น ใช้ OST แบคทีเรียกลายพันธุ์ *Pseudomonas putida* T-57 สายพันธุ์ TODE1 เป็นตัวเร่งชีวภาพภายใต้ระบบการผลิตแบบสองเฟส โดยมี 1-เดคานอลเป็นเฟสที่สอง การเติมกลีเซอรอล, Mn^{2+} หรือ Fe^{2+} ในแต่ละสภาวะการผลิตสามารถเพิ่มผลผลิตของ 3MC อย่างมีนัยสำคัญ จากนั้น ภายใต้สภาวะการผลิตที่เหมาะสมซึ่งได้จากการออกแบบด้วย response surface methodology-based central composite design สามารถเพิ่มประสิทธิภาพการผลิตโดยรวมของ 3MC ได้ถึง 34 มิลลิโมลาร์ ซึ่งเป็นความเข้มข้นที่สูงที่สุดเมื่อเทียบกับงานวิจัยที่ผ่านมา ประการที่สอง สำหรับการผลิตทางชีวภาพของสาร 3-ไฮดรอกซีโพรพิโอแนลดีไฮด์ (3-HPA) ซึ่งเป็นสารเคมีที่ชอบน้ำนั้นได้ใช้ระบบการผลิตแบบเฟสเดียว ในการคัดเลือกแบคทีเรียเจ้าเรือนเพื่อทำวิศวกรรมพันธุศาสตร์นั้น แบคทีเรีย 7 สายพันธุ์ถูกคัดเลือกบนพื้นฐานของความสามารถในการทนตัวทำละลายอินทรีย์ชอบน้ำได้หลายชนิด เช่น อะซิโตไนไตรและ โพรพิโอแนลดีไฮด์ จากนั้น ศึกษาปัจจัยที่มีผลต่อการแสดงออกของยีนใน OST แบคทีเรียเพื่อการผลิตสาร 3-HPA แม้ว่าลักษณะสมบัติของ โปรโมเตอร์ บริเวณจับของไรโบโซม และแหล่งของยีน ถูกนำมาพิจารณาและปรับเปลี่ยนแล้ว ผลผลิตของ 3-HPA ที่ได้จากการผลิตของแบคทีเรียรีคอมบิแนนท์ *Bacillus subtilis* BKS2-P2/3 หรือ *P. putida* T-57 ยังมีปริมาณน้อย ประการสุดท้ายของการศึกษา ได้แก่ กลไกการทนทานต่อสารไฮโดรคาร์บอนประเภทตัวทำละลายอินทรีย์ชอบน้ำของ OST แบคทีเรีย เนื่องจากข้อมูลดังกล่าวมีอยู่น้อย แต่เป็นสิ่งสำคัญสำหรับการประยุกต์ใช้ต่อไปในอนาคต การศึกษากลไกการทนทานต่อสารดังกล่าว ได้ศึกษาในแบคทีเรีย *Exiguobacterium* sp. SBH81 ซึ่งสามารถทนทานต่ออะซิโตไนไตรความเข้มข้นสูง ผลการศึกษาแสดงให้เห็นว่า กลไกการทนทานมีความเกี่ยวข้องกับการเพิ่มขนาดของเซลล์เพื่อลดปริมาณสารที่แบคทีเรียรับเข้าสู่เซลล์ รวมทั้ง การขับตัวทำละลายอินทรีย์ออกจากเซลล์ด้วยระบบเอฟฟลัก (ชนิด RND และ MATE) โดยสรุป ผลการศึกษาทั้งหมดนี้แสดงตัวอย่างการพัฒนาแบคทีเรียแพลตฟอร์มสำหรับการผลิตสารเคมีไฮโดรคาร์บอนที่ไม่ชอบน้ำและชอบน้ำโดยใช้ OST แบคทีเรียดัดแปลงพันธุกรรม ข้อมูลด้านบทบาทที่สำคัญของกลไกการทนต่อตัวทำละลายอินทรีย์ กลไกในระดับโมเลกุลสำหรับการแสดงออกของยีน และกระบวนการผลิตทางชีวภาพของตัวเร่งชีวภาพโดย OST แบคทีเรีย ซึ่งได้อธิบายไว้ในวิทยานิพนธ์นี้ จะเป็นข้อมูลที่สำคัญสำหรับการพัฒนาการผลิตสารเคมีไฮโดรคาร์บอนอุตสาหกรรมด้วยกระบวนการทางเทคโนโลยีชีวภาพต่อไป

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AJIRAPORN KONGPOL : BIOPRODUCTION OF 3-HYDROXYPROPIONALDEHYDE AND 3-METHYLCATECHOL BY ORGANIC SOLVENT-TOLERANT BACTERIA. ADVISOR : ASSOC. PROF. ALISA VANGNAI, Ph.D., CO-ADVISOR : PROF. JUNICHI KATO, Ph.D., 164 pp.

In the present day, biotechnological production of industrial chemicals has become significantly important and has several advantages over the chemical processes. Nevertheless, the toxicity of substrates and/or hydrocarbon products may limit the survival and catalytic activity of biocatalyst. Organic solvent-tolerant (OST) bacteria are bacteria that can thrive in the presence of high concentrations of hydrocarbon and solvent as a result of their tolerance mechanisms. Therefore, OST bacteria and a suitable bioproduction system were explored for hydrophobic and hydrophilic chemical bioproduction in this study. Firstly, for bioproduction of 3-methylcatechol (3MC) as a hydrophobic chemical, it was carried out by a mutant OST *Pseudomonas putida* T-57 strain TODE1 under a two-phase system, where 1-decanol was used as a second phase. The supplementation with either glycerol, Mn^{2+} or Fe^{2+} significantly enhanced the 3MC productivity. Based upon the response surface methodology-based central composite design optimized conditions, the overall 3MC production was produced up to 34 mM, which is the highest yield reported so far. Secondly, for bioproduction of 3-hydroxypropionaldehyde (3-HPA) as a hydrophilic chemical, it was explored by hydrophilic OST bacteria under a single-phase system. Seven hydrophilic OST bacteria were isolated based on their tolerance capability to various types of toxic hydrophilic organic solvent, including acetonitrile and propionaldehyde. To apply these bacteria as a bioproduction host, the transformation system and genetic manipulation of hydrophilic OST bacteria were established for 3-HPA bioproduction. Despite the fact that effect of promoter, ribosome binding site and source of structural gene were taken into consideration and modified, a low titer of 3-HPA was obtained when either *Bacillus subtilis* BKS2-P2/3 or *P. putida* T-57 was used as a bacterial host. Because the information of tolerance mechanisms of hydrophilic OST bacteria is scarce, but it is important for further applications. The mechanisms study was conducted with the bacterium with high tolerance to acetonitrile, *Exiguobacterium* sp. SBH81. The results may suggest that its tolerance mechanisms rely on increase of cell size to lower solvent partitioning into cells and the extrusion of solvents through the efflux system (RND and MATE system). In conclusion, this study provides examples of the development of bacterial platforms for hydrophobic and hydrophilic chemical production using genetically engineered OST bacteria. The important roles of OST mechanism of the biocatalytic host, molecular mechanism for gene expression as well as bioproduction process described in this study would be important information for further development of chemical production by biotechnological processes.

Field of Study : Biotechnology Student's Signature ....

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NOMENCLATURE

CFU	=	Colony forming unit
ml	=	Milliliter
µl	=	Microliter
µg	=	microgram
O.D.	=	Optical density
rpm	=	Revolution per minute
min	=	Minute
^{o}C	=	Degree of Celsius
cm	=	centimeter
et al.	=	ET. Alii (latin), and others
g	=	Gravitation acceleration
h	=	hour
l	=	liter
M	=	molar
mM	=	millimolar
mg	=	milligram
min	=	minute
MW	=	molecular weight

CHAPTER I

INTRODUCTION

Contents

1.1 Background and rationale of this dissertation

1.2 Objectives

1.3 Hypothesis

1.4 Scope of study

1.5 Expected outcome

1.6 Thesis organization

1.1 Background and rationale of this dissertation

Chemical production become more important in many industrial, especially their bioproduction has been increasing (Weber et al., 2010). Due to biotechnological production has several advantages compared to the chemical process such as energy saving, higher specificity to reaction/or substrate, and yields have been shown to be higher than those achieved by chemical synthesis. However, the toxicity of substrates and/or products towards bacterial producer may limit the accumulation of product in the bioproduction process. Organic solvent-tolerant (OST) bacteria are a unique group of microorganisms that can thrive in the presence of high concentrations of solvent as a result of their tolerance mechanisms (Sardessai and Bhosle, 2002) and bioproduction system have been attempted to overcome this toxicity and increase the yield for biotechnological production. The possible biotechnological production processes using OST bacteria as producer of chemical bioproduction, of which substrates and/or products may have toxic effects to bacterial producer are a two-phase system for hydrophobic chemicals and a single-phase system for hydrophilic chemicals. For example, a two-phase system for hydrophobic chemicals bioproduction of 3-methylcatechol (3MC) is possible by the conversion of aromatic hydrocarbons such as toluene into catechol, which substrate toluene and 3MC product directly affected to the bacteria producer growth. A single-phase system for hydrophilic chemicals bioproduction of 3-hydroxypropionaldehyde (3-HPA), as a key precursor of polymer production and several industrial chemicals, the toxicity of 3-HPA formed in the broth medium during the fermentation processes is a main limitation on 3-HPA microbial production (Vollenweider and Lacroix, 2004). Due to 3-HPA production was produced from glycerol conversion, crude glycerol as by-

product from biodiesel production has been used as substrate for the production of 3-HPA at a significantly low cost in recently (Dobson et al., 2012). Nevertheless an impurity in crude glycerol is a limitation on bacteria producer of 3-HPA production.

Because of their advantages, many reports have been explored to screen, isolate and characterize novel OST bacteria, to investigate tolerance mechanisms (Kongpol et al., 2008), to develop gene manipulation system (Kataoka et al., 2012) and, to apply as host cell for bioproduction. The goal of this research were to isolate and characterize bacteria able to tolerant organic solvent at high concentration, to develop bacterial host cells for production of 3-HPA and 3MC on using a recombinant OST bacterium as a whole-cell biocatalyst and to investigate the suitable bioproduction process of 3-HPA and 3MC production.

1.2 Objectives

This research work focuses on five objectives as follows:

1.2.1 To screen, isolate and characterize the OST bacteria as a recombinant host.

1.2.2 To investigate solvent tolerance mechanisms and to improve tolerance by adaptation.

1.2.3 To develop a transformation system and gene manipulation method of the selected OST bacteria.

1.2.4 To clone and over express of genes involve in 3-HPA and/or 3MC production in the selected bacterial host.

1.2.5 To develop and optimize efficient bioconversion system.

1.3 Hypothesis

The substrates and/or products toxicity directly affects to bacteria producer used in the bioproduction process. This implies that bacteria, which can thrive in the presence of high concentrations of substrates and/or products (OST bacteria), might be used as a whole-cell biocatalyst for chemical biotechnological industry as well as bioremediation.

1.4 Scope of study

1.4.1 Screening, isolation and characterization of OST bacteria and investigation their tolerance mechanism

1.4.2 Development of a transformation system and gene manipulation method for the selected OST bacterium as a gene expression host

1.4.3 Construction of an expression vector for bioproduction from OST bacteria

1.4.4 Determination and optimization of product formation from recombinant OST bacteria

1.4.5 Development of suitable bioprocesses for efficient fermentation/ bioconversion system

1.5 Expected outcome

Isolated OST bacteria and its mechanisms will be useful for further information of bioremediation and biotechnology. Moreover, the genetic manipulation and bioproduction of OST bacteria as a whole-cell biocatalyst will efficiently be usage for biotechnological industry.

1.6 Dissertation organization

This dissertation comprises of six chapters. First, Chapter I provide the introduction part of this research. The theoretical background and literature review are described in chapter II and the research methodology is explained in chapter III. In part of results and discussion, it can be divided into 2 parts. Firstly, chapter IV describes the results and discussion of "Hydrophobic organic solvent of 3-methylcatechol (3MC) bioproduction from organic solvent-tolerant bacteria using a two-phase system". Second the results and discussion of "Hydrophilic organic solvent of 3-hydroxypropionaldehyde (3-HPA) bioproduction from organic solvent-tolerant bacteria using a single-phase system" is demonstrated in Chapter V. Finally, the conclusion is orderly described in Chapter VI.

CHAPTER II

THEORETICAL BACKGROUND AND LITERATURE REVIEWS

Contents

2.1 Organic solvent tolerant bacteria

2.1.1 Isolation of organic solvent-tolerant bacteria

2.1.2 Organic solvent-tolerance mechanisms

2.1.3 Application of organic solvent-tolerant bacteria

2.2 Hydrophobic organic solvent of 3-methylcatechol production

2. 3 Hydrophilic organic solvent of 3-hydroxypropionaldehyde production

2.1 Organic solvent tolerant bacteria

Organic solvent (referred to as solvent) can be classified by the property of solubility in water into three groups as showed in Table 2.1. The log P_{ow} value of commonly used organic solvent was shown in Table 2.2 (Laane et al., 1987). Hydrophilic organic solvent which log $P \leq 2$, such as acetonitrile, 3-hydroxypropionaldehyde and 3-methylcatechol have been extensively used in analytical setting and as solubilizing agents in the plastic industry, and pharmaceutical industries.

An organic solvent even at very low concentration (0.1 %, v/v) has destructive effects on microorganicsm cells due to their toxicity. Then microorganisms that can grow in high concentration of solvent are solvent-tolerant (OST) bacteria (Sardessai and Bhosle, 2002).

Table 2.1 Maximal solubility of organic solvents in water versus log *P* value of organic solvent

log P value	Solubility in water (20ºC) (wt %)
log P ≤ 2	> 4.0
2< log P < 4	0.04 - 0.4
log P ≥ 4	< 0.04

Table 2.2 log *P* value of commonly used organic solvent (Laane et al., 1987)

Solvents	log *P*	Solvents	log *P*	Solvents	log *P*
1 dimethylsulfoxide	-1.30	38 m-phthalic acid	1.5	75 xylene	3.1
2 dioxane	-1.10	39 triethylamine	1.6	76 cychohexane	3.2
3 N,N-dimetylformamide	-1.00	40 benzylacetate	1.6	77 benzophenone	3.2
4 methanol	-0.76	41 butylacetate	1.7	78 propoxybenzene	3.2
5 3-hydroxypropionaldehyde	-0.59	42 3-methylcatechol	1.8	79 diethylphthalate	3.3
6 acetonitrile	-0.33	43 chloropropane	1.8	80 nonanol	3.4
7 ethanol	-0.24	44 acetophenone	1.8	81 decanone	3.4
8 acetone	-0.23	45 hexanol	1.8	82 hexane	3.5
9 acetic acid	-0.23	46 nitrobenzene	1.8	83 propylbenzene	3.6
10 ethoxyethanol	-0.22	47 heptanone	1.8	84 butylbenzoate	3.7
11 methylacetate	0.16	48 benzoic acid	1.9	85 methylcyclohexane	3.7
12 propanol	0.28	49 dipropylether	1.9	86 ethyloctanoate	3.8
13 propionic acid	0.29	50 hexanoic acid	1.9	87 dipentylether	3.9
14 butanone	0.29	51 chloroform	2.0	88 benzylbenzoate	3.9
15 hydroxybenzylethnol	0.40	52 benzene	2.0	89 decanol	4.0
16 tetrahydrofuran	0.49	53 methylcyclohexanol	2.0	90 heptane	4.0
17 diethylamine	0.64	54 methoxybenzene	2.1	91 cymene	4.1
18 ethyacetate	0.68	55 mehylbenzoate	2.2	92 pentylbenzoate	4.2
19 pyridine	0.71	56 propylbutylamine	2.2	93 diphenylether	4.3
20 butanol	0.80	57 pentylacetate	2.2	94 octane	4.5
21 pentanone	0.80	58 dimethylphthalate	2.3	95 undecanol	4.5
22 butyric acid	0.81	59 octanone	2.4	96 ethyldecanoate	4.9
23 diethylether	0.85	60 heptanol	2.4	97 dodecanol	5.0
24 benzylethanol	0.90	61 toluene	2.5	98 nanane	5.1
25 cyclohexanone	0.96	62 ethylbenzoate	2.6	99 dibutylphthalate	5.4
26 methylpropionate	0.97	63 ethoxybenzene	2.6	100 decane	5.6
27 dihydroxybenzene	1.00	64 dibutylamine	2.7	101 undecane	6.1
28 methylbutylamine	1.20	65 pentylpropionate	2.7	102 dipentylphthalate	6.5
29 propylacetate	1.20	66 chlorobenzene	2.8	103 dodecane	6.6
30 ethylchloride	1.30	67 octanol	2.8	104 oleylalchohol	7.0
31 pentanol	1.30	68 butybytyrate	2.8	105 dihexylphthalate	7.5
32 hexanone	1.30	69 nonanone	2.9	106 tetradecane	7.6
33 benzylformate	1.30	70 dibutyether	2.9	107 hexadecane	8.8
34 phenylethanol	1.40	71 styrene	3.0	108 dioctylphthalate	9.6
35 chclohexanol	1.50	72 tetrachloromethane	3.0	109 butyloleate	9.8
36 methycyclehexanone	1.50	73 pentane	3.0	110 didecylpththalate	11.7
37 phenol	1.50	74 ethylbenzene	3.1	111 dilaurylphthalate	13.7

2.1.1 Isolation of organic solvent-tolerant bacteria

Recently, many researchers have been explored to screen, isolate and characterize novel solvent-tolerant bacteria. The first report of an organic-solvent-tolerant bacterium was by Inoue and Horikoshi in 1989 (Inoue and Horikoshi, 1989). A large number of the reported organic-solvent-tolerant bacteria are *Pseudomonas* strains (i.e., *P. putida*, *P. aeruginosa*, *and P. fluorescens*), *Pseudomonas putida* IH-

2000 is from soil origin (Inoue and Horikoshi, 1989)), whereas *P. putida* DOT-T1E has been isolated from wastewater (Ramos et al., 1995). Organic-solvent-tolerant *Pseudomonas* strains have been isolated from mud samples, garden, forest and humus (Inoue and Horikoshi, 1989; Kim *et al*., 1998). Kato et al. report that organic-solvent-tolerant bacteria are over 100 times more abundant in deep-sea mud samples than in terrestrial soils. Other Gram negative solvent-tolerant bacteria such as *E. coli* mutants tolerant to cyclohexane and *p*-xylene have been constructed from parent strains having a solvent sensitive phenotype (Kato *et al*., 1996).

Moreover, strains of Gram-positive bacteria like *Bacillus*, *Rhodococcus* and *Arthrobacter* tolerant to benzene and *Deinococcus geothermalis* tolerant to broad range of solvents have been reported (Kato *et al*., 1996; Kongpol *et al*., 2008; Paje *et al.,* 1997). Most of the Gram-positive organic-solvent-tolerant microbial strains such as *Bacillus* DS-1906 and DS-994 and *Arthrobacter* ST-1 have been isolated from deep sea and marine mud samples. In addition, other organic-solvent-tolerant species like *Flavobacterium* DS-711 and yeast (*Candida* Y-40) have also been obtained from marine mud, indicating a greater biodiversity of such bacteria in the marine environment (Kato *et al*., 1996). Many reports have been explored hydrophobic OST bacteria, while little information of hydrophilic OST has been conducted as showed in Table 2.3.

Table 2.3 Some reported strains of organic-solvent-tolerant bacteria

Organism	**Tolerated solvents**	**Reffence**
Pseudomonas putida IH-2000	Heptanol, toluene	Inoue and Horikoshi 1989
Pseudomonas putida PpG1 (mutant)	Toluene	Shima *et al*.1991
Pseudomonas putida Idaho	Dimethylphthalate, toluene	Cruden *et al*.1992
Pseudomonas aeruginosa ST-001	Heptanol, toluene	Aono *et al*.1994
Pseudomonas patida S12	Dimethylphthalate, toluene	Weber and de Bont 1996
Pseudomonas aeruginosa LST-03	Toluene	Ogino *et al*. 1995
Pseudomonas putida DOT-T1E	Toluene	Ramos *et al*. 1995
Sphingomonas aromaticivorans B0695	Toluene, naphtalene, xylenes, p-cresol, fluorene, biphenyl, dibenzothiophene	Fredrickson *et al*. 1995
Arthrobacter ST-1	Benzene	Kato *et al*. 1996
Rhodococcus strain 33	Benzene	Paje *et al*. 1997
Bacillus	Toluene	Isken and de Bont 1998
Pseudomonas putida GM62, GM73	Toluene	Kim *et al*. 1998
Pseudomonas sp. Strain GM80		
Bacillus cereus strain R1	Toluene	Matsumoto *et al.* 2002
Bacillus sp. BC1	Chloroform	Sardessai and Bhosle 2004
Rhodococcus opacus	Benzene, toluene, styrene, xylene, ethylbenzene, propylbenzene, octane, decane	Na *et al*. 2005
Staphylococcus sp. Strain ZZ1	Toluene	Zahir *et al*. 2006

2.1.2 Organic solvent-tolerance mechanisms

Mechanisms enabling bacteria to survive otherwise toxic concentrations of an organic solvents comprise. “Adaptation” is defined as a change in cell physiology and/ or composition to adjust to the environment without the means of genetic modifications (mutations). Mechanisms enabling bacteria to survive otherwise toxic concentrations of organic solvents comprise: rigidification of the cell membrane, change in the membrane’s protein content/composition, active excretion of the solvent, adaptation of the energetic status, changes in cell wall and outer membrane composition, modification of the cell surface properties, morphological changes, and metabolization or transformation of the solvent. The tolerance mechanisms of Gram negative and Gram positive OST bacteria were described as Table 2.4 (Sardessai and Bhosle, 2002). Mechanisms playing a potential role in solvent-tolerance were discussed in two reviews previously (Isken and de Bont, 1998; Sikkema *et al*., 1994); Isken and de Bont 1998); however, it is a combination of diverse mechanisms that eventually leads to efficient solvent tolerance ((Isken and de Bont, 1996; Kabelitz *et al*., 2003).

Table 2.4 Tolerance mechanisms of Gram negative and Gram positive bacteria

Bacteria	Mechanisms
Gram-negative bacteria e.g., some strains of *Pseudomonas* and certain *E. coli* mutants	**Known mechanisms:** 1) modifications in cell envelope to increase cell membrane rigidity and decrease permeability: a) *cis-trans* isomerisation of membrane fatty acids by *cis-trans* iomerase b) decreased cell surface hydrophobicity c) changes in chemical composition/proportions of membrane lipids and proteins 2) increased rate of membrane repair enzymes 3) special solvent-inactivating enzymes 4) active efflux of solvents by means of solvent molecules sdhering to them 5) production of phage shock protein (stress protein in *E. coli*)
Gram-positive bacteria: e.g., some strains of *Bacillu*s, *Rhodococcus*, *Arthrobacter*	**Proposed mechanisms:** 1) protection rendered by endospores? 2) induction of general stress regulon, leading to the production of general stress proteins? 3) solvent deactivating or emulsifying enzymes?

2.1.3 Application of organic solvent-tolerant bacteria

Due to OST bacteria advantages, OST bacteria are being used for their potential in many applications such as bioremediation and biotechnological industry.

(1) Use of industrial biochemicals and biological host cell systems as factories to produce industrial bioproducts

There is considerable interest in the development of technology for the production of value-added chemicals from hydrophobic substrates such as petroleum hydrocarbons by using biocatalysts. Enantiospecific oxidation and reduction of aromatic and aliphatic hydrocarbons have especially attracted much interest, because many important chemicals can be produced by these reactions. Since cofactors and their regeneration are required for oxidation and reduction reactions, whole cells are favored as biocatalysts. Therefore, solvent-tolerant bacteria as hosts and genes encoding enzymes that catalyze reactions of interest are necessary for the development of bioconversion processed for hydrophobic substrates. Some microorganisms found in soil and water is known to have the capacity to metabolize a wide range of aromatic hydrocarbons as sole source of carbon and energy. Most of these bacteria have been demonstrated to belong to pseudomonas.

(2) Bioremediation and wastewater treatment

The persistence of benzene, toluene, and xylene in contaminated sites is indicative of the lack of natural systems that can efficiently degrade these compounds. Although many aromatic hydrocarbon degrading strains have been isolated, they are usually solvent sensitive and degradation of aromatic hydrocarbons occurs only when these compounds are supplied at low concentrations. There is considerable interest in the isolation of microbes able to thrive in high concentrations of organic solvents,

because such organisms can be used as vehicles in the elimination of low molecular weight aromatics that are highly carcinogenic even in ppm amounts. Since most natural contaminated sites are saturated with solvents such as benzene, toluene, etc., organic solvent tolerant bacteria with the requisite catabolic potential can be of vital importance in cleanup operations.

2.2 Hydrophobic organic solvent of 3-methylcatechol production

Catechols and its derivatives such as 3-methylcatechol (3MC) are important in pharmaceutical production process and valuable precursors in the production of stains and synthetic flavors (Shirai, 1986). Chemical production of 3-substituted is complex and hampered resulting in mixtures of a low overall yield and different catechols.

In general 3-substituted catechols conversion model has been studies from bioproduction of 3MC from toluene (Husken et al., 2002). Microorganisms are known to produce catechols from toluene as showed in Table 2.5, which all 3MC bioproduction are *P. putida*. In this production, the substrates from toluene and 3MC product are toxic to bacteria producer. To overcome this problem, solid adsorption materials have been used in the production process for removal of these compounds from the cells (Prpich P.G. and Daugulis A.J., 2007). Other possible solution for this problem is to scavenge the toxic substrate/or product from the cells using a second liquid extraction phase in the production process. Examples of such processes are production of *P. putida* in a two-phase as show in Table 2.5. Production of 3MC from *P. putida* was conversed from toluene under todC1C2BAD gene cluster as showed in Figure 2.1.

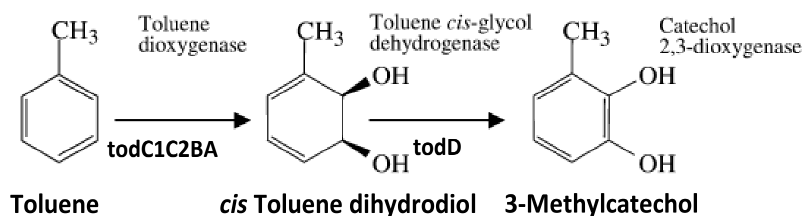

Figure 2.1 Conversion of toluene into 3-methylcatechol by *P. putida*

Table 2.5 Comparison of 3MC production from toluene by engineering bacteria using various types of organic phase for two-phase systems in batch fermentation.

Microorganism	Production system	References
P. putida MC2	Single phase	(Hüsken et al., 2001)
P. putida MC2	Single phase	(Husken *et al.*, 2002)
P. putida DS10	1-Octanol	(Wery et al., 2000)
P. putida MC2	1-Octanol	(Husken E. Leonie *et al.*, 2001)
P. putida TODE1	1-Butano, oleyl alcohol	(Faizal *et al.*, 2007)

2.3 Hydrophilic organic solvent of 3-hydroxypropionaldehyde production

3-Hydroxypropionaldehyde (3-HPA) forms, together with HPA-hydrate and HPA-dimer, a dynamic, multi-component system (HPA system) used in food preservation, as a precursor for many modern chemicals such as acrolein, acrylic acid, and 1,3-propanediol (1,3- PDO), and for polymer production. 3-HPA can be obtained both through traditional chemistry and bacterial fermentation. To date, 3-HPA has been produced from petrochemical resources as an intermediate in 1, 3-PDO. In vivo, glycerol is converted in one enzymatic step into 3-HPA. The 3-HPA-producing

Lactobacillus reuteri is used as a probiotic in the health care of humans and animals. The biotechnological production of 3-HPA from renewable resources is desirable both for use of 3-HPA in foods and for the production of bulk chemicals. The main challenge will be the efficient production and recovery of pure 3-HPA (Vollenweider and Lacroix, 2004).

2.3.1 Traditional chemical production of 3-hydroxypropionaldehyde

The raw materials for the multistep synthesis are obtained from the petrochemical industry. Two chemical processes are known to produce 3-HPA as an intermediate in 1, 3- PDO production: the Degussa and Shell processes (Vollenweider and Lacroix, 2004). The Degussa process begins with the catalytic transformation of propylene into acrolein, which is hydrated to 3-HPA and further reduced to 1, 3-PDO. Although propylene is inexpensive, the separation of the different molecules is and acrolein is toxic. The 1, 3-PDO yield of this process is only 43%. The Shell process begins with ethylene, forming ethylene oxide, which is transformed by a hydroformylation reaction under high pressure (150 bar) with syngas to 3-HPA. Ethylene is inexpensive and the intermediate products are not toxic, but 3-HPA has to be extracted from the organic phase. No information on the yields for 3-HPA production is available in the literature.

2.3.2 Biotechnological production of 3-hydroxypropionaldehyde

Biotechnological production has several advantages compared to the chemical process. First, 3-HPA production can be carried out in glycerol-containing aqueous solution at room temperature or at 37°C under normal pressure. The transformation

from glycerol to 3-HPA is a one-step enzymatic catalysis (Figure 2.2) and the yields have been shown to be higher than achieved by chemical synthesis (Vollenweider *et al*., 2003).

There are six genera of bacteria that are able to ferment glycerol into 3-HPA has been identified: *Bacillus*, *Klebsiella*, *Enterobacter, Citrobacter*, *Clostridium* and *Lactobacillus* as shown in Table 2.6. The enzyme responsible for the formation of 3-HPA from glycerol, glycerol dehydratase, is known to be cobamide-dependent. 3-HPA is normally an intracellular intermediate that does not accumulate but is reduced by an NAD^+-dependent oxidoreductase to 1, 3-PDO, the end-product of glycerol fermentation.

However, the toxicity of 3-HPA towards bacteria limits the accumulation of this compound in the fermentation broth medium. As show in Table 2.6, two methods have been attempted to overcome this toxicity and increase the yield for biotechnological production. In the first method, 3-HPA is entrapped by semicarbazide (Vancauwenberge et al., 1990), such that 3-HPA becomes unavailable. This reduces its toxic affect and prevents its further reduction to 1, 3-PDO, increasing the yield of 3-HPA production. This method resulted in a very high total production of 621 mM 3-HPA. Although this method is very promising, the recovery of 3-HPA has never been reported. The second method uses *Lactobacillus reuteri*, a species shown to sustain large amounts of 3-HPA produced from glycerol. However, although *L. reuteri* is very resistant to high concentrations of 3-HPA, its viability does decrease when 3-HPA is produced in large quantities. To further optimize biotechnological production, bacterial strains that is both more efficient and capable of tolerating large amounts of 3-HPA need to be developed.

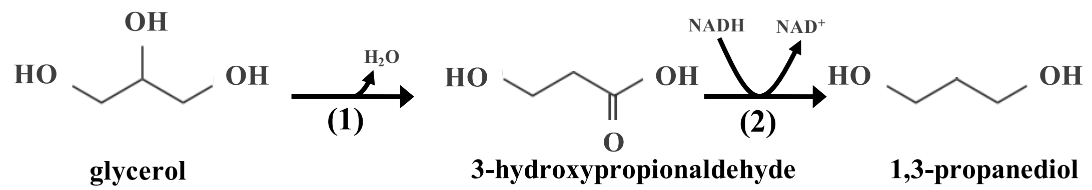

Figure 2.2 Bacterial conversion of glycerol to 1, 3-PD: (1) glycerol dehydratase, (2) 1, 3-PD dehydrogenase

Table 2.6 Summary of conversion of glycerol into 3-HPA

Bacteria	Fermentation/ Conversion	3-HPA production condition	Yield and/or productivity	Reference
Lactobacillus	Anaerobic glycerol fermentation	A two step process, consisting of biomass production (resting cells) of *L. reuteri* DSMZ 20016 follwed by glycerol biotransformation to 3-HPA 3-HPA production was optimal at pH 6 and in the presence of 300 mM glycerol *L. reuteri* cell viability was not significantly affected at concentrations below 35 mM over a period of at least 2 hrs	35 mM	(Bauer et al., 2010)
		L. reuteri NRRL B-14171 was immobilized in spherical calcium alginate beads.	90 mM 80 mM (free cell)	(Zamudio-Jaramillo et al., 2009)
		Resting cell bioconversion of *L. reuteri* ATCC 53608 (two-step process)	235 mM	(Doleyres et al., 2005)
		3-HPA accumulaton in resting cells of *L. reuteri* (ATCC 5360) in two-step fermentation	170 mM	(Lüthi-Peng et al., 2002)
		L. reuteri 1063 were grow in 250 mM glycerol in distilled water, and incubated under anaerobic conditions (nitrogen sparging) at 37°C. In some studies, 2 M glucose was added together with the glycerol.	150 mM	(Talarico et al., 1988)
		Lactobaillus brevis B22 and *Lactobacillus buchneri* B190		
		3-HPA production by glycerol dehydratase from *Lactobacillus* sp. NRRL B-1720	7 g/L (95 mM)	(Slininger et al., 1983)
Bacillus		Formation of acrolein from glycerol by *Bacillus welchii*	-	Humphreys ., 1924
Klebsiella	Aerobic glycerol fermentation	Whole cells of *K. pneumoniae* NRRL B-199 (ATCC8724) suspended in semicarbazide hydrochloride solution. Glycerol solutions of 30 g/liter Production condition at 28°C, shaken at 150 rpm.	13.1 g/L (177 mM)	(Slininger et al., 1983)
	Aerobic conversion of glycerol	24, 72 and 48-h of *K. pneumoniae* ATCC8724 cells harvested for resuspension in the 20 to 50 g/liter of glycerol and suspended in buffer supplemented with semicarbazide.	13.9 g/L (220 mM)	(Slininger and Bothast, 1985)
	Aerobic conversion of glycerol	*K. pneumoniae* NRRL B-4011. The culture sequence was as follows: stock -> seed -> cell production -> 3-HPA production. Under optimum conditions (28°C; 40 g of semicarbazide hydrochloride per liter, 70 g of glycerol per liter; and pH 6.0), 14.5 g of cells per liter	46 g/L (621 mM)	(Vancauwenberge et al., 1990)
	Aerobic glycerol fermentation	*K. pneumoniae* TUAC01 5 L fermentation, 50 g/L initial glycerol, pH 6.8 and 37ºC, 250 rpm	12.57 mM	(Hao et al., 2008)

Table 2.6 Summary of conversion of glycerol into 3-HPA (continued)

Bacteria	Fermentation/ Conversion	3-HPA production condition	Yield and/or productivity	Reference
Citrobacter		1,3-PDO production		(Daniel and Gottschalk, 1992)
		1,3-PDO production		(Anand and Saxena, 2012)
Enterobacter		*Enterobacter agglomerans*, end of the fermentation using 480 mM glycerol	29 mM	(Barbirato et al., 1996)
Clostridium		Production of 1,3-propanediol from glycerol by *C. butyricum*	-	(González-Pajuelo et al., 2005)
***E. coli* RB**		1,3-Propanediol production by *E. coli* expressing genes from the *K. pneumoniae dha* regulon The transformed *E. coli* AG1/pTC1 was able to grow and produce 1,3-PD under anaerobic conditions on ST medium containing 2 g of glycerol per liter and 2 g of DHA per liter	the yield of 1,3-PD from glycerol was 0.46 mol/mol.	(Tong et al., 1991)
		E. coli K-12 ER2925 two-stage fermentation process	1,3-PDO production from glycerol	(Tang et al., 2009)

CHAPTER III

MATERIALS AND METHODOLOGY

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Materials

Equipments, chemicals and enzymes

3.1 Equipments

DNA electrophoresis: Gelmate 2000, Toyobo, Japan.

DNA sequencer CEQ 8000 version 7.0: Backmen coulter, USA

Electro Cell Manipulator, model ECM 630, BTX Molecular Delivery Systems, Harvard Apparatus Inc., CA, USA.

Gel Document: Model Gel Doc^{TM} XR+, Bio-Rad, USA.

T100^{TM}Thermal Cycler: Bio-Rad, USA.

HPLC: Shimazu, Japan

Hyperclone column 5u BDS C18, 130A 250x4.6 m: Phenomenex, USA

Incubator shaker: Innova 4000, New Brunswick Scientific, USA.

MyCycler Thermal Cycler: Bio-Rad, USA.

Protein electrophoresis: Model Mini-protein II Cell, Bio-Rad, USA.

Refrigerated centrifuge: Model 5804R, Eppendrof, USA.

Scanning Electron Microscope: Model JSM-5900, Jeol, Japan

Spectrophotometer: Model DU800, Beckman Coulter, USA.

Ultrasonic: Banderlin, Germany.

3.2 Chemicals and solvents

(1) Chemicals

Acrolein: Singma, USA

Acrylamide: Merck, Germany.

Agar: Scharlau Microbiology, Spain.

Agarose: Seakem, USA.

Ampicillin: Sigma, USA.

Bovine serum albumin (BSA): Sigma, USA.

5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside (X-gal): Fermentas, USA.

Buffer-saturated phenol: Invitrogen, New Zealand.

Citric acid: LAB-SCAN, Thailand.

Crude glycerol from biodiesel: gift from Verasuwan Company, Thailand.

Ethidium bromide: Sigma, USA.

Ethylenediamine tetraacetaic acid (EDTA): Fluka, Switzerland.

Glucose: Fluka, Switzerland.

Glycerol: Univar, Australia.

Hydrochloric acid: LAB-SCAN, Thailand.

Kanamycin: Sigma, USA.

Lysozyme: Sigma, USA.

Magnesium chloride: Ajax finechem, Australia.

3-Methylcatechol: Sigma, USA.

N,N,N'N'-tetramethylaene ethylene diamine (TEMED): BDH, England.

o-Nitrophenyl-beta-galactopyranoside: Wako, Japan.

Orthovanadate: Nacalai tesque, Japan.

Paroxitine: Toronto Research chemical Inc, Canada.

PAβN: Sigma, Japan.

Phenol: BDH, England.

Sodium carbonate: BDH, England.

Sodium dodecyl sulfate (SDS): Sigma, USA.

Sucrose: Ajax finechem, Australia.

Tetracycline: Sigma, USA.

Tryptone: Himedia, India.

D, L-Tryptophane: Sigma, USA.

Vitamin B12: Sigma, USA

Yeast extract: Scharlau Microbiology, Spain.

(2) Solvents

Acetonitrile (99.9 % purity): Fluka, Germany.

β-mercaptoethanol (99.5 % purity): Sigma, USA.

n-Butanol: Fluka (99.5 % purity), Germany.

Butyraldehyde (99.5 % purity): Fluka, Germany.

Butyl acetate (99.5 % purity): Fluka, Germany.

Benzene (99 % purity): Fluka, Germany.

Chloroform (99.5 % purity): Merck, Germany.

Cyclohexane (99.8 % purity): Fluka, Germany.

n-Decane (98 % purity): Fluka, Germany.

n-Decanol (98 % purity): Sigma, USA.

Diethyl ether (99.5 % purity): Fluka, Germany.

Diethylphthalate (96 % purity): Fluka, Germany.

Dimethyl sulfoxide (99.5 % purity): Sigma, USA.

Diethylformamide (99.5 % purity): Fluka, Germany.

Ethyl acetate (99.9 % purity): LAB-SCAN, Thailand.

Ethanol (99.8 % purity): Carlo Erba Reagenti, Italy.

Ethylbenzene (99 % purity): Fluka, Germany.

n-Heptane (99 % purity): Fluka, Germany.

n-Heptanol (99 % purity): Fluka, Germany.

n-Hexane (98 % purity): Fisher Scientific, United

Methanol (99.5 % purity): Fluka, Germany.

n-Octane (99 % purity): Fluka, Germany.

Pentanol (99.5 % purity): Fluka, Germany.

Propanol (99.5 % purity): Fluka, Germany.

Propionaldehyde (99.5 % purity): Sigma, USA.

Styrene (99 % purity): Fluka, Germany.

Toluene (99.5 % purity): Fluka, Germany.

m-Xylene (99 % purity): Fluka, Germany.

o-Xylene (98 % purity): Fluka, Germany.

p-Xylene (98 % purity): Fluka, Germany.

3.3 Enzymes and Kits

DNA marker: 1 kb GeneRuler™ (#SM 0311), Fermentas, USA.

DNA marker: 100 bp GeneRuler™ (#SM 0241), Fermentas, USA.

Fast Plasmid Mini Kit: Eppendorf, USA.

Ligation high: Toyobo, Japan.

Lysozyme: Sigma, USA.

QIAquick® Gel extraction Kit (Cat. No. 28704): Qiagen, Germany.

Restriction enzymes: Toyobo, Japan.

RNase A: USBiological, USA.

Long Taq polymerase: Fermentas, USA.

Culture medium, bacterial strains and plasmids

3.4 Culture medium

(1) Luria-Bertani medium (LB)

LB medium in 1 liter of distilled water consisted of 10 g tryptone, 5 g yeast extract and 10 g NaCl. Subsequently, it was adjusted the solution of pH of 7 and autoclaved at 121ºC for 15 minutes. For solid media, 1.5% agar was added into the LB medium.

(2) Mineral salt basal medium (MSB)

The MSB medium consisted of media and trace element (Faizal et al., 2005). Media in 1 liter of distilled water consisted of 4.3 g K_2HPO_4, 3.4 g KH_2PO_4, 2.0 g $(NH_4)_2SO_4$, and 0.34 g $MgCl_2•6H_2O$. For trace element, in 1 liter contained 0.001 g $MnCl_2•4H_2O$, 0.006 g $FeSO_4•7H_2O$, 0.026 g $CaCl_2•2H_2O$, 0.002 g $NaMoO_4$, 0.1 mg $ZnCl_2$, 0.01 mg $CoCl_2•6H_2O$, 0.01 mg $CuSO_4$, 0.001 mg $NiSO_4$ and 0.0001 mg Na_2SeO_4. When it was supplemented with yeast extract (0.1 % (w/v)) and glucose (3.5 g/l), it was abbreviated as MSBYG.

(3) SOC medium

SOC medium in 100 ml of distilled water consisted of 2 g tryptone, 0.5 g yeast extract, 1 ml 1M NaCl, 0.25 ml 1 M KCl and 1 ml 2M Mg^{2+} stock and 1 ml 2M glucose. Tryptone, yeast extract, NaCl and KCl were adjusted to 97 ml of distilled water. After autoclaved, 2 M Mg^{2+} stock and 2 M glucose were prepared by dissolving of 20.33 g $MgCl_2$. $•6H_2O$ and 24.65 g $MgSO_4•7H_2O$ in 100 ml distilled water and filter sterile.

(4) BS medium

One liter of BS medium used in this study was prepared by dissolving of 4 g/L $(NH_4)_2SO_4$, 0.69 g/L $K_2HPO_4{\bullet}3H_2O$, 0.25 g/L KH_2PO_4, 0.2 g/L $MgSO_4$, 1.5 g/L yeast extract, 30 or 50 g/L glycerol and, 1 ml of trace element solution.

The trace element solution (1 liter) contained 100 mg $MnSO_4{\bullet}4H_2O$, 70 mg $ZnCl_2$, 35 mg $Na_2MoO_4{\bullet}2H_2O$, 60 mg H_3BO_3, 200 mg $CoCl_2{\bullet}6H_2O$, 29.28 mg $CuSO_4{\bullet}5H_2O$, 25 mg $NiCl_2{\bullet}6H_2O$ and 0.9 ml 37% HCl. 40% (w/w) NaOH was used to adjust the pH of solution.

3.5 Bacterial strains and growth conditions

Pseudomonas putida T-57 and mutant strain TODE1 were kindly provided by Professor Dr. Junichi Kato (Hiroshima University, Japan). *P. putida* T-57 was grown at 30ºC with shaking at 200 rpm in LB medium supplemented with appropriate antibiotics; 50 $\mu g.ml^{-1}$. The mutant strain TODE1, an deletion-insertion mutant of the 3-methylcatechol 2,3-dioxygenense gene (*todE*) was constructed by inserting a kanamycin resistant gene cassette into the chromosomal *todE* gene of *P. putida* T-57 was also grown in LB medium at 30ºC with checking condition.

Escherichia coli DH5α was used as a host for recombinant plasmid. *E. coli* was grown at 37ºC with shaking condition in LB medium.

3.6 Plasmids

The plasmid map was shown in Appendix A.

(1) Cloning vector

pGEM-T easy vector: Amp^r, cloning vector (Promega, USA)

pBluescript II SK(-) plasmid: Amp^r, cloning vector

pUC118: Amp^r, general cloning vector

(2) Promoter activity plasmid (Kataoka et al., 2012)

The promoter activity test plasmids of *B. subtilis* and an expression vectors were kindly supplied by Professor Dr. Junichi Kato (Hiroshima University, Japan) as shown in Appendix A.

pHP2N: Tet^r, pHY300PLK containing P2N promoter

pHP2L: Tet^r, pHY300PLK containing P2L promoter

pHPTet: Tet^r, pHY300PLK containing Tet promoter

pHPKm: Tet^r, pHY300PLK containing Km promoter

pHPSpac: Tet^r, pHY300PLK containing Spac promoter

pHPXyl: Tet^r, pHY300PLK containing Xyl promoter

pHP43: Tet^r, pHY300PLK containing 43promoter

pHPXyl-Km: Km^r, pHY300PLK containing Xyl promoter

(3) An expression vector of *B. subtilis*

pHY300PLK: Tet^r, promoterless cloning vectors for *B. subtilis*

(4) An expression vector of *P. putida*

pUCP18: Km^r, an expression vector containing lac promoter

pMEPT: Km^r, an expression vector containing tac promoter

Reagent stock solutions and buffers

3.7 Reagent stock solutions

Ampicillin: 100 mg ml^{-1}dissolved in sterile deionized water

EM solution: 0.5 M sorbitol, 0.5 M manitol and 10% (w/v) glycerol

EP solution: 250 mM sucrose, 1 mM HEPES, 1 mM $MgCl_2$ and 10% (w/v) glycerol

Fehling reagent: mixture of Fehling reagent A (8.65g of $CuSO_4 \cdot 5H_2O$ in 75 ml of water) and Fehling reagent B (86.5 g of sodium citrate and 50 g of anhydrous Na_2CO_3 in 400 ml H_2O) for 4:1 ratio

HSMG buffer: 1 mM HEPES (pH 7.0), 250 mM sucrose, 1mM $MgCl_2$, 10% (w/v) glycerol

Kanamycin: 50 mg ml^{-1}dissolved in sterile deionized water

3-Methylcatechol: dissolved in 99.9% ethanol

ONPG: 4 mg ml^{-1} dissolved in sterile deionized water

RNase A solution: 20 mg ml^{-1} dissolved in sterile deionized water

Protein determination

Solution A (Lowry): 2% Na_2CO_3 in 0.1 M NaOH containing 0.5% SDS

Solution B (Lowry): 0.5% $CuSO_4 \cdot 5H_2O$ in 1% potassium sodium tartrate

Solution C (Lowry): Phenol reagent (Folin-Ciocalteu's reagent)

Tetracyclin: 20 mg ml^{-1} dissolved in 50% (v/v) ethanol

X-gal: 80 mg ml^{-1} dissolved in N, N-dimethylformamide

Tryptophan solution: 2.05 g D, L-tryptophane, 4.17 ml concentrated hydrochloric acid and 2.5 ml toluene and adjust volume 1 litter with distilled water.

Z-buffer: 4.27 g Na_2HPO_4, 2.75 g $NaH_2PO_4 \cdot H_2O$, 0.375 g KCl, 0.125 g $MgSO_4 \cdot 7H_2O$, 0.014 ml ß-mercaptoethanol in 500 ml deionized water (pH 7.0)

3.8 Buffers

TAE buffer: 40 mM Tris-HCl, 40 mM acetate and 1 mM EDTA (pH 8.0)

Phosphate buffer: 1M of phosphate buffer containing of 87.09 g K_2HPO_4 and 68.04 g KH_2PO_4 in 500 ml deionized water (pH 8.0)

TE buffer: 10 mM Tris-HCl and 1 mM EDTA (pH 8.0)

Methodology

The experimental methodologies are described in order of the experiments performed. All of the methods used in this dissertation can be simplified as a flow chart below:

Method flow **Result flow**

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3.9-3.15 General techniques in genetic engineering
3.16-3.20 Determination of proteins, enzymes and products formation

Method flow	Result flow
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↓

Method flow	Result flow
Hydrophilic organic solvent of 3-hydroxypropionaldehyde (3-HPA) bioproduction from OST bacteria using a single-phase system • **Screening and characterization of OST bacteria as host cell for valued chemical production** 3.22 Screening and isolation of OST bacteria 3.23 Characterization of hydrophilic OST Gram positive bacteria • **Development of a transformation system and gene manipulation method for the selected OST bacterium** 3.24 Development of a transformation system and gene manipulation method for the selected hydrophilic OST Gram positive bacteria as a gene expression host • **3-HPA bioproduction from glycerol by OST Gram positive *B. subtilis* BKS2-P2/3 and Gram negative *P. putida* T-57 using a single-phase system** 3.25 Screening and isolation of 3-HPA producing bacteria for source of gene 3.26 Cloning of *dha*B genes cluster for production of 3-HPA by OST *B. subtilis* BKS2-P2/3 3.27 Gene expression and product formation determination in recombinant OST *B. subtilis* BKS2-P2/3 3.28 3-HPA bioproduction from glycerol by OST Gram negative *P. putida* T-57 • **Characterization of hydrophilic OST bacteria** 3.29 Characterization of hydrophilic OST Gram positive *Exiguobacterium* sp. SBH81	**CHAPTER V**

General techniques in genetic engineering

3.9 Bacterial genomic DNA extraction

The selected bacterial isolates were grown in LB medium at 37°C and shaking at 200 rpm for 18 h. Cells were collected by centrifugation at 4ºC, 12000 rpm for 10 min and resuspended in TE buffer. RNaseA and lysozyme solutions were added at 300 µl and 100 µl, respectively then incubated at 37ºC for 30 min. After incubation, 1/10 volume of 10% (w/v) SDS and 20 mg proteinase K were added and incubated at 37ºC for 1 h. Cetyltrimethylammonium bromide (CTAB) (10%, w/v) containing 0.7 M NaCl was added with 1/10 volume and further incubated at 65ºC for 20 min. Then, an equal volume of phenol: chloroform: isoamyl alcohol (25: 24: 1) was added and mixed thoroughly. The mixture was then centrifuged at 12000 rpm for 15 min. The upper layer was collected and 0.6 volume of 2-propanol was added and mixed thoroughly. The DNA obtained was washed twice with 70% (v/v) ethanol and briefly dried DNA pellet in vacuum desiccator. The DNA pellet was then dissolved in an appropriate volume of deionized water and stored at -20ºC until use.

3.10 Bacterial identification by determination of 16s ribosomal DNA gene sequence analysis

Genomic DNA from individual bacteria strains was extracted by Method 3.9. The 16S rDNA gene fragment was amplified from the genomic DNA of each bacteria by polymerase chain reaction (PCR) using the bacterium-specific primer: forward primer (63f) (5' CAGGCCTAACACATGCAAGTC 3') and a reverse primer (1387r) (5' GGGCGGWGTGTACAAGGC 3') (Marchesi et al., 1998). PCR reaction conditions were as follows: 30 cycles of 1 min at 95ºC, 30 sec at 55ºC and 1.3 min at

72ºC. The fragments (1,300 base pairs) were extracted by QIAquick® Gel extraction Kit and then cloned into pGEM®-T Easy vector (Promega, Madison,WI, USA) (3,015 base pairs) and transformed in competent *E. coli* DH5α cells. Recombinant plasmid was isolated using restriction enzyme digestion. The insert in recombinant plasmid was then sequenced by the Macrogen Inc. (Seoul, Korea) and compared to the most similar sequences with Clustal W in NCBI database (http://www.ncbi.nlm.nih.gov).

3.11 Nucleotide sequence analysis

K. pneumoniae GF37j7 isolated in this study was used as source of *dha*B gene cluster, and then its *dha*B gene was sequenced and aligned. The *dha*B gene in pGEM-T easy vector was double digested with *Xho*I-*Eco*RI, *Eco*RI-*Bam*HI and *Eco*RI-*Pst*I and single digested with *Bam*HI and *Pst*I. Each fragment was ligated into pUC118 plasmid and transformed in *E. coli* DH5α. The randomized *dha*B gene fragments were subjected to DNA sequencing using CEQ800 (Backmen coulter). Then the nucleotide sequences were assembled by SEQUENCHER (Gene Codes Cooperation). Homology search analysis and gene fragments alignment were performed using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/).

3.12 Plasmid extraction

The solution was used as follow: **Solution I :** 50 mM glucose, 25 mM Tris-HCl (pH 8.0), 10 mM EDTA (pH 8.0), **Solution II :** 100 µl 1 N NaOH, 100 µl 10% (w/v) SDS and 800 µl deionized water, **Solution III :** 60ml 5M potassium acetate and 11.5 ml glacial acetic acid and 28.5 ml deionized water.

(1) Gram negative bacteria

Plasmids were extracted from *E. coli* DH5α and *P. putida* T-57 using the modified alkaline lysis method or the Fast Plasmid Mini Kit (Eppendrof, USA). For the modified alkaline lysis method, cells were grown in 5 ml LB medium and then collected by centrifugation at room temperature. Solution I (250 μl) was added and resuspended by vortex, followed with Solution II (350 μl) and mixed gently by inversion. The precipitates formed were then removed by centrifugation at 12000 rpm for 15 min. The supernatant was collected and transferred to new eppendrof and equal volume of cold 2-propanol was then added and incubated on ice for 1 h. The plasmid DNA precipitated was collected by centrifugation at 12000 rpm at 4ºC for 15 min. The pellet was washed twice with 70% (v/) ethanol and dried with vacuum desiccators. The plasmid was then dissolved in an appropriate in deionized water and kept at -20ºC until use.

(2) Gram positive bacteria

B. subtilis plasmid was extracted according to the method of Voskuil and Chambliss in 1993 (Voskuil and Chambliss, 1993). Briefly, overnight culture (5 ml of high copy number plasmid and 10 ml of low copy number plasmid) was collected by centrifugation. The pellet was suspended in 200 μl 20 mg/ml lysozyme and incubated at 37ºC for 2 h. Solution II (350 μl) was added and resuspended by vortex, followed with ice-cold Solution II (350 μl) and mixed gently by inversion. The precipitates formed were then removed by centrifugation at 12000 rpm for 15 min. The supernatant was collected and transferred to new microtube and equal volume of cold phenol: chloroform: isoamyl (25:24:1) was then added and mixed by vortex for 10 min. The mixture was then centrifuged at 12000 rpm at 4ºC for 5 min. The upper

layer was collected and equal volume of 2-propanol was added and mixed by inverting. The plasmid DNA precipitated was collected by centrifugation at 12000 rpm at 4ºC for 5 min. The pellet was washed twice with 70% (v/) ethanol and dried with vacuum desiccators. The plasmid was then dissolved in an appropriate in deionized water supplemented with 20µg/ml RNaseA and kept at -20ºC until use.

3.13 DNA manipulation techniques

For restriction enzyme digestion, DNA ligation and other DNA modification were performed according to the supplier's instructions. Polymerase chain reaction (PCR) was carried out in 50 µl of reaction volume using T100™ Thermal Cycler (Bio-Rad, USA). Agarose gel electrophoresis was performed with 0.8-1.5% (w/v) agarose in TAE buffer and DNA extracted from gel was purified using a QIAquick® Gel extraction Kit.

3.14 Preparation of *E. coli* competent cells for heat shock method

Overnight grown culture (200 µl) of *E. coli* DH5α was inoculated into 100 ml sterile LB medium, and then was grown at 37ºC, 200 rpm until OD_{600} reached 0.4-0.6. The culture was incubated on ice for 30 min. The cells were collected by centrifugation at 5000 rpm, 4ºC for 10 min. The pellet was resuspended with equal volume of cold 0.1 M $MgCl_2$ and centrifuged at 5000 rpm, 4ºC for 10 min. A supernatant was discarded then the pellet was resuspended in 10 ml cold 0.1 M $CaCl_2$ and incubated on ice for 30-60 min. After incubation, the cell suspension was centrifuged at 5000 rpm, 4ºC for 10 min. A supernatant was discarded then the pellet was resuspended in 1.0 ml cold 0.1 M $CaCl_2$ and 0.4 ml sterile glycerol. The cell

suspension was aliquot 100µl/tube on ice prior to immediate drop into liquid nitrogen. The competent cells were stored at -80ºC until use.

The plasmid was mixed with cold cell suspension in microtube and place on ice for 30 min. The mixture was incubated at 42ºC for 60 seconds and place on ice for 2 min. The cell suspension was transferred into a new sterile tube containing 1 ml SOC medium. The shocked cells were incubated at 37ºC with shacking condition for 1 h and spreaded onto the LB agar plate containing appropriated antibiotic (ampicillin, 100µg/ml; tetracycline, 10µg/ml; kanamycin, 50µg/ml). The plate was incubated at 37ºC for overnight.

3.15 Preparation of *P. putida* T-57 competent cells for electroporation method

P. putida T-57 competent cells were prepared as described (Faizal et al., 2005). *P. putida* T-57 was grown in LB medium with shaking at 30ºC, overnight. The overnight grown culture was inoculated to 50 ml LB with 250 mM sucrose and incubated at 30ºC with shaking condition. After 3 h cultivation, bacteria culture was incubated on ice for 30 min and harvested cell by centrifugation at 4ºC. Finally, suspended cell in 1.25 ml of ice-cold HSMG buffer (see 3.7).

For electroporation, the competent cell (195 µl) was mixed with plasmid (5 µl), and then incubated on ice for 30 min. Cell suspended was transferred into ice cold 0.2 cm electroporation gap cuvette. The mixture was electroporated with apparatus as follow; 50 µF, 300Ω of the pulse controller unit and 1.5 kV/ cm, time constant 12-13 msec. After that, the electroporated cell was transferred into 4 ml SOC medium and incubated at 30ºC shaking at 200 rpm for 5 h. The generated cells were then spreaded

onto LB plate containing appropriate antibiotic (kanamycin, 50µg/ml) and incubated at 30ºC for overnight.

Determination of proteins, enzymes and products formation

3.16 Protein determination

Protein concentration was determined by a modified Lowry's method with bovine serum albumin (BSA) as the standard protein. The samples were diluted with deionized water to 0.4 and 2.0 ml of fresh mixed solution A and B (A: B, 50:1) (see in 3.7) which were added and rapidly mixed. The mixture was incubated at 30ºC for 10 min. Subsequently, 0.2 ml of solution C was added, rapidly mixed and incubated at 30ºC for 30 min. Finally, the absorbance of clear blue-color solution was measured at 750 nm by UV-Visible spectrophotometer. BSA calibration curve was used to analyze absorbance of protein concentration results as shown in Appendix B.

3.17 β-Galactosidase activity assay

β-Galactosidase activity was determined according to the method previously reported (Rygus and Hillen, 1991). Briefly, cells were grown in LB medium containing an appropriate antibiotic to reach OD_{600} of 1.0. If the induction was needed, the inducer was added when OD_{600} was at 0.3 until OD_{600} of 1.0. Then cells cultures (0.5 ml) were spin and resuspended with Z-buffer (see in 3.7) with the same volume (measure OD_{600}) and permeabilized with toluene (2%v/v). The samples were incubated at 30ºC for 10 min before ONPG solution addition (the reaction was started) and continuous incubated at 30ºC for 30 min. and stopped the reaction after sufficient yellow color. After that, 0.25 ml 1M Na_2CO_3 was added and vortex for 5

min. to remove debris and toluene. Recorded the absorbance at 420 nm and 550 nm for each tube and calculated the units of activity using equation shown below:

Miller Units = [OD420- (1.57 x OD550) / OD600 x Time x volume] x 1000

3.18 Glycerol dehydratase activity assay

Glycerol dehydratase activity was determined according to the method of Raj, 2008 (Raj et al., 2008) with colorimetric method that can be monitored by a UV-Visible spectrophotometer with an absorbance at 560 nm. Cells were grown in LB containing an appropriate antibiotic to OD_{600} of 1.0. If the induction was needed, the inducer was added when OD_{600} was at 0.4-0.6 until OD_{600} of 1.0 then cells were harvested with centrifugation at 5000 rpm, 4ºC for 15 min. Cell pellets were washed twice with 35 mM phosphate buffer pH 8.0 and resuspended in the same buffer pH 8.0 supplemented with 50 mM KCl. Resuspended cells were disrupted using sonicator for 5 times with power 10, 5 sec for 2 cycles. The cell debris was then removed by centrifugation at 12,000 rpm at 4ºC for 15 min. The obtained supernatant was designated as intracellular cell-free extracts for glycerol dehydratase activity. Glycerol dehydratase enzyme activity reaction was initiated by adding 50 mM glycerol and 15 μM coenzyme B12 at 37ºC for 10 min. The reaction was terminated after 10 min by adding an equal volume of 100 mM citric acid. 3-HPA formation from reaction mixture was determined by the method 3.19.

3-HPA formed in the reaction mixture was dehydrated to acrolein, which reacts subsequently with tryptophane to form a purple complex that can monitor by UV-Visible spectrophotometer with absorbance at 560 nm. Since 1 mol of 3-HPA was dehydrated to 1 mol of acrolein, the absorbance data were expressed in terms of 3-

HPA concentration. One unit of glycerol dehydratase activity was defined as the amount of enzyme required to form 1 µmol of 3-HPA/min.

3.19 Detection of 3MC product

Qualitative and quantitative analyses of 3MC production were performed using High performance Liquid Chromatography (HPLC). Aqueous phase or organic phase samples were harvested to remove cells by centrifugation and mixed with acetonitrile at ratio 1:10 (v/v) then filtered through with 0.45 µm of nylon syringe filter.

The prepared samples were analyzed by a reversed phase HPLC with a UV detector at a wavelength of 240 nm. The separation was performed on Hyperclone 5u BDS C18 HPLC column using a mixture of acetonitrile: water (70:30, v/v) as a mobile phase with a flow rate 1 ml/min. The injection volume was 20 µl. The retention time of 3MC under the condition test was 2.9-3.0 min.

3MC calibration curve was used to analyze chromatogram of HPLC result in order to calculate the concentration of product as shown in Appendix C.

3.20 Detection of 3-HPA product

3-HPA formation was determined by colorimetric method from reaction of tryptophane and acrolein dehydrated from 3-HPA, which presented in a purple complex (absorbance at 560 nm). To investigate 3-HPA formation in bacterial culture, samples (3 ml) were added with concentrate HCl (6 ml) and tryptophane solution (1.5 ml), and then incubated at 40ºC for 20 min. After reaction, samples were centrifuged for cells removing and the supernatant was measured at absorbance 560 nm. To

calculate 3-HPA concentration, an absorbance 560 nm was used to compare with acrolein standard curve as shown in Appendix D.

Hydrophobic organic solvent of 3-methylcatechol (3MC) bioproduction from OST bacteria using a two-phase system

3.21 3MC bioproduction from toluene by OST Gram negative *P. putida* T-57 mutant strain TODE1

3.21.1 Preparation of *P. putida* TODE1 for 3MC production

P. putida TODE1 cell preparation for 3MC production was determined according to the method previously reported (Faizal et al., 2007). Briefly, TODE1 cells grown in LB medium at 30ºC for overnight with shacking condition (200 rpm) were inoculated into MSB medium supplemented with 2 g/l glucose. Cell culture was incubated at 30ºC, 200 rpm until OD_{600} of 0.5, toluene was added with vapor phase for cells induction and continuous incubated at the same condition to OD_{600} of 1.0. Cells were harvested by centrifugation and washed twice with saline solution and re-suspended with 10 ml MSB medium appropriate some nutrients and various pH. This cells preparation was used for 3MC production in this research.

3.21.2 Production of 3MC with a single-phase and a two-phase system

(1) A single-phase system: *P. putida* TODE1 cells in MSB medium pH 7.0 obtained from the method 3.21.1 added with toluene (production substrate) and 1-butanol (cofactor substrate) in the vapor phase and incubated at 30ºC, 150 rpm for 22 h. After 22 h, 3MC was determined according to the method 3.19.

(2) A two-phase system: cells in MSB medium pH 7.0 obtained from the method 3.21.1 added with toluene and 1-butanol as vapor phase, and 1 ml of 1-decanol was directly added as organic phase. Subsequently, they were incubated at 30ºC at 150 rpm. After 22 h, 3MC was determined from aqueous phase and organic phase according to the method 3.19.

3.21.3 Optimization of 3MC production from different conditions with a two-phase system

To investigate the optimum conditions for 3MC production, effects of agitation speed pH and temperature were investigated in two-phase system of 1-decanol with different condition as follows:

(1) Effect of agitation speed. Cells prepared as described 3.21.1 in MSB were tested at medium pH 7.0 in the presence of toluene and 1-butanol as vapor phase and 1 ml of 1-decanol was as an organic phase. The mixture was incubated at 30ºC for 22 and 50 h before 3MC production was analyzed by HPLC. The effect of agitation speed was evaluated at agitation speed of 150, 200 and 280 rpm.

(2) Effect of pH. Cells from 3.21.1 in MSB medium were tested at various pH (6.5, 7.0, 7.5 and 8.0) in the presence of toluene and 1-butanol as vapor phase and 1 ml of 1-decanol was as an organic phase. The mixture was incubated at 30ºC with 150 rpm for 22 and 50 h.

(3) Effect of temperature (ºC). Cells from 3.21.1 in MSB medium pH 7.0 were tested in the presence of toluene and 1-butanol as vapor phase and 1 ml 1-decanol was as an organic phase. The mixture was incubated at various temperatures of 28, 30, 35 and 37ºC with 150 rpm for 22 and 50 h.

3MC formations from aqueous and organic phases were determined according to the method 3.19.

3.21.4 Phase ratio of aqueous and organic phase of 1-decanol on 3MC production

TODE1 cells in MSB medium was tested in the presence of toluene and 1-butanol as vapor phase and 1-decanol as direct addition with different volume ratio of 10:1, 10:3 and 10:5. The mixture was incubated at 30ºC shacking with 200 rpm for 22 h and 50 h before 3MC production was analyzed by HPLC from aqueous and organic phases by according to the method 3.19.

3.21.5 Effect of addition of toluene and 1-butanol on 3MC production in a two-phase system

To investigate the effect of addition of production substrate (toluene) and growth substrate (1-butanol) on 3MC production in two-phase system of *P. putida* TODE1, supplementation of toluene and 1-butanol at various concentrations were determined. The cell culture was prepared in MSB medium pH 7.0 supplemented with 1 ml of 1-decanol, and directly added at various condition of toluene (vapor, 1, 5, 10, 20, 40, 47 and 60 mM), and 1-buatanol (vapor, 5, 10 and 50 mM). The supplementation of toluene and 1-butanol as vapor were used as control to compare with directly addition. Cell culture was incubated at 30ºC shaking with 200 rpm for 22 h and 50 h. 3MC formations from aqueous and organic phase were determined after 22 h according to method 3.19.

3.21.6 Effect of addition of some nutrients and divalent ions on 3MC production in a two-phase system

To investigate the effect of addition of some nutrients and divalent ions on 3MC production in a two-phase system of *P. putida* TODE1, supplementation of various concentrations of nutrients and divalent ions were determined. The cell culture was incubated at 30ºC shacking with 200 rpm for 22 h and 50 h under toluene and 1-butanol as vapor and 1 ml of 1-decanol, the MSB medium containing different nutrients and divalent ions as following.

Addition of nutrients: glucose (3, 6 and 11 mM), glycerol (3, 6 and 12 mM), crude glycerol from biodiesel (3, 6 and 12 mM), succinate (5, 10 and 20 mM) and acetate (5, 10 and 20 mM).

Addition of divalence ions: $MgCl_2$ (5, 10 and 20 mM), $CaCl_2$ (1, 5 and 10 mM), $MnCl_2$ (0.01, 0.025 and 0.05 mM) and $FeSO_4$ (0.05, 0.1 and 0.5 mM). 3MC formations from aqueous and organic phase were determined after 22 h according to method 3.19.

3.21.7 Optimization of 3MC production under two-phase system by central composite designs (CCD) and response surface methodology (RSM)

The optimum nutrients and divalent ions provided highly 3MC production yield by *P. putida* TODE1 from the method 3.21.6 were investigated in this study. The results showed that the major variable influencing the 3MC production were glycerol, $MnCl_2$ and $FeSO_4$. Then these components were chosen for RSM. RSM-based CCD was employed to determine the optimal conditions of the pre-experiments to improve 3MC production. The level of each factor and the sign matrix

are given in Table 3.3 and 3MC production was then investigated. The low, medium and high levels of each variable were designated as -1, 0 and 1, respectively (Table 3.1).

Table 3.1 The CCD design and response surface methodology

Order	Type	$MnCl_2$ (mM)	$FeSO_4$ (mM)	Glycerol (mM)
1	1	0	0	0
2	1	0.05	0	0
3	1	0	0.4	0
4	1	0.05	0.4	0
5	1	0	0	8
6	1	0.05	0	8
7	1	0	0.4	8
8	1	0.05	0.4	8
9	-1	0	0.2	4
10	-1	0.05	0.2	4
11	-1	0.025	0	4
12	-1	0.025	0.4	4
13	-1	0.025	0.2	0
14	-1	0.025	0.2	8
15	0	0.025	0.2	4
16	0	0.025	0.2	4
17	0	0.025	0.2	4
18	0	0.025	0.2	4
19	0	0.025	0.2	4
20	0	0.025	0.2	4

Hydrophilic organic solvent of 3-hydroxypropionaldehyde (3-HPA) bioproduction from OST bacteria using a single-phase system

- **Screening and characterization of hydrophilic OST bacteria as host cell for 3-HPA production**

3.22 Screening and isolation of OST bacteria

Soil, water and sea water samples were taken from contaminated area, hot spring area, agricultural area, activated sludge and natural area in vary province, Thailand. Approximately 5 g of samples were incubated in a 50-ml screw-capped vial containing each organic solvent in a small tube with each organic solvent: log P_{ow} > 2.0 (toluene (2.5), styrene (3.0) and cyclohexane (3.2)) and with directly addition (0.1%, v/v): log P_{ow} < 2.0 (butaraldehyde (0.88), butanol (0.8) and propionaldehyde (0.54)) for 1 week at 30 ºC, 37 ºC, 45ºC. After acclimation, 1 g of samples was dispersed in 4 ml of LB medium supplemented with 0.1% (v/v) each organic solvent as mention before and incubation at various temperatures for overnight. Serial dilutions of samples culture were plated out on LB plates. Colonies obtained were reinoculated into the LB medium supplemented with 0.1% (v/v) various organic solvent to confirm the tolerance.

To isolate OST bacteria as a host cell for 3-HPA production, bacteria from propionaldehyde enrichment and other enrichment solvent were investigated for propionaldehyde tolerance. Cells were grown in LB medium to a mid-log phase before addition of propionaldehyde at various concentrations (1% - 3% (v/v)), incubate until 6 h at 37ºC with shacking condition. Cell survivals as CFU/ml were used for determined cells tolerance to propionaldehyde.

3.23 Characterization of hydrophilic OST Gram positive bacteria

3.23.1 Propionaldehyde tolerance

Hydrophilic OST Gram positive bacteria was grown until the mid-log phase at 37°C in 20 ml LB medium, after that 1% to 30 % (v/v) of propionaldehyde was directly added into the bacterial culture. Cells were incubated in the presence of the organic solvent for 6 h and cell survival was determined as colony-forming units (CFUs) per milliliter on LB agar plate.

3.23.2 Pure glycerol and crude glycerol from biodiesel utilization

Cell utilization of glycerol was demined by 2 methods; 1) Cells ability to utilize pure glycerol was carried out in MSB medium pH 7.0 supplemented with various concentrations of glycerol at 37ºC with shaking condition. Cell growth was measured with optical density at 600 nm OD_{600}; 2) Cell ability to utilize and tolerance crude glycerol from biodiesel was monitored by measuring of cell survival (CFUs).

- **Development of a transformation system and gene manipulation method for the selected OST bacterium**

3.24 Development of a transformation system and gene manipulation method for the selected Gram positive OST bacterium as a gene expression host

3.24.1 Transformation of hydrophilic OST Gram positive bacteria

The selected isolates were grown in LB medium at 37°C at different stages of growth monitored by OD_{600} (i.e. 0.3-0.4 for early-exponential phase; 0.6-0.7 for mid-exponential phase and 0.9-1.0 for late-exponential phase). Cells were hold on ice for 30 min before harvesting, and washed four times with ice-cold electroporation media

(sterile distilled water, glycerol solution [10% v/v] followed by EM solution or EP solution buffer, and concentrated cells to 150-fold (Turgeon et al., 2006). Then, competent cells (100 µl) were mixed with pHY300PLK plasmid DNA at various concentrations (100, 200, 500, and 1000 ng/µl) and kept on ice for 30 min. Electroporation was performed in 2-mm gapped electroporation cuvette at 50 µF, 300 Ω with various pulse strengths (1-12 kV/cm) using Electro Cell Manipulator. Pulsed cells were immediately diluted with 1 ml of SOC medium and incubated during recovery period with shaking (120 rpm) for 3 h before spreading on LB medium agar plate supplemented with tetracycline (10 µg ml-1) or kanamycin (5 µg ml-1) at 37ºC.

3.24.2 Promoter activity test

To select promoter with different strength (activity) for gene construction of 3-HPA production, several promoters (Kataoka et al., 2012) were investigated in *B. subtilis* BKS2-P2/3. Plasmid pHY300PLK derivative containing each promoter::*lac*Z transcription was transformed into *B. subtilis* BKS2-P2/3 according to method 3.25.1. Cells were grown to a late-exponential phase and OD600 of ~1.0, harvested, washed with EP solution and electrophorated with the following conditions: 50 µF, 300 Ω with pulse strengths 1-2 kV/cm. β-galactosidase activity was determined from recombinant *B. subtilis* BKS2-P2/3 by the method 3.17. High promoter activity was selected for further study.

Table 3.2 The promoter used in this study (Kataoka et al., 2012)

Promoter	Sequence source	Name/Definition & Functioning source	
P2N	pNCMO2	P2 promoter	- functions in *Brevibacillus* sp.
P2L	pHY300PLK	A temperature inducible	- functions in *Bacillus subtilis*
Tet	pUC4K	Tetracycline promoter	- functions in *Bacillus* sp.
Km	pDG148	Kanamycin promoter	- functions in *Rhodococcus* sp.
Spac	*Bacillus subtilis*	*Bacillus subtilis*	
Xyl	pNCMO2	*Bacillus megeterium*	
43	pWH1520	*Bacillus subtilis*	

• **3-HPA bioproduction from glycerol by OST Gram positive *B. subtilis* BKS2-P2/3 and Gram negative *P. putida* T-57 using a single-phase system**

3.25 Screening and isolation of 3-HPA producing bacteria for source of gene

Primary screening: Soil samples from various natural areas in Thailand were mixed with MSB medium containing of 2% (w/v) glycerol and then incubated at 37ºC with shaking and static conditions. Serial dilution of samples cultures were spreaded on MSB containing of 2% (w/v) glycerol plate. Colonies obtained were kept in glycerol stock at -80ºC.

Secondary screening: To screen aldehyde and 3-HPA producing bacteria, bacterial isolated from method 3.21 were grown in MSB medium supplemented with 2% (w/v) glycerol for 37ºC overnight. For aldehyde producing bacteria, 1 ml of bacterial culture was mixed with 0.5 ml Fehling solution and boiling at 95ºC for 5 min. Aldehyde formation was determined from the demonstrated color of reaction,

red or green color mean the presence of aldehyde whereas a clear or slightly blue color meant aldehyde did not present.

For 3-HPA producing bacteria, bacterial cultures were investigated according to the method 3.20.

3.26 Cloning of *dha*B genes cluster for production of 3-HPA by OST *B. subtilis* BKS2-P2/3

3.26.1 Amplification of *dha*B genes for production of 3-HPA by OST *B. subtilis* BKS2-P2/3

Amplify a *dha*B cluster (4.5 kb) from genomic DNA of *K. pneumoniae* GF37j7, *K. pneumoniae* ATCC13883 and *C. butyricum* ATCC13983 with the primer (Table 3.3). The forward primer and the reverse primer contained restriction size of *Kpn*I and *Eco*RI, respectively, which contained various types of Ribosome Binding Site (RBS). PCR reaction conditions were as follows: 30 cycles of 30 sec at 95ºC, 30 sec at 55ºC and 3 min at 68ºC with KOD-plus neo (Toyobo, Japan) as DNA polymerase.

3.26.2 Construction of an expression vector containing *dha*B genes cluster for production of 3-HPA in OST *B. subtilis* BKS2-P2/3

To construct an expression vector containing *dha*B cluster using a backbone and promoter from pHYPxyl for production of 3-HPA by selected OST *B. subtilis* BKS2-P2/3, each *dha*B fragment from various types of RBS was ligated in the pHYPxyl with *Kpn*I and *Eco*RI restriction size (referred as pHPxyl-KdhaB to pHPxyl-K2dhaB).

Table 3.3 Forward and reverse primers used in this study.

Primers	Sequence
***dha*B gene sequencing**	
dhaB-F	
dhaB-R	
K-dhaB gene forward and reverse primers	
KdhaB-F1	GCGGTACC**AGGAGG**ACAGCT**ATG**AAAAGATCAAAACGATTTGCAGTACTG
KdhaB-F2	CG GGTACC AAA**GCGTGGTG**TTTTT**ATG**AAAAGATCAAAACGATTTGC
KdhaB-F3	CG GGTACC AAA**GCGTGGTG**TTTTTTT**ATG**AAAAGATCAAAACGATTTGC
KdhaB-F4	GC GGTACC **GAGC**CGATGAACA**ATGA**AAAGATCAAAACGATTTGCAGTA
KdhaB-F5	GCGGTACC**AGGAGG**CGATGAACA**ATG**AAAAGATCAAAACGATTTGCAGTA
KdhaB-F6	GC GGTACC **GGAGG**CGATGAACA**ATG**AAAAGATCAAAACGATTTGCAGTA
KdhaB-F7	GC GGTACC **GGAA**CGATGAACA**ATG**AAAAGATCAAAACGATTTGCAGTA
KdhaB-F8	GC GGTACC **GGAG**CGATGAACA**ATG**AAAAGATCAAAACGATTTGCAGTA
KdhaB-F9	GC GGTACC **GAGG**CGATGAACA**ATG**AAAAGATCAAAACGATTTGCAGTA
KdhaB-F10	GC GGTACC **AGGA**CGATGAACA**ATG**AAAAGATCAAAACGATTTGCAGTA
KdhaB-F11	GC GGTACC **AAGG**CGATGAACA**ATG**AAAAGATCAAAACGATTTGCAGTA
KdhaB-R	CC GAATTC TTAATTCGCCTGACCGGCCAGT
***orfX* gene forward and reverse primers**	
orfX-F	GAAGAATTCGAGGGGACCGTCATGTCGCTTTCACCGCCAG
orfX-R	GCGTCTAGATCAGTTTCTCTCACTTAACGGC

Underlined nucleotides indicate the *Kpn*I, *Eco*RI and *Xba*I restriction enzyme site, respectively. Bold underlined and bold nucleotides indicate the RBS sequence and the translational start codon.

3.27 Gene expression and product formation determination in recombinant OST *B. subtilis* BKS2-P2/3

3.27.1 Recombinant OST *B. subtilis* BKS2-P2/3 gene expression

The recombinant of *B. subtilis* BKS2-P2/3 was grown in LB medium at 37ºC shaking at 150 rpm. The culture was induced with different concentration of IPTG (0. 0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 mM) at 0.4-0.6 OD_{600}. After 4h of induction, cells were harvested and centrifuged at 4ºC. The cell pellets were washed twice with 35 mM potassium phosphate buffer (pH 8.0) and resuspended in 35 mM of the same solution supplemented with 50 mM KCl. The cells were disrupted using sonicator at 10 sec,

10% power for 2 cycles. The cell lysates were centrifuged at 4ºC and the supernatants were used for SDS-PAGE (see 3.16) and analyzed an enzyme activity (see 3.18).

3.27.2 3-HPA production from glycerol by OST *B. subtilis* BKS2-P2/3

To determine 3-HPA production from recombinant *B. subtilis* TBKS2-P2/3, recombinant cells were cultured in 100 ml BS medium at pH 8.0 supplemented with 500 mM glycerol at shacking condition until the density of cells reached approximately to OD_{600} of 0.6-0.9. Grown cells were harvested and resuspended in 20 ml of the same medium. 3-HPA production was initiated by adding 48 μM of co-enzyme B12 and 0.1 mM of IPTG and then incubated at 37ºC under shacking condition. The 3-HPA assay was determined base on the colorimetric method (see 3.20).

3.28 3-HPA bioproduction from glycerol by OST Gram negative *P. putida* T-57

3.28.1 Amplification of *dha*B genes and its reactivator for production of 3-HPA by OST *P. putida* T-57

To clone *dha*B genes (*dha*B1, *dha*B2, *dha*B3 and *orf*Z) and *orf*X, the *K. pneumoniae* GF37j7 and ATCC13883 genomic DNA were used as a source of genes. The *dha*B genes containing various types of RBS (referred as K-dhaB) were amplified from *K. pneumonia* GF37j7 and the orfX gene was amplified separately with its native RBS because of location in the reverse orientation to *dha*B genes cluster in the *K. pneumoniae* genome. Primers were used in this work as show in Table 3.3. PCR reaction conditions were as follows: 30 cycles of 20 sec at 94ºC, 30 sec at 60ºC and 4.5 min at 68ºC with long taq polymerase (Fermentus).

3.28.2 Construction of an expression vector containing *dha*B genes cluster for production of 3-HPA in OST *P. putida* T-57

To express of *dha*B and *orf*X, the pUCP18 or pMEPT vector with lac and tac promoters were used in this study. Details about the construction of plasmids are shown in Figure 3.1. The KdhaB to K10dhaB fragment with *Kpn*I and *Eco*RI restriction sites, and the *orf*X fragment with *Eco*RI and *Xba*I restriction sites were ligated in the pBluescript II SK(-) plasmid (referred as pBSKdhaB to pBSK10dhaB and pBS-orfX respectively).

pBSKdhaB to pBSK10dhaB were digested with *Kpn*I and *Eco*RI and the 4.5 kb fragment and sub-cloned into pUCP18 or pMEPT plasmid. The obtained plasmid was referred to as pUKdhaB to pUK10dhaB and pMKdhaB to pMK10dhaB, respectively. pBS-orfX was digested with *Eco*RI and *Xba*I and the ~0.36 kb fragment was sub-cloned into pUKdhaB to pUK10dhaB and pMKdhaB to pMK10dhaB. The obtained plasmid, containing *dha*B123::*orf*Z and *orf*X in the pUCP10 and pMEPT, are referred to as pUKdX to pUK10dX and pMKdX to pMK10dX, respectively.

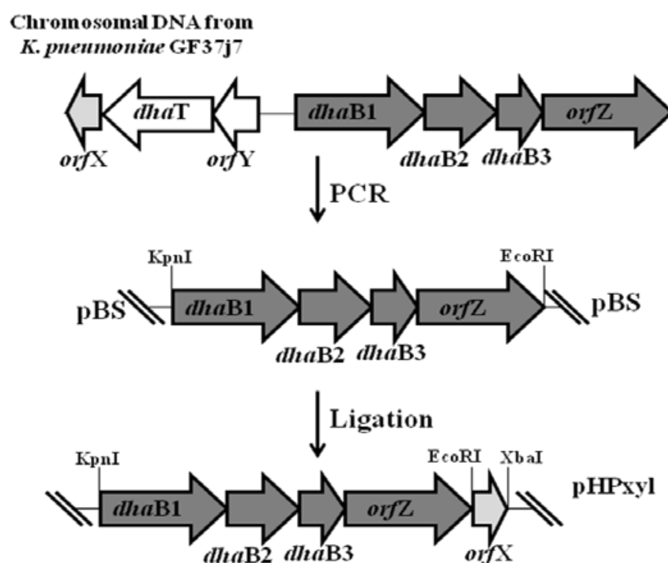


Figure 3.1 Construction of an expression vector for OST *P. putida* T-57

3.28.3 Protein expression and determination of glycerol dehydratase enzyme activities of *P. putida* T-57 recombinant

The *P. putida* T-57 recombinant strains were grown in LB medium at 30ºC shaking at 150 rpm. The cultures were induced with different concentrations of IPTG (0. 0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 mM) at 0.4-0.6 OD_{600}. After 4 h of induction, cells were harvested and centrifuged at 4ºC. The cell pellets were washed twice with 35 mM potassium phosphate buffer (pH 8.0) and resuspended in 35 mM in the same solution supplemented with 50 mM KCl. The cells were disrupted using a sonicator at 10 sec, 10% power for 2 cycles. The cell lysates were centrifuged at 4ºC and the supernatants were used for SDS-PAGE (see 3.16). Enzyme activities were analyzed (see 3.18).

2.28.4 3-HPA production from glycerol by recombinant *P. putida* T-57 in shake-flask condition

To determine the potential of the recombinant strains for 3-HPA production, recombinant *P. putida* T-57 was cultured in 100 ml BS medium at pH of 8.0 supplemented with 500 mM glycerol at shaking condition until the density of cells approximately reached to OD_{600} of 0.4-0.6. Grown cells were harvested and resuspended in 20 ml of the same medium. 3-HPA production was initiated by adding 48 µM of co-enzyme B12 and 0.1 mM of IPTG and incubated at 37ºC under shaking condition. The 3-HPA assay was determined base on the colorimetric method (see 3.20).

- **Characterization of hydrophilic OST bacteria**

3.29 Characterization of OST Gram positive marine bacterium, *Exiguobacterium* sp. SBH81

3.29.1 *Exiguobacterium* sp. SBH81 growth characteristic

To investigate *Exiguobacterium* sp. SBH81 growth characteristic, it was grown in LB medium under various conditions, i.e. at pH of 3 to12, temperature of 5 to 60ºC and salinity of 0 to13% (w/v) of NaCl.

3.29.2 Solvent tolerance and utilization of *Exiguobacterium* sp. SBH81

To further analyze the OST properties of these strains, cell survival and optical density at 600 nm (OD_{600}) were measured after the addition of various log P_{ow} value of organic solvents with two methods.

(1) Cells were grown until the mid-log phase at 37 °C in 20 ml LB medium after that 5% to 10 % (v/v) of each compound of organic solvent was directly added in the bacterial culture. Cells were incubated in the presence of organic solvent for 12 h and cell survival was determined as colony-forming units (CFUs) per milliliter.

(2) To test cell tolerance to solvent under growing condition, acetonitrile at various conditions (1% - 25% (v/v)) was added together with cells culture in MSBYG medium at 37ºC with shacking condition. Cell growth was determined as cell density at OD_{600}.

Cell ability to utilize acetonitrile was determined in MSB medium supplemented with acetonitrile vapor at 37ºC with shacking condition. Cell growth was monitored by measuring OD_{600}.

3.29.3 *Exiguobacterium* sp. SBH81 acetonitrile adaptation

Cells were grown at 37°C for 12 h in MSBYG medium supplement with 4% (v/v) acetonitrile and reinoculated several times (10, 20 and 30 cycles) in the same condition to obtain acetonitrile adapted cells. After that, adapted cells were determined the ability of tolerance and utilization of acetonitrile at 37°C in MSBYG and MSB medium, respectively.

3.29.4 Efflux pumps inhibitors (EPI) examination

To investigate effect of EPI on acetonitrile tolerance of *Exiguobacterium* sp. SBH81, non-adapted and acetonitrile adapted cells were cultivated at 37°C in the MSB medium supplemented with acetonitrile vapor without and with each EPI: 10

mM orthovanadated, 0.1 mM paroxitine and 0.1 mM PAßN. Cell growth was measured with optical density at 600 nm OD_{600}.

3.29.5 *Exiguobacterium* sp. SBH81 cells morphology

Morphology of non-adapted and adapted *Exiguobacterium* sp. SBH81 (method 3.29.3) were investigated using scanning electron microscope (SEM) (JSM-5900, JEOL, Japan).

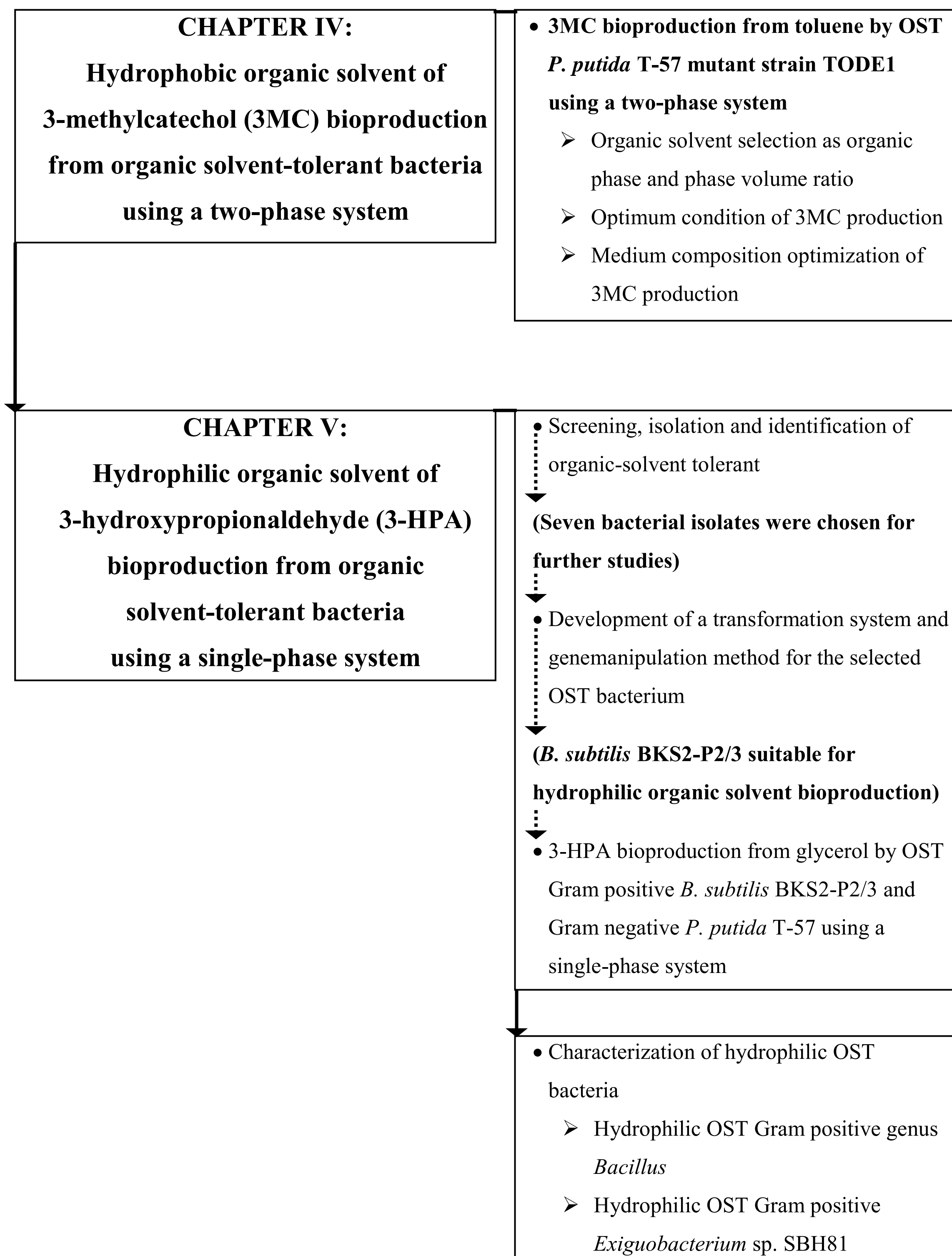
3.29.6 BATH assay

Adapted and non-adapted *Exiguobacterium* sp. SBH81 cells were grown in MSBYG medium without and with 5% (v/v) acetonitrile at 37°C, shaking at 180 rpm for 12 hours. Cells culture were harvested by centrifugation and washed twice with 0.85% (w/v) NaCl after that it was resuspended in same solution until OD_{600} rich to 1.0. Cells cultures were mixed with *n*-tetradecane (5:1 (v/v)) for 30 sec and stand for 20 min. $OD6_{00}$ of aqueous phase was determined by measuring at 0 h (A0) and after 24 h (A1). Cells' adhering was calculated with equation as follows;

$$\text{Cells adhering (A)} = [(A0-A1)/A0] \times 100$$

Conceptual framework of results and discussion

The results and discussion of this dissertation presented in order as shown in the following flow chart.



CHAPTER IV

RESULTS AND DISCUSSION

ON

Hydrophobic organic solvent of 3-methylcatechol (3MC) bioproduction from organic solvent-tolerant bacteria using a two-phase system

Contents

4.1 Solvent selection for enhance 3MC production in a two-phase system

4.2 Effect of phase ratio of aqueous and 1-decanol as an organic phase on 3MC production

4.3 Optimum condition of 3MC productions

4.4 Influence of various type of compound on 3MC production

4.5 Central composite design (CCD) and response surface methodology (RSM) for 3MC production

4.6 3MC production from toluene by TODE1 strain in optimized medium

Hydrophobic organic solvent bioproduction of 3-methylcatechol (3MC) is possible by the conversion of aromatic hydrocarbons such as toluene into catechols, which substrate toluene and product 3MC directly affected to the bacteria producer growth. Therefore, *P. putida* T-57 exhibited high tolerance to various types of organic solvent with log P_{ow} range from 0.8 to 5.6 was used in this study. According to the report of Faizal *et.al* (Faizal et al., 2007), *P. putida* T-57 mutant strain TODE1, which lack of *tod*E gene (Figure 4.1) was carried out accumulation of 3MC from toluene under simple a two-phase system. To enhance the production of 3MC from toluene, several strategies have been widely used such as establishing mathematical model, optimizing fermentative operating condition. In this study the optimizing fermentative condition and suitable fermentation medium on 3MC production from toluene were investigated in Gram negative OST *P. putida* TODE1.

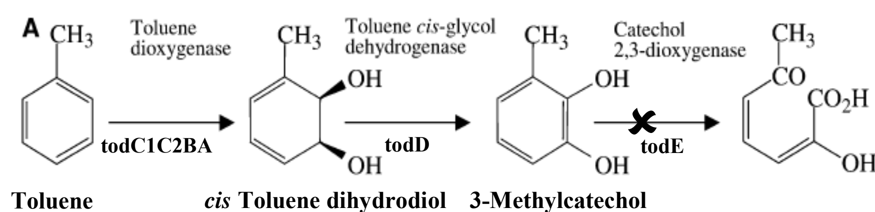


Figure 4.1 3MC production pathways from *P. putida* TODE1

4.1 Solvent selection for enhance 3MC production in a two-phase system

3MC production in a single phase system by *P. putida* was previously studied and only a few productivity (14 mM) was obtained (Hüsken et al., 2001). 3MC at low concentration (5 mM) and substrate toluene (2.5 mM) affected to bacteria producer growth. Production of 3MC in a two-phase system was investigated and found that

3MC production was higher than that in the a single phase system (Wery et al., 2000). This indicated that a two-phase system is likely to play the important role on 3MC production from toluene. The second phase system acts as a reservoir, absorbing excess concentration of toxic product/substrate from the aqueous phase. Examples of organic solvent that were used for second phase system for 3MC production are shown in Table 4.1.

To reduce the toxicity of product 3MC and substrate toluene toward bacteria producer, solvent selection as an organic phase was investigated in this dissertation. The organic solvent in this dissertation was selected from the property as described in the Table 4.1 (i.e. ester group that are low log *P* value (butylbutyrated), and aliphatic alcohol that are high log *P* value (oleyl alcohol with 1-butanol and 1-decanol with 1-butanol). The results showed that, the highest 3MC production was produced from a two-phase system of 1-decanol with 1-butanol, oleyl alcohol with 1-butanol, and butylbutyrate with 1-butanol, respectively (Figure 4.2).

The low log *P* value (2.8) and aliphatic alcohol of 1-octanol investigated as an organic phase for 3MC production by *P. putida* according to Husken in 2001 and Wery in 2000 (Husken E. Leonie et al., 2001; Wery et al., 2000). Moreover, Faizal *et al.* (Faizal et al., 2007) found that 1-octanol significantly affected to toluene dioxygenase activity (TDO) of *P. putida* TODE1. Therefore, butylbutyrate with same log *P* value of 1-octanol (2.8) but containing ester group was investigated to determine the effect of low log *P* value on 3MC production from toluene by strain TODE1. The results showed that the production of 3MC from toluene by *P. putida* TODE1 was decreased. It was suggested that low log *P* value might not be suitable as an organic phase of 3MC production under a two-phase system.

In case of high log *P* value (>4.0) and long chain alkanes, it was found that 3MC did not partition to the organic phase whereas the high log *P* value (>4.0) and polar functional group of ester such as BES and DEHP demonstrated the partitioning of 3MC to them which in accordance with Prpich and Daugulis's report (Prpich P.G. and Daugulis A.J., 2007), however noted that they are hazardous compounds. In addition, the high log *P* value (>4.0) and aliphatic alcohol such as oleyl alcohol (7.7) showed high 3MC production by TODE1 strain (Faizal et al., 2007). In this study, aliphatic alcohol 1-decanol with log *P* value of 4.0 produced high concentration of 3MC from toluene by TODE1 strain. It was suggested that 1-decanol was suitable as an organic phase for 3MC production from toluene of TODE1 strain. Then it was selected for further study.

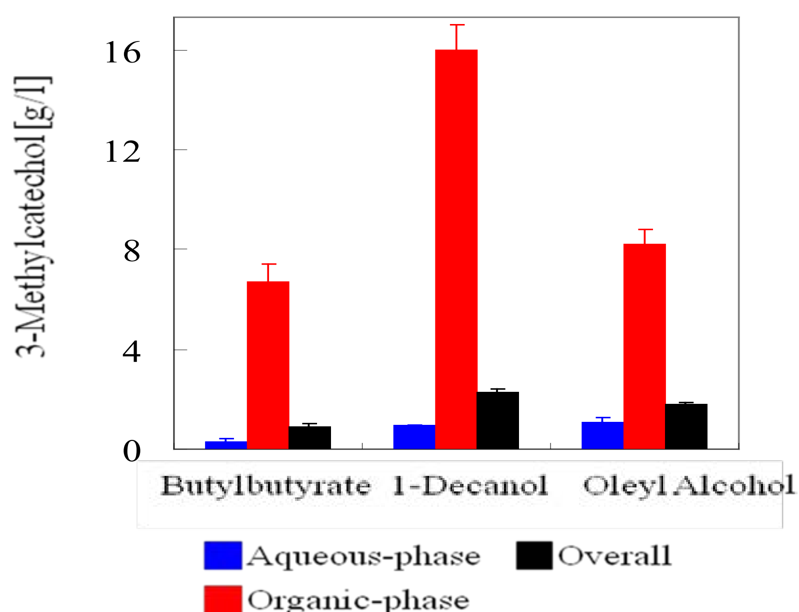


Figure 4.2 The 3MC production from toluene in a two-phase system of strain TODE1 under various type of organic solvent.

Table 4.1 Characteristics for solvent selection as organic phase in a two-phase system of 3MC production from toluene by *P. putida*.

Solvents	Log P_{ow}	Property	Description	Reference
1-octanol	2.8	Aliphatic alcohol	3MC production with aqueous/1-octanol from toluene by *P. pudida*	(Husken E. Leonie et al., 2001; Wery et al., 2000)
1-octanol	2.8	Aliphatic alcohol low log P_{ow}	Control with previous report and low log P_{ow}	(Prpich P.G. and Daugulis A.J., 2007)
1-dodecanol	4.7	Aliphatic alcohol, log P_{ow} higher than 1-octanol	Possesses log *P* value sufficiently high enough to support biological system, but high melting point (24ºC) (difficult to work on it, then not selected)	
bis (2-ethylhexyl) sebacate (BES)	10	Esters, polar functional group and high log *P* values	More favorable log *P* value for use in biological system. Not carbon/energy source for *P. putida*	
bis (2-ethylhexyl) phthalate (BEHP or DEHP)	8.4		Absorb 3MC, but	
silicone oil	-	Effective immiscible liquid in two-phase systems	Did not have 3MC partitioning	
decane	5.2	Alkanes, brancing or chain length would affect affinity for 3MC and based on high log P_{ow}	Did not absorb towards 3MC	
iso-octane	4.0			
dodecane	6.2			
1-dodecane	6.1			
1-octanol	2.8	Aliphatic alcohol, low log *P* value	Affect to TDO activity of mutant strain TODE1	(Faizal et al., 2007)
1-butanol + oleyl alcohol	7.7	Aliphatic alcohol, High log *P* value	Low effect to TDO activity and improve 3MC production, melting point 13-19 °C	
butylbutyrate	2.8	Ester group, low log *P* value	Affect to TDO activity of mutant strain TODE1	This study
1-butanol + 1-decanol	4.0	Aliphatic alcohol, High log *P* value	Good 3MC uptake, high log *P* value, use for carbon & energy source for *P. putida* TODE1, low melting point (6.4ºC)	
1-butanol + oleyl alcohol	7.7	Aliphatic alcohol, High log *P* value	Control with publication and high log P_{ow}	

4.2 Effect of phase ratio of aqueous and 1-decanol as an organic phase on 3MC production

To improve overall 3MC production by a two-phase system, the effect of aqueous phase/organic phase (1-decanol) ratio was investigated. The aqueous phase/1-decanol ratios were varied from 2 to 10 (v/v) (aqueous phase: 1-decanol phase ratio of 10:1, 10: 3 and 10:5). The results showed that the phase ratio of 10:1 clearly provided the highest overall 3MC production followed by the ratio of 10:3 and 10:5, respectively (Figure 4.3). This might have been caused by the toxicity towards TODE1 due to high volume of 1-decanol. Moreover, the higher volume of organic phase might obstruct the O_2 diffusion to aqueous phase that affected to 3MC production by TODE1.

The effect ratio of aqueous and organic phase depended on production condition, organic solvent as organic phase such as high production of 3MC under phase ratio 8: 2 of aqueous: 1-octanol (Wery et al., 2000) and phase ratio 10:30 of aqueous: BES (Prpich P.G. and Daugulis A.J., 2007). The phase ratio of 10:1 was selected for further study.

Figure 4.3 The production of 3MC from toluene by TODE1 strain under various phase ratio of aqueous and 1-decanol as an organic phase.

4.3 Optimum condition of 3MC productions

Different conditions such as agitation speed, pH and temperature were investigated overall 3MC production from toluene by strain TODE1under a two-phase system of 1-decanol. The maximum overall 3MC production was found in agitation speed of 200 rpm, temperature of 30ºC and pH of 7.0 to 7.5 (Figure 4.4). Therefore this condition was selected for further study.

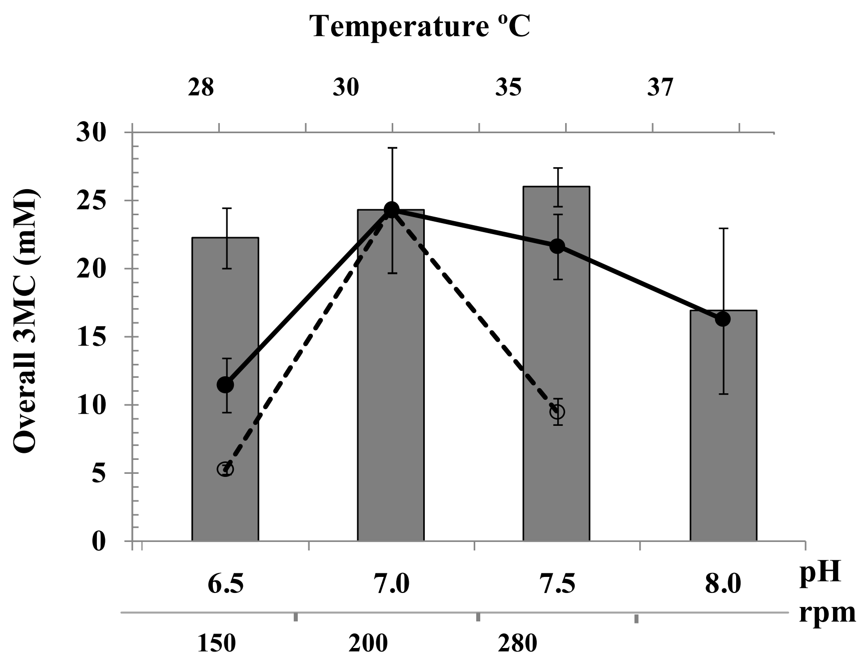


Figure 4.4 Optimization of 3MC production from toluene under a two-phase system. The symbols were represented agitation speed (dash line), temperature (solid line) and pH (bar graph).

4.4 Influence of various type of compound on 3MC production

P. putida T-57 mutant strain TODE1 was not able to utilize toluene as a growth substrate because of lack of *tod*E gene. Therefore, additions of carbon and energy sources were required for 3MC production by *tod*E mutant strain. To optimize the suitable fermentation medium, the addition of substrate toluene, carbon and energy source, and divalent ions at various conditions on 3MC production was determined (Table 4.2-4.3).

Addition of substrate, determination of 3MC production under various concentration of substrate toluene was investigated (Table 4.2). The results showed that, the production of 3MC increased when the concentration of toluene increased to 40 mM which equal concentration in normal condition that provided toluene by vapor whereas 3MC production decreased when more provided than 40 mM of toluene. This suggested that the concentration of toluene over 40 mM might be toxic to TODE1 strain leading to lower 3MC production. Then provided toluene as vapor was selected for further study.

Additions of cofactor source from 1-butanol, Faizal *et al*. reported that 1-butanol supply as vapor involved in 3MC production of TODE1 strain (Faizal et al., 2007). Therefore, various concentration of 1-butanol was directly added and compared with vapor supply in normal condition. The result showed that 1-butanol directly added did not significantly affect to 3MC production. 1-Butanol provided as vapor (normal condition) was enough for 3MC production (Table 4.2), and then it was selected for further study.

Additions of carbon and energy source, five compounds were used in this study. Addition of C6 carbon such as glucose was investigated by Faizal *et al.* (Faizal et al., 2007) that it was repressed TDO activity. Then to investigate the effect of carbon and energy sources on 3MC production, succinate (C4), glycerol (C3), and acetate (C2) were used to determine 3MC production compared with C6 carbon of glucose. Interestingly, glycerol (C3) showed significantly increasing on maximum overall 3MC production of 25 to 27 mM at 22 h and 50 h, respectively. In addition, the addition of succinate provided the increased overall 3MC of 20 mM at production time of 22 h and acetate added was shown increased of overall 3MC of 23.9 mM at

the lastly production (50 h) (Table 4.2). This result may suggest that C2 to C4 carbon and energy source did not repress TDO activity of TODE1 strain.

Effect of four divalent ions (Mg^{2+}, Ca^{2+}, Fe^{2+} and Mn^{2+}) supplementation on overall 3MC production by TODE1 strain was investigated, and without supplementation (normal condition) was performed for comparison. This was the first research descripts on the effect of divalent ions on 3MC production from toluene by *P. putida* under a two-phase system (Table 4.3). It was found that 3MC significantly increased from 23.8 to 29.6 and 25.9 to 26.6 mM, with production time of 22 and 50 h, respectively, when all concentration of Fe^{2+} and Mn^{2+} were supplemented into the medium. In case of Mg^{2+} supplementation, 25.3 mM 3MC was produced at 20 h. Nevertheless, the 3MC production was inhibited for supplemented Ca^{2+} in medium.

As can be seen, the comparison of with and without supplementation (normal condition), 54.9% and 23.1 % maximum overall 3MC significant increasing when glycerol and succinate were supplemented into the medium at production time of 22 h and it was decreased significantly at production time of 50 h (Table 4.4). While supplemented with acetate showed a miner affects to overall 3MC production. For the supplementation of divalent ions, 56.0%, 45.3% and 59.7% maximum overall 3MC were show significantly increased from supplementation of Mg^{2+}, Fe^{2+} and Mn^{2+} at production of time 22 h, respectively, and it decreased at production time of 55 h. On the other hand, supplemented with 1-butanol, glucose and Ca^{2+} clearly decreased overall 3MC production.

Table 4.2 Results of the pre-experiment of 3MC production under different each parameter

Compound		22 h			50 h		
		Aqueous phase	Organic phase	Overall	Aqueous phase	Organic phase	Overall
Normal condition*		8.7±1.1	91.8±4.6	16.2±3.5	15.1±2.7	116.8±11.4	24.5±2.8
Production substrate (mM)							
Toluene	1	0.7±0.1	8.3±1.0	1.4±0.1	0.4±0.1	5.7±0.6	0.9±0.1
	5	2.9±0.2	30.5±0.9	5.4±0.2	1.6±0.0	22.8±0.8	3.5±0.1
	10	5.7±0.7	52.8±3.1	10.0±0.9	2.4±0.6	37.5±2.4	5.6±0.7
	20	3.1±1.8	25.8±1.0	10.7±0.6	3.1±1.8	25.8±1.0	15.3±1.8
	40	3.3±1.2	25.1±0.6	18.6±0.0	3.3±1.2	25.1±0.6	25.4±0.8
	47	2.6±1.0	20.8±2.0	13.7±1.2	2.6±1.0	20.8±2.0	22.4±2.6
	60	2.4±1.0	20.1±5.5	13.6±3.0	2.4±1.0	20.1±5.5	23.0±1.0
Cofactor source							
1-Butanol	5	4.6±2.0	52.2±1.4	6.9±3.1	3.9±0.5	53.0±5.3	8.4±0.9
	10	3.8±0.2	29.1±5.6	6.1±0.7	2.1±0.9	34.1±6.5	5.0±1.4
	50	2.3±0.2	22.6±2.3	4.1±0.4	1.2±0.2	18.4±2.3	2.8±0.4
Carbon and energy source							
Glucose	3	3.0±0.8	76.2±3.1	9.6±1.0	4.5±0.7	55.7±0.4	9.1±0.7
	6	6.1±0.0	81.3±7.7	13.0±0.7	2.5±0.4	41.5±5.9	6.0±0.1
	11	3.5±0.2	69.3±6.5	9.5±0.8	1.8±0.4	27.5±8.5	4.1±1.1
Glycerol	3	14.6±0.8	131.0±3.1	25.2±1.0	18.3±0.7	88.3±0.4	24.6±0.7
	6	13.1±3.6	125.7±9.2	22.6±5.1	20.3±0.8	96.4±0.9	27.2±0.7
	11	10.7±0.8	103.9±1.2	19.2±0.8	19.8±0.3	95.0±5.2	26.6±0.8
Acetate	5	9.8±0.4	99.4±3.1	17.9±1.0	19.6±0.7	91.7±0.4	26.1±0.7
	10	8.1±0.7	86.7±0.1	15.2±0.6	19.3±0.6	91.9±3.6	25.9±0.2
	20	8.1±1.2	91.5±6.6	15.4±2.1	15.2±0.2	69.4±6.4	17.6±0.7
Succinate	5	5.3±4.2	89.8±1.2	8.9±2.0	13.1±0.7	77.7±0.4	20.9±0.7
	10	11.0±1.5	108.3±8.1	19.8±2.1	17.5±0.7	87.9±0.4	23.9±0.7
	20	9.8±0.5	121.7±1.4	20.0±0.3	4.1±0.3	73.9±0.5	10.4±0.3

*Normal condition mean 3MC production without supplementation of energy source and divalent ions under a two-phase system of 1-decanol, toluene and 1-butanol was supplied as vapor phase.

Table 4.3 Results of the pre-experiment of 3MC production under different each addition of divalent ions

Compound		22 h			50 h		
		Aqueous phase	Organic phase	Overall	Aqueous phase	Organic phase	Overall
Normal condition		8.7±1.1	91.8±4.6	16.2±3.5	15.1±2.7	116.8±11.4	24.5±2.8
Divalence ions							
Mg^{2+}	5	8.8±1.1	71.8±4.2	14.5±1.4	6.3±1.1	79.8±5.2	13.0±1.5
	10	18.9±1.5	98.2±1.7	25.3±2.6	9.6±1.2	112.1±8.6	18.9±1.9
	20	12.3±1.0	106.2±3.8	20.9±1.3	6.8±0.6	90.5±2.5	14.4±0.8
Ca^{2+}	1	3.0±0.5	76.2±5.9	9.6±0.1	6.3±0.3	76.3±3.3	12.7±0.5
	5	6.1±0.0	81.3±7.7	13.0±0.7	6.0±0.2	112.1±5.3	15.7±0.7
	10	3.5±0.2	69.3±6.5	9.5±0.8	6.8±1.3	90.5±8.7	14.4±2.0
Fe^{2+}	0.05	12.2±0.4	110.2±2.0	21.2±0.6	20.2±1.3	121.2±0.2	29.4±1.2
	0.10	13.6±0.7	123.5±3.2	23.6±1.0	20.1±1.2	124.5±2.9	29.6±1.4
	0.50	12.3±3.3	120.4±9.7	21.4±5.0	19.9±2.8	123.2±9.2	29.3±1.8
Mn^{2+}	0.01	13.7±0.3	124.7±2.2	23.8±0.5	17.7±0.3	89.5±3.6	24.3±0.1
	0.025	15.3±0.7	132.3±5.0	25.9±1.1	19.2±0.4	100.9±3.4	26.6±0.7
	0.05	13.6±1.7	107.2±7.7	22.1±0.8	18.3±0.8	93.3±1.9	25.1±0.6

*Normal condition mean 3MC production without supplementation of energy source and divalent ions under a two-phase system of 1-decanol, toluene and 1-butanol was supplied as vapor phase.

Table 4.4 The comparison of with and without supplemented with compound on 3MC production from toluene by TODE1 strain under a two-phase system of 1-decanol.

Compound		22 h	50 h
Normal condition		100	100
Carbone and energy source (mM)			
	5	42.19	34.12
Butanol	10	37.43	20.48
	50	25.53	11.29
	3	59.33	37.25
Glucose	6	79.88	24.68
	11	58.55	16.82
	3	**154.90**	**100.62**
Gylcerol	**6**	**139.28**	**111.26**
	11	**118.30**	**108.81**
	5	**110.49**	**106.82**
Acetate	10	93.60	**105.63**
	20	94.78	72.10
	5	54.74	85.42
Succinate	10	**122.18**	97.61
	20	**123.13**	42.62
Divalence ions (mM)			
	5	89.35	53.10
Mg^{2+}	10	**156.04**	77.12
	20	**128.43**	58.97
	1	59.33	51.81
Ca^{2+}	5	79.88	64.10
	10	58.55	58.97
	0.05	**130.26**	**119.99**
Fe^{2+}	0.10	**145.26**	**120.80**
	0.50	**131.61**	**119.57**
	0.01	**146.75**	99.15
Mn^{2+}	0.025	**159.65**	**108.60**
	0.05	**136.34**	**102.51**

*Normal condition mean 3MC production without supplementation of energy source and divalent ions under a two-phase system of 1-decanol, toluene and 1-butanol was supplied as vapor phase.

4.5 Central composite design (CCD) and response surface methodology (RSM) for 3MC production

As pre-experiments glycerol, Fe^{2+} and Mn^{2+} significantly affect on overall 3MC production, and then it was further optimized by using central composite design (CCD) and response surface methodology (RSM). Their central point's chosen for experimental designs were: 4 mM glycerol, 0.2 mM Fe^{2+}, and 0.025 mM Mn^{2+}, respectively. The CCD design and corresponding experimental responses at various production time were listed in Table 4.5. In this study, the *t*-test and *P*-value were used to identify the effect of each factor on 3MC production (Table 4.6). A *P*-value below 0.05 indicates that the responses are significant. In this case, glycerol and Fe^{2+} had significant effects on overall 3MC production ($P < 0.05$) at both of production time of 20 and 40h. While Mn^{2+} had significant effects at production time of 40h. The fitness of response was checked by coefficient of determination R^2 and Adj R^2, which production time of 22h was calculated to be 80.36% and 62.68%., respectively and production time of 40h was 92.19% and 85.16%, respectively. Higher than 90% of R^2 was considered as very high correlation. The effects of glycerol and Fe^{2+} on overall 3MC production were also checked by the 3D response surface and 2D contour plots. The 3D curve and 2D contour plots exhibited the main relationship between glycerol combined with Fe^{2+}, glycerol combined with Mn^{2+}, and Fe^{2+} combined with Mn^{2+} of production time of 22h and 40h (Figure 4.5 - 4.10). According to the plots, the optimal concentrations of glycerol and Fe^{2+} were 4 mM and 0.4 mM, respectively.

Table 4.5 The design and results of 3MC production at different removal time base on CCD

Standard order	Type	Factor (mM)			Removal time (h)			
		Mn^{2+}	Fe^{2+}	Glycerol	22	30	40	50
1	1	0	0.0	0	12.47	16.96	18.24	17.32
2	1	0.05	0.0	0	10.86	13.37	19.78	20.62
3	1	0	0.4	0	10.00	12.20	23.36	21.58
4	1	0.05	0.4	0	8.45	14.41	25.14	22.43
5	1	0	0.0	8	9.37	24.66	24.88	26.00
6	1	0.05	0.0	8	9.57	10.69	18.48	16.72
7	1	0	0.4	8	14.88	13.43	26.90	14.25
8	1	0.05	0.4	8	10.74	14.50	16.45	17.56
9	-1	0	0.2	4	13.50	10.45	24.16	20.36
10	-1	0.05	0.2	4	8.94	5.51	21.37	20.84
11	-1	0.025	0.0	4	12.94	16.05	18.75	17.89
12	-1	0.025	0.4	4	12.72	11.56	25.13	23.38
13	-1	0.025	0.2	0	8.30	17.07	9.62	13.11
14	-1	0.025	0.2	8	9.47	16.97	6.49	14.55
15	0	0.025	0.2	4	11.52	13.71	18.43	20.61
16	0	0.025	0.2	4	9.75	13.71	18.60	19.17
17	0	0.025	0.2	4	10.21	15.71	16.22	18.39
18	0	0.025	0.2	4	12.10	11.34	19.82	18.96
19	0	0.025	0.2	4	12.62	10.78	20.21	18.90
20	0	0.025	0.2	4	10.42	16.69	17.57	18.78

Table 4.6 The coefficients and their significance for response surface model.

Term	Coef.		Standard error		*t*-Value		*P*-Value	
	22h	40h	22h	40h	22h	40h	22h	40h
Constant	12.215	17.3	0.990	1.73	12.337	9.974	0.000	0
Mn	-32.540	-458.6	59.730	104.45	-0.545	-4.39	0.598	0.001
Fe	-21.260	-38.5	7.470	13.06	-2.847	-2.952	0.017	0.014
Glycerol	0.859	5	0.370	0.65	2.302	7.709	0.044	0
Mn*Mn	224.000	10264	1072.570	1875.47	0.209	5.473	0.839	0
Fe*Fe	43.750	139.7	16.760	29.3	2.611	4.769	0.026	0.001
Glycerol*Glycerol	-0.137	-0.5	0.040	0.07	-3.274	-7.077	0.008	0
Mn*Fe	-107.000	-95.3	78.610	137.45	-1.361	-0.693	0.203	0.504
Mn*Glycerol	-0.975	-25.2	3.930	6.87	-0.248	-3.669	0.809	0.004
Fe*Glycerol	1.806	-1.6	0.490	0.86	3.677	-1.908	0.004	0.085

R^2= 80.36% of 22h and R^2= 92.19% of 40h

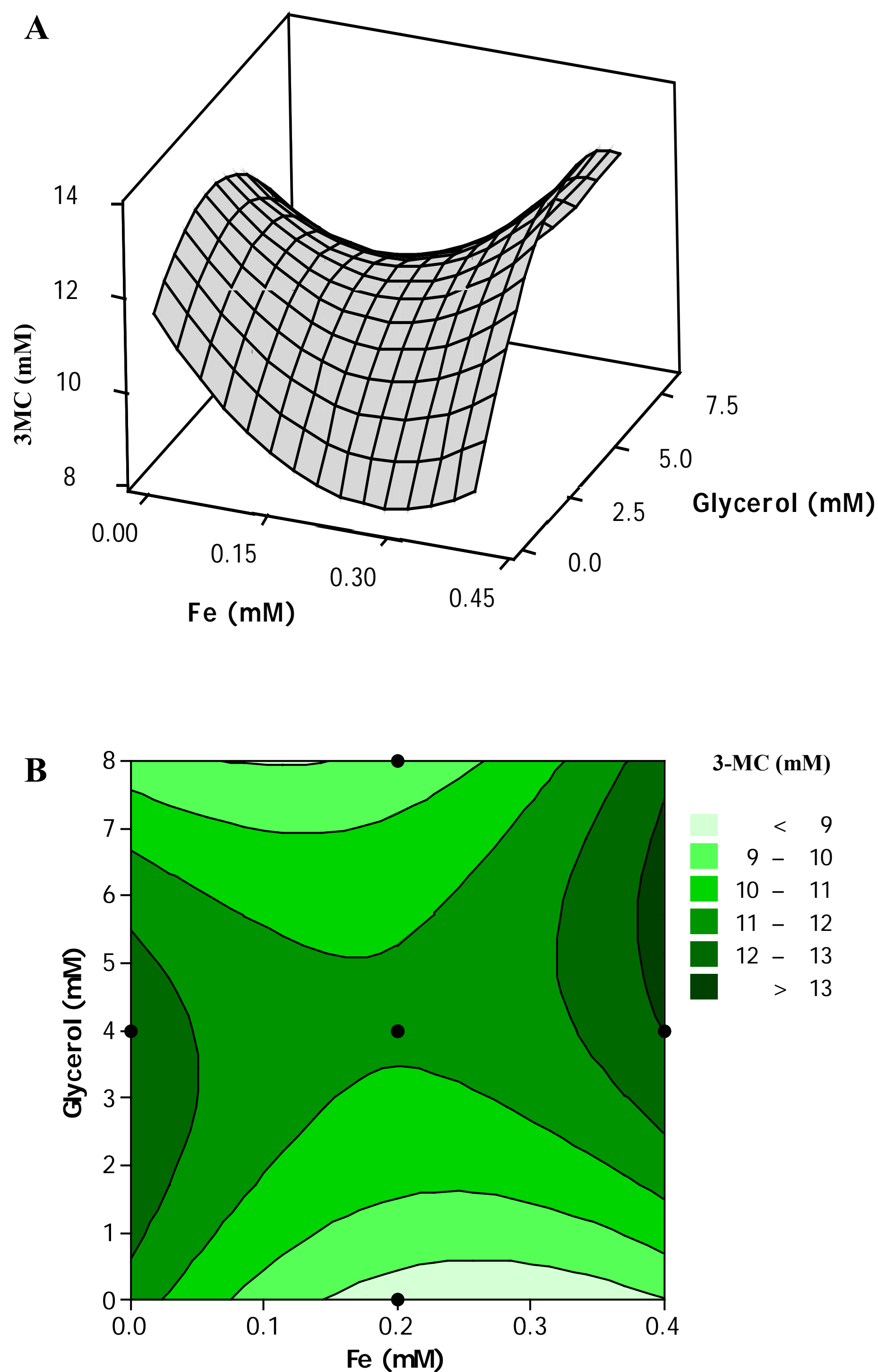


Figure 4.5 The dimension response surface (A) and contour plots (B) of glycerol combined with Fe^{2+} for the maximum 3MC production at production time of 22 h removal time.

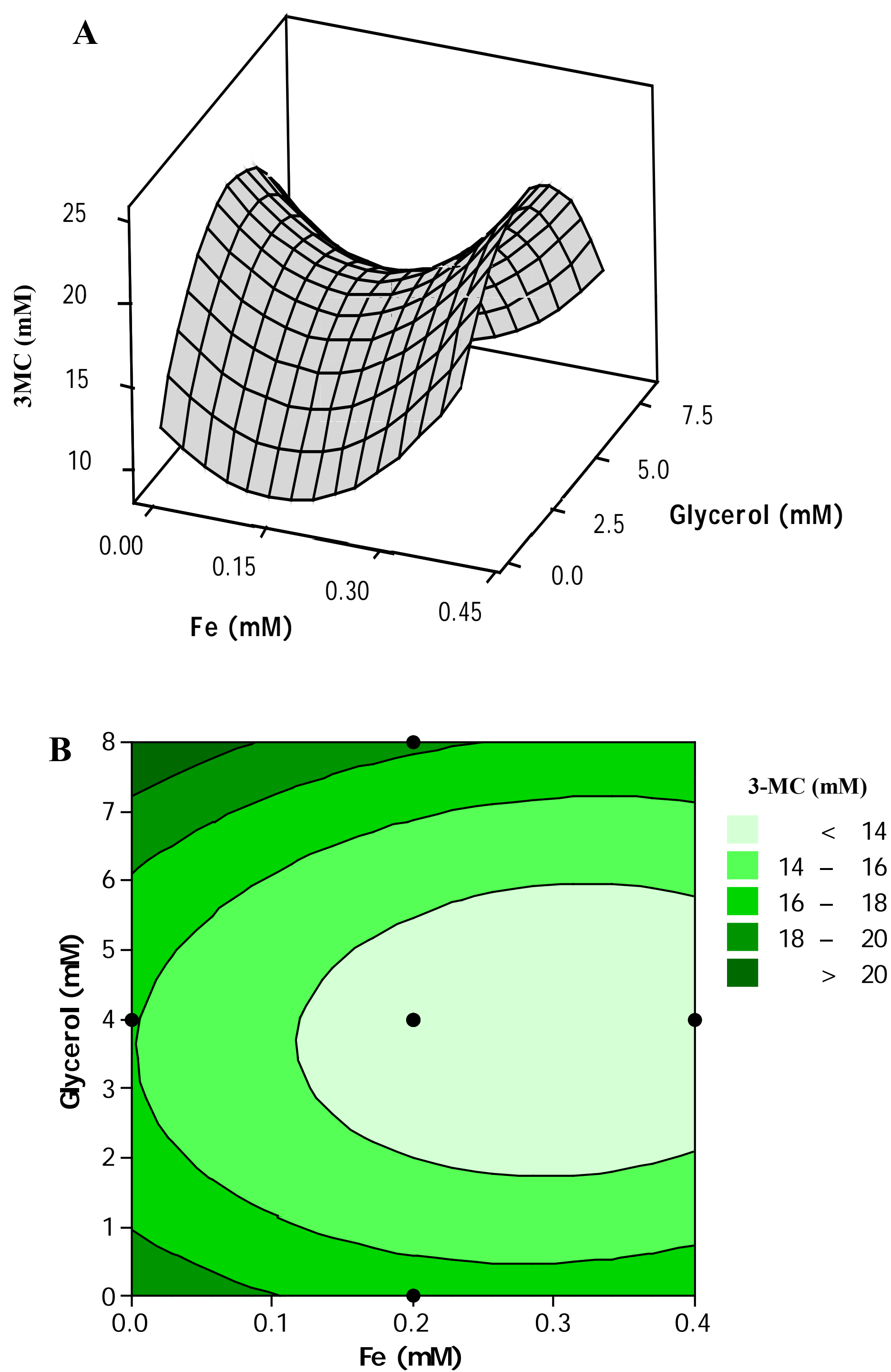


Figure 4.6 The dimension response surface (A) and contour plots (B) of glycerol combined with Fe^{2+} for the maximum 3MC production at production time of 40 h removal time.

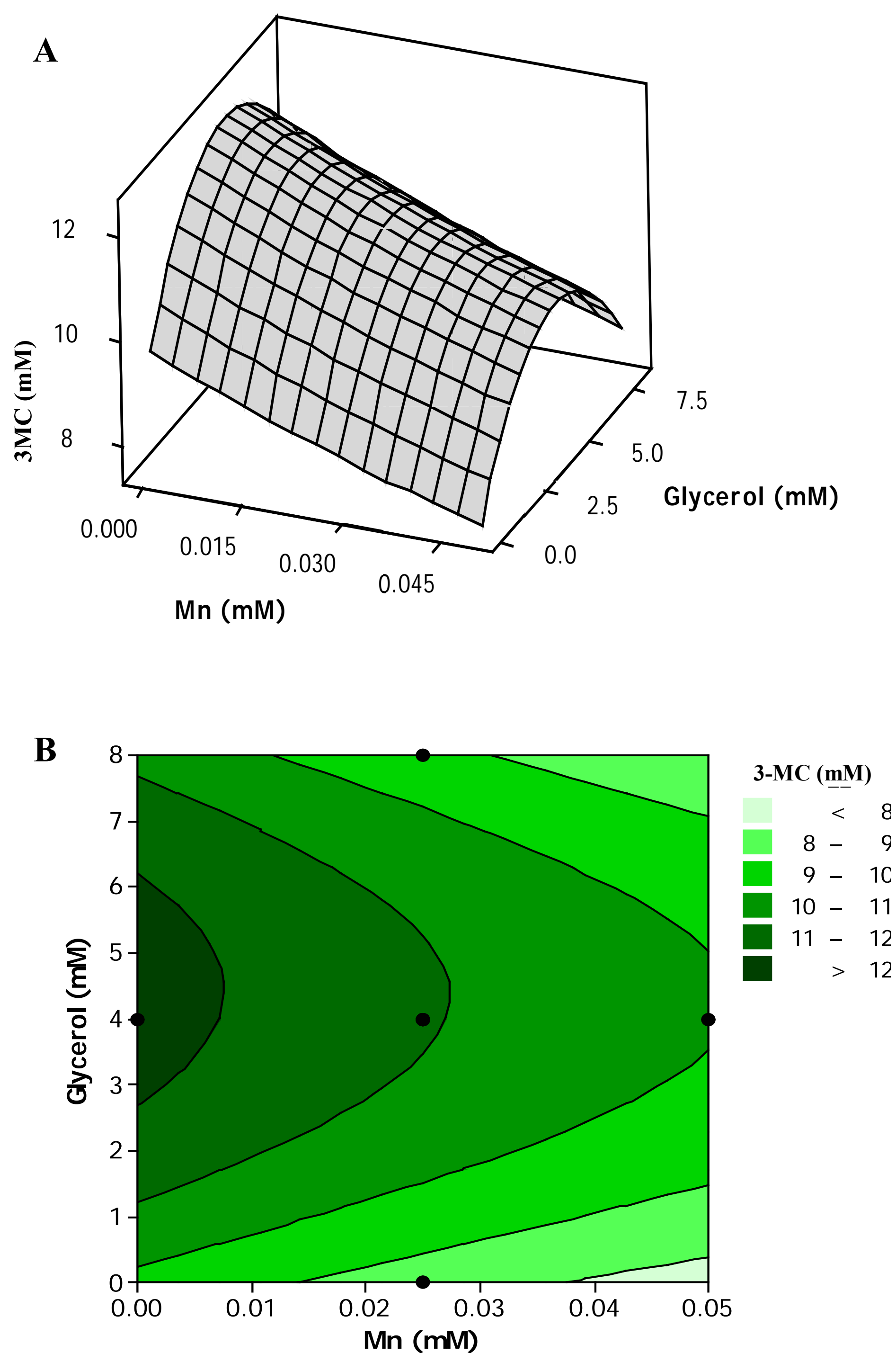


Figure 4.7 The dimension response surface (A) and contour plots (B) of glycerol combined with Mn^{2+} for the maximum 3MC production at production time of 22 h removal time.

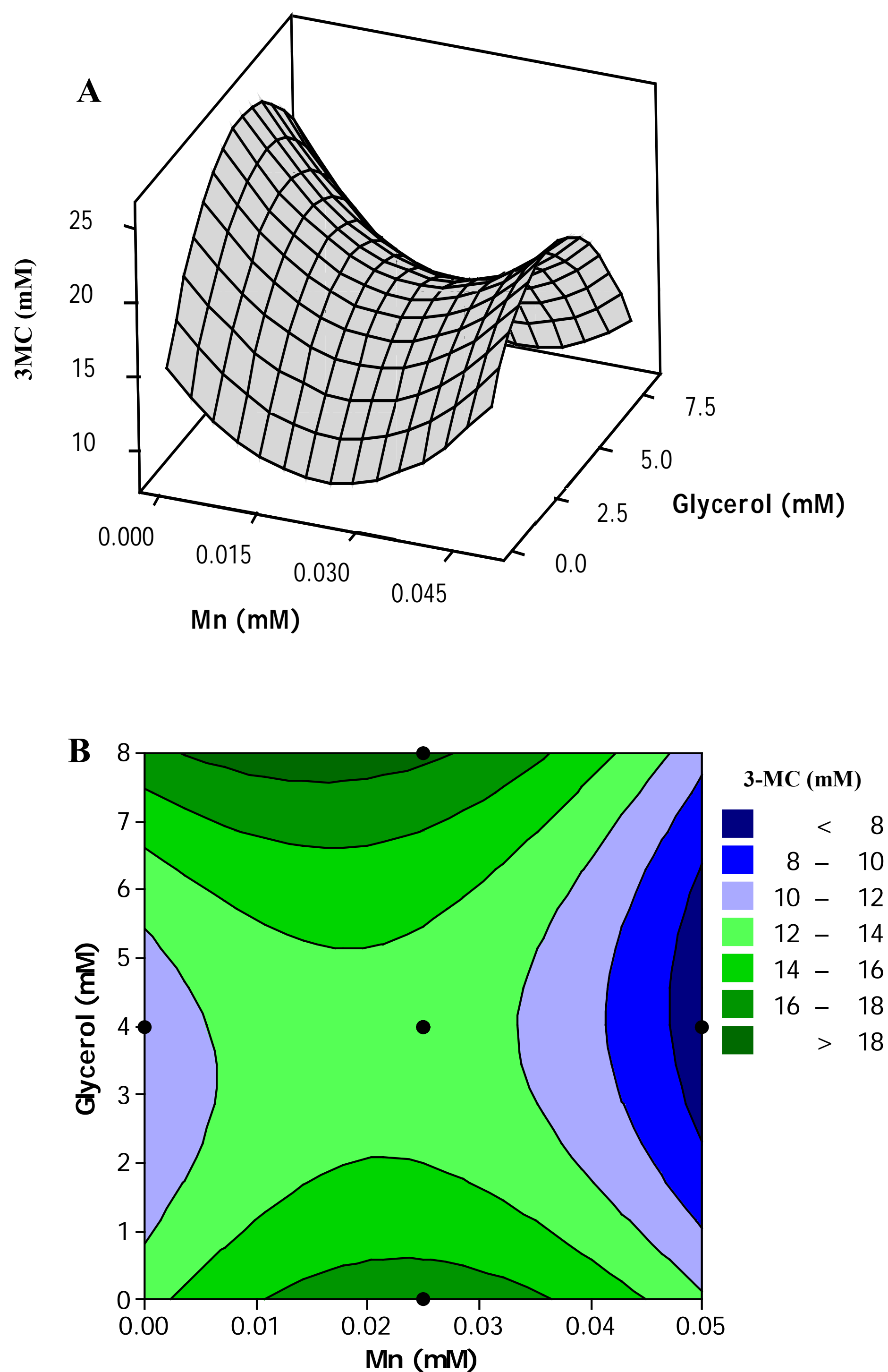


Figure 4.8 The dimension response surface (A) and contour plots (B) of glycerol combined with Mn^{2+} for the maximum 3MC production at production time of 40 h removal time.

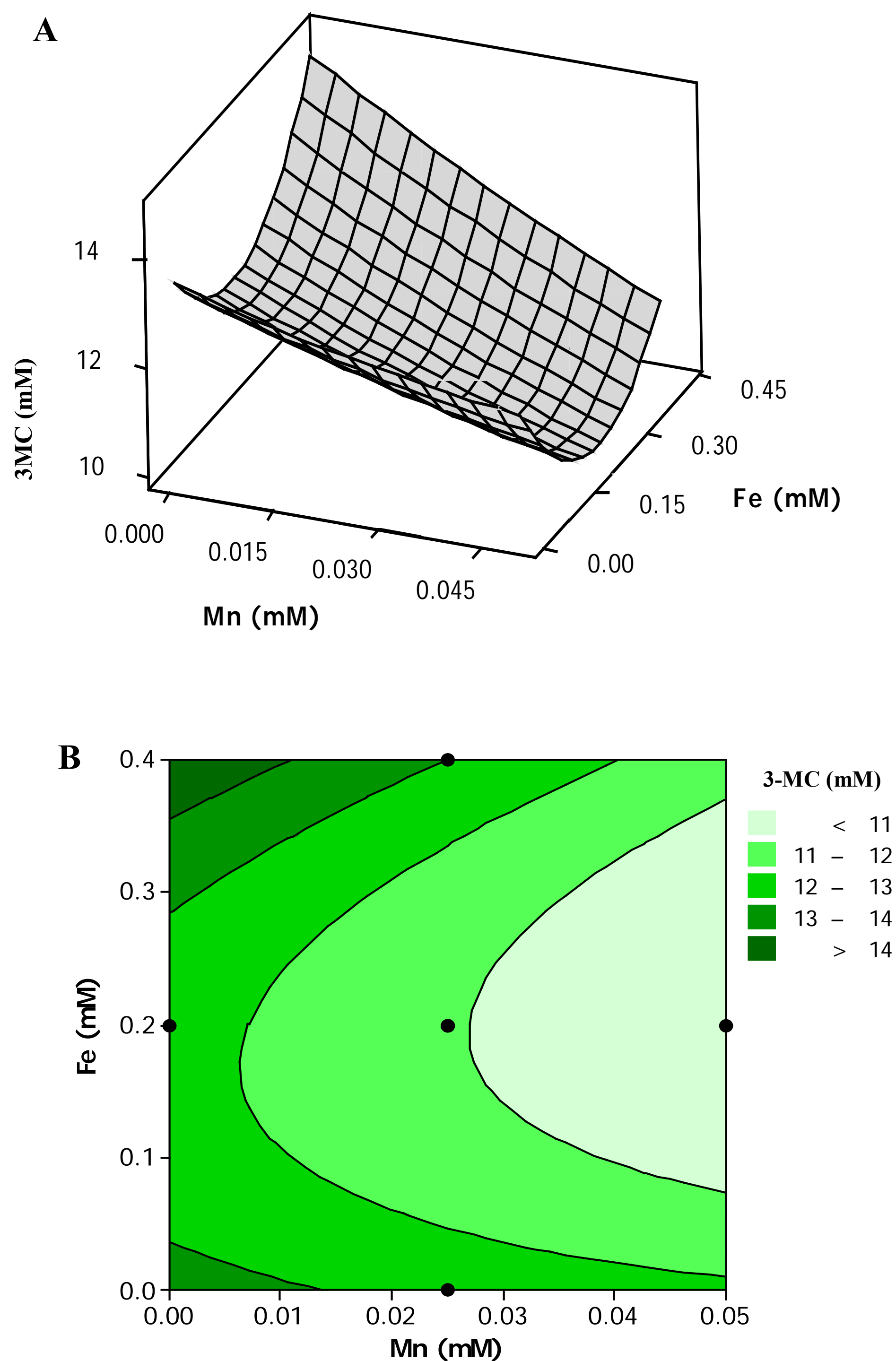


Figure 4.9 The dimension response surface (A) and contour plots (B) of Fe^{2+} combined with Mn^{2+} for the maximum 3MC production at production time of 22 h removal time.

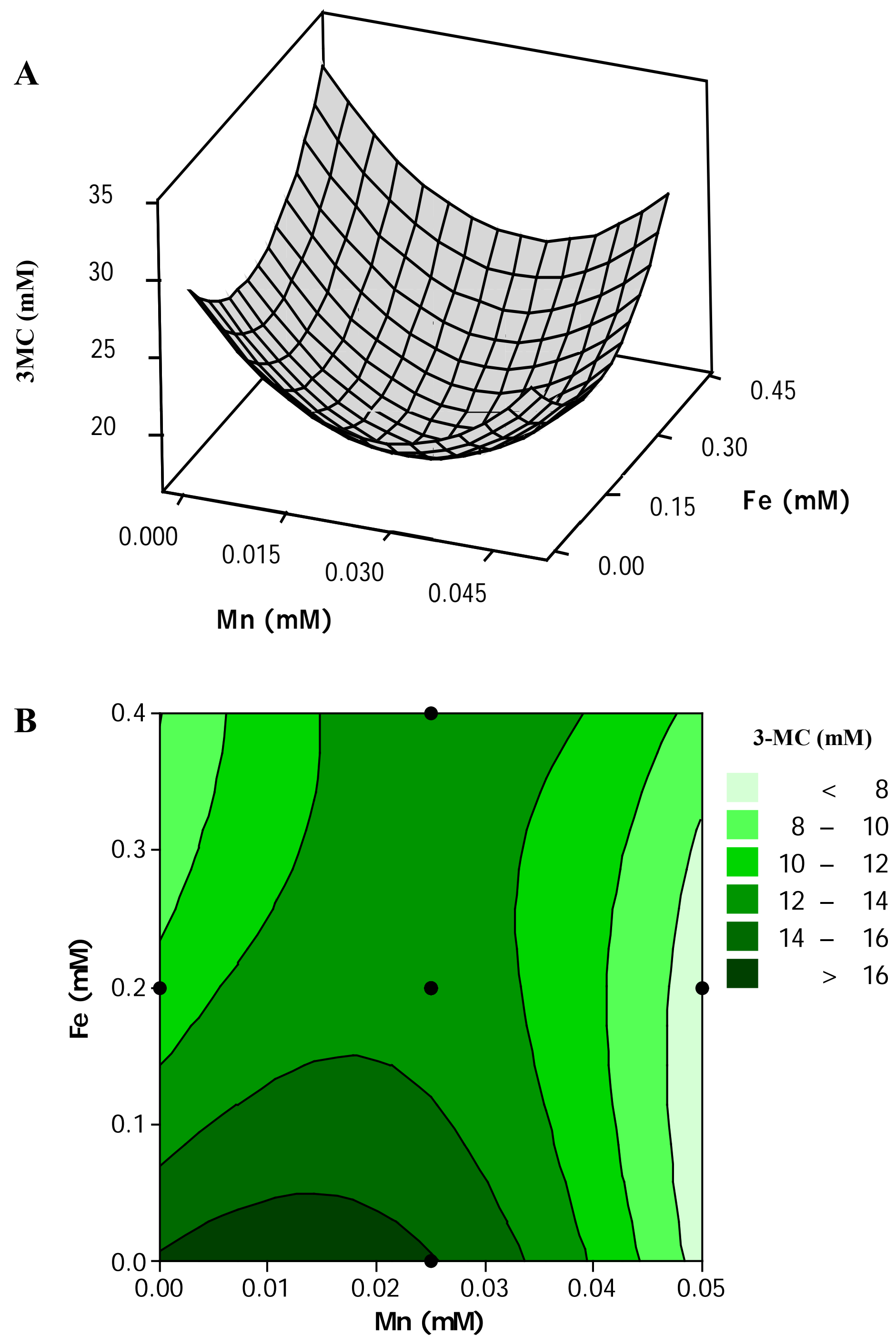


Figure 4.10 The dimension response surface (A) and contour plots (B) of Fe^{2+}combined with Mn^{2+} for the maximum 3MC production at production time of 40 h removal time.

4.6 3MC production from toluene by TODE1 strain in optimized medium

Base on the results of optimized medium, the optimum composition for 3MC production from toluene by TODE1 strain under a two-phase system of 1-decanol as an organic phase was MSB medium supplemented with 4 mM glycerol and 0.4 mM $FeS0_4$ (optimized medium). The corresponding predicted maximum overall 3MC production was 33.42 mM at production time of 40 h which it was higher than that reported previously. Therefore to confirm the predicted maximum, the production of 3MC was investigated in optimized and non-optimized medium using a two-phase system of 1-decanol under toluene and 1-butanol supplied as vapor in triplicate testes. The production of 3MC in batch cultivation of optimized medium and non-optimized medium were produced maximum overall 3MC at 50 h of 24.53 and 34.0 mM, respectively (Figure 4.11).

The maximum overall 3MC production was obtained at 50h of 34 mM, with was clearly in compared with predicted maximum value of 33.42 mM and higher than that reported previously (Table 4.7). Therefore, this result indicated the optimized medium favored the production of 3MC from toluene by TODE1 strain using a two-phase system of 1-decanol.

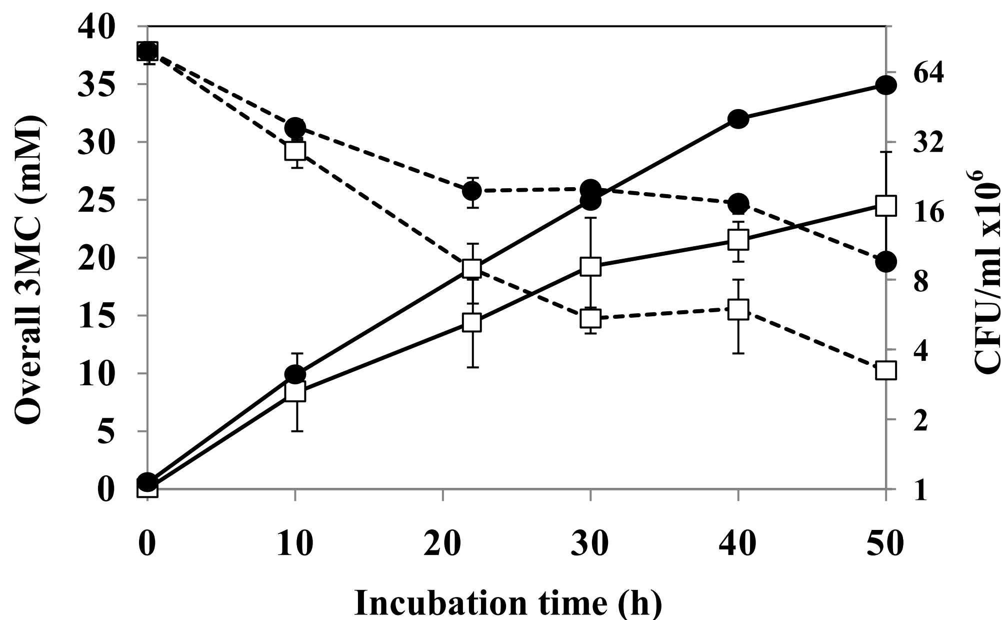


Figure 4.11 Time course of 3MC production from toluene using a two-phase system of 1-decanol in batch fermentation by mutant strain TODE1 base on the optimized medium (closed circle) compared with normal condition (open square). The production of 3MC (solid line) and cell survival CFU.ml-1 (dashed line) were examined.

Table 4.7 Comparison of 3MC production from toluene by engineering bacteria using various types of organic phase for a two-phase system in batch fermentation.

Microorganism	**Production system**	**Overall 3MC (mM)**			**References**
		Aqueous phase	**Organic phase**	**Overall**	
P. putida MC2	Single phase	14.0	-	-	(Hüsken et al., 2001)
P. putida MC2	Single phase	14.2	-	-	(Husken et al., 2002)
P. putida DS10	1-Octanol	7	11.5	8.3	(Wery et al., 2000)
P. putida MC2	1-Octanol	3.8	83	19.6	(Husken E. Leonie et al., 2001)
P. putida TODE1	1-Butano, oleyl alcohol	16.1	107.3	24.4	(Faizal et al., 2007)
***P. putida* TODE1**	**1-Butanol + 1-decanol + glycerol + Fe^{2+}**	**20.1**	**124.5**	**34**	**Present study**

CHAPTER V

RESULTS AND DISCUSSION

ON

Hydrophilic organic solvent of 3-hydroxypropionaldehyde (3-HPA) bioproduction by organic solvent-tolerant bacteria using a single-phase system

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5.9.1 Organic solvent tolerance and utilization

5.9.2 Cell adaptation to acetonitrile

5.9.3 Acetonitrile tolerance mechanisms

Screening, isolation of OST bacteria as host-cells for hydrophilic organic solvent bioproduction

5.1 Screening, isolation and identification of hydrophilic OST bacteria

OST bacteria were isolated from soil, water and seawater. Soil, water and seawater samples were collected from the oil and hydrocarbon contaminated area, hot spring area, agricultural area and natural area (Table 5.1). Enrichment technique was applied to increase quantity of organic OST bacteria. Bacterial screening was then performed on LB medium at various temperatures of 30ºC, 37ºC and 45ºC under a direct addition of organic solvent (0.1%, v/v). One hundred and four bacteria isolates that grow under directly added of organic solvent. The bacterial isolates could be divided into 2 groups according to different log P_{ow} value of enrichment organic solvents (Table 5.1): Group I bacteria tolerate to organic solvent with log P_{ow} 2.0-3.0; Group II bacteria tolerate to organic solvent with log P_{ow} < 2.0. To isolate OST bacteria for bacterial host cell as biocatalyst of 3-HPA and 3MC have log P_{ow} of -1.093 and 1.8, respectively; therefore isolation and characterization of Group II were focused. Within these groups II of OST bacteria, seven bacteria isolate having comparatively good growth in the presence of high concentration of propionaldehyde (similar property of 3-HPA) (Table 5.2) were chosen for further studies. Therefore, seven bacteria isolates chosen were identified using 16S rDNA sequence analysis. It was found that most of them were genus *Bacillus* and only strain SBH81 was *Exiguobacterium* sp. Therefore, characterization of the selected OST bacterial isolates were separated into two groups according to bacteria genus, that is genus *Bacillus,* and *Exiguobacterium* sp (Table 5.3).

Table 5.1 Source of samples used for screening and bacterial isolates obtained from each source

Temperature (ºC)	Sample types		Sample sources	Enrichment solvents	Isolation number
30	Water	Sea water	Chaum, Phetchaburi	1-Butanol (0.80)	SL1, SL2
				Butyraldehyde (0.88)	SL3, SL4, SL5, SL6, SL7, SL8, SL9, SL10, SL11, SL12
				Styrene (3.0)	SL13, SL14, SL15, SL16, SL17
				Toluene (2.5)	SL18, SL19, SL20, SL21
37	Water	Sea water	G1	Butyraldehyde (0.88)	GRSW1-BA1
			G2		GRSW2-BA1, GRSW2-BA2
			Krabi 1		KBSW1-BA1
			Krabi 2		KBSW2-BA1
			Songkhla		SKSW1-BA1, SKSW1-BA2, SKSW1-BA3
			Trang		TRSW1-BA1, TRSW1-BA2, TRSW1-BA3
			Nakhon Si Thammarat		NTSW1-BA1, NTSW1-BA2
			G2	Propionaldehyde (0.59)	GRSW2-P1/1, GRSW2-P1/2
			Krabi 1		KBSW1-P1, KBSW1-P2, KBSW1-P3
			Krabi 2		**KBSW2-P1**, KBSW2-P2
			Cha-Um, Phetchaburi		W8-P1
			Hau-Hin, Prachaupkirikhun		W9-P1
		Fresh water	Rangsit canal, Bangkok		BKW1-P1
		Wastewater	Paper mill industry, Kanchanaburi		KJW1-P1, KJW1-P2, KJW1-P3
			Leachate from landfilde, Ratchaburi		RBW1-P1/1, RBW1-P1/2, **RBW1-P3**
	Soil	Natural area	Rangsit canal, Bangkok		BKS-P1, BKS-P2/2
		Compost	Commercial		**COM1, COM2**, COM3
		Activated sludge	Jotun company, Bang-Chang, Bankok		BKS2-P1, BKS2-P2/1,**BKS2-P2/3**
		Agricultural area	Sugarcane farm, Pathum Thani		PTS1-P1
			Cassava farm, Nakhon Ratchasima		NRS1-P1
			Parawood farm, Krabi		KBS1-P1
			Quava farm, Pathum Thani		PTS2-P1
			Grape farm, Nakhon Ratchasima		NRS2-P1
			Linchy farm, Nakhon Ratchasima		NRS3-P1
			Cassava farm, Kanchanaburi		**KJS1-P1**, KJS1-P2
			Cassava farm, Pathum Thani		PRS3-P1

The bolded numbers were the bacterial isolate chosen for further studies

Table 5.1 Source of samples used for screening and bacterial isolates obtained from each source (continued)

Temperature (ºC)	Sample types		Sample sources	Enrichment solvents	Isolation number
45	Water	Sea water	Bangsan, Chon Buri	1-Butanol (0.80)	SBH01, SBH02/1, SBH02/2, SBH03, **SBH81**, SBH82, SBH83, SBH84, SBH85
				Butyraldehyde (0.88)	SBDH81, SBDH82, SBDH83, SBDH84, SBDH85
				Toluene (2.5)	ST01, ST02
				Styrene (3.0)	SSH81, SSH82
			Chaum, Phetchaburi	1-Butanol (0.80)	SWH1, SWH2, SWH3, SWH4
				Butyraldehyde (0.88)	SWH5
				Toluene (2.5)	SWH6, SWH7, SWH8
				Styrene (3.0)	SWH9
		Hot spring area	Krabi	Toluene (2.5)	4/1, 6/4, 13, 36, 45
				Cyclohexane (3.2)	21, 24, 24/1, 3C4, 3C5

The bolded numbers were the bacterial isolate chosen for further studies

Table 5.2 Secondary screening of hydrophilic OST bacteria

No.	[PA] %	CFUs after exposed with PA (h)			
		0	1	3	6
1	**KJW1-P1**				
	0	+++	+++	+++	+++
	1	+++	+++	+++	+
	2	+++	+	+	+
	3	+++	+	+	+
2	**KJW1-P3**				
	0	++++++	++++++	++++++	++++++
	1	++++++	+++++	+++++	++++++
	2	++++++	+	+	+
	3	++++++	+	+	+
3	**KJS1-P1**				
	0	+++++	+++++	+++++	+++++
	1	+++++	+++++	+++	++
	2	+++++	+++	++	++
	3	+++++	++	++	++
4	**W8-P1**				
	0	+++	+++	+++	++
	1	+++	+++	+++	+
	2	+++	+	+	+
	3	+++	+	+	+
5	**KBSW1-P2**				
	0	+++++	+++++	+++++	+++++
	1	+++++	+++++	+++	+
	2	+++++	+	+	+
	3	+++++	+	+	+
6	**KBSW1-P1**				
	0	++++++	++++++	++++++	++++++
	1	++++++	++++	+	+
	2	++++++	-	+	+
	3	++++++	-	+	+
7	**KBSW1-P3**				
	0	+++++	+++++	+++++	+++++
	1	+++++	++++++	+++	+
	2	+++++	+	+	+
	3	+++++	+	+	+
8	**KBSW2-P1**				
	0	+++++	+++++	+++++	+++++
	1	+++++	+++++	++++	++++
	2	+++++	+++	+++	++
	3	+++++	++	++	++
9	**KBSW2-P2**				
	0	++++++	++++++	++++++	++++++
	1	++++++	++++++	+++++	+
	2	++++++	+	+	+
	3	++++++	+	+	+
10	**GRSW2-P1/1**				
	0	+++++	+++++	+++++	+++++
	1	+++++	+++++	+++++	+
	2	+++++	+++++	+	+
	3	+++++	+	+	+
11	**GRSW2-P1/2**				
	0	+++++	+++++	+++++	+++++
	1	+++++	+++++	+++++	+
	2	+++++	++	+	+
	3	+++++	+	++	+
12	**BKS1-P1**				
	0	++++++	++++++	++++++	++++++
	1	++++++	++++++	++++++	+
	2	++++++	+	+	+
	3	++++++	+	+	+
13	**BKW1-P1**				
	0	++++++	++++++	++++++	++++++
	1	++++++	+++++	++++++	+
	2	++++++	+	+	+
	3	++++++	+	+	+
14	**COM2**				
	0	+++++	+++++	++++++	+++++
	1	+++++	++++++	+++++	+++++
	2	+++++	+++++	++	+++
	3	+++++	+++	+	++

The shaded text were the bacterial isolates chosen for further studies

Table 5.2 Secondary screening of hydrophilic OST bacteria **(continued)**

No.	[PA] %	CFUs after exposed with PA (h)			
		0	1	3	6
15	**KJW1-P2**				
	0	+++++	++++++	++++++	++++++
	1	+++++	+++++	+++++	-
	2	+++++	+++	-	-
	3	+++++	-	-	-
16	**RBW1-P3**				
	0	++++++	++++++	++++++	++++++
	1	++++++	++++++	+++	+++
	2	++++++	+++++	+++	+++
	3	++++++	+++	+++	+++
17	**BKS2-P2/3**				
	0	+++	+++	+++	+++
	1	+++	+++	+++	+++
	2	+++	+++	+++	+++
	3	+++	++	++	++
18	**W9-P1**				
	0	++++	+++++	+++++	+++++
	1	++++	+++	+++++	+++
	2	++++	+++	-	-
	3	++++	-	-	-
19	**RBW1-P1/1**				
	0	++++++	++++++	++++++	++++++
	1	++++++	++++++	++++++	+++++
	2	++++++	+	+	+
	3	++++++	+	+	+
20	**RBW1-P1/2**				
	0	++++++	++++++	++++++	++++++
	1	++++++	+++++	+++++	+++++
	2	++++++	+	+	+
	3	++++++	+	+	+
21	**BKS1-P2/2**				
	0	++++++	++++++	+++++	++++++
	1	++++++	+++++	+++++	+
	2	++++++	+	+	+
	3	++++++	+	+	+

No.	[PA] %	CFUs after exposed with PA (h)			
		0	1	3	6
22	**COM1**				
	0	+++++	+++++	+++++	++++++
	1	+++++	+++++	+++++	+++++
	2	+++++	+++	+++	+++
	3	+++++	++	++	++
23	**COM3**				
	0	+++	++++	+++	++++
	1	+++	+++	+++	+++
	2	+++	+	+	+
	3	+++	+	+	+
24	**BKS2-P1**				
	0	++++++	++++++	++++++	++++++
	1	++++++	++++++	++++++	++++++
	2	++++++	+	+	+
	3	++++++	+	+	+
25	**BKS2-P2/1**				
	0	++++++	++++++	++++++	++++++
	1	++++++	++++++	+++++	+
	2	++++++	++	+	+
	3	++++++	+	+	+
26	**PTS1-P1**				
	0	++++++	++++++	++++++	++++++
	1	++++++	++++++	++++++	++++++
	2	++++++	+	+	+
	3	++++++	+	+	+
27	**NRS1-P1**				
	0	++++++	++++++	++++++	++++++
	1	++++++	++++++	+	+
	2	++++++	+	+	+
	3	++++++	+	+	+
28	**KBS1-P1**				
	0	++++++	++++++	++++++	++++++
	1	++++++	++++++	+	+
	2	++++++	+	+	+
	3	++++++	+	+	+

The shaded text were the bacterial isolates chosen for further studies

Table 5.3 Hydrophilic OST bacteria isolates chosen for further study

Bacterial isolates	% Similarity	Bacterial identification
KJS1-P1	99%	*Bacillus* sp.
KBSW2-P1	99%	*Bacillus* sp.
COM1	98%	*Bacillus subtilis*
COM2	98%	*Bacillus subtilis*
RBW1-P3	96%	*Bacillus cereus*
BKS2-P2/3	99%	*Bacillus subtilis*
SBH81	100%	*Exiguobacterium* sp.

5.2 Characterization of hydrophilic OST bacteria

5.2.1 Propionaldehyde tolerance test

The effect of propionaldehyde on growth and tolerance of bacterial isolates chosen were monitored after cell exposure to various concentrations of propionaldehyde. Cells were grown to late log phase (about 6 h) before propionaldehyde was added. Cell growth and cell survival was assessed by viable cell number ($CFU.ml^{-1}$). Cell survival after propionaldehyde shock were displayed in Figure 5.1, showing a relationship between cell number and the amount of propionaldehyde added (1, 2, 5, 10, 20 and 30%, v/v) to the media compared to that in the absence of propionaldehyde. The results demonstrated that all six isolates from genus *Bacillus* exhibited tolerance to high concentration of propionaldehyde up to 10% (v/v) within 6 h. Although at the concentration over 10% (v/v) of propionaldehye, cells were adversely affected by the propionaldehyde exposure in that cell viability was decreased. Therefore all isolates were chosen for bacterial host cell of 3-HPA production.

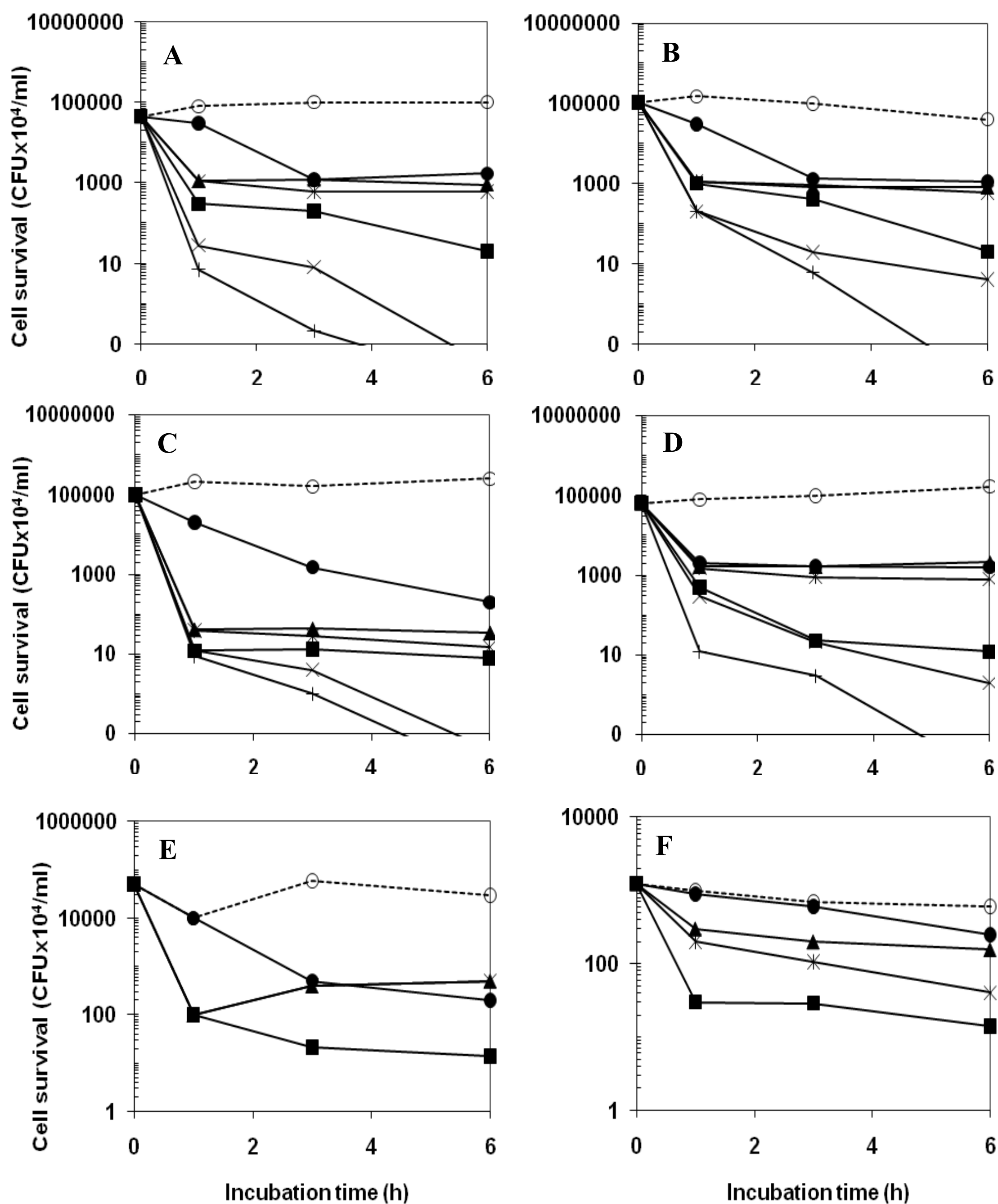


Figure 5.1 Propionaldehyde tolerance test of genus *Bacillus* bacterial isolates KJS1-P1 (A), KBSW2-P1 (B), COM1 (C), COM2 (D), RBW1-P3 (E) and BKS2-P2/3 (F) exposed with various concentration of propionaldehyde (1, (●); 2, (▲); 5, (✱); 10, (■); 20, (✕); and 30, (✚)% (v/v)) compared to that in the absence.

5.2.2 Glycerol utilization

To apply hydrophilic OST bacterial isolates chosen as host cell for 3-HPA production from glycerol, cell ability to utilize glycerol are important criteria for bacteria selection. Cell growth under MSB medium supplemented with various concentrations of glycerol was monitored by measuring OD_{600}. Cell growths in medium contained glycerol were displayed in Figure 5.2, showing a relationship between growth and the amount of glycerol added (1, 3, and 5%, w/v) to the media. The results demonstrated that isolate RBW1-P3 showed a small growth under the medium supplemented with glycerol. When glycerol concentration was increased, growth of isolate RBW1-P3 was decreased. On the other hand, five isolates of KJS1-P1, KBSW2-P1, COM1, COM2, and BKS2-P2/3 could well utilize high concentration of glycerol.

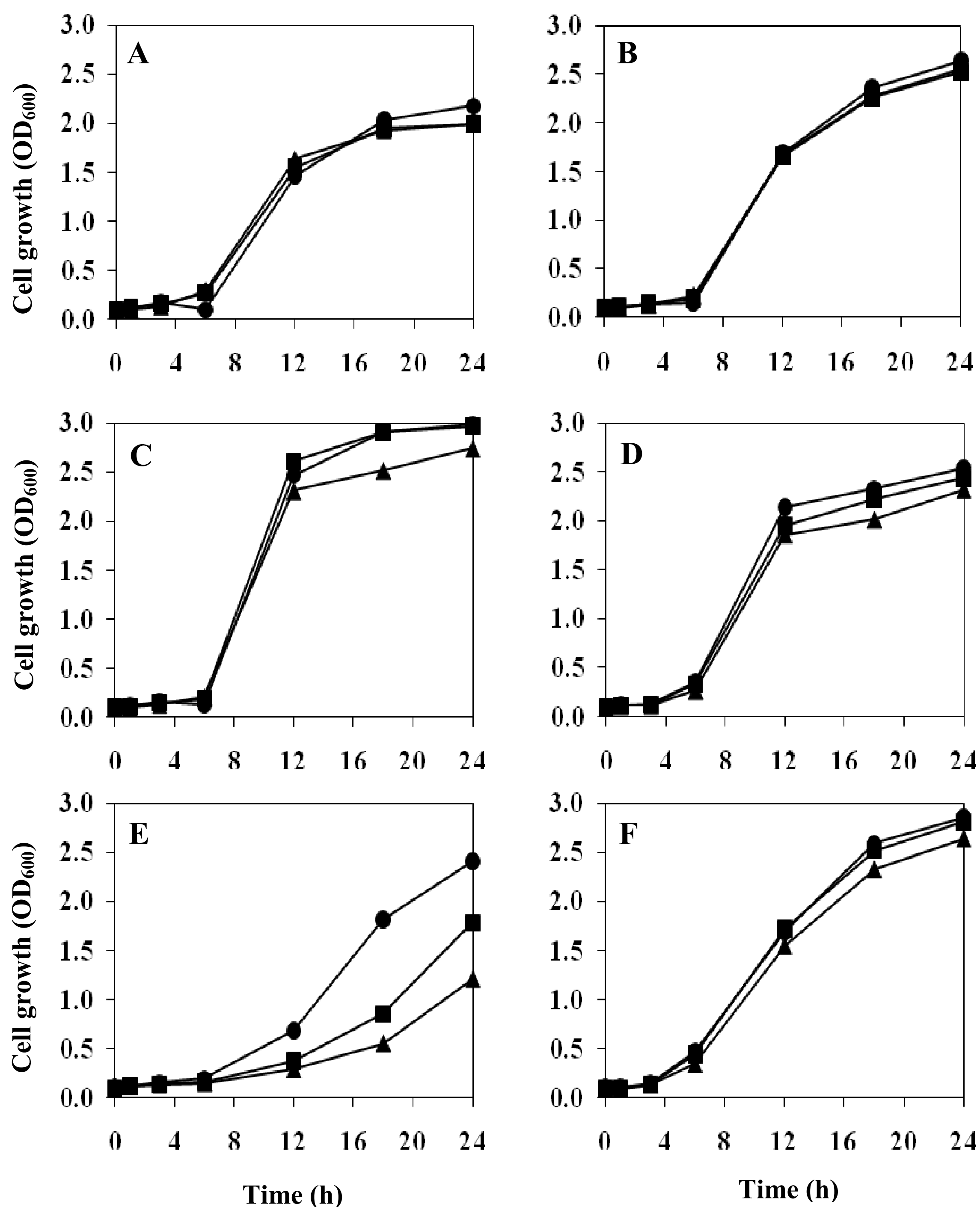


Figure 5.2 Glycerol utilization of bacterial isolates KJS1-P1 (A), KBSW2-P1 (B), COM1 (C), COM2 (D), RBW1-P3 (E) and BKS2-P2/3 (F) at various concentrations of 1 (●), 3 (■) and 5% (▲) (w/v).

5.2.3 Crude glycerol from biodiesel utilization and tolerance

To apply crude glycerol as by-product from biodiesel production for 3-HPA production by hydrophilic OST Gram positive bacteria, cell ability to utilize and tolerance to impurity of crude glycerol are important for production. Cell growth under MSB medium supplemented with various concentrations of crude glycerol was monitored by viable cell number ($CFU.ml^{-1}$). Cell utilization and tolerance in medium contained crude glycerol were displayed in Table 5.4, showing a relationship between growth and the amount of crude glycerol added (1, 3, 5, and 10%, w/v) to the media. The results demonstrated that all isolates exhibited high utilization and tolerance to impurity of crude glycerol from biodiesel up to 10%, w/v.

Table 5.4 Cells survival of the selected hydrophilic OST Gram positive genus *Bacillus* from crude glycerol from biodiesel

Crude glycerol concentration (%)	Incubation time (h)				
	0	1	3	6	12
KJS1-P1					
0	++++	++++	++++	++++	++++
1	++++	++++	++++	++++	++++
3	++++	++++	++++	++++	++++
5	++++	++++	++++	++++	++++
10	++++	++	++	++	++
KBSW2-P1					
0	++++	++++	++++	++++	++++
1	++++	++++	++++	++	++
3	++++	++	++	++	++
5	++++	++	++	++	++
10	++++	++	++	++	++
COM1					
0	++++	++++	++++	++++	++++
1	++++	++	++++	++++	++++
3	++++	++	++	++	++
5	++++	++	++	++	++
10	++++	++	++	++	++
COM2					
0	++++	++++	++++	++++	++++
1	++++	+++	+++	+++	+++
3	++++	+++	+++	+++	+++
5	++++	+++	+++	+++	+++
10	++++	+++	+++	+++	+++
RBW1-P3					
0	+++	+++	+++	+++	+++
1	+++	++	++	++	++
3	+++	++	++	++	++
5	+++	++	++	++	++
10	+++	++	++	++	++
BKS2-P2/3					
0	+++	+++	+++	+++	+++
1	+++	++	++	++	++
3	+++	++	++	++	++
5	+++	++	++	++	++
10	+++	++	++	++	++

Cells were grown until mid-log phase in LB medium after that crude glycerol from biodiesel (1-10%, w/v) was directly added to the cell culture. Cells survival was represented by ++, CFU ≥1-20 x10^4; +++, CFU ≥20-50x10^4; ++++, CFU ≥100x10^6.

Development of a transformation system and gene manipulation method for the selected OST bacterium

The Gram positive OST bacteria (7 isolates), were isolated as high tolerance to hydrophilic organic solvent, and good growth in the present of glycerol and crude glycerol from biodiesel. As their property, the Gram positive OST bacteria was applied as heterologous host for production of hydrophilic organic solvnent of 3-HPA. Then the development of transformation system and gene manipulation method for 3-HPA production was investigated in this Chapter.

5.3 Genetic transformation of selected Gram positive OST bacteria

Cell ability to transform and manipulate of the selected Gram positive OST bacteria are important for the development of an alternative host for 3-HPA production. It is known that many Gram positive bacteria are difficult to transform. Then several cell pretreatment and transformation protocol were investigated for each Gram positive OST bacteria. Plasmid pHY300PLK used for transformation with various conditions such as cell preparation (growth phase, cell density, electroporation buffer), and transformation system (electroporation condition, plasmid concentration, recovery medium, and recovery period). From the result (Table 5.5), bacterial strains SBH81, KJS1-P1, KBSW2-P1, COM1, COM2, and RBW1-P3 could not transform or manipulate. It may probably from the unique membrane characteristics of OST bacteria may affect to transformation. On the other hand, electroporation was successfully applied for strain KBS2-P2/3. The competent cell of KBS2-P2/3 was prepared in LB medium to late exponential phase with OD_{600} of 0.9, and with ice-cold EP solution. Plasmid DNA of pHY300PLK was then introduced at 300 ng to the

competent cells (300 μl), and chilled on ice for 30 min before electroporation was performed at 50 μF, 300 Ω, with the optimized field strength at 2.0 $kV \cdot cm^{-1}$, a time constant of 4.7 ± 0.1 ms. To recovery cell, the SOC medium was immediately added in pulsed cell and incubated for 5 h. After that, the generated cell was spread on LB medium agar plates including an appropriate antibiotic. From this result, hydrophilic OST Gram positive *B. subtilis* BKS2-P2/3 was chosen as host cell for 3-HPA production.

Table 5.5 Electroporation result of selected Gram positive OST bacteria

Bacterial isolates	**Electroporation result**
Exiguobacterium sp. SBH81	-
Bacillus sp. KJS1-P1	-
Bacillus sp. KBSW2-P1	-
Bacillus subtilis COM1	-
Bacillus subtilis COM2	-
Bacillus cereus RBW1-P3	-
***Bacillus subtilis* BKS2-P2/3**	+

5.4 Determination of promoter strength for an expression vector in *B. subtilis* BKS2-P2/3

Bioproduction capability of industrial chemical using heterologous host is depend on promoter system. A suitable promoter for efficient production of recombinant gene products is considered based on its strength and controllability (i.e. inducibility) at an indicated time or condition (Timmis, 1994). In this study, the several promoters of *Bacillus* sp. and Gram positive bacteria (Table 5.6), which kindly provided by Prof. Junichi Kato (Hiroshima University, Japan), were introduced into pHY300PLK, an *E. coli*- *Bacillus* shuttle vector, and their activity was then assessed by measuring β-galactosidase reporter gene activity.

The promoter could be classified into two groups, the first group of promoters consisted of constitutive promoters including: P43, a well-characterized promoter that is functional during both exponential and stationary growth phases (Wang and Doi, 1984); PKm, a promoter of the kanamycin resistance gene (Masai et al., 1995); and P2N, a strong promoter that functions in *Brevibacillus choshinensis*. The second group comprised inducible promoters, consisting of: P2L, a temperature inducible promoter (Li et al., 2007); PTetL, a strong promoter of the *tet*L gene encoding efflux-mediated tetracycline resistance in *Streptococcus, Enterococcus*, and *Bacillus* (Butaye et al., 2003); PSpac, an IPTG-inducible promoter (Vagner et al., 1998); and PXylA, a xylose-inducible promoter originated from *B. megaterium*. The expression activity of an inducible promoter in BKS2-P2/3was tested at each optimal inducible condition

The result found that (Figure 5.3), the activity of P2N, P2L, PTet, PKm, PSpac, and P43 in BKS2-P2/3 was slightly higher than the basal activity of the wild type and harboring an original vector pHY300PLK. On the other hand, a significant

level of β-galactosidase activity was observed in the transformants harboring PXyl, where the activity was 104 miller units.

Table 5.6 The test promoter used in this study (Kataoka et al., 2012)

Promoter	Sequence source	Name/Definition & Functioning source	
P2N	pNCMO2	P2 promoter	- functions in *Brevibacillus* sp.
P2L	pHY300PLK	A temperature inducible	- functions in *Bacillus subtilis*
PTet	pUC4K	Tetracycline promoter	- functions in *Bacillus* sp.
PKm	pDG148	Kanamycin promoter	- functions in *Rhodococcus* sp.
PSpac	*Bacillus subtilis*	*Bacillus subtilis*	
PXyl	pNCMO2	*Bacillus megeterium*	
P43	pWH1520	*Bacillus subtilis*	

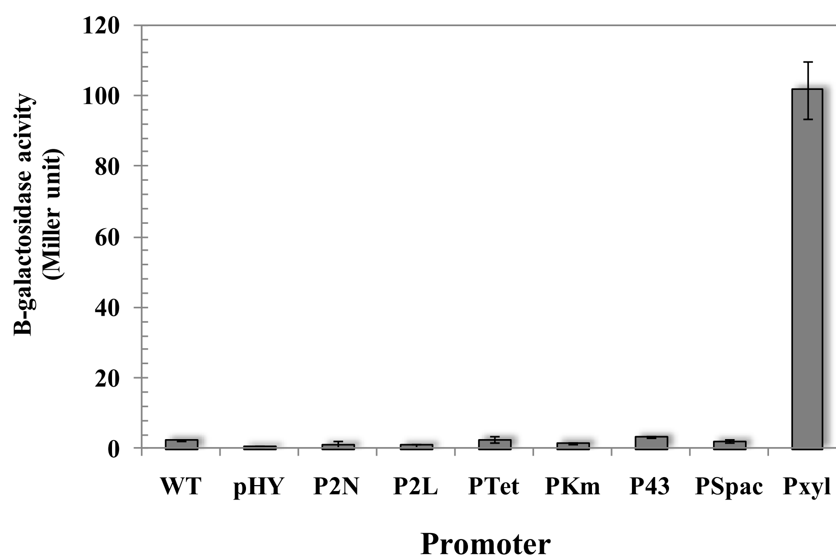


Figure 5.3 Promoter activities of Gram positive OST *B. subtilis* BKS2P2/3

5.5 Propionaldehyde tolerance of recombinant Gram positive OST *B. subtilis* BKS2-P2/3

The prior to any further genetic engineering, solvent tolerance of the transformants was reaffirmed. According to Kataoka *et al*., 2012 (Kataoka et al., 2012) found that recombinant Gram positive OST *B. subtilis* GRSW2-B1was showed cross-activity with bacterial tolerance to broad range of organic solvent resulting of tetracycline resistance gene (*tet*L) system. In this study, recombinant Gram positive OST *B. subtilis* BKS2-P2/3 harboring pHY300PLK contain *lac*Z gene and PXyl as promoter was tested propionaldehyde tolerance. As Figure 5.4, propionaldehyde tolerance of wild type and recombinant BKS2-P2/3 showed not significant cell survival as CFU/ml. It was suggested that *tet* L resistance system might not affected on propionaldehyde tolerance of Gram positive OST *B. subtilis* BKS2-P2/3.

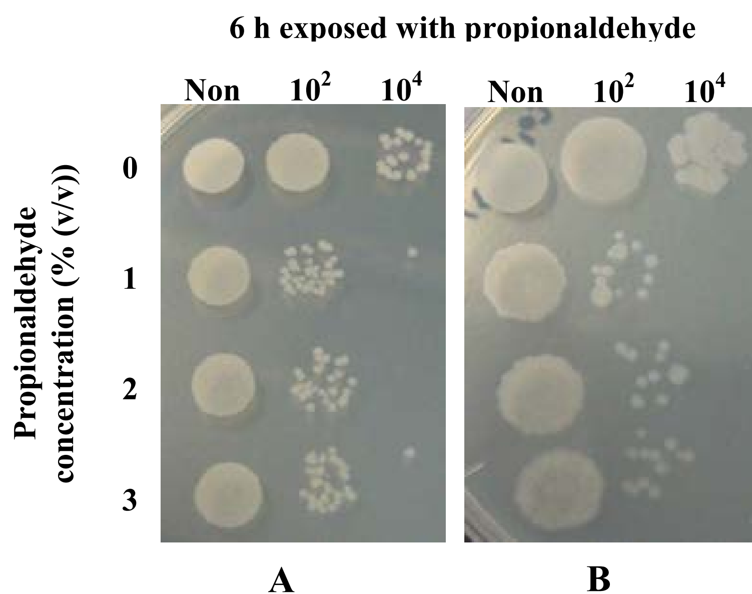


Fig 5.4 Propionaldehyde tolerance of *B. subtilis* BKS2P2/3 (A) wild type and recombinant (B)

Hydrophilic organic solvent of 3-HPA bioproduction from glycerol by OST bacteria using a single-phase system

5.6 Selection of *dha*B gene source

To screen 3-HPA producing bacteria as a source of *dha*B gene, it was divided into 2 parts as follow.

The primary screening, 3-HPA producing bacteria were isolated from soil determining the glycerol utilization. In this step, MSB medium containing with glycerol was applied to isolate bacteria that able to produce 3-HPA under aerobic and static conditions. Seventeen bacterial isolates were obtained from the primary screening (Table 5.7) however that seven isolates were high growth in the presence of glycerol under aerobic and static conditions. Therefore, seven isolated bacteria were carried out to confirm the ability of aldehyde and 3-HPA production for further study.

The secondary screening step, seven isolates were investigated the aldehyde production and 3-HPA production determination. And the results show that, all strains were identified as *Klebsiella pneumoniae* that showed ability to produced aldehyde under aerobic and static conditions. Among, seven aldehyde producing bacterial isolates, only strain GF37j7 was shown 3-HPA formation. To investigate 3-HPA production of *K. pneumoniae* GF37j7 and to select as source of gene, cell was grown in MSB medium supplemented with 3% (w/v) glycerol under aerobic condition. It was found that, GF37j7 could produce approximately 2.5 mM 3-HPA (Figure 5.5). Even though *K. pneumoniae* GF37j7 only yield a low concentration but there is less information of *K. pneumonia*e accumulated and flowed out 3-HPA to medium. Then *K. pneumoniae* GF37j7 was selected for source of gene for 3-HPA production by using OST bacteria as a host cells.

Glycerol dehydratase can be separated into 2 type that are B12-depentdent; glycerol dehydratase which enzyme need vitamin B12 as cofactor, and B12-independent glycerol dehydratase; vitamin B12 is un-necessary for the activity.

Moreover, *K. pneumonia* ATCC13883 could produce glycerol B12-dependent and *C. butyricum* ATCC13983 of glycerol B12-independent were selected as gene source (*dha*B gene cluster) together with *K. pneumoniae* GF37j7. Due to *K. pneumoniae* GF37j7 was isolated in this study, and then its *dha*B gene cluster was determined sequence similarity compared with other strain. The randomized *dha*B gene fragments were assembled by SEQUENCHER (Gene Codes Cooperation) with the Method 3.11. Briefly, primers KdhaB-F and KdhaB-R (Table 3.2) for amplification of *K. pnumoniae* GF37j7 were designed and analyzed by clone Manager (Scientific and Education Software) from the conserved regions of glycerol dehydratase (*dha*B). Nucleotide sequences of primers were deduced from the conserved region. The *dha*B gene (4.5 kb) was cloned in pGEM-T easy vector and was double digested with *Xho*I-*Eco*RI, *Eco*RI-*Bam*HI and *Eco*RI-*Pst*I and single digested with *Bam*HI and *Pst*I. Each fragment was ligated into pUC118 plasmid and transformed in *E. coli* DH5α. The randomized *dha*B gene fragments were subjected to DNA sequence. Nucleotides sequence contigs were assembled using the SEQUENCER^{TM} program. The BLASTX results each randomized contigs show 98% similarity to *K. pnumoniae* MGH78578 as shown in Figure 5.6.

Table 5.7 Screening of 3-HPA producing bacteria by glycerol utilization test under aerobic and static condition and Fehling test

Bacterial isolate	**Glycerol utilization**		**Fehling test**	**3-HPA formation**	**Bacterial identification**
	Aerobic	**Static**			
GF37j1	++++	++	ND	ND	ND
GF37j2	+++	+	ND	ND	ND
GF37j3	++++	+	ND	ND	ND
GF37j4	+++	+	ND	ND	ND
GF37j5	++	+	ND	ND	ND
GF37j6	+++	+++	+	-	*Klebsiella pneumoniae*
GF37j7	++++	++++	+	+	*Klebsiella pneumoniae*
GA37H1	++++	++++	+	-	*Klebsiella pneumoniae*
GA37H2	+++	+++	+	-	*Klebsiella pneumoniae*
GA37H3	++++	++	ND	ND	ND
GA37H4	++++	++	ND	ND	ND
GA37H5	++++	++	ND	ND	ND
GA37H6	++++	+++	+	-	*Klebsiella pneumoniae*
GF37y1	++	+	ND	ND	ND
GF37y2	+++	++	ND	ND	ND
GF37y3	++++	++++	+	-	*Klebsiella pneumoniae*
GF37y4	++++	++++	+	-	*Klebsiella pneumoniae*

Glycerol utilization was represented by +/-; +, $0.2 \leq$ OD600 <0.5; ++, $0.5 \leq$ OD600 <1.0; +++, $1.0 \leq$ OD600< 1.5; ++++, OD600>1.5. Fehling test and 3-HPA formation were represented by +, positive; ND, not determined. The bolded isolated was the bacterial isolate chosen for further study.

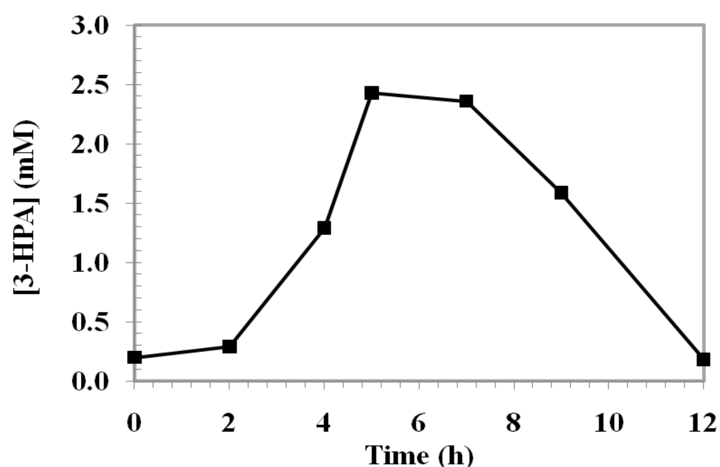


Figure 5.5 3-HPA productions by *K. pneumoniae* GF37j7 under aerobic condition.

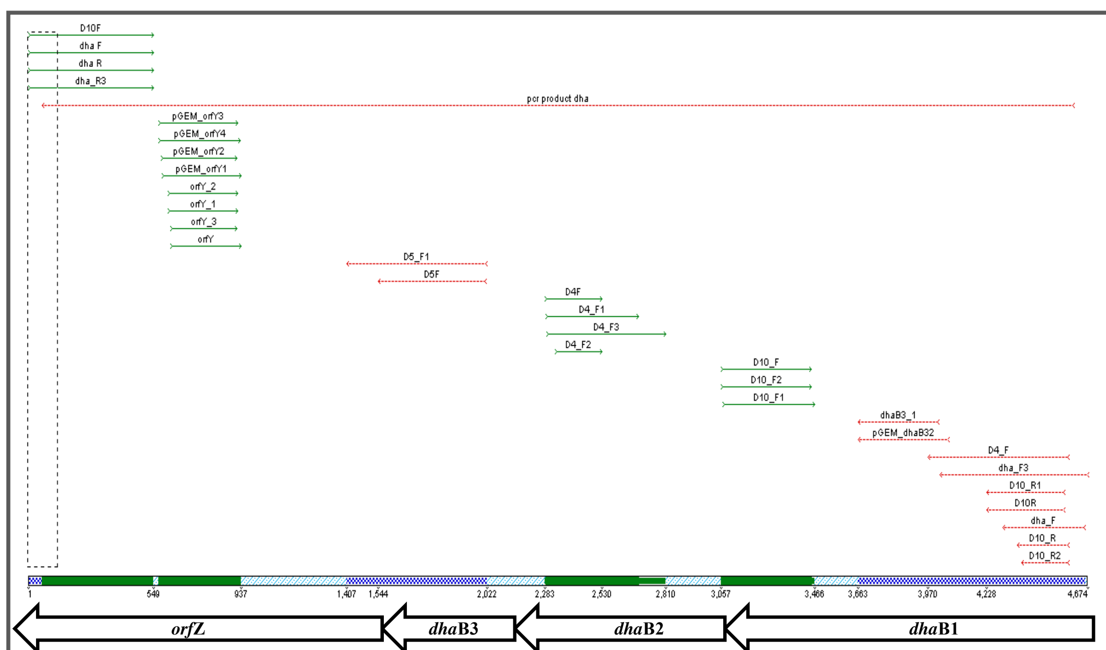


Figure 5.6 Contigs of *dha*B nucleotide sequencing from *K. pneumoniae* GF37j7 were assembled using the SEQUENCHER™ program.

5.7 Construction an expression vector for 3-HPA production by OST Gram positive *B. subtilis* BKS2-P2/3

As the determination of promoter system, an expression pHY300PLK contained PXyl promoter (referred as pHPxyl) showed significant *β*-galactosidase activity in hydrophilic OST Gram positive *B. subtilis* BKS2-P2/3, and then it was selected as an expression vector for 3-HPA production.

5.7.1 Gene cloning and factors involving in 3-HPA production by OST Gram positive *B. subtilis* BKS2-P2/3

Various factors were affected expression of the heterologous proteins in Gram positive bacteria such as promoter strength, which involve in transcription step; ribosome binding site (RBS) and its distance; that show important role in translation of mRNA. In this study, the improvement of transcription level, the pHPxyl showed high promoter strength in BKS2-P2/3 was selected as an expression vector. In addition, there is less information of ribosome binding site (RBS) and its distance in Gram positive bacilli. In this study, three RBS and its distance was used for enhance *dha*B gene cluster translation in BKS2-P2/3 as shown in Table 5.8.

Table 5.8 Various types of RBS and its distance from Gram positive bacteria used in this study.

RBS	**Sequence**	**Distances**	**Start codon**	***dha*B gene name**	**Description/ Reference**
RBS	**AGGAGG**	ACAGCT	ATG	**KJdhaB/KAdhaB /or CdhaB**	Conserve RBS
RBS1	AAA**GCGTGGTG**	TTTTT	ATG	**KJ1dhaB/or KA1dhaB /or C1dhaB**	*B. subtilis* RBS (Shaw et al., 2005)
RBS2	AAA**GCGTGGTG**	TTTTTTT	ATG	**KJ2dhaB/or KA2dhaB /orC2dhaB**	

5.7.2 Construction of *dha*B genes cluster involved in 3-HPA production by OST Gram positive *B. subtilis* BKS2-P2/3

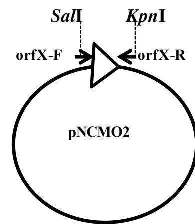
The *dha*B gene containing various types of RBS from *K. pneumoniae* GF37j7 (*dhaB*1, *dhaB*2, *dhaB*3, *orf*Z; referred as KJdhaB), *K. pneumoniae* ATCC13883 *(dhaB*1, *dhaB*2, *dhaB*3, *orf*Z; referred as KAdhaB), and *C. butyricum* ATCC13983 (dhaB1, dhaB2; referred as C-dhaB) as shown in Table 5.9 were amplified using primer in Table 3.2.

The construction of an expression vector containing KJdhaB, KAdhaB, and CdhaB gene, respectively was described as Figure 5.7 and 5.8, respectively. Briefly, in Figure 5.7, the KJdhaB /or KAdhaB fragments (4.5 kb) containing various types of RBS with *Kpn*I and *Eco*RI restriction sites were ligated in the pHPxyl plasmid (referred as pHPxyl-KdhaB). The resulting plasmid is referred as pHPxyl-KJdhaB to pHPxylKK2dhaB, pHPxyl-KAdhaB to pHPxylKA2dhaB, and pHPxylCdhaB to pHPxylC2dhaB, respectively as Table 5.9. An expression vector was constructed with a tetracycline resistance cassette-containing vector pHPxyl with xyl promoter.

The expression plasmids were transformed into Gram positive OST *B. subtilis* BKS2-P2/3 by electroporation technique. The bacteria strains and plasmids used in this study are presented in Table 5.9.

Table 5.9 Strains and plasmids used in this work.

Strains and plasmids	Description	Source
Strains		
C. butyricum ATTC13983	Source for C-*dha*B	Provided from Prof. Kato
*K. pneumonia*e ATCC13883	Source for KA-*dha*B	Provided from Prof. Kato
*K. pneumonia*e GF37j7	Source for KJ-*dha*B	This study
E. coli DH5α	Cloning host	Novagen
B. subtilis BKS2-P2/3	Expression host	This study
Plasmids		
pBluescript II SK(-)	Cloning vector; Amp^r	Stratagene
pHY300PLK	*E. coli-Bacillus* shuttle vector; Expression vector; Tet^r	(Ishiwa and Shibahara 1985)
KJdhaB gene	*dha*B gene cluster from *K. pneumonia*e GF37j7	This study
pHPxyl KJdhaBX	KJ*dha*B::*orf*X gene containing RBS in pHPxyl	Further study
pHPxyl KJ1dhaBX	KJ*dha*B::*orf*X gene containing RBS1 in pHPxyl	Further study
pHPxyl KJ2dhaBX	KJ*dha*B::*orf*X gene containing RBS2 in pHPxyl	Further study
KAdhaB gene	*dha*B gene cluster from *K. pneumonia*e ATCC13883	This study
pHPxyl KAdhaBX	KA*dha*B:: *orf*X gene containing RBS in pHPxyl	Further study
pHPxyl KA1dhaBX	KA*dha*B::*orf*X gene containing RBS1 in pHPxyl	Further study
pHPxyl KA2dhaBX	KA*dha*B::*orf*X gene containing RBS2 in pHPxyl	Further study
CdhaB gene	*dha*B gene cluster from *C. butyricum* ATTC13983	This study
pHPxyl CdhaBX	C*dha*B:: *orf*X gene containing RBS in pHPxyl	Constructed in this study
pHPxyl C1dhaBX	C*dha*B::*orf*X gene containing RBS1 in pHPxyl	Constructed in this study
pHPxyl C2dhaBX	C*dha*B::*orf*X gene containing RBS2 in pHPxyl	Constructed in this study



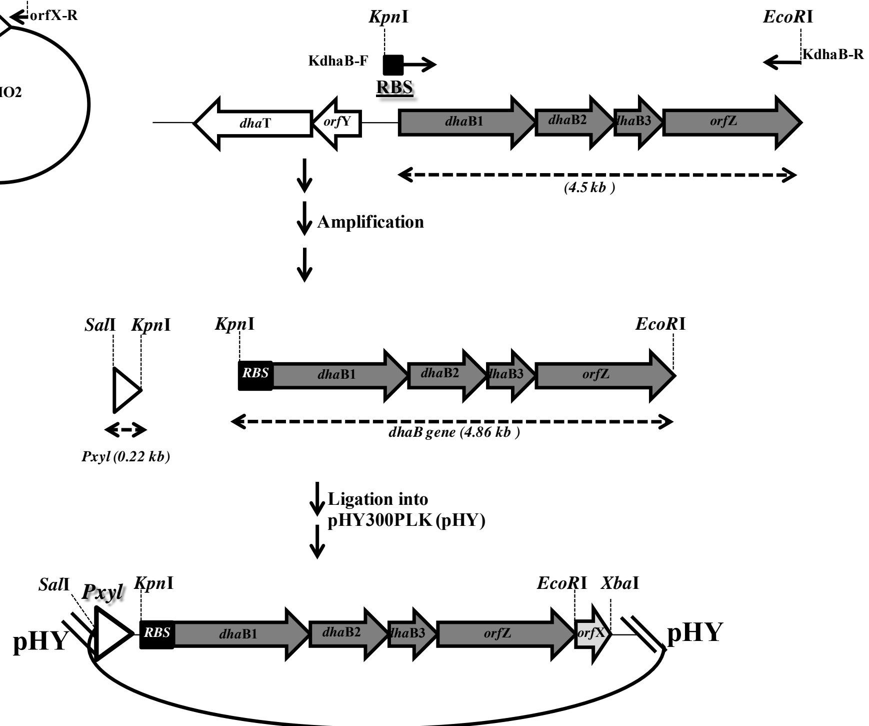
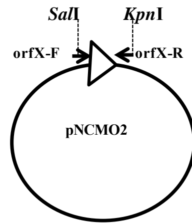


Figure 5.7 Construction of an expression vector for *B. subtilis* BKS2-P2/3 using *dha*B gene from *K. pneumoniae*.

Preparation of Pxyl promoter

Preparation of *dha*B gene

Chromosomal DNA from *C. Butyricum* ATCC13883



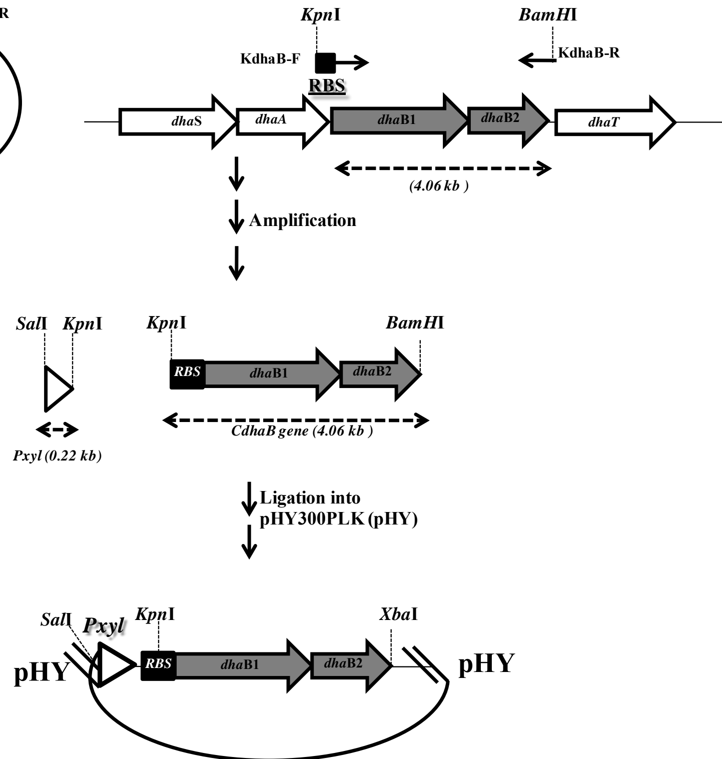


Figure 5.8 Construction of an expression vector for *B. subtilis* BKS2-P2/3 using *dha*B gene from *C. butyricum.*

5.7.3 Gene expression and 3-HPA production from OST Gram positive *B. subtilis* BKS2-P2/3

After being transformed constructed plasmid from Table 5.9 into *B. subtilis* BKS2-P2/3, an expression vector were expressed under the control of xyl promoter within the vector pHY300PLK. Only an expression vector harboring *dha*B gene with various types of RBS from *C. butyricum* (CdhaB) was success from the tranformation in *B. subtilis* BKS2-P2/3. The recombinant *B. subtilis* BKS2-P2/3 was determined gene expression.

(1) *Protein expression*: as Figure 5.9 the SDS-PAGE analysis of crude protein fractions of recombinant *B. subtilis* BKS2-P2/3 harboring pHPxyl-CdhaBX, pHPxyl-C1dhaBX, and pHPxyl-C2dhaBX, respectively, after induction with 0.1% (w/v) xylose in LB for 4 h. Glycerol dehydratase protein from *C. butyricum* was about 85 and 35 kD (indicated by arrow), as the SDS-PAGE results of crude protein fraction did not detect protein molecular mass corresponded to glycerol dehydrates protein.

(2) *Enzyme activity assay*: according to previous reports of B12-independent glycerol dehydratase from *C. butyricum* was sensitive to oxygen, and then it is difficult to handle this test. Therefore B12-independent glycerol dehydratase from *C. butyricum* in recombinant *B. subtilis* BKS2-P2/3 did not determined.

(3) *3-HPA formation*: To evaluate the potential of *B. subtilis* BKS2-P2/3 recombinant for 3-HPA production, the recombinant strains were cultivated with anaerobic condition in BS medium at pH 8.0 under batch experiment. Production of 3-HPA was investigated at 37ºC from 500 mM glycerol, 1% (w/v) xylose at cell density OD_{600} of 0.7-0.9 (Figure 5.10). The result shown that 3-HPA production by

the heterologous expression of *B. subtilis* BKS2-P2/3 provided less concentration of 3-HPA at 0.068 mM.

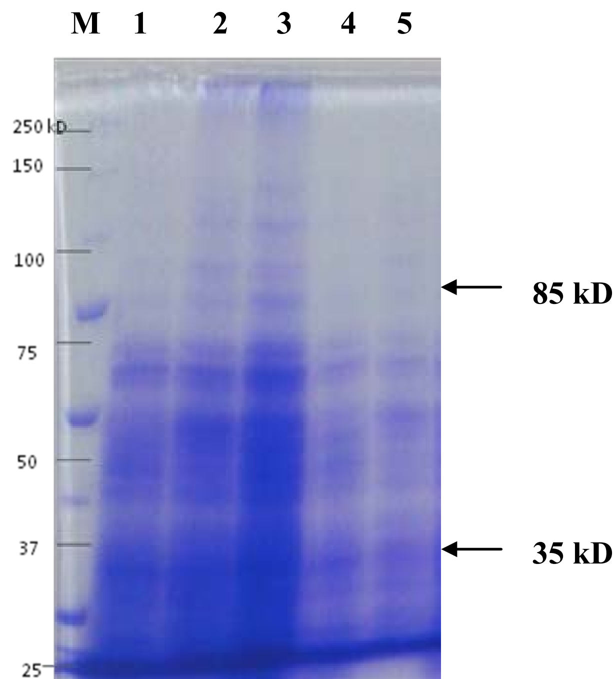


Figure 5.9 Protein over-expression profile of glycerol dehydratase (indicated by arrow) from *C. butyricum* of recombinant BKS2-P2/3; lane 1 (wild type), lane 2 (pHPxyl), lane 3 (pHPxyl-CdhaB), lane 4 (pHPxyl-C1dhaB, and lane 5 (pHPxyl-C2dhaB).

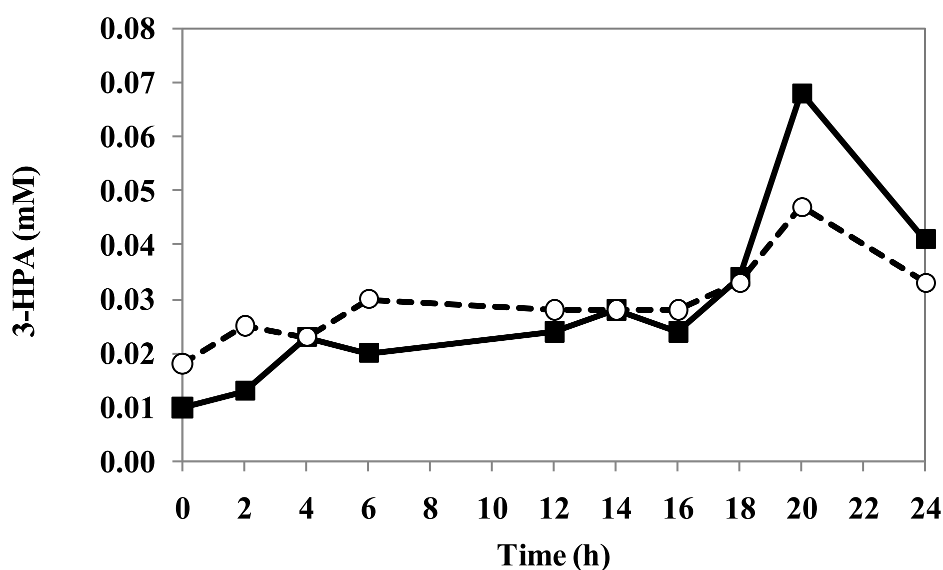


Figure 5.10 Bioproduction of 3-HPA from recombinant BKS2-P2/3 harboring pHPxyl-CdhaB (■) and pHPxyl empty vector (○), respectively.

5.8 Construction an expression vector for 3-HPA production by OST Gram negative *P. putida* T-57

Development of a transformation system and gene manipulation method of the selected OST Gram positive bacteria as a gene expression host for 3-HPA production was investigated at the beginning of this study. However, the recombinant Gram positive OST bacteria produced low concentration of 3-HPA from glycerol.

Due to the limitation of Gram positive OST bacteria, Gram negative OST bacterium was chosen for production of 3-HPA, which well know in genetic manipulation and transformation system.

OST Gram-negative *P. putida* T-57

As screening and isolation of OST bacteria results in this study, all of OST bacteria obtained as Gram positive. Various reports have investigated Gram negative OST bacteria, especially *Pseudomonas* and *E. coli*, and focus on their tolerance to hydrophobic organic solvents. However, *P. putida* T-57 was isolated and characterized as OST bacteria, and exhibited high tolerance to various types of organic solvent with log P_{ow} range from 0.8 to 5.6 (Faizal et al., 2005). According to advantages of Gram negative bacteria, Gram negative OST bacteria *P. putida* T-57 was chosen for 3-HPA production. Gram negative OST *P. putida* T-57 was kindly provided by Professor Dr. Junichi Kato, Hiroshima University, Japan.

5.8.1 Gene cloning and factors involving in 3-HPA production by OST Gram negative *P. putida* T-57

As gene selection section, *C. butyricum* ATCC13983 and two strain of *K. pneumoniae* ATCC13883 and GF37j7, respectively, were chosen as gene sources for 3-HPA production by hydrophilic OST Gram positive *B. subtilis* BKS2-P2/3. Because *C. butyricum* glycerol dehydratase enzyme are difficultly handle in aerobic condition, then the glycerol dehydratase and its reactivate encoding *dha*B and *orf*X gene, respectively were amplified and cloned to *P. putida* T-57 as gene source from *K. pneumoniae*.

5.8.2 Construction of *dha*B genes cluster involved in 3-HPA production by OST Gram negative *P. putida* T-57

Many factors affecting expression of the heterologous proteins in Gram positive OST bacteria as described before such as copy number, RBS sequence. It has been generally observed that RBS properly located 5' before AUG initiation codon, plays an important role in formation of the initiation complex. The RBS sequence search priority is base on the following six variations: GGAGG, GGAA, GGAG, GAGG, AGGA, and AAGG (Chang et al., 2006). Aligned spacing of the SD sequences generally varies from 5 to 13 bases, with optimal spacing of about 8 to 10 bases for *E. coli* (Paik et al., 2006). Therefore variations on the consensus sequence of RBS with optimal spacing of about 9 bases for Gram negative and Gram positive of was investigated in this study.

The *dha*B (*dhaB*1, *dhaB*2, *dhaB*3, *orf*Z) genes containing various types of RBS (referred as K-dhaB) as in Table 5.10 were amplified from *K. pneumoniae* ATCC 13883. *K. pneumonia* GF37j7 was used as a source of *orf*X gene; it was amplified separately with native RBS because of location in the reverse orientation to *dha*B genes cluster in the *K. pneumoniae* genome. Primers used in this work are shown in Table 3.2.

The construction methods were similar to that for Gram positive method (as described in 5.7.2). Briefly, in Figure 5.11, the KdhaB fragment (4.5 kb) with *Kpn*I and *Eco*RI restriction sites, and the *orf*X (0.36 kb) fragment with *Eco*RI and *Xba*I restriction sites were ligated in the pBluescript II SK (-) plasmid (referred as pBSKdhaBX). The fragment KdhaBX (*dhaB*1, *dhaB*2, *dhaB*3, *orf*Z, and *orf*X) gene with various types of RBS (Table 5.10) were amplified from the pBSKdhaBX

plasmid by PCR using primer in Table 3.2 with *Kpn*I and *Xba*I restriction site, respectively. An expression vector was constructed with a kanamycin resistance cassette-containing vector pUCP18 and pMEPT with lac and tac promoter, respectively. The resulting plasmid is referred to as pUKdhaB to pUK10dhaB and pMKdhaB to pMK10dhaB, respectively as Table 5.11.

The expression plasmids were transformed into Gram negative OST *P. putida* T-57 by electroporation technique. The bacteria strains and plasmids used in this study are presented in Table 5.11. An expression vector of pUCP18 (pUKdhaBX, pUK1dhaBX, and pUK2dhaBX) and pMEPT (pMKdhaBX, pMK1dhaBX, and pMK2dhaBX) were constructed in this study.

Table 5.10 Various types of RBS and its distance from Gram negative and positive bacteria used in this study.

RBS	**Sequence**	**Distances**	**Start codon**	***dha*B gene name**	**Description/Reference**
RBS	**AGGAGG**	ACAGCT	ATG	**KdhaB**	Conserve RBS
RBS1	AAA**GCGTGGTG**	TTTTT	ATG	**K1dhaB**	*B. subtilis* RBS
RBS2	AAA**GCGTGGTG**	TTTTTTT	ATG	**K2dhaB**	(Shaw et al., 2005)
RBS3	**GAGC**	CGATGAACA	ATG	**K3dhaB**	*K. pneumonia dha*B RBS
RBS4	**AGGAGG**	CGATGAACA	ATG	**K4dhaB**	Conserve RBS
RBS5	**GGAGG**	CGATGAACA	ATG	**K5dhaB**	
RBS6	**GGAA**	CGATGAACA	ATG	**K6dhaB**	
RBS7	**GGAG**	CGATGAACA	ATG	**K7dhaB**	(Chang et al., 2006)
RBS8	**GAGG**	CGATGAACA	ATG	**K8dhaB**	
RBS9	**AGGA**	CGATGAACA	ATG	**K9dhaB**	
RBS10	**AAGG**	CGATGAACA	ATG	**K10dhaB**	

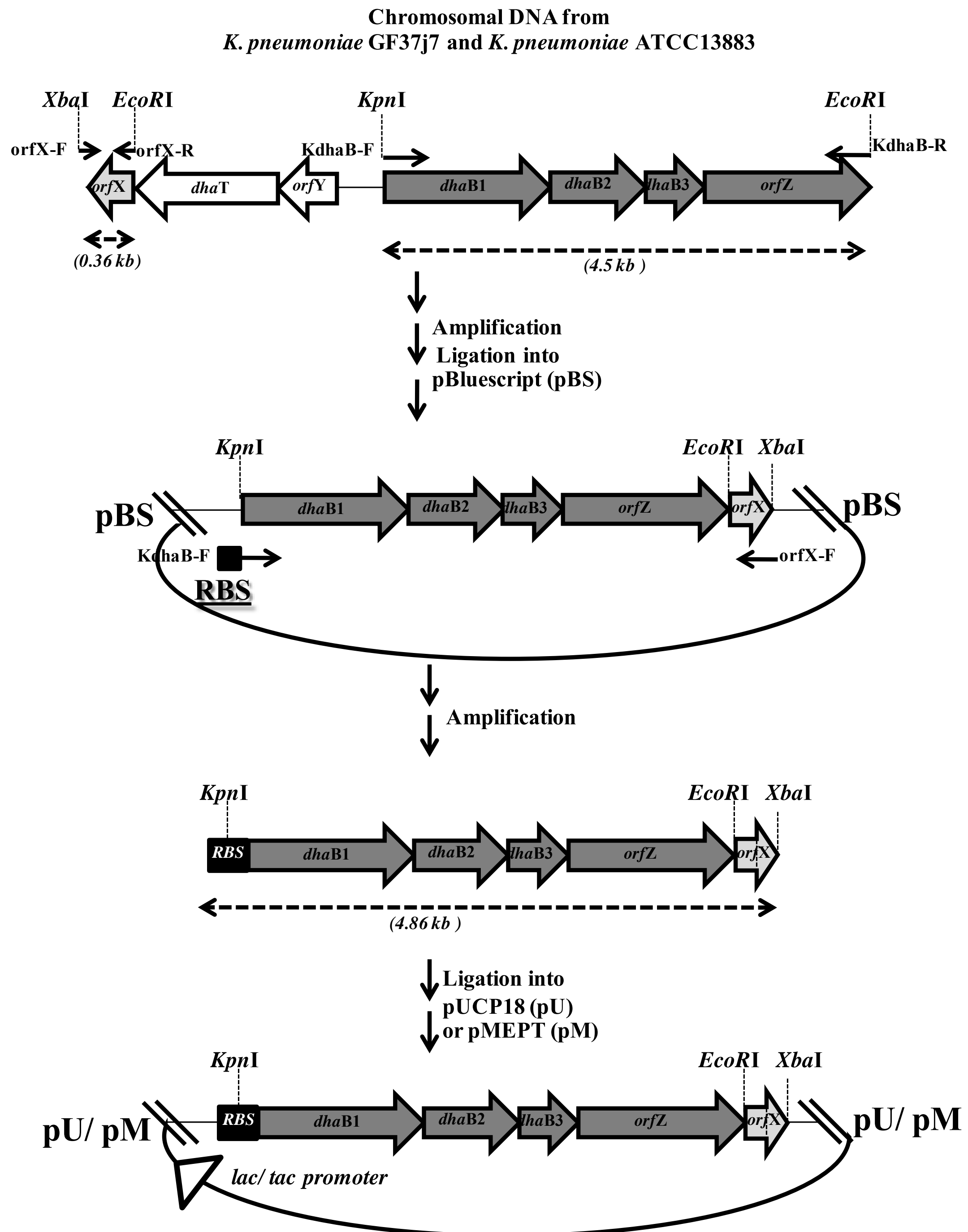


Figure 5.11 Cloning of *dha*B123::*orf*Z and *orf*X gene (KdhaBX) for Gram negative OST bacteria *P. putida* T-57.

Table 5.11 Strains and plasmids used in this work.

Strains and plasmids	Description	Source
Strains		
K. pneumoniae ATCC13883	Source for K-*dha*B	ATCC
K. pneumoniae GF37j7	Source for K-*dha*B and *orf*X	This study
E. coli DH5α	Cloning host	Novagen
P. putida T-57	Expression host	Provided from Prof. Kato
Plasmids		
pBluescript II SK(-)	Cloning vector	Stratagene
pUCP18	Expression vector; lac promoter, Amp^r, Km^r	
pUKdhaBX	K-*dha*B::*orf*X gene containing RBS in pUCP18	Constructed in this study
pUK1dhaBX	K-*dha*B::*orf*X gene containing RBS1 in pUCP18	Constructed in this study
pUK2dhaBX	K-*dha*B::*orf*X gene containing RBS2 in pUCP18	Constructed in this study
pUK3dhaBX to pUK10dhaBX	K-*dha*B:: *orf*X gene containing RBS3 to RBS10 in pUCP18	Further study
pMEPT	Expression vector; tac promoter, Amp^r, Km^r	
pMKdhaBX	K-*dha*B:: *orf*X gene containing RBS in pMEPT	Constructed in this study
pMK1dhaBX	K-*dha*B:: *orf*X gene containing RBS1 in pMEPT	Constructed in this study
pMK2dhaBX	K-*dha*B:: *orf*X gene containing RBS2 in pMEPT	Constructed in this study
pMK3dhaBX to pMK10dhaBX	K-*dha*B:: *orf*X gene containing RBS3 to RBS10 in pMEPT	Further study

5.8.3 Gene expression and 3-HPA production from OST Gram negative *P. putida* T-57

(1) Protein expression

After being transformed into *P. putida* T-57, *dha*B gene of KdhaBX, K1dhaBX, K2dhaBX contained RBS, RBS1, and RBS2 sequence as in Table 5.11 were expressed under the control of lac and tac promoter within the vector pUCP18 and pMEPT. In Figure 5.12 the SDS-PAGE analysis of crude protein fractions of *P. putida* T-57 harboring pUCP18- KdhaBX, pUCP18- K1dhaBX, and pUCP18-

K2dhaBX, respectively, after induction with 1 mM IPTG in LB for 4 h. The SDS-PAGE results of pUMEPT- KdhaBX, pUMEPT- K1dhaBX, and pUMEPT - K2dhaBX, respectively, were similar to the an expression vector pUCP18 described.

The SDS-PAGE results of crude protein fraction from pUCP18 (pUKdhaBX to pUK2dhaBX) indicated that the molecular mass of the expressed recombinant product was about 64 kDa, 20 kDa, and 14 kDa (indicated by an arrow), which corresponded to the glycerol dehydratase protein as reported in previous literature (Wang et al., 2007). All six recombinants showed the low protein expression.

(2) Enzyme activity assay

Glycerol dehydratase activity in recombinant *P. putida* T-57 strains containing pUKdhaB, pUK1dhaB, pUK2dhaB, pMKdhaB, pMK1dhaB and pMK2dhaB were assayed and were shown in Figure 5.13. The recombinant strains were grown in LB medium at 30ºC shaking at 150 rpm. The cultures were induced with different concentrations of IPTG (0. 0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 mM) at 0.4-0.6 OD_{600} for 4 h. The KdhaBX activities of various types of RBS in an expression vector of pUCP18 and pMEPT were determined using method reported from Raj *et al.* and Rathasingh *et al.,* (Raj et al., 2008; Rathnasingh et al., 2009) with slight modifications. Briefly, glycerol dehydratase activity was initiated by adding 50 mM glycerol and 15 µM coenzyme B12, and then incubated at 37ºC. The reaction was terminated after 1 min by adding an equal volume of 100 mM citric acid. 3-HPA formed in the reaction mixture was reacted with tryptophan solution to form a purple complex. The purple complex was measured colorimatrically at an absorbance of 560 nm. It was shown no difference in glycerol dehydratase activity from both of all recombinant strains in LB under various concentrations of IPTG for 4 h. It is possible that the heterologous

enzyme expression activity may depend on various factors such as concentration of inducer, stage of growth before induced with inducer, and induction time. Glycerol dehydratase activity of *P. putida* T57 recombinant was shown at low level, which may because the enzyme activity was detected under un-suitable condition.

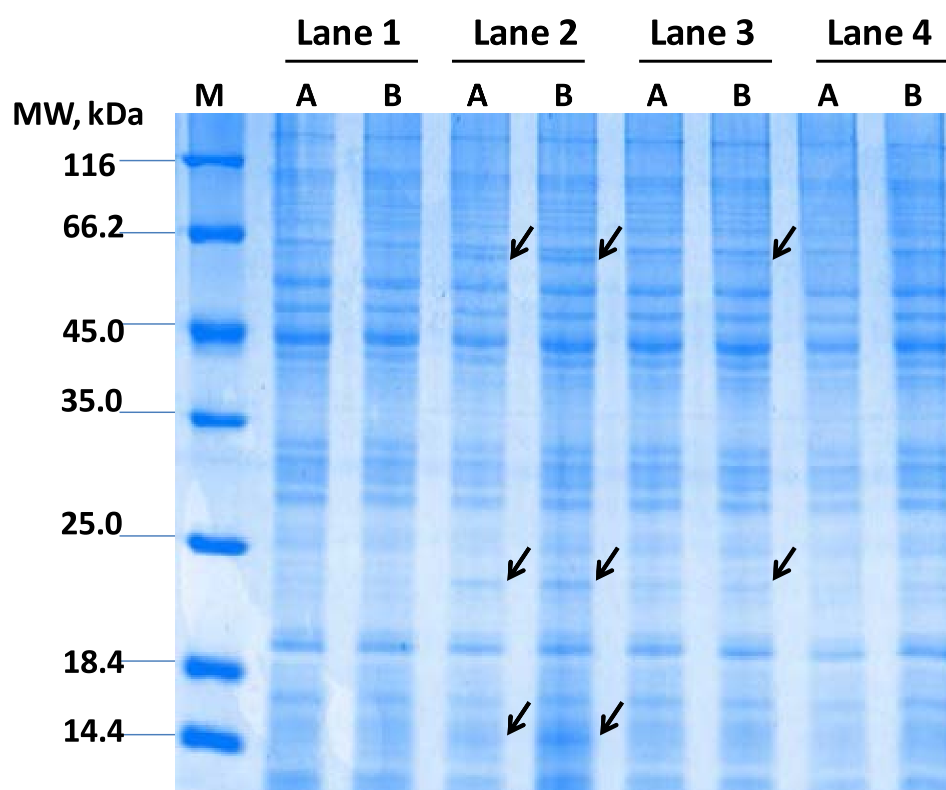


Figure 5.12 SDS-PAGE analysis of the expressed recombinant protein from the recombinant *P. putida* T-57 harboring pUCP18; pUKdhaBX (lane2A), pUK1dhaBX (lane3B), and pUK2dhaBX (lane4A), respectively, the recombinant protein from an expression vector pMEPT; pMKdhaBX (lane2B), pMK1dhaBX (lane3B), and pMK2dhaBX (lane4B), respectively. The empty vector of pUCP18 (lane1A) and pMEPT (lane1B) were used as control of protein expression.

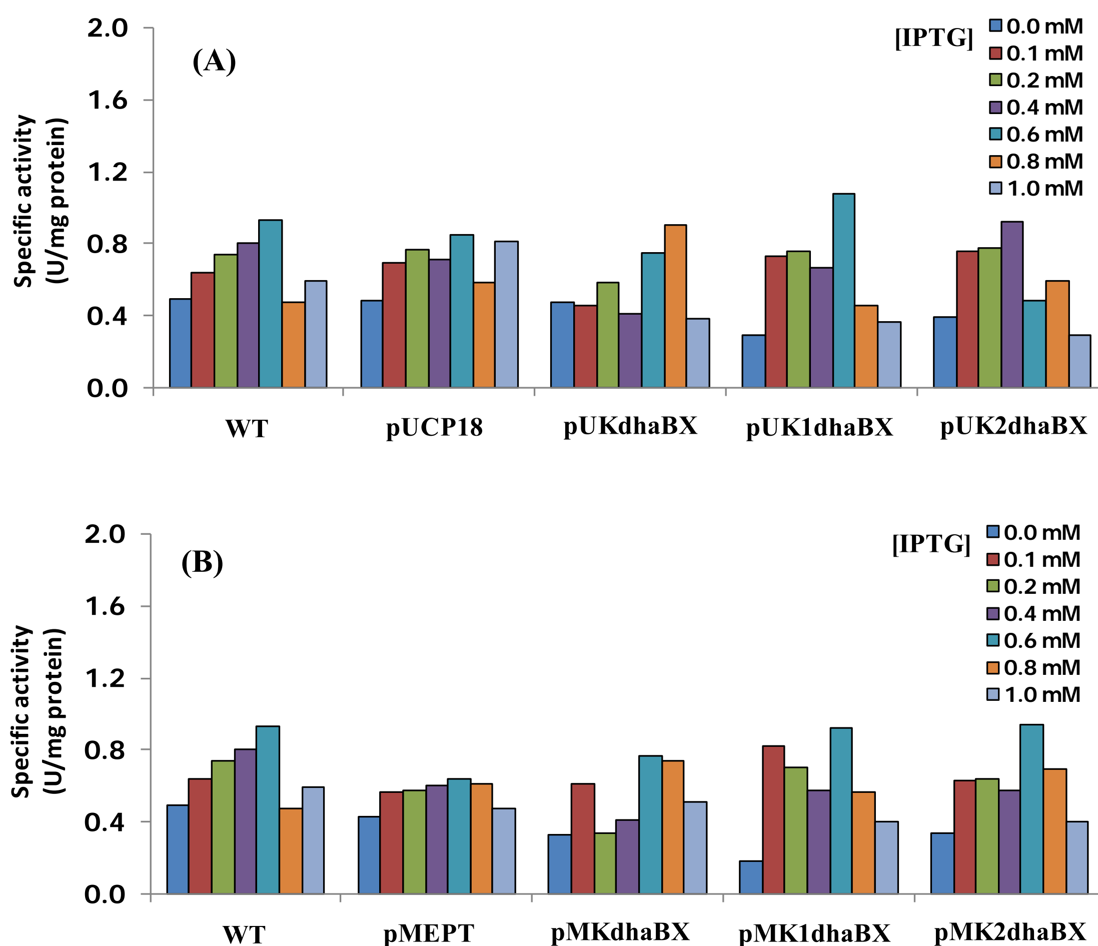


Figure 5.13 Specific activity of glycerol dehydratase of the recombinant *P. putida* T-57 after induction with various concentrations of IPTG (0-1.0 mM) for 4 h.

(3) 3-HPA bioconversion from glycerol

To evaluate the potential of *P. putida* T-57 recombinants for 3-HPA production, the recombinant strains were cultivated aerobically in BS medium at pH 8.0 under batch experiment. Production of 3-HPA was produced at 37ºC from 500 mM glycerol, 48 µM co-enzyme B12 and 0.1 mM IPTG at difference stage of growth at cell density OD_{600} of 0.4 to 0.6 and 0.7-0.9 (Figure 5.14). The result shown that 3-

HPA production by the heterologous expression of *P. putida* T-57 provided a maximum concentration of 3-HPA at 0.84 mM from cell density OD_{600} of 0.7-0.9 and 0.68 mM from cell density of 0.4-0.6 with the recombinant plasmid pMKdhaBX and pUKdhaBX, respectively.

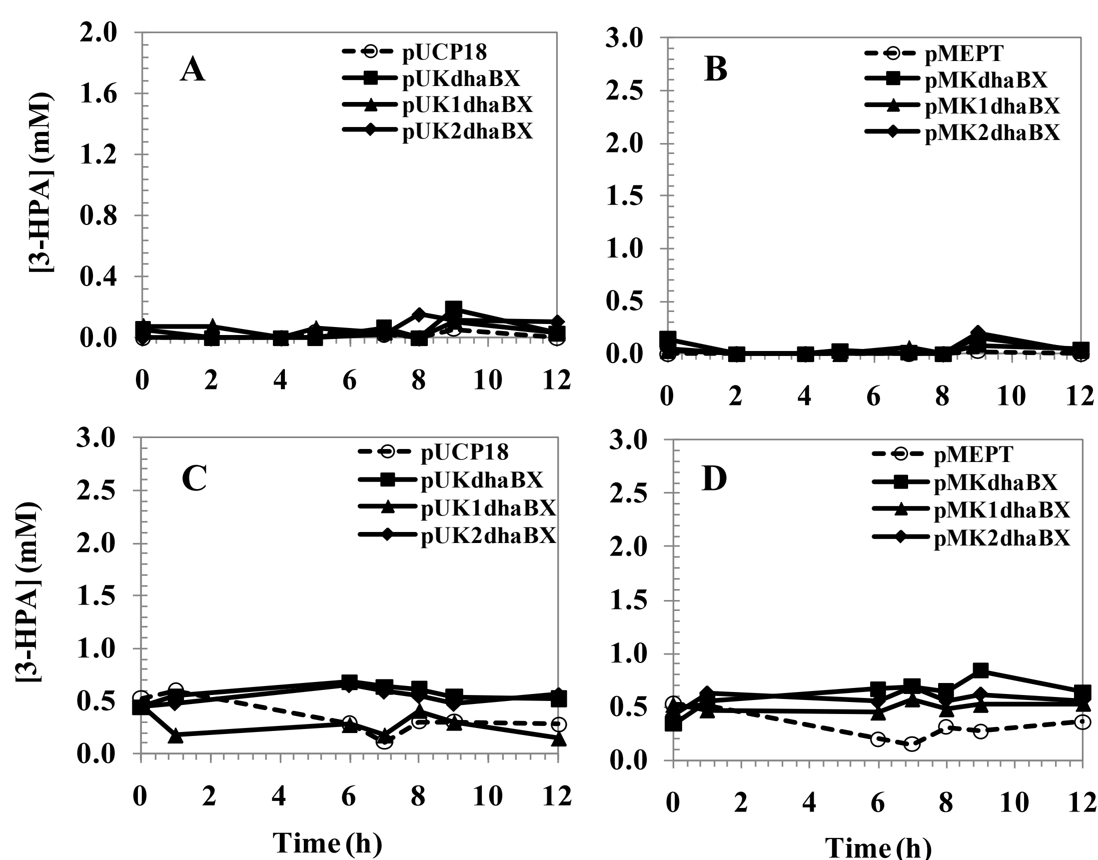


Fig 5.14 3-HPA production from glycerol of recombinant *P. putida* T-57 with cell density OD_{600} of 0.4 to 0.6 (A, B) and 0.7 to 0.9 (C, D) from the recombinant plasmid pUKdhaBX to pUK2dhaBX (A. C) and pMKdhaBX to pMK2dhaBX (B, D), respectively.

Characterization of hydrophilic OST bacteria

5.9 Characterization of hydrophilic OST Gram positive *Exiguobacterium* sp. SBH81

Bacterial isolate SBH81 was obtained from 0.1% (v/v) butanol enrichment at 45°C of seawater samples. The isolate SBH81 had only moderate butanol tolerance but that it was highly tolerant to relatively hydrophilic organic solvents such as propanol, propionaldehyde, acetonitrile and dimethylsulfoxide. As this unique property, it was chosen for further study. The strain SBH81 is Gram-positive bacteria, non spore-forming, and rod shape, motile and formed orange-pigmented, spherical colony on the LB medium. Beside, amylase/protease/cellulose was found positive. It was identified by biochemical analysis as *Exiguobacterium acetylicum* and the 16S rRNA gene sequence analysis indicated that strain SBH81 is *Exiguobacterium* sp. The genus *Exiguobacterium* had been isolated from wide range habitats including hot and cold environments which included psychrotrophic, mesophilic and moderate thermophilic species (Vishnivetskaya and Kathariou, 2005). In addition, it was reported as salt tolerant (Tan et al., 2009) and alkaliphilic bacteria (Joshi et al., 2008). While *Exiguobacterium* sp. strain SBH81 was able to grow at high temperature ranging from 20-50°C but no growth at 5°C; broad range of pH ranging from pH 4-12 and salt content from 0-10% (w/v) (Figure 5.15). The optimum growth was determined with a specific growth rate at 40°C; pH 8-9 and salt concentration 0-6 % (w/v) in LB medium having specific growth rate 0.29, 0.21 and 0.22 h^{-1} respectively. It was suggested that *Exiguobacterium* sp. strain SBH81 was a moderately thermo-tolerant, alkaliphilic and halo-tolerant bacterium.

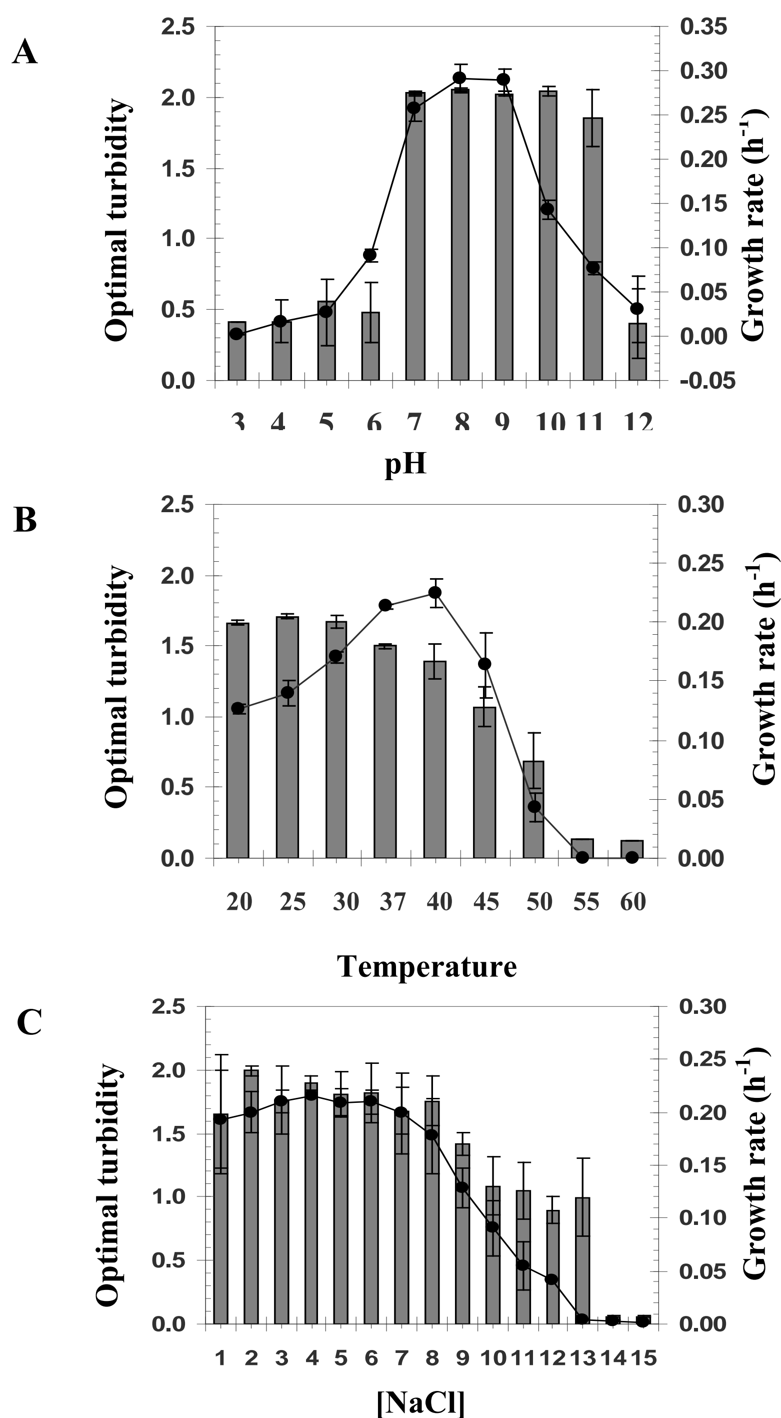


Figure 5.15 Optimal growth condition of *Exiguobacterium* sp. SBH81. LB medium was also used for determining growth at different pH values (3-12) (A), at various temperatures (5-60°C) (B), and in the presence of various concentration of NaCl (0-13 %) (C). The optimal growth and growth rate represented in bar graph and line graph, respectively. Data are means of the results from at least three individual experiments.

5.9.1 Organic solvent tolerance and utilization

The log P_{ow}, which represents the ability of a solvent's partition coefficient in a standard 1-octanol water mixture, a reliable index for the toxicity of solvents (Inoue and Horikoshi, 1991). Previous reports have reported that the log P_{ow} between 1.0-4.0 (Sardessai and Bhosle, 2002) or 1.5-4.0 (Ramos et al., 2002), which toxic for microorganisms. To investigate solvent tolerance ability of *Exiguobacterium* sp. strain SBH81, cells were grown until mid-log phase after that 5-10 % (v/v) of various organic solvents was added directly in the culture medium. Cell viability was examined when it was exposed to solvents with different log P_{ow} values. However, the results were demonstrated that *Exiguobacterium* sp. strain SBH81 exhibited tolerance towards various types of solvent with a broad range of log P_{ow} value (Table 5.12). The log P_{ow} more than 3.0 such as decane, heptane, octane, decanol, hexane, diethylphathalate and lower than 1.0 such as propanol, diethyl ether, acetonitrile, ethanol, methanol, demethylformamide and diehyl sulfoxide were less toxic for *Exiguobacterium* sp. strain SBH81. While it exhibited low tolerance to log P_{ow} between 3.0- 1.0 such as benzene and 1-butanol and weakly tolerance to cyclohexane and xylene.

The previous reported *Exiguobacterium gaettbuli* strain LJ8 was screened from seawater could tolerant to benzene, toluene, ethylbenzene and *p*-xylene (Wang et al., 2008). In contrast, *Exigoubacterium* sp. SBH81 could not grow in the present of toluene and ethylbenzene. Organic solvent tolerance is believed to be a strain specific proper and its own intrinsic tolerance level for organic solvents and is also influenced by environmental factors (Huertas et al., 1998; Kobayashi et al., 1998).

In addition, studies of genus *Exiguobacterium* mainly focused on the characteristics of resistance to extreme conditions (Rodrigues et al., 2009; Rodrigues et al., 2008; Vishnivetskaya et al., 2007) and the environmental pollutant treatment (Alam and Malik, 2008; Anderson and Cook, 2004; Lopez et al., 2005). There has been no report on solvent tolerant by genus *Exiguobacterium*, except *Exiguobacterium gaettbuli* (Wang et al., 2008). Therefore, *Exigoubacterium* sp. SBH81 tolerant to broad range of organic solvents was reported.

OST bacteria have been more reported in Gram negative such as *Pseudomonas putida* and *Enterobacter aerogenes* (Neumann et al., 2005a) and Gram positive bacteria such as *Rhodococcus opacus*, *Dienococcus geothermalis* and *Brevibacillus agri* (Kongpol et al., 2008; Kongpol et al., 2009; Na et al., 2005) at various types and log P_{ow} values. There is few reports have been focused on log P_{ow} value lower than 1.0 especially acetonitrile, which nitrile is stable chemical and most nitriles are high toxic compounds (Sorokin et al., 2007). Nonetheless, it wildly used for synthesis of amines, amides, carboxylic acids, esters, aldehydes and ketones compounds (Banerjee and Sharma, 2002) and also widespread in the environment.

The ability of *Exiguobacterium* sp. SBH81 to tolerate of acetonitrile was determined by demonstrating the ability of cells grow at 37°C in LB medium pH 8.0 in the presence of different concentrations of acetonitrile. It was found that, *Exiguobacterium* sp. SBH81 could tolerate acetonitrile at 6% (v/v), but not at 7% (v/v) (Figure 5.16). Furthermore, the ability of *Exiguobacterium* sp. SBH81 to utilize was also investigated in MSB medium at 37°C in the presence of acetonitrile vapor. The results demonstrated *Exiguobacterium* sp. SBH81 utilized acetonitrile as a sole carbon and nitrogen source (Figure 5.17).

To date, acetonitrile utilization has been reported in the *Paracoccus* sp. SKG and *Serratia marcescens* MSK1 Santoshkumar *at el*., 2010), *Natronocella acetinitrlica* (Sorokin et al., 2007), *Rhodococcus pyridinivorans S85-2* and *Brevundimonas diminuta AM10-C-1* (Kohyama et al., 2006), *Comamonas* sp. (Manolov et al., 2005), *Pseudomonas pseudoalcaligenes* (Luque-Almagro *at el*., 2005) and *Rhodocuccus erytropolis* (Brandao *at el*., 2003), but it was poor reported about acetonitrile tolerance and limited investigation towards understanding the mechanisms of acetonitrile tolerance.

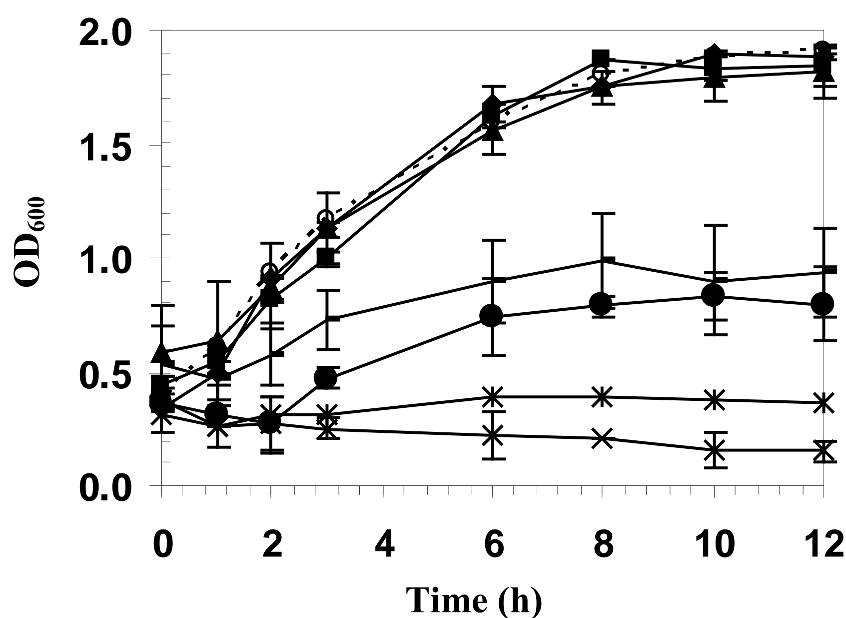


Figure 5.16 Acetonitrile tolerant of *Exiguobacterium* sp. SBH81. Cells were grown in LB medium supplement with acetonitrile at various concentration (◆, 1%; ■, 2%; ▲, 3%; ●, 5%; ▪, 6%; ✶, 7%; ×, 10% (v/v)) compared with absence of solvent (-○-). Data are means of the results from at least four individual experiments.

Table 5.12 Solvents tolerance of *Exiguobacterium* sp. SBH81

		Cells survival (CFUs)			
Organic solvents	**Log P_{ow}**	**Solvent at 5% (v/v)**	**Solvent at 10% (v/v)**	**Solvent at 20% (v/v)**	**Solvent at 30% (v/v)**
Decane	5.40	+++	+++	+++	+++
Heptane	4.70	+++	+++	+++	+++
Octane	4.50	+++	+++	+++	+++
Decanol	4.23	+	+	+	+
Hexane	3.50	+	+	+	+
Diethylphathalate	3.30	+++	+++	+++	+++
Cyclohexane	3.20	+	+	+	+
Xylene	3.10	w	w	w	w
Ethylbenzene	3.10	-	ND	ND	ND
Styrene	3.00	-	ND	ND	ND
Toluene	2.50	-	ND	ND	ND
Heptanol	2.30	-	ND	ND	ND
Benzene	2.00	++	++	++	++
Chloroform	2.00	-	ND	ND	ND
Butyl acetate	1.80	-	ND	ND	ND
Pentanol	1.51	-	ND	ND	ND
Butyraldehyde	0.88	-	ND	ND	ND
1-Butanol	0.80	++	-	-	-
Ethyl acetate	0.70	-	ND	ND	ND
Pyridine	0.65	-	ND	ND	ND
Acetaldehyde	0.63	-	ND	ND	ND
Propionaldehyde	0.59	+	ND	ND	ND
Propanol	0.25	++++	+++	-	-
Diethyl ether	0.12	+++	ND	ND	ND
Acetone	-0.24	+++++	+++++	++++	-
Acetonitrile	-0.30	+++	+++	-	-
Ethanol	-0.33	+++	+++	-	-
Methanol	-0.77	+++++	+++++	+++++	+++
Dimethylformamide	-0.87	+++++	++++	w	-
Dimethyl sulfoxide	-1.35	+++++	+++++	++++	++++
Non		+++++	+++++	+++++	+++++

Cells were grown until mid-log phase in LB medium after that solvent (5-30% (v/v)) was directly added to the cell culture and incubated for 6 h. Cells survival was represented by +/- and w symbols; -, no growth; w, $CFU < 10^2$; +, $10^2 \leq CFU < 10^4$; ++, $10^4 \leq CFU < 10^6$; +++, $1x10^8 \leq CFU < 50x10^8$; ++++, $50x10^8 \leq CFU < 100x10^8$; +++++, $CFU \geq 100x10^8$; ND; not determined.

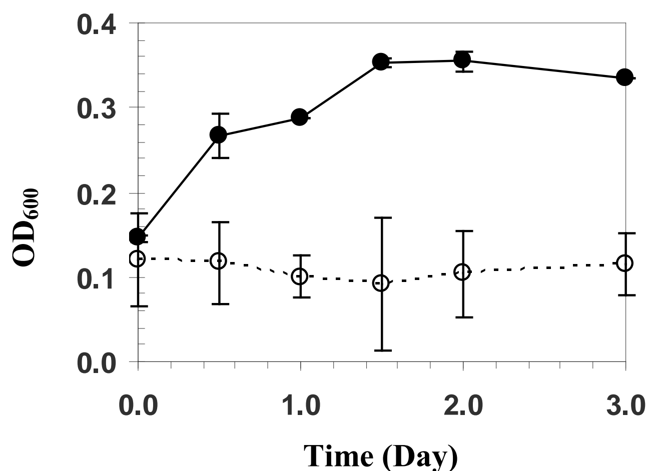


Figure 5.17 Utilization of acetonitrile as a growth substrate of *Exiguobacterium* sp. SBH81. Cells were grown at 37°C for 3 days in the presence of acetonitrile vapor (●) compared with absence of solvent was carried out as a control (-○-) in MSB medium. Data are means of the results from at least three individual experiments.

5.9.2 Cell adaptation to acetonitrile

Pseudomonas strains such as *P. putida* Idaho and *P. putida* S12 have been shown to adapt to high solvent concentration of *p*-xylene and styrene, respectively (Cruden et al., 1992; Weber et al., 1993). *Exiguobacterium* sp.SBH81 was not only resistant to acetonitrile, but also utilized acetonitrile as a sole carbon and nitrogen source described above. Furthermore, cell tolerance and utilization to acetonitrile was enhanced by adapting cells in LB medium supplemented with 4 % (v/v) acetonitrile for 10-30 cycles. Interestingly 10, 20 and 30 serial adaptation cultivations improved tolerance of strain SBH81 to acetonitrile had increased at 11, 53 and 60 %, respectively, when compared with non-adapted cells (Figure 5.18 A, B). Subsequently, the improvement of acetonitrile tolerance of *Exiguobacterium* sp.

SBH81 adapted cells was found to be stable characteristic even after growing in medium without acetonitrile serially transferred (Figure 5.18 C, D).

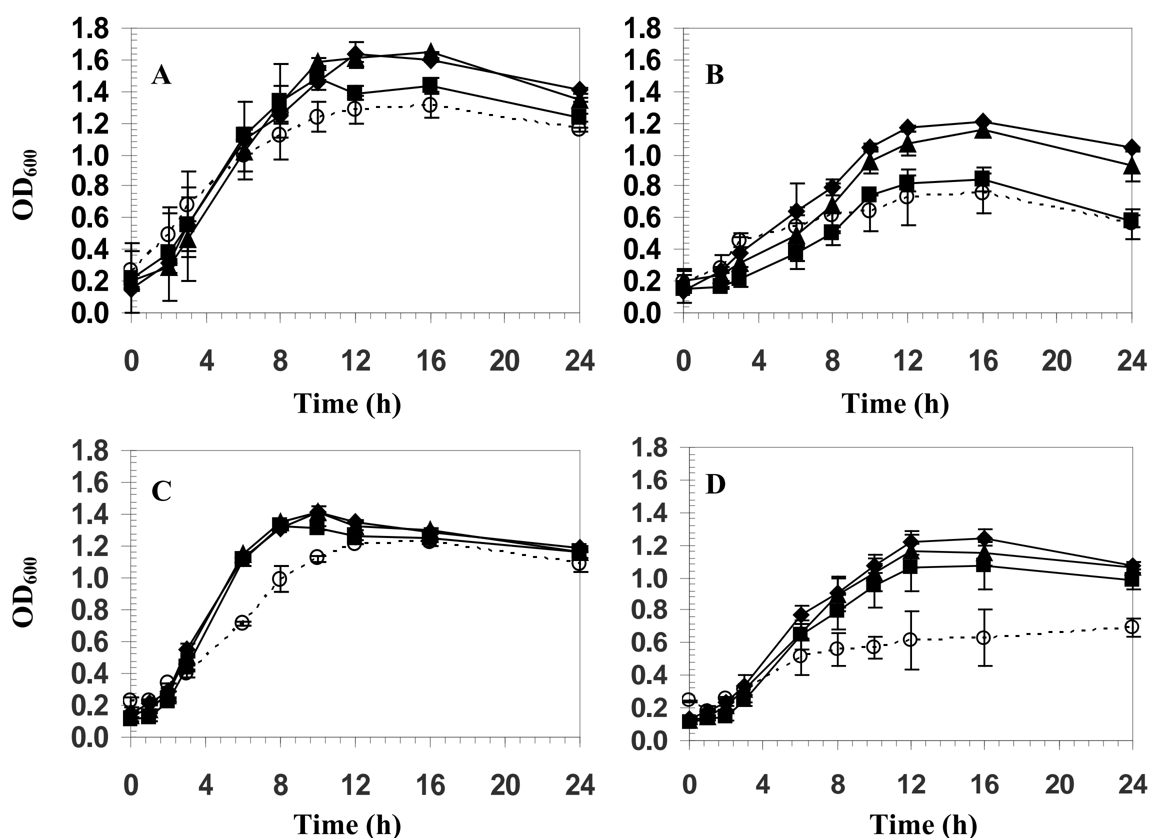


Figure 5.18 Effect of *Exiguobacterium* sp. SBH81 adapted cells to acetonitrile tolerant and its stability. Adapted cells were prepared in LB medium supplement with 4% (v/v) of acetonitrile and incubate at 37 °C for 12 h and re-culture in the same condition for 0 (○), 10 (■), 20 (▲) and 30 (◆) times. For adapted cells stability, cells were grown in LB medium absent acetonitrile in the same condition for 2 times. Effect of adaptive cells to acetonitrile tolerance (A, B) and its stability (C, D) were estimated in MSBYG medium supplement with 5% (100 mM) (A, C) and 6% (v/v) (110 mM) (B, D) of acetonitrile. Data are means of the results from at least three individual experiments.

5.9.3 Acetonitrile tolerance mechanisms

Several mechanisms proven to enable bacteria to thrive in toxic organic solvent such as utilization ability as growth substrate, morphological adaptation and efflux pump system, which have been conducted in Gram negative bacteria. Although little information of tolerance mechanisms has been described in Gram positive bacteria, then mechanism studies were investigated in *Exiguobacterium* sp. SBH81.

(1) Utilization or degradation of acetonitrile

The ability of 30 cycle-adapted and non-adapted cell were also investigated utilization of acetonitrile in MSB medium supplemented with acetonitrile vapor. It was found that 30 cycle-adapted and non-adapted cell were shown small amount of turbidity in MSB medium with acetonitrile as carbon source. It was obvious that the adapted cells improved acetonitrile utilization at 37.3% when compared with non-adapted cells (Figure 5.19).

(2) Active transport of solvents from the membrane into the environment by energy-consuming efflux systems

The enhancement of acetonitrile tolerance and utilization of *Exiguobacterium* sp. SBH81 is remarkable when compared with adapted cells described previously. Therefore, the question arises why adapted cell help the resistance of acetonitrile. The first possibility can be supported by the observation that the efflux pump system was induced when cells exposed with acetonitrile. Previous reports have reported that *srpABC* operon for the efflux of organic solvents is induced by organic solvents in *Pseudomonas putida* (Kieboom et al., 1998b).

Then three efflux pumps inhibitors were used to investigate the nature of efflux pumps system for acetonitrile tolerance: (1) orthovanadate as a specific inhibitor of ABC families; (2) paroxitine, which is an inhibitor of the resistance nodulation cell division families (RND); (3) PAßN, which is the multidrug and toxic compound extrusion families (MATE) and the RND families (Marquez, 2005). Cells were cultivated in MSB medium supplement with and without various types of efflux inhibitors in the presence of acetonitrile vapor (Figure 5.20). The amount of cell turbidity in the presence of orthovanadate was not significant different when compared with that in the absence efflux inhibitor. These observations indicated that, the ABC families were less effect involved in acetonitrile tolerance in *Exiguobacterium* sp. SBH81. The amount of cells turbidity in the presence of paroxitine and PAßN were one and two fold respectively lower than that in the absence of efflux inhibitor. The results may suggest that acetonitrile molecules were accumulated inside of cells, and not pumped out into the environment in the presence of efflux inhibitors conditions. Solvent are known to accumulate in and disrupt the bacterial cell membrane thus affecting the structure and functional integrity of the cell (Sikkema et al., 1995). Cells turbidity in the presence of paroxitine and PAßN were decreased. This data clearly shows that the MATE and RND families ones of the mechanisms for acetonitrile tolerance of *Exiguobacterium* sp. SBH81, while the ABC families have less effect on acetonitrile tolerance. An export system was detected in Gram-positive such as the ABC-type transporter was involved in toluene efflux in *Bacillus cereus* R1 (Matsumoto et al., 2002) and RND families are one of the main mechanisms resistance to organic solvent tin Gram-negative bacteria (Kieboom et al., 1998a).

Furthermore, the transformation or degradation of toxic organic solvent into non-toxic compounds is involved in the organic solvent-tolerance. Biodegradation is the major adaptive response for benzene-tolerant *Rhodococcus* strain (Paje et al., 1997). However, many OST bacteria can grow in the presence of solvents without degrading then strain SBH81 could not increased cell turbidity in the presence of efflux inhibitor (paroxitine and PAßN) while, increased accumulation of substrate in cells. It was suggested that, degradation is not main mechanism of acetonitrile tolerance of strain SBH81.

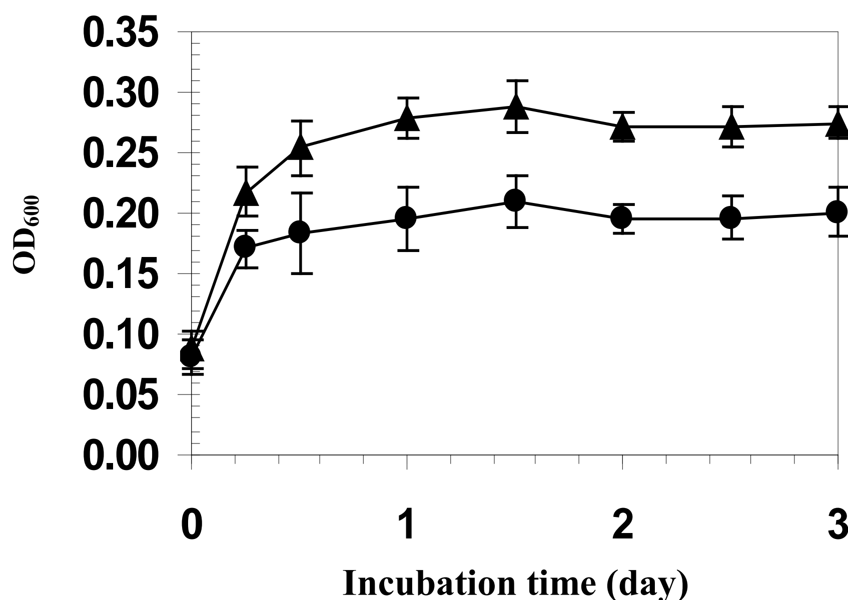


Figure 5.19 Acetonitrile utilization of 30 cycle-adapted (▲) and non-adapted (●) cells were estimated in MSB medium in the presence of acetonitrile as vapor phase. Data are means of the results from six individual experiments.

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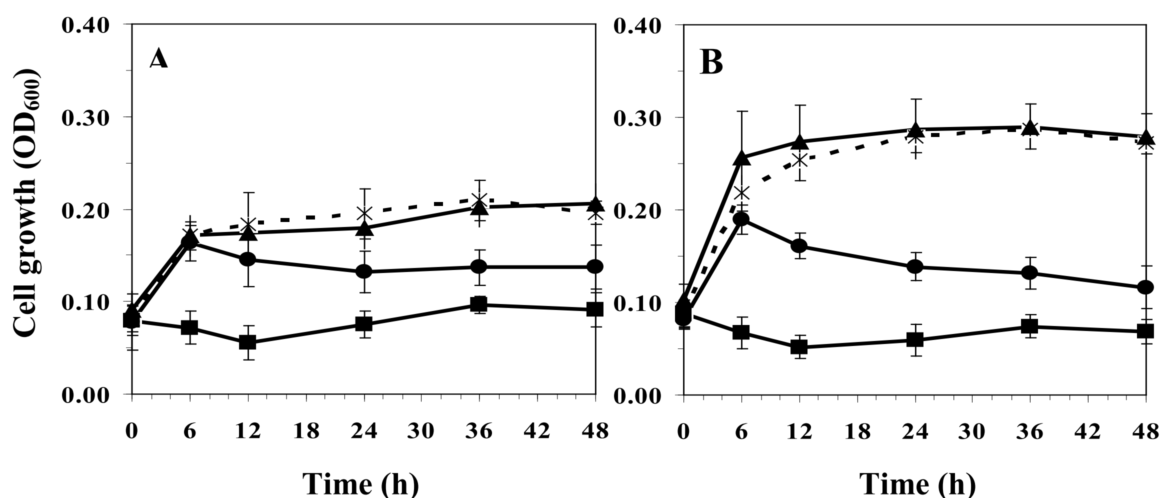
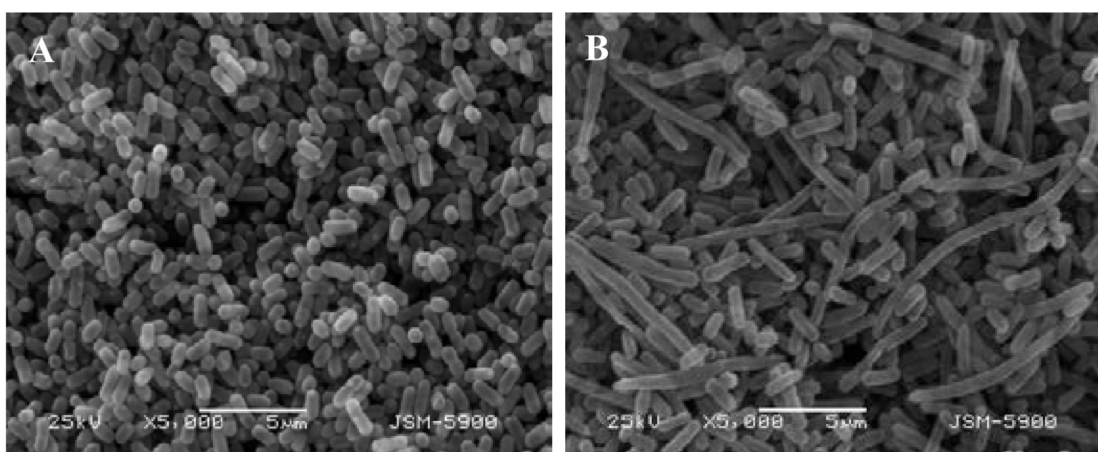


Figure 5.20 Effect of efflux pump inhibitor (EPI) to viability of *Exiguobacterium* sp. SBH81 in the presence of acetonitrile vapor. Non-adapted cells (**A**) and 30-cycle acetonitrile-adapted cells (**B**) were grown in MSB medium supplemented with acetonitrile vapor without EPI (✱) and with EPI: PAβN (0.1 mM) (■), paroxitine (0.1 mM) (●), and orthovanadate (10 mM) (▲). Effect of EPI on cell growth in MSBYG medium in the absence of acetonitrile is shown in the inset. Data are means of the results from at least six individual experiments.

(3) Morphological adaptation

Little is known about morphological changes in bacteria as a response to solvents, although there are a number of reports that cell sizes increase when exposed to toxic organic compounds. Major factor responsible for these changes seems to be the surface-to-volume ratio of the cells. Changes in cell morphology and surface characteristics have been commonly described in Gram-negative bacteria as adaptive responses to environmental stresses such as *P. putida* and *Enterobacter* sp., increased in size when exposed to phenol, 4-chlorophenol, and 1-butanol (Neumann et al., 2005b). However, decrease in cell size has been reported for *Enterobacter* sp. when grown on 1-butanol as the sole carbon and energy source. To investigate cell morphology of Gram positive *Exiguobacterium* sp. SBH81, SEM analysis was used in this study. Non-adapted SBH81 was shown to have a short rod shape with a surface per volume (S/V) ratio of 8.002±0.724, whereas adapted cells increased their size (i.e. increased length with a constant radius) and a reduced S/V ratio to 6.906±0.100 (Figure 5.21). The relative reduction of the cell S/V ratio as a response to acetonitrile exposure reduces the attachable cell surface to solvent, lowering solvent diffusion and partitioning into the cell membrane, therefore providing SBH81 higher tolerance to acetonitrile.



Cell dimension	Non-adapted cells	Acetonitrile-adapted cells*
Width (μm)	0.629 ± 0.043	0.617 ± 0.068
Length (μm)	1.282 ± 0.247	3.243 ± 0.243
Radius (μm)	0.314 ± 0.022	0.318 ± 0.034
Volume (μm^3)	0.397 ± 0.127	1.031 ± 0.198
Surface (μm^2)	3.179 ± 0.712	7.118 ± 0.884
Surface/Volume (μm^{-1})	8.002 ± 0.724	6.906 ± 0.100

Figure 5.21 Morphology and dimension of *Exiguobacterium* sp. SBH81. Scanning electron micrographs show non-adapted cells (A) and 30-cycle acetonitrile-adapted cells (B). Cells were grown in MSBYG medium at 37 ºC for 24 h. Cell dimension was the average value from 150 individual cells. Scale bars, 5 μm.

(4) Cell surface characteristics

Modification of cells hydrophobicity has been stated as another cell adaptation mechanism to solvent exposure. Nevertheless, the cell surface property of non-adapted and 30-cycle-adapted SBH81, examined using a BATH test, suggested a moderate hydrophobic cell surface with % hydrophobicity values of 45±7 and 43±8, respectively (Figure 5.22). This result suggested that modification of the cell surface may not involve in acetonitrile of *Exiguobacterium* sp. SBH81.

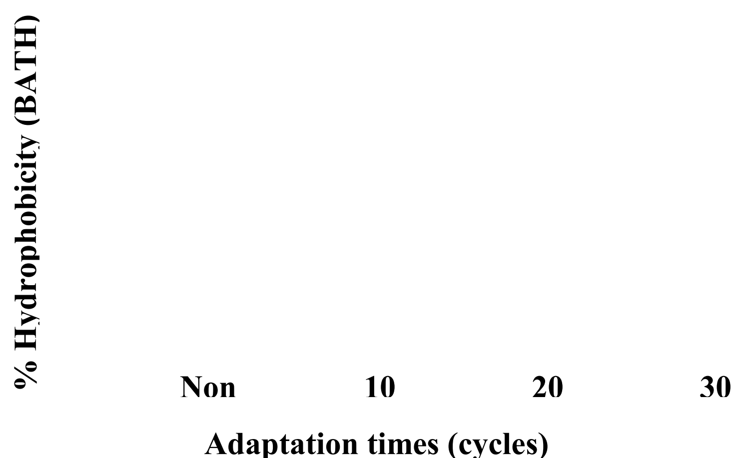


Figure 5.22 Adapted and non-adapted *Exiguobacterium* sp. SBH81 were investigated cells hydrophobicity by using BATH assay. A value of > 50% indicates a relatively strongly hydrophobic cell surface; 20-50% indicates a moderately hydrophobic cell surface; and < 20% indicates a hydrophilic cell surface. Data are means of the results from at least three individual experiments.

CHAPTER VI

CONCLUSION

Chemical production become more important in many industrial, especially their bioproduction has been increasing. Due to biotechnological production has several advantages compared to the chemical process such as energy saving, higher specificity to reaction/or substrate, and yields have been shown to be higher than those achieved by chemical synthesis. However, the toxicity of substrates and/or products towards bacterial producer may limit the accumulation of product in the bioproduction process. Organic solvent-tolerant (OST) bacteria are a unique group of microorganisms that can thrive in the presence of high concentrations of solvent as a result of their tolerance mechanisms and bioproduction system have been attempted to overcome this toxicity and increase the yield for biotechnological production. The possible biotechnological production processes using OST bacteria as producer of chemical bioproduction, of which substrates and/or products may have toxic effects to bacterial producer are a two-phase system for hydrophobic chemicals and a single-phase system for hydrophilic chemicals. Because of their advantages, this study were isolated and characterized bacteria able to tolerant organic solvent at high concentration, developed bacterial host cells for production of 3-HPA and 3MC on using a recombinant OST bacterium as a whole-cell biocatalyst and investigated the suitable bioproduction process of 3-HPA and 3MC production.

The hydrophobic chemical of 3MC bioproduction from toluene of Gram negative OST *P. putida* mutant strain TODE1 was carried out under a two-phase

system. Introduction of a two- phase of 1-decanol improved 3MC production by strain TODE1. Investigation of suitable production of a two-phase system illustrated that addition of glycerol and divalent ions of Mg^{2+}, Mn^{2+} and Fe^{2+} significant enhanced 3MC production up to 50%. In the RSM-based CCD optimization condition, optimal concentrations of glycerol and Fe^{2+} were 4 mM and 0.4 mM, respectively, the overall 3MC production was produced up to 34 mM which is higher than that of previous report. The hydrophobic chemical of 3MC bioproduction under a two-phase of 1-decanol system and optimized condition are suitable for usage in biotechnological industry.

The hydrophilic chemical bioproduction was started from screening and characterization of hydrophilic OST bacteria because less information of hydrophilic OST bacteria reported. Hydrophilic OST bacteria were isolated from soil, water and sea water using enrichment technique with various type of hydrophilic organic solvent. It exhibited high tolerance to various type of toxic hydrophilic organic solvent, including propionaldehyde, 1-butanol and acetonitrile, at high concentration. Within these part seven bacteria isolate, 6 strains of *Bacillus* and 1 strain of *Exiguobacterium* having comparatively good growth in the presence of high concentration of hydrophilic organic solvent and good growth in the present of glycerol and crude glycerol from biodiesel. Cell ability to transform and manipulate are important for the development of an alternative host for chemical production. Using electroporation technique was successfully applied for OST Gram positive *B. subtilis* BKS2-P2/3. As the determination of promoter system, an expression pHY300PLK contained PXyl promoter showed significant β-galactosidase activity in

BKS2-P2/3, it was suggested that BKS2-P2/3 suitable for hydrophilic organic solvent bioproduction.

Among of hydrophilic OST bacteria was isolated, *Exiguobacterium* sp. SBH81 was shown unique characteristic on acetonitrile tolerance. So far, there has been poor information of acetonitrile tolerance and limited investigation towards understanding the mechanisms of acetonitrile tolerance. *Exiguobacterium* sp. SBH81 was shown tolerate to high concentration of acetonitrile up to 6% (v/v). Investigation of acetonitrile mechanism was showed that tolerance mechanisms does not rely on solvent inactivation processed or modification cell surface characteristics, but rather, increase of the cell size lowers solvent partitioning into cells and the extrusion of solvents through the efflux system. A test using efflux pump inhibitors suggested that secondary transporters, i.e. resistance nodulation cell division (RND) and the multidrug and toxic compound extrusion (MATE) family, are involved in acetonitrile tolerance in this strain. In addition, its acetonitrile tolerance ability could be stably and significantly enhanced by repetitive growth in the presence of toxic acetonitrile. The fact that SBH81 has high solvent tolerance to various types of hydrophilic organic solvent, and is susceptible to solvent tolerance enhancement; make it attractive as a genetic host for biotechnological production and bioremediation applications.

REFERENCES

Alam, M.Z., and Malik, A. (2008). Chromate resistance, transport and bioreduction by *Exiguobacterium* sp. ZM-2 isolated from agricultural soil irrigated with tannery effluent. *Journal of Basic Microbiology* 48: 416-420.

Anand, P., and Saxena, R.K. (2012). A comparative study of solvent-assisted pretreatment of biodiesel derived crude glycerol on growth and 1,3-propanediol production from *Citrobacter freundii*. *New Biotechnology* 29: 199–205.

Anderson, C.R., and Cook, G.M. (2004). Isolation and characterization of arsenate-reducing bacteria from arsenic-contaminated sites in New Zealand. *Current Microbiology* 48: 341-347.

Banerjee, and Sharma (2002). The nitrile-degrading enzymes: current status and future prospects. *Applied Microbiology and Biotechnology* 60: 33-44.

Barbirato, F., Grivet, J.P., Soucaille, P., and Bories, A. (1996). 3-Hydroxypropionaldehyde, an inhibitory metabolite of glycerol fermentation to 1,3-propanediol by enterobacterial species. *Applied and Environmental Microbiology* 62: 1448-1451.

Bauer, R., Toit, M.d., and Kossmann, J. (2010). Influence of environmental parameters on production of the acrolein precursor 3-hydroxypropionaldehyde by *Lactobacillus reuteri* DSMZ 20016 and its accumulation by wine *lactobacilli*. *International Journal of Food Microbiology* 137: 28-31.

Butaye, P., Cloeckaert, A., and Schwarz, S. (2003). Mobile genes coding for efflux-mediated antimicrobial resistance in Gram-positive and Gram-negative bacteria. *International Journal of Antimicrobial Agents* 22: 205-210.

Chang, B., Halgamuge, S., and Tang, S.-L. (2006). Analysis of SD sequences in completed microbial genomes: Non-SD-led genes are as common as SD-led genes. *Gene* 373: 90-99.

Cruden, D.L., Wolfram, J.H., Rogers, R.D., and Gibson, D.T. (1992). Physiological properties of a *Pseudomonas* strain which grows with p-xylene in a two-phase (organic-aqueous) medium. *Applied and Environmental Microbiology* 58: 2723-2729.

Daniel, R., and Gottschalk, G. (1992). Growth temperature-dependent activity of glycerol dehydratase in *Escherichia coli* expressing the *Citrobacter freundii dha* regulon. *FEMS Microbiology Letters* 100: 281-285.

Dobson, R., Gray, V., and Rumbold, K. (2012). Microbial utilization of crude glycerol for the production of value-added products. *Journal of Industrial Microbiology & Biotechnology* 39: 217-226.

Doleyres, Y., Beck, P., Vollenweider, S., and Lacroix, C. (2005). Production of 3-hydroxypropionaldehyde using a two-step process with *Lactobacillus reuteri. Applied Microbiology and Biotechnology* 68: 467-474.

Faizal, I., Dozen, K., Hong, S., C., Kuroda, A., Takiguchi, N., Ohtake, H., Takeda, K., Tsunekawa, H., and Kato, J. (2005). Isolation and characterization of solvent-tolerant *Pseudomonas putida* strain T-57, and its application to biotransformation of toluene to cresol in a two-phase (organic-aqueous) system. *Journal of Industrial Microbiology and Biotechnology* 32: 542-547.

Faizal, I., Ohba, M., Kuroda, A., Takiguchi, N., Ohtake, H., Honda, K., and J., K. (2007). Bioproduction of 3-methylcatechol from toluene in a two-phase (organic-aqueous) system by a genetically modified solvent-toleant *Pseudomonas putida* strain T-57. *Journal of Environmental Biotechnology* 7: 39-44.

González-Pajuelo, M., Andrade, J., and Vasconcelos, I. (2005). Production of 1,3-Propanediol by *Clostridium butyricum* VPI 3266 in continuous cultures with high yield and productivity. *Journal of Industrial Microbiology Biotechnology* 32: 391-396.

Hao, J., Wang, W., Tian, J., Li, J., and Liu, D. (2008). Decrease of 3-hydroxypropionaldehyde accumulation in 1,3-propanediol production by over-expressing *dha*T gene in *Klebsiella pneumoniae* TUAC01. *Journal of Industrial Microbiology Biotechnology* 35: 735-741.

Huertas, M., Duque, E., Marques, S., and Ramos, J.L. (1998). Survival in soil of different toluene-degrading *Pseudomonas* strains after solvent shock. *Applied and Environmental Microbiology* 64: 38–42.

Husken, E., L.,, De bont, A.M., J.,, Beef, T., Johannes, T., and Wery, J. (2002). Optimisation of microbial 3-methylcatechol production as ffected by culture conditions. *Biocatalysis and Biotransformation* 20*:* 57-61.

Husken E. Leonie, Dalm C.F. Marcella, Tramper Johannes, Wery Jan, de-Bont A.M. Jan, and Rik, B. (2001). Integrated bioproduction and extraction of 3-methylcatechol. *Journal of Biotechnology* 88: 11–19.

Hüsken, L.E., Beeftink, R., de Bont, J.A.M., and Wery, J. (2001). High-rate 3-methylcatechol production in *Pseudomonas putida* strains by means of a novel expression system. *Applied Microbiology and Biotechnology* 55: 571-577.

Inoue, A., and Horikoshi, K. (1989). A *Pseudomonas* thrives in high concentrations of toluene. *Nature* 338: 264 - 266.

Inoue, A., and Horikoshi, K. (1991). Estimation of solvent-tolerance of bacteria by the solvent parameter log *P*. *Journal of Fermentation and Bioengineering* 71: 194-196.

Isken, S., and de Bont, J.A. (1996). Bacterai tolerant to organic solvents. *Extremophiles* 2: 229-238.

Isken, S., and de Bont, J.A. (1998). Bacteria tolerant to organic solvents. *Extremophiles* 2: 229-238.

Joshi, A.A., Kanekar, P.P., Kelkar, A.S., Shouche, Y.S., Vani, A.A., Borgave, S.B., and Sarnaik, S.S. (2008). Cultivable bacterial diversity of alkaline Lonar lake, India. *Microbial Ecology* 55: 163-172.

Kabelitz, N., Sntos, P.M., and Heipieper, H.J. (2003). Effect of aliphatic alcohols on growth and degree o saturation of membrane lipids in *Acinetobacter calcoaceticus*. *FEMS Microbiology Letter* 2: 223-227.

Kataoka, N., Tajima, T., Kato, J., Rachadech, W., and Vangnai, S., Alisa, (2012). Development of butanol-tolerant *Bacillus subtilis* strain GRSW2-B1 as a potential bioproduction host. *AMB Express* 1: 10-20.

Kato, C., Inoue, A., and Horikoshi, K. (1996). Isolating and characterizing deep-sea marine microorganisms. *TIBTECH* 14: 6-12.

Kieboom, J., Dennis, J.J., de Bont, J.A.M., and Zylstra, G.J. (1998a). Identification and molecular characterization of an efflux pump involved in *Pseudomonas putida* S12 solvent tolerance. *The Journal of Biological Chemistry* 273: 85-91.

Kieboom, J., Dennis, J.J., Zylstra, G.J., and de Bont, J.A. (1998b). Active efflux of organic solvents by *Pseudomonas putida* S12 is induced by solvents. *Journal of Bacteriology* 180: 6769–6772.

Kim, K., Lee, S., Lee, K., and Lim, D. (1998). Isolation and characterization of toluene-sensitive mutants from the toluene-resistant bacterium *Pseudomonase putida* GM73. *Journal of Biotechnology* 180: 3692-3696.

Kobayashi, H., Yamamota, M., and Aono, R. (1998). Appearance of a stress-response protein, phage-shock protein A, in *Escherichia coli* exposed to hydrophobic organic solvents. *Microbiology* 144: 353-359.

Kohyama, E., Yoshimura, A., Aoshima, D., Yoshida, T., Kawamoto, H., and Nagasawa, T. (2006). Convenient treatment of acetonitrile-containing wastes using the tandem combination of nitrile hydratase and amidase-producing microorganisms. *Applied Microbiology and Biotechnology* 72: 600-606.

Kongpol, A., Kato, J., and Vangnai, A.S. (2008a). Isolationand characterization of*Deinococcus geothermalis* T27, a slightly thermophilicand organic solvent-tolerant bacteriumable to survive in the presence of high concentrations of ethyl acetate. *FEMS Microbiology Letters* 286: 227-235.

Kongpol, A., Pongtharangkul, T., Kato, J., Honda, K., Ohtake, H., and Vangnai, A.S. (2009). Characterization ofan organic-solvent-tolerant *Brevibacillus agri* strain 13 able to stablilize solvent/water emulsion. *FEMS Microbiology Letters* 297: 225-233.

Laane, C., Boeren, S., Vos, K., and Veeger, C. (1987). Rules for optimization of biocatalysis in organic solvents. *Biotechnology and Bioengineering* 23: 81-87.

Li, W., Li, H.X., Ji, S.Y., Li, S., Gong, Y.S., Yang, M.M., and Chen, Y.L. (2007). Characterization of two temperature-inducible promoters newly isolated from *B. subtilis*. *Biochemical and Biophysical Research Communications* 13: 1148-1153.

Lopez, L., Pozo, C., Rodelas, B., Calvo, C., Juarez, B., Martinez-Toledo, M.V., and Gonzalez-Lopez, J. (2005). Identification of bacteria isolated from an oligotrophic lake with pesticide removal capacities. *Ecotoxicology* 14: 299-312.

Lüthi-Peng, Q., Schärer, S.S., and Puhan, Z.P. (2002). Production and stability of 3-hydroxypropionaldehyde in *Lactobacillus reuteri*. *Applied Microbiology and Biotechnology* 60: 73-80.

Manolov, T., Kristina, H., and Benoit, G. (2005). Continuous acetonitrile degradation in a packed-bed bioreactor. *Applied Microbiology and Biotechnology* 66: 567-574.

Marchesi, J.R., Sato, T., Weightman, A.J., Martin, T.A., Fry, J.F., Hiom, S.J., and Wade, W.G. (1998). Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16S rRNA. *Applied and Environmental Microbiology* 64: 795-799.

Marquez, B. (2005). Bacteria efflux systems and efflux pumps inhibitors. *Biochimie* 87: 1137-1147.

Masai, E., Yamada, A., Healy, J.M., Hatta, T., Kimbara, K., Fukuda, M., and Yano, K. (1995). Characterization of biphenyl catabolic genes of gram-positive

polychlorinated biphenyl degrader *Rhodococcus* sp. strain RHA1. *Applied and Environmental Microbiology* 61: 2079-2085.

Matsumoto, M., de Bont, J.A., and Isken, S. (2002). Isolation and characterization of the solvent-tolerant *Bacillus cereus* strain R1. *Journal of Bioscience and Bioengineering* 94: 45-51.

Na, K.S., Kuroda, A., Takiguchi, N., Ikeda, T., Ohtake, H., and Kato, J. (2005). Isolation and characterization of benzene-tolerant *Rhodococcus opacus* strains. *Journal of Bioscience and Bioengineering* 99: 378-382.

Neumann, G., Kabelitz, N., Zehnsdorf, A., Miltner, A., Lippold, H., Meyer, D., Schmid, A., and Heipieper, H.J. (2005a). Prediction of the adaptability of *Pseudomonas putida* DOT-T1E to a second phase of a solvent for economically sound two-phase biotransformations. *Applied and Environmental Microbiology* 71: 6606-6612.

Neumann, G., Veeranagouda, Y., Karegoudar, T.B., Sahin, O., Mäusezahl, I., Kabelitz, N., Kappelmeyer, U., and Heipieper, H.J. (2005b). Cells of *Pseudomonas putida* and *Enterobacter* sp. adapt to toxic organic compounds by increasing their size. *Extremophiles* 9: 163-168.

Paik, S.-Y., Ra, S., Kyung, , Cho, S., Hoon, , Koo, B., Kwang, , Baik, S., Hyung, , Lee, C., Myung,, Yun, W., Jong, , and Choi, W., Jang, (2006). The Influence of the nucleotide sequences of random shine-salgarno and spacer region on bovine growth hormone gene expression. *The Journal of Microbiology* 44: 64-71.

Paje, M.L., Neilan, B.A., and Couperwhite, L. (1997a). A *Rhodococus* species that trives on medium saturated with liquid benzene. *Microbiology* 143: 2975-2981.

Prpich P.G., and Daugulis A.J. (2007). Solvent selection for enhanced bioproduction of 3-methylcatechol in a two-phase partitioning bioreactor. *Biotechnology and Bioengineering* 97: 536-543.

Raj, S.M., Rathnasingh, C., Jo, J.-E., and Park, S. (2008). Production of 3-hydroxypropionic acid from glycerol by a novel recombinant *Escherichia col*i BL21 strain. *Process Biochemistry* 43: 1440-1446.

Ramos, J.L., Duque, E., Gallegos, M., Godoy, P., Ramos-Gonzalez, M., Rojas, A., Teran, W., and Segura, A. (2002). Mechanisms of solvent tolerance in Gram-negative bacteria. *Annual Review of Microbiology* 56: 743–768.

Ramos, J.L., Duque, E., Huertas, M.J., and Haïdour, A. (1995). solation and expansion of the catabolic potential of a *Pseudomonas putida* strain able to grow in the presence of high concentrations of aromatic hydrocarbons. *Biotecnology* 177: 3911-3916

Rathnasingh, C., Raj, S.M., Jo, J.-E., and Park, S. (2009). Development and evaluation of efficient recombinant *Escherichia coli* strains for the production of 3-hydroxypropionic acid from glycerol. *Biotechnology and Bioengineering* 104: 729-739.

Rodrigues, D.F., da, C.J.E., Ayala-Del-Rio, H.L., Pellizari, V.H., Gilichinsky, D., Sepulveda-Torres, L., and Tiedje, J.M. (2009). Biogeography of two cold-adapted genera: *Psychrobacte*r and *Exiguobacterium*. *The ISME Journal* 3: 658-665.

Rodrigues, D.F., Ivanova, N., He, Z., Huebner, M., Zhou, J., and Tiedje, J.M. (2008). Architecture of thermal adaptation in an *Exiguobacterium sibiricum* strain isolated from 3 million year old permafrost: a genome and transcriptome approach. *BMC Genomics* 9: 547.

Rygus, T., and Hillen, W. (1991). Inducible high-level expression of heterologous genes in *Bacillus megaterium* using the regulatory elements of the xylose-utilization operon. *Applied Microbiology and Biotechnology* 35, 594-599.

Sardessai, Y., and Bhosle, S. (2002a). Tolerance of bacteria to organic solvents. *Research in Microbiology* 153: 263-268.

Shaw, G.-C., Wu, M.-Y., Lee, T.-R., and Hsu, C.-W. (2005). The influence of nucleotide sequences at and near ribosome-binding site on translational efficiency of the *Bacillus subtilis* rho gene. Biochimica et Biophysica Acta (BBA) - *Gene Structure and Expression* 1729: 10-13.

Shirai, K. (1986). Screening of microorganisms for catechols production from benzen. *Agricultural and biological chemistry* 50: 2875-2880.

Sikkema, J., de Bont, J.A., and Poolman, B. (1994). Interaction of cyclic hydroxarbons with biological membranes. *Biological Chemistry* 269: 8022-8028.

Sikkema, J., Debont, J.A.M., and Poolman, B. (1995). Mechanisms of membrane toxicity of hydrocarbons. *FEMS Microbiology Reviews* 59: 201-222.

Slininger, P.J., and Bothast, R.J. (1985). Optimizing aerobic conversion of glycerol to 3-hydroxypropionaldehyde. *Applied and Environmental Microbiology* 50: 1444-1450.

Slininger, P.J., Bothast, R.J., and Smiley, K.L. (1983). Production of 3-Hydroxypropionaldehyde from glycerol. *Applied and Environmental Microbiology* 46: 62-67.

Sorokin, D.Y., van Pelt, S., Tourova, T.P., Takaichi, S., and Muyzer, G. (2007). Acetonitrile degradation under haloalkaline conditions by *Natronocella acetinitrilica* gen. nov., sp. nov. *Microbiology* 153: 1157-1164.

Talarico, T.L., Casas, I.A., Chung, T.C., and Dobrogosz, W.J. (1988). Production and isolation of reuterin, a growth inhibitor produced by *Lactobacillus reuteri*. *Antimicrobial agents and chemotherapy* 32: 1854-1858.

Tan, L., Qu, Y.Y., Zhou, J.T., Li, A., and Gou, M. (2009). Identification and characteristics of a novel salt-tolerant *Exiguobacterium* sp. for azo dyes decolorization. *Applied Biochemistry and Biotechnology* 159: 728-738.

Tang, X., Tan, Y., Zhu, H., Zhao, K., and Shen, W. (2009). Microbial conversion of glycerol to 1,3-propanediol by an engineered strain of *Escherichia coli. Applied and Environmental Microbiology* 75: 1628-1634.

Timmis, K.N. (1994). Designing microorganisms for the treatment of toxic wastes. *Annual Review of Microbiology* 48: 525-557.

Tong, I.T., Liao, H.H., and Cameron, D.C. (1991). 1,3-Propanediol production by *Escherichia coli* expressing genes from the *Klebsiella pneumoniae dha* regulon. *Applied and Environmental Microbiology* 57: 3541-3546.

Turgeon, N., Laflamme, C., Ho, J., and Duchaine, C. (2006). Elaboration of an electroporation protocol for *Bacillus cereus* ATCC 14579. *Journal of Microbiological Methods* 67: 543-548.

Vagner, V., Dervyn, E., and Ehrlich, S.D. (1998). A vector for systematic gene inactivation in *Bacillus subtilis*. *Microbiology* 144: 3097-3104.

Vancauwenberge, J.E., Slininger, P.J., and Bothast, R.J. (1990). Bacterial conversion of glycerol to beta-hydroxypropionaldehyde. *Applied and Environmental Microbiology* 56: 329-332.

Vishnivetskaya, T.A., and Kathariou, S. (2005). Putative transposases conserved in *Exiguobacterium* isolates from ancient Siberian permafrost and from contemporary surface habitats. *Applied and Environmental Microbiology* 71: 6954-6962.

Vishnivetskaya, T.A., Siletzky, R., Jefferies, N., Tiedje, J.M., and Kathariou, S. (2007). Effect of low temperature and culture media on the growth and freeze-thawing tolerance of *Exiguobacterium* strains. *Cryobiology* 54: 234-240.

Vollenweider, S., Grassi, G., Konig, I., and Puhan, Z. (2003). Purification and structural characterization of 3-hydroxypropionaldehyde and its derivatives. *Journal of agricultural and food chemistry* 51: 3287-3293.

Vollenweider, S., and Lacroix, C. (2004). 3-Hydroxypropionaldehyde: applications and perspectives of biotechnological production. *Applied microbiology and biotechnology* 64: 16-27.

Voskuil, M., and Chambliss, H., Glenn, (1993). Rapid isolation and sequencing of purified plasmid DNA from *Bacillus subtilis*. *Applied and Environmental Microbiology* 59: 1138-1142.

Wang, F., Qu, H., Tian, P., and Tan, T. (2007). Heterologous expression and characterization of recombinant glycerol dehydratase from *Klebsiella pneumoniae* in *Escherichia coli*. *Biotechnology Journal* 2: 736-742.

Wang, L., Qiao, N., Sun, F., and Shao, Z. (2008). Isolation, gene detection and solvent tolerance of benzene, toluene and xylene degrading bacteria from nearshore surface water and Pacific Ocean sediment. *Extremophiles* 12: 335-342.

Wang, P.Z., and Doi, R.H. (1984). Overlapping promoters transcribed by *bacillus subtilis* sigma 55 and sigma 37 RNA polymerase holoenzymes during growth and stationary phases. *The Journal of Biological Chemistry* 259: 8619-8625.

Weber, C., Farwick, A., Benisch, F., Brat, D., Dietz, H., Subtil, T., and Boles, E. (2010). Trends and challenges in the microbial production of lignocellulosic bioalcohol fuels. *Applied Microbiology and Biotechnology* 87: 1303-1315.

Weber, F.J., Ooijkaas, L.P., Schemen, R.M.W., Hartmans, S., and de Bont, J.A. (1993). Adaptation of *Pseudomonas putida* S12 to high concentrations of styrene and other organic solvents. *Applied and Environmental Microbiology* 59: 3502-3504.

Wery, J., Mendes da Silva, D.I., and de Bont, A.M., J. (2000). A genetically modified solvent-tolerant bacterium for opimized production of a toxic fine chemical. *Applied Microbiology and Biotechnology* 54: 180-185.

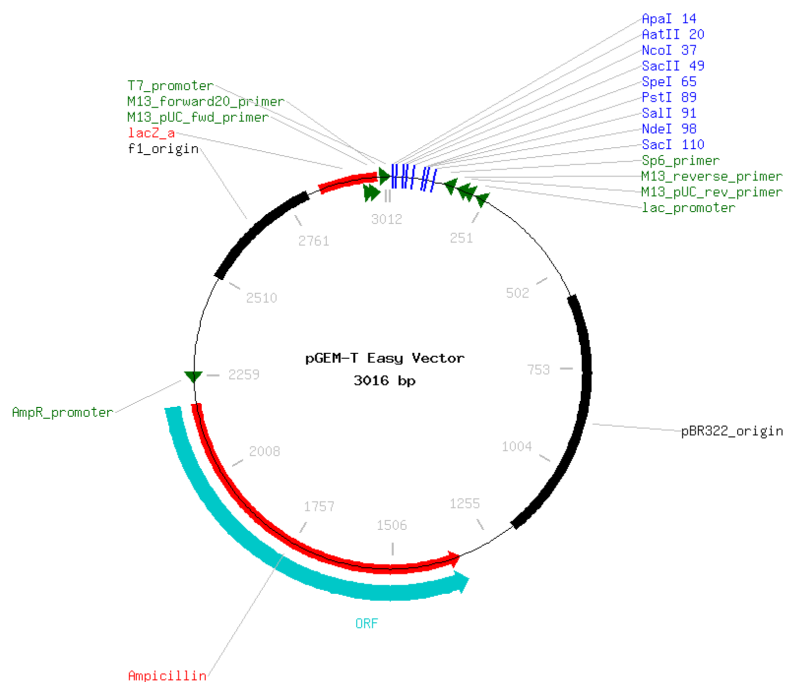
Zamudio-Jaramillo, M.A., Hernandez-Mendoza, A., Robles, V.J., Mendoza-Garcia, P.G., Espinosa-de-los-Monteros, J.J., and Garcia, H.S. (2009). Reuterin production by *Lactobacillus reuteri* NRRL B-14171 immobilized in alginate. J*ournal of Chemical Technology and Biotechnology* 84: 100–105.

APPENDICES

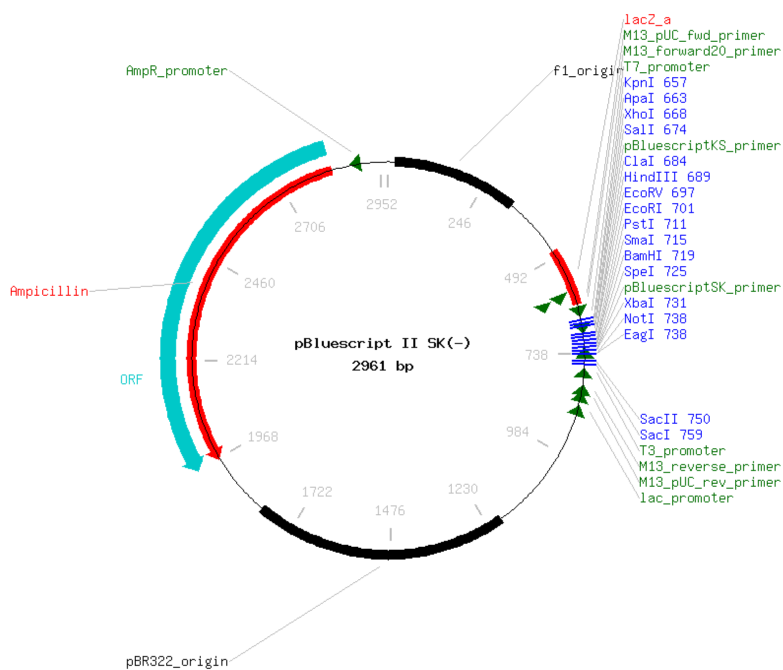
APPENDIX A

Plasmid map

(1) pGEM-T easy vector

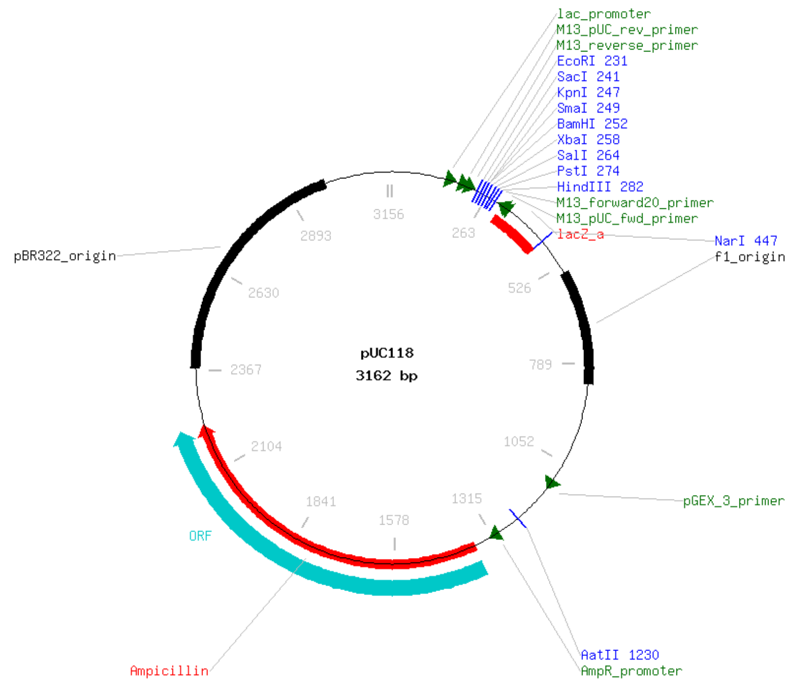


(2) pBluescript II SK (-) plasmid

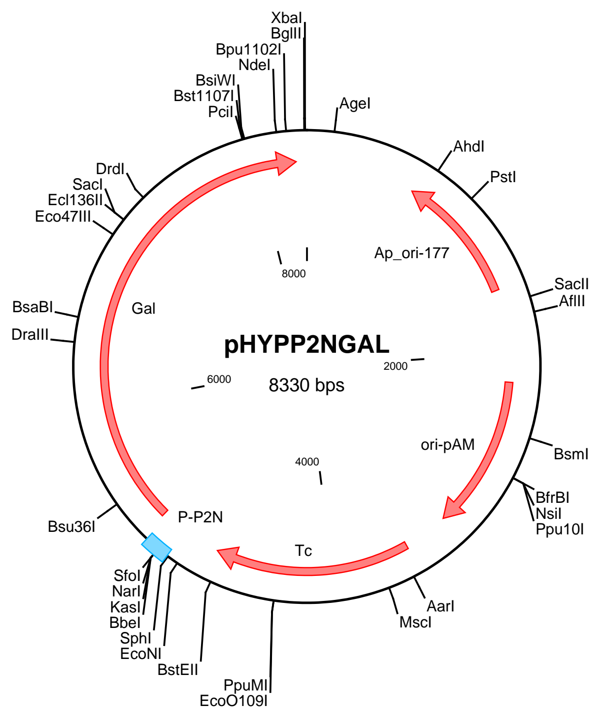


APPENDIX A (continued)

(3) pUC118

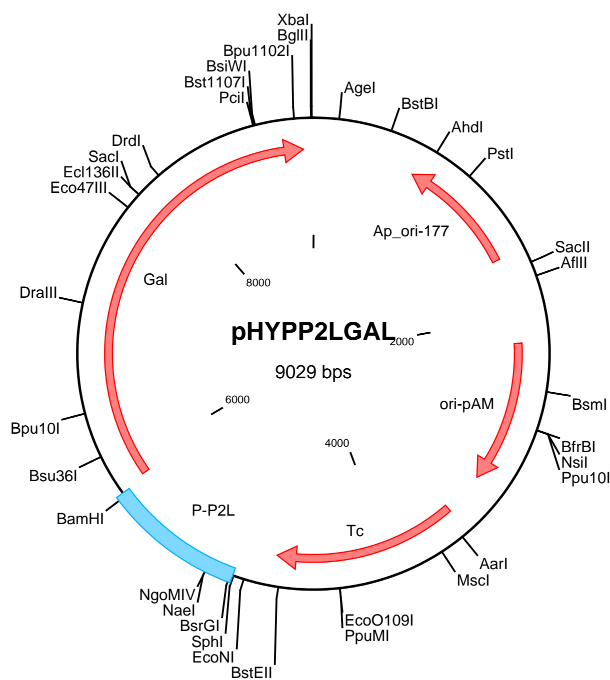


(3) pHP2N

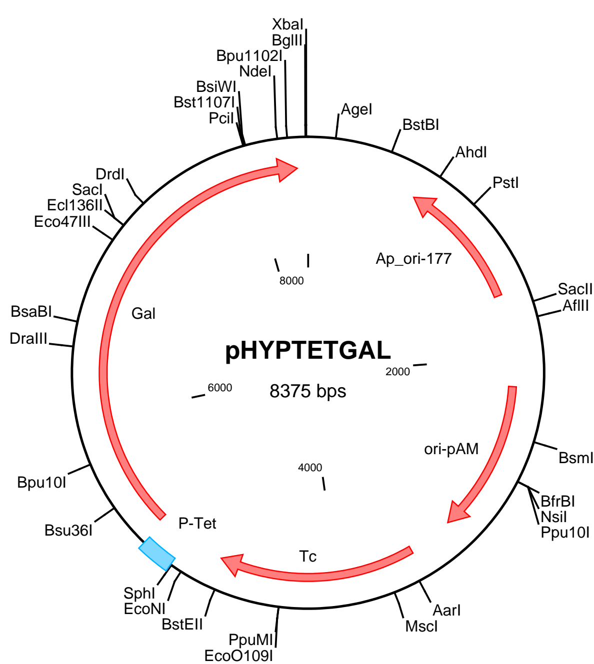


APPENDIX A (continued)

(4) pHP2L

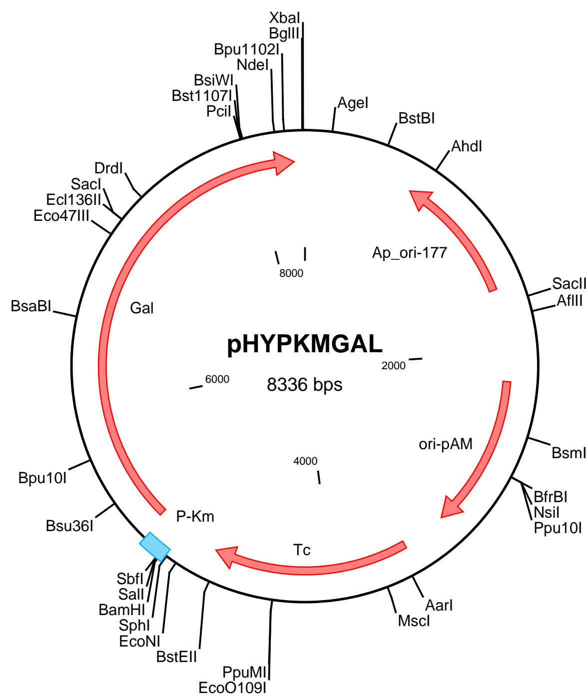


(4) pHPTet

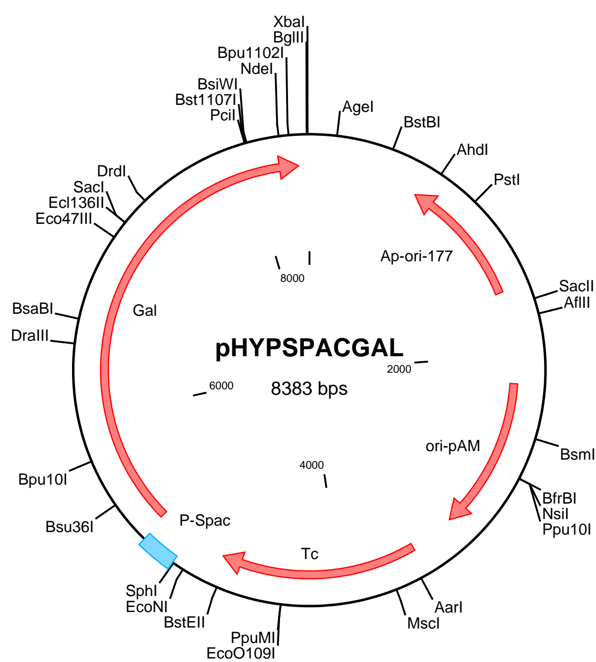


APPENDIX A (continued)

(5) pHPKm

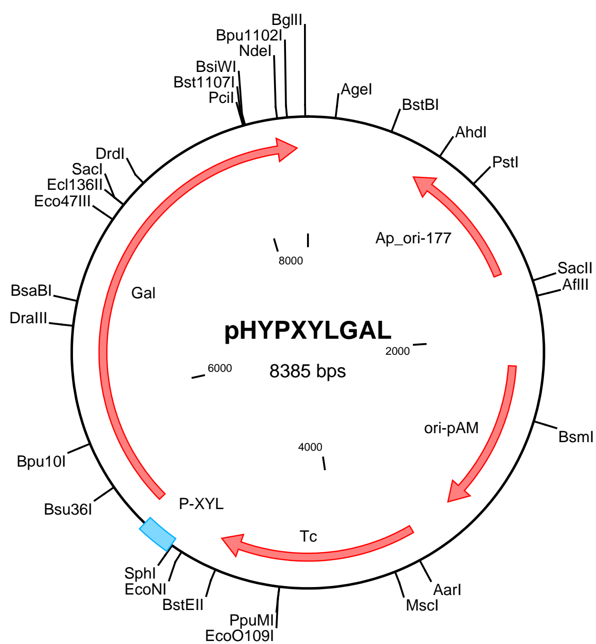


(6) pHPSpac

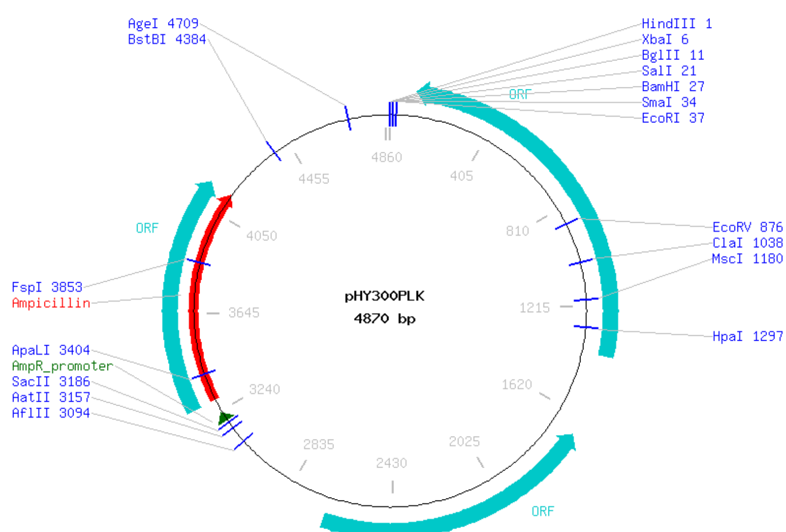


APPENDIX A (continued)

(7) pHPXyl

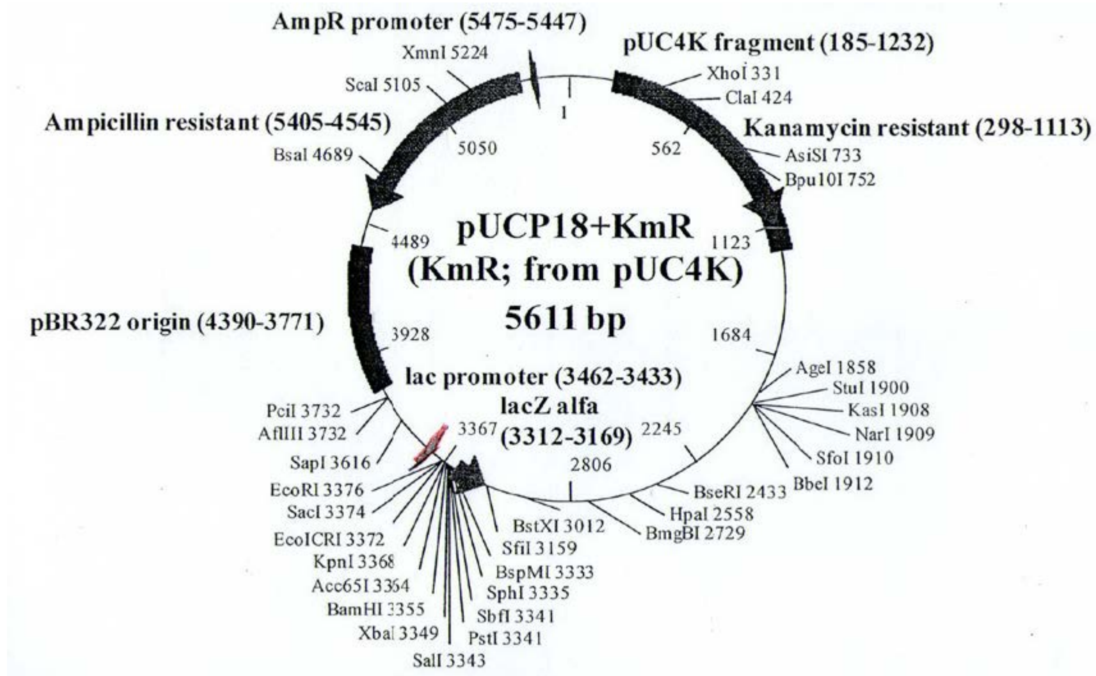


(8) pHY300PLK

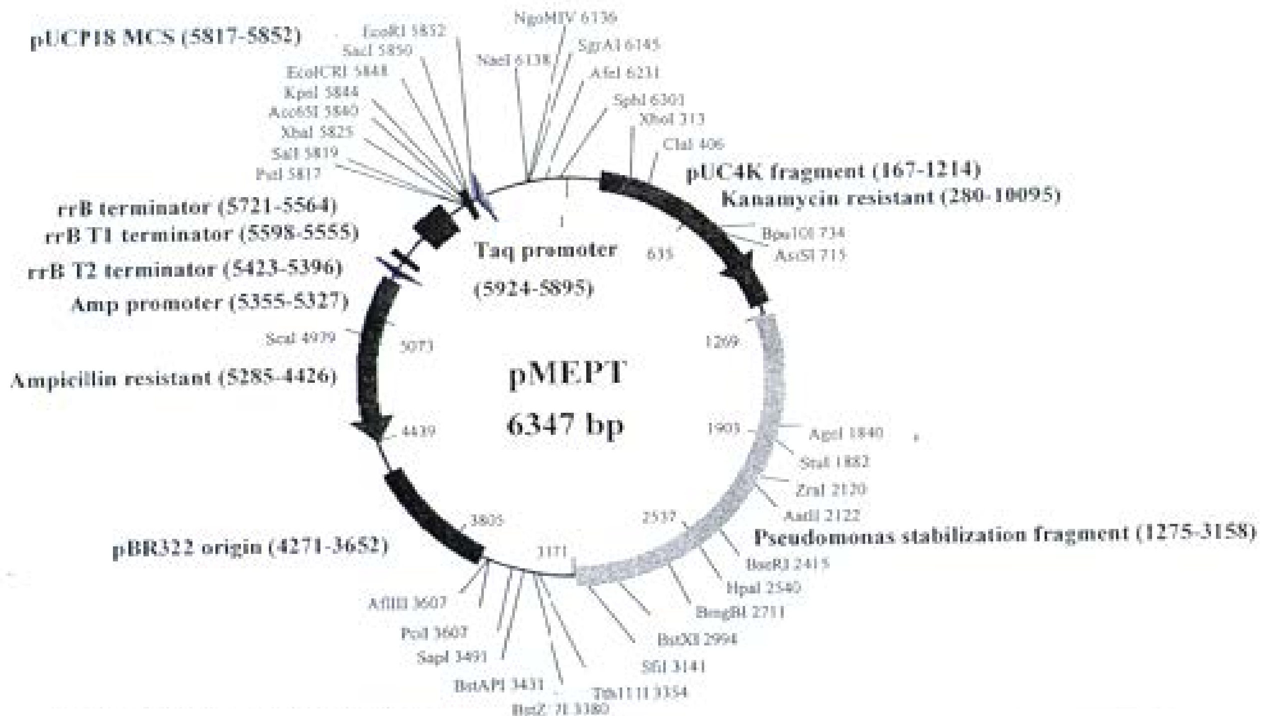


APPENDIX A (continued)

(9) pUCP18



(10) pMEPT



APPENDIX B

BSA standard curve

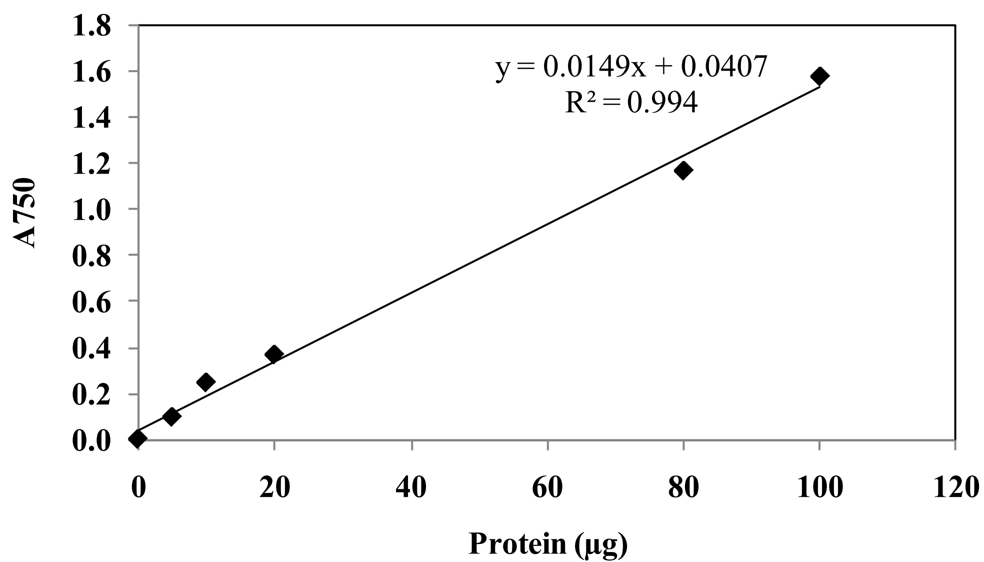


Figure B Standard curve of BSA from modified Lowry method used to determine cell protein.

APPENDIX C

3-Methylcatechol (3MC) standard curve

Standard curve was used to analyze chromatogram of HPLC result in order to calculate the concentration of 3MC product. The 3MC standard was freshly prepared in ethanol as a stock solution at 100 mM. Then, 3MC at various concentrations was obtained by doing a serial dilution from 3MC stock solution. The 3MC residue was determined by using the same Method as described in 3.20. The standard curve of 3MC showed in Figure D1.

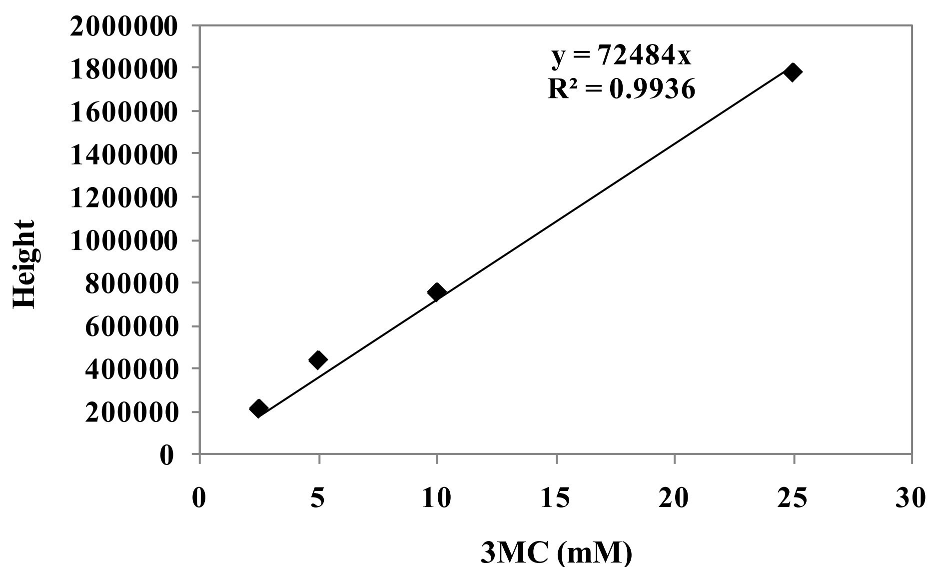


Figure C1 3MC standard curve for calculation 3MC concentration.

$$\text{The amount of 3MC} = \frac{\text{Peak height}}{72484}$$

APPENDIX C (continued)

Retention time of 3MC

3MC was analyzed using reverse phase HPLC equipped with a UV detector (at 240 nm). The separation was performed at 28ºC on a C18 column using an acetonitrile: water mixture (70: 30, v/v) as a mobile phase at a flow rate of 1 ml/min. the injection volume was 20 µl. The retention times of 3MC standard and 3MC in aqueous phase and in 1-decanol phase under condition tested were 3.002, 2.991, and 2.984, as showed in Figure D2, D3, and D4, respectively.

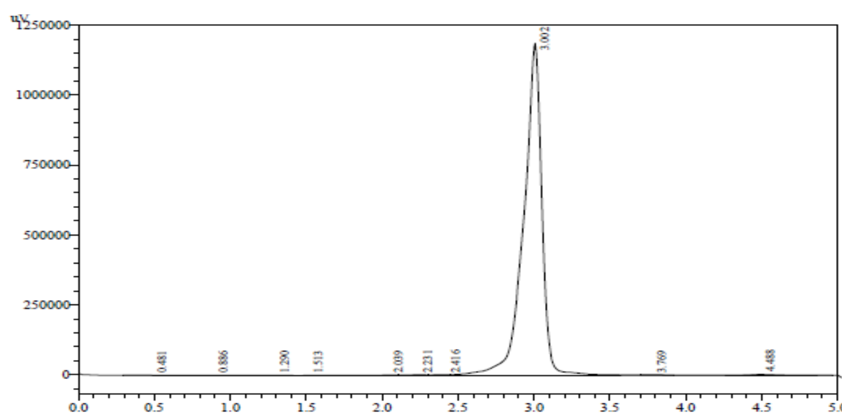


Figure C2 HPLC chromatogram of 3MC standard at concentration100 mM. The retention time of 3MC was 3.002.

APPENDIX C (continued)

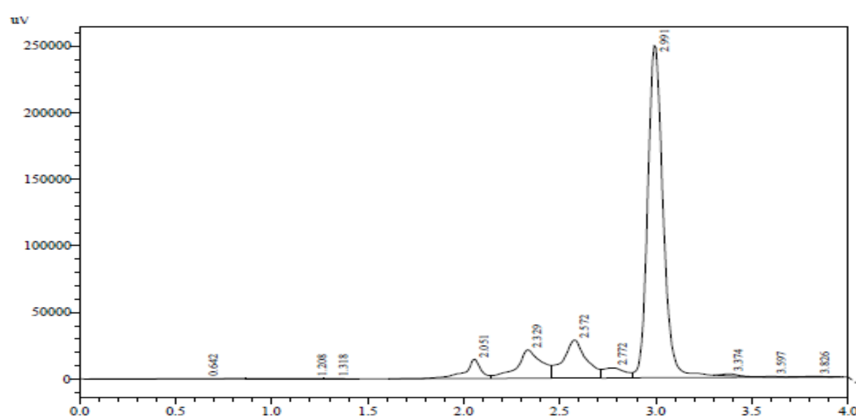


Figure C3 HPLC chromatogram of 3MC in aqueous phase. The retention time of 3MC was 2.991.

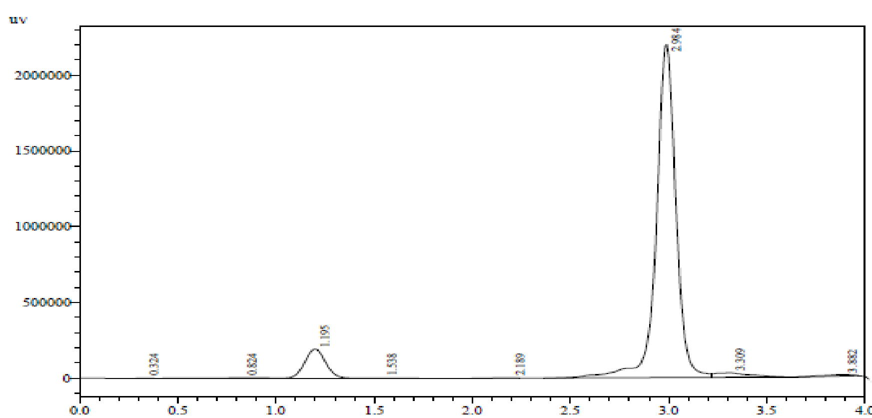


Figure C4 HPLC chromatogram of 3MC in 1-decanol phase. The retention time of 3MC was 2.984.

APPENDIX D

Acrolein standard curve

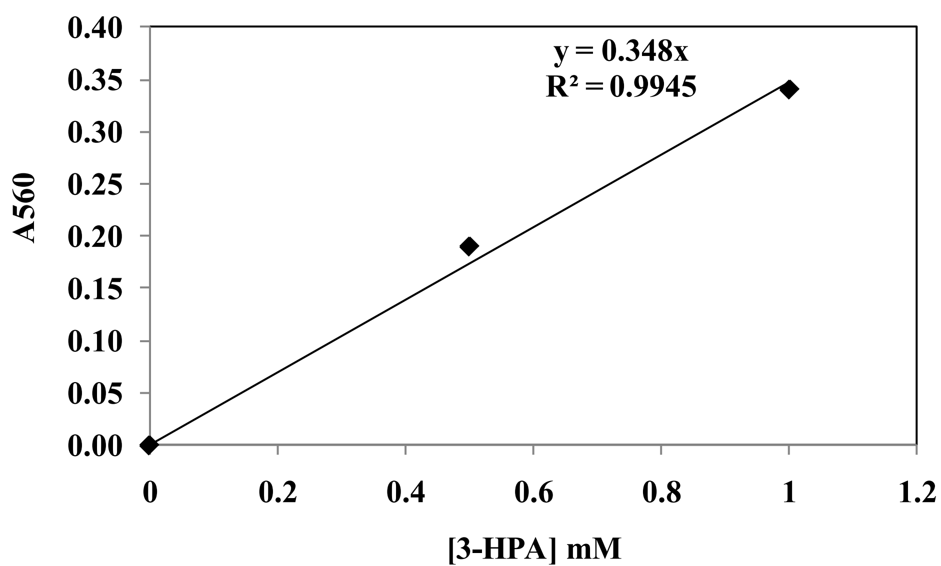


Figure D Acrolein standard curve for calculation 3-HPA concentration in sample mixture.

BIOGRAPHY

Miss Ajiraporn Kongpol was born on June 1982, in Krabi province, Thailand. She received Bachelor's Degree in Applied biology and Microbiology, Faculty of science, Suan Sunandha Rajaphat University in 2005. She finished Master Degree study in Biotechnology Program, Faculty of Science, Chlulalongkorn University, and Bangkok, Thailand in 2008. And then follow with Ph. D. Degree in same program of master degree, during her study, the output of her research work can be summarized as follows:

1. **Ajiraporn Kongpol**, Junichi Kato, and Alisa S. Vangnai. (2008) Isolation and characterization of *Deinococcus geothermalis* T27, a slightly thermophilic and organic solvent-tolerant bacterium able to survive in the presence of high concentrations of ethyl acetate. *FEMS Microbiology Letters*. 286, 277-235. Impact factor = 2.247
2. **Ajiraporn Kongpol,** Thunyarat Pongtharangkul, Junichi Kato, Kohsuke Honda, Hisao Ohtake & Alisa S. Vangnai. (2009) Characterization ofan organic-solvent-tolerant *Brevibacillus agri* strain13 able to stabilize solvent/water emulsion. *FEMS Microbiology Letters*. 297, 225-233. Impact factor = 2.247
3. **Ajiraporn Kongpol**, Junichi Kato, Takahisa Tajima & Alisa S. Vangnai. (2010) Characterization of acetonitrile-tolerant marine bacterium *Exiguobacterium* sp. SBH81 and its tolerance mechanism.

   Impact factor = 2.248