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# Biocatalysis for oxidation of naphthalene to 1-naphthol: liquid-liquid biphasic systems and solvent tolerant strains

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# BIOCATALYSIS FOR OXIDATION OF NAPHTHALENE TO 1-NAPHTHOL: LIQUID-LIQUID BIPHASIC SYSTEMS AND SOLVENT TOLERANT STRAINS

by

Bhaskara Janardhan Garikipati Satya Venkata

An Abstract

Of a thesis submitted in partial fulfillment of the requirements
for the Doctor of Philosophy degree in Chemical and Biochemical
Engineering in the Graduate College of
The University of Iowa

May 2009

Thesis Supervisor: Associate Professor Tonya L. Peeples

# ABSTRACT

Biocatalysis involves the use of enzymes to perform stereo- and enantio-specific reactions. One of the reactions where biocatalysis is a valuable technology is oxidation of naphthalene to 1-naphthol using Toluene *ortho*-Monooxygenase (TOM) variant TmoA3 V106A, also known as TOM-Green. Whole-cell biocatalysis in a water-organic solvent biphasic system was used to minimize naphthalene and 1-naphthol toxicity, and to increase substrate loading. Recombinant *Escherichia coli* TG1 cells expressing TOM-Green were used for biphasic biocatalysis and lauryl acetate gave best results among the solvents tested. On a constant volume basis, 8 - fold improvement in 1-naphthol production was achieved using biphasic systems compared to biotransformation in aqueous medium. The organic phase was optimized by studying the effects of organic phase ratio and naphthalene concentration in the organic phase. The efficiency of biocatalysis was further improved by application of a solvent tolerant strain *Pseudomonas putida* S12. *P. putida* S12 is solvent tolerant owing to its two adaptive mechanisms: outer membrane modification and solvent extrusion using solvent resistant pump srpABC. *P. putida* S12, in addition to its tolerance to various organic solvents, showed better tolerance to naphthalene compared to *E. coli* TG1 strain expressing TOM-Green. Application of solvent tolerant *P. putida* S12 further improved 1-naphthol productivity by approximately 42%. Solvent tolerance of *P. putida* S12 was further analyzed by transferring its tolerance to a solvent sensitive *E. coli* strain by transfer of solvent resistant pump srpABC genes. Engineered *E. coli* strain bearing srpABC genes either in low-copy number plasmid or high-copy number plasmid grew in the presence of a saturated toluene concentration. Engineered *E. coli* strains were also more tolerant to

toxic solvents, e. g., decanol and hexane, compared to the control *E. coli* strain without srpABC genes. The expression of solvent resistant pump genes was confirmed by Reverse Transcriptase PCR analysis. The main drawbacks of biocatalysis for production of chemicals were addressed and approaches to minimize the drawbacks have been presented. The production of 1-naphthol was significantly improved using biocatalysis in liquid-liquid biphasic systems.

Abstract Approved: _____
Thesis Supervisor

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Title and Department

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Date

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Iowa City, Iowa

CERTIFICATE OF APPROVAL

PH. D. THESIS

This is to certify that the Ph. D. thesis of

Bhaskara Janardhan Garikipati Satya Venkata

has been approved by the Examining Committee for the thesis requirement for the Doctor of Philosophy degree in Chemical and Biochemical Engineering at the May 2009 graduation.

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# INTRODUCTION

## Background

Biocatalysis involves the use of enzymes to perform chemical reactions. Enzymes catalyze various reactions, often at ambient temperatures, mild conditions and in an environmentally benign way. In addition, most enzymes carry out reactions with high chemo-, regio- and enantio-selectivity thereby reducing the formation of unwanted by-products. Application of enzymes in biocatalytic processes is an emerging technology that has potential in various industries. Although most applications have been for low-volume high value products such as pharmaceutical drugs and fine chemicals, bulk chemicals such as ethanol and fructose have been produced by fermentation at more than million tons per year (Thayer 2001). The emergence of recombinant DNA technology has improved the efficiency of biocatalytic processes, thereby making it economically feasible in various applications.

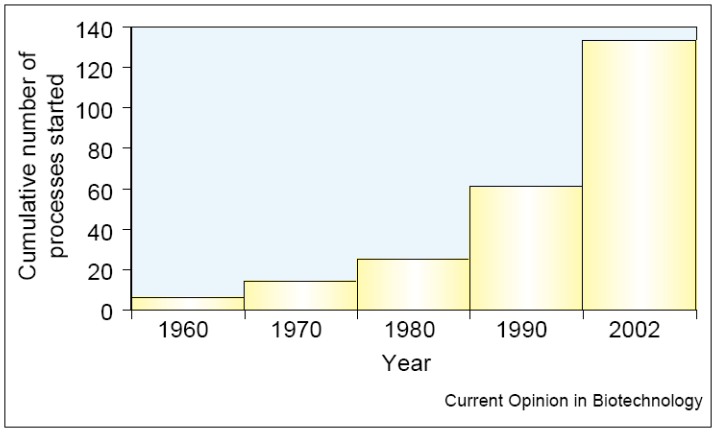



Figure 1.1. Cumulative number of bioprocesses that have been started on an industrial scale (Straathof et al. 2002)

Biocatalysis has become a standard technology in various industries, and there have been increasing number of bioprocesses running on a commercial scale as shown in Figure 1.1 (Straathof et al. 2002). By 2050, it is expected that 30% of the chemicals business could be from biotransformations (van Beilen et al. 2003). However, the dependence of many enzymes on costly cofactors, such as NADPH or NADH, complicates their utilization. The application of whole cell biocatalysis offers an economical way for recycling the cofactor. Commercially, there are processes where both isolated enzymes and whole cells were used, for example in the production of 6-amino penicillanic acid and indigo, respectively, as shown in Figure 1.2 (Tramper 1996).

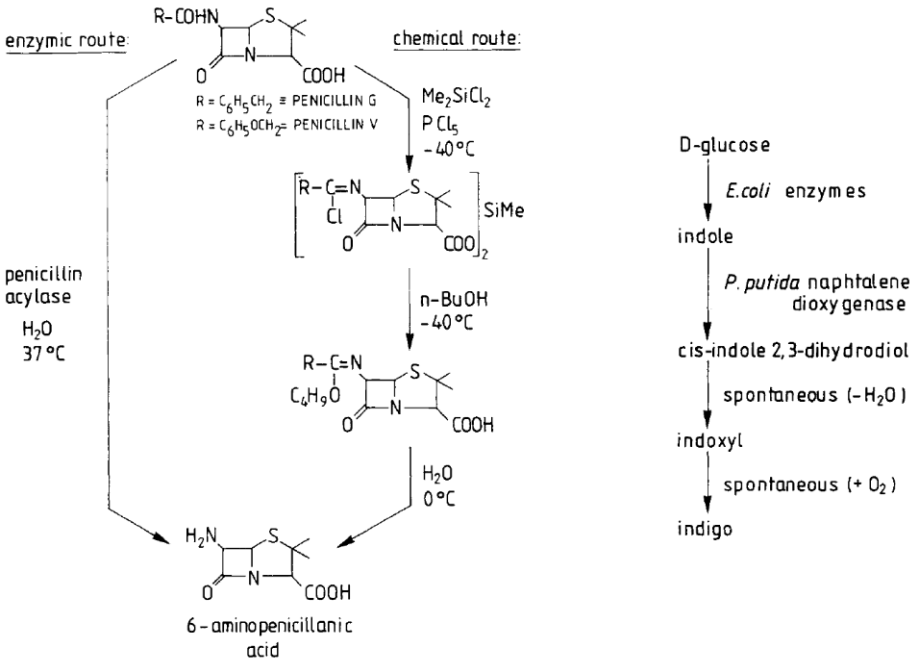



Figure 1.2. Commercial processes using isolated enzymes and whole-cells (Tramper 1996)

Straathof *et al.* (2002) have analyzed about 134 industrial biotransformations and revealed that hydrolases (44%) and redox (30%) biocatalysts are the most prominent, as shown in Figure 1.3. They also revealed that whole-cells are more popular compared to isolated enzymes, as shown in Figure 1.4. The biocatalysts were also used in immobilized form, to achieve higher stability and recycle efficiency of the biocatalyst.

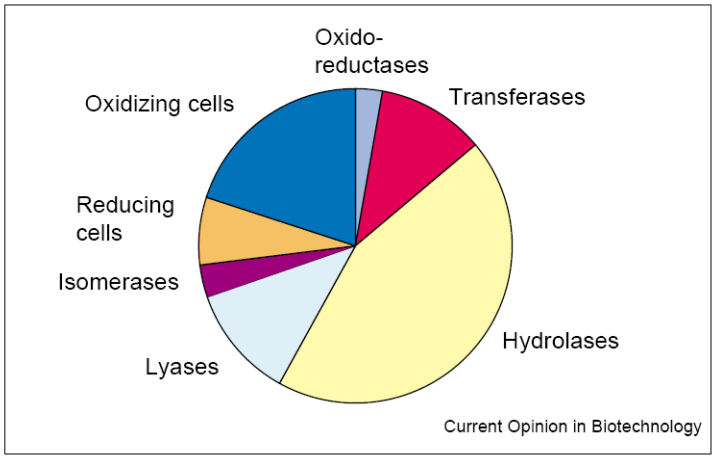



Figure 1.3. Enzyme types used in industrial biotransformations (Straathof et al. 2002)

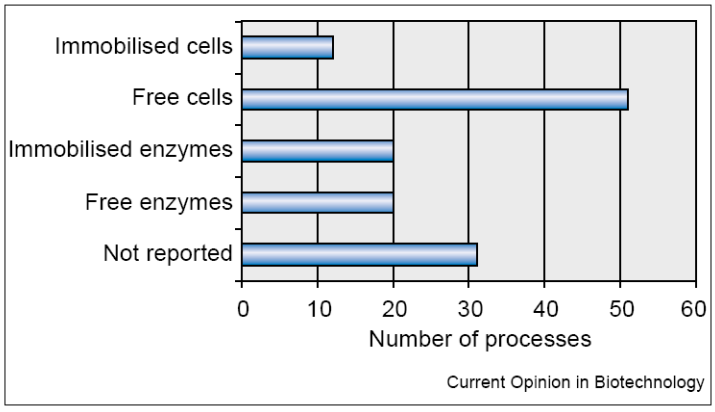



Figure 1.4. Use of enzymes or whole-cells in industrial biotransformations (Straathof et al. 2002)

## Oxidation of Naphthalene

One of the reactions where biocatalysis is a useful technology is oxidation of naphthalene. Oxidation of naphthalene produces singly hydroxylated products, 1-naphthol and 2-naphthol, and doubly hydroxylated products, naphthalene dihydrodiols, which have wide applications in dyes, perfumes and pigments industries. 1-naphthol is also used for the manufacture of an insecticide, Carbaryl (1-naphthyl methylcarbamate) (Back 1965; Kulkarni et al. 1991) sold under the brand name Sevin by Union Carbide, and in the manufacture of Propranolol (Weglicki 2000), a pharmaceutical drug used for hypertension. The traditional chemical manufacture of 1-naphthol involves the use of toxic reagents and harsh conditions. 1-Naphthol is produced commercially by Union Carbide and Figure 1.5 shows the flow sheet for 1-naphthol production (Ajiboye 2007).

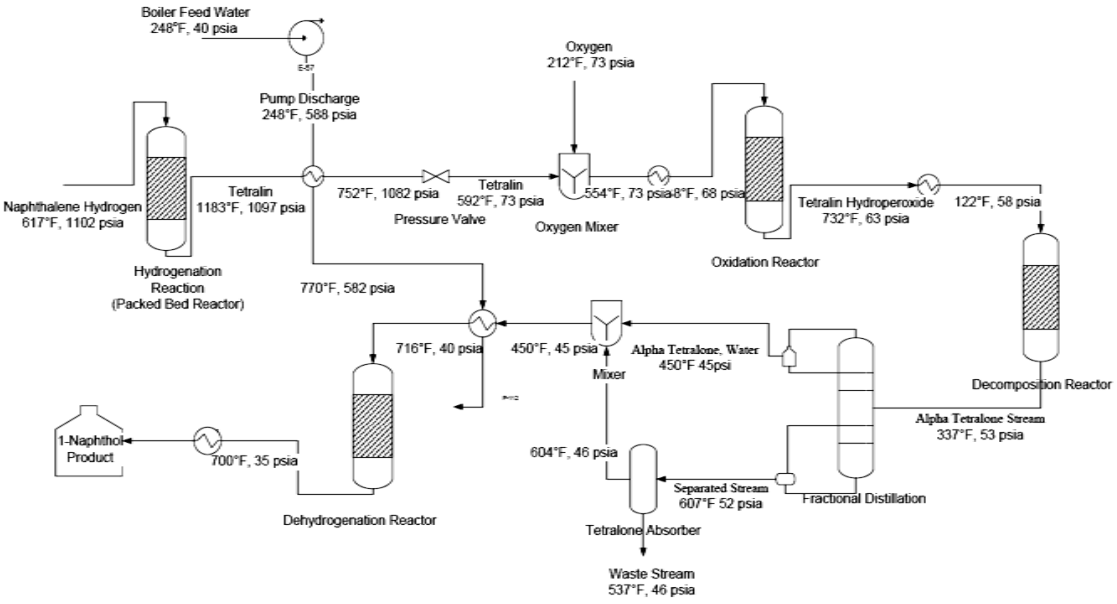



Figure 1.5. 1-Naphthol production from naphthalene (Union Carbide Process) (Ajiboye 2007)

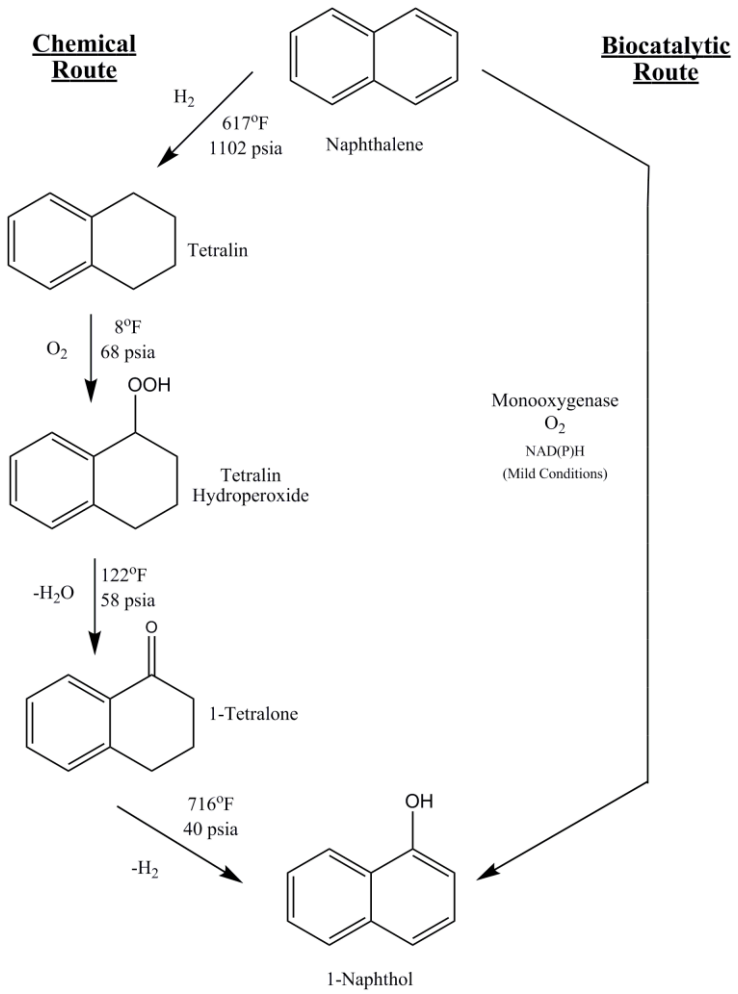



Figure 1.6. Comparison of chemical and biocatalytic routes for 1-naphthol production (Ajiboye 2007)

The Union Carbide process uses nickel, copper or platinum as catalyst and the environmental analysis of the process, done by Ajiboye (2007), reveals that it has high fish toxicity index (47%) and high human toxicity ingestion index (53%). The process has high emissions of 1, 2-dihydronapthalene and 1-tetralol acid.

Biocatalysis offers a 'Green Chemistry' approach for this reaction by the use of monooxygenases for oxidation of naphthalene to 1-naphthol. Figure 1.6 shows a comparison of chemical and biocatalytic routes for 1-naphthol production.

Monooxygenases use molecular oxygen and NAD(P)H as a cofactor under mild-conditions. Biocatalytic route involves a single-step reaction compared to multi-step reactions involved in chemical route. Biocatalytic route eliminates the formation of byproducts by the use of monooxygenase with high regio- & chemo-selectivities. Whole-cells expressing monooxygenase can be used for 1-naphthol production to recycle costly cofactor NAD(P)H, thereby eliminating the need for addition of costly cofactors.

The overall goal of this project is the oxidation of naphthalene to 1-naphthol to achieve high product yields with low downstream processing costs in an environmentally benign way. Although biocatalysis shows promise for oxidation of naphthalene, there are two major limitations in improving the yields for 1-naphthol production. First, naphthalene and its oxidation products are toxic to the cells. These compounds are hydrophobic and accumulate in the bacterial membranes, thereby causing deleterious effects on the microorganism and decreasing the enzymatic activity. Second, naphthalene is poorly soluble in the water making it less bioavailable to the microorganism. Some of these problems are evident from Figure 1.7, which shows 1-naphthol synthesis using Toluene *ortho*-Monooxygenase (TOM) and its variant TOM-Green at different substrate concentrations (Canada et al. 2002). Although the enzymatic activity was increased significantly for TOM-Green, as shown in the Figure 1.7, the optimum substrate concentration was low due to the toxic effects of the substrate.

## Hypothesis and Specific Aims

The overall goal of the project is to achieve high 1-naphthol productivities by minimizing the drawbacks of biocatalysis. High 1-naphthol productivities can be

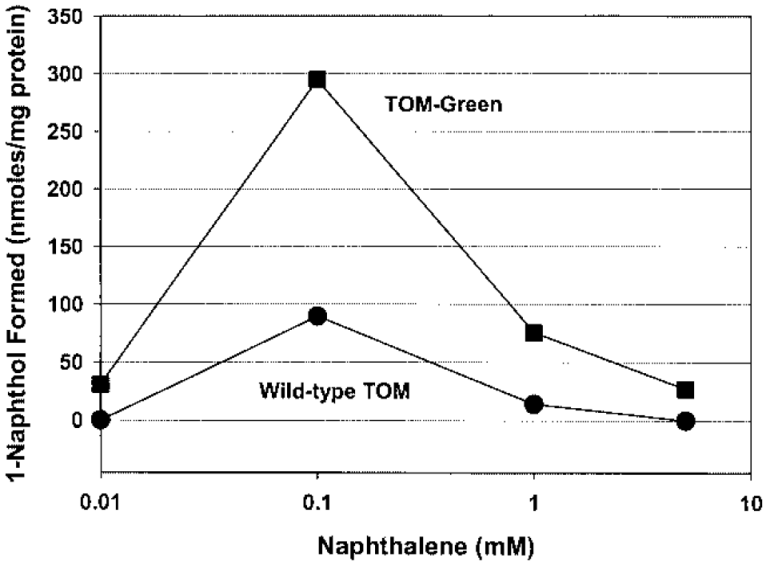



Figure 1.7. Effect of naphthalene concentration on enzyme activity (Canada et al. 2002)

achieved by designing a process which allows for high substrate loading in addition to maintaining low concentrations of substrate and product in the vicinity of the biocatalyst. Such a process can be achieved by application of a water-organic solvent biphasic system for oxidation of naphthalene (Daugulis 1997; Leon et al. 1998). The use of a second phase of an organic solvent not only increases the substrate concentrations and maintains low concentrations of toxic compounds in the aqueous phase, but also enhances productivity by in-situ product recovery, and helps in easier recovery of the product and the biocatalyst with a decrease in downstream processing costs (Bruce and Daugulis 1991; de Bont 1998). Biphasic reactions have been applied earlier to improve productivities of various reactions, involving toxic substrates and/or toxic products (Collins and Daugulis 1999; Mathys et al. 1999; Morrish et al. 2008; Prpich and Daugulis 2007b; Tao et al. 2005a) and for easier recovery of the product (Leon et al. 2005; Newman et al. 2006) However, biphasic systems have limitations associated with solvent

toxicity. Many organic solvents are toxic to microorganisms, thereby decreasing the stability and activity of the biocatalyst in biphasic media. Although application of solid adsorbents as a second phase for product removal have been applied earlier, they have limitations associated with adsorption capacity and selectivity (Prpich and Daugulis 2007a). Application of thermoplastic polymers instead of organic solvents has been shown recently to have better advantages (Morrish and Daugulis 2008; Prpich and Daugulis 2007a). However, biphasic water-organic solvent reactions have been well demonstrated and have advantages associated with the availability of vast number of solvents with diverse properties (Daugulis 1997). Therefore, a solvent tolerant strain is required to express the enzyme for biocatalysis. Solvent tolerant strains have various adaptive mechanisms to survive in the presence of toxic organic solvents. Many solvent tolerant strains have been isolated (Heipieper et al. 2007; Kieboom et al. 1998a; Ramos et al. 2002; Rojas et al. 2001; Segura et al. 1999; Zahir et al. 2006). One of such strains upon which extensive physiological studies were performed to study solvent tolerance is *Pseudomonas putida* S12. *P. putida* S12 survives in presence of toxic organic solvents by extruding solvents from the cell membrane by utilizing a proton-dependent solvent efflux pump (Kieboom et al. 1998a). Although *P. putida* S12 has various advantages for naphthalene oxidation in a biphasic system, *P. putida* S12 has a few drawbacks to be a potential host for biocatalysis. *P. putida* S12 has a styrene monooxygenase and is known to metabolize some organic solvents, such as styrene, heptanol and octanol (Weber et al. 1993). This can lead to the formation of unwanted products which complicate the downstream process. Moreover, this strain has not been fully sequenced and there could be other enzymes which compete to form unwanted byproducts. Considering these

drawbacks, if an *E. coli* strain could be genetically modified to achieve solvent tolerance, then it can be a better host for biocatalysis. Engineering solvent tolerance in *E. coli* can be done by expressing solvent resistant pump genes from *P. putida* S12. The following **Specific Aims** of the project will be necessary to achieve the goal:

1. Oxidation of naphthalene in liquid-liquid biphasic systems
2. Application of *P. putida* S12 for oxidation of naphthalene in liquid-liquid biphasic systems
3. Engineering solvent tolerance in *E. coli*

*Chapters 2, 3, and 4* discuss in detail the scope, experiments performed and achievements related to the specific aims 1, 2, and 3 respectively. The lines of research address some of the major drawbacks associated with production of chemicals using biocatalysis. The approach used herein can be applied to any biocatalytic process where microorganisms are used for production of chemicals. The work in this dissertation aids in developing processes for the production of chemicals in an economical and environmentally benign way. *Chapter 5* states the key conclusions of this work and *Chapter 6* provides some future work ideas to further improve the process.

# CHAPTER 2
# WHOLE-CELL BIOCATALYSIS FOR 1-NAPHTHOL PRODUCTION IN A LIQUID-LIQUID BIPHASIC SYSTEM

## Introduction

Biocatalysis has emerged as an important technology in industrial organic synthesis for the production of chemical synthons and high value products (Pollard and Woodley 2007; Schmid et al. 2001; Straathof et al. 2002). Biocatalysis offers the advantage of performing reactions under mild conditions and provides an environmentally benign approach for chemical reactions (Azerad 2001; Tao et al. 2005a). Oxygenases are a class of enzymes that have great potential and versatility to catalyze reactions that are generally not accessible by chemical routes with high regio-, stereo- and enantio-selectivity (Buhler and Schmid 2004; Nolan and O'Connor 2008; Urlacher and Schmid 2006; van Beilen et al. 2003). Oxygenases introduce either one or two atoms of molecular oxygen into organic molecules using NADH or NADPH as a cofactor. To eliminate the addition of costly cofactor, whole-cells expressing oxygenases are generally used (Schmid et al. 2001; van Beilen et al. 2003).

One of the potential applications of biocatalysis utilizing oxygenases is the oxidation of naphthalene to 1-naphthol. 1-Naphthol has wide applications in the manufacture of dyes, drugs, insecticides, perfumes and surfactants (Back 1965; Canada et al. 2002; Kirk-Othmer 1999). Oxidation of naphthalene to 1-naphthol was reported by England *et al.* (1998) using Cytochrome $P450_{cam}$ and its Y96 mutants. One of the Y96 mutants, Y96F, gave maximum naphthalene degradation rates and coupling efficiency defined as percentage of cellular cofactor utilized for the reaction. Tao *et al.*(2005b) have

compared the reaction rates and regioselectivities of various wild-type and modified monooxygenases for the oxidation of naphthalene to 1-naphthol. Toluene 4-monooxygenase (T4MO) of *Pseudomonas mendocina* KR1 (Whited and Gibson 1991) showed maximum activity for naphthalene oxidation but had poor regioselectvity producing 52% 1-naphthol and 48% 2-naphthol. Among the monooxygenases tested, the best enzyme for the oxidation of naphthalene to 1-naphthol was a toluene *ortho*-monooxygenase (TOM) variant TmoA3 V106A, also known as TOM-Green. TOM was isolated from *Burkholderia cepacia* G4, and consists of a $(\alpha\beta\gamma)_2$ hydroxylase (from *tom*A1A3A4) with two catalytic oxygen-bridged binuclear iron centers, a NADH-oxidoreductase (from *tom*A5), a protein (from *tom*A2) involved in electron transfer between oxidoreductase and hydroxylase, and a relatively unknown subunit (from *tom*A0) (Newman and Wackett 1995; Shields et al. 1995). The enzyme has 64% overall DNA identity to toluene/benzene 2-monooxygenase of *Pseudomonas sp.* strain JS150 and 54% overall DNA identity to phenol hydroxylase form *Pseudomonas* CF600 (Canada et al. 2002). TOM-Green was produced by directed evolution of TOM with one amino acid change in the alpha-subunit of the hydroxylase (Canada et al. 2002; Rui et al. 2004). TOM-Green retained high regioselectivity (98%) and was 7-fold faster than wild-type TOM. The apparent $V_{max}$ and $K_m$ for cells expressing TOM-Green to convert 5 mM naphthalene was calculated by Rui et al. (2004) as 4.5 nmol. $min^{-1}$. mg of $protein^{-1}$ and 13.5 μM, respectively.

A considerable effort was made to identify and characterize oxidative biocatalysts for 1-naphthol production (Canada et al. 2002; England et al. 1998; Newman and

Wackett 1995; Rui et al. 2004; Rui et al. 2005; Shields et al. 1995; Tao et al. 2005b; Tao et al. 2004; Whited and Gibson 1991). However, the process is not economically feasible owing to very low optimum naphthalene concentration [0.1 mM (Canada et al. 2002), less than its solubility of 0.23 mM (0.03 g/L) (Perry and Green 1997)] and toxicity of both naphthalene and 1-naphthol (Tao et al. 2005a; Wilson et al. 1996). The substrate loading has to be increased and the toxicity of both naphthalene and 1-naphthol has to be minimized to make the process feasible. As a consequence, biotransformations in water-organic biphasic media have been developed (Collins and Daugulis 1999; Daugulis 1997; Freeman et al. 1993; Leon et al. 1998; van Beilen et al. 2003; Witholt et al. 1990; Wubbolts et al. 1996). The use of a second phase of organic solvent not only increases substrate loading but also maintains low concentration of toxic compounds in the aqueous phase and enables *in-situ* recovery of the product, thereby decreasing processing costs. The choice of organic solvent is critical in achieving the benefits of biphasic media. Two main criteria for solvent selection are high distribution coefficient for the product and biocompatibility with microorganisms (Brink and Tramper 1985; Bruce and Daugulis 1991). Biocompatibilty is generally correlated with logP of the solvent, which is the logarithm of the partition coefficient in octanol-water system, and organic solvents with logP value greater than 4 are generally biocompatible with microorganisms (Laane et al. 1987). However, the correlation of activity with logP is specific to the microorganism and the critical logP for the microorganism has to be identified for selection of biocompatible solvents (Inoue and Horikoshi 1991). Innoue et al. (1991) observed that the solvent critical logP for *E. coli* is 3.4 to 3.8, depending on the strains tested. In order to eliminate effects of solvent toxicity, solid-liquid two phase systems were also used

where either solid adsorbents or thermoplastic polymers were used to enhance productivities (Held et al. 1999; Morrish and Daugulis 2008; Prpich and Daugulis 2007a). The stability of cells in the presence of organic solvents can also be improved by immobilization, which also allows for biocatalyst reuse (Hocknull and Lilly 1990; Keweloh et al. 1989), or by application of solvent tolerant microorganisms (Heipieper et al. 2007; Husken et al. 2001; Rojas et al. 2004).

Biphasic reactions have been used earlier for reactions involving a toxic substrate and/or product to enhance productivities (Mathys et al. 1999; McIver et al. 2008; Morrish et al. 2008; Prpich and Daugulis 2007b; Tao et al. 2005a). Biphasic reactions also have application in the production of volatile compounds where volatile amorpha-4,11-diene produced by metabolically engineered *E. coli* strain was captured using a second phase of dodecane (Newman et al. 2006). Oxidation of naphthalene was also improved using biphasic reactions (Harrop et al. 1992; McIver et al. 2008; Sello et al. 2004; Tao et al. 2005a). Tao *et al.* (2005a) applied a biphasic system for 2-naphthol and phenol production using toluene 4-moooxygenase (T4MO) and its variant TmoA I100A. They achieved 10- to 21- fold improvement in 2-naphthol and phenol concentrations using dioctyl phthalate as the organic solvent. McIver *et al.* (2008) have used naphthalene dioxygenase to oxidize naphthalene to *cis*-(1R, 2S)-1,2-naphthalene dihydrodiol using dodecane as the organic solvent. 1.7 g/gCDW/h diol productivity was obtained in the first six hours using 20% organic phase ratio and the cellular activity was maintained for four cycles using immobilized cells. In spite of significant improvements achieved by using a biphasic system for various reactions, its application to 1-naphthol production has not yet

been explored. Considering high toxicity of both naphthalene and 1-naphthol (Tao et al. 2005a), biphasic reactions can improve the productivities. In this work, a biphasic system was applied to improve 1-naphthol productivities using whole-cells of *E. coli* expressing the TOM-Green enzyme. Organic solvents were screened and suitable solvents were identified for high 1-naphthol productivity. The organic phase was optimized by studying the effects of naphthalene concentration and organic phase ratio. The stability of biocatalyst for recycle was also tested.

## Materials and Methods

*Chemicals and Bacterial Strain.* Dodecane, lauryl acetate, naphthalene, 1-naphthol, and sodium alginate were purchased from Sigma (St. Louis, MO). Dioctyl phthalate and $CaCl_2$ were purchased from Fischer Scientific (Hanover Park, IL). Luria-Bertani (LB) broth was purchased from Difco (Lawrence, KS).

*Escherichia coli* TG1 pBS(Kan)TOM-Green expressing TOM-Green enzyme was kindly donated by Thomas K. Wood (Texas A&M University). The plasmid pBS(kan)TOM-Green expresses TOM-Green (*tom*A012345) from a *lac* promoter. The *lac* promoter yields constitutive expression of TOM-Green genes due to high copy number of plasmid and lack of *lac*I represser (Canada et al. 2002; Tao et al. 2004).

*Toxicity Experiments.* The cells were grown until early log-phase in 250 mL shake flasks and 5 mL of growing cells were added to each 20 mL sterile screw-cap vials. Four different concentrations of naphthalene and 1-naphthol, 0.05 g/L, 0.1 g/L, 0.5 g/L and 1 g/L, in 50 µL dimethyl formamide (DMF) were added to the growing cells and the

growth was monitored by optical density (OD) at 660 nm. Due to low solubility of naphthalene and 1-naphthol in water, a cosolvent DMF was used to suspend the compound in aqueous phase. A positive control was performed where 50 µL DMF was added without naphthalene or 1-naphthol.

*Cell Viability Determination.* A Becton Dickinson LSR II Flow Cytometer at the University of Iowa Flow Cytometry Facility was used to measure cell viability. The Molecular Probes LIVE/DEAD *Bac*Light Bacterial Viability Kit L-7012 was used for analysis. Aqueous phase samples were collected after 3 h of the biotransformation for the analysis. The flow cytometry procedure provided with the viability kit was used to prepare samples for analysis. Cells were washed twice with 0.85% NaCl solution and diluted to ~0.03 OD at 660 nm. SYTO 9 and propidium iodide dyes were added to the samples and incubated in dark for 15 minutes before analysis. The live cells fluoresce green and the dead cells fluoresce red.

*HPLC Analysis.* High performance liquid chromatography (HPLC) was used to quantify 1-naphthol and naphthalene. An 1100 Series Agilent HPLC with a PDA detector and a supelcosil LC-PAH 5 µm column (25 cm x 4.6 mm) at room temperature was used for the analysis. Aqueous samples were diluted (1/2) in acetonitrile. Organic samples were directly injected without dilution. Both organic and aqueous samples were centrifuged to separate cell debris and were filtered with 0.2 µm PTFE filters.

The mobile phase consisted of deionized water with 0.2% (v/v) glacial acetic acid and optima grade acetonitrile with a gradient elution. The gradient for water / acetonitrile was 65/35 (v/v) at t = 0 min; linear gradient to 100/0 at t = 7 min; linear gradient to 65/35 at t = 8 min; equilibration to t = 10 min. The mobile phase flow rate was 1.5 mL/min and the injection volume was 10 µL. Naphthalene and 1-naphthol were analyzed at 272 nm and detected at 5.7 and 3.9 minutes of retention time, respectively. The integrated areas of the elution peaks were used to calculate the concentration of naphthalene and 1-naphthol in each phase.

*Distribution Coefficient Measurement.* 500 µL of different organic solvents with 0.75 g/L 1-naphthol was added to 500 µL of phosphate buffer in a 2 mL microcentrifuge tube. The two phases were mixed by vortexing for 30 sec for five times with 1 min intervals. The two phases were analyzed using HPLC. Distribution coefficient ($K_D$) was calculated as the ratio of 1-naphthol concentration in organic phase to its concentration in aqueous phase.

*Biotransformation.* All biotransformations were conducted in 250 mL Erlenmeyer flasks with 50 mL working volume at 30$^{o}$C and 200 rpm. Fresh LB medium was inoculated with over-night grown culture of *E. coli* TG1 pBS(kan)TOM-Green cells. Cells were grown to late log-phase (OD at 660nm ~1.6) when LB medium appears green in color due to the production of indigo and isatin (Canada et al. 2002; Eaton and Chapman 1995). Cells were harvested by centrifugation (~10, 000 ×g), washed with phosphate buffer at pH 7.2 (PB) and resuspended in 50% volume of PB to increase cell density. The

medium was supplemented with 20 mM glucose and 100 mg/L kanamycin. Resuspended cells (30 mL) were used for cell dry weight measurement. For aqueous biotransformation, 0.5 g/L naphthalene was added to 50 mL resuspended cells using a cosolvent dimethyl formamide due to low water solubility of naphthalene. For biphasic biotransformations, desired volume of an organic solvent with known concentration of dissolved naphthalene was added to aqueous phase of resuspended cells give a final volume of 50 mL. Aqueous and organic phase samples were taken at 3, 6, 24 and 48 h and analyzed using HPLC. The results shown are an average of three identical experiments.

*Organic Phase Optimization.* Four different organic phase ratios of lauryl acetate, 20, 40, 60, and 80%, were added to aqueous phase, and for each organic phase ratio, four different concentrations of naphthalene, 20, 40, 60, and 70 g/L, were used. The formation of 1-naphthol in the lauryl acetate phase was monitored using HPLC.

*Immobilization.* *E. coli* TG1 pBS(kan)TOM-Green cells (360 mL) were grown to late log-phase (OD at 660 nm of ~1.6) and harvested by centrifugation at ~10,000 ×g for 10 minutes. The cells were washed with Tris buffer at pH 7.2. The cells for each experiment were immobilized together and later divided into 6 separate flasks. A 3% sodium alginate solution was prepared in 120 mL of deionized water. A 1% $CaCl_2$ solution as the gelation agent was prepared in deionized water. Both the sodium alginate and the $CaCl_2$ solution were autoclaved at 121$^{o}$C for 15 minutes. The sodium alginate solution was allowed to cool to room temperature and the $CaCl_2$ solution was cooled to 4$^{o}$C. The pelleted cells were resuspended in 25 mL of sterilized deionized water. The cells and

sodium alginate solution were mixed by stirring for five minutes on a stir plate. The mixture was added drop wise into the stirred gelation agent using a 60 mL syringe with an 18 gauge needle. The mixture was stirred for 1 hour to harden. The resulting calcium alginate beads were 1-2 mm in diameter. After hardening, the beads were removed from the solution and washed twice with sterilized deionized water. The immobilized cells were divided equally among six sterile flasks with 19.5 g of beads each.

*Immobilized Cells Biotransformation.* The immobilized biocatalyst was suspended in 250 mL Erlenmeyer flasks with 50 mL working volume. The beads were suspended in 30 mL of Tris-HCl buffer at pH 7.2 supplemented with 20 mM glucose and 100 mg/L kanamycin. The solvent phase (20 mL) is added to begin the reaction and the flasks were shaken at 200 rpm and 37$^{o}$C. The HPLC analysis was done on samples of the solvent phase.

## Results and Discussion

*Substrate and Product Toxicity.* Whole-cells of *E. coli* TG1 expressing TOM-Green were used for oxidation of naphthalene to 1-naphthol. The toxicity of both naphthalene and 1-naphthol to *E. coli* TG1 strain expressing TOM-Green is shown in Figure 2.1. Naphthalene inhibited cell growth even at a low concentration of 0.05 g/L. The inhibition of growth increased as the concentration of naphthalene increased to 0.5 g/L, and there was no growth observed for 1 g/L naphthalene concentration. The inhibitory effect of 1-naphthol is greater than that of naphthalene with no growth observed even for 0.5 g/L 1-naphthol. These results are comparable to similar work done earlier (Tao et al. 2005a).

Therefore, maintaining low concentrations of substrate and product is critical in maintaining the cell viability/activity for the reaction.

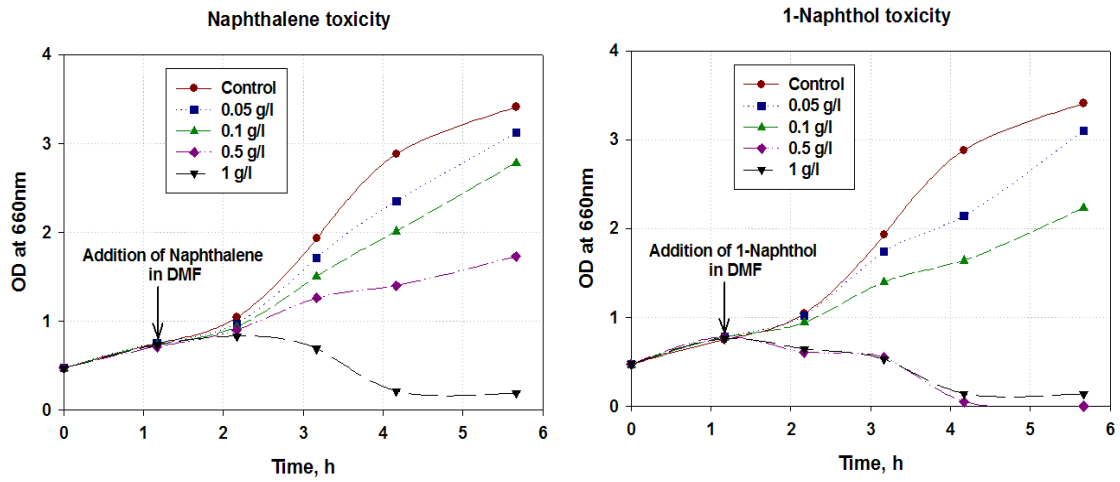



Figure 2.1. Toxicity of naphthalene and 1-naphthol to *E. coli* cells expressing TOM-Green. (Cells grown in LB medium at 37°C)

*1-Naphthol Production.* Whole-cells of *E. coli* TG1 expressing TOM-Green was used to oxidize naphthalene to 1-naphthol, as shown in the Figure 2.2. The TOM-Green enzyme

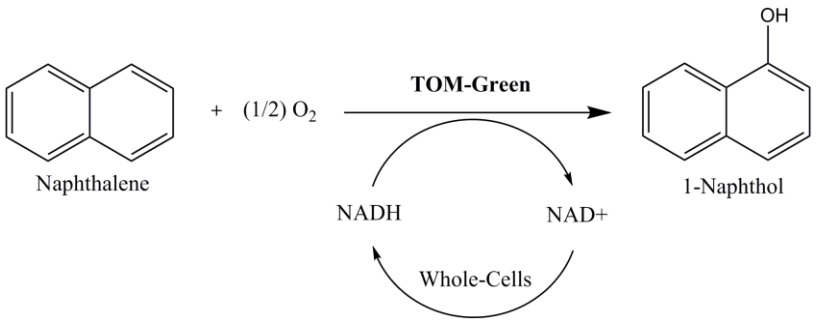
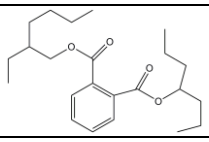
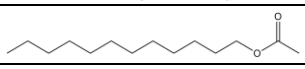



Figure 2.2. Oxidation of naphthalene to 1-naphthol using whole cells of TOM-Green

uses molecular oxygen and NADH as cofactor. Oxidation of naphthalene to 1-naphthol was performed in an aqueous medium and 0.04 g/L 1-naphthol was obtained after 24 h. To improve 1-naphthol production, biphasic biotransformations were performed with

40% organic phase and 40 g/L naphthalene dissolved in the solvent. The choice of organic solvent is critical in achieving maximum benefits from biphasic reactions. Three solvents, dodecane, dioctyl phthalate and lauryl acetate, were chosen for screening. Table 2.1 shows the distribution coefficients and structures of the three solvents used for 1-naphthol production. Dodecane (McIver et al. 2008; Sello et al. 2004) and dioctyl phthalate (Sello et al. 2004; Tao et al. 2005a) were used earlier to improve 1,2-naphthalene dihydrodiol and 2-naphthol productivities, respectively. Lauryl acetate was chosen because it is slightly polar and has high logP value. Lauryl acetate and dioctyl phthalate have high distribution coefficients for 1-naphthol. However, dodecane has a low distribution coefficient for 1-naphthol. All three solvents have logP values above 4 and the biocompatibility of the solvents was confirmed by assaying cell viability using flow cytometry, as shown in the Figure 2.2. *E. coli* pBS(kan)TOM-Green cell viability was approximately 99% after 3 h exposure to 40% of organic phase with any of the three solvents, dodecane, lauryl acetate or dioctyl phthalate.

Table 2.1. Distribution coefficient of the three solvents for 1-naphthol

| Organic Solvent | Structure | logP* | % in Organic Phase | $K_D$ |
|---|---|---|---|---|
| Dodecane |  | 6.1 | 77.5 | 3.4 |
| Dioctyl Phthalate |  | 8.7 | 98.5 | 66 |
| Lauryl Acetate |  | 7.0 | 98.4 | 61 |

* logP values were obtained from Laane et al. (1989)

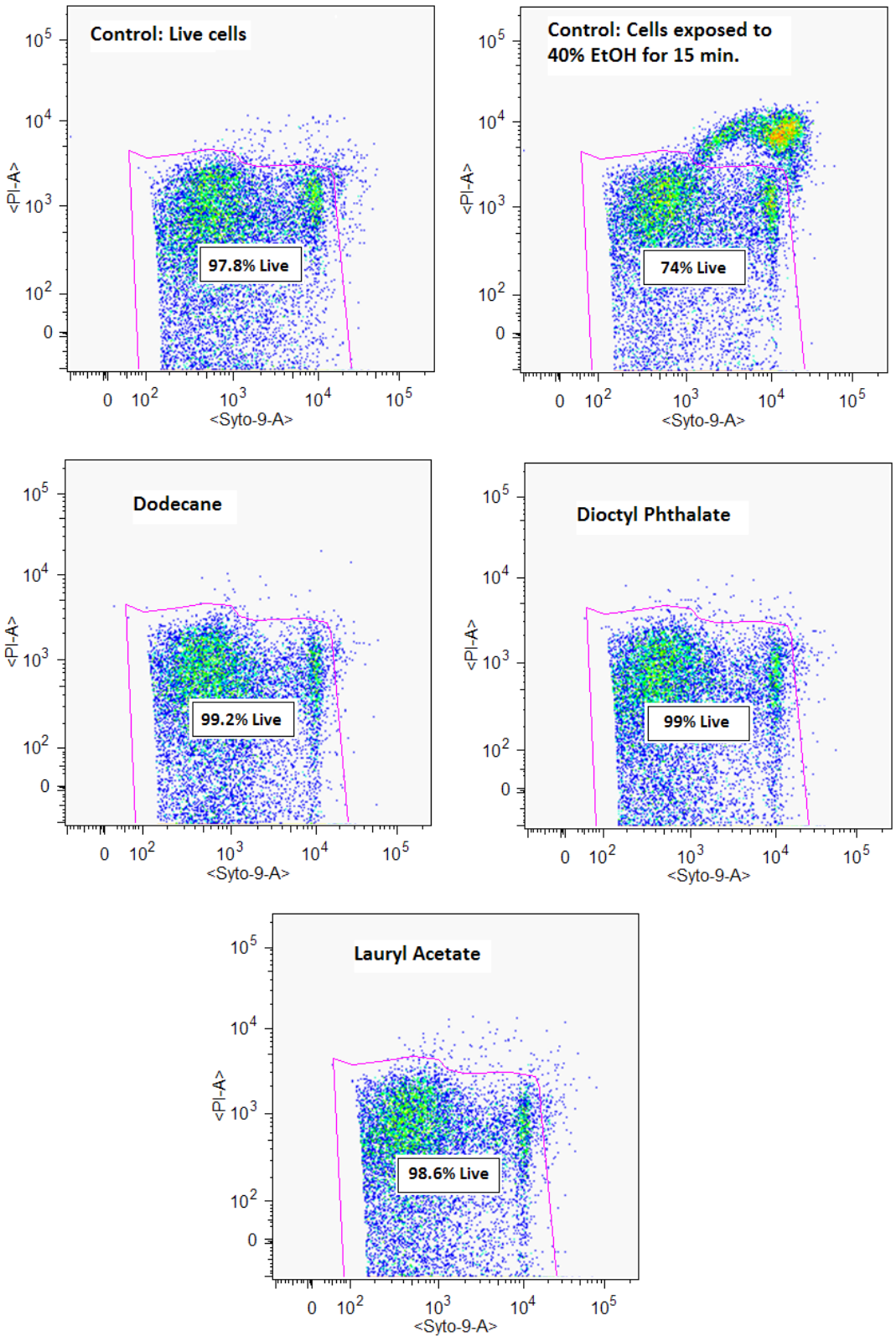



Figure 2.2. *E. coli* pBS(kan)TOM-Green viability in presence of 40% organic solvents.

Biphasic biotransformations were performed using the three solvents. Figure 2.3 shows 1-naphthol concentration in the organic phase and 1-naphthol productivity expressed as g of 1-naphthol formed per g of cell dry weight (CDW). When dodecane was used, most of 1-naphthol was formed within 3 h of biotransformation, and the cells lost their activity thereafter. However, when either lauryl acetate or dioctyl phthalate was used, 1-naphthol was formed at a steady rate for 6 h, and then 1-naphthol was formed at a slower rate up to 48 h. The formation of 1-naphthol was significantly higher using either lauryl acetate or dioctyl phthalate compared to dodecane. After 48 h, higher concentrations of 1-naphthol were achieved for lauryl acetate and dioctyl phthalate [approximately10 times (0.724 ± 0.03 g/L) and 7 times (0.52 ± 0.022 g/L) higher, respectively] compared to 1-naphthol concentrations achieved using dodecane (0.075 g/L).

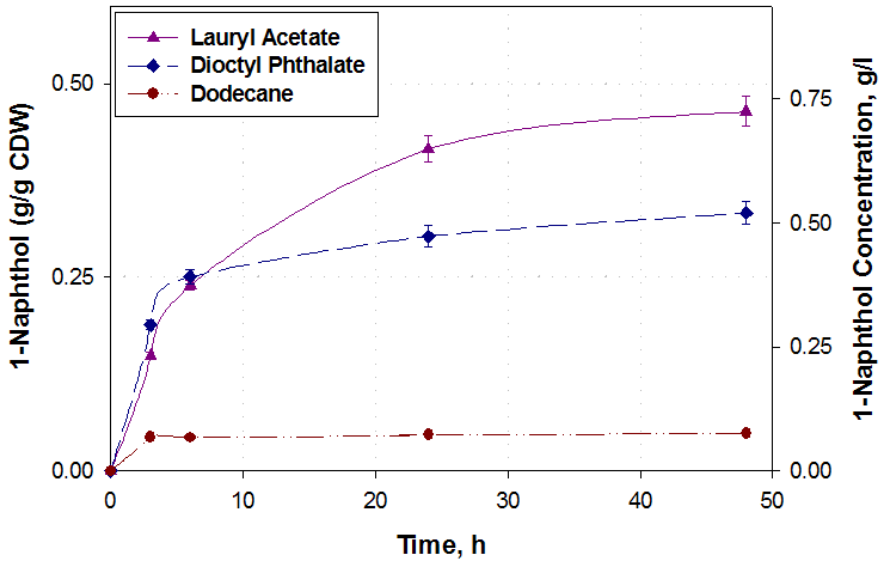



Figure 2.3. 1-Naphthol formation in biphasic media using *E. coli* cells expressing TOM-Green Reaction Conditions: 40% organic phase with 40 g/L naphthalene, 30°C and 200 rpm (average of three independent experiments)

High distribution coefficient of either lauryl acetate or dioctyl phthalate for 1-naphthol improves the efficiency of partitioning toxic 1-naphthol from aqueous phase. Higher 1-naphthol productivities obtained using either lauryl acetate or diocytl phthalate compared to dodecane suggest that the dynamics of 1-naphthol partitioning into the organic phase plays a major role in maintaining cellular activity and improving 1-naphthol productivity. Moreover, the inefficient partition of 1-naphthol results in accumulation of the toxic product in aqueous phase, thereby lowering cellular activity and 1-naphthol productivity. Comparing lauryl acetate and dioctyl phthalate, lauryl acetate gave slightly higher concentrations of 1-naphthol. Although both solvents have high distribution coefficients for 1-naphthol, dioctyl phthalate has high viscosity and will need higher mixing time to reach equilibrium. Therefore, lauryl acetate gave the best results among the solvents tested, producing 0.72 ± 0.03 g/L 1-naphthol at a productivity of 0.46 ± 0.02 g/g CDW after 48 h.

*Optimization of Organic Phase.* After identification of lauryl acetate as the optimal solvent among the tested solvents, the organic phase ratio and naphthalene concentration have to be optimized to achieve the best results. Different organic phase ratios and naphthalene concentrations were tested using lauryl acetate for 1-naphthol production, and their effect is shown in the Figure 2.4. 1-Naphthol productivity was increased with either an increase in naphthalene concentration from 20 to 70 g/L in the organic phase or an increase in the organic phase ratio from 20 to 60%. Higher naphthalene concentrations in the organic phase will allow more naphthalene to partition into the aqueous phase, thereby increasing naphthalene bioavailability. A higher organic phase ratio also favors

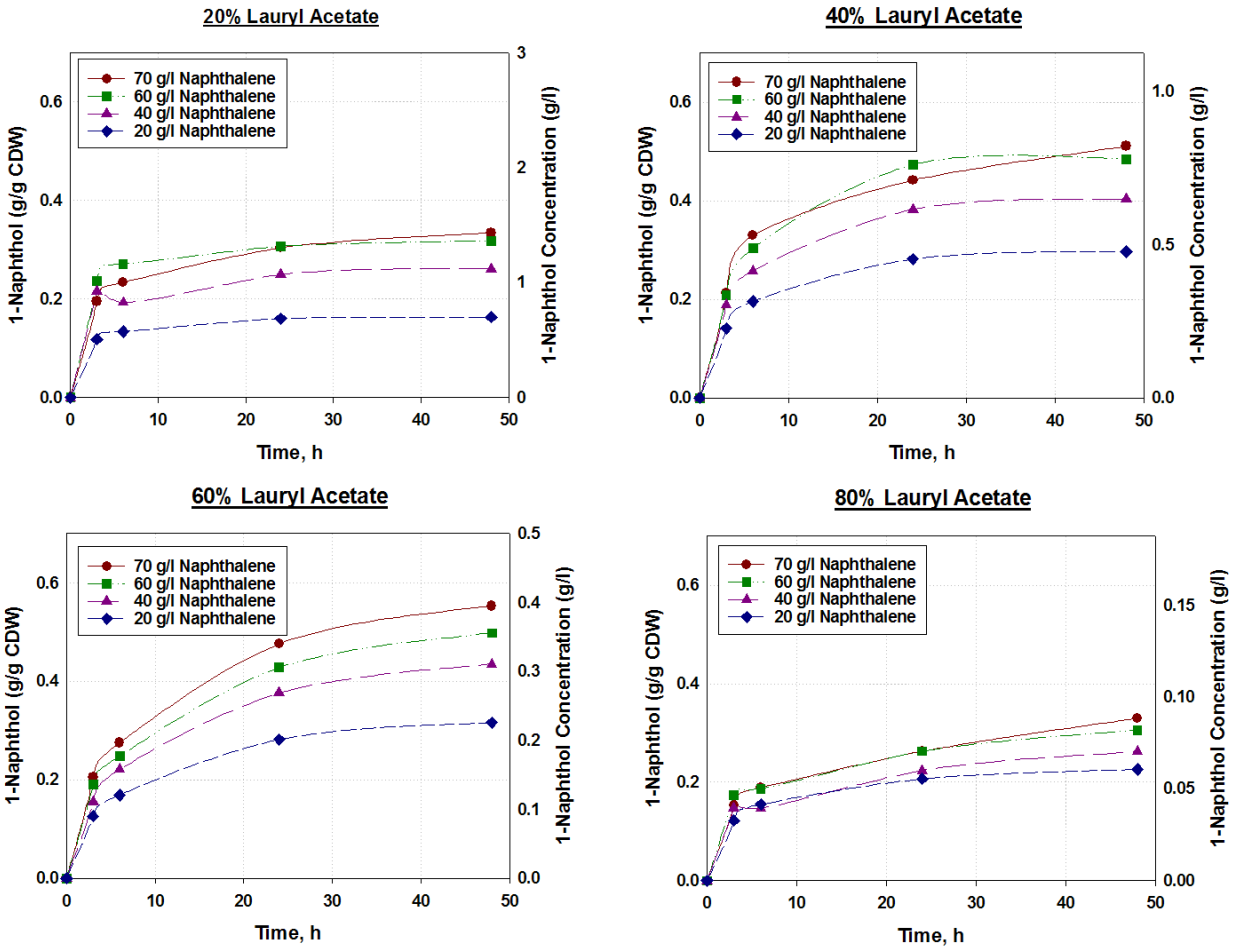



Figure 2.4. Effect of naphthalene concentration and organic phase ratio on 1-naphthol formation using Lauryl Acetate as the organic solvent. Reaction conditions: total working volume of 50 mL at 30$^{o}$C and 200rpm.

the reaction by allowing better partitioning of 1-naphthol, thereby minimizing its toxicity and improving productivity. Improvement in 1-naphthol productivity with an increase in organic phase ratio was also observed earlier (Tao et al. 2005a). However, 1-naphthol productivity decreased when the organic phase ratio increased from 60 to 80%. For the different reaction conditions tested, the highest 1-naphthol concentration of 1.43 g/L and the highest 1-naphthol productivity of 0.55 g/gCDW were achieved.

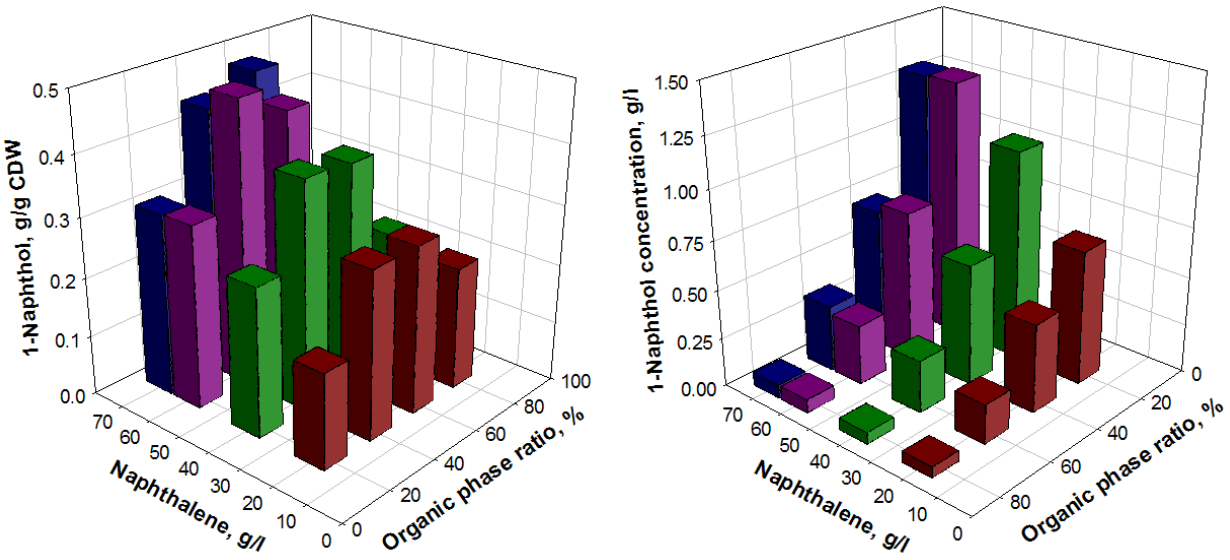



Figure 2.5. Optimization of organic phase for 1-naphthol formation using *E. coli* cells expressing TOM-Green with lauryl acetate as the organic solvent. Reaction conditions: reaction time of 24 h at 30°C and 200rpm.

Although 1-naphthol productivity increases with increasing organic phase ratio, 1-naphthol concentration in the organic phase decreases. Therefore, the reaction conditions can be optimized either for high 1-naphthol productivity, which impacts upstream processing costs, or for high 1-naphthol concentration in organic phase, which impacts downstream processing costs. In order to find the optimum reaction conditions, the effect of naphthalene concentration and organic phase ratio after 24 h is shown in Figure 2.5. Although high organic phase ratio and naphthalene concentration improve productivity, the results shown in Figure 2.5 demonstrate that the optimum conditions for the reaction can be lower than the maximum organic phase ratio or naphthalene concentration without significant loss in productivity. Optimum conditions for high 1-naphthol productivity were 40% organic phase ratio and 60 g/L naphthalene. Optimum conditions for high 1-naphthol concentration were 20% organic phase ratio and 60 g/L naphthalene. Table 2.2 shows the amount of 1-naphthol formed for each condition and percent naphthalene

conversion to 1-naphthol. Maximum conversion of 3.5% was achieved for 20 g/L naphthalene and 20 % organic phase ratio. At optimum conditions for high 1-naphthol productivity and high 1-naphthol concentration in organic phase, naphthalene conversions of 1.3% and 2.3%, respectively, were achieved.

Table 2.2. Naphthalene conversions at different naphthalene concentrations in organic phase and organic phase ratios (total reaction volume of 50 mL)

| Naphthalene Concentration (g/L) | Organic Phase Ratio (%) | 1-Naphthol (mg) | Conversion |
|---|---|---|---|
| 20 | 20 | 7.0 | 3.5% |
| | 40 | 9.5 | 2.4% |
| | 60 | 6.8 | 1.1% |
| | 80 | 2.4 | 0.3% |
| 40 | 20 | 11.2 | 2.8% |
| | 40 | 13.0 | 1.6% |
| | 60 | 9.3 | 0.8% |
| | 80 | 2.8 | 0.2% |
| 60 | 20 | 13.6 | 2.3% |
| | 40 | 15.6 | 1.3% |
| | 60 | 10.7 | 0.6% |
| | 80 | 3.3 | 0.1% |
| 70 | 20 | 14.3 | 2.0% |
| | 40 | 16.4 | 1.2% |
| | 60 | 11.8 | 0.6% |
| | 80 | 3.5 | 0.1% |

*Recycle of Biocatalyst.* Recycle of biocatalyst improves process economics. Recycle experiments were performed to test the stability of the cells. The stability of both free and immobilized cells in calcium alginate was tested. Immobilized cells produced ~40% 1-naphthol compared to free cells (0.196 ± 0.014 g 1-naphthol /gCDW for immobilized cells compared to 0.49 ± 0.01 g 1-naphtol/gCDW for free cells after 6 h biotransformation at 40% organic phase ratio and 60 g/L naphthalene). The decrease in immobilized cell activity could be because of mass transfer limitations introduced due to

the calcium alginate beads. Similar loss in activity of immobilized cells compared to free cells was also observed earlier (McIver et al. 2008).

The reaction time for recycle experiments is critical in determining the stability of biocatalyst considering its exposure to toxic products, toxic substrates and organic solvents. Optimum conditions of 40% organic phase ratio and 60 g/L naphthalene for 1-naphthol productivity were used for the recycle. Considering the time course of 1-naphthol production at optimum reaction conditions (from Figure 2.4), 1-naphthol was produced at essentially a linear rate up to 6 h and at a reduced rate thereafter. Therefore, two reaction times of 6 h and 12 h were chosen to test the stability of cells at the two reaction rates. The percentage of activity retained by either free or immobilized cells for 4 cycles is shown in the Figure 2.6. For 6 h recycle, free cells showed higher activity retention and ~ 40% activity of free cells was retained compared to ~ 20% for immobilized cells for the second run. The activities decreased further for third and fourth runs. For 12 h recycle, both free and immobilized cells lost most of their activity with only 20% activity retention for the second run. Similar recycles for biphasic biotransformation have been performed earlier (McIver et al. 2008) to produce non-toxic *cis*-1,2-naphthalene dihydrodiol, and most of the activity was retained up to four runs for 6 h recycle. However, in this reaction, the product 1-naphthol is very toxic and could be the main reason for loss in activity during recycle (Tao et al. 2005a). Moreover, diffusional limitations for immobilized cells may result in 1-naphthol accumulation in the beads which would adversely affect immobilized cell activity (Husken 2002).

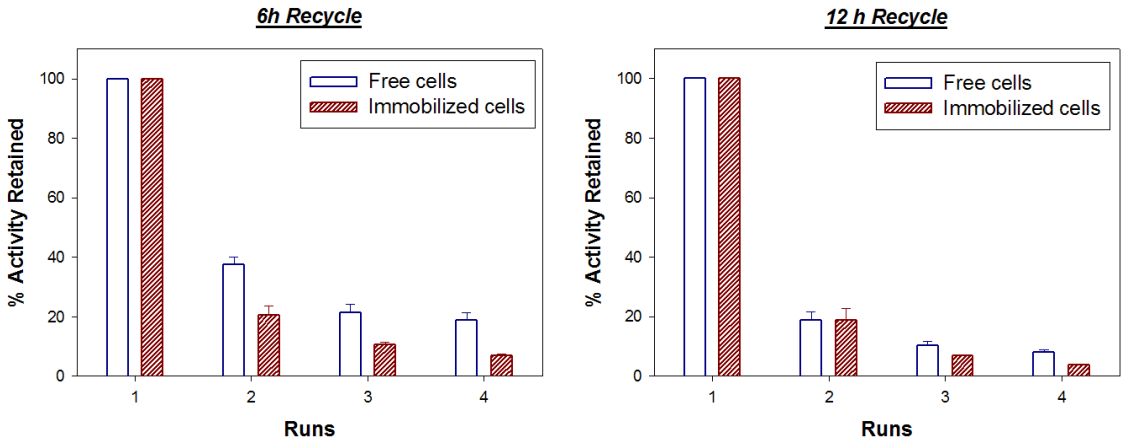



Figure 2.6. Recycle of biocatalyst after 6 and 12 h using both free and immobilized cells. Reaction conditions: 30°C and 200rpm (average of three identical experiments)

*Discussion.* Whole-cells expressing TOM-Green enzyme were used for 1-naphthol production in a biphasic system. On a constant volume basis, 8 - fold improvement in 1-naphthol production was achieved using lauryl acetate as a second phase in a biphasic system compared to biotransformation in aqueous medium [16.4 mg 1-naphthol (at 70 g/L naphthalene and 40% organic phase ratio) compared to 2 mg (0.04 g/L) for 50 ml reactions]. The 1-naphthol concentrations achieved in the organic phase and naphthalene conversions in this work are comparable to results obtained in the literature for a similar compound 2-naphthol (Tao et al. 2005a). Although higher product concentrations and conversions were achieved for oxidation reactions in a biphasic system (Buhler et al. 2003; Prpich and Daugulis 2007b), high toxicity of 1-naphthol is the main limiting factor to achieve higher product concentrations. Additional organic solvents can be tested based on biocompatibility and high selectivity for 1-naphthol. Solvent tolerant strains can be used to express the enzyme to improve biocatalyst stability in presence of solvents that are generally toxic to microorganisms (Heipieper et al. 2007). Solvent tolerant strains

have been used earlier for production of toxic products such as 3-methyl catechol in the presence of toxic organic solvents (Heipieper et al. 2007; Husken et al. 2001; Rojas et al. 2004). Application of solid-liquid biphasic systems have also been demonstrated recently for production of 3-methyl catechol and carveol (Morrish and Daugulis 2008; Prpich and Daugulis 2007a), and may be useful in enhancing 1-naphthol productivities.

## Conclusions

Whole-cells of *E. coli* expressing TOM-Green were used for oxidation of naphthalene to 1-naphthol in biphasic media. This is the first report of applying biphasic media to oxidation of naphthalene to 1-naphthol. Biphasic media, with decreased product toxicity by in-situ product removal into the organic phase and increased substrate loading, increases 1-naphthol productivity. Among the solvents tested, lauryl acetate gave the best results, i.e., producing ~ 0.72 g/L 1-naphthol in the organic phase at a productivity of 0.46 g/gCDW after 48 h for 40% organic phase with 40 g/L naphthalene. The effects of organic phase ratio and naphthalene concentration on 1-naphthol production was investigated. Highest 1-naphthol concentration of 1.43 g/L in organic phase and highest productivity of 0.55 g/gCDW were achieved by varying organic phase ratio and naphthalene concentration. The recycle ability of the biocatalyst was tested using both free cells and immobilized cells. Free cells were better than immobilized cells for 6 h recycle retaining 40% of activity compared to 20% activity retention for immobilized cells. However, there was significant loss in activity for both free and immobilized cells for 6 and 12 h which could be attributed to product toxicity. The production of 1-naphthol was improved economically and environmentally by the use of biocatalysis and liquid-liquid biphasic reactions. On a constant volume basis, 6-fold improvement in 1-naphthol

production was achieved using biphasic media compared to biotransformation in aqueous medium. Both solvent and substrate naphthalene could be recycled. However, the product concentrations have to be increased to at least 50 – 100 g/L to make the process industrially feasible (Pollard and Woodley 2007). More work will be done in increasing the product concentrations by the use of high cell densities and better expression hosts that are more stable in presence of toxic environments.

# CHAPTER 3
# APPLICATION OF SOLVENT TOLERANT STRAIN *PSEUDOMONAS PUTIDA* S12 TO 1-NAPHTHOL PRODUCTION

## Introduction

Whole-cell biocatalysis in water-organic solvent biphasic systems has become a standard technology for production of chemical synthons and high value products (Collins and Daugulis 1999 1997; Endoma et al. 2002; Leon et al. 1998; Pollard and Woodley 2007; Schmid et al. 2001; Schoemaker et al. 2003; van Beilen et al. 2003; Witholt et al. 1990; Wubbolts et al. 1996). Productions of various compounds, such as 3-methyl catechol (Husken et al. 2001; Prpich and Daugulis 2007b), 1,2-naphthalene dihydrodiol (Harrop et al. 1992; McIver et al. 2008; Sello et al. 2004), carvone (Morrish et al. 2008), octanol (Mathys et al. 1998), and amorpha-4,11-diene (Newman et al. 2006), have been enhanced using biphasic reactions. One of the reactions where application of biphasic systems can be a valuable tool is oxidation of naphthalene to 1-naphthol. 1-Naphthol has wide applications in the manufacture of dyes, drugs, insecticides, perfumes and surfactants (Back 1965; Kirk-Othmer 1999). However, the production of 1-naphthol by biocatalysis is mainly limited by the toxicity of both naphthalene and 1-naphthol to microorganisms, and by the poor solubility of substrate naphthalene in aqueous medium (Canada et al. 2002; Garikipati et al. 2009; Tao et al. 2005a; Wilson et al. 1996). Application of biphasic systems minimizes these drawbacks by maintaining low concentrations of both naphthalene and 1-naphthol in the aqueous phase, increasing substrate loading and shifting thermodynamic equilibrium by *in-situ* product removal (Brink and Tramper 1985; Bruce and Daugulis 1991). A Toluene *ortho*-monooxygenase (TOM) variant TmoA3 V106A, also known as TOM-Green due to the conversion of LB

medium into green color, oxidizes naphthalene to 1-naphthol with a high rate and regio-selectivity (Canada et al. 2002; Rui et al. 2004; Tao et al. 2005b). TOM-Green is a three component enzyme consisting of a hydroxylase, an oxido-reductase and an electron transfer protein, and it utilizes molecular oxygen and NAD(P)H as cofactor for oxidation of naphthalene (Canada et al. 2002; Newman and Wackett 1995; Shields et al. 1995).

Although biphasic systems have various advantages, there are drawbacks associated with organic solvent toxicity (Leon et al. 1998, inoue, 1991). The toxicity of solvents is generally correlated with the logarithm of partition coefficient in an octanol-water system (logP). Organic solvents with logP greater than four are considered biocompatible with microorganisms (Laane et al.; Leon et al. 1998). However, this is a general assumption and the critical logP value above which a solvent is toxic to bacteria ranges from 3.1 to 7 depending on the bacterial strain (Inoue and Horikoshi 1991). The toxic organic solvents have damaging effects on the microorganism by impairment of vital functions, by facilitating loss of ions, metabolites, lipids, and proteins, and by inhibition of membrane protein functions (Bruce and Daugulis 1991; Leon et al. 1998). Solid-liquid systems with either solid adsorbents or thermoplastic polymers have been used earlier to eliminate toxic effects of solvents (Held et al. 1999; Morrish and Daugulis 2008; Prpich and Daugulis 2007a). However, considering various benefits of liquid-liquid biphasic systems, a solvent tolerant microorganism should be employed to fully realize its potential (Heipieper et al. 2007). Solvent tolerant microorganisms have adaptive mechanisms to survive in the presence of toxic organic solvents (de Bont 1998; Isken and de Bont 1998; Segura et al. 1999; Weber and deBont 1996; Wery et al. 2001). The

presence of energy-dependent efflux systems that selectively pump organic solvents out of the cell membrane were identified (Isken and DeBont 1996; Kieboom et al. 1998a; Ramos et al. 2002; Rojas et al. 2001; Rojas et al. 2003). One of the strains for which extensive physiological studies were performed to study solvent tolerance is *Pseudomonas putida* S12 (Isken and DeBont 1996; Isken et al. 1999; Kieboom and de Bont 2001; Kieboom et al. 1998a; Kieboom et al. 1998b; Volkers et al. 2006; Weber et al. 1993). *P. putida* S12 is known to tolerate saturated concentrations of organic solvents because of two adaptive mechanisms: outer membrane modification and extrusion of solvents. Isomerization of *cis*- to *trans*- unsaturated fatty acids in the outer membrane makes the membrane more rigid, thereby reducing the influx of solvents (von Wallbrunn et al. 2003). Extrusion of the solvent from the cell membrane is facilitated by an energy dependent efflux pump (Kieboom et al. 1998a). Kieboom et al. (1998a) have identified and characterized the srpABC genes encoding a proton-dependent multidrug efflux pump responsible for organic solvent tolerance in *P. putida* S12. The srpABC pump belongs to RND family (Moreira et al. 2004; Nikaido 1996; Putman et al. 2000) of pumps, which is composed of three protein components, an inner membrane transporter (*srp*B), an outer membrane channel (*srp*C) and a periplasmic linker (*srp*A) (Kieboom et al. 1998a). Active efflux of solvents in *P. putida* S12 was induced only by solvents and not by antibiotics or heavy metals (Isken and De Bont 2000; Kieboom and de Bont 2001; Kieboom et al. 1998b).

Solvent tolerant strains have previously been used for the production of toxic chemicals in biphasic systems (Heipieper et al. 2007; Nijkamp et al. 2005; Rojas et al.

2004; Wery et al. 2000). *P. putida* S12 was used for enhancing the production of cinnamic acid (Nijkamp et al. 2005) and 3-methyl catechol (Wery et al. 2000). The catabolic potential of *P. putida* S12 was also improved by the transfer of toluene, naphthalene and biphenyl degradation plasmids (Marconi et al. 1997). Application of solvent tolerant strain *P. putida* S12 can be beneficial for 1-naphthol production considering high toxicity of 1-naphthol. *Escherichia coli* TG1 strain expressing TOM-Green was used earlier for production of 1-naphthol in biphasic systems (Garikipati et al. 2009). Maximum 1-naphthol concentration of 1.43 g/l and highest 1-naphthol productivity of 0.55 g/gCDW were achieved using lauryl acetate as the solvent. It was observed that minimizing 1-naphthol toxicity and efficient partition of 1-naphthol into the organic phase were the critical factors for improving 1-naphthol production. Therefore, in this work, we evaluated the use of a solvent tolerant organism as a host strain for production of 1-naphthol in biphasic systems. The tolerance of *P. putida* S12 to naphthalene and 1-naphthol was studied. 1-Naphthol production using a relatively toxic solvent, decanol, was also analyzed. Moreover, 1-naphthol productivities using both lauryl acetate and decanol were compared between *P. putida* S12 and *E. coli* host strains.

## Materials and Methods

*Chemicals and Bacterial strains.* Decanol, lauryl acetate, naphthalene and 1-naphthol were purchased from Sigma (St. Louis, MO). Luria-Bertani (LB) broth was purchased from Difco (Lawrence, KS). *Escherichia coli* TG1 pBS(kan)TOM-Green expressing TOM-Green enzyme (Canada et al. 2002; Garikipati et al. 2009) was kindly donated by Dr. Thomas K. Wood (Texas A&M University). This strain expresses TOM-Green

constitutively and has kanamycin resistance genes in its plasmid. *P. putida* S12 was purchased from ATCC (ATCC # 700801) (Kieboom et al. 1998a). All *Pseudomonas* strains were grown at 30$^{o}$C and *E. coli* strains were grown at 37$^{o}$c in LB medium.

*P. putida* S12 was transformed with pBS(kan)TOM-Green plasmid by conjugation using pRK2013 plasmid (Sambrook et al. 1989). *E. coli* TG1 pBS(kan)TOM-Green cells were routinely grown in LB medium supplemented with 100 mg/L kanamycin at 37$^{o}$C and 200 rpm. *P. putida* S12 pBS(kan)TOM-Green were routinely grown in LB medium supplemented with 100 mg/L kanamycin at 30$^{o}$C and 200 rpm. Unless specified, *P. putida* S12 with or without pBS(kan)TOM-Green was routinely grown in presence of 3 mM toluene to induce solvent tolerance by expression of solvent resistant pump genes (Kieboom et al. 1998b).

*Solvent Tolerance Experiments.* Cells were grown in 125 mL shake flasks with 25 mL working volume till early log-phase. 3 mM and 6 mM toluene was added to growing cells and the growth was monitored by measuring optical density (O.D.) at 600nm using a Beckman UV spectrometer.

*Naphthalene and 1-Naphthol Toxicity Experiments.* The cells were grown until early log-phase in 250 mL shake flasks and 5 mL of growing cells were added to each 20 mL sterile screw-cap vials. Four different concentrations of naphthalene and 1-naphthol, 0.05 g/L, 0.1 g/L, 0.5 g/L and 1 g/L, in 50 µL dimethyl formamide (DMF) were added to the growing cells and the growth was monitored by optical density (OD) at 660 nm. Due to

low solubility of naphthalene and 1-naphthol in water, a cosolvent DMF was used to suspend the compound in aqueous phase. A positive control was performed where 50 µL DMF was added without naphthalene or 1-naphthol.

*Biotransformation.* All biotransformations were conducted in 250 mL Erlenmeyer flasks with 50 mL working volume at 30°C and 200 rpm. Fresh LB medium was inoculated with over-night grown culture of either *E. coli* TG1 pBS(Kan)TOM-Green or *P. putida* S12 pBS(kan)TOM-Green cells. Cells were grown until late log-phase (OD at 660 nm ~1.6) when cells produce indigo and isatin, compounds which make the LB medium appear green in color (Canada et al. 2002; Eaton and Chapman 1995). Cells were harvested by centrifugation (~10,000 ×g), washed with phosphate buffer (PB) at pH 7.2 and resuspended in 50% volume of PB to increase cell density. The medium was supplemented with 20 mM glucose and 100 mg/L kanamycin. Resuspended cells (30 mL) were used for cell dry weight (CDW) measurement. For biphasic biotransformations, 20 mL organic solvent (40% organic phase ratio) with desired naphthalene concentration was added to 30 mL resuspended cells to get a final volume of 50 mL. Aqueous and organic phase samples were taken at 3, 6, 24 and 48 h and analyzed using HPLC (Garikipati et al. 2009). The results shown are an average of three identical experiments.

## Results and Discussion

*Solvent Tolerance.* The *P. putida* S12 is a solvent tolerant strain that can survive saturated concentrations of toxic solvents. Figure 3.1 shows the growth curves of *P. putida* S12, *E. coli* JM109 (DE3) pDTG141 and *P. sp.* NCIB 9816-4 in the absence or presence of either 3 or 6 mM toluene, where 6 mM toluene is its saturated concentration in water. The *P. sp.*

Strain NCIB 9816-4 is the wild-type strain bearing genes for Naphthalene dioxygenase (NDO) and *E. coli* JM109 (DE3) pDTG141 is the recombinant strain bearing the plasmid carrying NDO genes. *E. coli* JM109 (DE3) pDTG141 growth was slightly inhibited in the presence of 3mM toluene. However, in presence of 6mM toluene, the cell density decreased for ~ 2 hours and increased thereafter. The overall yield in 6mM toluene was less compared to the control experiment where no toluene was added. The increase in cell

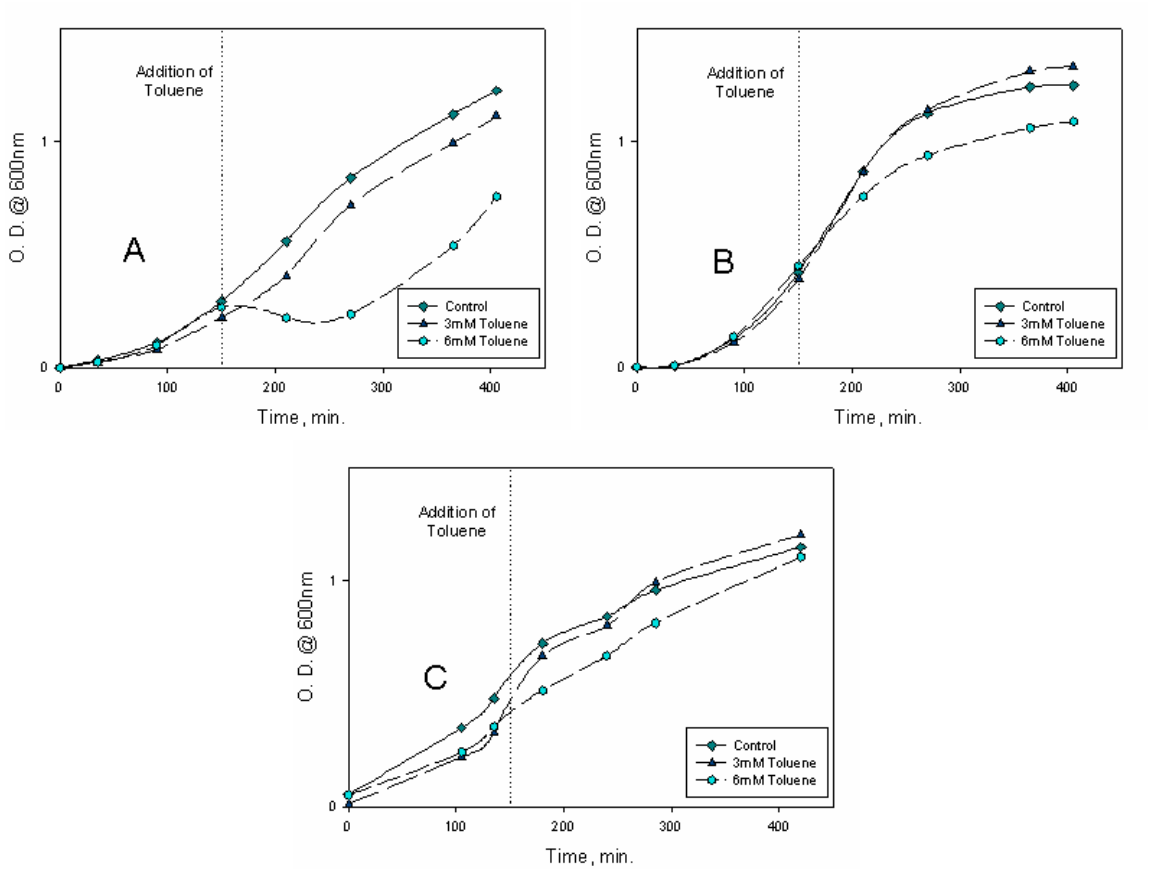



Figure 3.1. Solvent tolerance of (A) *E. coli* JM109 (DE3) pDTG141, (B) *Pseudomonas sp.* Strain NCIB 9816-4, (C) *Pseudomonas putida* S12.

density for *E. coli* growing in 6mM toluene after ~2 hours of toluene addition can be attributed to the evaporation of toluene as the flasks were not air-tight. The experiment was repeated and the increase was not observed in the repeated experiment with air-tight

flasks. *Pseudomonas* strains are generally more tolerant to organic solvents, and the growth of *P. sp.* NCIB 9816-4 was not inhibited by 3mM toluene but was inhibited in the presence of 6mM toluene. However, the growth of *P. putida* S12 was not inhibited in either 3 or 6 mM toluene owing to its tolerance to toxic solvents. Solvent tolerance of *P. putida* S12 was further studied by growing it in the presence of a second phase of organic solvent with phase ratios of 2% and 10%. Four organic solvents with logP ranging from 2.5 to 4.5 were added to the growth medium along with the inoculum and the growth of *P. putida* S12 was tested after 24 h. The growth was observed in all the conditions within 24h, as shown in Table 3.1.

Table 3.1. Growth of adapted *P. putida* S12 in various organic solvents with different phase ratios. (+ indicates growth within 24 h)

| Solvent | Log P | 2% | 10% |
|---|---|---|---|
| Toluene | 2.5 | + | + |
| Cyclohexane | 3.2 | + | + |
| Hexane | 3.5 | + | + |
| Octane | 4.5 | + | + |

*Substrate and Product Toxicity.* *P. putida* S12 was transformed with pBS(kan)TOM-Green plasmid from *E. coli* TG1 pBS(kan)TOM-Green strain by conjugation. The expression of TOM-Green enzyme was confirmed by conversion of LB medium into green color by the transformed microorganism. *P. putida* S12 pBS(kan)TOM-Green was grown in the presence of different concentrations of naphthalene and 1-naphthol, and the growth curve is shown in Figure 3.2. Inhibition of growth was observed for naphthalene concentrations greater than or equal to 0.1 g/L. The inhibition increased with increasing

concentrations of naphthalene up to 1 g/L and slight growth was still observed for 1 g/L naphthalene. The inhibitory effect of 1-naphthol is greater than that of naphthalene and the growth was inhibited for low concentration of 0.05 g/L 1-naphthol. For 0.5 g/L or more of 1-naphthol addition, the growth of the microorganism ceased within 4 h of 1-naphthol addition.

A similar study was performed earlier on *E. coli* TG1 pBS(kan)TOM-Green strain (Garikipati et al. 2009). The growth of the *E. coli* strain was inhibited even for 0.05 g/L naphthalene, and no growth was observed for 1 g/l naphthalene. The growth of the *E. coli* strain was also inhibited by 0.05 g/L 1-naphthol, and no growth was observed for 0.5 g/L 1-naphthol. *P. putida* S12 showed more tolerance to naphthalene compared to the *E. coli* strain. However, toxicity of 1-naphthol was similar for both the strains. *P. putida* S12 has impairment of the cell membrane and its properties, but also because of their metabolites,

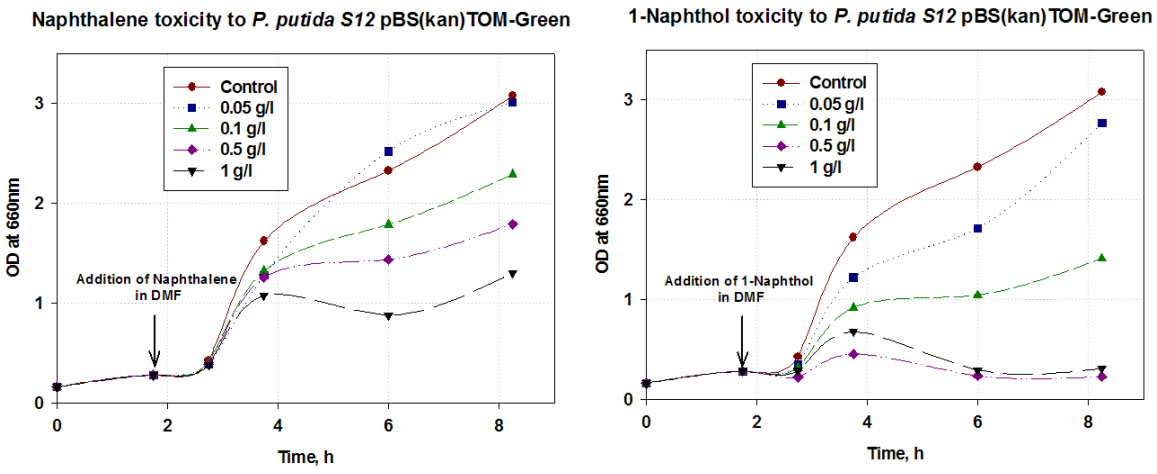



Figure 3.2. Toxicity of naphthalene and 1-naphthol to *P. putida* S12 cells expressing TOM-Green. (Cells grown in LB medium at 30°C)

such as 1,2-naphthoquinone and other unstable oxidized products (Pumphrey and Madsen 2007; Wilson et al. 1996). Therefore, a biphasic system should be employed to maintain low concentrations of these compounds in aqueous phase.

*1-Naphthol Production.* Oxidation of naphthalene to 1-naphthol was performed in a biphasic system. *P. putida* S12 pBS(kan)TOM-Green strain, which showed a greater tolerance to naphthalene and 1-naphthol, was used for the biotransformation to further improve 1-naphthol productivities. 1-Naphthol production using either *E. coli* pBS(kan)TOM-Green or *P. putida* S12 TOM-Green is shown in Figure 3.3 for different conditions. The results show that 1-naphthol concentration increased by three times when dodecane (logP 6.6) was used as a second phase for both the strains. Dodecane is not toxic to the microorganisms but organic solvents such as n-octane (logP 4.5) and cyclohexane (logP 3.2) are relatively more polar and are toxic to microorganisms. Hence, the concentration of 1-naphthol decreased for *E. coli* TG1 pBS(kan)TOM-Green when these solvents were used. However, 1-naphthol concentration for *P. putida* S12 pbs(kan)TOM-Green was still higher when n-octane and cyclohexane were used, which could be attributed to its stability in presence of toxic organic solvents. Moreover, the concentration of 1-naphthol achieved using *P. putida* S12 pBS(kan)TOM-Green was higher for either aqueous or biphasic system compared to *E. coli* TG1 pBS(kan)TOM-Green. This increase in 1-naphthol concentration for *P. putida* S12 pBS(kan)TOM-Green could be because of its better tolerance to naphthalene.

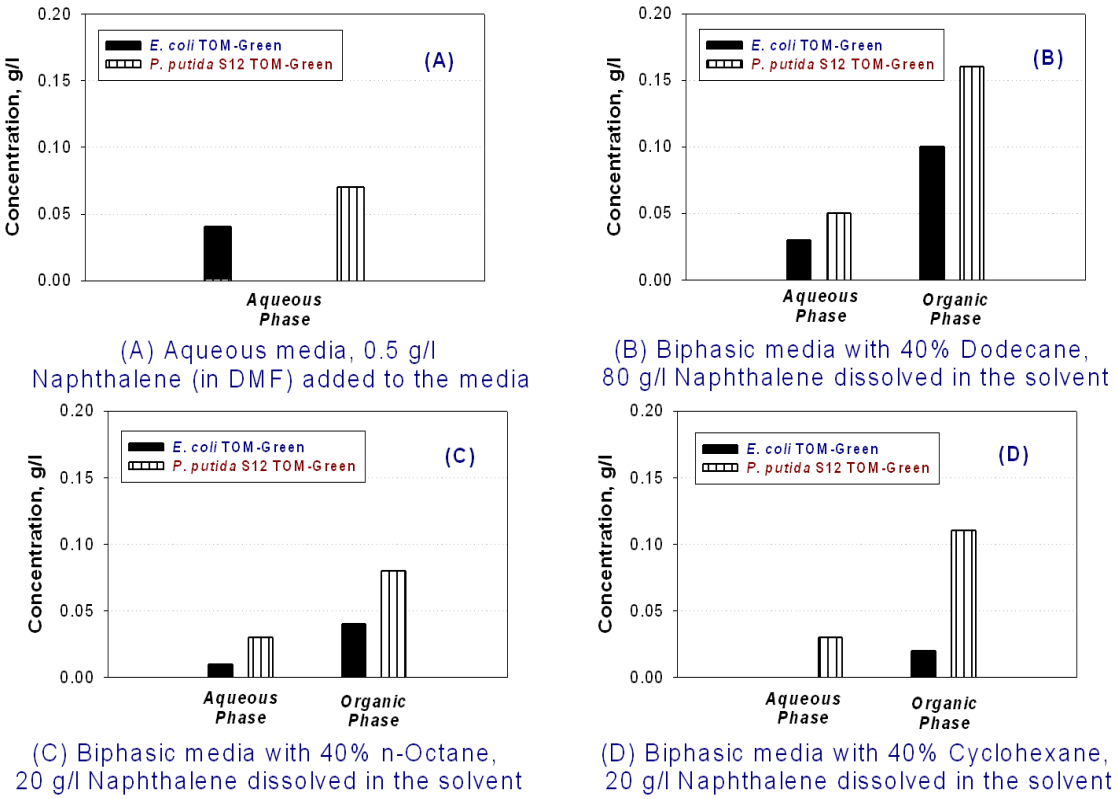
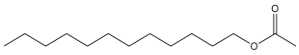
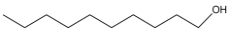



Figure 3.3. Oxidation of naphthalene to 1-naphthol using whole cells expressing TOM-Green enzyme

All the three solvents tested are non-polar and have low affinity for 1-naphthol. Therefore, more solvents which have high distribution coefficient for 1-naphthol have to be tested to enhance 1-naphthol productivities. Lauryl acetate was earlier shown to be a good solvent for 1-naphthol production and the optimum conditions were 40% organic phase ratio and 60 g/L naphthalene (Garikipati et al. 2009). Lauryl acetate in similar conditions was used for 1-naphthol production using *P. putida* S12 pBS(kan)TOM-Green. Due to the solvent tolerance of *P. putida* S12, a slightly toxic solvent decanol (logP of 4) was also tested for 1-naphthol production in the biphasic system. Table 3.2 shows the distribution coefficients of the two solvents for 1-naphthol, and both the solvents have good distribution coefficients.

Table 3.2. Distribution coefficient of lauryl acetate and decanol for 1-naphthol

| Organic Solvent | Structure | logP* | % in Organic Phase | $K_D$ |
|---|---|---|---|---|
| Lauryl Acetate |  | 7.0 | 98.4 | 61 |
| Decanol |  | 4.0 | 98.5 | 64 |

* logP values were obtained from Laane et al. (1989)

Biphasic biotransformations were performed using the two solvents. Figure 3.4 shows 1-naphthol concentration in the organic phase and 1-naphthol productivity based on cell dry weight for both *P. putida* S12 and *E. coli* strains. Biotransformation with *E. coli* using lauryl acetate was done earlier (Garikipati et al. 2009) and shown here for comparison. 1-Naphthol concentrations and productivities were much higher for the *P. putida* S12 strain compared to the *E. coli* strain when lauryl acetate was used. More tolerance towards naphthalene and 1-naphthol for *P. putida* S12 (From Figure 3.2) contributes to increased stability of the microorganism exposed to these toxic substances, and therefore higher productivities were achieved. 1-Naphthol productivity was improved by approximately 42% by using the *P. putida* S12 instead of the *E. coli* to express TOM-Green (0.66 ± 0.01 g 1-naphthol/gCDW or 1.28 ± 0.02 g 1-naphthol/L for *P. putida* S12 compared to 0.46 ± 0.02 g 1-naphthol/gCDW or 0.72 ± 0.03 g 1-naphthol/L for *E. coli* after 48 h). When decanol was used instead of lauryl acetate, *P. putida* S12 produced a similar amount of 1-naphthol compared to lauryl acetate. However, the *E. coli* strain produced less than 0.01 g/L 1-naphthol when decanol was used.

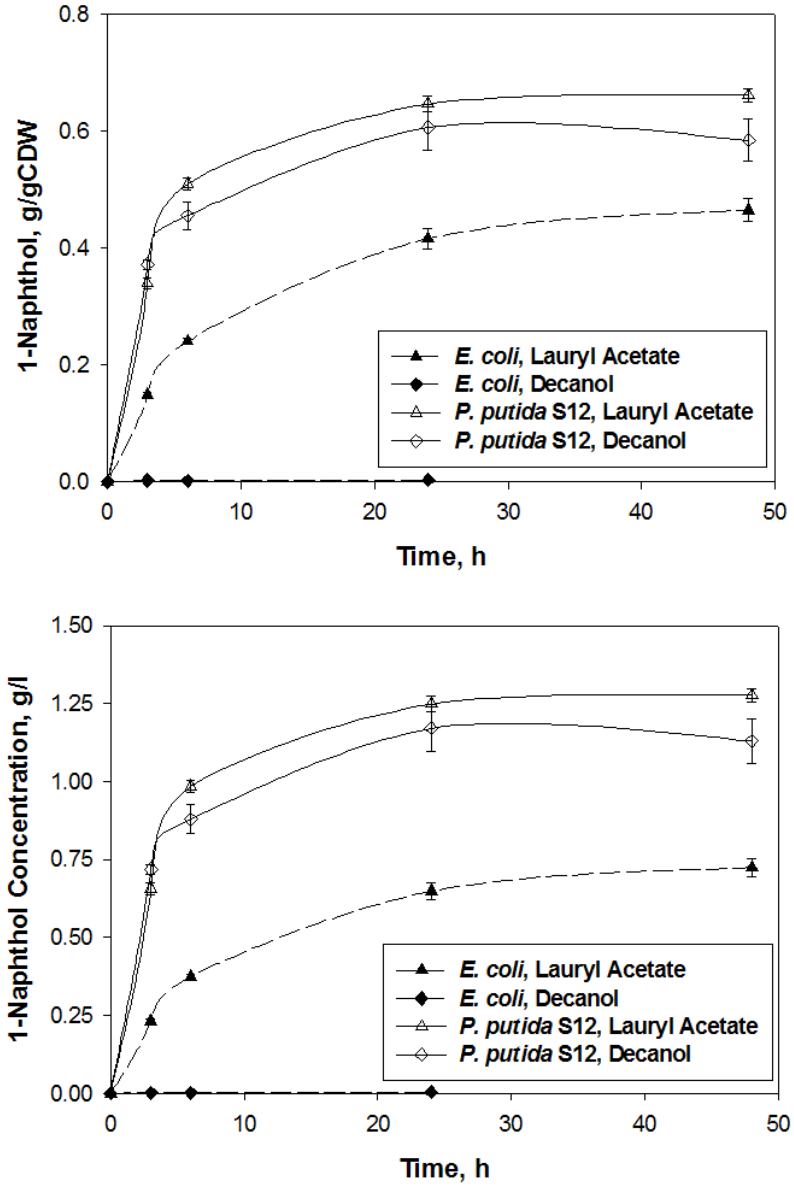



Figure 3.4. 1-Naphthol formation in biphasic media using *E. coli* and *P. putida* S12 cells expressing TOM-Green. (a) 1-naphthol concentration in organic phase, (b) 1-naphthol productivity based on cell dry weight. Reaction Conditions: 40% organic phase with 60 g/L naphthalene, 30$^{o}$C and 200 rpm (averages of three identical experiments, error bars represent standard deviation)

*Solvent Toxicity.* In order to understand the reason for low productivity for *E. coli* when decanol was used as a solvent, cell density after three hours of biotransformation was measured for both solvents. Percent cell density of *E. coli* and *P. putida* S12 strains in

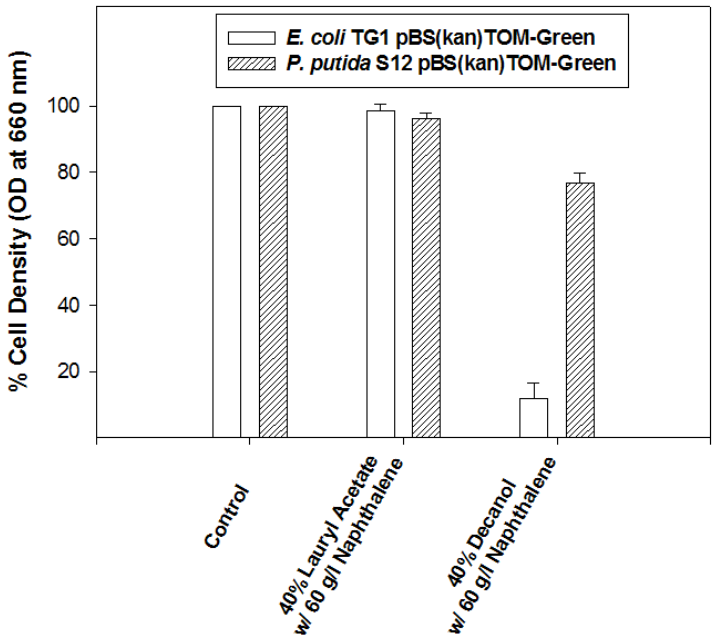



Figure 3.5. Toxicity of solvents lauryl acetate and decanol. Reaction conditions: 30°C and 200rpm, after 3 h (average of two identical experiments)

both the solvents is shown in Figure 3.5. Percent cell density is the ratio of optical density (absorbance at 660 nm) of the aqueous phase from the biphasic system to the optical density of an aqueous cell culture control in the absence of the solvent and substrate. Lauryl acetate has a logP of 6.6 and is generally not toxic to the cells. Therefore, both the *P. putida* S12 and *E. coli* biotransformations retained most of their cell density. However, decanol has logP of 4, and therefore the *E. coli* biotransformations lost most of their cell density when decanol was used. The *P. putida* S12 biotransformations had maintained a higher percent cell density in decanol owing to the solvent tolerance of the strain. Therefore, low productivity of the *E. coli* strain in decanol could be because of solvent toxicity. Application of the solvent tolerant *P putida* S12 strain to express TOM-Green enzyme increased the range of solvents to work with for biphasic whole-cell biocatalysis. A less expensive solvent such as decanol (approximate price from Sigma, ~ $20 per KG

of decanol compared to ~ $90 per KG of lauryl acetate) can be used instead of lauryl acetate to get similar productivities.

## Conclusions

Application of a solvent tolerant strain *P. putida* S12 for 1-naphthol production in biphasic systems was evaluated. *P. putida* S12, in addition to its tolerance to various toxic organic solvents, showed higher tolerance towards naphthalene and 1-naphthol compared to *E. coli*. The solvent tolerant strain *P. putida* S12 was transformed with pBS(kan)TOM-Green plasmid to constitutively express TOM-Green enzyme. *P. putida* S12 pBS(kan)TOM-Green was used to oxidize naphthalene to 1-naphthol in biphasic systems, and the results were compared to that obtained using *E. coli* TG1 pBS(kan)TOM-Green. Application of *P. putida* S12 improved 1-naphthol productivity by approximately 42% (1.28 ± 0.02 g 1-naphthol/L or 0.66 ± 0.01 g 1-naphthol/gCDW for *P. putida* S12 compared to 0.72 ± 0.03 g 1-naphthol/L or 0.46 ± 0.02 g 1-naphthol/gCDW for *E. coli* after 48 h). Moreover, *P. putida* S12 produced a similar amount of 1-naphthol when lauryl acetate was replaced with a less expensive solvent decanol. *E. coli* produced less than 0.01 g/L 1-naphthol due to the toxicity of decanol. Therefore, 1-naphthol productivity in biphasic system was further improved by application of the solvent tolerant strain, *P. putida* S12. These results also demonstrate the potential to improve the economics of 1-naphthol production by the use of a less expensive solvent to achieve similar productivity.

# CHAPTER 4
# ENGINEERING SOLVENT TOLERANCE IN *ESCHERICHIA COLI* USING srpABC GENES FROM *PSEUDOMONAS PUTIDA* S12

## Introduction

Many bacteria are resistant to various organic solvents despite their toxic effects. The toxicity of organic solvents is generally correlated with logP, which is the logarithm of the partition coefficient of solvent in an octanol-water system (Laane et al. 1987; Leon et al. 1998). Solvents with logP greater than 4 are considered biocompatible to microorganisms. However, solvents with logP less than or equal to 4 are toxic to microorganisms (Leon et al. 1998). The organic solvents diffuse through the cell membrane and settle in the hydrophobic region, thereby disrupting the membrane functions (Rajagopal 1996; Sikkema et al. 1995). To compensate for these effects, some bacteria have adaptive mechanisms to survive in harsh environments (de Bont 1998; Isken and de Bont 1998; Ramos et al. 2002; Segura et al. 1999; Weber and deBont 1996; Wery et al. 2001). Microorganisms generally adapt by membrane modifications which decrease their membrane fluidity or by degradation of the organic solvents to non-toxic products. These mechanisms partially compensate for the toxic effects of organic solvents and continuous removal of the organic solvents from the membrane is required (Kieboom et al. 1998a). Microorganisms with energy-dependent efflux systems which selectively pump the organic solvents out of the cell membrane were identified (Isken and DeBont 1996; Kieboom et al. 1998a; Rojas et al. 2001; Rojas et al. 2003). One of the such microorganisms that has been extensively studied is *Pseudomonas putida* S12 (Isken and DeBont 1996; Isken et al. 1999; Kieboom and de Bont 2001; Kieboom et al. 1998a; Kieboom et al. 1998b; Volkers et al. 2006; Weber et al. 1993). The export system in *P.*

*putida* S12 is a solvent resistant pump (srp) belonging to the resistance-nodulation-cell division (RND) family, which are proton dependent multidrug efflux systems composed of three protein components that together span the inner and outer membranes of gram-negative bacteria (Kieboom et al. 1998a; Moreira et al. 2004; Nikaido 1996; Putman et al. 2000). This kind of pump exports a large variety of compounds, but the solvent resistant pump identified in *P. putida* S12 is shown to be specific only to organic solvents (Isken et al. 1997; Kieboom and de Bont 2001; Kieboom et al. 1998b).

Whole-cell biocatalysis in organic media has become a standard technology for the production of high-value compounds. For higher productivities, solvent tolerant strains are required to keep the biocatalyst stable in presence of toxic organic solvents (Leon et al. 1998). Although *P. putida* S12 has various advantages as a stable host for biocatalysis, it has some drawbacks which may limit its application. Specifically *P. putida* S12 has a styrene monooxygenase and is known to metabolize some organic solvents, such as styrene, heptanol and octanol (Weber et al. 1993). This can lead to the formation of unwanted products which complicate downstream processing. The *P. putida* S12 strain also has a multidrug efflux pump (antibiotic resistance pump arpABC) which confers resistance to various antibiotics, and the pump is known to be induced in the presence of organic solvents (Isken et al. 1997; Kieboom and de Bont 2001). Moreover, this strain has not been fully sequenced and there could be other enzymes which compete to form unwanted byproducts. Considering these drawbacks, engineering solvent tolerance in the *Escherichi coli* strain could create a better host for biocatalysis.

Genetic modifications to solvent-sensitive *E. coli* strains have been done earlier (Aono 1998; Aono et al. 1994; Asako et al. 1997; White et al. 1997). Over expression of transcriptional activators, such as *mar*A producing multiple antibiotic resistance phenotype (Asako et al. 1997), *rob*A encoding global regulatory protein, and *sox*S encoding regulatory protein controlling the superoxide response regulon (Li and Demple 1994), increased solvent tolerance of *E. coli*. The increased solvent tolerance in *E. coli* due to these activators was attributed to increased expression levels of acrAB, which is a multi-drug efflux pump (White et al. 1997). Efflux pump acrAB has low substrate specificity and extrudes both antibiotics and solvents, thereby conferring solvent tolerance to *E. coli* (Nikaido 1996). Similar work was done by Aono et al. (1994) where an uncharacterized organic solvent tolerance gene *ost*A from a hexane tolerant *E. coli* was over expressed in solvent sensitive *E. coli* to confer resistance to hexane. Although these modifications improved solvent tolerance in *E. coli*, expression of a major solvent tolerance mechanism, such as solvent resistant pump srpABC in *P. putida* S12, was not done before. Kieboom et al. (1998a) has shown earlier that solvent tolerance in *P. putida* S12 can be transferred to a solvent sensitive *P. putida* strain by expression of srpABC genes. The transfer of resistance to various antibiotics was also demonstrated by inserting genes for a similar multi-drug efflux pump, MexAB-OprM, from *P. aeruginosa* into *E. coli* by Srikumar et al. (1998). Therefore, expression of srpABC genes could create solvent tolerant *E. coli* which can be a better host for biocatalysis. In this work, srpABC genes were cloned and inserted into *E. coli* to transfer solvent tolerance.

## Materials and Methods

*Chemicals.* Dodecane, Toluene, and decanol were purchased from Sigma (St. Louis, MO). Hexane and octane were purchased from Fischer Scientific (Hanover Park, IL). Luria-Bertani (LB) broth was purchased from Difco (Lawrence, KS).

*Bacterial Strains.* All *E. coli* strains were routinely grown in Luria-Bertani (LB) at 37$^{o}$C and 200 rpm. 100 mg/L ampicillin was added to the broth whenever required. *P. putida* S12 was purchased from American Type Culture Collection (ATCC # 700801) (Kieboom et al. 1998a) and was routinely grown in LB broth at 30$^{o}$C and 200 rpm.

*Cloning srpABC genes.* The srpABC genes amplification procedure is shown in Figure 4.1. Total genomic DNA of *P. putida* S12 was isolated using Qiagen DNAeasy tissue kit. The genomic DNA fragment with srpABC genes and *srp* promoter was amplified by PCR using Expand Long Template PCR System from Roche. The primers used for PCR were 5'- CTG ATT GGT TCA TAT CTT TCC TCT G - 3' and 5'- CAT ATC TTT CTC ATT GTC CTG CAA G -3' which amplify a 6253 kb fragment starting from 55 bp to 6307 bp (GenBank Accession # AF029405 for 6590 bp sequence for srpABC (Kieboom et al. 1998a)). The plasmids pJG001 and pJG002 were constructed by inserting PCR amplified srpABC genes into pBR322 and pUC19, respectively.

The DNA fragment with srpABC genes without *srp* promoter was cloned from pJG001 plasmid. The forward primer used was 5'-TCT AGA CTA CCG CAT TAC GAT TCA G-3' and the reverse primer was 5'-GAA TTC ACA GCA TCG CCA GTC ACT A-

3'. The srpABC genes were ligated into pBR322 in reverse alignment and the primers amplify the region from 4229 to 10036 in the reverse pJG001 DNA sequence.

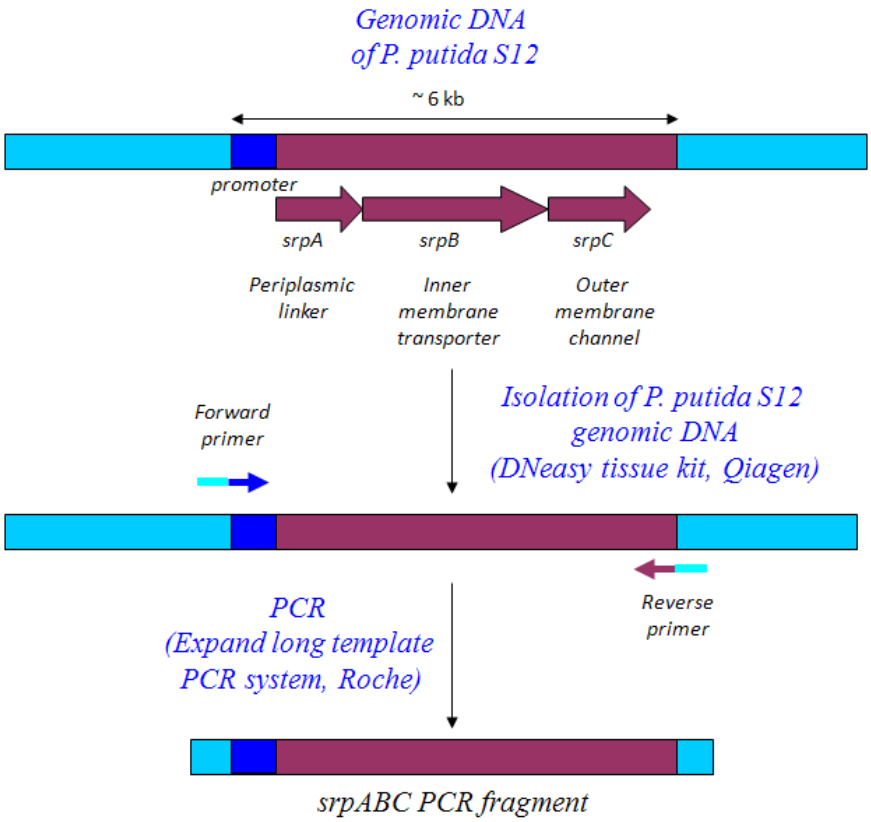



Figure 4.1. srpABC fragment amplification

Agarose gel electrophoresis was performed using 1% agarose gels in TBE buffer and the DNA was stained using ethidium bromide solution (Sambrook et al. 1989). DNA restriction and ligation (New England Biolabs, Ipswich, MA), genomic DNA and plasmid isolation (Qiagen, Valencia, CA), and PCR (Roche, Basel, Switzerland) were performed as suggested by the respective supplier.

*Transformation and Selection.* Electroporation was performed using Electromax DH10B cells (Invitrogen). Transformation by electroporation was done as recommended by the

supplier (Invitrogen). In order to identify transformants with pJG001 plasmid, single colonies from LB-Amp agar plates were simultaneously plated on LB-Amp and LB-Tet agar plates. Colonies growing only on LB-Amp plates and not on LB-Tet plates were selected as positive colonies. In order to identify transformants with pJG002, blue and white screening was used, and white colonies were selected as positive colonies. *E. coli* DH10B was also transformed with pUC19 plasmid and is used as the control strain for comparison. DNA sequencing was performed at University of Iowa DNA sequencing facility. Ten primers were designed to sequence the srpABC genes using primer walk.

*Protein Expression.* Cell extract was obtained by using lysis buffer (Kumar and Worobec 2002), containing 2% sodium dodecyl sulfate (SDS), 4% dithiothreitol (DTT), 10% glycerol and 1M Tris. Cells were harvested from 1.5 mL of overnight culture and resuspended in 100 µL lysis buffer. The cell lysate was centrifuged at maximum speed on a benchtop microcentrifuge for 10 min. The supernatant was mixed with two times concentrated sample loading buffer for SDS-PAGE. In order to prepare membrane protein fraction, Readyprep Protein Extraction Kit – Membrane I from BioRad was used. The samples for SDS-PAGE were prepared as suggested by the supplier. Cell pellet was obtained by centrifugation of 3 mL of overnight culture. Cells were lysed by sonication with five 30 sec pulses and 1 min interval on ice. The hydrophobic fraction was isolated and 100 µL of hydrophobic fraction was purified using Readyprep 2D cleanup kit. Protease inhibitors were added during cleanup. The protein pellet obtained after cleanup was suspended in 20 µL Laemmli Buffer supplemented with 5% v/v of β-mercaptoethanol (50 µL β-mercaptoethanol to 950 µL buffer).

SDS-PAGE was performed using 4-20% stacked Tris-HCL Ready gel for cell extract and 7.5% Tris-HCL Ready gel for membrane proteins. 10 µL of sample was added to each well and electrophoresis was performed at 200 V for ~45 min using SDS running buffer. After electrophoresis, gel was washed twice with deionized water and stained in 0.1% coomassie blue solution for 1 h. After staining the gel, the gel was again washed with deionized water and destained overnight.

*Solvent Toxicity Experiments.* The toxicity of five different solvents (dodecane, octane, hexane, decanol and toluene) was tested on the engineered strains. Overnight culture was diluted ten times and 5 mL of diluted culture was added to each 20 mL sterile screw-cap vial. 1% v/v of organic solvent was added to the cells and the growth was monitored using optical density measured at 660 nm on a UV-Vis spectrometer.

*Reverse Transcriptase (RT)-PCR analysis.* Qiagen RNeasy Protect Bacteria Mini kit was used to isolate RNA from Bacterial cultures. The cells were diluted to a final optical density at 600 nm of 0.25 (~$2.5 \times 10^8$ cells). Cells lysis was performed using enzymatic (lysozyme) lysis and Qiagen Proteinase K digestion as suggested by the supplier (Qiagen). Residual DNA was digested using DNase I set from Qiagen as suggested. Primers were designed to amplify ~ 1 kb DNA fragment of srpA, srpB, and srpC genes. The primers and the amplified fragment sizes are shown in Table 4.1. Qiagen one-step PCR kit was used to amplify DNA fragments from isolated RNA.

Table 4.1. Primers and amplified fragment sizes for each of srpABC genes for RT-PCR analysis

| Gene | Amplified Fragment size | Primer & Position* | DNA Sequence |
|---|---|---|---|
| srpA | 1016 bp | Forward Primer at 331 bp | 5' – CCT TAA CTG CGC TGA TGC TA – 3' |
| | | Reverse Primer at 1347 bp | 5' – CAC GTT GAA CAC CTT CGG TA – 3' |
| srpB | 824 bp | Forward Primer at 1992 bp | 5' – GCC TTA TGC CAT GCG TAT CT – 3' |
| | | Reverse Primer at 2816 bp | 5' – GGT ACG AAT ACA GCC GAC AG – 3' |
| srpC | 897 bp | Forward Primer at 4995 bp | 5' – TGG TGA CTG CCT ACG AGT TG – 3' |
| | | Reverse Primer at 5892 bp | 5' – CGT TGA GCA TCC AGC ACT GT – 3' |

* Position corresponding to Accession # AF029405

## Results

*Construction of Plasmids with srpABC Genes.* The solvent resistant pump srpABC genes that are responsible for solvent tolerance in *P. putida* S12 were amplified by PCR. The yield and specificity of PCR for amplification of srpABC genes was optimized, as shown in Figure 4.2. Lanes A-E in Figure 4.2 correspond to different PCR runs with either 10% dimethyl sulfoxide (DMSO) or 10% betaine to amplify genes from two different isolated genomic samples of *P. putida* S12. From the Figure 4.2, addition of 10% DMSO improved specificity of PCR. The PCR fragment was sequenced to confirm the presence of srpABC genes.

The membrane proteins encoded by srpABC genes could be toxic to the microorganisms (Miroux and Walker 1996; Srikumar et al. 1998) and therefore, srpABC

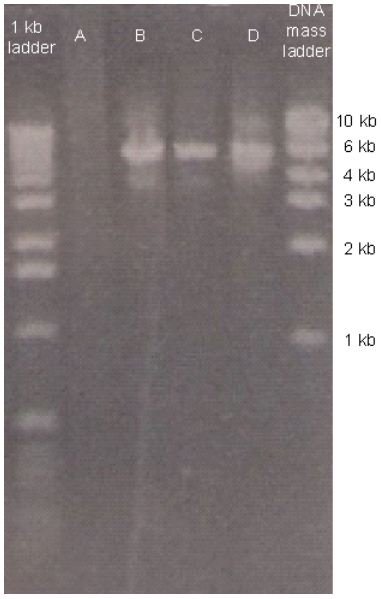



Figure 4.2. DNA gel electrophoresis picture showing srpABC genes (~6 kb). Lanes A & C have 10% DMSO, B & D have 10% betaine, and lanes A & B and lanes C & D are PCR runs from two different isolated genomic DNA samples from *P. putida* S12

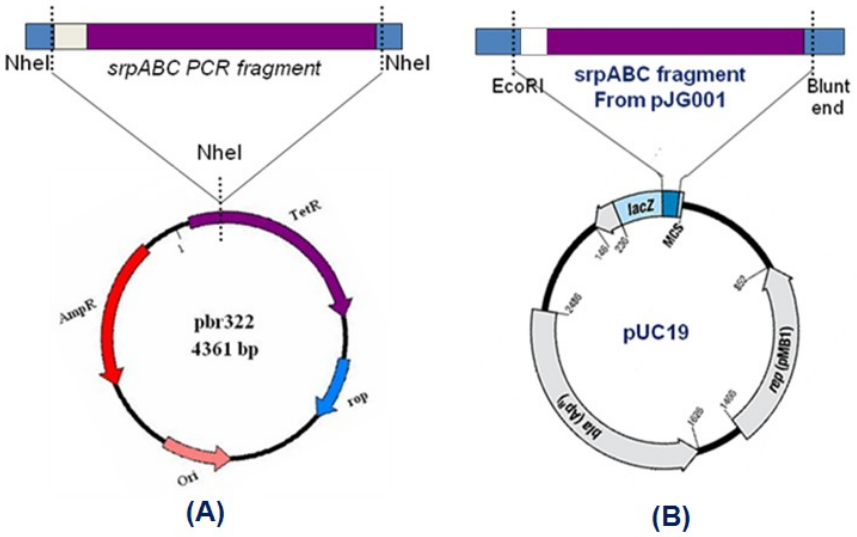



Figure 4.3. Insertion of srpABC genes into (A) a low-copy number plasmid, pBR322 and (B) a high-copy number plasmid, pUC19. (Plasmid maps for pUC19 and pBR322 were obtained from New England Biolabs)

genes were inserted into two plasmids, a low-copy number plasmid pBR322 and a high-copy number plasmid pUC19, as shown in the Figure 4.3. The PCR fragment and

pBR322 plasmid were separately digested with NheI enzyme. The digested vector and PCR fragment were ligated using a T4 ligase, and the Figure 4.4 shows the agarose gel for ligation mix. The ligated vector with PCR fragment along with vector religated product was observed. In order to eliminate vector religation, the digested pBR322 was further dephosphorylated using Shrimp Alkaline Phosphatase (SHP), from Fermentas, as suggested by the supplier and ligated with PCR fragment. Figure 4.5 shows the agarose gel for ligation mix with dephosphorylated vector. The vector religation was eliminated and the vector ligated with srpABC genes (pJG001, 10246 bp) was obtained. The inserted fragment cleaves the tetracycline resistance gene in the pJG001 plasmid.

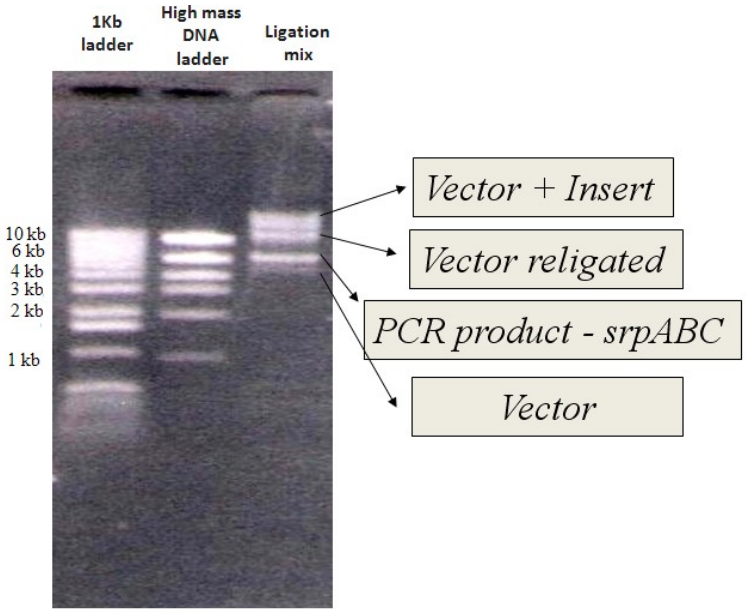



Figure 4.4. Ligation of srpABC PCR fragment into pBR322

In order to insert srpABC genes into a high copy number plasmid, plasmid pJG001 was double digested with EcoRI and PshAI to isolate the srpABC genes with the *srp* promoter. Plasmid pUC19 was double digested with EcoRI and HincII in the multiple cloning site. Figure 4.6A shows agarose gel of digested plasmids pJG001 and pUC19.

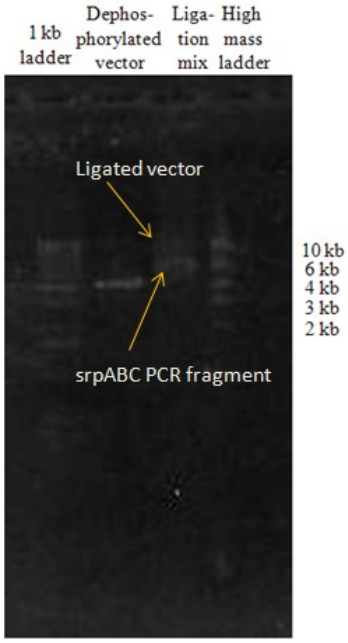



Figure 4.5. Ligation of srpABC PCR fragment into dephosphorylated pBR322

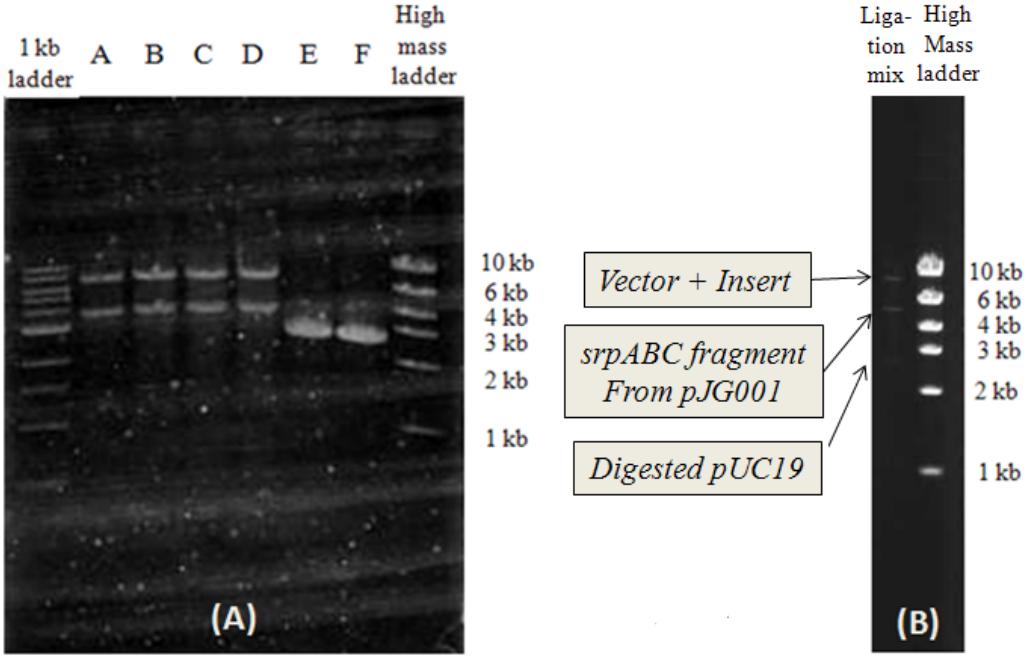



Figure 4.6. Construction of pJG002 plasmid. (A) Restriction of pJG001 and pUC19. Lanes A-D show double digested pJG001 with EcoRI and PshAI to produce ~6.5 kb fragment consisting of srpABC genes. Lanes E & F show double digested pUC19 with EcoRI and HincII. (B) Ligation of srpABC into pUC19 plasmid with purified restriction fragments

Double digestion of pJG001 yields a ~6.5 kb fragment consisting of srpABC genes. The srpABC fragment and digested pUC19 fragment were gel purified using QIAquick gel extraction kit from Qiagen. The purified fragments were ligated using T4 ligase, and Figure 4.6B shows the gel electrophoresis of ligation mix with ligated plasmid (pJG002, 9254 bp).

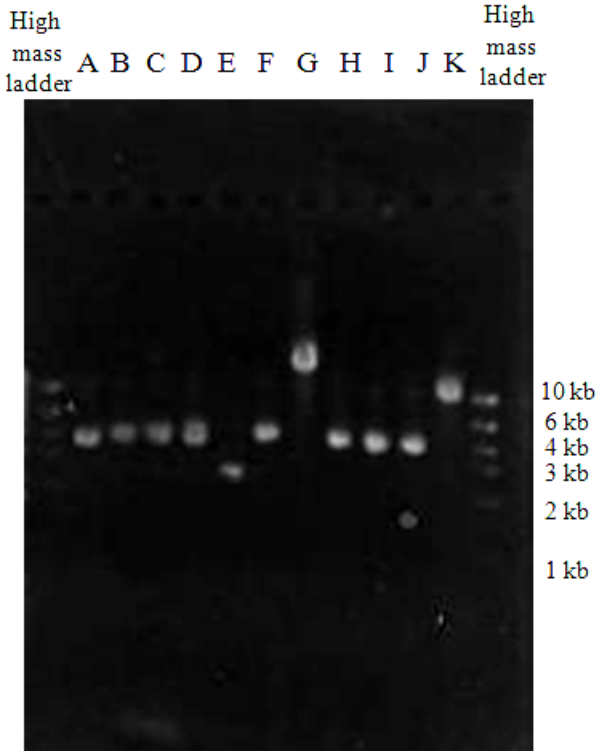



Figure 4.7. Isolation of plasmids from 11 positive colonies for pJG001 transformation

*Insertion of pJG001 and pJG002 into* E. coli. Plasmids pJG001 (srpABC in pBR322) and pJG002 (srpABC in pUC19) were constructed and transformed into *E. coli* DH10B cells by electroporation. Positive transformants with pJG001 were selected and plasmids were isolated, as shown in Figure 4.7. Colonies G and K had plasmids with size 10 kb or greater. Further restriction analysis was performed as shown in Figure 4.8. Colonies G and K show fragments of srpABC and pBR322 after NheI restriction. However,

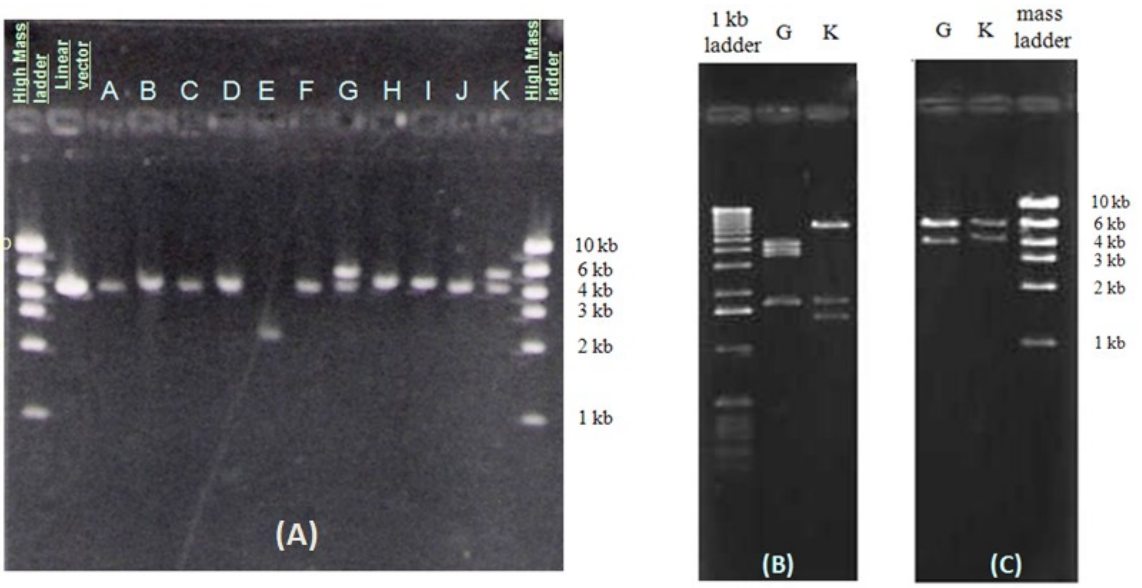



Figure 4.8. Screening for transformants with pJG001 (A) NheI restriction of 11 positive colonies. Colonies G and K showed the bands for the plasmid (~4 kb) and srpABC genes insert (~6 kb). Further analysis was performed on the plasmid of colonies G and K, (B) SalI restriction of colonies G and K, (C) NdeI restriction of colonies G and K. Colony K has the plasmid showing the restriction fragments corresponding to the predicted DNA sequence of pJG001.

restriction analysis with SalI and NdeI revealed that only colony K has the pJG001 plasmid. The presence of srpABC genes was further confirmed by DNA sequencing of the plasmid isolated from colony K. Positive transformants with pJG002 were also isolated and restriction analysis was performed, as shown in Figure 4.9. Colony I has a plasmid with ~10 kb and further restriction analysis reveals that it has the pJG002 plasmid. The presence of srpABC genes was further confirmed by DNA sequencing of the plasmid isolated from colony I.

*Solvent Tolerance.* The solvent tolerance of *E. coli* strains with pJG001 and pJG002 was tested by growing the strains in saturated concentration of toluene (Kieboom et al. 1998a). Cells were growing both on LB agar plates with saturated vapor of toluene and in

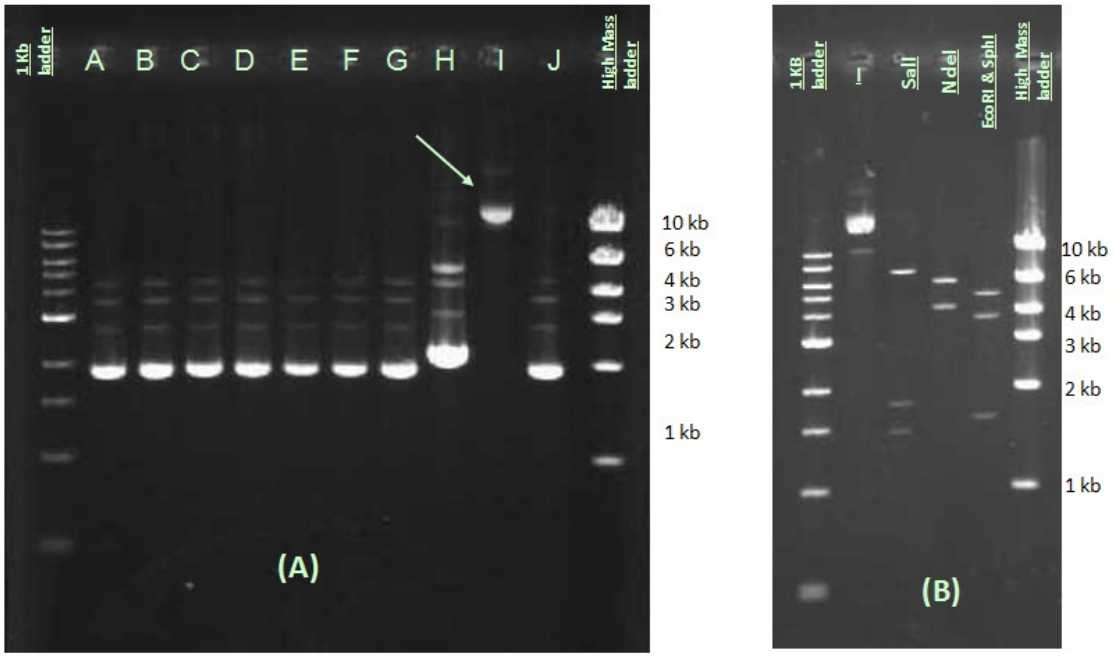



Figure 4.9. Screening for transformants with pJG002 (A) Isolated plasmids of 12 positive colonies. Colony I has a plasmid with the required size and further analysis was performed (B) SalI, NdeI and double digest with EcoRI & SphI of colony I show the restriction fragments corresponding to the predicted DNA sequence of pJG002.

LB liquid medium with saturated concentration (6.2 mM (Kieboom et al. 1998b), which is 0.0012 % v/v for 5 mL cells in 20 mL vial) of toluene. However, the control *E. coli* strain with pUC19 plasmid did not grow either on LB agar plates or in LB liquid medium with saturated concentration of toluene. Therefore, *E. coli* cells with pJG001 and pJG002 were more tolerant to toluene compared to control strain. This difference in growth patterns could be attributed to the solvent resistant pump srpABC. Similar results were obtained earlier when srpABC genes were transferred into a solvent-sensitive *P. putida* strain (Kieboom et al. 1998a). To further study the solvent tolerance of the engineered *E. coli* strains, cells were grown until early log phase and 1% v/v of different organic solvents was added. The growth of the cells was measured by optical density at 660 nm at 6 h and 18.5 h, and the results are shown in Figure 4.10.

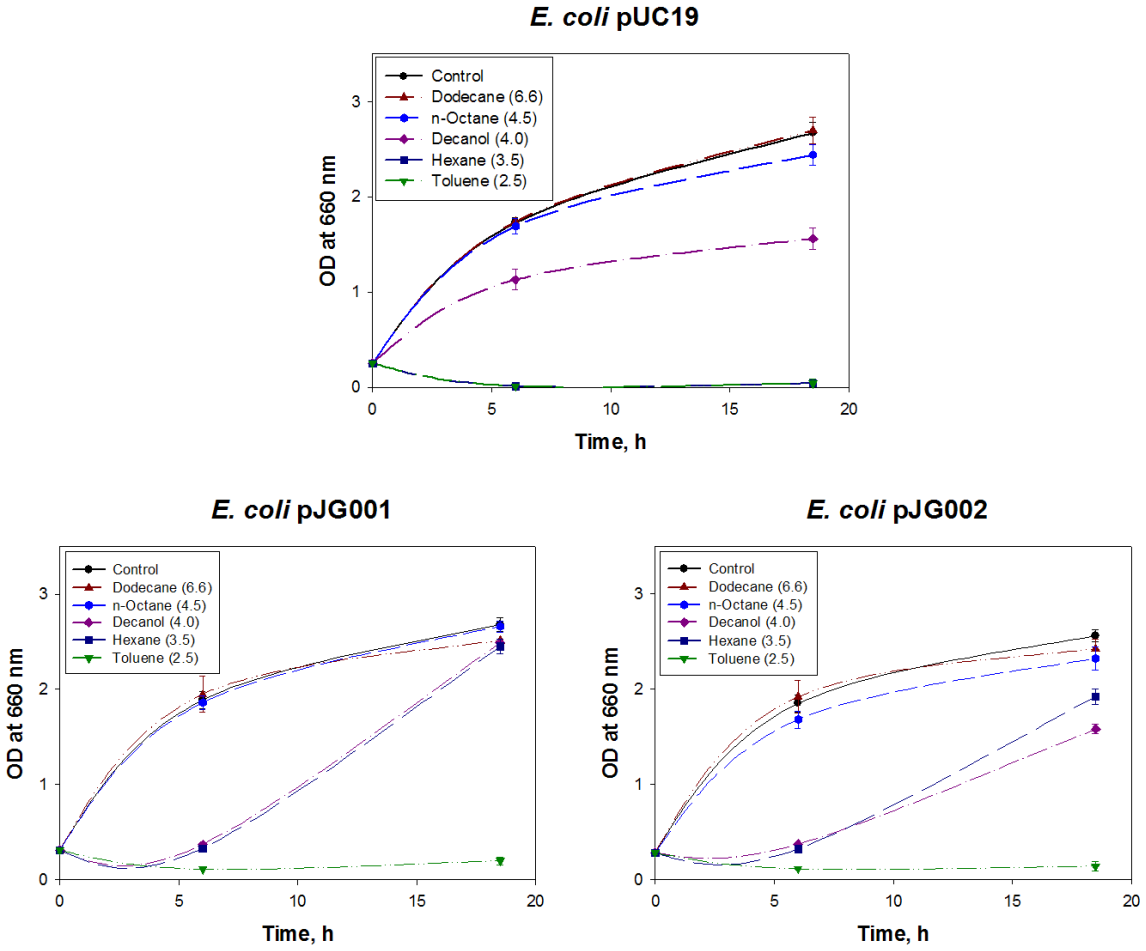



Figure 4.10. Growth of *E. coli* strains with pUC19, pJG001 and pJG002 in presence of 1% v/v of various organic solvents (with their logP values in parenthesis) at 37 $^{o}$C. (Average of three independent experiments)

Solvents dodecane and octane have logP values greater than 4 and are generally not toxic to microorganisms (Laane et al. 1987; Leon et al. 1998). Therefore, the growth of *E. coli* strains was not inhibited in presence of these solvents. However, solvents decanol, hexane and toluene are toxic to microorganisms. The toxicity of solvents increases, as its logP value decreases, from decanol to hexane to toluene. The growth of the control strain, *E. coli* bearing pUC19 plasmid, was inhibited in the presence of decanol and no growth was observed in the presence of hexane and toluene. The growth

of *E. coli* strains with pJG001 or pJG002 was inhibited within 6 h for decanol and hexane. However, growth was recovered after 6 h for both the strains. The cell density of *E. coli* pJG001 in hexane or decanol after 18.5 h was similar to that of the control where no solvent was added. The cell density of *E. coli* pJG002 in hexane or decanol was slightly less than that of the control after 18.5 h. Therefore, both the strains *E. coli* pJG001 and *E. coli* pJG002 were tolerant to hexane and decanol. However, neither of the engineered *E. coli* strains grew in 1% v/v of toluene.

The presence of organic solvents leads to ineffective metabolism due to the uncoupling effects of the solvent and leads to increased phospholipid biosynthesis in microorganisms (Heipieper et al. 1994; Isken et al. 1999; Pinkart and White 1997). Moreover, the presence of a proton-dependent solvent resistant pump srpABC further increases the energy requirements of the cell in presence of these solvents, thereby reducing the yield of the cells (Isken and DeBont 1996; Kieboom et al. 1998a). Therefore, both strains *E. coli* pJG001 and pJG002 had a low cell density at 6 h in the presence of decanol and hexane. However, the srpABC pump helps the cells to adapt in presence of these solvents and hence, the cell density of both the strains increased after 6 h and reached levels similar to the control by 18.5 h. Among the two engineered *E. coli* strains, *E. coli* with pJG002 showed a slower growth rate which could be attributed to higher energy requirement due to the expression of energy dependent srpABC proteins from a high-copy number plasmid. Comparing engineered *E. coli* with *P. putida* S12, which grows in presence of 1% v/v of toluene (Weber et al. 1993), *E. coli* will be more susceptible to solvents due to their membrane permeability. *P. putida* S12 modifies its

outer membrane by *cis*-to-*trans* isomerization, thereby decreasing its membrane permeability (von Wallbrunn et al. 2003). The higher membrane permeability of *E. coli* increases solvent influx and therefore compromises the effect of the solvent efflux pump in the engineered *E. coli* strains.

*Expression of Solvent Resistant Pump Proteins.* The expression of *srp*ABC proteins in the engineered *E. coli* strains has to be analyzed. The molecular weights of *srp*A, *srp*B and *srp*C are 51 kDa, 114 kDa and 41 kDa, respectively (Kieboom, 1998a). The cell extract of *E. coli* pJG001 along with *P. putida* S12 and *E. coli* pBR322 was analyzed for protein expression, and Figure 4.11 shows the SDS-PAGE gel after electrophoresis. The *srp*ABC proteins were not identified due to the presence of numerous native proteins in the size range of *srp*ABC proteins for each strain. In order to further analyze protein expression, membrane proteins from engineered *E. coli* strains were isolated using Readyprep Protein Extraction Kit – Membrane I from BioRad. Figure 4.12 shows SDS-PAGE analysis of membrane protein fractions of *P. putida* S12, *E. coli* pJG001, *E. coli* pJG002 and *E. coli* pUC19 (control). The *srp*ABC proteins were not identified even from SDS-PAGE analysis of membrane fractions.

*Reverse Transcriptase (RT) - PCR Analysis.* RT-PCR analysis was performed to further investigate the expression of srpABC genes. Cells were grown in presence or absence of 1% v/v hexane for ~18 h and RNA was isolated using Qiagen RNeasy Protect Bacteria Mini kit. Primers were designed to amplify ~ 1 kb fragment from each of srpABC genes. A negative control, *E. coli* pUC19, and a positive control, *P. putida* S12 grown in

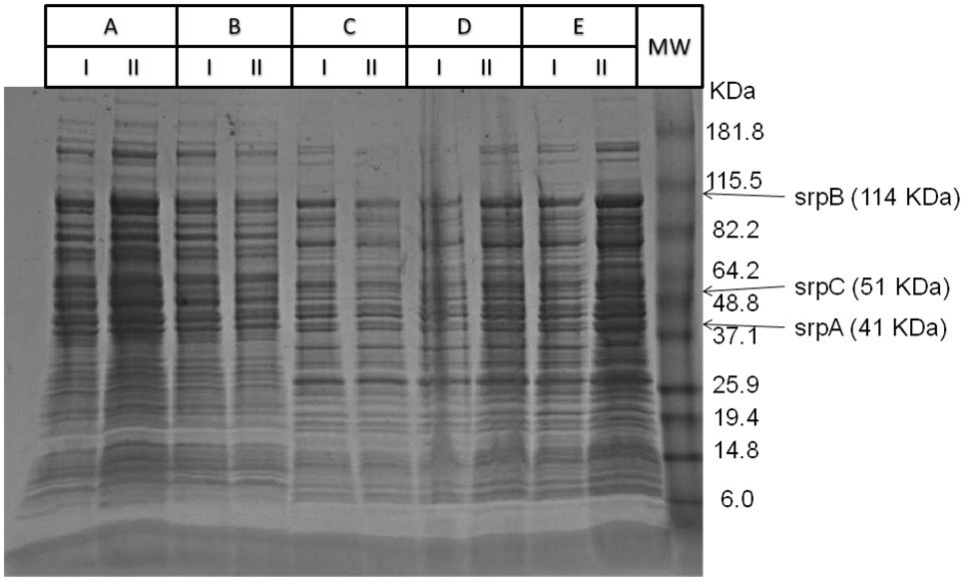



Figure 4.11. SDS-PAGE using 4-20% stacked Tris-HCL Ready gel of cell extract from, A – *P. putida* S12 grown in presence of 6 mM toluene, B – *P. putida* S12 grown in absence of toluene, C – *E. coli* pJG001 grown in presence of 6 mM toluene, D – *E. coli* pJG001 grown in absence of toluene, and E – *E. coli* pBR322 (control) grown in absence of toluene. I and II represent two dilutions, where I is (1/2) sample and II is (1/6) sample. MW represent Benchmark Prestained Protein Ladder from Invitrogen.

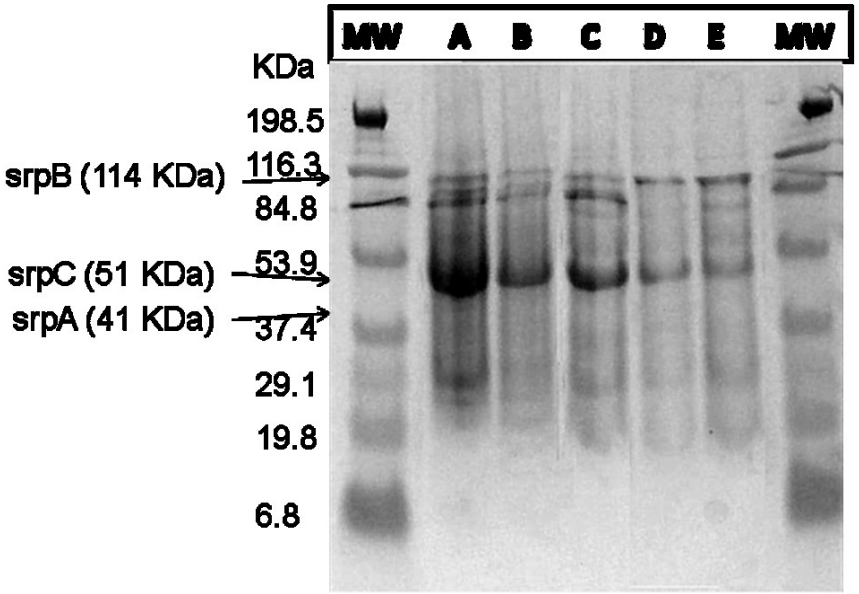



Figure 4.12. SDS-PAGE analysis of mebrane proteins isolated from A – *P. putida* S12 grown in 6 mM toluene, B – *P. putida S12*, C – *E. coli pJG001*, D – *E. coli pJG002*, and E – *E. coli* pUC19, where B, C , D and E were grown in absence of toluene. MW represent Prestained SDS-PAGE standards – Broad range from BioRad.

presence of 1% v/v hexane, were used for comparison. The agarose gel pictures of amplified products are shown in Figure 4.13 for srpA, srpB and srpC. The negative control doesn't show amplified product bands as it doesn't have srpABC genes. *E. coli* pJG001, *E. coli* pJG002 and *P. putida* S12 show amplified product bands for srpA, srpB and srpC, proving the expression of srpABC genes in engineered *E. coli* strains. Therefore, the solvent tolerance of engineered *E. coli* strains is due to the solvent resistant pump srpABC. Engineered *E. coli* strains with pJG001 or pJG002 show amplified product bands even in the absence of hexane proving that the expression of srpABC genes is constitutive.

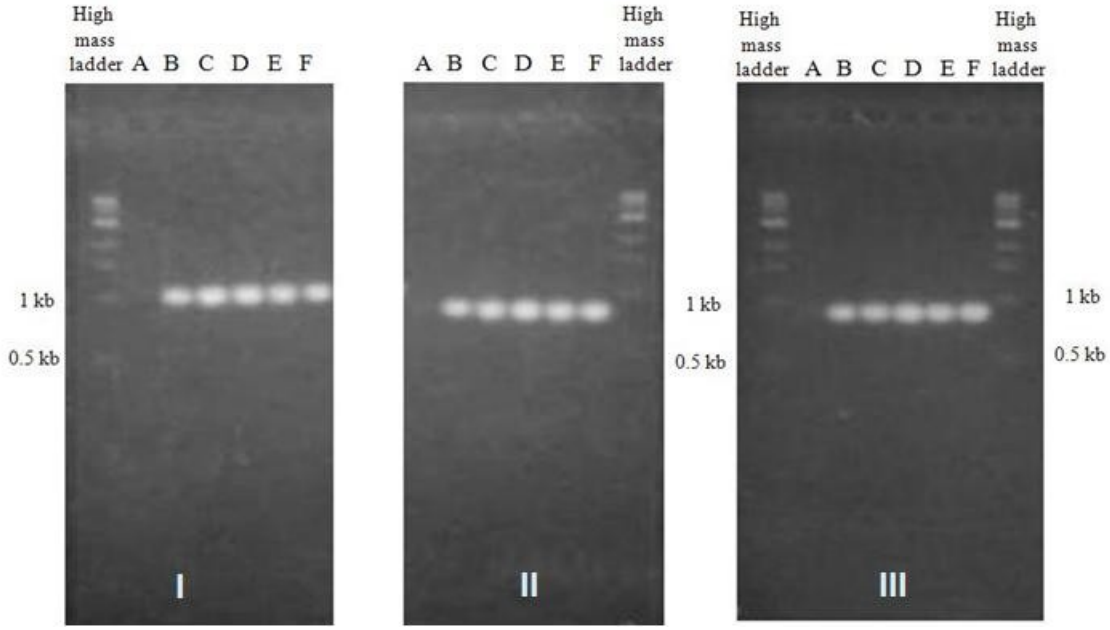



Figure 4.13. RT-PCR analysis for expression of srpABC genes. A – *E. coli* pUC19 (negative control), B – *E. coli* pJG001, C – *E. coli* pJG001 in presence of 1% v/v hexane, D – *E. coli* pJG002, E – *E. coli* pJG002 in presence of 1% v/v hexane, F – *P. putida* S12 grown in presence of 1% v/v hexane (positive control). I, II, and III show analysis for srpA, srpB and srpC genes, respectively.

*T7 Promoter for srpABC Gene Expression.* The expression of srpABC genes in the engineered *E. coli* strains was constitutive due to the absence of regulatory proteins *srp*RS that control expression from *srp* promoter (Wery et al. 2001). Solvent tolerance of engineered *E. coli* strains can be improved by controlled expression of srpABC genes. Therefore, srpABC genes were introduced downstream to T7 promoter to regulate their expression. The srpABC genes without *srp* promoter were amplified from pJG001 plasmid. Figure 4.14 shows agarose gel with PCR product and its restriction analysis to confirm the presence of srpABC genes without *srp* promoter. The PCR product was inserted into the TA cloning site of pCR XL TOPO cloning vector using TOPO XL cloning kit from Invitrogen. Although Expand Long Template PCR System uses a mix of Taq and proofreading polymerases, it produces approximately 80% sticky A ends. The

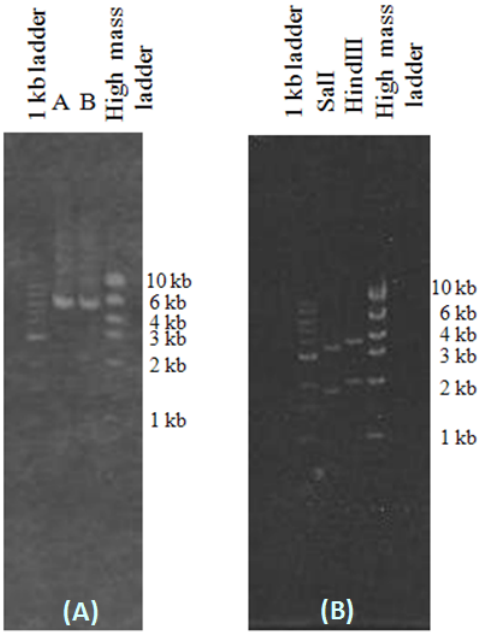



Figure 4.14. PCR for srpABC genes without *srp* promoter and PCR product restriction analysis. (A) A and B are identical PCR reactions with 10% DMSO, (B) Restriction of PCR product to confirm the presence of srpABC genes

ligated plasmid with srpABC genes was named as pJG003. Transformation was performed using Electromax DH10B cells using electroporation. The vector has a *cccd*B lethal gene and transformants with unligated vector will not survive. Therefore, colonies that grow on ampicillin were isolated as positive colonies. Figure 4.15 shows isolation and restriction analysis of plasmids from 10 positive colonies. Colony E has a plasmid with required size. However, restriction analysis reveals that srpABC genes were ligated in reverse orientation to T7 promoter. More colonies were screened, but a transformant with pJG003 plasmid (srpABC genes in orientation with T7 promoter) was not identified.

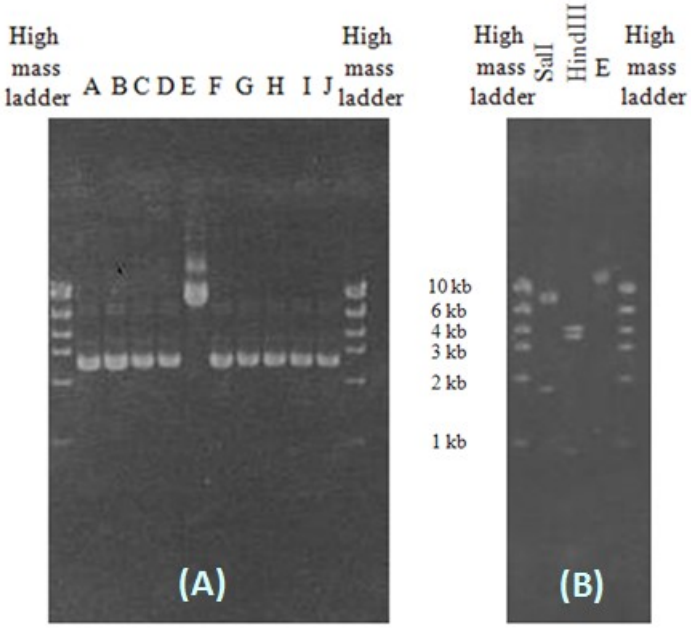



Figure 4.15. Screening for transformants with pJG003. (A) Isolation of plasmids from 10 positive colonies. Colony E has a plasmid with required size and further analysis was performed, (B) Restriction with SalI and HindIII to confirm presence of srpABC genes in reverse orientation from T7 promoter in colony E

## Conclusions

The solvent resistant pump (srpABC) genes from *P. putida* S12 were cloned and inserted into a solvent sensitive *E. coli* strain to transfer solvent tolerance. Recombinant

*E. coli* strains bearing either plasmid pJG001 or pJG002, containing srpABC genes in low-copy number or high-copy number respectively, grew in the presence of a saturated concentration of toluene. Both the strains were more tolerant to 1% v/v of toxic solvents decanol and hexane, reaching similar cell density as the control where no solvent was added. The expression of srpABC genes was confirmed by Reverse Transcriptase (RT) – PCR analysis. The solvent tolerance of *P. putida* S12 through a major solvent tolerance mechanism of a proton-dependent solvent resistant pump srpABC was successfully transferred to a solvent sensitive *E. coli* strain. The engineered *E. coli* strain could serve as a better biocatalyst in presence of toxic organic solvents. More work will be done to analyze the expression of the srpABC proteins from the two plasmids. The pump activity will be analyzed in the two strains to identify the best expression system for the srpABC genes.

# CHAPTER 5
# CONCLUSIONS

Whole-cells expressing TOM-Green enzyme was used for 1-naphthol production. A liquid-liquid biphasic system, with decreased product toxicity by in-situ product removal into the organic phase and increased substrate loading, was used to improve 1-naphthol productivity. *E. coli* pBS(kan)TOM-Green was used as the biocatalyst for 1-naphthol production, and lauryl acetate gave best results among the solvents tested. The production of 1-naphthol was optimized by studying the effect of naphthalene concentration in the organic phase and the organic-to-aqueous phase ratio. Highest 1-naphthol concentration of 1.43 g/L in the organic phase and highest productivity of 0.55 g/gCDW were obtained by optimization of the organic phase. The recycle ability of the biocatalyst was tested in both free and immobilized form. The biocatalyst in either free or immobilized form has low stability losing more than 60% activity for second run of recycle which could be because of 1-naphthol toxicity. On a constant volume basis, 8-fold improvement in 1-naphthol production was achieved using biphasic media relative to biotransformation in aqueous medium.

1-Naphthol productivity was further improved by the application of solvent tolerant host *P. putida* S12 to express TOM-Green enzyme. *P. putida* S12, in addition to its tolerance to various toxic organic solvents, showed more tolerance towards naphthalene compared to *E. coli*. Application of *P. putida* S12 improved 1-naphthol productivity by approximately 42% compared to *E. coli* using lauryl acetate as the solvent. Improvement in 1-naphthol production using *P. putida* S12 with lauryl acetate as

the solvent could be because of its higher tolerance to naphthalene. Production of 1-naphthol was also tested using decanol, and decanol was observed to be toxic to *E. coli*. However, *P. putida* S12 strain produced similar productivities of 1-naphthol compared to that using lauryl acetate. The results demonstrate that *P. putida* S12 is a better host for biocatalysis owing to its tolerance to various toxic compounds. Moreover, *P. putida* S12 enabled using a less expensive solvent, decanol, to produce similar amount of 1-naphthol compared to the solvent lauryl acetate.

Engineering solvent tolerance in *E. coli* was also performed to create a better host for whole-cell biocatalysis in biphasic systems. The solvent resistant pump srpABC genes from *P. putida* S12 were cloned and inserted into a solvent sensitive *E. coli* strain to transfer solvent tolerance. *E. coli* strain with plasmid pJG001 or pJG002, containing srpABC genes in a low-copy number or a high-copy number plasmid respectively, grew in the presence of saturated concentration of toluene. Both the engineered *E. coli* strains were more tolerant to 1% v/v of toxic solvents decanol and hexane reaching similar cell densities as the control where no solvent was added. The expression of srpABC genes was confirmed by Reverse Transcriptase (RT) – PCR analysis. Controlled expression of srpABC genes may improve solvent tolerance, and plasmid with srpABC genes without *srp* promoter downstream to T7 promoter was constructed and transformed into *E. coli*. However, transformant with srpABC genes in reverse orientation to T7 promoter was only identified. The solvent tolerance of *P. putida* S12 through a major solvent tolerance mechanism of a proton-dependent solvent resistant pump srpABC was successfully transferred to a solvent sensitive *E. coli* strain.

The production of 1-naphthol was improved environmentally by using biocatalysis to replace traditional chemical method involving multiple reactions and harsh conditions. The production of 1-naphthol was enhanced by addressing the limitations of biocatalysis. Limitations of low solubility of substrate, and toxicity of both the substrate and product were minimized by using liquid-liquid biphasic systems. The stability of biocatalyst in biphasic system was also improved by the application of solvent tolerant strain *P. putida* S12. The amount of 1-naphthol produced was significantly improved by process modifications. However, 1-naphthol concentrations have to be further improved to about 50 to 100 g/L to make the process industrially feasible. The process modifications presented herein will help in addressing problems associated with production of valuable products using biotransformations. The work served as an experience and motivation for four undergraduate students, and also benefited collaborators at Prairie View A & M University. These research efforts have resulted four manuscripts, five presentations as national conferences and several presentations to industrial partners at Center for Environmentally Beneficial Catalysis (CEBC) (see *Appendix F*).

# CHAPTER 6
# FUTURE WORK

Previous chapters demonstrate the application of biphasic systems and solvent tolerant microorganisms for 1-naphthol production. Significant contributions were made to improve the efficiency and economics of 1-naphthol production. However, more work is needed to make the process commercially viable. The product concentrations have to be increased to at least 50 – 100 g/l to make the process industrially feasible (Pollard and Woodley 2007). Suitable techniques to efficiently recover and purify the product have to be identified. Moreover, the overall process for 1-naphthol production has to be designed including recycle of both substrate and organic solvent to minimize the cost. In order to improve product concentrations and productivities based on the results shown in the previous chapters, following are some key areas to explore.

## Media Formulation for Biphasic Systems

Oxidation of naphthalene to 1-naphthol was performed using resting cells in phosphate buffer supplemented with glucose (*Chapters 2 & 3*). The presence of a second phase of organic solvent has detrimental effects on the microorganism. Therefore, additional nutrients can be supplied to the microorganism to facilitate its repair, thereby improving its stability (Rajagopal 1996). Moreover, the presence of organic solvents results in the loss of ions across the cell membrane and Inoue et al.(1991) added various metal ions to the medium to test the growth of the microorganism in presence of solvents. They observed an increase in growth of the microorganism for more than 2 mM $Mg^{+2}$ or more than 0.5 mM $Ca^{+2}$ ions addition. Therefore, effects of other nutrients, such as a

nitrogen source (amino acids or ammonium sulfate) or metal ions, on the stability of the microorganism in presence of organic solvents should be evaluated. Media supplements can also be added to the biotransformation after 12 or 24 h to enhance the stability/viability of the microorganism.

## Immobilization and Recycle

Recycle of the biocatalyst is necessary to improve the efficiency and economics of the process. Immobilization improves the stability of the biocatalyst by minimizing direct contact of cells with the organic solvents thereby reducing phase toxicity (Leon et al. 1998). However, immobilization introduces mass transfer limitations associated with transport of substrate and product across the immobilization matrix. Recycle of *E. coli* pBS(kan)TOM-Green cells was performed (*Chapter 2*) and most of the activity was lost for both free and immobilized cells after first run. Recycle was performed for 6 and 12 h. However, cell viability has to be determined at different time periods of biotransformation to identify the best time for recycle. Immobilized cells lost more activity than free cells which could be because of toxic product accumulation in the calcium alginate beads. However, accumulation of the product has to be proven by experiments. Carefully designed experiments have to be performed to study the transport of substrate and product in the bead. Smaller beads can be used to reduce mass transfer limitations (Leon et al. 1998). Immobilization matrix can be modified by addition of surfactants or modification of bead surface (Leon et al. 1998). The partition coefficient of product and substrate into various immobilization matrices has to be calculated to identify the best immobilization method for 1-naphthol production. *P. putida* S12 showed more tolerance to 1-naphthol and may have better retention of activity for recycle. Both

free and immobilized cells of *P. putida* S12 pBS(kan)TOM-Green can be recycled to test the stability of this strain.

## Bioreactor Studies

Scaling up the biphasic system and control of the parameters, such as pH, dissolved oxygen and glucose, within a bioreactor may enhance productivities. Maintaining optimum conditions and required concentration of nutrients is necessary to maintain the viability/activity of the microorganism. Moreover, the process has to be optimized to determine the best conditions, such as glucose concentration, nutrient requirement, dissolved oxygen, and agitation, to achieve maximum productivity. High cell densities can be used in the bioreactor to further improve the productivities.

## Organic Solvent Screening

Organic solvent selection is one of main criteria to realize the potential of biphasic systems. Additional solvents which are biocompatible and have good distribution coefficient for the product need to be screened. Computational approaches can be used to identify potential solvents using programs, such as Extractant Screening Program (ESP) (Bruce and Daugulis 1991), to decrease the time required for screening. ESP is a computer program that calculates the distribution coefficient for different solvents by the UNIFAC method and ranks the solvents based on the features required for biphasic system, such as distribution coefficient, biocompatibility, price, etc. Mixtures of solvents can also be used instead of pure solvents to get the required polarity and biocompatibility.

## Application of *P. putida* S12 to Diol production

Oxidation of naphthalene to *cis*-1,2-naphthalene dihydrodiol was done earlier (McIver et al. 2008). *P. putida* S12 is more tolerant to naphthalene than *E. coli* strains and its application may enhance diol production. However, *P. putida* S12 has an antibiotic resistance pump which confers resistance to various antibiotics, such as ampicillin, chloramphenicol, tetracycline etc. (Kieboom and de Bont 2001). The catabolic plasmids containing naphthalene or toluene dioxygenase genes have ampicillin resistance markers. Therefore, insertion of these catabolic plasmids into *P. putida* S12 is limited due to the absence of antibiotic pressure to retain the plasmid. In order to transfer these genes, either the plasmids have to be modified with different markers or the antibiotic resistance pump genes have to be knocked out. Preliminary work in modification of naphthalene dioxygenase plasmid with kanamycin resistance gene was done (see *Appendix B*). Application of solvent tolerant *P. putida* S12 strain also allows for the use of polar solvents, which are generally toxic, to recover the polar product *in-situ*.

## Application of Solid-Liquid Biphasic System

Liquid-liquid biphasic systems have various advantages to perform reactions involving toxic substrate and/or product. However, liquid-liquid biphasic systems have drawbacks associated with solvent toxicity. Although application of solvent tolerant strains improve productivity (as shown in *Chapter 3*), use of a solid phase instead of organic solvent could have additional advantages (Prpich and Daugulis 2007a). Solid adsorbents have been used earlier for *in-situ* removal of toxic products. Preliminary work in exploring the use of resins for 1-naphthol production was done (see *Appendix D*). However, use of solid adsorbents is limited due to low selectivity for product compared

to the substrate (Held et al. 1999). Recently, thermoplastic polymers have been used to enhance productivities (Morrish and Daugulis 2008; Prpich and Daugulis 2007a). Thermoplastic polymers are analogous to solvents in absorption of compounds compared to adsorption mechanism (Prpich and Daugulis 2007a). Therefore, production of 1-napthol in biotransformations with different thermoplastic polymers can be performed for comparison to liquid-liquid biphasic reactions. This study will identify the best process for production of toxic 1-naphthol.

## srpABC Expression and Activity Analysis

Engineering solvent tolerance was performed by transforming a solvent-sensitive *E. coli* strain either with a low-copy number (pJG001) or a high-copy number (pJG002) plasmid containing srpABC genes (*Chapter 4*). The expression of the srpABC genes was confirmed by Reverse Transcriptase – PCR analysis. The expression of srpABC genes from pJG001 and pJG002 have to be further studied and compared to identify best expression system. The *E. coli* pJG001 showed slightly better tolerance to solvents than the strain with pJG002 plasmid. This could be because of higher energy requirements for over expressed srpABC proteins from a high copy number plasmid. However, this explanation has to be proven by analyzing the activity of the pump. The activity of srpABC proteins can be analyzed by calculating the amount of organic solvent, such as toluene, accumulated in the cell membrane in the presence and absence of proton conductor carbonyl cyanide m-chlorophenylhydrazone (CCCP). The solvent efflux pump srpABC is a proton-dependent pump and the presence of CCCP disrupts proton motive force thereby rendering the pump inactive. The activity measurement in presence and absence of CCCP is generally used to analyze energy dependent pumps (Nikaido 1996).

A similar assay was also used earlier (Isken and DeBont 1996) for *P. putida* S12 where cells were incubated in presence of $^{14}$C-labeled toluene and the accumulation was measured by radioactivity. Similar activity analysis for the two engineered *E. coli* strains can help in confirming srpABC expression and in identifying the best expression system.

The solvent tolerance of *E. coli* strains with srpABC genes was demonstrated in Chapter 4. However, more detailed analysis regarding the tolerance to different solvents has to be performed in order to compare with other solvent tolerant *E. coli* strains in the literature (Aono 1998; Asako et al. 1997; White et al. 1997). The tolerance of *E. coli* strain to other solvents with logP between 3.5 (hexane) and 2.5 (toluene), such as cyclohexane, p-xylene etc., has to be analyzed. The engineered *E. coli* strain with active solvent efflux pump will always be more susceptible to solvents than *P. putida* S12 strain because the *Pseudomonas* strain has an additional adaptive mechanism of outer membrane modification. The higher membrane permeability of the *E. coli* strain increases solvent influx, thereby compromising the effect of solvent efflux pump. In order to further improve tolerance of engineered *E. coli* strain, the srpABC proteins can be over expressed using *lac* promoter instead of using native *srp* promoter. Expression of srpABC genes was done earlier by Kieboom et al.(1998a) in a solvent-sensitive *P. putida* JK1 strain. They observed that the strain had better tolerance when genes were expressed using *lac* promoter compared to expression of genes in opposite orientation to *lac* promoter. A transformed *E. coli* strain with srpABC genes in reverse orientation with T7 promoter was identified (*Chapter 4*). Further work has been done to isolate srpABC genes from transformed plasmid and to insert it into pUC19 in orientation with T7

promoter (see *Appendix B*). The plasmid has to be transformed into *E. coli* to get controlled expression of the genes. Moreover, the srpABC genes are downstream to the *lac* promoter in pJG002 plasmid. Induction of srpABC genes can be done by addition of IPTG to over express srpABC proteins. However, over expression of these proteins can be lethal to microorganisms (Miroux and Walker 1996; Srikumar et al. 1998).

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# APPENDIX A. MICROORGANISMS AND GROWTH

## Growth of Microorganisms

The characteristics of various microorganisms and plasmids are shown in Table A. 1. The *P. putida* S12 strains are routinely grown at 30°C and *E. coli* strains were

Table A. 1. Microorganisms and Plasmids used

| Strains and/or Plasmid | Relevant Characteristics* | Source/Reference |
|---|---|---|
| *E. coli* TG1 pBS(kan)TOM-Green | $Kan^r$, TOM-Green | (Canada et al. 2002) |
| *E. coli* HB101 pRK2013 | Helper plasmid for mobilization of non-self-transmissible plasmids (Conjugation) | (Figurski and Helinski 1979) |
| *P. putida* S12 | Solvent resistant pump srpABC, Antibiotic resistant pump arpABC | ATCC # 700801 (Kieboom et al. 1998a) |
| *P. putida* S12 pBS(kan)TOM-Green | Solvent resistant pump srpABC, Antibiotic resistant pump arpABC, $Kan^r$, TOM-Green | This work |
| *E. coli* DH10B | Electrocompetent cells | Invitrogen |
| pBR322 | Low-copy number, $Amp^r$, $Tet^r$ | New England Biolabs |
| pJG001 | Low-copy number, derived from pBR322 by insertion of PCR amplified srpABC (cleaving $Tet^r$ gene) in reverse orientation, $Amp^r$ | This work |
| pUC19 | High-copy number, $Amp^r$ | New England Biolabs |
| pJG002 | High-copy number, derived from pUC19 by insertion of srpABC genes (cut from pJG001) in multiple cloning site, $Amp^r$ | This work |

* $Kan^r$ is Kanamycin resistance, $Amp^r$ is ampicillin resistance, $Tet^r$ is tetracycline resistance, Antibiotic resistance pump arpABC confers resistance to various antibiotics such as ampicillin, tetracycline, chloramphenicol etc. (Kieboom and de Bont 2001)

routinely grown at 37°C in LB medium supplemented with required antibiotics. All strains were grown in an incubator shaker at 200 rpm. The optical density measurements were done on Beckman UV spectrometer with sufficient dilutions at 660 nm to get the reading in its accuracy range of 0.1 to 0.5.

## Cell Dry Weight (CDW) measurement

Cell dry weight measurement was done by harvesting the cells from 20 mL culture by centrifugation (4000 xg, 10 min) in a 50 mL centrifuge tube. The cells in the tube were dried in a oven at 40°C overnight. The cells were scraped from the tube onto a filter paper and dried at 105°C until the weight of the cells was constant.

## Luria-Bertani (LB) Medium

Add 25 g LB (Luria-Bertani from Miller) broth containing 10 g tryptone, 5 g yeast extract and 10 g NaCl to 1 L deionized water. Dissolve the broth by mixing and autoclave the solution. After autoclaving, add 100 mg/L of the required antibiotics (ampicillin or kanamycin). For agar plates, add 10 g LB-Agar (form Miller) containing 2.5 g Tryptone, 1.25 g Yeast Extract, 2.5 g NaCl and 3.75 g Agar to 250 ml deionized water. Autoclave the solution and cool it to ~55°C. Add suitable amount of antibiotics and pour into the plates.

## Phosphate Buffer (PB)

800 mM phosphate buffer was prepared by adding 5.44 g of $KH_2PO_4$ and 6.96 g of $K_2HPO_4$ to 100 ml of deionized water. Adjust pH to 7.2 with KOH.

**Modified Hutner's Mineral Base Medium (MSB)**

Add 40 mL of Solution A, 10 mL of Solution B and 5 mL of Solution C to 945 mL deionised water and autoclave. A white precipitate will form but will redissolve after cooling. For agar plates (1 L), add the above amounts of solutions A, B and C to 445 mL $dH_2O$. Add 17 g Noble Agar to 500 mL $dH_2O$. Autoclave separately and mix the two solutions after autoclaving. Adjust pH to 7.2.

*Solution A:* Phosphate Buffer

Prepare 1 M $Na_2HPO_4$ (142 g/L) and 1 M $KH_2PO_4$ (136 g/L). Add 2.4 parts of 1 M $Na_2HPO_4$ per part of 1 M $KH_2PO_4$ to get a solution with pH 7.3. To make 500 mL Solution A with pH 7.3, Add 150 mL 1 M $KH_2PO_4$ and 350 ml 1 M $Na_2HPO_4$.

*Solution B:* Hutner's Concentrated Base (Mciver 2008)

Add all the components in specified quantities shown in Table A. 2 to 1 liter of deionised water.

*Solution C:* (20% Ammonium Sulfate)

Add 10 g $(NH_4)_2SO_4$ in 50 ml deionized water

Table A. 2. Solution B – Modified Hutner's Mineral Base (Mciver 2008)

| Component* | Amount |
|---|---|
| Nitrilotriacetic acid (NTA+free acid) | 20 g |
| $MgSO_4$ anhydrous | 28.9 g |
| $CaCl_2.2H_2O$ | 6.67 g |
| $(NH_4)_6Mo_7O_{24}.4H_2O$ | 18.5 mg |
| $FeSO_4.7H_2O$ | 198 mg |
| Metals 44 (see Table A.2) | 100 mL |
| $H_2O$ | To 1 L |

*Dissolve NTA separately in 600 mL of water and neutralize with KOH (14.6 g KOH); add other components and dissolve in order given. Adjust to pH 6.8 before making to final volume of 1 Liter. When pH is near 6.8, color of solution changes from deep yellow to straw color.

Table A. 3. Metals 44 - Hutner's Minerals (Mciver 2008)

| Component | Weight |
|---|---|
| EDTA (free acid) | 2.5 g |
| $ZnSO_4.7H_2O$ | 10.95 g |
| $FeSO_4.7H_2O$ | 5 g |
| $MnSO_4.H_2O$ | 1.54 g |
| $CuSO_4.5H_2O$ | 392 mg |
| $Co(NO_3)_2.6H_2O$ | 250 mg |
| $Na_2B_4O_7.10H_2O$ | 177 mg |
| $H_2O$ | Make to final volume of 1 L |
| Add few drops concentrated $H_2SO_4$ to retard precipitation | |

# APPENDIX B. CLONING

## Polymerase Chain Reaction (PCR)

PCR reactions were performed in 200 µL vials with a 10 µL, 50 µL or 100 µL volume of sample. Expand Long Template PCR System from Roche was used for the amplification. Genomic DNA fragment with srpABC genes and *srp* promoter was amplified by PCR using Expand Long Template PCR System from Roche. The primers used for PCR were 5'- CTG ATT GGT TCA TAT CTT TCC TCT G - 3' and 5'- CAT ATC TTT CTC ATT GTC CTG CAA G -3' which amplify a 6253 kb fragment from 55 bp to 6307 bp (GenBank Accession # AF029405 for 6590 bp sequence for srpABC (Kieboom et al. 1998a)). Figure B.1 shows the PCR method used.

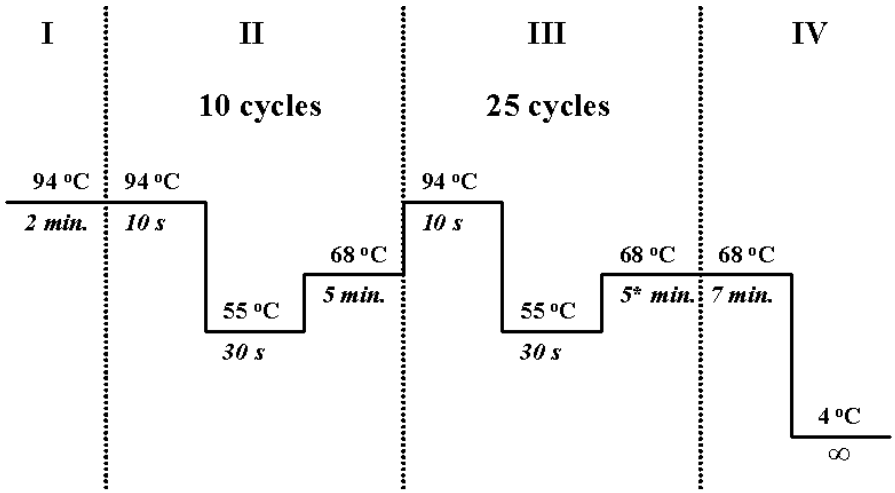


Figure B.1. PCR method for cloning srpABC along with *srp* promoter from genomic DNA of *P. putida* S12

DNA fragment with srpABC genes without *srp* promoter was cloned from pJG001 plasmid. The forward primer used was 5'-TCT AGA CTA CCG CAT TAC GAT TCA

G-3’ and the reverse primer was5’-GAA TTC ACA GCA TCG CCA GTC ACT A-3’. The srpABC genes were ligated into pBR322 in reverse alignment and the primers amplify the region from 4229 to 10036 in the reverse pJG001 DNA sequence. Figure B.2 shows the PCR method used for amplification.

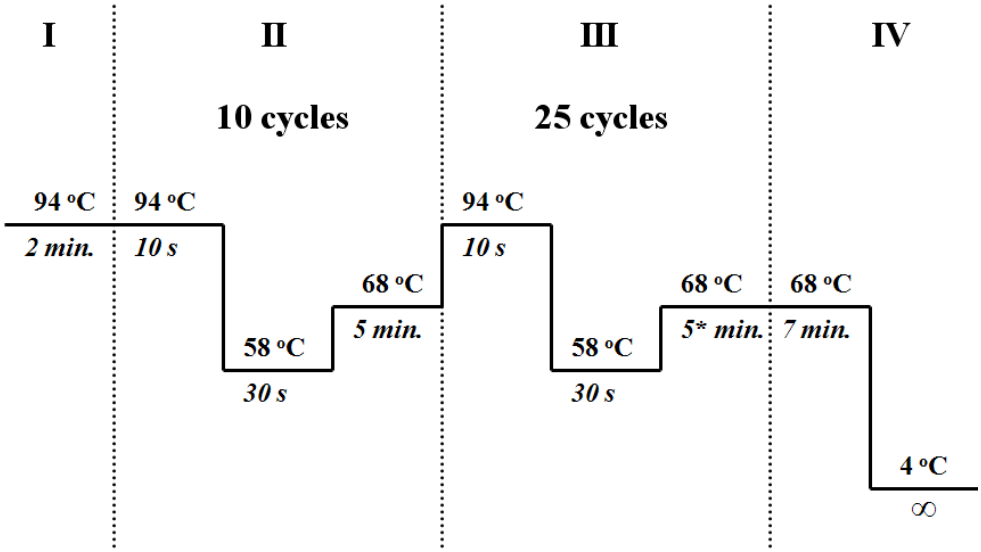


Figure B.2. PCR method for cloning srpABC without *srp* promoter from pJG001 plasmid

## DNA Gel Electrophoresis

*Preparation of 10x TBE buffer:* Add 108 g Tris Base, 55 g boric acid and 9.3 g $Na_4EDTA$ to 1 L deionized water.

*Preparation of 1% agarose gel:* 250 mg agarose was added to 25 mL TBE buffer and heated till agarose was completely dissolved. The liquid was then poured into the gel casting tray with comb (either 8 well or 14 well) and allowed to solidify in a refrigerator (~2 h).

*Preparation of 6x DNA loading buffer:* Add 1.2 g Ficoll 400, 1.2 ml 0.5 mM $Na_2EDTA$, 300 ul 20% SDS stock, 60 ul 0.5% bromphenol blue, 60 ul 0.5% xylene cyanol and water to a final volume of 10 ml.

*Electrophoresis Procedure:* After the gel solidifies, the comb was removed carefully. The gel was placed in the electrophoresis chamber with the wells towards the negative electrode (black) so that DNA migrates towards positive electrode (red). The gel was covered with TBE buffer and the DNA samples in 1X loading buffer were loaded carefully. The electrodes were connected to the power supply and a suitable method was used (80V & 45 min for ~6kb fragment). After electrophoresis, the gel was stained in 0.5 ug/mL Ethidium Bromide for about 20 min and DNA bands were viewed using UV transilluminator.

## Transformation by Conjugation

*P. putida* S12 (acceptor), *E. coli* TG1 pBS(kan)TOM-Green (donor), and *E. coli* HB101 pRK2013 (helper plasmid) were grown until early log phase. The three cultures were mixed to have acceptor to donor to helper plasmid ratio of 1 : 6 : 1 to get 25 ml media. The mixed culture was grown at 30$^{o}$C and 200 rpm for 6-8 hours and 2 ml of it was used to inoculate 25 ml of MSB supplemented with 100 µl styrene and 100 ppm kanamycin. *P. putida* S12 has styrene monooxygenase and can grow on styrene as sole carbon source. Therefore, the cells growing on MSB agar plates in styrene vapor were the transformants of *P. putida* S12 with pBS(kan)TOM-Green. The cells from MSB were plated on fresh LB agar plates supplemented with 100 ppm kanamycin.

## Other Engineered *E. coli* Strains

*E. coli pJG001* and *E. coli* pJG002 were identified (Chapter 4). Similar strains with srpABC genes along with *E. coli* pJG001 and pJG002 were also identified but not fully characterized. *E. coli* DH10B strains bearing plasmids which were derived from pUC19 and have srpABC genes similar to *E. coli* pJG002 are GJ38C, GJ38E and GJ38J named after my initials, lab notebook number and page number. Plasmid (pJG003) with srpABC genes without *srp* promoter downstream to T7 promoter was constructed and a transformant (GJ317E) with srpABC genes in reverse orientation to T7 promoter was isolated. In order to create a plasmid with srpABC genes downstream to T7 promoter, the plasmid from isolated transformant was double digested with XhoI and SacI, and pUC19 was double digested with SalI (compatible ends with XhoI) and SacI. The digested fragments were gel purified and ligated to construct pJG003 with srpABC genes in orientation with T7 promoter. The plasmid was transformed into *E. coli* using electroporation. However, no transformant with the required plasmid was identified.

## Transformation of *P. putida* S12 with NDO genes

*P. putida* S12 was transformed with pDTG141 plasmid (McIver et al. 2008) encoding genes for naphthalene dioxygenase (NDO) and ampicillin resistance using chemical treatment method. However, no transformants with pDTG141 were obtained because of native ampicillin resistance in *P. putida* S12 due to its antibiotic pump (Kieboom et al. 2001). The antibiotic pump in *P. putida* S12 confers resistance to various antibiotics. However, it doesn't confer resistance to kanamycin and was observed to stably express genes from a plasmid with kanamycin marker (*P. putida* S12 pBS(kan)TOM-Green). Therefore, plasmid pDTG141 was modified by inserting

kanamycin resistance gene from pBS(kan)TOM-Green plasmid. Plasmid pDTG141 was double digested with AhdI and DraI to isolate NDO genes. Plasmid pBS(kan)TOM-Green was double digested with AhdI and PsiI (compatible ends with DraI) to isolate its kanamycin resistance gene. The isolated fragments were gel purified and ligated using T4 ligase. The constructed plasmid with NDO and kanamycin resistance genes was transformed into electrocompetent *P. putida* S12 by electroporation. Electrocompetent *P. putida* S12 was prepared as suggested by Sambrook et al. (1989). 50 mL of cells with OD at 660 nm of ~1 were harvested by centrifugation and washed twice with 40 mL of ice cold 10% glycerol. Cells were kept on ice during the preparation. Cells were resuspended in 0.5 mL 10% glycerol and used for electroporation. However, no transformants with required plasmid were identified.

# APPENDIX C. HPLC ANALYSIS

High performance liquid chromatography (HPLC) was used to quantify 1-naphthol and naphthalene. An 1100 Series Agilent HPLC with a PDA detector and a supelcosil LC-PAH 5µm column (25cm x 4.6mm) at room temperature was used for the analysis. Aqueous samples were diluted (1/2) in acetonitrile. Organic samples were directly injected without dilution. Both organic and aqueous samples were centrifuged to separate cell debris and were filtered with 0.2 µm PTFE filters. The calibration curves for 1-naphthol and naphthalene are shown in Figure C.1.

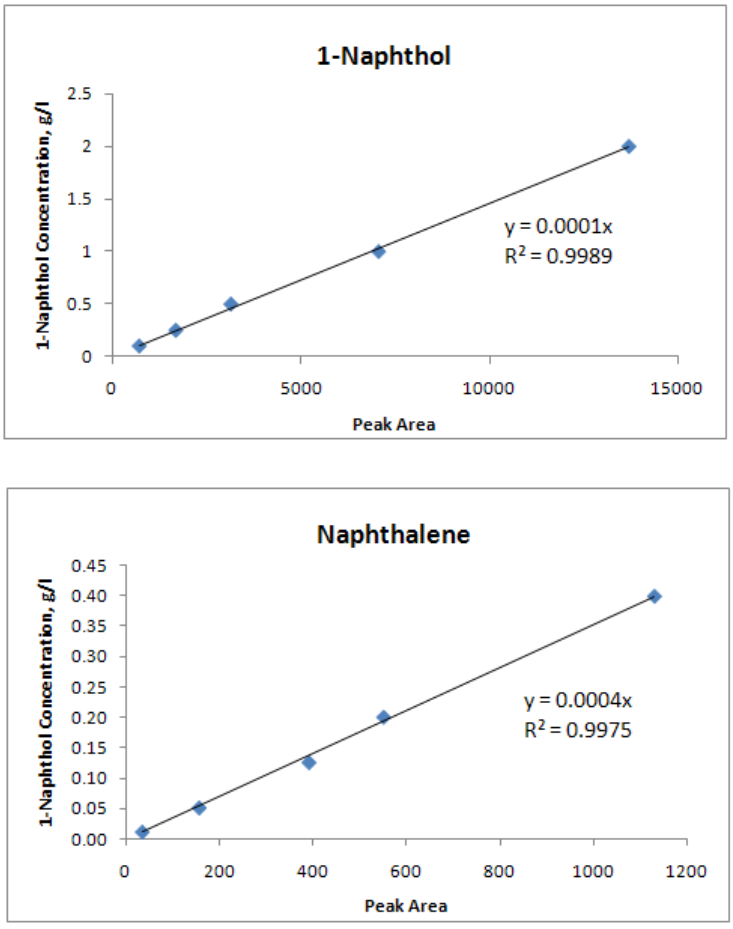


Figure C.1. Calibration curves for 1-naphthol and naphthalene

The mobile phase consisted of deionized water with 0.2% (v/v) glacial acetic acid and optima grade acetonitrile with a gradient elution. The gradient for water / acetonitrile was 65/35 (v/v) at t = 0 min; linear gradient to 100/0 at t = 7 min; linear gradient to 65/35 at t = 8 min; equilibration to t = 10 min. The mobile phase flow rate was 1.5 ml/min and the injection volume was 10 µl. Naphthalene and 1-naphthol were analyzed at 272 nm and detected at 5.7 and 3.9 minutes of retention time respectively. The integrated areas of the elution peaks were used to calculate the concentration of naphthalene and 1-naphthol in each phase.

# APPENDIX D. APPLICATION OF RESIN

Amberlite XAD7HP and Dowex Optipore L493 adsorbents (obtained from Sigma-Aldrich) were used in preliminary experiments for 1-naphthol production (Mciver 2008). The XAD 7HP adsorbent has acrylic ester matrix with surface area of 450 $m^2/g$. The L 493 resin has styrene-DVB matrix with surface area of 1100 $m^2/g$. Biotransformations were performed in 20 ml screw-cap vials with 5 ml working volume. *E. coli* pBS(kan)TOM-Green cells were grown till late log phase and cell were harvested by centrifugation, washed and resuspended in half the volume of phosphate buffer. Two different amounts of the two resins, 100 and 200 mg, were added to 3 ml of resuspended culture. Lauryl acetate was used as the solvent with 40% organic phase ratio and 60 g/l naphthalene. Lauryl acetate with naphthalene was added to aqueous phase and the reaction was performed for 24 h. After 24 h, the resin was separated by filtration, and the organic and aqueous phases were analyzed by HPLC. 1-Naphthol was extracted from the resin by suspending it in 10 ml methanol and shaking overnight on an orbital shaker. The extracted 1-naphthol in methanol was analyzed by HPLC. The amount of 1-naphthol in organic phase and methanol is shown in Figure D.1. The control in Figure D.1 represents data for biotransformation where no adsorbent was added. The results do not follow any particular trend and the total amount of 1-naphthol produced is similar for all the conditions. The color of the resin suspended in aqueous phase was changed to brown which could be because of 1-naphthol adsorption. However, most of the resin used in biotransformations was settled at the water-organic solvent interface and there was no color change indicating that it didn't adsorb 1-naphthol.

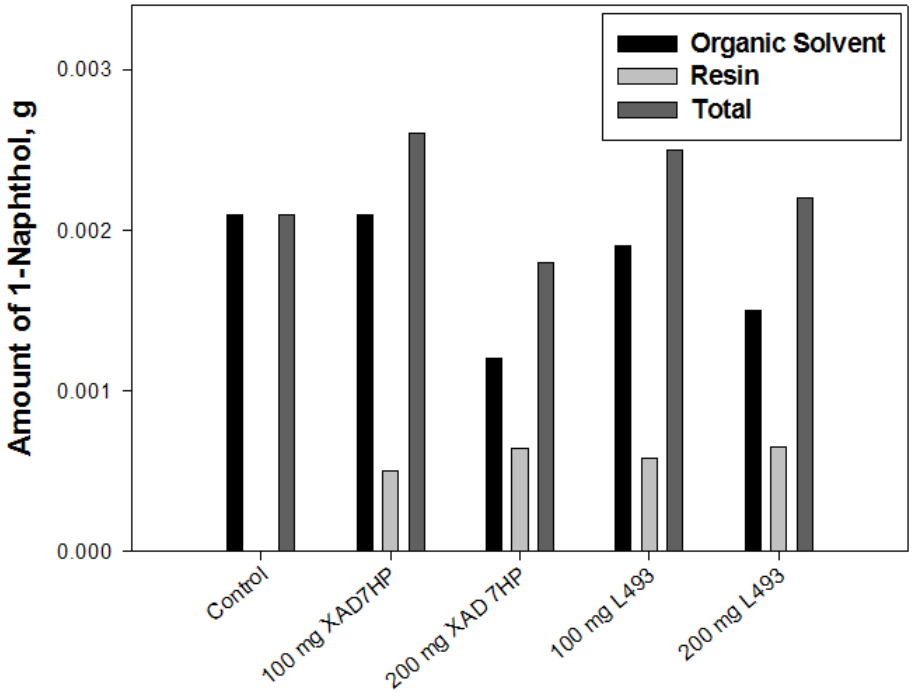


Figure D.1. 1-Naphthol formation in biphasic system with different resins

# APPENDIX E. PRODUCTION OF (+)-(1R,2S)-*cis*-1,2-NAPHTHALENE DIHYDRODIOL (NDHD)

Recombinant *E. coli* pDTG141 strain expressing naphthalene dioxygenase (NDO) was grown until OD at 660nm reaches ~1 and 40% organic solvent containing 40g/l naphthalene was added. Four different organic solvents were used. NDHD slightly partitions into decanol, dioctyl phthalate and lauryl acetate, but it doesn't partition into dodecane phase. The total NDHD formed in both phases is shown in the Figure E.1. Best results were obtained using dodecane (McIver et al. 2008).

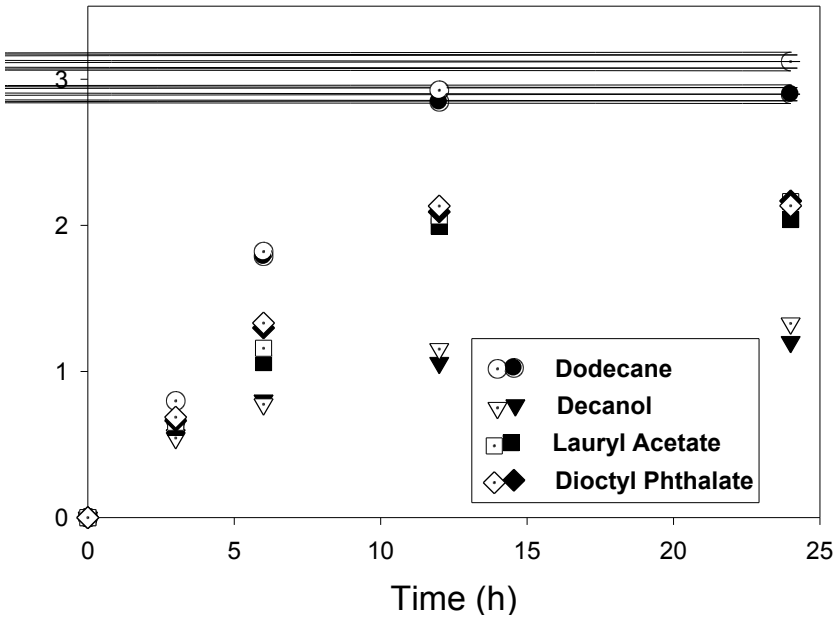


Figure E.1. Production of (+)-(1R,2S)-*cis*-1,2-naphthalene dihydrodiol in biphasic system

*E. coli* pDTG141 cell viability was analyzed using Baclight live and Dead assay kit from molecular probes and Flow Cytometry. Baclight live/dead assay kit has two dyes, propidium iodide (PI) which is impermeable to cell membrane, and Syto-9 which is permeable to cell membrane. Live cells are stained by only Syto-9 dye, and dead cells are stained mainly by PI. However, there is a population which are stained by both dyes and represents cells which are uncharacterized in terms of viability. Cells were grown till OD

1 and four different solvents were added. Cells incubated with solvents for 3 h at 37 $^{o}$C and 200 rpm. Flow cytometry results are shown in Figure E.2. Cells had good viability in solvents dodecane, dioctyl phthalate, and lauryl acetate but had only 47% viability in decanol due to its toxicity.

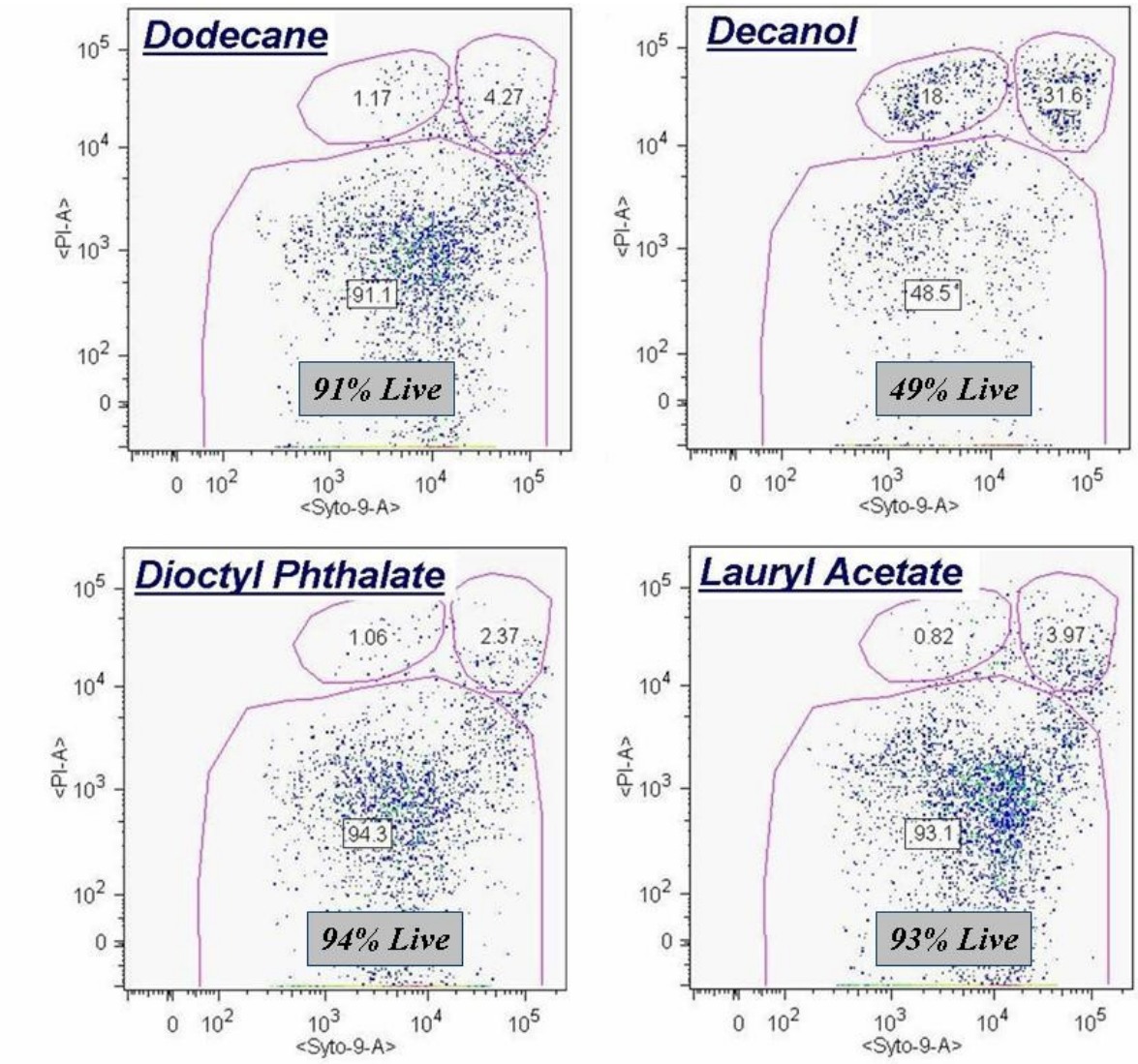


Figure E.2. Flow cytometry results for viability of *E. coli* pDTG141 in presence of 40% of different organic solvents after 3 h

# APPENDIX F. PRESENTATIONS AND PUBLICATIONS

## Conference Presentations

*CBB Annual Conference 2008, Iowa City, IA.* "Whole-cell Biocatalysis in Biphasic Media for Oxidation of Naphthalene".

*AIChE/ACS Spring National Meeting 2008, New Orleans, LA.* "Whole-cell Biocatalysis for Oxidation of Naphthalene in Biphasic Media".

*CBB Annual Conference 2007, Iowa City, IA.* "Whole-cell Biocatalysis in Biphasic Media".

*AIChE Conference 2006, San Francisco, CA.* "Solvent tolerant microbial system for biocatalysis".

*CBB Annual conference 2005, Iowa City, IA*. "Chemo-enzymatic Baeyer-Villiger oxidation to produce racemic lactones".

## Publications

McIver, A.; Garikipati, J.; Bankole, K. S.; Gyamerah, M.; Peeples, T. Oxidation of Naphthalene to *cis*-1,2-Naphthalene Dihydrodiol using Naphthalene Dioxygenase in Biphasic Media. *Biotechnol. Prog.,* 2008, 24 (3): 593-598.

Garikipati, J.; McIver, A.; Peeples, T. Oxidation of Naphthalene to 1-Naphthol using Biocatalysis in Biphasic Media. Submitted to *Applied and Environmental Microbiology.*

Garikipati, J.; Peeples, T. Solvent Tolerant *Pseudomonas putida* S12 strain for Oxidation of Naphthalene in Biphasic Media. Submission in Progress.

Garikipati, J.; Peeples, T. Engineering Solvent Tolerance into *Escherichia coli* by expression of srpABC genes from *Pseudomonas putida* S12. Submission in Progress.