Solvent Resistance Pumps of *Pseudomonas putida* S12: applications in 1-Naphthol production and biocatalyst engineering

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Abstract

The solvent resistance capacity of *Pseudomonas putida* S12 was applied by using the organism as a host for biocatalysis and through cloning and expressing solvent resistant pump genes into *Escherichia coli*. *P. putida* S12 expressing toluene ortho monooxygenase (TOM-Green) was used for 1-naphthol production in a water-organic solvent biphasic system. Application of *P. putida* S12 improved 1-naphthol production per gram cell dry weight by approximately 42% compared to *E. coli*. Moreover, *P. putida* S12 enabled the use of a less expensive solvent, decanol, for 1-naphthol production. The solvent resistant pump (srpABC) genes of *P. putida* S12 were cloned into a solvent sensitive *E. coli* strain to transfer solvent tolerance. Recombinant strains bearing srpABC genes in either a low-copy number or a high-copy number plasmid grew in the presence of saturated concentration of toluene. Both of the recombinant strains were more tolerant to 1% v/v of toxic solvents, decanol and hexane, reaching similar cell density as the no-solvent control. Reverse-transcriptase analysis revealed that the srpABC genes were transcribed in engineered strains. The results demonstrate successful transfer of the proton-dependent solvent resistance mechanism and suggest that the engineered strain could serve as more robust biocatalysts in media with organic solvents.

Keywords:

solvent resistant pump (srp), srpABC, *P. putida* S12, solvent tolerance

Abbreviations

acrAB	gene encoding multidrug efflux pump
arpABC	gene encoding multidrug efflux pump
CDW	cell dry weight
DMF	dimethyl formamide
OD ₆₆₀	optical density measured at 660 nm
LB	Luria-Bertani medium
MexAB-OprM,	gene encoding multi-drug efflux pump from <i>Pseudomonas aeruginosa</i>
logP _{ow}	logarithm of partition coefficient in octanol-water system
marA	gene encoding multiple antibiotic resistance transcriptional activator
ostA	organic solvent tolerance gene
PB	phosphate buffer
pBS(kan)TOM-Green	plasmid bearing Toluene ortho monooxygenase gene and kanamycin resistance
pJG001	low copy number plasmid with srpABC
pJG002	high copy number plasmid with srpABC
RND	resistance-nodulation-division family of genes
robA	gene encoding global regulatory protein
soxS	gene encoding controller the superoxide response regulon
srpABC	gene encoding solvent resistance pumps
srpA	gene encoding solvent resistance periplasmic linker
srpB	gene encoding solvent resistance inner membrane transporter
srpC	gene encoding solvent resistance outer membrane channel
TBE	Tris-Borate-EDTA buffer

1. 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; Daugulis, 1997; Endoma et al., 2002; 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). Production of various compounds, such as 3-methyl catechol (Husken et al., 2001; Prpich and Daugulis, 2007), 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), amorphous-4,11-diene (Newman et al., 2006), have been enhanced using biphasic reactions.

Although biphasic systems have various advantages, there are drawbacks associated with organic solvent toxicity (Leon et al., 1998, Inoue, 1991). The toxicity of a solvent is generally correlated with the logarithm of its partition coefficient in an octanol-water system ($\log P_{ow}$) (Laane et al.; Leon et al., 1998). The critical $\log P_{ow}$ value above which a solvent is toxic to a bacterium ranges from 3.1 to 7 depending on the bacterial strain (Inoue and Horikoshi, 1991). Toxic organic solvents have damaging effects on microorganisms by impairing vital functions, by facilitating loss of ions, metabolites, lipids, and proteins, and by inhibiting membrane protein functions (Bruce and Daugulis, 1991; Leon et al., 1998). Therefore, a solvent tolerant microorganism should be employed to fully realize the potential of whole-cell biocatalysis in biphasic environments (Heipieper et al., 2007).

Solvent tolerant microorganisms have adaptive mechanisms to survive in the presence of toxic organic solvents (de Bont, 1998; Dunlop et al., 2011; Harrison and Dunlop, 2012; Isken and de Bont, 1998; Segura et al., 1999; Weber and deBont, 1996; Wery et al., 2001). Extensive physiological studies of solvent tolerance in *Pseudomonas putida* S12 have demonstrated that this organism is able to tolerate saturated concentrations of organic solvents because of two adaptive mechanisms: *cis*- to *trans*-unsaturated fatty acids in the outer membrane making the membrane more rigid (von Wallbrunn et al., 2003) and extrusion of the solvent from the cell membrane facilitated by an efflux pump (Kieboom et al., 1998a). Energy-dependent efflux systems which selectively pump organic solvents out of the cell membrane (Isken and DeBont, 1996; Kieboom et al., 1998a; Ramos et al., 2002; Rojas et al., 2001; Rojas

et al., 2003) have been identified in a variety of organisms. Kieboom et al.(1998a) have identified and characterized the solvent resistant pump *srpABC* encoding a proton-dependent multidrug efflux pump responsible for organic solvent tolerance in *P. putida* S12. The *srpABC* pump belongs to RND (resistance-nodulation-division) family (Moreira et al., 2004; Nikaido, 1996; Putman et al., 2000) of pumps which is composed of three protein components, an inner membrane transporter (*srpB*), an outer membrane channel (*srpC*) and a periplasmic linker (*srpA*) (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).

Recently Dunlop et al (2011) have reported the application of heterologously expressed RND efflux pumps to enhance solvent-tolerance in microbial strains for biofuel application (Dunlop, 2011; Dunlop et al., 2011). Although a variety of alcohols induce expression of the pumps, this induction does not result in increased tolerance in all cases. The variability in tolerance has been attributed to varying abilities to export alcohols. The pumps appear to be less effective at exporting short-chain alcohols (Dunlop, 2011), and hence, strains modified with efflux pumps may show reduced solvent tolerance upon heterologous expression. These findings suggest that for strain development and optimization, the specific solvent systems should be tested to identify the suitability of the engineered system for new applications. Here we report the effectiveness of the *P. putida* S12 and its efflux pumps in the presence of solvents in biphasic systems useful for naphthol production.

The production of chemicals which can be toxic to microbes is often achieved by the application of microbes in biphasic systems (Heipieper et al., 2007; Nijkamp et al., 2005; Rojas et al., 2004; Wery et al., 2000). Specifically *P. putida* S12 has been applied to enhance the production of cinnamic acid (Nijkamp et al., 2005), 3-methyl catechol (Wery et al., 2000) and phenol (Wierckx et al., 2005). Another alcohol product, 1-naphthol, has wide applications in the manufacture of dyes, drugs, insecticides, perfumes and surfactants (Back, 1965; Canada et al., 2002). The toxicity of this alcohol makes it an interesting model compound for tolerance studies. Other researchers have applied various wild-type

and modified monooxygenases to the production of alcohols as chemical synthons (Tao et al., 2005; Whited et al., 1994). The toluene *ortho*-monooxygenase (TOM) variant TmoA3 V106A, also known as TOM-Green, was generated by mutational activity on a native monooxygenase and has been demonstrated to work effectively in 1-naphthol production (Rui et al., 2004). *Escherichia coli* TG1 pBS(kan)TOM-Green strain expressing TOM-Green constitutively was previously used 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/g CDW 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. Based on these studies, *P. putida* S12 would be expected to be a beneficial host for 1-naphthol production making the recombinant system more resilient to the high toxicity of naphthalene and 1-naphthol. Therefore, in this work, we evaluated the use of *P. putida* S12 as a host strain for production of 1-naphthol in biphasic systems.

Although *P. putida* S12 has various advantages as a stable host for biocatalysis, it has some drawbacks which may limit its application. *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. *P. putida* S12 strain also has a multidrug efflux 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). One strategy for biocatalysis is the transfer of solvent tolerance mechanisms to a better-characterized production strain such as *E. coli*.

Genetic modifications to solvent-sensitive *E. coli* strains for solvent tolerance is an active area of research to identify strains that would be useful for a variety of industrial processes (Aono, 1998; Aono et al., 1994; Asako et al., 1997; White et al., 1997; Dunlop 2011, Rojas 2001, Zeng 2012). Overexpression of transcriptional activators, such as *marA* producing multiple antibiotic resistance phenotype (Asako et

al., 1997), *robA* encoding global regulatory protein, and *soxS* 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 levels of expression 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 conferring solvent tolerance to *E. coli* (Nikaido, 1996). Similar work was done by Aono et al.(1994) where an uncharacterized organic solvent tolerance gene *ostA* from a hexane tolerant *E. coli* was overexpressed 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 the solvent resistant pump *srpABC* from *P. putida* S12, has only recently been reported. Kieboom et al.(1998a) showed 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, *srpABC* gene expression is of interest in improving solvent tolerance in *E. coli*, which might result in a better host for biocatalysis. This avenue is being explored in biocatalyst development for fuels production (Dunlop et al., 2011; Harrison and Dunlop, 2012). Here, we report that *srpABC* genes were cloned and inserted into *E. coli* to transfer solvent tolerance to media suitable for naphthol conversion.

2. Materials and Methods

2.1. Chemicals.

Dodecane, toluene, decanol, naphthalene and 1-naphthol 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). All other chemicals and reagents were also commercially available.

2.2. Bacterial Strains.

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). The strain expresses TOM-Green constitutively and carries kanamycin resistance genes in its plasmid. *Pseudomonas putida* S12 (ATCC 700801) was purchased from ATCC, Manassas, VA, USA (Kieboom et al., 1998a). *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°C and 200 rpm. *P. putida* S12 pBS(kan)TOM-Green cultures were routinely grown in LB medium supplemented with 100 mg/L kanamycin and 3 mM 1-hexanol at 30°C and 200 rpm. 1-Hexanol was added to the medium to induce solvent tolerant genes in *P. putida* S12 (Kieboom et al., 1998b).

2.3. Naphthalene and 1-Naphthol Toxicity Experiments.

Shake flask cultures were grown until early log-phase (approximately 2 hours post inoculation). 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 dimethyl formamide (DMF) were added to growing cells. Growth was monitored by optical density at 660 nm (OD_{660}).

2.4. Biphaseic Biotransformations.

The experimental procedure for biotransformation has been previously described (Garikipati et al., 2009). All biotransformations were conducted in 250 ml Erlenmeyer flasks with 50 ml working volume at 30°C and 200 rpm. Fresh LB medium supplemented with 100 mg/L kanamycin 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 ($OD_{660} \sim 1.6$) 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 ($\sim 10,000$ g), washed with phosphate buffer (PB) at pH 7.2 and

resuspended in 50% volume of PB to increase cell density. The biotransformation buffer 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 containing 60 g/L dissolved naphthalene was added to 30 mL resuspended cells for a 40% organic phase ratio in a 250 mL Erlenmeyer flask. Biotransformation was performed at 30°C, and both aqueous and organic phase samples were taken at 3, 6, 24 and 48h for HPLC analysis (Garikipati et al., 2009). Data were generated from three identical experiments.

2.5. Construction of plasmids with *srpABC* genes.

The procedure for amplifying *srpABC* genes is shown in Figure 1. Total genomic DNA of *Pseudomonas putida* S12 was isolated using Qiagen DNAeasy tissue kit. The genomic DNA fragment with *srpABC* genes and the *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 no. [AF029405](#) for 6590 bp sequence for *srpABC* (Kieboom et al., 1998a)). The PCR fragment and pBR322 plasmid were digested with *NheI* enzyme and ligated using T4 ligase to construct pJG001. The inserted fragment cleaves tetracycline resistance gene in pBR322 plasmid. Plasmid pJG001 was cut with *EcoRI* and *PshAI* to isolate *srpABC* genes with the *srp* promoter. Plasmid pUC19 was digested with *EcoRI* and *HincII* in the multiple cloning site and the digested pJG001 fragment was ligated into pUC19 to construct pJG002. 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). Other molecular procedures including 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 kit or reagent supplier.

2.6. Transformation of *E. coli* with *srp* genes and Selection.

The plasmids were transformed into Electromax DH10B *E. coli* cells (Invitrogen) using electroporation. Transformation 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.

2.7. 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 660 nm of 0.25 ($\sim 2.5 \times 10^8$ 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 1. The Qiagen one-step PCR kit was used to amplify DNA fragments from isolated RNA.

2.8. Solvent Toxicity Experiments.

The toxicity of five different solvents (dodecane, octane, hexane, decanol and toluene) was tested on the engineered strains. For each solvent tested, the cells were grown until early log-phase, and solvent was added to 5 mL of growing cells in a 20 ml screw cap vial. The volume of solvent added resulted in a 1% v/v of organic solvent relative to aqueous medium. Optical density of the aqueous phase was measured at 660 nm using UV-Vis spectrophotometer to monitor the growth of the cells.

3. Results

3.1. *P. putida* S12 Tolerance and Biotransformations.

Tolerance of *P. putida* S12 pBS(Kan)TOM-Green to naphthalene and 1-naphthol was analyzed by adding different concentrations of either naphthalene or 1-naphthol to growing cells of *P. putida* S12. Cell growth was monitored by measuring optical density (OD) at 660 nm, as shown in Figure 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. Inhibition of growth by 1-naphthol was higher than naphthalene, and growth was inhibited for 1-naphthol concentration of 0.05 g/L or higher. At 0.5 g/L or higher 1-naphthol concentration, growth ceased within 3h of 1-naphthol addition.

P. putida S12 pBS(kan)TOM-Green strain was used for 1-naphthol production in a biphasic system. The solvent-buffer system for *P. putida* S12 had a 40% organic phase ratio with 60 g/l naphthalene dissolved in the organic phase. Solvents evaluated included lauryl acetate and decanol. Both the solvents have high distribution coefficients for 1-naphthol, as shown in Table 2. Figure 3 shows 1-naphthol concentration in the organic phase and 1-naphthol productivity based on cell dry weight versus time for both *P. putida* S12 and *E. coli* strains. The results shown are an average of three identical experiments. Biotransformation with *E. coli* TG1 using lauryl acetate was done earlier (Garikipati et al., 2009) and is shown here for comparison.

The comparison of naphthalene conversions and amount of 1-naphthol obtained for the two strains is shown in Table 3. 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* TG1 strain produced less than 0.01 g/L 1-naphthol when decanol was used. Cell density after three hours of biotransformation was measured for both solvents. Percent cell density of *E. coli* and *P. putida* S12 strains in both the solvents is shown in Figure 4. 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. *P. putida* S12 and *E. coli* biotransformations retained most of their cell density when lauryl acetate was used as the organic solvent. However, *E. coli* biotransformations lost

about 80% of cell density compared to only 20% reduction in cell density for *P. putida* S12 when decanol was used as the organic solvent.

3.2. Insertion of *srpABC* genes into *E. coli*.

The solvent resistant pump genes (*srpABC*) responsible for solvent tolerance in *P. putida* S12 were amplified by PCR. The membrane proteins encoded by *srpABC* genes could be toxic to the microorganisms (Miroux and Walker, 1996; Srikumar et al., 1998) and therefore, *srpABC* genes were inserted into two plasmids, a low-copy number plasmid pBR322 and a high-copy number plasmid pUC19, as shown in Figure 1b. Plasmids pJG001 (*srpABC* in pBR322) and pJG002 (*srpABC* in pUC19) were constructed and transformed into *E. coli* DH10B cells by electroporation. Positive transformants were selected based on antibiotic markers, plasmids isolated and insertion of *srpABC* genes was confirmed by restriction analysis and DNA sequencing.

3.3. Reverse Transcriptase (RT) - PCR Analysis.

RT-PCR analysis was performed to investigate the expression of *srpABC* genes. Cells were grown in presence or absence of 1% v/v hexane until late log phase and RNA was isolated using Qiagen RNeasy Protect Bacteria Mini kit. Primers were designed to amplify about 1 kb fragment from each of *srpABC* genes. A negative control, *E. coli* pUC19, and a positive control, *P. putida* S12 grown in presence of 1% v/v hexane, were used for comparison. The agarose gel pictures of amplified products for *srpA*, *srpB* and *srpC* genes are shown in Figure 5. The negative control does not show amplified product bands as it does not 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. 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. The expression of *srpABC* genes in the engineered *E. coli* strains was constitutive due to the absence of regulatory proteins *srpRS* that control expression from *srp* promoter (Wery et al. 2001).

3.4. Solvent Tolerance

The solvent tolerance of *E. coli* strains with pJG001 and pJG002 was tested by growing the strains in saturated concentrations of toluene (Kieboom et al., 1998a). The engineered strains grew on LB agar plates with saturated vapor of toluene and in LB liquid medium with saturated concentration of toluene, which is 6.2 mM (Kieboom et al., 1998b)). However, the negative control *E. coli* strain with pUC19 plasmid did not grow either on LB agar plates or in LB liquid medium under toluene saturation. Therefore, *E. coli* cells transformed with *srpABC* genes were more tolerant to toluene compared to the control strain. 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 6.

Growth of all *E. coli* strains was not inhibited in presence of solvents dodecane or octane. Growth of the control strain *E. coli* pUC19 was inhibited in the presence of solvents decanol, hexanol or toluene. The growth of *E. coli* strains with pJG001 or pJG002 was inhibited during the first 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 grown without solvent addition. 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.

4. Discussion

The tolerance of *P. putida* S12 pBS(Kan)TOM-Green to either naphthalene or 1-naphthol was investigated. 1-Naphthol was more toxic to cell growth compared to naphthalene, which is similar to earlier studies done on *E. coli* TG1 pBS(kan)TOM-Green strain (Garikipati et al., 2009; McIver et al.,

2008). However, the *P. putida* S12 pBS(Kan)TOM-Green strain showed more tolerance to naphthalene where its growth recovered after 4h exposure to 1 g/L naphthalene but *E. coli* TG1 pBS(kan)TOM-Green did not grow at 1 g/L naphthalene. The toxicity of 1-naphthol was comparable for both the strains. Higher tolerance to substrate naphthalene will make *P. putida* S12 pBS(Kan)TOM-Green a better biocatalyst for oxidation of naphthalene. One stabilizing factor may be that the *P. putida* S12 solvent resistant efflux pump *srpABC* which could give the host more tolerance to these toxic compounds. However, toxicity of naphthalene and 1-naphthol is not only due to the impairment of cell membrane and its properties, but also to metabolites such as 1,2-naphthoquinone and other unstable oxidized products (Pumphrey and Madsen, 2007; Wilson et al., 1996). A biphasic system should be employed to maintain low concentrations of these compounds in the aqueous phase.

P. putida S12 pBS(kan)TOM-Green strain was used for 1-naphthol production in a biphasic system. Lauryl acetate was earlier shown to be a good solvent for 1-naphthol production, and the optimum conditions were a 40% organic phase ratio and 60 g/l naphthalene (Garikipati et al., 2009). 1-Naphthol concentrations and productivities were much higher for the *P. putida* S12 strain compared to the *E. coli* TG1 strain when lauryl acetate was used, which could be because of the organism's higher stability in toxic environments. 1-Naphthol productivity was improved by approximately 42% by using the *P. putida* S12 instead of the *E. coli* TG1 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* TG1 after 48 h at 40% organic phase ratio and 60 g/L dissolved naphthalene).

When decanol was used in the biphasic system, *P. putida* S12 produced a similar amount of 1-naphthol compared to lauryl acetate. In contrast, *E. coli* TG1 strain produced less than 0.01 g/L 1-naphthol with a decanol solvent phase. 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, and percent cell density was evaluated. Lauryl acetate has $\log P_{ow}$ of 6.6 and is generally not toxic to the cells. However, decanol has $\log P_{ow}$ of 4, and the *E. coli* biotransformations lost most of their cell density when decanol was used. The *P. putida* S12 biotransformations retained most of the specific

cell density in decanol owing to the solvent tolerance of the strain. Hence, 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 obtain similar productivities.

Solvent resistant pump genes *srpABC* were transferred into *E. coli* strain either on a low-copy or high-copy number plasmid, pJG001 or pJG002 respectively. The growth of the engineered strains was tested in the presence of various solvents. Solvents dodecane and octane have $\log P_{ow}$ values greater than 4 and are generally not toxic to microorganisms (Laane et al., 1987; Leon et al., 1998). Therefore, the growth of wild-type and engineered *E. coli* strains was not inhibited in presence of these solvents. However, solvents decanol, hexane, and toluene are toxic to microorganisms. The $\log P_{ow}$ value decreases and the toxicity increases for the solvents from decanol to hexane to toluene. Therefore, the growth of the control strain, *E. coli* bearing pUC19 plasmid, was inhibited in presence of these solvents. However, the growth of engineered strains with *srpABC* genes had similar cell density as the control without any solvent, with longer lag phase. The presence of organic solvents leads to ineffective metabolism due to the uncoupling effects of the solvent and lead 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 low cell density at 6 h in the presence of decanol and hexane. However, the *srpABC* pump helps the cells to adapt in the presence of these solvents and hence, the cell density of both the strains increased after 6 h and reached levels similar to the control at 18.5 h. Among the two engineered *E. coli* strains, *E. coli* with pJG002 showed 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 these strains with *P. putida* S12 strain,

which grows in presence of 1% v/v of toluene (Weber et al., 1993), *E. coli* strains 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* strain increases solvent influx compromising the effect of solvent efflux pump. Multiple tolerance mechanisms can give synergistic effects in improving tolerance of a microorganism (Dunlop et al., 2011) which makes *P. putida* S12 more tolerant to toxic organic solvents.

5. 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*. *P. putida* S12 was transformed with pBS(kan)TOM-Green plasmid and was used to improve 1-naphthol production by approximately 42%. Moreover, 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 can be used instead of lauryl acetate to get similar productivities. The results demonstrate the potential of *P. putida* S12 to improve the productivity and economics of 1-naphthol production.

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 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 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.

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References

- Aono, R., (1998) Improvement of organic solvent tolerance level of *Escherichia coli* by overexpression of stress-responsive genes. *Extremophiles* 2, 239-248.
- Aono, R., Negishi, T., Nakajima, H., (1994) Cloning of Organic-Solvent Tolerance Gene Osta That Determines N-Hexane Tolerance Level in *Escherichia-Coli*. *Applied and Environmental Microbiology* 60, 4624-4626.
- Asako, H., Nakajima, H., Kobayashi, K., Kobayashi, M., Aono, R., (1997) Organic solvent tolerance and antibiotic resistance increased by overexpression of *marA* in *Escherichia coli*. 63, 1428-1433.
- Back, R., (1965) Carbamate Insecticides, Significant Development sin Eight Years with Sevin Insecticides. *Journal of Agricultural and Food Chemistry* 13, 198-199.
- Bruce, L.J., Daugulis, A.J., (1991) Solvent Selection-Strategies for Extractive Biocatalysis. *Biotechnology Progress* 7, 116-124.

- Canada, K.A., Iwashita, S., Shim, H., Wood, T.K., (2002) Directed evolution of toluene ortho-monooxygenase for enhanced 1-naphthol synthesis and chlorinated ethene degradation. *Journal of Bacteriology* 184, 344-349.
- Collins, L.D., Daugulis, A.J., (1999) Benzene/toluene/p-xylene degradation. Part I. Solvent selection and toluene degradation in a two-phase partitioning bioreactor. 52, 354-359.
- Daugulis, A.J., (1997) Partitioning bioreactors. 8, 169-174.
- de Bont, J.A.M., (1998) Solvent-tolerant bacteria in biocatalysis. *Trends in Biotechnology* 16, 493-499.
- Dunlop, M.J., (2011) Engineering microbes for tolerance to next-generation biofuels. *Biotechnology for Biofuels* 4, 1-9.
- Dunlop, M.J., Dossani, Z.Y., Szmidt, H.L., Chu, H.C., Lee, T.S., Keasling, J.D., Hadi, M.Z., Mukhopadhyay, A., (2011) Engineering microbial biofuel tolerance and export using efflux pumps. *Molecular Systems Biology* 7, 487-487.
- Eaton, R.W., Chapman, P.J., (1995) Formation of indigo and related-compounds from indolecarboxylic acids by aromatic acid-degrading bacteria - Chromogenic reactions from cloning genes encoding dioxygenases that act on aromatic-acids. *Journal of Bacteriology* 177, 6983-6988.
- Endoma, M.A., Bui, V.P., Hansen, J., Hudlicky, T., (2002) Medium-scale preparation of useful metabolites of aromatic compounds via whole-cell fermentation with recombinant organisms. *Organic Process Research & Development* 6, 525-532.

- Garikipati, S.V.B.J., McIver, A.M., Peeples, T.L., (2009) Whole-cell Biocatalysis in Biphasic Media for 1-Naphthol Production. *Applied and Environmental Microbiology* 75, 6545-6552
- Harrison, M.E., Dunlop, M.J., (2012) Synthetic feedback loop model for increasing microbial biofuel production using a biosensor. *Frontiers in microbiology* 3, 360-360.
- Harrop, A.J., Woodley, J.M., Lilly, M.D., (1992) Production of Naphthalene-Cis-Glycol by *Pseudomonas-Putida* in the Presence of Organic-Solvents. *Enzyme and Microbial Technology* 14, 725-730.
- Heipieper, H.J., Neumann, G., Cornelissen, S., Meinhardt, F., (2007) Solvent-tolerant bacteria for biotransformations in two-phase fermentation systems. *Applied Microbiology and Biotechnology* 74, 961-973.
- Heipieper, H.J., Weber, F.J., Sikkema, J., Keweloh, H., Debont, J.A.M., (1994) Mechanisms of Resistance of Whole Cells to Toxic Organic-Solvents. *Trends in Biotechnology* 12, 409-415.
- Husken, L.E., Beeftink, R., de Bont, J.A.M., Wery, J., (2001) High-rate 3-methylcatechol production in *Pseudomonas putida* strains by means of a novel expression system. *Applied Microbiology and Biotechnology* 55, 571-577.
- Inoue, A., Horikoshi, K., (1991) Estimation of Solvent-Tolerance of Bacteria by the Solvent Parameter Log-P. *71*, 194-196.

- Isken, S., de Bont, J.A.M., (1998) Bacteria tolerant to organic solvents. *Extremophiles* 2, 229-238.
- Isken, S., De Bont, J.A.M., (2000) The solvent efflux system of *Pseudomonas putida* S12 is not involved in antibiotic resistance. *Applied Microbiology and Biotechnology* 54, 711-714.
- Isken, S., DeBont, J.A.M., (1996) Active efflux of toluene in a solvent-resistant bacterium. *Journal of Bacteriology* 178, 6056-6058.
- Isken, S., Derks, A., Wolffs, P.F.G., de Bont, J.A.M., (1999) Effect of organic solvents on the yield of solvent-tolerant *Pseudomonas putida* S12. *Applied and Environmental Microbiology* 65, 2631-2635.
- Isken, S., Santos, P., deBont, J.A.M., (1997) Effect of solvent adaptation on the antibiotic resistance in *Pseudomonas putida* S12. *Applied Microbiology and Biotechnology* 48, 642-647.
- Kieboom, J., de Bont, J.A.M., (2001) Identification and molecular characterization of an efflux system involved in *Pseudomonas putida* S12 multidrug resistance. *Microbiology-Uk* 147, 43-51.
- Kieboom, J., Dennis, J.J., de Bont, J.A.M., Zylstra, G.J., (1998a) Identification and molecular characterization of an efflux pump involved in *Pseudomonas putida* S12 solvent tolerance. *Journal of Biological Chemistry* 273, 85-91.

- Kieboom, J., Dennis, J.J., Zylstra, G.J., de Bont, J.A.M., (1998b) Active efflux of organic solvents by *Pseudomonas putida* S12 is induced by solvents. *Journal of Bacteriology* 180, 6769-6772.
- Laane, C., Boeren, S., Vos, K., Veeger, C., (1987) Rules for Optimization of Biocatalysis in Organic-Solvents. *Biotechnology and Bioengineering* 30, 81-87.
- Leon, R., Fernandes, P., Pinheiro, H.M., Cabral, J.M.S., (1998) Whole-cell biocatalysis in organic media. *Enzyme and Microbial Technology* 23, 483-500.
- Li, Z.Y., Dimple, B., (1994) SoxS, an Activator of Superoxide Stress Genes in *Escherichia-Coli* - Purification and Interaction with DNA. 269, 18371-18377.
- Mathys, R.G., Kut, O.M., Witholt, B., (1998) Alkanol removal from the apolar phase of a two-liquid phase bioconversion system. Part 1: Comparison of a less volatile and a more volatile in-situ extraction solvent for the separation of 1-octanol by distillation. *Journal of Chemical Technology and Biotechnology* 71, 315-325.
- McIver, A.M., Garikipati, S., Bankole, K.S., Gyamerah, M., Peeples, T.L., (2008) Microbial oxidation of naphthalene to cis-1,2-naphthalene dihydrodiol using naphthalene dioxygenase in biphasic media. *Biotechnology Progress* 24, 593-598.
- Miroux, B., Walker, J.E., (1996) Over-production of proteins in *Escherichia coli*: Mutant hosts that allow synthesis of some membrane proteins and globular proteins at high levels. *Journal of Molecular Biology* 260, 289-298.

- Moreira, M.A.S., de Souza, E.C., de Moraes, C.A., (2004) Multidrug efflux systems in gram-negative bacteria. *Brazilian Journal of Microbiology* 35, 19-28.
- Morrish, J.L.E., Brennan, E.T., Dry, H.C., Daugulis, A.J., (2008) Enhanced Bioproduction of Carvone in a Two-Liquid-Phase Partitioning Bioreactor With a Highly Hydrophobic Biocatalyst. 101, 768-775.
- Newman, J.D., Marshall, J., Chang, M., Nowroozi, F., Paradise, E., Pitera, D., Newman, K.L., Keasling, J.D., (2006) High-level production of amorpha-4,11-diene in a two-phase partitioning bioreactor of metabolically engineered *Escherichia coli*. 95, 684-691.
- Nijkamp, K., van Luijk, N., de Bont, J.A.M., Wery, J., (2005) The solvent-tolerant *Pseudomonas putida* S12 as host for the production of cinnamic acid from glucose. *Applied Microbiology and Biotechnology* 69, 170-177.
- Nikaido, H., (1996) Multidrug efflux pumps of gram-negative bacteria. *Journal of Bacteriology* 178, 5853-5859.
- Pinkart, H.C., White, D.C., (1997) Phospholipid biosynthesis and solvent tolerance in *Pseudomonas putida* strains. 179, 4219-4226.
- Pollard, D.J., Woodley, J.M., (2007) Biocatalysis for pharmaceutical intermediates: the future is now. *Trends in Biotechnology* 25, 66-73.
- Prpich, G.P., Daugulis, A.J., (2007) Solvent selection for enhanced bioproduction of 3-methylcatechol in a two-phase partitioning bioreactor. 97, 536-543.

- Pumphrey, G.M., Madsen, E.L., (2007) Naphthalene metabolism and growth inhibition by naphthalene in *Polaromonas naphthalenivorans* strain CJ2. 153, 3730-3738.
- Putman, M., van Veen, H.W., Konings, W.N., (2000) Molecular properties of bacterial multidrug transporters. *Microbiology and Molecular Biology Reviews* 64, 672.
- Ramos, J.L., Duque, E., Gallegos, M.T., Godoy, P., Ramos-Gonzalez, M.I., Rojas, A., Teran, W., Segura, A., (2002) Mechanisms of solvent tolerance in gram-negative bacteria. *Annual Review of Microbiology* 56, 743-768.
- Rojas, A., Duque, E., Mosqueda, G., Golden, G., Hurtado, A., Ramos, J.L., Segura, A., (2001) Three efflux pumps are required to provide efficient tolerance to toluene in *Pseudomonas putida* DOT-T1E. *Journal of Bacteriology* 183, 3967-3973.
- Rojas, A., Duque, E., Schmid, A., Hurtado, A., Ramos, J.L., Segura, A., (2004) Biotransformation in double-phase systems: Physiological responses of *Pseudomonas putida* DOT-T1E to a double phase made of aliphatic alcohols and biosynthesis of substituted catechols. *Applied and Environmental Microbiology* 70, 3637-3643.
- Rojas, A., Segura, A., Guazzaroni, M.E., Teran, W., Hurtado, A., Gallegos, M.T., Ramos, J.L., (2003) In vivo and in vitro evidence that TtgV is the specific regulator of the TtgGHI multidrug and solvent efflux pump of *Pseudomonas putida*. *Journal of Bacteriology* 185, 4755-4763.
- Rui, L.Y., Kwon, Y.M., Fishman, A., Reardon, K.F., Wood, T.K., (2004) Saturation mutagenesis of toluene ortho-monooxygenase of *Burkholderia cepacia* G4 for enhanced 1-naphthol

- synthesis and chloroform degradation. *Applied and Environmental Microbiology* 70, 3246-3252.
- Sambrook, J., Fritsch, E.F., Maniatis, T., (1989) *Molecular Cloning: A Laboratory Manual*. 2nd ed.
- Schmid, A., Dordick, J.S., Hauer, B., Kiener, A., Wubbolts, M., Witholt, B., (2001) Industrial biocatalysis today and tomorrow. *Nature* 409, 258-268.
- Schoemaker, H.E., Mink, D., Wubbolts, M.G., (2003) Dispelling the myths - Biocatalysis in industrial synthesis. 299, 1694-1697.
- Segura, A., Duque, E., Mosqueda, G., Ramos, J.L., Junker, F., (1999) Multiple responses of Gram-negative bacteria to organic solvents. *Environmental Microbiology* 1, 191-198.
- Sello, G., Bernasconi, S., Orsini, F., Tansi, M., Galli, E., Di Gennaro, P., Bestetti, G., (2004) Organic phase effect in the biphasic bioconversion of substituted naphthalenes by engineered E-coli containing P-fluorescens N3 dioxygenase. *Journal of Molecular Catalysis B-Enzymatic* 29, 181-186.
- Srikumar, R., Kon, T., Gotoh, N., Poole, K., (1998) Expression of *Pseudomonas aeruginosa* multidrug efflux pumps MexA-MexB-OprM and MexC-MexD-OprJ in a multidrug-sensitive *Escherichia coli* strain. 42, 65-71.
- Tao, Y., Bentley, W.E., Wood, T.K., (2005) Phenol and 2-naphthol production by toluene 4-monooxygenases using an aqueous/dioctyl phthalate system. *Applied Microbiology and Biotechnology* 68, 614-621.

- van Beilen, J.B., Duetz, W.A., Schmid, A., Witholt, B., (2003) Practical issues in the application of oxygenases. *Trends in Biotechnology* 21, 170-177.
- von Wallbrunn, A., Richnow, H.H., Neumann, G., Meinhardt, F., Heipieper, H.J., (2003) Mechanism of cis-trans isomerization of unsaturated fatty acids in *Pseudomonas putida*. *Journal of Bacteriology* 185, 1730-1733.
- Weber, F.J., deBont, J.A.M., (1996) Adaptation mechanisms of microorganisms to the toxic effects of organic solvents on membranes. *Biochimica Et Biophysica Acta-Reviews on Biomembranes* 1286, 225-245.
- Weber, F.J., Ooijkaas, L.P., Schemen, R.M.W., Hartmans, S., Debont, J.A.M., (1993) Adaptation of *Pseudomonas-Putida* S12 to High-Concentrations of Styrene and Other Organic-Solvents. *Applied and Environmental Microbiology* 59, 3502-3504.
- Wery, J., da Silva, D.I.M., de Bont, J.A.M., (2000) A genetically modified solvent-tolerant bacterium for optimized production of a toxic fine chemical. *Applied Microbiology and Biotechnology* 54, 180-185.
- Wery, J., Hidayat, B., Kieboom, J., de Bont, J.A.M., (2001) An insertion sequence prepares *Pseudomonas putida* S12 for severe solvent stress. *Journal of Biological Chemistry* 276, 5700-5706.
- White, D.G., Goldman, J.D., Demple, B., Levy, S.B., (1997) Role of the *acrAB* locus in organic solvent tolerance mediated by expression of *marA*, *soxS*, or *robA* in *Escherichia coli*. 179, 6122-6126.

- Whited, G.M., Corey Downie, J., Hudlicky, T., Fearnley, S.P., Dudding, T.C., Olivo, H.F., Parker, D., (1994) Oxidation of 2-methoxynaphthalene by toluene, naphthalene and biphenyl dioxygenases: structure and absolute stereochemistry of metabolites. *Bioorganic & Medicinal Chemistry* 2, 727-734.
- Wierckx, N.J.P., Ballerstedt, H., de Bont, J.A.M., Wery, J., (2005) Engineering of solvent-tolerant *Pseudomonas putida* S12 for bioproduction of phenol from glucose. *Applied and Environmental Microbiology* 71, 8221-8227.
- Wilson, A.S., Davis, C.D., Williams, D.P., Buckpitt, A.R., Pirmohamed, M., Park, B.K., (1996) Characterisation of the toxic metabolite(s) of naphthalene. *Toxicology* 114, 233-242.
- Witholt, B., Desmet, M.J., Kingma, J., Vanbeilen, J.B., Kok, M., Lageveen, R.G., Eggink, G., (1990) Bioconversions of aliphatic-compounds by *Pseudomonas oleovorans* in multi-phase bioreactors - background and economic-potential. *Trends in Biotechnology* 8, 46-52.
- Wubbolts, M.G., FavreBulle, O., Witholt, B., (1996) Biosynthesis of synthons in two-liquid-phase media. *Biotechnology and Bioengineering* 52, 301-308.

Table 1. Primers and amplified fragment sizes for each of srpABC genes for RT-PCR analysis

<u>Gene</u>	<u>Amplified Fragment size</u>	<u>Primer & Position*</u>	<u>DNA Sequence</u>
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

Table 2. Distribution coefficient of lauryl acetate and decanol for 1-naphthol

<u>Organic Solvent</u>	<u>Structure</u>	<u>logP[*]</u>	<u>% in Organic Phase</u>	<u>K_D</u>
Lauryl Acetate		7.0	98.4	61
Decanol		4.0	98.5	64

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

Table 3. Comparison of naphthalene conversions and amount of 1-naphthol obtained for the two strains tested for 24 h reaction time (total reaction volume of 50 mL)

<u>Biocatalyst</u>	<u>Solvent</u>	<u>1-Naphthol</u> <u>(mg)</u>	<u>Conversion</u>
<i>E. coli</i> TG1 pBS(kan)TOM-Green	Lauryl Acetate	15.2*	1.3*%
	Decanol	0.1	< 0.01%
<i>P. putida</i> S12 pBS(kan)TOM-Green	Lauryl Acetate	25.0	2.1%
	Decanol	23.4	2.0%

* from Garikipati et al.(Garikipati et al., 2009)

Figure 1a. *srpABC* fragment amplification

Figure 1b. Insertion of *srpABC* genes into a low-copy number plasmid, pBR322 (A) and a high-copy number plasmid, pUC19 (B)

Naphthalene toxicity to *P. putida* S12 pBS(kan)TOM-Green

1-Naphthol toxicity to *P. putida* S12 pBS(kan)TOM-Green

Figure 2. Toxicity of naphthalene and 1-naphthol to *P. putida* S12 cells expressing TOM-Green.

(Cells grown in LB medium at 30°C)

Figure 3. 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°C and 200 rpm (average of three identical experiments)

Figure 4. Toxicity of solvents lauryl acetate and decanol. Reaction conditions: 30°C and 200rpm, after 3 h (average of two identical experiments)

Figure 5. 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.

Figure 6. 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 °C. (Average of three independent experiments)