Appl Microbiol Biotechnol (2005) 68: 614–621
DOI 10.1007/s00253-005-1939-9

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Phenol and 2-naphthol production by toluene 4-monooxygenases using an aqueous/dioctyl phthalate system

Received: 23 November 2004 / Revised: 25 January 2005 / Accepted: 8 February 2005 / Published online: 2 March 2005

Abstract A two-phase system is developed here for converting: (1) benzene to phenol and (2) naphthalene to 2-naphthol, using whole cells expressing wild-type toluene 4-monooxygenase (T4MO) and the alpha subunit variant TmoA I100A from *Pseudomonas mendocina* KR1. Using the T4MO TmoA I100A variant, the solubility of naphthalene was enhanced and the toxicity of the naphthols was prevented by the use of a water/dioctyl phthalate (80:20, vol%) system which yielded 21-fold more 2-naphthol. More than 99% 2-naphthol was extracted to the dioctyl phthalate phase, dihydroxynaphthalene formation was prevented, 92% 2-naphthol was formed, and 12% naphthalene was converted. Similarly, using 50 vol% dioctyl phthalate, an initial concentration of 3.0 g l^{-1} (39 mM), and wild-type T4MO, a 51±9% conversion of benzene was obtained and phenol was produced at a purity of 97%. Relative to the one-phase system, there was a 12-fold reduction in the formation of the byproduct catechol.

Introduction

In recent years, biocatalysis has become an increasingly important technology for producing medium-priced, oxidized aromatic and aliphatic compounds such as epoxides and acids for the chemical industry (Miller and Peretti 2002). The specificity and the regio- and stereo-selectivity of enzymatic transformations, combined with the mild reaction conditions and the use of inexpensive reagents, represent sound advantages for biocatalysis (Schmid et al. 2001a). One recent commercial example is the production of an intermediate for an antilipolytic drug from the oxidation of 2,5-dimethylpyrazine to 5-methylpyrazine-2-carboxylic acid using whole cells of *Pseudomonas putida* expressing xylene monooxygenase (Schmid et al. 2001a).

Phenol and naphthol compounds are important precursors for the manufacture of many dyes, drugs, perfumes, insecticides, and surfactants (Howe-Grant 1991). World production for phenol is huge (6,600,000 t $year^{-1}$) and that of 1-naphthol and 2-naphthol is around 15,000 t $year^{-1}$ and 50,000 t $year^{-1}$, respectively (Howe-Grant 1991; Niwa et al. 2002). Industrially, more than 90% of the world phenol production is manufactured by a three-step cumene process (Howe-Grant 1991) that relies on the co-production of acetone and has a low phenol yield (Niwa et al. 2002). Hence, attempts to direct conversion of benzene to phenol by one-step reactions have been investigated (Niwa et al. 2002). Oxidation of benzene to phenol using nitrous oxide has been achieved (>40% conversion, 95% selectivity). However, this process at present is not economically feasible (Vulpescu et al. 2004). The remaining significant challenge in the chemical synthesis of phenol is the low conversion of benzene per pass of these processes (<16%; Niwa et al. 2002; Vulpescu et al. 2004).

Current commercial methods for manufacturing 2-naphthol include oxidation and aromatization of tetralin (a narcotic; Lewis 1993), caustic fusion of sodium 1-naphthalenesulfonate with sodium hydroxide in a five-step reaction, and hydroxylation of 2-isopropylnaphthalene (Howe-Grant 1991). The selectivity for 2-naphthol based on naphthol isomer distribution using those chemical methods is 90% or better. However, chemical synthesis is hampered by toxic reagents such as naphthalene-1-sulfonic acid, a high concentration of acids such as hydrogen fluoride, complicated, multiple-step reactions, extreme temperature conditions, and the co-production of acetone (Howe-Grant 1991). For example, in order to obtain high selectivity (98%), a fluorosulfuric acid-sulfuryl chloride fluoride solution at −60°C to −78°C was used for 2-naphthol production

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from naphthalene (Olah et al. 1991). To our knowledge, there has been no domestic producer of 2-naphthol in the United States since 1982, when the hydroperoxidation process ceased production (Howe-Grant 1991) due to environmental problems and China became the major supplier. It is predicted production of 2-naphthol will be reduced if China intensifies its environmental protection policies, and the impact of a supply shortage may spread to the global market (Cheng 1999).

Wild-type toluene 4-monooxygenase (T4MO) from *P. mendocina* KR1 is a highly active enzyme which hydroxylates toluene successively to *p*-cresol and 4-methylcatechol (Tao et al. 2004a) and oxidizes benzene to phenol, catechol, and 1,2,3-trihydroxybenzene (Tao et al. 2004b), although it was originally reported T4MO only oxidizes toluene and benzene to singly hydroxylated products (Whited and Gibson 1991). Furthermore, wild-type T4MO is not regiospecific for naphthalene, producing 50% of each naphthol isomer from naphthalene (Tao et al. 2005). Recently, we reported the first biocatalytic synthesis of 2-naphthol, based on an engineered T4MO enzyme (T4MO TmoA I100A; Tao et al. 2005). Using saturation mutagenesis, one variant with a single amino acid substitution, I100A, in the alpha subunit, was formed which enabled this enzyme to oxidize naphthalene primarily to 2-naphthol (92%). The 2-naphthol product was further oxidized to dihydroxynaphthalenes (Tao et al. 2005). *Escherichia coli* TG1 expressing T4MO TmoA I100A had an apparent V_{max} of 9±1 nmol min^{-1} mg^{-1} protein toward naphthalene, compared with 15±5 nmol min^{-1} mg^{-1} protein for the wild-type enzyme.

By exploring the use of a two-phase system, we sought to reduce the toxicity of the product 2-naphthol. Substituted phenols generally have antimicrobial properties (Meyer et al. 2003), and 1-naphthol has been found to be toxic to microorganisms (Sello et al. 2004), human colorectal tumor cells (Fluck et al. 1984), and human hepatic microsomes (Wilson et al. 1996). The cytotoxicity of 1-naphthol may be mediated by the formation of 1,2- or 1,4-naphthoquinone (Wilson et al. 1996).

Due to the low aqueous solubility of the substrate naphthalene (0.03 g l^{-1}; Perry and Chilton 1973), only low dissolved concentrations of the substrate may be used in a single-phase system. Fortunately, the addition of a second organic phase may be used to reduce the substrate toxicity and serve as a substrate reservoir (Wubbolts et al. 1994). In such two-liquid-phase systems, cells are grown or maintained in an aqueous medium containing water-soluble growth substrates (e.g., sugars and inorganic salts), while the hydrophobic substrates are mostly dissolved in an immiscible organic solvent phase.

Although T4MO is highly active and regiospecific toward aromatic substrates, its successive hydroxylations in a single-phase system make it impractical for the phenolic intermediates phenol and 2-naphthol (2-naphthol is converted to dihydroxynaphthalenes, phenol to catechol; Tao et al. 2005, 2004b). There are several studies indicating that two-phase systems may prevent the further conversion of products, because the organic solvent often serves as an extractant for hydrophobic oxidation products and the product is not further converted by the cells (Schmid et al. 1998). For example, *iso*-octane was used to prevent further conversion of phenylacetaldehyde to phenylacetic acid (Celik et al. 2004), and dioctyl phthalate (DOP; did not affect bacterial growth) was used to direct product formation towards one specific product (aldehyde, acid, alcohol), depending on the reaction conditions (Buhler et al. 2002a,b). Also, *n*-dodecane was used as the second phase for producing *cis*-diol derivatives from naphthalene (Sello et al. 2004).

In this paper, we optimized the one-step conversion of benzene to phenol and naphthalene to 2-naphthol, using whole-cell biocatalysts expressing wild-type T4MO and T4MO TmoA I100A in a two-phase system. This allowed efficient production of phenol from benzene and 2-naphthol from naphthalene under conditions of reduced substrate and product toxicity. The two-phase approach facilitated naphthalene solubility, reduced 2-naphthol toxicity, and allowed selective accumulation of the phenolic products in the organic phase, which facilitated product recovery and prevented the further oxidation of naphthol and phenol to dihydroxylated products. This is the first report of using toluene monooxygenases in two-liquid phase systems for synthesizing industrially-significant, phenolic compounds, and this represents one of the most efficient catalysts for converting benzene to phenol.

Materials and methods

Chemicals

Benzene, phenol, catechol, dodecane, and naphthalene were purchased from Fisher Scientific Co. (Fairlawn, N.J.). 1-Naphthol, 2-naphthol, and DOP [di (2-ethylhexyl) phthalate, a suspected carcinogen which must be treated with caution] were purchased from Sigma-Aldrich Chemical Co. (Milwaukee, Wis.). All materials used were of the highest purity available and were used without further purification.

Bacterial strains and growth conditions

E. coli TG1 strains (Sambrook et al. 1989) with: (1) pBS (Kan) (Canada et al. 2002), henceforth TG1/pBS(Kan), (2) pBS(Kan)T4MO (Tao et al. 2004b), henceforth TG1/T4MO, (3) pBS(Kan)T4MOTmoAI100A (Tao et al. 2005), henceforth TG1/T4MOI100A, and (4) pBS(Kan)TpMO expressing wild-type toluene *p*-monooxygenase (TpMO) from *Ralstonia pickettii* PKO1 (Fishman et al. 2004), henceforth TG1/TpMO, were routinely cultivated at 37°C with shaking at 250 rpm in a C25 incubator shaker (New Brunswick Scientific Co., Edison, N.J.) in Luria–Bertani (LB) medium (Sambrook et al. 1989) supplemented with kanamycin (Kan) at 100 μg ml^{-1} to maintain the plasmid.

Toxicity of naphthalene, 1-naphthol, 2-naphthol, and phenol

The toxicity of naphthalene, 1-naphthol, 2-naphthol, and phenol was determined with TG1/pBS(Kan) (no monooxygenase control) and TG1/T4MOI100A incubated in LB medium at 37°C. After reaching the mid-exponential growth phase, the cultures were subdivided into 20-ml aliquots in 250-ml flasks. Naphthalene (final concentrations of 0, 0.013, 0.026, 0.13, 1.66, 9.73, 19.97 g l^{-1}), 2-naphthol (0, 0.03, 0.07, 0.12, 0.14, 0.22, 0.29 g l^{-1}), 1-naphthol (0, 0.03, 0.07, 0.12, 0.14, 0.22, 0.29 g l^{-1}), or phenol (0, 0.02, 0.05, 0.09, 0.14, 0.19 g l^{-1}) was added. All the substrates were added using a constant 100 μl of dimethyl formamide (0.5 vol%). Control experiments demonstrated that 0.5 vol% dimethyl formamide was not toxic. The 20-ml cultures were incubated at 37°C and shaken at 250 rpm for a period of 8 h. The growth of the cultures was determined by optical density at 600 nm (OD_{600}), using a UVmini-1240 spectrophotometer (Shimadzu, Kyoto, Japan). The toxicity of dodecane or DOP to *E. coli* with different amounts of naphthalene (0, 2.43, 9.98, 19.97 g l^{-1}) and 2-naphthol (0, 0.07, 0.14, 0.36, 0.72, 1.44 g l^{-1}) was determined by a similar procedure. After splitting an exponentially growing TG1/pBS(Kan) culture, an equal volume of organic phase (20 ml) was added and incubation was continued.

2-Naphthol and phenol production in two-phase systems

Experiments were conducted by diluting overnight cells to an OD_{600} of 0.05–0.2 and growing to an OD_{600} of 1.4. The cultures were used directly without washing, except that cells were centrifuged and suspended in smaller volumes of LB to increase the cell density. To find a suitable solvent and to investigate the best aqueous/DOP ratio, experiments were performed in 250-ml flasks containing 40 ml of exponentially grown cultures (OD_{600} 1.4) with various amounts of solvents. Large head spaces and normal polyethylene stoppers were used to supply ample oxygen. To determine the best naphthalene concentration for 2-naphthol production in the two-phase system, 25 ml of exponentially grown cells (OD_{600} 1.4) and 25 ml of DOP were transferred to 250-ml flasks and a concentration of 2.05–48.61 g l^{-1} naphthalene (if all in the organic phase) was used. The reaction cultures were shaken at 250 rpm and incubated at 37°C. A sample of 1.5 ml was taken every 30 min to 22 h. After separation of the organic layer from the aqueous cell-containing phase by centrifugation, the organic phase and the water phase were diluted 25-fold by ethyl acetate and water, respectively, and were injected into a high-pressure liquid chromatograph (HPLC).

To produce phenol from benzene using DOP as a second organic phase, 25 ml of exponentially grown cells and 25 ml of DOP were transferred to a 250-ml flask and a concentration of 3.05 g l^{-1} and 12.19 g l^{-1} benzene (if all in the organic phase) was used (benzene added directly without a diluent). Similarly, reaction cultures were incubated at 37°C and shaken at 250 rpm. A sample of 1.5 ml was taken every 30 min to 17 h. The diluted samples were analyzed by HPLC (ethyl acetate added to the organic phase, water added to the aqueous phase).

Analytical methods

Reverse-phase HPLC was performed on a Waters 515 solvent delivery system (Waters Corp., Milford, Mass.) coupled to a Waters 996 photodiode array detector. Samples were injected by a Waters 717plus autosampler and analyzed using a Zorbax SB-C8 column (5 μm, 4.6×250.0 mm, Agilent Technologies). The injection volume was 20 μl. The elution gradient for detecting naphthalene and naphthol oxidation products was performed with water (0.1% formic acid) and acetonitrile (55:45 for 0–5 min, gradient to 20:80 at 16 min, gradient to 55:45 at 20 min) as a mobile phase, at a flow rate of 1 ml min^{-1}. For phenol and catechol products from benzene, the gradient elution was performed with water (0.1% formic acid) and acetonitrile (70:30 for 0–8 min, gradient to 40:60 at 15 min, gradient to 70:30 at 20 min) as the mobile phase, at a flow rate of 1 ml min^{-1}. All compounds were identified both by comparing their retention times and UV-visible spectra with those of authentic standards and by co-elution with standards. 1-Naphthol was monitored at a maximum wavelength of 294 nm, 2-naphthol was monitored at a maximum wavelength of 330 nm, and both phenol and catechol were monitored at 270 nm.

Results

Toxicity of naphthalene, naphthols, phenol, dodecane, and DOP

To investigate the limitations of the single-aqueous-phase system, product toxicities were measured. The growth of TG1/pBS(Kan) continued without inhibition in the presence of 0.01–0.13 g l^{-1} naphthalene, but significant growth inhibition was observed with 1.66–20 g l^{-1} naphthalene (Fig. 1a) in water. Both 1-naphthol and 2-naphthol were more toxic than naphthalene and exhibited a similar toxicity to cells: both 0.03 g l^{-1} 1-naphthol (data not shown) and 0.03 g l^{-1} 2-naphthol (Fig. 1c) reduced TG1/pBS(Kan) growth, but the cells reached a final cell density similar to that of the non-naphthol controls in 8 h. However, concentrations of 0.07–0.22 g l^{-1} 1-naphthol (data not shown) and 2-naphthol (Fig. 1c) significantly inhibited cell growth, and above 0.22 g l^{-1} no further growth was observed. Hence, single-phase systems cannot produce more than 0.22 g l^{-1} 1-naphthol or 2-naphthol. Phenol (0.02–0.19 g l^{-1}) did not inhibit cell growth in LB medium.

TG1/pBS(Kan) and TG1/T4MOI100A cells grew well in the presence of both 50 vol% dodecane and DOP in LB medium. Cells reached the same density as non-solvent

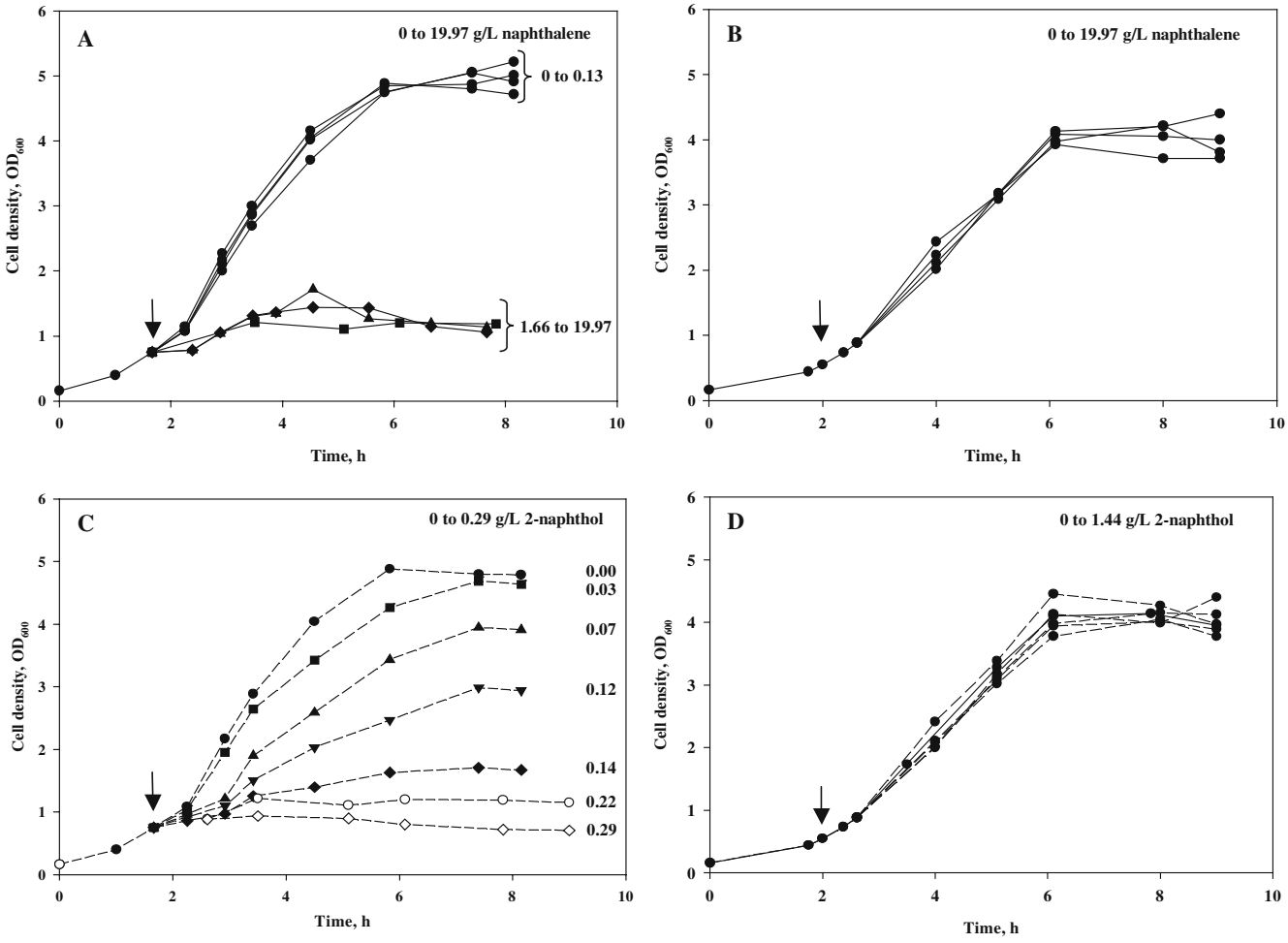

Fig. 1 Growth of TG1/pBS(Kan) in the presence of naphthalene in a single phase of LB (**a**), with two phases (50 vol% DOP) (**b**), in the presence of 2-naphthol in a single phase of LB (**c**), and with a two-phase system (50 vol% DOP) (**d**). The *arrows* indicate the addition of naphthalene at 0–19.97 g l^{-1} (**a**), naphthalene at 0–19.97 g l^{-1} (**b**), 2-naphthol at 0–0.29 g l^{-1} (**c**), and 2-naphthol at 0–1.44 g l^{-1} (**d**). *L* Liters

controls 6 h after the addition of the solvents. The addition of 2.43–20 g l^{-1} naphthalene (Fig. 1b) and 0.07–1.44 g l^{-1} 2-naphthol (Fig. 1d) to DOP also did not affect cell growth.

Single-phase naphthalene reaction

Since naphthalene up to 0.13 g l^{-1} did not inhibit cell growth in the single-phase system, this concentration was used. From 0.13 g l^{-1} naphthalene, TG1/T4MOI100A rapidly formed 0.09 g l^{-1} 2-naphthol (92% pure) after 0.5 h of incubation, which was sequentially transformed to dihydroxynaphthalenes, leading to a 2-naphthol percentage of only 50% in the product mixtures at 1.8 h. Using a higher initial naphthalene concentration (2.05 g l^{-1}), further transformation of 2-naphthol was reduced: dihydroxylated products only accounted for 15% of total products after 0.5 h and 2-naphthol was 73%. However, only 0.07±0.007 g l^{-1} 2-naphthol was produced from 2.05 g l^{-1} substrate (3.2% conversion). The reduction in dihydroxylation was likely due to the toxicity from both the naphthalene and naphthol products.

Organic solvent selection

Dodecane (Sello et al. 2004) and DOP (Buhler et al. 2002b) were tested as solvents for 2-naphthol production in shake-flasks. Neither solvent inhibited growth of TG1/T4MOI100A. At a phase fraction of 20 vol%, cells showed an overall 7-fold higher 2-naphthol concentration in the presence of DOP versus dodecane (Fig. 2), and the overall (water + organic) 2-naphthol concentration was 0.26 g l^{-1} vs 0.04 g l^{-1}. At a phase fraction of 20 vol%, 35±6% 2-naphthol remained in the aqueous phase for the dodecane/LB system, while for the DOP/LB system, 99% 2-naphthol was extracted to the organic phase (Fig. 2). Thus, DOP extracted 2-naphthol better.

Further hydroxylation of 2-naphthol to dihydroxylated products was reduced 4- to 5-fold in the DOP/LB system relative to dodecane/LB and single-phase systems, due to

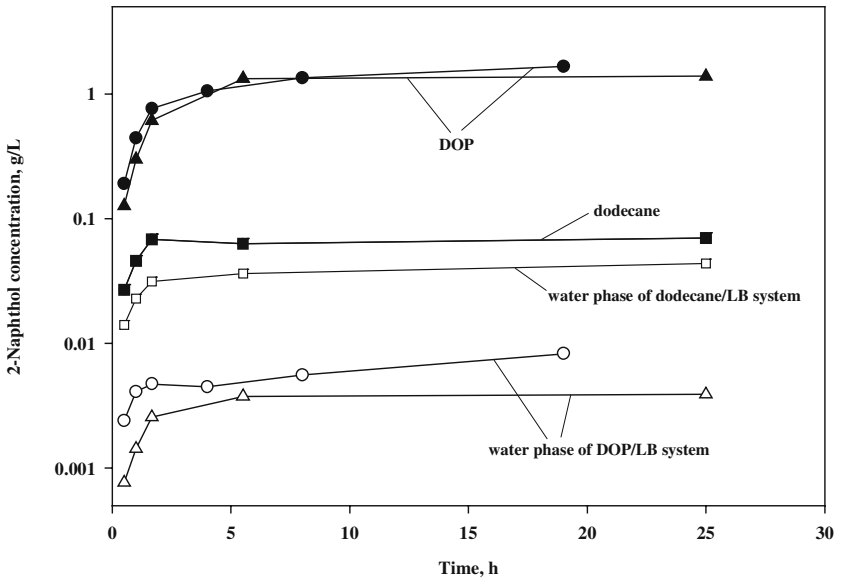

Fig. 2 2-Naphthol partitioning in water/DOP (80:20, vol%) during synthesis with TG1/T4MOI100A using 19.97 g l^{-1} naphthalene, showing 2-naphthol concentrations in the DOP phase (•, ▲) and in the water phase (○, △), also 2-naphthol concentrations in the dodecane phase (▪) and in the water phase (□). Results of two independent experiments are depicted for the DOP/LB system

the low 2-naphthol concentration in the aqueous phase for the DOP/LB system. Thus, DOP was chosen as the second liquid phase. It is noted that dihydroxylated products such as 2,6-dihydroxynaphthalene were partitioned completely in the aqueous phase using both solvents.

Naphthalene concentration and 2-naphthol production

To study product formation at high substrate concentrations, naphthalene (in DOP) at concentrations of 2.05–48.61 g l^{-1} was reacted in batch with a phase fraction of 50 vol% DOP. The highest initial 2-naphthol formation rate and 2-naphthol concentration after 4 h was obtained at a naphthalene concentration of 20 g l^{-1} (Fig. 3); hence, 20 g l^{-1} was chosen for the subsequent shake-flask experiments. The rate enhancement in the higher concentration was possibly due to the higher concentrations of naphthalene in the aqueous phase. However, the naphthalene conversion decreased with increasing initial substrate concentration, from 4.7±1.4% to 0.5±0.04% (Fig. 3). With a low naphthalene concentration (2.05 g l^{-1}), no significant difference in the 2-naphthol concentration was detected for the single- and two-phase systems.

DOP phase fraction

Using DOP as an organic phase and 20 g l^{-1} naphthalene, the organic phase was varied from 20 vol% to 80 vol% of the total liquid volume. Table 1 shows the highest 2-naphthol concentration in DOP was obtained at 20 vol%, which was 21-fold higher than the single-phase system (1.53±0.14 g l^{-1} vs 0.07±0.007 g l^{-1}). On a constant total volume basis, 4.3-fold more 2-naphthol was formed in the two-phase system using 20 vol% DOP than in the single phase (13.2 mg vs 3.1 mg, in 50-ml systems). However, the system with the phase ratio of 80 vol% DOP gave the highest total 2-naphthol in 24 h (31.4 mg of 2-naphthol) and the highest initial product formation rate (Table 1), but at the cost of lower conversion. Hence, the phase ratio substantially affects the conversion of naphthalene.

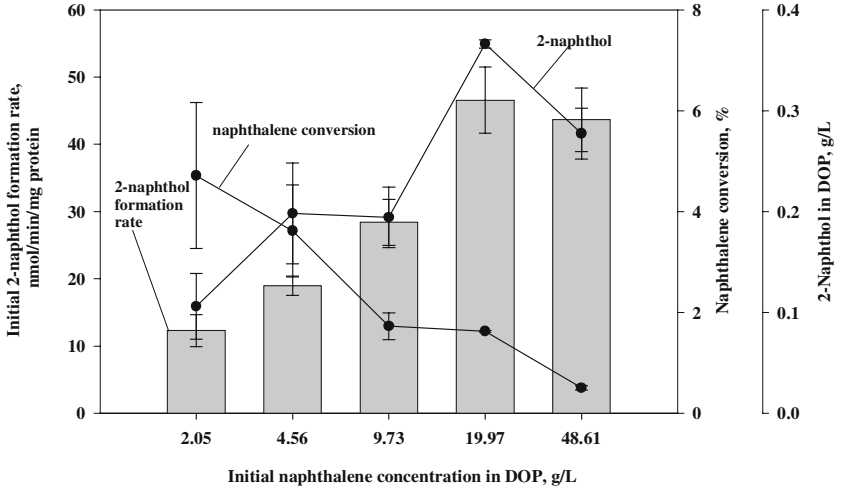

Fig. 3 Initial 2-naphthol formation rate (*bar graph*), naphthalene conversion (•), and 2-naphthol production (•) as a function of naphthalene concentration at a phase ratio of 50 vol% DOP, using TG1/T4MOI100A (4 h reaction, higher concentration seen with longer contact; Table 1)

Table 1 2-Naphthol (2-NP) synthesis in the two-phase DOP/LB system using TG1/T4MOI100A. A concentration of 2.05 g l^{-1} naphthalene was used for 0% DOP (water solubility taken as 0.03 g l^{-1}; Perry and Chilton 1973), and 19.97 g l^{-1} naphthalene was used for 20–80% (v/v) DOP. Cell density was OD_{600} 1.4. 2-NP concentration was determined after 24 h. 2-NP formation rate for the two-phase system was determined based on the 2-NP concentration in the DOP phase, and the initial rates here are approximately equal to the overall rates, including both phases, since 99% of the 2-NP was extracted to the DOP phase. – Not applicable or not determined

Organic phase fraction (v/v, %)	Volume (ml)		2-NP concentration (g l^{-1})		2-NP production (mg)		2-NP formation rate (nmol min^{-1} mg^{-1} protein)	Conversion (%)	Dihydroxylated product (mg)
	LB	DOP	LB	DOP	LB	DOP			
0	40	0	0.07±0.007	–	2.5±0.1	–	20.3±0.5	3.2	0.5
20	40	10	0.006±0.003	1.53±0.14	0.2±0.1	13±2	35±1	6.7	0
50	40	40	0.002±0.001	0.56±0.01	0.1±0.0	17±3	50±5	2.4	0
80	40	160	–	0.23	–	31.4	63±1	1.0	0

Using high cell densities (OD_{600} 8.0) and 20 vol% DOP, 2-naphthol was formed from naphthalene by the mutant enzyme T4MO TmoA I100A at 108±50 mg $l_{aqueous}^{-1}$ h^{-1}. 2-Naphthol accounted for 92.0±0.1% of the final product mixture in the DOP phase, and 1-naphthol was the only by-product (8.1±0.4%).

Phenol production from benzene using single- and two-phase systems

Using a single aqueous phase with 0.08 g l^{-1} benzene (0.23 g l^{-1} benzene added if all in the liquid phase; Dolfing et al. 1993) and TG1/T4MOI100A (OD_{600} 2.2), products of benzene oxidation were not seen until after 25 min, which could be due to the toxicity of benzene to the cells. Phenol at 0.05±0.02 g l^{-1} was produced with 0.003±0.001 g l^{-1} by-product catechol in 0.73 h, and 0.10 g l^{-1} phenol and 0.04 g l^{-1} catechol were produced in 1.7 h. Therefore, 20–45% of the benzene was transformed, but phenol only accounted for 39–78% of the products after 1.7 h. Since benzene and phenol are equally good substrates for T4MO (Tao et al. 2004b), phenol produced from benzene was rapidly consumed and converted to catechol, such that in 3 h only 8% phenol remained. Thus, the reaction proceeds rapidly and it is hard to accumulate the intermediate phenol.

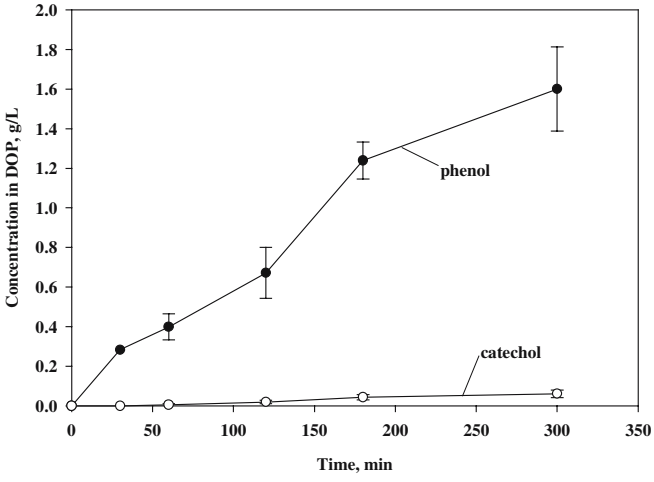

Fig. 4 Phenol synthesis from benzene (3.05 g l^{-1}) with TG1/T4MO, using 50 vol% DOP and OD_{600} 6 cells. One standard deviation is shown. • Phenol concentration in the DOP phase, ○ catechol concentration in the DOP phase

The advantage of the two-phase (DOP/LB) system over the one-phase system was demonstrated with TG1/T4MOI100A cells using 50 vol% DOP and 3.05 g l^{-1} benzene. Unlike the single-phase system, there was no lag phase for phenol production in the two-phase system, and 0.59±0.06 g l^{-1} phenol was produced in 4.5 h with a purity of 96%. Since most of the phenol product (90±1%) was extracted into the DOP phase, further oxidation of phenol to catechol (Tao et al. 2004b) was reduced 12-fold (respectively, 96% vs 8% phenol remained in the two-phase and one-phase systems after 3 h of reaction) as was also seen for naphthalene oxidation in the DOP/LB two-phase system.

The conversion of benzene to phenol was also examined with 3.05 g l^{-1} and 12.19 g l^{-1} benzene in the 50 vol% DOP system using TG1/T4MO and TG1/TpMO. For wild-type T4MO, both concentrations (3.05 g l^{-1}, 12.19 g l^{-1}) gave similar phenol formation rates and final concentrations (similar for TpMO), but TG1/T4MO produced 2- to 3-fold higher phenol concentrations than both TG1/T4MOI100A and TG1/TpMO. From 3.05 g l^{-1}, 51±9% benzene was consumed (based on product formation in both liquid phases) by TG1/T4MO and 43±6% benzene was converted to phenol (Fig. 4). The by-product catechol was accumulated at 3–4% in both the organic phase and the total system; and, therefore, phenol accounted for 97% of the total products in the organic phase.

Discussion

This work shows clearly that, using a two-phase system, T4MO and its variant TmoA I100A produced phenol and 2-naphthol with high purity (97%, 92% in the organic phase) and 10- to 21-fold higher concentrations (1.88–2.88 g l^{-1}) than the single-phase system, respectively. Previously, two-phase systems have also been used to direct the reaction chemistry for the accumulation of exclusively one product (Buhler et al. 2002a,b; Celik et al. 2004). The application of two-phase systems for aromatic hydroxylations using oxygenases has also been reported recently: 2,3-dihydroxybiphenyl was produced from 2-hydroxybiphenyl with

purified 2-hydroxybiphenyl 3-monooxygenase in a water/decanol (20:80, vol%) system, with a conversion of 15% (Schmid et al. 2001b), and less than 25% toluene was converted to 3-methylcatechol using whole cells expressing toluene dioxygenase (Husken et al. 2001). Cells expressing xylene monooxygenase made exclusively 3,4-dimethylbenzaldehyde (purity 89%) from pseudocumene with a conversion of 39% (Buhler et al. 2002a). Our results of 12% and 51% conversion for naphthalene and benzene oxidation are comparable with these reported values.

Here, biocatalysis has a number of advantages over chemical catalysis. The enzymes display good regiospecificity and allow for the production of 2-naphthol in a one-step reaction under environmentally friendly conditions. The two-phase system using whole-cell TG1/T4MOI100A is comparable wth (although greener than) the chemical routes for 2-naphthol production, since 12±0.4% naphthalene conversion was obtained here for producing 2-naphthol with 92% purity, whereas the chemical method requires the use of super acid and −60°C to −78°C conditions to produce 82–98% pure 2-naphthol with 17–50% naphthalene conversion (Olah et al. 1991).

Niwa et al. (2002) showed a palladium-based catalytic membrane reactor forms 95% phenol from benzene in a one-step reaction with oxygen, hydrogen, and in situ generated H_2O_2, with 3% benzene conversion per pass. The low yield of phenol was due to the unfavorable side-reactions of hydrogen and benzene that formed water and CO_2 (Vulpescu et al. 2004). Consequently, this interesting palladium membrane reactor for phenol production is still far from commercial. Current chemical methods in general give low conversions of benzene. However, 51±9% benzene conversion and 97% selectivity for phenol were obtained here, using the whole cell biocatalyst TG1/T4MO in the two-phase DOP/LB system.

Comparing the two-phase systems for phenol and 2-naphthol production shown here, the production of phenol from benzene was much more efficient than the production of 2-naphthol. The low solubility of naphthalene in the water phase could be one of the limiting factors. Furthermore, phenol is much less toxic than 2-naphthol for the biocatalyst in the aqueous phase. Although, in the two-phase system, 2-naphthol and naphthalene did not inhibit cell growth, there could be activity damage to the cells due to prolonged contact with those toxic compounds. Approximately 88% naphthalene did not react and remained in the DOP phase. However, the solvent and substrate could be recycled. The DOP phase is easy to separate from the aqueous phase, and its high boiling point (366°C) makes it less volatile than the substrates and products (e.g., 181°C for phenol), which facilitates distillation and pervaporation for product separation and recovery (Mathys et al. 1998, 1999).

Acknowledgements This research was supported by the National Science Foundation (BES-0124401) and the United States Environmental Protection Agency.

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