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BIOTECHNOLOGICAL PRODUCTS AND PROCESS ENGINEERING

Utilization of hydrophobic bacterium *Rhodococcus opacus* B-4 as whole-cell catalyst in anhydrous organic solvents

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Abstract *Rhodococcus opacus* strain B-4, which has recently been isolated as an organic solvent-tolerant bacterium, has a high hydrophobicity and exhibits a high affinity for hydrocarbons. This bacterium was able to survive for at least 5 days in organic solvents, including *n*-tetradecane, oleyl alcohol, and *bis*(2-ethylhexyl) phthalate (BEHP), which contained water less than 1% (*w*/*v*). The biocatalytic ability of *R. opacus* B-4 was demonstrated in the essentially nonaqueous BEHP using indigo production from indole as a model conversion. By the catabolism of oleic acid for NADH regeneration, indigo production increased up to 71.6 μg ml^{-1} by 24 h.

Keywords Organic solvent-tolerant bacteria · *Rhodococcus opacus* · Whole-cell catalyst · Nonaqueous media

Introduction

Enzymes can catalyze reactions with high chemo-, regio-, and stereoselectivities under benign conditions, which makes them useful for chemical syntheses. Biocatalytic conversions are now widely recognized as practical alternatives to traditional chemical syntheses (Schmid et al. 2001; Schoemaker et al. 2003). Many enzymes can work in organic solvents containing little or no water (Akkara et al. 1999; Carrea and Riva 2000; Klibanov 2001; Hudson et al. 2005). Hence, the use of organic solvents as reaction media has the potential to greatly expand the repertoire of biocatalytic transformations.

The thermodynamics of enzymatic reactions in organic solvents is much different from that in aqueous media. For instance, most hydrolases catalyze the hydrolysis of esters to corresponding acids and alcohols in water, whereas they preferentially catalyze esterification and transesterification in anhydrous environments (García-Alles and Gotor 1998; Zaks and Klibanov 1985). More importantly, enzymatic selectivities, including chemo-, regio-, and stereoselectivities, can be markedly altered and sometimes even reversed by organic solvents (Wescott and Klibanov 1994; Carrea et al. 1995). Additionally, enzymatic conversions in organic solvents containing little or no water are potentially more beneficial to chemical syntheses than those in conventional aqueous/organic biphase systems because they can simplify product recovery in downstream processes.

Successful examples of biocatalytic conversions in nonaqueous environments using enzymes including lyophilized lipases, esterases, and proteases, some of which are now used in large-scale industrial processes, have been reported (Akkara et al. 1999; Carrea and Riva 2000). However, most of these biocatalytic conversions involve relatively simple, single-step hydrolytic enzymes. One of the challenges in this area is the utilization of more complex enzymatic reactions involving multicomponent enzymes, cofactor regeneration, and multistep enzymatic conversions in organic solvents. Therefore, attention has been paid to the development of a whole-cell catalyst that functions in nonaqueous environments (Klibanov 2001).

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Since the isolation of the toluene-tolerant bacterium *Pseudomonas putida* by Inoue and Horikoshi (1989), numerous organic solvent-tolerant microorganisms have been isolated from a variety of natural habitats (Kato et al. 1996; Sardessai and Bhosle 2002; Paje et al. 1997). Their unique physiology has attracted the attention of researchers working on the extremophiles, the bioremediation of petroleum contaminants, and the biosynthesis of hydrophobic compounds. *Rhodococcus opacus* strain B-4, which has recently been isolated from a gasoline-contaminated soil sample, is highly tolerant to a variety of organic solvents including *n*-alkanes and monoaromatics (Na et al. 2005a). *R. opacus* B-4 cells are also able to enter the oil phase when they are grown in aqueous/organic biphasic systems. In this study, we report that *R. opacus* B-4 cells are able to retain their metabolic activity even in essentially anhydrous organic solvents and to mediate biosynthesis as a whole-cell catalyst.

Materials and methods

Microorganisms and culture conditions

R. opacus B-4 (Na et al. 2005a), *Rhodococcus erythropolis* PR4 (Komukai-Nakamura et al. 1996), and *P. putida* T-57 (Faizal et al. 2005) were used in this study. The rhodococcal strains were cultivated on tryptic soy broth (TSB, Difco Laboratories, Detroit, MI, USA) for 24 h. *P. putida* T-57 was grown on Luria–Bertani medium for 12 h. Precultures were grown overnight in test tubes (16× 150 mm) containing 5 ml of the medium at 30°C on a reciprocal shaker (150 rpm). The precultures were inoculated into 500-ml Erlenmeyer flasks containing 50 ml of fresh medium (a 1% inoculum) and incubated at 30°C with shaking (150 rpm). For test-tube cultures, the precultures were inoculated into the test tubes containing 5 ml of fresh medium and incubated under the same conditions. Cells were harvested by centrifugation at 8,000×*g* for 5 min at room temperature. After discarding the supernatant, the cells were centrifuged again at 8,000×*g* for 1 min, and the remaining supernatant was completely removed by pipetting. The resulting cell pellets were dispersed in organic solvents.

Construction of plasmids

To express arbitrary genes in *R. opacus* B-4, an expression vector plasmid (pROPtac) was constructed on the basis of a DNA fragment containing a chloramphenicol resistance gene and the *Escherichia coli* replication origin of pHSG396 (Takara Bio, Kyoto, Japan). This plasmid contained the *tac* promoter followed by a unique cloning site and the terminator sequence of pKK223-3 (Amersham Pharmacia, Buckingshire, UK), and the rhodococcal replicon genes, namely, *repA* and *repB*, of pRHK1 (Hirasawa et al. 2001). The isolation of the chromosomal DNA from *R. opacus* B-4 and the electroporation of the bacterium were performed as described previously (Na et al. 2005b). The genes encoding four components of benzene dioxygenase, namely, *bnzA1*, *bnzA2*, *bnzA3*, and *bnzA4* (Na et al. 2005a), were amplified from the chromosomal DNA of *R. opacus* B-4 by PCR using the following oligonucleotide primers: the sense primer 5′-CCGAATTCATGACTGACGTGCAATGTGA-3′ (the *Eco*RI site is underlined) and the antisense primer 5′-AAAAGCTTTCATGCCGCTGTTGTTCTCTC-3′ (the *Hin*dIII site is underlined). The amplicon was digested with *Eco*RI and *Hin*dIII and then ligated to the corresponding restriction sites of pROPtac. The resulting plasmid was designated as pROPtacBnzA.

Hydrophobicity assay

Cell-surface hydrophobicity was assessed by the bacterial adhesion to hydrocarbon (BATH) test (Dorobantu et al. 2004). The assays were carried out using a modified protocol of Neufeld et al. (1980). Washed cells of *R. opacus* B-4, *R. erythropolis* PR4, or *P. putida* T-57 were suspended in 0.85% NaCl to give an optical density at 600 nm (OD_{600}) of 1.0. An aliquot of the cell suspension (5 ml) and 0.2 ml of an organic solvent were placed in a test tube (16×150 mm), mixed by vortexing for 30 s, and then left to stand for 15 min to separate the oil phase from the aqueous phase. Then, the OD_{600} of the aqueous phase was measured using a U-2000 spectrophotometer (Hitachi, Tokyo, Japan). Cell-surface hydrophobicity was also determined using a G-1-1000 contact angle meter (Erma, Tokyo, Japan). *n*-Tetradecane was used as an organic solvent in the measurement of the three-phase contact angle.

Viability and bioconversion assays

R. opacus B-4 cells, which were collected from a 5-ml test-tube culture, were suspended in 2 ml of an organic solvent in a screw-capped tube (16×150 mm). The cell suspension was incubated with shaking (180 rpm) at 30°C. After incubation for 1 to 5 days, an aliquot of the suspension was sampled, and the cells were collected by centrifugation. The cells were resuspended in an adequate volume of 0.85% NaCl and then plated onto TSB agar plates. The plates were incubated at 30°C for 3 days for colony formation.

For bioconversion assay, unwashed *R. opacus* B-4 transformants, which were collected from a 5-ml test-tube culture, were suspended in 2 ml of an organic solvent

containing 5 mg ml^{-1} indole. The reaction was performed at 30°C for 12 h with reciprocal shaking at 180 rpm. After removing the cells by centrifugation, indigo production was assessed by measuring the OD_{600} of the supernatant.

Determination of extracellular water content

R. opacus B-4 cells were suspended at a concentration of 10 g wet cells l^{-1} in a solution containing 2.5 mg ml^{-1} blue dextran (average molecular weight, 20,000; Sigma). The cells were collected by centrifugation, resuspended in an appropriate volume (typically 4 ml for 1 g of wet cell pellet) of 0.85% NaCl and then collected again by centrifugation. The final concentration of blue dextran in the resulting supernatant was determined by measuring the absorbance at 620 nm. Extracellular water content in the pellet was estimated using

$$X = VC_{\mathrm{d}}/2.5 \quad (1)$$

where X is the extracellular water content (milliliter) in the pellet, V is the volume of 0.85% NaCl (milliliter) used for suspending *R. opacus* cells, and $C_{\mathrm{d}}/2.5$ is the ratio of the final concentration to the initial concentration (milligram per milliliter) of blue dextran. The total amount of water in the pellet was estimated by subtracting the dry cell weight from the wet cell weight.

Metabolizing activity in organic solvent

Typically, 570 mg of an *R. opacus* B-4 wet cell pellet, which contained 32% (*v*/*w*) extracellular water and 44% (*v*/*w*) intracellular water, was suspended in *bis*(2-ethylhexyl) phthalate (BEHP) to give a cell concentration of 2.5% (*w*/*v*), and oleic acid (Wako Pure Chemical, Kyoto, Japan) was added to the cell suspension at a final concentration of 10 mg ml^{-1}. Ten milliliters of the mixture was transferred into a 50-ml baffled flask, which was then incubated at 30°C on an orbital shaker at 170 rpm. An aliquot of the mixture was sampled at time intervals of 3 h and diluted 100-fold with acetonitrile containing 0.1 mg ml^{-1} linoleic acid as an internal standard. After removing the cells by centrifugation, fatty acids in the supernatant were fluorescence-labeled with 9-anthryldiazomethane (Funakoshi, Tokyo, Japan) as described by Nakagawa and Waku (1985) and then analyzed by HPLC on a Cosmosil 5C18AR-II column (4.6×50 mm, Nacalai Tesque, Kyoto, Japan). The mobile phase was composed of acetonitrile, water, and orthophosphate (90/10/0.1, pH 2.5), and the flow rate was adjusted to 1 ml min^{-1} for elution. The labeled fatty acids were detected with a fluorescence detector (excitation at 365 nm, emission at 412 nm). Blind assays were carried out using *R. opacus* B-4 cells that had been subjected to heating at 90°C for 5 min.

Indigo production

Approximately 570 mg of *R. opacus* B-4 (pROPtacBnzA) wet cells was suspended in BEHP at a concentration of 2.5% (*w*/*v*). Indole was added to the cell suspension to give a concentration of 9 mg ml^{-1}. Oleic acid was then added at a concentration of 10 mg ml^{-1}. Ten milliliters of the mixture was placed in a 50-ml Erlenmeyer flask and incubated at 30°C with orbital shaking at 150 rpm. An aliquot of the mixture was sampled at time intervals of 3 h, and after removing the cells by centrifugation, indigo concentration was determined spectrophotometrically at 600 nm.

Results

Behavior of bacterial cells in organic solvents

Three bacterial strains, namely, *R. opacus* B-4, *R. erythropolis* PR4, and *P. putida* T-57, were examined for their affinities for organic solvents. *R. erythropolis* PR4 is a rhodococcal strain with a high tolerance to various hydrocarbons. *P. putida* T-57 is a Gram-negative organic solvent-tolerant bacterium. These bacteria showed markedly different affinities for *n*-tetradecane (Fig. 1a). *P. putida* T-57 showed essentially no affinity for *n*-tetradecane. By contrast, *R. opacus* B-4 and *R. erythropolis* PR4 were dispersible in the organic solvent. Microscopic observation revealed that they

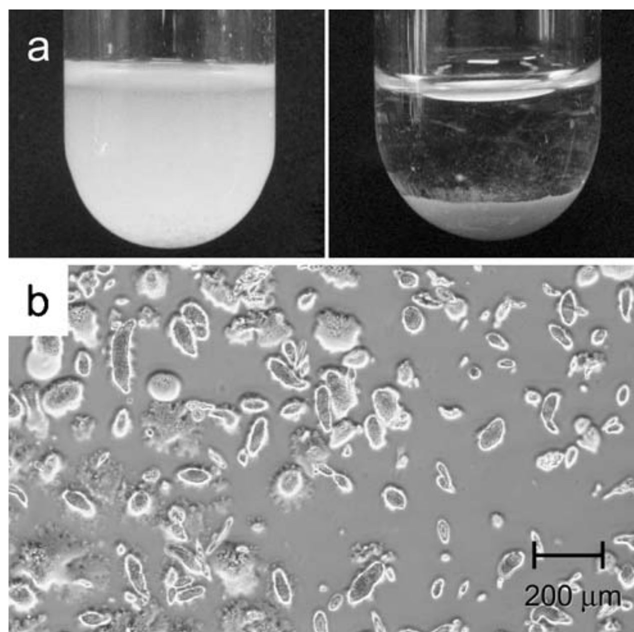

Fig. 1 Bacterial cell suspensions in *n*-tetradecane. **a** Cells harvested from 5 ml of the culture were mixed with 1 ml of *n*-tetradecane by vortexing for 30 s at a maximum rotation speed. *R. opacus* B-4 cells were dispersible in *n*-tetradecane (*left*), whereas *P. putida* cells showed no affinity for the organic solvent (*right*). **b** Cell aggregates of *R. opacus* B-4 in essentially water-free *n*-tetradecane

were dispersed in the oil phase in which they formed small cell aggregates (Fig. 1b). The cell aggregates settled out of the solvent when the mixture was left to stand. Similarly, *R. opacus* B-4 and *R. erythropolis* PR4 were also dispersible in BEHP. In this case, however, the cell aggregates did not settle out of the organic solvent even after the mixture was left to stand for 12 h. This is probably due to the differences in specific gravity and viscosity between BEHP [0.99 kg m^{-3}; 80 mPa·s (20°C)] and *n*-tetradecane [0.75 kg m^{-3}; 2.3 mPa·s (20°C)]. Cell-surface hydrophobicity was estimated by the BATH test and the three-phase contact angle measurement (Table 1). The BATH test revealed that *R. opacus* B-4 had a higher cell-surface hydrophobicity than *R. erythropolis* PR4 and *P. putida* T-57. Because the BATH test provides only preliminary data for surface hydrophobicity, three-phase contact angle measurement was also carried out with the three bacterial strains. *R. opacus* B-4 as well as *R. erythropolis* PR4 showed markedly high three-phase contact angles. These results show the significant relationship between the cell-surface hydrophobicity and the affinity of the cells for organic solvents. *R. opacus* B-4, which showed the highest cell-surface hydrophobicity, was used for further studies.

Cell viability and biocatalytic ability in organic solvents

The hydrophobicity of an organic solvent is given by the logarithm of the partition coefficient in a mixture of *n*-octanol and water (log P_{ow}) (Leo et al. 1971). A solvent with a lower log P_{ow} shows a higher toxicity to microorganisms (Isken and de Bont 1998). Figure 2 shows the cell viabilities of *R. opacus* B-4 in organic solvents having different log P_{ow} values. Among the *n*-alkanes tested, the solvent with a lower log P_{ow} was shown to be more toxic to *R. opacus* B-4. *R. opacus* B-4 was not tolerant to *n*-decanol (log P_{ow}=4.0), but it showed a significant viability in cyclohexane (log P_{ow}=3.4). *R. opacus* B-4 cells were also able to survive in essentially water-free BEHP and oleyl alcohol for at least 5 days. It has been reported that *R. opacus* B-4 was able to grow on TSB in the presence of 10% (*v*/*v*) monoaromatics, including benzene, toluene, xylene, and ethylbenzene (Na et al. 2005a). However, *R. opacus* B-4 was unable to survive in essentially water-free monoaromatics.

The biocatalytic ability of *R. opacus* B-4 was investigated using the conversion of indole to indigo as a model reaction. The genes encoding large and small subunits of benzene dioxygenase, ferredoxin, and NADH-ferredoxin reductase of *R. opacus* B-4 were expressed constitutively using the *tac* promoter in pROPtacBnzA. Indigo production by the *R. opacus* transformants was significantly affected by the organic solvent employed as the reaction medium (Fig. 2). No significant relationship was observed between cell viability and indigo production. No detectable amount of indigo was produced in middle- and long-chain *n*-alkanes despite their low toxicities to *R. opacus* B-4. This is probably due to the low solubility of indole in such *n*-alkanes. Although indole showed high solubility in BEHP and oleyl alcohol, its solubility in *n*-alkanes was less than 1% (*w*/*v*). By contrast, the *R. opacus* transformants produced indigo in oleyl alcohol and BEHP. When oleyl alcohol was used as the reaction medium, the reaction mixture turned green. The reaction mixture showed an absorbance peak at approximately 400 nm in addition to the absorbance peak at 600 nm, which corresponded to indigo, suggesting the presence of an unknown by-product. No peak at 400 nm was detected with BEHP. Because indigo production was most effective in BEHP, this organic solvent was employed as the reaction medium for further studies.

Table 1 Cell surface hydrophobicity

Strain	BATH (%)[a] (*n*=3)		Contact angle (°) (*n*=10)
	n-Tetradecane	BEHP	
R. opacus B-4	26.3±0.25	74.7±4.0	154±1.8
R. erythropolis PR4	83.5±7.9	81.0±1.5	160±2.1
P. putida T-57	106±0.54	114±0.54	19.6±2.3

[a] 100×(OD_{600} of aqueous phase after mixing)/(OD_{600} of aqueous phase before mixing). The values are averages ± standard deviations.

Oleic acid consumption in BEHP

Before assay for oleic acid consumption, extracellular water content in wet cell pellets was carefully determined using a blue dextran solution. When *R. opacus* B-4 cells were grown in 50 ml of TSB medium until the stationary phase (OD_{600}≒12) in a 500-ml Erlenmeyer flask and harvested by centrifugation at 8,000×*g* for 5 min, the average wet weight of the cell pellet was 572±34 mg (*n*=5). The total amount of water averaged 440±7 mg (*n*=5). Then, the amount of extracellular water in the cell pellet was estimated to be 186±37 mg (*n*=5) using Eq. 1. Thus, when the cell pellet was resuspended in an anhydrous organic solvent at a concentration of 2.5% (*w*/*v*), it was estimated that the reaction mixture contained an extracellular water content of approximately 0.8% (*w*/*v*).

Preliminary assimilation tests suggested that *R. opacus* B-4 was able to utilize oleic acid as a favorable carbon and energy source that is soluble in organic solvents (data not shown). When *R. opacus* B-4 cells were incubated in BEHP containing 1% (*w*/*v*) oleic acid, oleic acid concentration decreased significantly with time as shown in Fig. 3. The consumption rate was nearly constant during the 12-h incubation. Approximately 64% of the initial amount of oleic

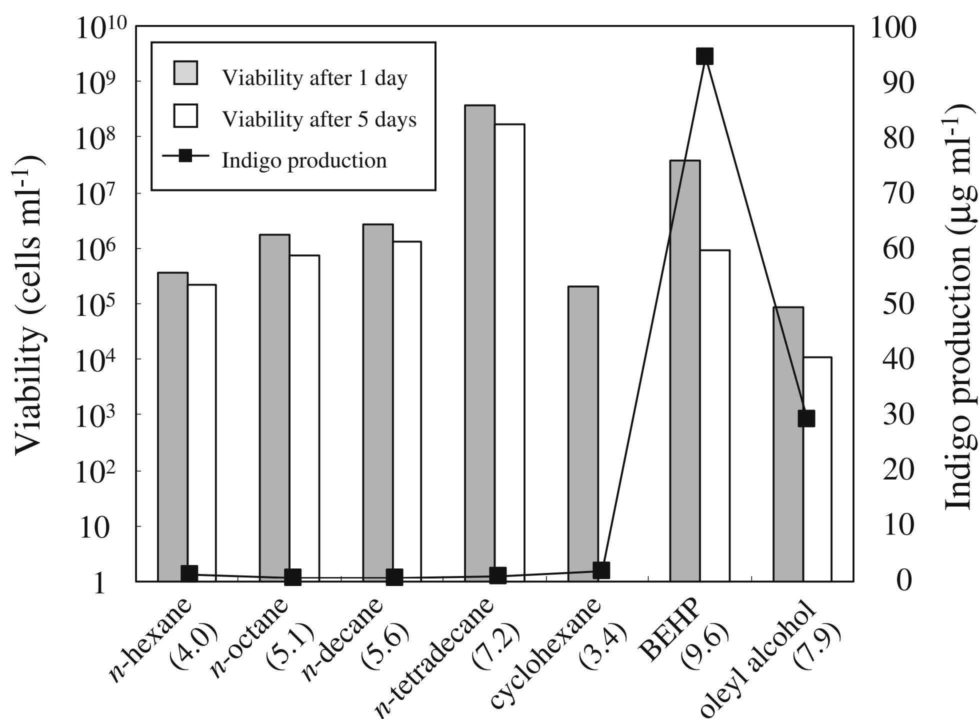

Fig. 2 Cell viability of and indigo production by *R. opacus* B-4 in organic solvents. Cell viability was determined on the basis of the number of colonies formed on TSB agar plates. The initial concentration of the bacterial suspension was 2.35×10^9 cells ml^{-1}. The log P_{ow} of each organic solvent is given *in parentheses*. No colony formed after 1 day of incubation for the following organic solvents: ethanol (log P_{ow}=−0.30), isopropanol (0.71), butanol (0.8), hexanol (2.0), octanol (2.9), decanol (4.0), cyclohexanol (1.2), benzene (2.1), toluene (2.5), *p*-xylene (3.1), ethyl acetate (0.73), and dimethyl sulfoxide (−1.4)

acid was consumed by 12 h. No significant decrease in the amount of oleic acid was observed in blind tests with heat-killed cells.

Indigo production

Because benzene dioxygenase requires NADH as a cofactor, NADH regeneration is essential for hydroxylation. It has been shown that introduction of an NADH regeneration system significantly improved indigo production (Doukyu et al. 2003). We examined the effect of the addition of oleic acid as an energy source on indigo production from indole by *R. opacus* B-4 transformants in BEHP (Fig. 4). The rates of indigo production were essentially the same for the initial 6 h regardless of whether oleic acid was present. However, the difference in the rate of indigo production became significant after 6 h. In the presence of oleic acid,

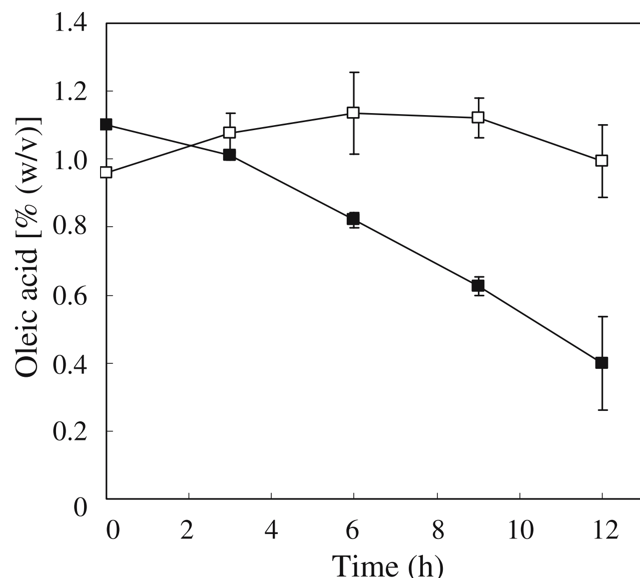

Fig. 3 Oleic acid consumption by *R. opacus* B-4 in BEHP. Intact (*filled squares*) and heat-killed (*empty squares*) cells of *R. opacus* B-4 were incubated in BEHP with 10 mg ml^{-1} oleic acid. The data are shown as averages±standard deviations (*n*=3)

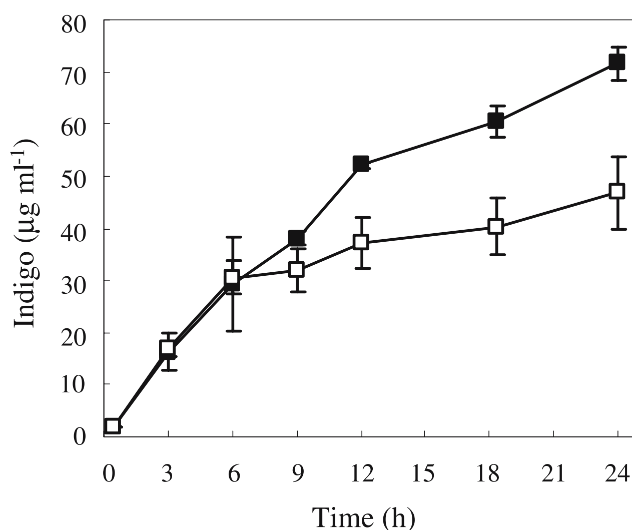

Fig. 4 Indigo production from indole by *R. opacus* B-4 in BEHP. Experiments were carried out with (*filled squares*) or without (*empty squares*) 10 mg ml^{-1} oleic acid. The data are shown as averages ± standard deviations (*n*=3)

the indigo concentration reached 71.6 μg ml^{-1} by 24 h, which was approximately 1.5-fold higher than that observed in the absence of oleic acid.

Discussion

BEHP is neither accessible nor toxic to bacteria because of its high hydrophobicity and low solubility in water. *R. opacus* B-4 was unable to grow on BEHP as the sole carbon source (data not shown). Several microorganisms have been found in toxic anhydrous organic solvents such as dimethyl sulfoxide (Fedorka-Cray et al. 1988) and diesel fuel (DeGray and Killian 1962). These indicate that some microorganisms can survive in essentially nonaqueous environments. However, little information is available on the biocatalytic ability of living cells in organic solvents. The data presented in this study demonstrate that *R. opacus* B-4 can survive and exhibit significant biological activity in essentially nonaqueous environments with less than 1% (*w*/*v*) water.

Several attempts have been made to use metabolically active cells in organic solvents. For example, intact cells of *E. coli*, *Acinetobacter calcoaceticus*, and *Saccharomyces cerevisiae* have been immobilized in surfactant-mediated, water-in-oil reverse micelles (Haring et al. 1985; Pfammatter et al. 1989). According to these previous reports, microorganisms were viable for at least 1 day and maintained their enzyme activities for a longer period of time. Dias et al. (1994) reported that the enzymatic side-chain cleavage of β-sitosterol proceeds in pure BEHP using intact or immobilized cells of *Mycobacterium* sp. NRRL B-3805 as biocatalysts. This side-chain cleaving reaction involves multiple enzymatic conversions, and some of them required NADPH or NADH. The cofactors are supplied by the catabolism of the cleaved side chain, and consequently the reaction proceeds continuously (Szentirmai 1990). Interestingly, the viability of *Mycobacterium* sp. cells extended in a pure organic solvent system compared with an aqueous/organic biphasic system (de Carvalho et al. 2004). This evidence suggests a potential advantage of the single organic solvent system over the conventional biphasic system.

R. opacus B-4 transformants carrying pROPtacBnzA catalyzed the oxidation of indole to indigo in anhydrous organic solvents. The transformants were able to catabolize oleic acid in the organic solvent and thus regenerate NADH. The addition of oleic acid to the reaction medium was effective in sustaining the reaction and consequently improved product yield. This approach is potentially applicable to other cofactor-dependent enzymatic reactions. An alternative potential approach may be the coexpression of NADH- or NADPH-generating enzymes such as glucose dehydrogenase (Kataoka et al. 2003) and formate dehydrogenase (Kula and Kragl 2000; Tishkov et al. 1999). However, it should be mentioned that the substrates for these enzymes, including glucose and formate, are insoluble in organic solvents. Therefore, enzymes such as the 2-propanol dehydrogenase of *Rhodococcus ruber* (Kosjek et al. 2004) and the hydrogenase of *Pyrococcus furiosus* (Mertens et al. 2003) may be good-candidate coupling enzymes.

The hydrophobic feature of *Rhodococcus* may be attributed to its unique cell envelope, which contains many mycolic acids (Sutcliffe 1998; Sokolovská et al. 2003). When *R. opacus* B-4 was grown in the presence of 1 μg ml^{-1} isoniazid, which specifically inhibits the biosynthesis of mycolic acids, the three-phase contact angle of *R. opacus* B-4 cells decreased to 109±0.86°. This preliminary observation indicates that mycolic acids play an important role in the cell hydrophobicity of *R. opacus* B-4. A detailed study of the association between the cell-envelope structure and the behavior of *R. opacus* B-4 in organic solvents is now in progress.

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