Process Biochemistry 48 (2013) 838–843

Contents lists available at SciVerse ScienceDirect

Process Biochemistry

journal homepage: www.elsevier.com/locate/procbio

Use of hydrophobic bacterium *Rhodococcus rhodochrous* NBRC15564 expressed thermophilic alcohol dehydrogenases as whole-cell catalyst in solvent-free organic media

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ARTICLE INFO

Article history:
Received 28 January 2013
Received in revised form 22 March 2013
Accepted 26 March 2013
Available online 12 April 2013

Keywords:
Thermostable enzyme
Hydrophobic bacteria
Alcohol dehydrogenase
Organic media
Whole-cell catalysis
Rhodococcus rhodochrous

ABSTRACT

The hydrophobic bacterium *Rhodococcus rhodochrous* NBRC15564 was employed as a whole-cell biocatalyst to examine its potential for bioconversion in solvent-free organic media. The genes encoding two different thermostable alcohol dehydrogenases (ADH_{Tt1} and ADH_{Tt2}) of *Thermus thermophilus* HB27 were expressed in *R. rhodochrous* cells. To inactivate indigenous mesophilic enzymes in *R. rhodochrous*, transformant cells were heated at 70 °C for 10 min. Heat-treated hydrophobic wet cells were used for the bioconversion of 2,2,2-trifluoroacetophenone (TFAP) to α-(trifluoromethyl) benzyl alcohol (TFMBA) as a model reaction with ADH_{Tt1}. NADH, which was supplied in aqueous solution, was regenerated by converting cyclohexanol to cyclohexanone by ADH_{Tt2}. All reactions were performed by suspending heat-treated cells in solvent-free organic media consisting of 3.7 M TFAP and 4.8 M cyclohexanol (1:1, v/v ratio) at 60 °C. When 800 mg heat-treated *R. rhodochrous* cells were dispersed in 2 mL of solvent-free organic media (400 mg cells/mL), the product concentration reached about 3.6 M TFMBA by 48 h with a total NADH turnover number of approximately 900. The overall productivity was 190 mol TFMBA/kg cells/h.

1. Introduction

Biocatalysis has emerged as an important technology for producing specialty and commodity chemicals [1–5]. Enzymes have great potential and versatility for catalyzing reactions with high regio-, stereo-, and enantio-selectivities, which are important in industrial organic synthesis, but difficult to achieve by chemical means [6–9]. Since enzymes can catalyze chemical reactions under mild conditions, they have a potential to provide an environmentally benign approach to industrial chemical processes [10]. In enzymatic processes, coenzyme requirements often make the reaction prohibitive [5,11]. On the other hand, whole cells have been used to regenerate cofactors such as NADH and NADPH. Whole cells can be readily removed from reaction mixtures by centrifugation in downstream processes [5].

Many organic solvents are toxic to metabolically active cells. In addition, the complete removal of water from the reaction system results in the loss of biocatalytic activity in living cells [5,11,12]. To minimize the toxicity of organic solvents, biocatalytic processes in aqueous-organic biphasic media have been developed [4,13–19]. The presence of an organic phase increases substrate solubility, while minimizing the toxicity of chemicals to living cells in the aqueous phase [4,19,20]. However, a small interfacial area limits the mass transfer of the substrate and product.

Organic monophasic media could be an alternative to aqueous-organic biphasic systems, when both substrate and product are water-insoluble or unstable in water [21]. The use of solvent-free organic media seems to be potentially beneficial for product recovery and waste disposal. Some enzymes can catalyze reactions in organic media [4,19]. For example, Mutti and Kroutil [22] have recently reported the asymmetric amination of ketones using lyophilized crude cell-free extracts in an organic solvent. Thermophilic enzymes often show resistance to high or low pH, detergents, and organic solvents [23–28].

In the present study, the hydrophobic bacterium *Rhodococcus rhodochrous* NBRC15564, which expressed thermophilic alcohol dehydrogenases (designated ADH_{Tt1} and ADH_{Tt2}) of *Thermus thermophilus* HB27, was examined for its bioconversion activity in solvent-free organic media. This bacterium showed high affinity for water-immiscible hydrophobic chemicals and could be well dispersed in organic media [29]. To inactivate indigenous mesophilic enzymes, *R. rhodochrous* transformants were heated at 70 °C for 10 min. The bioconversion of 2,2,2-trifluoroacetophenone (TFAP) to α-(trifluoromethyl) benzyl alcohol (TFMBA) was employed as a model reaction with ADH_{Tt1} (Fig. 1). NADH was regenerated by converting cyclohexanol to cyclohexanone with ADH_{Tt2}. Cyclohexanol, but not isopropyl alcohol, was employed as a substrate for NADH regeneration, because volatile chemicals are difficult to use

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http://dx.doi.org/10.1016/j.procbio.2013.03.022

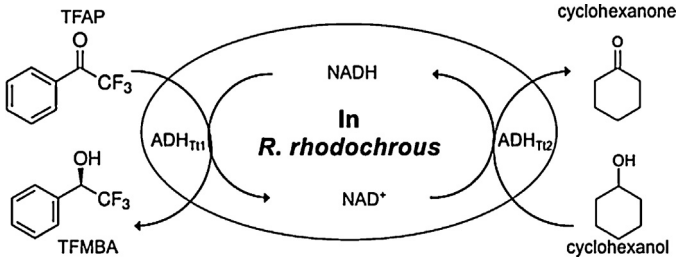

Fig. 1. Bioconversions of 2,2,2-trifluoroacetophenone (TFAP) to α-(trifluoromethyl) benzylalcohol (TFMBA) and cyclohexanol to cyclohexanone using ADH_{Tt1} and ADH_{Tt2}, respectively.

in organic media at 60 °C. ADH_{Tt1} was the thermophilic alchol dehydrogenase of *T. thermophilus* HB27, which has been described by Pennacchio et al. [30]. ADH_{Tt1} was employed as a highly enantioselective short-chain NAD(H)-dependent alchol dehydrogenase. ADH_{Tt2} was a homolog of alcohol dehydrogenase of *Thermus* sp. ATN1, which could effectively use cyclohexanol to regenerate NADH [31,32].

All the reactions were performed in solvent-free organic media consisting of 3.7 M TFAP and 4.8 M cyclohexanol (a 1:1, v/v ratio) at 60 °C.

2. Materials and methods

2.1. Chemicals

TFAP, TFMBA, and (*R*)-(−)-TFMBA were purchased from Tokyo Chemical Industry, Japan. (*S*)-(+)-TFMBA was obtained from Sigma–Aldrich (MO, USA). Cyclohexanol, cyclohexanone, cyclohexane, *n*-tetradecane, *n*-hexadecane, and toluene were purchased from Nakalai (Kyoto, Japan). NADH was obtained from Wako (Osaka, Japan). All the chemicals used in the present study were of analytical grade.

2.2. Bacterial strains and plasmids

R. rhodochrous NBRC15564 was obtained from the Biological Resource Center, National Institute of Technology and Evaluation. *Escherichia coli* B strain BL21 (DE3) (Novagen, Germany), *Rhodococcus opacus* B-4 [33], and *Rhodococcus erythropolis* PR4 [34] were used in the bacterial adhesion to hydrocarbon (BATH) assay. *E. coli* DH5α was used as the molecular cloning host. To clone the alcohol dehydrogenase gene of *T. thermophilus* HB27 (adh_{Tt1}) [30], a DNA fragment was amplified by PCR with the primers 5′-ttcatatgggccttttcgctggcaa and 5′-ttgaattcctacaccggccgccccgccatcatg (the *Nde*I and *Eco*RI restriction sites are underlined, respectively). *T. thermophilus* HB27 genomic DNA was used as the template. The amplified DNA fragment was digested with *Eco*RI and cloned into *Eco*RI- and *Eco*RV-digested pBR322. The DNA fragment containing adh_{Tt1} was digested with *Nde*I and *Eco*RI and subcloned into pTipQT2 [35] or pET-21a(+) (Novagen, Germany).

To clone the adh_{Tt2} gene from *T. thermophilus* HB27 (GenBank accession no. YP_004007), which encodes a homolog of alcohol dehydrogenase of *Thermus* sp. ATN1, a DNA fragment was amplified by PCR with the primers 5′-aacatatgagggccgtggtctac and 5′-ttggatcctcagggcacgagggcgacctt (the *Nde*I and *Bam*HI restriction sites are underlined, respectively) [31,32]. The amplified DNA fragment was digested with *Bam*HI and cloned into *Bam*HI- and *Eco*RV-digested pBR322. The DNA fragment containing adh_{Tt2} was digested with *Nde*I and *Bam*HI and subcloned into pTipRC2 [35].

2.3. Growth conditions and cell preparation

E. coli was grown in LB medium at 37 °C. Other bacterial strains were grown in 3% TSB medium (Becton, Dickinson and Company, USA) at 30 °C. Agar (1%) was added when a solid medium was used. When needed, 100 μg of ampicillin or 15 μg of tetracycline or 25 μL of chloramphenicol (Wako, Japan) were added to 1 mL of the culture medium. To induce protein synthesis, 1 mM IPTG (Wako, Japan) or 20 μg/mL thiostrepton (Sigma–Aldrich, USA) was added. *R. opacus* B-4, *R. erythropolis* PR4, and *R. rhodochrous* NBRC15564 were transformed by the method of Na et al. [36]. For heat treatment, cells were washed with 0.1 M Tris–HCl (pH 8.0), resuspended in the same buffer at 100 mg cells/mL and heated at 70 °C for 10 min. Cell pellets were obtained by centrifugation at 8000 × *g* for 10 min and stored at 4 °C. The stored cell could be used for at least 7 months. The extracellular water content of cell pellets was estimated using the method described by Yamashita et al. [29]. The total amount of water in cell pellets was estimated by subtracting the dry pellet weight from the wet pellet weight.

2.4. BATH assay

BATH assay was performed by the method of Hamada et al. [37]. Cells were washed with 0.85% NaCl and resuspended in 4 mL of the same solution at an OD_{660} of 1.0. After adding 400 μL of *n*-tetradecane, *n*-hexadecane or toluene, the cell suspension was gently vortexed for 1 min and left to stand at room temperature for 1 h. BATH (%) was calculated from the initial and final OD_{600}s in the aqueous phase as

$$\text{BATH}\,(\%) = 100 \times \frac{\text{initial OD}_{660} - \text{final OD}_{660}}{\text{initial OD}_{660}}$$

2.5. Enzyme assay

Crude ADH_{Tt1} activity in the aqueous medium was assayed by measuring the change in absorbance at 340 nm of NADH at 60 °C. To obtain crude ADH_{Tt1}, 100 mg/mL bacterial cells were sonicated using an ultrasonic disruptor (UD-201: Tomy, Japan), heated at 70 °C for 10 min and centrifuged at 8000 × *g* for 10 min. Crude enzyme assay was performed by adding 10 μL of supernatant to 1 mL of a preheated assay mixture containing 2 mM TFAP and 0.2 mM NADH. The NADH aqueous solution was prepared using 100 mM Tris–HCl (pH 8.0). The absorption coefficient of NADH at 340 nm was 6.22 mM/cm. ADH_{Tt1} activity in the whole cells was assayed by adding 200 mg of the heat-treated cells to 2 mL of the assay mixture containing 37 mM TFAP, 9.3 M cyclohexane, and 4 mM NADH. NADH was dissolved with ultrapure water (Milli-Q) at 250 mM, and 32 μL of 250 mM NADH was added to the reaction mixture at a final concentration of 4 mM.

2.6. SDS-PAGE

After disrupting 100 mg/mL *R. rhodochrous* cells, which expressed ADH_{Tt1} and ADH_{Tt2}, by sonication, the samples were heated at 70 °C for 10 min and centrifuged at 10,000 × *g* for 2 min. About 50 μL of the supernatant was mixed with 50 μL of Sample Buffer (100 mM Tris–HCl (pH 6.8), 20% (v/v) glycerol, 12% (v/v) β-mercaptoethanol, and 4% SDS and 0.004% bromophenol blue) and boiled for 3 min. About 20 μL of each sample was subjected to SDS-PAGE.

2.7. Bioconversion

Various amounts of heat-treated wet *R. rhodochrous* cells were suspended in 2 mL of 3.7 M TFAP and 4.8 M cyclohexanol (1:1, v/v ratio). NADH was supplied in an aqueous solution at the start of incubation at 60 °C. NADH was dissolved with Milli-Q at 250 mM. When the final NADH concentration in the reaction mixture was 0.4, 4.0 or 40 mM, 3.2, 32 or 320 μL water was brought into the 2 mL organic media, respectively. After centrifugation, the supernatant was determined using a gas chromatography system (GC-2014, Shimadzu, Kyoto, Japan) with a flame ionization detector. GC analysis was performed with a DB-17 column (30 m × 0.250 mm × 0.25 μm, J&W Scientific, USA) and nitrogen at 70 kPa as the carrier gas. Column temperature was initially kept at 90 °C for 3 min, increased to 240 °C at a rate of 20 °C/min and kept at 240 °C for 3 min. Both the injector and detector temperatures were set at 260 °C. Chiral GC was performed with a MEGA-DEX DMP β column (25 m × 0.250 mm × 0.25 μm, MEGA S.N.C., Italy) and nitrogen at 70 kPa as a carrier gas [30]. Column temperature was initially kept at 130 °C for 2 min, increased to 150 °C at a rate of 2.5 °C/min and kept at 150 °C for 3 min.

3. Results

3.1. Effect of heat treatment on bioconversion

The affinity of bacterial cells to water-immiscible hydrophobic chemicals was examined by BATH assay (Table 1). The BATH assay showed that *R. rhodochrous* had the highest affinity for the water-immiscible hydrophobic chemicals among the bacterial strains examined. To study the effect of heat treatment on cell hydrophobicity, BATH assay was also performed with cells that had been subjected to heating at 70 °C for 10 min. Except in the case of *E. coli*, no significant change was observed in the results of BATH assay before and after heat treatment. *R. rhodochrous* NBRC15564, which exhibited the highest affinity to hydrophobic chemicals, was used for further study.

Interestingly, the heat-treated *R. rhodochrous* cells showed better performance in the conversion of TFAP to TFMBA than the untreated cells (Fig. 2). It has been reported that a high temperature plays a significant role in the proper folding or oligomeric structural formation of thermostable enzymes [38]. However, no

Table 1
Effect of heat treatment on bacterial affinity to water-immiscible hydrophobic chemicals.

	BATH (%)					
	n-Tetradecane		*n*-Hexadecane		Toluene	
	Unheated	Heated	Unheated	Heated	Unheated	Heated
R. opacus B-4	93 ± 1	90 ± 1	95 ± 1	95[a]	95[a]	93[a]
R. erythropolis PR4	41 ± 2	40 ± 4	46 ± 2	56 ± 1	50[a]	43 ± 1
R. rhodochrous NBRC15564	98 ± 1	98 ± 1	97 ± 1	98 ± 1	93 ± 2	100[a]
E. coli BL21 (DE3)	3.0 ± 1	15[a]	7.5 ± 4	17[a]	35 ± 1	8.2 ± 1

Before and after heat treatment at 70 °C for 10 min, the cells were washed with 0.85% NaCl and resuspended in 4 mL of the same solution (OD_{660} = 1.0). After adding of 400 μL of *n*-tetradecane, *n*-hexadecane or toluene, the cell suspension was gently vortexed for 1 min and left to stand at room temperature for 1 h. The data represent the mean ± standard deviation of three independent experiments.

[a] The standard deviation was less than 1.

significance difference was detected between the bioconversion activities of crude ADH_{Tt1} and ADH_{Tt2} before and after heat treatment at 70 °C for 10 min (data not shown). Thus, the heat treatment may have improved the membrane permeability of *R. rhodochrous* cells, since the estelic lipid in the cell membrane is heat-sensitive [39]. Kashihara et al. [40] have reported that heat treatment at 70 °C can cause cell wall destabilization, forming openings on the cell membrane in bacterial cells.

3.2. Effect of cell hydrophobicity on bioconversion

E. coli is an excellent host in terms of easy handling and availability of molecular tools [22]. When crude ADH_{Tt1} was prepared from 200 mg of bacterial cells, the total enzyme activity was significantly higher in *E. coli* than in *R. rhodochrous* and *R. erythropolis* (Fig. 3a). However, heat-treated whole cells of *R. rhodochrous* showed better performance in the bioconversion of TFAP to TFMBA in organic media than those of *E. coli* (Fig. 3b). In addition, TFMBA production increased with increasing cell mass of *R. rhodochrous* or *R. erythropolis* from 200 to 800 mg. The % conversion of TFAP to TFMBA was not proportional to bacteria cell mass. This was likely to reflect that bacterial cells were not freely dispersed in organic media. Previously, we have showed that the hydrophobic bacterium *R. opacus* formed small cell aggregates in organic media [29]. Since *E. coli* cells showed essentially no affinity to organic media, increasing the cell mass failed to increase TFMBA production. These results suggest the advantage of hydrophobic cells as a whole-cell catalyst in organic media.

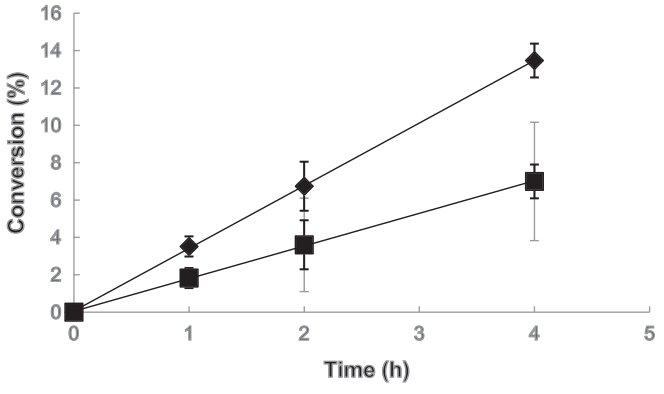

Fig. 2. Effect of heat treatment on the conversion of TFAP to TFMBA. A mass of 200 mg heat-treated (diamonds) or untreated wet cells (squares) were added to the reaction mixture containing 2 mL of 3.7 M TFAP and 4.8 M cyclohexanol. At the start of incubation at 60 °C, 32 μL of aqueous 250 mM NADH was added to the mixture. The final concentration of NADH was 4 mM. Error bars represent the standard deviations of three independent reactions.

3.3. Improvement in TFMBA production

Expression of ADH_{Tt1} and ADH_{Tt2} in *R. rhodochrous* was confirmed by SDS-PAGE (Fig. 4). SDS-PAGE analysis showed the production of 27- and 36-kDa proteins corresponding to ADH_{Tt1} and ADH_{Tt2}, respectively. Pennacchio et al. [30] have reported that ADH_{Tt1} activity increases with increasing temperature up to 73 °C. When the crude ADH_{Tt1} and ADH_{Tt2} activities were examined in the present study, both the enzymes showed the highest activity at 70 °C (data not shown). However, these enzyme activities were assessed in aqueous environments. To study the effects of

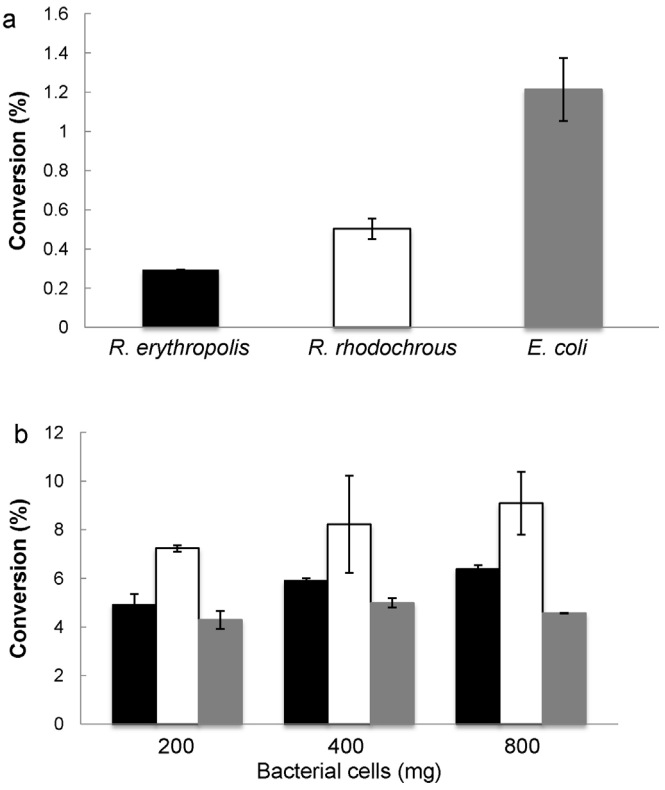

Fig. 3. The percent conversion of TFAP to TFMBA by crude ADH_{Tt1} (a) and ADH_{Tt1}-expressing whole cells (b). To obtain crude ADH_{Tt1}, 200 mg of *R. erythropolis*, *R. rhodochrous*, or *E. coli* cells was sonicated using an ultrasonic disruptor, heated at 70 °C for 10 min and centrifuged at 8000 × *g* for 10 min. Crude enzyme assay was performed by adding 10 μL of the cell-free supernatant to 1 mL of an aqueous solution containing 2 mM TFAP and 0.2 mM NADH at 60 °C. The data is given by % conversion of 2 mM TFAP to TFMBA after 1 min of incubation. Whole-cell ADH_{Tt1} activity in organic media was assayed by adding 200, 400 or 800 mg of heat-treated *R. erythropolis* (black), *R. rhodochrous* (white), or *E. coli* (gray) wet cells to 2 mL of an organic solution containing 37 mM TFAP, 9.3 M cyclohexane, and 4 mM NADH. The data is given by % conversion of 37 mM TFAP to TFMBA after 1 h of incubation. Error bars represent the standard deviations of three independent reactions.

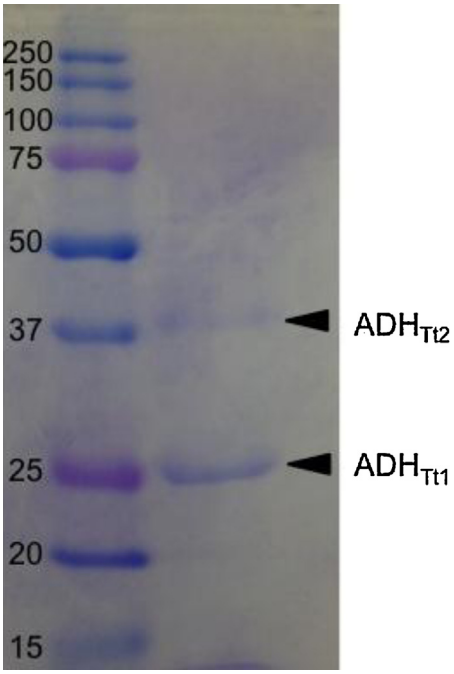

Fig. 4. SDS-PAGE of *T. thermophilus* HB27 ADH_{Tt1} and ADH_{Tt2} expressed in *R. rhodochrous* cells. Left lane, molecular mass markers; right lane, ADH_{Tt1} and ADH_{Tt2} of *Thermus thermophilus* HB27 expressed in *R. rhodochrous* cells. The arrows indicate ADH_{Tt1} and ADH_{Tt2}, respectively.

temperature on ADH_{Tt1} and ADH_{Tt2} activities in organic media, the bioconversion of TFAF to TFMBA was conducted using heat-treated *R. rhodochrous* cells at various temperatures from 50 to 70 °C (Fig. 5). The results showed that the bioconversion rate was significantly high at 60 °C, compared to those observed at 50 and 70 °C. This optimal temperature was approximately 10 °C lower in the organic media than those detected with crude ADH_{Tt1} and ADH_{Tt2} in aqueous environments. However, the reason is unclear at this time. For further study, TFAF bioconversion was conducted at 60 °C.

To study the effect of cell concentration on TFMBA production, bioconversion assay was performed using various cell concentrations (Fig. 6). The product concentration at 48 h increased from 0.94 to 3.6 M with increasing cell mass from 200 to 800 mg (100–400 mg/L). Heat-treated *R. rhodochrous* cells failed to exhibit bioconversion of TFAP, unless NADH was added to the reaction

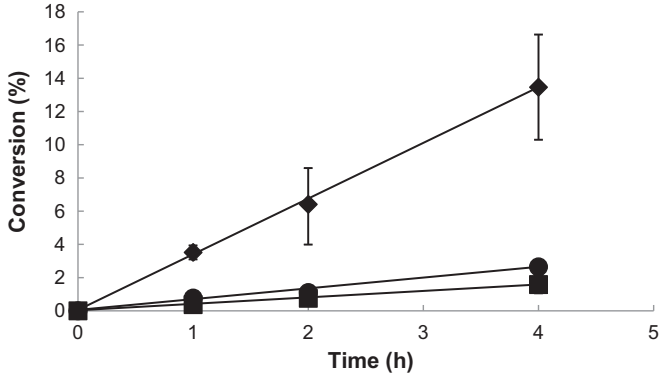

Fig. 5. Effect of temperature on the conversion of TFAP to TFMBA. A mass of 200 mg heat-treated wet cells was added to 2 mL of the reaction mixture containing 3.7 M TFAF and 4.8 M cyclohexanol. Bioconversion of TFMF to TFMBA was performed at 50 (squares), 60 (diamonds), and 70 °C (circles). At the start of incubation, 32 μL of water containing 250 mM NADH was added to the reaction mixture. Error bars represent the standard deviations of three independent reactions.

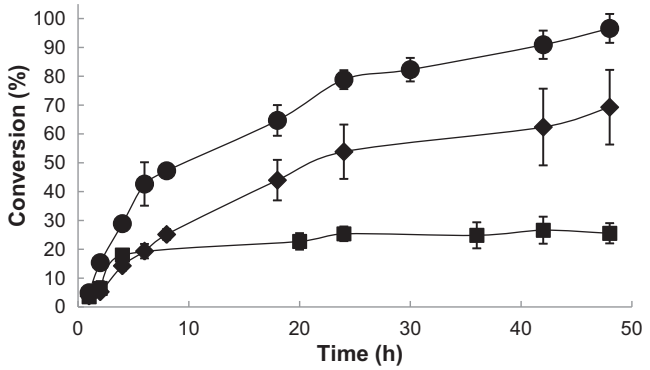

Fig. 6. Effect of cell concentration on the conversion of TFAP to TFMBA. A mass of 200 (squares), 400 (diamonds), or 800 mg (circles) heated-treated wet *R. rhodochrous* cells was added to 2 mL of the reaction mixture consisting of 3.7 M TFAF and 4.8 M cyclohexanol. At the start of incubation at 60 °C, 32 μL of Milli-Q water containing 250 mM NADH was added to the reaction mixture. Error bars represent the standard deviations of two to five independent reactions.

mixture (Table 2). With increasing NADH concentration from 0.4 to 4.0 mM, the conversion of TFAP to TFMBA increased from 64 to 97% in 48 h (Fig. 7). However, no significant improvement was observed with increasing NADH concentration from 4.0 to 40 mM. When the NADH concentration was 40 mM, 320 μL of aqueous solution containing 250 mM NADH was added to the organic media. This indicated that the volume of water added to the organic media was approximately ten times more than that at 4.0 mM NADH. When 320 μL aqueous solution containing 25 mM NADH was add to the 2 mL reaction mixture (a final concentration of 4.0 mM NADH), the conversion of TFAP to TFMBA was decreased by 7%, compared to the addition of 32 μL aqueous solution containing 250 mM NADH (Table 2). When 97% of 3.7 M TFAP was converted to TFMBA, the overall turnover number of NADH was approximately 900.

4. Discussion

Many organic solvents are toxic to metabolically active microbial cells [5]. The use of aqueous-organic two-phase media is one possible solution to this problem [4,5,13–19]. On the other hand, the use of solvent-tolerant microorganisms, including *Pseudomonas*, *Bacillus*, and *Rhodococcus* species, is beneficial for whole-cell bioconversion in two-phase media [19]. Organic monophase media could be an alternative to aqueous-organic

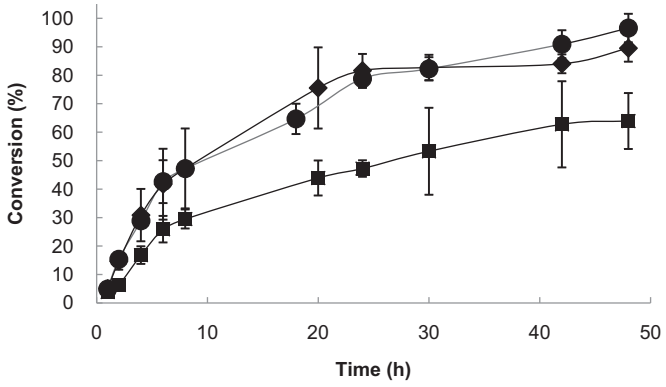

Fig. 7. Effect of NADH addition on the conversion of TFAP to TFMBA. A mass of 800 mg of heated-treated wet cells was added to 2 mL of a reaction mixture containing 3.7 M TFAF and 4.8 M cyclohexanol. At the start of incubation at 60 °C, 3.2 (squares), 32 (circles), or 320 μL (diamonds) of Milli-Q water containing 250 mM NADH was added to the reaction mixture. The NADH concentrations in the reaction mixture were 0.4 (squares), 4.0 (circles), and 40 mM (diamonds). Error bars represent the standard deviations of two to four independent reactions.

Table 2
Effect of the addition of water containing NADH on the conversion of TFAP to TFMBA.

NADH supply	NADH concentration in the reaction mixture (mM)	Conversion of TFAP to TFMBA by 48 h (%)
No addition of NADH	0	Not detected
Addition of 32 μL water containing 250 mM NADH	4.0	97%
Addition of 320 μL water containing 25 mM NADH	4.0	90%
Addition of 320 μL water containing 250 mM NADH	40	90%

A mass of 800 mg of heated-treated wet cells was added to 2 mL of a reaction mixture consisting of 3.7 M TFAP and 4.8 M cyclohexanol (1:1, v/v ratio). At the start of incubation at 60 °C, 32 μL water, 32 μL aqueous 250 mM NADH (the final concentration of 4.0 mM NADH in the reaction mixture), 320 μL aqueous 25 mM NADH (the final concentration of 4.0 mM NADH), or 320 μL aqueous 250 mM NADH (the final concentration of 40 mM NADH) was added to the reaction mixture.

two phase systems, when both substrate and product are water-insoluble or unstable in water [21]. In previous studies [21,29,41,42], organic solvents have been used to dissolve water-immiscible hydrophobic substrates for monophase media. The conversion of cholesterol to cholest-4-ene-3-one has been performed using *Nocardia* species in carbon tetrachloride [41] and *Bacillus subtilis* AF 333249 in toluene [42]. Yamashita et al. [29] have reported that the hydrophobic bacterium *R. opacus* B-4 could convert indole to indigo in an essentially water-free *bis* (2-ethylhexyl) phthalate (BEHP).

Lyophilized *E. coli* has also been used for bioconversion in organic media [21,43,44]. Lyophilized *E. coli* cells, which overexpress an alcohol dehydrogenase from *Rhodococcus ruber*, have been used in a micro-aqueous system with 99% (v/v) isopropanol [43]. Lyophilized *E. coli* cells, which express the *Candida parapsilosis* carbonyl reductase, have been applied to a reaction medium containing only water-immiscible hydrophobic substrates. This system could convert 300–500 g/L acetophenone to (*S*)-phenylethanol with a product yield of 98% or greater in 14 days [44]. Lyophilized *Saccharomyces cerevisiae* has also been used for the reduction of keto esters in organic media [45]. Rather et al. [46] have reported the use of lyophilized cells of the thermotolerant yeast *Pichia etchellsii* for the synthesis of long-chain alkyl glycosides. In these systems, biocatalytic activity increased with increasing water activity. Scholz et al. [21] have shown that living *E. coli* cells exhibit a higher activity to produce chiral cyanohydrins from various aldehydes and hydrogen cyanide than lyophilized *E. coli* cells. Although lyophilization is useful for improving cell permeability, it seems costly and time-consuming. In the present study, the hydrophobic bacterium *R. rhodochrous* was used as the biocatalyst in solvent-free organic media without cell lyophilization. By suspending heat-treated *R. rhodochrous* cells in organic media consisting of the hydrophobic substrates TFAP and cyclohexanol, approximately 3.6 M TFMBA was produced from TFAP in 48 h (Fig. 7). This is the first report on the use of hydrophobic bacterial cells for bioconversion in solvent-free organic media.

Hydrophobic bacteria could be well dispersed in organic media [29]. Species in the mycolata family of actinomyces, including *Rhodococcus* species, contain mycolic acids in their cell wall. This is likely responsible for their cell surface hydrophobicity [47]. In the present study, *R. rhodochrous* NBRC15564 was used as the whole-cell biocatalyst, since it showed the highest affinity to hydrophobic chemicals among the bacterial strains examined. The TFMBA yield by *R. rhodochrous* increased in increasing cell concentration in organic media (Fig. 3b). By contrast, no significant increase in the yield of TFMBA was detected with the hydrophilic *E. coli* cells. This implies the advantage of hydrophobic cells over hydrophilic cells as whole-cell catalysts in solvent-free organic media. Cell-surface hydrophobicity may favor the interaction between bacterial cells and hydrophobic compounds. This likely allows *R. rhodochrous* to exhibit a high bioconversion activity in solvent-free organic media. In addition, the cell pellets of *R. rhodochrous* could be readily removed from the reaction mixture after 48 h of incubation by centrifugation ($800 \times g$ for 10 min). No slurry or gel-like material was detected with the supernatant by visual observation. When 800 mg *R. rhodochrous* cells were collected from 2 mL aqueous cell suspension by centrifugation at $8000 \times g$ for 10 min, the resulting cell pellet contained about 10% (w/w) extracellular water (data not shown). This indicates that about 80 μL water (equivalent to 80 mg water) was removed into the cell pellet from the 2 mL cell suspension. Assuming that the extracellular space of cell pellet was filled with the organic media, product loss from 2 mL organic media due to cell separation could be approximately 5%.

Alcohol dehydrogenases display low to moderate enantioselectivities in aqueous monophase systems [44]. Pennacchio et al. [30] have shown that purified ADH_{Tt1} exhibits more than 93% enantioselectivity in the conversion of TFAP to (*R*)-(−)-TFMBA in aqueous environments. In the present study, ADH_{Tt1} in *R. rhodochrous* cells showed an approximately 52% enantioselectivity in the organic media. Scholz et al. [21] have reported that wet *E. coli* cells showed a lower enantioselectivity for 2-chloromandelonitrile, 2-fluoromandelonitrile and 2-furaldehyde than lyophilized cells. del Olmo et al. [48] have also shown that *S. cerevisiae* could carry out the asymmetric aldol condensation between 4-nitrobenzaldehyde and acetone with the highest enantioselectivity of 45% ee at 2.5% of water in organic media. In this case, the enantioselectivity decreased from 45 to 5% with increasing water content from 2.5 to 50%. Taking together, it cannot be ruled out that the water brought into the reaction mixture caused a reduction of the enantioselectivity of ADH_{Tt1} in organic media in the present study. Further study is needed to clarify the effect of water content on enantioselectivity of enzymes in organic media. Unfortunately, due to loss of ADHs activity, heat-treated cells could not be re-used in the present model system.

Heat treatment is likely to improve membrane permeability in addition to the inactivation of indigenous mesophilic enzymes. Restiawaty et al. [49] have reported that heat-treated *E. coli* recombinants could effectively convert glycerol to glycerol-3-phosphate using polyphosphate as the extracellular phosphate donor. The improvement in membrane permeability by heat treatment allowed high-molecular-weight polyphosphates to diffuse into the cytoplasmic space where they were used in glycerol phosphorylation catalyzed by thermostable polyphosphate kinase. Moreover, bioconversion at elevated temperatures has several advantages such as the prevention of contamination, the decrease in viscosity, and the enhancement of diffusion [50]. However, it should be noted that volatile chemicals are difficult to use as substrate in organic media at elevated temperature. This was the reason that cyclohexanol, but not isopropanol, was employed as the substrate for NADH regeneration by ADH_{Tt2} in the present study.

In summary, 97% of 3.7 M TFAP was converted to TFMBA using 800 mg heat-treated *R. rhodochrous* wet cells after 48 h in solvent-free organic media consisting of 3.7 M TFAP and 4.8 M cyclohexanol (1:1, v/v ratio). The final product concentration reached about 3.6 M TFMBA at an NADH turnover number of approximately 900. The use of solvent-free organic media is potentially beneficial, since organic solvents are often toxic, costly and harmful to the environment.

Acknowledgment

This work was supported in part by a grant-in-aid for scientific research from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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