Journal of Bioscience and Bioengineering
VOL. 127 No. 2, 145–149, 2019

www.elsevier.com/locate/jbiosc

Expression of engineered carbonyl reductase from *Ogataea minuta* in *Rhodococcus opacus* and its application to whole-cell bioconversion in anhydrous solvents

Kohsuke Honda,[1,*] Tomohiro Ono,[1] Kenji Okano,[1] Ryoma Miyake,[2] Yasumasa Dekishima,[2] and Hiroshi Kawabata[2,3]

Department of Biotechnology, Graduate School of Engineering, Osaka University, 2-1 Yamadaoka, Suita, Osaka 565-0871, Japan,[1] Mitsubishi Chemical Corporation, Yokohama R&D Center, 1000 Kamoshida-cho, Aoba-ku, Yokohama 227-8502, Japan,[2] and API Corporation, 13-4 Uchikanda 1-chome, Chiyoda-ku, Tokyo 101-0047, Japan[3]

Received 28 March 2018; accepted 10 July 2018
Available online 31 July 2018

The carbonyl reductase from the methylotrophic yeast *Ogataea minuta* can catalyze the regio- and enantio-selective reduction of prochiral ketones to chiral alcohols, and is available for industrial manufacturing of statin drugs. We previously conducted a directed evolution experiment of the enzyme, and obtained a mutant (OCR_V166A) with improved tolerance to organic solvents. This expanded the applicability of the enzyme to the bioconversion of water-insoluble compounds (Honda et al., J. Biosci. Bioeng., 123, 673–678, 2017). In the present study, we expressed OCR_V166A in *Rhodococcus opacus* cells, which have a highly lipophilic surface structure and are dispersible in anhydrous organic solvents, and developed a whole-cell biocatalyst which can function in an organic-solvent-based reaction medium. The secondary alcohol dehydrogenase from *Thermoanaerobacter ethanolicus* (TeADH) was employed as an NADPH-regenerating enzyme and co-expressed with OCR_V166A in *R. opacus*. The whole-cell bioconversion of 2,2,2-trifluoroacetophenone to α-(trifluoromethyl)benzyl alcohol was performed in organic solvents, including isopropanol, isobutanol, and cyclohexanol, which served both as reaction media and as substrates for TeADH. The type of organic solvents markedly affected not only the product titer but also the enantio-purity of the product. When isobutanol was used as the reaction medium, the whole-cell biocatalyst showed higher stability than the isolated enzyme. Consequently, a high concentration (1 M) of the substrate was converted to the product with an overall conversion yield of 81% (mol/mol) in 24 h.

[**Key words:** Carbonyl reductase; Whole-cell biocatalysis; Thermal stability; Organic solvent; *Rhodococcus opacus*]

Carbonyl reductases and related enzymes are among the most intensively studied enzymes in the field of industrial biocatalysis. They catalyze the asymmetric reduction of prochiral ketones to enantiomerically pure secondary alcohols, and have been used for the biocatalytic manufacturing of chiral building blocks for pharmaceuticals, such as statins, a class of cholesterol-lowering drugs (1). Among these enzymes is a carbonyl reductase from the methylotrophic yeast *Ogataea minuta* (OCR), which is capable of catalyzing the regio- and enantio-selective reduction of a statin precursor using NADPH as the reducing cofactor (2). We recently performed a directed evolution experiment to improve OCR, and obtained a mutant enzyme with significantly improved stability (3). The mutant, which possesses the amino acid substitution V166A, had a half-life 6.1 times longer than that of the wild type at 50 °C. More importantly, the mutant (OCR_V166A) was shown to have improved tolerance to organic solvents; the calculated half-life of the enzyme at 50°C in the presence of 20% (v/v) dimethyl sulfoxide (DMSO) increased 11-fold to 13 h with the mutation. In a bioconversion assay in the presence of DMSO using 2,2,2-trifluoroacetophenone (TFAP) as a model substrate, the mutant was able to convert the substrate with a molar yield of 71%, whereas an equivalent concentration of the wild-type enzyme had a conversion yield of only 27%. However, similar to most statin drugs, TFAP is poorly soluble in aqueous solutions even in the presence of DMSO. Thus, only a modest concentration (60 mM, approximately 10 g/L) of TFAP could be used in our previous study. A possible approach to apply the enzyme to the conversion of higher concentration of TFAP and other water-insoluble substrates is its use in aqueous/organic two-phase systems (4,5). In two-phase systems, an aqueous solution containing biocatalysts is mixed with a water-immiscible organic solvent in which a high concentration of substrate has been dissolved. The substrate is slightly but continuously partitioned into the aqueous phase, converted by the biocatalyst, and then extracted back into the organic phase (6). The partitioning rate of the substrate into the aqueous phase increases in correlation with the specific interfacial area between the organic and aqueous phases as well as with the difference in the substrate concentration between these phases. It is therefore essential to maintain a sufficient specific interfacial area by vigorously mixing the organic and aqueous phases in order to achieve a high conversion rate when using two-phase systems.

Rhodococcus opacus strain B-4, which was originally isolated from gasoline-contaminated soil (7), has a lipophilic cell-surface structure and a high affinity for water-immiscible organic solvents in aqueous/

* Corresponding author. Tel.: +81 6 6879 7438; fax: +81 6 6879 7439.
E-mail address: honda@bio.eng.osaka-u.ac.jp (K. Honda).

1389-1723/$ – see front matter
https://doi.org/10.1016/j.jbiosc.2018.07.011

organic two-phase systems (8–10). Furthermore, wet cells of *R. opacus* are even dispersible in anhydrous solvents, where they form small cell aggregates. Owing to this unique feature, they can serve as a capsule to deliver enzymes into anhydrous reaction media. The use of this lipophilic whole-cell biocatalysis in organic solvents leads not only to a lower total volume of the reaction mixture but also to a larger specific interfacial area between the solvent and biocatalyst as compared to those in conventional aqueous/organic two-phase systems. In fact, we have previously demonstrated that high concentrations of water-insoluble substrates (3.7 M TFAP and 1.0 M ketoisophorone) can be converted with sufficient yields (97 mol% for TFAP, and 63 mol% for ketoisophorone) using *R. opacus* and another rhodococcal strain as whole-cell biocatalysts in anhydrous reaction media (11,12). In this study, we aimed to expand the applicability of OCR_V166A in the bioconversion of higher concentrations of water-insoluble substrates. To this end, we introduced and co-expressed genes encoding the mutant OCR and an $NADP^+$-dependent secondary alcohol dehydrogenase from *Thermoanaerobacter ethanolicus* (TeADH) in *R. opacus* and conducted whole-cell bioconversion in anhydrous reaction media.

MATERIALS AND METHODS

Microorganisms and plasmids Microorganisms and plasmids used in this study are listed in Table 1. DNA manipulation was performed according to standard protocols. Codon-optimized genes were synthesized by GeneArt (Thermo Fischer Scientific, Waltham, MA, USA). Codon optimization for the gene expression in *Escherichia coli* was done using the supplier's program. The codon-optimized gene encoding TeADH were digested with *Nde*I and *Xho*I and then inserted at corresponding sites in the plasmid pET21a (Merck Japan, Tokyo, Japan). The resulting plasmid was designated as pET-TeADH and transformed into *E. coli* BL21(DE3) cells. The vector used for the expression of OCR_V166A in *E. coli* was constructed as described previously (3). For the expression in *R. opacus*, genes encoding OCR_V166A and TeADH were codon-optimized using an online application (13). Both genes were digested with *Nde*I and *Xho*I, and inserted at corresponding sites in the plasmids pTip-QT2 and pTip-RC2 (14), yielding the following four plasmids: pQT-OCR_V166A, pRC-OCR_V166A, pQT-TeADH, and pRC-TeADH (Table 1). The plasmids were transformed into *R. opacus* cells through electroporation as described elsewhere (8).

Enzyme preparation Recombinant *E. coli* cells were cultivated at 37°C in LB medium supplemented with 100 μg mL^{-1} ampicillin. Gene expression was induced by adding 0.2 mM isopropyl-β-D-thiogalactopyranoside during the late log phase. The cells were further cultivated for another 4 h and harvested by centrifugation (8000× *g*, 10 min). The cells were then resuspended in 100 mM potassium phosphate (pH 7.0) and disrupted using a UD-201 ultrasonicator (Kubota, Osaka, Japan). After cell debris were removed by centrifugation (12,000× *g*, 10 min), the supernatant was used as a crude extract. Recombinant *R. opacus* cells were cultivated at 30°C in Tryptic Soy Broth (BD Biosciences, San Jose, CA, USA). Tetracycline and chloramphenicol were added at 20 μg mL^{-1} to ensure stable maintenance of the plasmid vectors. Thiostrepton (20 μg mL^{-1}) was added to a culture in its late log phase in order to induce gene expression, and the cells were further cultivated for another 17 h. The cells were then harvested, resuspended, and disrupted in the same manner as the *E. coli* cells used to prepare the crude extract.

TABLE 1. Microorganisms and plasmids used in this study.

Microorganism/Plasmid	Note	Source/Reference
E. coli DH5α	General cloning	Takara Bio (Shiga, Japan)
E. coli BL21(DE3)	Gene expression	Merck Japan
R. opacus B-4	Gene expression and bioconversion	NBRC 108011, Na et al. (7)
pET21a	Gene expression in *E. coli*, Am^R	Merck Japan
pET-TeADH	Expression of TeADH in *E. coli*	This study
pET-OCR_V166A	Expression of OCR_V166A in *E. coli*	3
pTip-QT2	Gene expression in *R. opacus*, Tc^R, θ type	14
pTip-RC2	Gene expression in *R. opacus*, Cm^R, rolling-cycle type	14
pQT-OCR_V166A	Expression of OCR_V166A in *R. opacus*	This study
pQT-TeADH	Expression of TeADH in *R. opacus*	This study
pRC-OCR_V166A	Expression of OCR_V166A in *R. opacus*	This study
pRC-TeADH	Expression of TeADH in *R. opacus*	This study

Enzyme assay The enzyme activity of OCR_V166A was determined as described previously (3), but with a reaction temperature of 60°C. Briefly, a mixture consisting of 100 mM potassium phosphate (pH 7.0), 0.2 mM NADPH, and an appropriate amount of the enzyme was incubated at 60°C for 2 min. The reaction was then initiated by adding a stock solution of TFAP (100 mM in DMSO) to a final concentration of 1 mM. The consumption of NADPH was spectrophotometrically monitored at 340 nm. The standard TeADH assay was performed using isopropanol as a substrate. A mixture containing 100 mM potassium phosphate (pH 7.0), 0.2 mM $NADP^+$, and an appropriate amount of the enzyme was incubated at 60°C for 2 min. The reaction was then initiated by adding isopropanol solution (1 M in H_2O) to a final concentration of 10 mM. The reaction rate was determined by monitoring NADPH formation at 340 nm.

Bioconversion Wet cells of recombinant *R. opacus* (150 mg wet weight) and 0.55 mg (0.74 μmol) of NADPH dissolved in 10 μL water were placed in a 1.5-mL microtube. A mixture of 68 μL (0.5 mmol) of TFAP and 432 μL of organic solvents (isopropanol, isobutanol, or cyclohexanol) was added to the tube, and the mixture was vortexed to suspend the cells. The mixture was then incubated at 60°C using a thermo-mixer (Eppendorf, Hamburg, Germany) with shaking at 1500 rpm (mixing orbit: 3 mm). After incubation, the mixture was centrifuged to remove the cells, and the supernatant was analyzed through gas chromatography (GC). GC analysis was performed using a chiral capillary column (MEGA-DEX DET-Beta, 0.25 mm × 25 m) as described previously (3).

RESULTS AND DISCUSSION

Identification and characterization of an NADPH-regenerating enzyme Carbonyl reductases require cofactors, such as NADH and NADPH, to reduce their substrates. Since these cofactors are expensive, the integration of another enzyme that can regenerate these cofactors through the $NAD(P)^+$-dependent dehydrogenation of a cheap sacrificial co-substrate is crucial for the enzymatic production of chiral alcohols (4). In our previous study, we used a glucose-1-dehydrogenase from the hyperthermophilic archaeon, *Sulfolobus solfataricus* (SsGDH) as the cofactor-regenerating enzyme for conversion with OCR_V166A (3). Many (hyper)thermophilic enzymes are known to be tolerant not only to high temperatures but also to organic-solvent stresses, although the mechanisms underlying these characteristics have not been elucidated (15,16). In fact, SsGDH was shown to have high tolerance to an organic solvent and to be able to retain 80% of its initial activity after incubation with 20% (v/v) DMSO for 4 h. However, glucose, the substrate of SsGDH, is soluble only in aqueous solutions, and thus cannot be used for cofactor-regeneration in non-aqueous reaction media. Therefore, we searched the literature for another thermophilic enzyme capable of dehydrogenating an oil-soluble sacrificial substrate for the regeneration of NADPH, which is the preferred cofactor of OCR_V166A. Through this search, we found an $NADP^+$-dependent secondary alcohol dehydrogenase from the thermophilic bacterium *T. ethanolicus* 39E (TeADH) (17). The gene encoding TeADH was then expressed in *E. coli*, and the recombinant enzyme was characterized. While TeADH had an optimum reaction temperature of at least 90°C (Fig. 1A), the enzyme had 25% of the maximum activity at even 60°C, at which OCR_V166A optimally works (3). This observation was consistent with a previously reported thermal profile of the enzyme (18). Isopropanol and isobutanol could serve as good substrates of TeADH, but the enzyme showed considerably lower activity when cyclohexanol was used as the substrates (Fig. 1B). The enzyme showed no reducing activity against TFAP (data not shown). The stability of the enzyme was assessed by measuring its residual activity after incubation with or without these solvents (Fig. 2A). Without the solvents, enzyme activity was retained or even improved by incubation at 60°C. Meanwhile, the residual activity of the enzyme

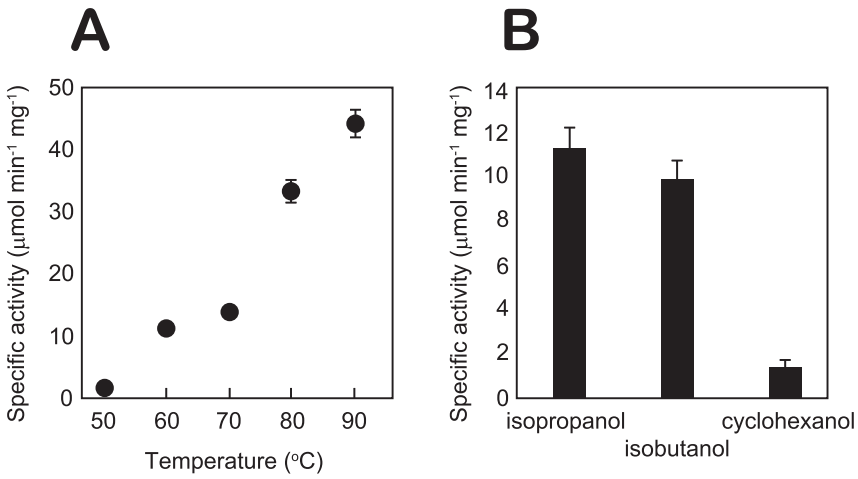

FIG. 1. Characterization of TeADH. (A) Thermal profile of the enzyme activity was assessed under standard conditions at the indicated temperatures. (B) Enzyme assays were performed using each indicated substrate at 10 mM concentration. The specific activity of the enzyme is expressed as the amount of NADPH produced per min per mg of total protein. Assays were performed in triplicate; means ± standard deviations (error bar) are shown.

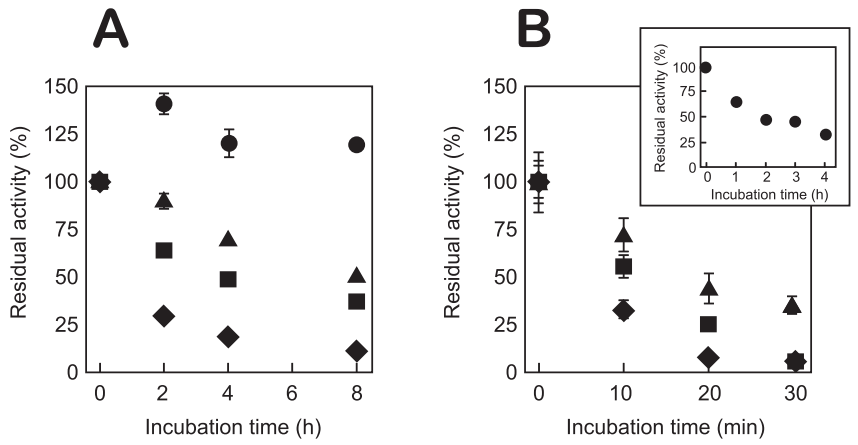

FIG. 2. Stability of TeADH (A) and OCR_V166A (B). Enzymes were incubated with 20% (v/v) isopropanol (square), isobutanol (diamond), and cyclohexanol (triangle) in 100 mM potassium phosphate (pH 7.0) at 60 °C with shaking at 1500 rpm. After incubation for the indicated time periods, the residual activity was determined. To determine the residual activity of TeADH, the enzyme was diluted at least 1000-fold in the assay mixture in order to reduce the effect of solvents carried over from the incubation mixture. The enzyme activity before incubation was defined as 100%. Enzyme stability without organic solvents are indicated by circles. The residual activity of OCR_V166A in the absence of organic solvents is separately shown in the inserted panel due to the difference in the time scale. Assays were performed in triplicate; means ± standard deviations (error bar) are shown.

decreased in a time-dependent manner upon incubation with organic solvents. The calculated half-lives of the enzyme in the presence of isopropanol, isobutanol, and cyclohexanol were estimated to be 5.9, 2.7, and 7.8 h, respectively. These half-lives were considerably longer than those of OCR_V166A under the same assay conditions (0.10, 0.10, and 0.32 h with isopropanol, isobutanol, and cyclohexanol, respectively; Fig. 2B). These observations indicate that TeADH is stable enough such that it does not limit the operation time of bioconversion with OCR_V166A when it was employed as a cofactor-regenerating enzyme.

Co-expression of OCR_V166A and TeADH The genes encoding OCR_V166A and TeADH were codon-optimized and co-expressed in *R. opacus* cells. Gene expression was achieved using two plasmid vectors, pTip-QT2 and pTip-RC2, which can autonomously replicate through the θ-type and rolling-cycle-type mechanisms, respectively, and thus are compatible with each other in rhodococcal cells (14). Two plasmid sets with different combinations of vectors and genes, namely pQT-OCR_V166A/pRC-TeADH and pRC-OCR_V166A/pQT-TeADH, were constructed and transformed into *R. opacus* cells. SDS-PAGE analysis of the crude extracts from these cells resulted in two extra protein bands, which were not found in the cell lysate of a control strain (Fig. 3). The estimated molecular sizes of these extra bands were similar to the calculated molecular weights of OCR_V166A (27 kDa) and TeADH (37 kDa). The specific enzyme activities in *R. opacus* cells harboring pQT-OCR_V166A/pRC-TeADH, and pRC-OCR_V166A/pQT-TeADH were 7.8 and 1.5, and 3.9 and 4.8 μmol min^{-1} mg^{-1} total protein, respectively. Regardless of encoding genes, pTip-QT2 resulted in higher protein expression levels than pTip-RC2. Similarly, Nakashima and Tamura (14) reported that a plasmid vector with the θ-type replication machinery showed comparable or higher protein expression levels than a rolling-cycle-type plasmid in rhodococcal strains, including *Rhodococcus erythropolis*, *Rhodococcus fascians*, and *R. opacus*. However, it remains unclear whether the differences in the expression levels were due to differences in transcriptional activities, plasmid stabilities, or the copy numbers of plasmids.

Bioconversion in anhydrous reaction media Wet cells of recombinant *R. opacus* with different combinations of plasmids (pQT-OCR_V166A/pRC-TeADH and pRC-OCR_V166A/pQT-TeADH) were subjected to bioconversion assays in anhydrous reaction media. OCR_V166A exhibits only modest enantio-selectivity to

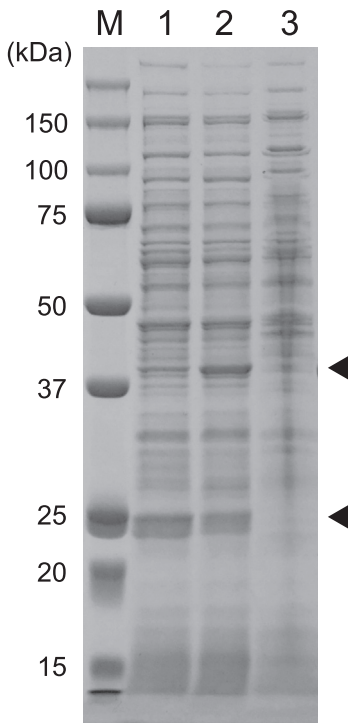

FIG. 3. SDS-PAGE analysis of the crude extracts of recombinant *R. opacus*. Crude extracts (approximately 15 mg total protein) were separated on a 12.5% (w/v) acrylamide gel. Proteins were visualized by staining with Coomassie Brilliant Blue. Lane M, molecular marker (Precision Plus Protein Standard, Bio-Rad, Hercules, CA, USA); lane 1, *R. opacus* with pQT-OCR_V166A/pRC-TeADH; lane 2, *R. opacus* with pRC-OCR_V166A/pQT-TeADH; lane 3, *R. opacus* with pTip-QT2/pTip-RC2. Protein bands corresponding to OCR_V166A and TeADH are indicated by arrowheads.

TFAP [about 40% *ee* for *R*-isomer; see Honda et al. (3)] as compared with that to the substrate for a statin manufacturing [a 3,5-diketoheptanoate ester derivative; see Hiraoka et al. (2)]. Nevertheless, TFAP was still employed as the model substrate owing to its high reactivity with OCR_V166A as well as its easy availability from commercial sources. Isopropanol, isobutanol, and cyclohexanol were used as both reaction media and sacrificial co-substrates for NADPH regeneration. Wet cells of recombinant *R. opacus* were mixed with a small volume of an NADPH solution and then dispersed in the solvents containing 1 M TFAP. Regardless of the plasmid combination, the use of isobutanol resulted in much higher product titers than those achieved using isopropanol and cyclohexanol as the reaction media (Table 2). The highest TFBA titer, which was 815 mM, was obtained when *R. opacus* cells harboring pRC-OCR_V166A/pQT-TeADH was used in combination with isobutanol. The recombinant cells with pRC-OCR_V166A, but without pQT-TeADH, produced only trace amount of TFBA (24.4 ± 7.8 mM) under the same reaction conditions, demonstrating that the bioconversion hardly proceeds without the cofactor regeneration mediated by TeADH (data not shown). Similarly, the low product titer in cyclohexanol was most probably due to the poor activity of TeADH towards this solvent (Fig. 1B). Meanwhile, the insufficient TFBA titer in isopropanol was unexpected because TeADH exhibited a slightly, but significantly, higher enzyme activity towards isopropanol than towards isobutanol. In addition, both OCR_V166A and TeADH showed higher tolerance to isopropanol than to isobutanol (Fig. 2). This unexpected result can be attributed to the higher dehydration ability of isopropanol. Isopropanol is a water-absorbing solvent, as indicated by its relatively low log P_{ow} (0.05) compared to that of isobutanol (0.8). Thus, when isopropanol is used as a reaction media, a large fraction of the inter- and intracellular water contained in the wet-cell pellet could be absorbed into the solvent phase, which in turn prevents the free diffusion of water-soluble enzymes and cofactors. It should be noted that not only the product titer but their enantio-purity was also markedly affected by the solvents. Whereas the use of isopropanol and isobutanol resulted in the similar enantio-purities ranging from 17% to 25% *ee* for the *R*-isomer, the enantio-selectivity of OCR_V166A was inverted in cyclohexanol (7.7–13% *ee* for *S*-isomer). We also performed the bioconversion assay in an aqueous reaction mixture with lower concentration of the solvents in order to investigate their effect on the enantio-selectivity of the enzyme in more detail. In this assay, cells harboring pRC-OCR_V166A and pQT-TeADH were incubated in 20 mM Tris–HCl (pH7.5) containing 3% (v/v) of 1 M TFAP solutions dissolved in isobutanol, isopropanol, and cyclohexanol. The solvents and substrate were apparently soluble in the reaction mixture at this concentration. When isopropanol and isobutanol were used, (*R*)-TFBA with enantio-purities of 45% and 55%, respectively, were obtained. These values were comparable to that obtained in our previous study (41% *ee* for *R*-isomer), in which the enzyme conversion was carried out in an aqueous medium containing 20% (v/v) dimethylsulfoxide (3). When the reaction was performed with 3% (v/v) cyclohexanol, OCR_V166A exhibited the opposite enantio-selectivity to that in the anhydrous solvent and (*R*)-TFBA with an enantio-purity of 17% *ee* was produced. These observations indicate that not only types of the solvent but their concentrations in the reaction mixture also has a significant impact on the enzyme enantio-selectivity. The biocatalytic conversion of water-insoluble chemicals in nearly and completely anhydrous solvents has been widely studied in the past few decades (19). Most of these studies focused on the use of lyophilized hydrolytic enzymes, such as lipases and esterases, and cannot be simply compared with whole-cell biocatalysis. However, the drastic alteration of enzyme enantio-selectivity in different organic solvents has often been observed in these studies (20). Several hypothetical mechanisms underlying this phenomenon have been proposed, such as differences in the solvation effects of organic solvents on substrates (21) and conformational changes in the enzymes caused by their interaction with solvents (22). Although these hypotheses appear to be valid for some specific combinations of enzymes and substrates, none of them are generally applicable in predicting the effects of different types of solvents on the selectivity of different classes of enzymes. The effect on the enantio-selectivity of enzymes, as well as the impact on enzyme activity and stability, can therefore be an important criterion in the screening of organic solvents used as reaction media for non-aqueous biocatalysis. The time profile of TFBA production in isobutanol showed that the whole-cell biocatalyst had a significantly higher operational stability than isolated enzymes (Fig. 4). OCR_V166A lost more than 95% of its activity in only 30 min when the free enzyme was incubated with isobutanol at 60 °C with (Fig. 2B). In contrast, bioconversion using the recombinant *R. opacus* cells was sustained for at least 12 h at the same temperature. This indicates that *R. opacus* cells can serve not only to deliver an enzyme into

TABLE 2. Bioconversion with recombinant *R. opacus* in different organic solvents.

Plasmids	Organic solvent	TFBA (mM)	Enantio-purity (% *ee*)[a]
pQT-OCR_V166A	Isopropanol	90 ± 1.5	22 ± 0.24 for (*R*)
pRC-TeADH	Isobutanol	510 ± 8.7	17 ± 0.81 for (*R*)
	Cyclohexanol	58 ± 7.1	13 ± 0.55 for (*S*)
pRC-OCR_V166A	Isopropanol	62 ± 7.6	25 ± 0.40 for (*R*)
pQT-TeADH	Isobutanol	815 ± 73	21 ± 0.12 for (*R*)
	Cyclohexanol	130 ± 2.2	7.7 ± 1.7 for (*S*)

[a] *ee*, enantiomer excess.

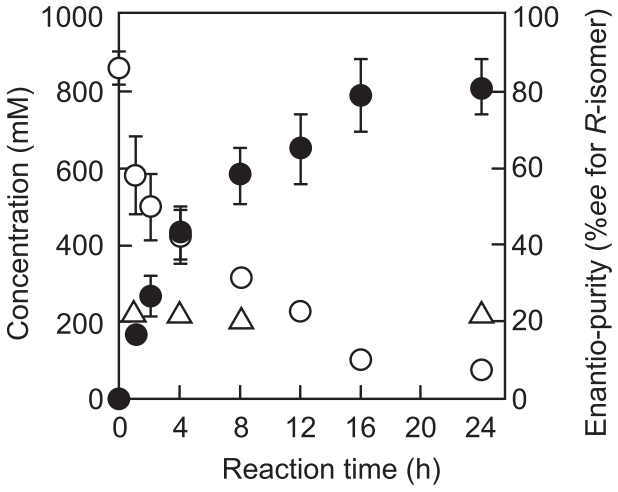

FIG. 4. Time profile of TFBA production with recombinant *R. opacus* harboring pRC-OCR_V166A/pQT-TeADH. Concentrations of TFAP (open circle) and TFBA (closed circle), and enantio-purity of the product (triangle) are shown. Assays were performed in triplicate; means ± standard deviations (error bar) are shown.

non-aqueous organic solvents, but also to relieve the negative effects of the solvents on enzyme stability, probably by preventing direct contact between the enzyme and the solvents.

Conclusion In the present study, the genes encoding OCR_V166A and TeADH were co-expressed in *R. opacus*, and the recombinant cells were used as lipophilic whole-cell biocatalysts in anhydrous reaction media. The concentration of the substrate used (1 M) and product titer (815 mM) were markedly increased compared to those achieved in an aqueous reaction medium using isolated enzymes [initial substrate concentration, 60 mM; final product titer, 43 mM; see Honda et al. (3)]. In addition, the whole-cell biocatalysts exhibited higher stability than the isolated enzyme. These observations demonstrated that lipophilic whole-cell biocatalysis can serve to improve the catalytic performances of OCR_V166A and related enzymes and to expand their applicability in the bioconversion of water-insoluble chemicals.

ACKNOWLEDGMENTS

This study is funded by Mitsubishi Chemical Corporation. RM, YD, and HK are employees of Mitsubishi Chemical Corporation. OCR, its derivatives, and a chemical manufacturing process using them are patented by Mitsubishi Chemical Corporation.

References

1. **Bornscheuer, U. T., Huisman, G. W., Kazlauskas, R. J., Lutz, S., Moore, J. C., and Robins, K.:** Engineering the third wave of biocatalysis, Nature, **485**, 185–194 (2012).
2. **Hiraoka, H., Ueda, M., and Hara, M.:** Novel carbonyl reductase, gene encoding it and process for producing optically active alcohols using the same, WO2003/078634 A1 (2003).
3. **Honda, K., Inoue, M., Ono, T., Okano, K., Dekishima, Y., and Kawabata, H.:** Improvement of operational stability of *Ogataea minuta* carbonyl reductase for chiral alcohol production, J. Biosci. Bioeng., **123**, 673–678 (2017).
4. **Kataoka, M., Kita, K., Wada, M., Yasohara, Y., Hasegawa, J., and Shimizu, S.:** Novel bioreduction system for the production of chiral alcohols, Appl. Microbiol. Biotechnol., **62**, 437–448 (2003).
5. **Goldberg, K., Schroer, K., Lütz, S., and Liese, A.:** Biocatalytic ketone reduction -a powerful tool for the production of chiral alcohols- part I: processes with isolated enzymes, Appl. Microbiol. Biotechnol., **76**, 237–248 (2007).
6. **Hamada, T., Maeda, Y., Matsuda, H., Sameshima, Y., Honda, K., Omasa, T., Kato, J., and Ohtake, H.:** Effect of cell-surface hydrophobicity on bacterial conversion of water-immiscible chemicals in two-liquid-phase culture systems, J. Biosci. Bioeng., **108**, 116–120 (2009).
7. **Na, K. S., Kuroda, A., Takiguchi, N., Ikeda, T., Ohtake, H., and Kato, J.:** Isolation and characterization of benzene-tolerant *Rhodococcus opacus* strains, J. Biosci. Bioeng., **99**, 378–382 (2005).
8. **Yamashita, S., Satoi, M., Iwasa, Y., Honda, K., Sameshima, Y., Omasa, T., Kato, J., and Ohtake, H.:** Utilization of hydrophobic bacterium *Rhodococcus opacus* B-4 as whole-cell catalyst in anhydrous organic solvents, Appl. Microbiol. Biotechnol., **74**, 761–767 (2007).
9. **Honda, K., Yamashita, S., Nakagawa, H., Sameshima, Y., Omasa, T., Kato, J., and Ohtake, H.:** Stabilization of water-in-oil emulsion by *Rhodococcus opacus* B-4 and its application to biotransformation, Appl. Microbiol. Biotechnol., **78**, 767–773 (2008).
10. **Hamada, T., Sameshima, Y., Honda, K., Omasa, T., Kato, J., and Ohtake, H.:** A comparison of various methods to predict bacterial predilection for organic solvents used as reaction media, J. Biosci. Bioeng., **106**, 357–362 (2008).
11. **Hibino, A. and Ohtake, H.:** Use of hydrophobic bacterium *Rhodococcus rhodochrous* NBRC15564 expressed thermophilic alcohol dehydrogenases as whole-cell catalyst in solvent-free organic media, Process Biochem., **48**, 838–843 (2013).
12. **Tsuji, N., Honda, K., Wada, M., Okano, K., and Ohtake, H.:** Isolation and characterization of a thermotolerant ene reductase from *Geobacillus* sp. 30 and its heterologous expression in *Rhodococcus opacus*, Appl. Microbiol. Biotechnol., **98**, 5925–5935 (2014).
13. **Puigbò, P., Guzmán, E., Romeu, A., and Garcia-Vallvé, S.:** OPTIMIZER: a web server for optimizing the codon usage of DNA sequences, Nucleic Acids Res., **35**, W126–W131 (2007).
14. **Nakashima, N. and Tamura, T.:** Isolation and characterization of a rolling-cycle-type plasmid from *Rhodococcus erythropolis* and application of the plasmid to multiple-recombinant-protein expression, Appl. Environ. Microbiol., **70**, 5557–5568 (2004).
15. **Owusu, R. K. and Cowan, D. A.:** Correlation between microbial protein thermostability and resistance to denaturation in aqueous:organic solvent two-phase systems, Enzyme Microb. Technol., **11**, 568–574 (1989).
16. **Atomi, H.:** Recent progress towards the application of hyperthermophiles and their enzymes, Curr. Opin. Chem. Biol., **9**, 166–173 (2005).
17. **Burdette, D. and Zeikus, J. G.:** Purification of acetaldehyde dehydrogenase and alcohol dehydrogenases from thermoanaerobacter ethanolicus 39E and characterization of the secondary-alcohol dehydrogenase (2 Adh) as a bifunctional alcohol dehydrogenase-acetyl-CoA reductive thioesterase, Biochem. J., **302**, 163–170 (1994).
18. **Burdette, D., Tchernajencko, V., and Zeikus, J. G.:** Effect of thermal and chemical denaturants on Thermoanaerobacter ethanolicus secondary-alcohol dehydrogenase stability and activity, Enzyme Microb. Technol., **27**, 11–18 (2000).
19. **Klibanov, A. M.:** Improving enzymes by using them in organic solvents, Nature, **409**, 241–246 (2001).
20. **Carrea, G., Ottolina, G., and Riva, S.:** Role of solvents in the control of enzyme selectivity in organic media, Trends Biotechnol., **13**, 63–70 (1995).
21. **Terradas, F., Teston-Henry, M., Fitzpatrick, P. A., and Klibanov, A. M.:** Marked dependence of enzyme prochiral selectivity on the solvent, J. Am. Chem. Soc., **115**, 390–396 (1993).
22. **Hirose, Y., Kariya, K., Sasaki, I., Kurono, Y., Ebiike, H., and Achiwa, K.:** Drastic solvent effect on lipase-catalyzed enantioselective hydrolysis of prochiral 1,4-dihydropyridines, Tetrahedron Lett., **33**, 7157–7160 (1992).