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Outline of Research Result

Development of a Water/Solvent-multiphase Bioprocess with Solvent Tolerant Bacteria

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Research aims

The use of organic solvents as reaction media has the potential to greatly expand the repertoire of biocatalytic transformations. Although it has been noticed that many enzymes can retain their activities in organic solvents containing little or no water, most of the work so far has involved relatively simple, hydrolytic enzymes[1]. Therefore, attention has been paid to the development of a whole-cell catalyst that functions in nonaqueous environments.

Rhodococcus opacus B4, which has recently been isolated from a gasoline-contaminated soil, is highly tolerant to a variety of organic solvents[2]. During the course of this study, it was found that the bacterial cells avidly adhere to the organic phase when they were cultured in organic-aqueous biphasic media. Interestingly, wet cells of the bacteria were also shown to be dispersible in anhydrous organic solvents. Here, it is reported that *R. opacus* B4 is able to retain their metabolic activity even in anhydrous organic solvents and to mediate bioconversion as a whole-cell catalyst.

Methods

Viability and bioconversion assays. R. opacus B4 was aerobically cultivated on Tryptic Soy Broth (TSB). Cells collected from 5 ml of the culture were suspended in 2 ml of an organic solvent in a screw-capped tube. The cell suspension was incubated with shaking at 30°C. After the incubation for 1 to 5 days, cells were collected by centrifugation, 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 assays, the genes encoding four components of benzene dioxygenase, namely, *bnzA1*, *bnzA2*, *bnzA3*, and *bnzA4*[2], were constitutively expressed under the *tac* promoter in a recombinant plasmid pROPtacBnzA. *R. opacus* B4 bearing the expression plasmid was grown in 5 ml of TSB, collected by centrifugation, and suspended in 2 ml of an organic solvent containing 5 mg ml^{-1} indole. Bioconversion of indole to indigo was performed at 30°C for 12 h with shaking.

Metabolizing activity in BEHP. Wet cells were suspended in *bis*(2-ethylhexyl) phthalate (BEHP) to give a cell concentration of 2.5% (w/v), and then oleic acid was added to the suspension at a final concentration of 10 mg ml^{-1}. Ten milliliters of the mixture was transferred into a 50-ml baffled flask, and incubated at 30°C with orbital shaking. An aliquot of the mixture was sampled at time intervals of 3 h. After removing the cells by centrifugation, remaining oleic acid in the supernatant was fluorescence-labeled with 9-anthryldiazomethane and determined by HPLC.

Indigo production. Wet cells of *R. opacus* B4 (pROPtacBnzA) were suspended in BEHP at a concentration of 2.5% (w/v). Indole and oleic acid were added to the cell suspension at concentrations of 9 and 10 mg ml^{-1}, respectively. Ten milliliters of the mixture was transferred into a 50-ml baffled flask and incubated at 30°C with orbital shaking. An aliquot of the mixture was sampled at time intervals of 3 h. After removing the cells by centrifugation, indigo production was assessed by measuring the absorbance at 600 nm of the supernatant.

Results

Viability and biocatalytic ability in organic solvents

Wet cells of *R. opacus* B4 were well dispersible in anhydrous 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}). A solvent with a lower log P_{ow} shows a higher toxicity to microorganisms[3]. Fig. 1 shows the cell viabilities of *R. opacus* B4 in organic solvents having different values of log P_{ow}. *R. opacus* B4 cells were able to survive in middle- and long-chain *n*-alkanes, BEHP, and oleyl alcohol for at least 5 days. It has been reported that *R. opacus* B4 was able to grow on a medium containing 10% (v/v) monoaromatics, including benzene, toluene, xylene, and ethylbenzene[2]. However, the bacterium was unable to survive in essentially water-free monoaromatics. The biocatalytic ability was also investigated using the conversion of indole to indigo as a model reaction (Fig. 1). No significant relationship was observed between cell viability and indigo production. Among the tested solvents, BEHP was most suitable for indigo production.

Metabolizing activity in BEHP

When *R. opacus* B4 cells were incubated in BEHP containing 1% (w/v) oleic acid, oleic acid concentration significantly decreased with time as shown in Fig. 2. The consumption rate was nearly constant during 12 h of incubation. Approximately 64% of the initial amount of oleic acid was consumed by 12 h. No significant decrease in the

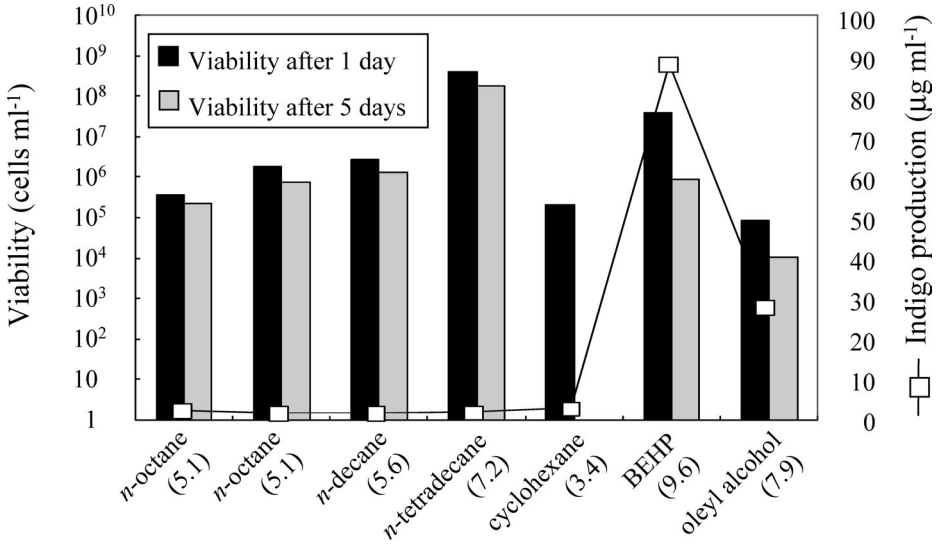

Fig. 1. Cell viability of and indigo production by *R. opacus* B4 in organic solvent. The initial concentration of the bacterial suspension was 2.35×10^9 cells ml^{-1}. The value of log P_{ow} is shown in parentheses.

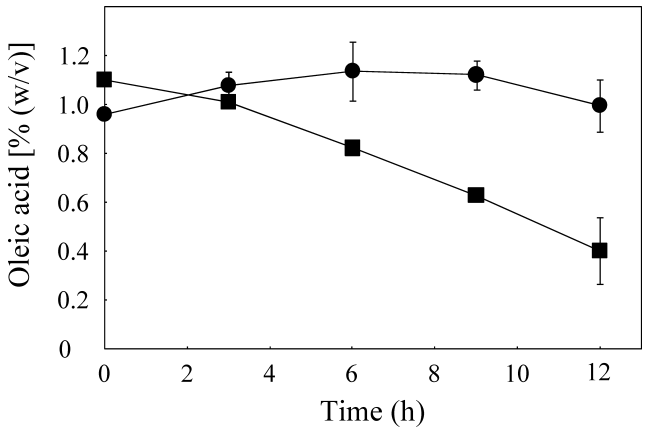

Fig. 2. Oleic acid consumption by intact (squares) or heat-killed (circles) cells of *R. opacus* B4 in BEHP.

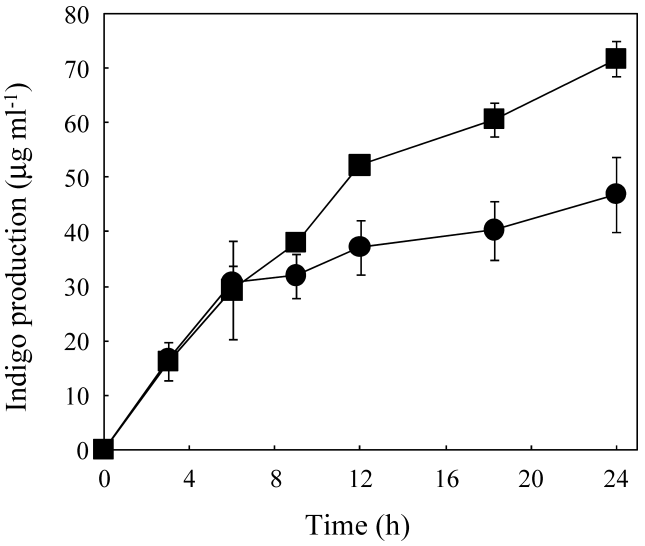

Fig. 3. Indigo production from indole by *R. opacus* B4 in BEHP. Experiments were performed with (squares) or without (circles) 10 mg ml^{-1} of oleic acid.

amount of oleic acid was observed in blind tests with heat-killed cells.

Indigo production

Because bioconversions catalyzed by benzene dioxygenase require NADH as a cofactor, NADH regeneration is essential for reactions. Bioconversion of indole to indigo by *R. opacus* B4 transformants was carried out in anhydrous BEHP by employing oleic acid as an oil-soluble energy source (Fig. 3). The rates of indigo production were not different 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 of incubation. In the presence of oleic acid, 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.

Conclusion

In the present study, the author developed a single-phase solvent culture technique using a solvent-tolerant bacterium *R. opacus* B4 as a whole-cell biocatalyst. *R. opacus* B4 has a high hydrophobicity and exhibits a high affinity for water-immiscible hydrocarbons. This bacterium was able to survive for at least 5 days in organic solvents, including *n*-tetradecane, oleyl alcohol, and BEHP. *R. oapcus* B4 transformants carrying pROPtacBnzA catalyzed the oxidation of indole to indigo in anhydrous BEHP. The transformants were able to catabolize oleic acid in BEHP and thus regenerate NADH. This approach is potentially applicable to other cofactor-dependent enzymatic conversions of water-immiscible compounds.

References

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