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Note

Asymmetric Reduction of Benzil to (*S*)-Benzoin with *Penicillium claviforme* IAM 7294 in a Liquid-Liquid Interface Bioreactor (L-L IBR)

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The asymmetric reduction of benzyl to (*S*)-benzoin with *Penicillium claviforme* IAM 7294 was applied to a liquid-liquid interface bioreactor (L-L IBR) using a unique polymeric material, ballooned microsphere (MS). The L-L IBR showed superior performance, as compared with suspension, organic-aqueous two-liquid-phase, and solid-liquid interface bioreactor (S-L IBR) systems, affording 14.4 g/l-organic phase of (*S*)-benzoin (99.0% ee).

Key words: *Penicillium*; benzoin; liquid-surface cultivation; interface bioreactor; asymmetric reduction

It is well known that many fungi catalyze the asymmetric reduction of various prochiral ketones.[1,2] Benzil is also reduced to optically active benzoin, a useful chiral auxiliary reagent, by some fungi, such as *Rhizopus* and *Rhizomucor*,[3] and also by some bacteria.[4–6] However, it has been difficult to produce high concentrations of optically active benzoin because of the low solubility of benzil, the toxicities of benzil and benzoin, and difficulties in the control of fungal morphology. The low solubility of substrates leads to a decrease in the reaction rate and a loss of substrate. The toxicities of substrates and products lead to a decrease in substrate concentration and product accumulation, and fast inactivation of biocatalysts.[7–10] It is also well known that the morphology of fungi, filamentous or pelleted form, is a very important factor in application to fermentation and bioconversion.[11,12]

Recently, we developed a liquid-surface immobilization (LSI) system as a novel fungal cultivation system and a liquid-liquid interface bioreactor (L-L IBR) as an efficient fungal bioconversion system using a unique micro-material, a ballooned microsphere (MS).[13] Fungal cells suspended in a liquid medium are effectively floated and immobilized on the surface of a liquid medium by the fast crowing flow of the enormous MS particles (diameter, 20–30 μm; density, 0.03–0.20). The immobilized fungal cells form a physically strong fungus-MS mat on the surface of the liquid medium during stationary cultivation. We expected that the LSI system would be useful in the production of enzymes, spores, and secondary metabolites (antibiotics and alkaloids), while the L-L IBR is widely applicable in the bioconversion of water-insoluble substrates. Indeed, the hydrolysis of 2-ethylhexyl acetate with *Absidia coerulea* IFO 4423 in the L-L IBR proceeded more efficiently than with emulsion, organic-aqueous two-liquid-phase, or solid-liquid interface bioreactor (S-L IBR) systems.[13] Furthermore, the strain produced much lipase in the LSI system, as compared with submerged cultivation.[13] Hence, we expected that the asymmetric reduction of benzil would proceed efficiently as compared with above-mentioned traditional systems (Fig. 1).

First, we screened the best fungus catalyzing the asymmetric reduction of benzil using the S-L IBR system,[14–17] consisting of a modified Sabouraud agar plate (volume, 10 ml; surface area, 7.1 cm^2)[13] and a 1 or 3% solution of benzyl in decane-hexyl ether (1:1; volume, 1 ml). Three pieces (approximately 2×2 mm^2) of each strain were inoculated on the surface of the agar plate with a long toothpick. Following 3 d of stationary precultivation, the benzil solution was applied onto the surface of the agar plate. After 3 d of stationary incubation, 1 ml of 1-butanol was added to the organic phase and the combined organic phase was directly analyzed by HPLC. The column was Zorbax RX-SIL (4.6 mm i.d. × 250 mm; Agilent Technologies, Santa Clara, CA), the column temperature was 25 °C, the eluent was hexane-2-propanol (7:3), the flow rate was 1.5 ml/min, and detection was done by UV_{234}. The retention times for benzil, benzoin, and hydrobenzoin were 5.67, 6.39, and 9.60 min, respectively. Each compound was determined by absolute calibration curve method, which confirmed sufficient quantification. The

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Abbreviations: LSI, liquid-surface immobilization; S-L IBR, solid-liquid interface bioreactor; L-L IBR, liquid-liquid interface bioreactor; MS, microsphere; ee, enantiomeric excess

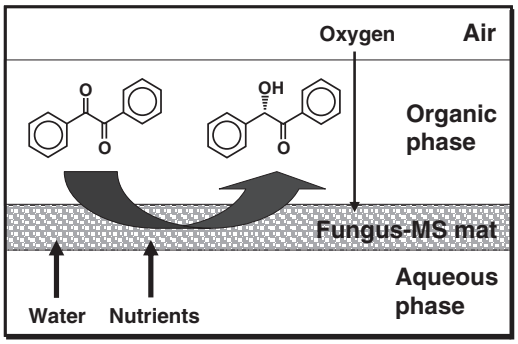

Fig. 1. Principle of the Asymmetric Reduction of Benzil to (*S*)-Benzoin in the Liquid-Liquid Interface Bioreactor (L-L IBR).

Cells of *P. claviforme* IAM 7294 were floated on the surface of a liquid medium by the aid of a microsphere (MS) to form a thick fungus-MS mat following stationary cultivation using nutrients and water in a liquid medium and oxygen in the atmosphere (liquid-surface immobilization system). The fungus-MS mat efficiently catalyzed the asymmetric reduction of benzil solubilized in a hexyl ether layer overlaid on the fungus-MS mat to (*S*)-benzoin.

combined organic phase was scarcely volatile, because the boiling points of decane and hexyl ether are 174 and 226 °C, respectively. The configuration of the product was determined by HPLC. The column was Chiralpak AD-H (2.6 mm i.d. × 250 mm; Daisel Chemical Industries, Osaka), the column temperature was 25 °C, the eluent was hexane-2-propanol (9:1), the flow rate was 0.8 ml/min, and detection was done by UV_{234}. The retention times for (*R*)- and (*S*)-benzoin were 8.25 and 10.86 min, respectively.

As shown in Table 1, many fungi, such as *Aspergillus* and *Penicillium*, reduced 1% benzil to afford (*S*)-benzoin in an organic phase at high conversion yield and ee, while some genera, such as *Chaetomium*, *Coriolus*, and *Schizophylum*, accumulated (*R*)-benzoin at moderate conversion yield and ee. Among the strains, *Penicillium claviforme* IAM 7294 was efficiently reduced 3% of benzil to afford 12.9 g/l of the organic phase of (*S*)-benzoin (95.2% ee) for 3 d incubation. On the other hand, *Rhizopus hangchao* IFO 4757, *Penicillium multicolor* f7147, and *Penicillium citrinum* IAM 7003 also accumulated over 12 g/l of the organic phase of (*S*)-benzoin, and the ee ranged from 76.9 to 89.3%. We selected *P. claviforme* IAM 7294 as the best strain for the production of (*S*)-benzoin.

In order to confirm the performance of the L-L IBR, the asymmetric reduction of benzil with *P. claviforme* IAM 7294 was examined by the suspension (submerged) and organic-aqueous two-liquid-phase systems, the S-L

Table 1. Fungal Strains Producing Optically Active Benzoin in the S-L IBR[a]

Strain	Benzoin		
	Concentration (g/l)[b]	Conversion yield (%)[c]	% ee
Aspergillus flavus IAM 2719	6.9	79	91.9 (*S*)
Aspergillus foetidus IAM 2393	6.5	71	96.2 (*S*)
Aspergillus oryzae var. *microsporus* O-14-5	6.8	69	91.6 (*S*)
Aspergillus parasiticus IAM 2723	6.6	96	93.2 (*S*)
Aspergillus parasiticus ATCC 15517	7.1	79	93.1 (*S*)
Aspergillus parasiticus IAM 2631	6.6	88	92.9 (*S*)
Aspergillus parasiticus IAM 2634	6.1	97	92.0 (*S*)
Aspergillus parasiticus IAM 2666	7.1	82	94.3 (*S*)
Aspergillus parasiticus IAM 2703	7.2	94	92.7 (*S*)
Aspergillus parasiticus IAM 2718	6.6	88	92.3 (*S*)
Cochliobolus abeanus FI-734	6.3	54	93.4 (*S*)
Penicillium citrinum IAM 7003	6.9	77	91.1 (*S*)
Penicilium claviforme IAM 7294	6.5	70	94.8 (*S*)
Penicillium funiculosum F-7084	7.1	77	95.8 (*S*)
Penicillium griseoroseum F-7209	6.1	65	90.4 (*S*)
Penicillium multicolor F-7147	6.7	68	93.1 (*S*)
Penicillium primulinum F-7231	7.0	82	93.6 (*S*)
Penicillium verrucosum F-7273	6.3	86	92.3 (*S*)
Phoma cucurbitacearum A-7	6.0	81	90.3 (*S*)
Pullularia pullulans F-24	6.9	78	91.7 (*S*)
Rhizopus hangchao IFO 4757	6.3	64	92.1 (*S*)
Aspergillus flavus WT-3-8	6.3	97	92.0 (*S*)
Chaetomium globosum ATCC 6205	5.0	71	85.2 (*R*)
Coriolus hirsutus IFO 4917	5.1	71	40.9 (*R*)
Schizophylum commune IFO 6505	3.7	62	47.5 (*R*)

[a]Three pieces (2 × 2 mm^2) of each fungal mat were inoculated on the surface of the agar plate whose surface area and volume were 7.1 cm^2 and 10 ml, respectively. After 3 d of stationary precultivation, 1 ml of a 1% solution of benzil in decane-hexyl ether (1:1) was applied onto a fungal mat. Cultivation was continued at 25 °C for 3 d.

[b]Concentration is expressed as content in the organic phase.

[c]The conversion yield is expressed as the % of produced benzoin per consumed benzil.

and L-L IBRs. Concerning the suspension and two-liquid-phase systems, 1 ml of a 3-d broth was inoculated to 50 ml of the liquid medium prepared in a 250-ml flask. After 1 d of precultivation at 25 °C, 5 ml of a 20% solution of benzil in DMSO and 250 µl of Tween-80 (suspension system) or 10 ml of a 1% solution of benzil in hexyl ether (two-liquid-phase system) were added to the 1-d broth, and cultivation was done at 25 °C with shaking (220 rpm) for 4 d. As for the S-L IBR, 200 µl of a 3-d broth was inoculated on the surface of a modified Sabouraud agar plate (volume, 10 ml; surface area, 7.1 cm^2) prepared in a glass vial (volume, 50 ml; diameter, 3 cm). Following stationary precultivation for 1 d, 2 ml of a 2% solution of benzil in hexyl ether was added onto a fungal mat formed during precultivation, and cultivation was done at 25 °C without shaking for 4 d. As for the L-L IBR, 200 µl of a 3-d broth was inoculated to a liquid medium containing 200 mg of MS (MFL-80GCA; $CaCO_3$-coated type; mean diameter, 20 µm; density, 0.2; Matsumoto Yushi-Seiyaku, Osaka), and precultivation was done at 25 °C without shaking for 1 d after vigorous mixing the inoculated mixture. After stationary precultivation, 2 ml of a 2% solution of benzil in hexyl ether was applied onto a MS-fungus mat formed during the precultivation, and cultivation was done at 25 °C without shaking for 4 d. The concentrations of benzil, (*S*)-benzoin, and hydrobenzoin in ethyl acetate extract from the broth (suspension system) or hexyl ether layer (two-liquid-phase, S-L, and L-L IBR systems), and ee of (*S*)-benzoin were determined by above-mentioned methods.

As shown in Fig. 2, the reduction of benzil did not proceed significantly in the suspension system due to the toxicity of benzil. The two-liquid-phase and S-L IBR systems exhibited moderate reducing activities to afford 9.6 (86.1% ee) and 10.2 g/l of the organic phase (96.8% ee) of (*S*)-benzoin, respectively. It was assumed that hexyl ether layer served as a reservoir for the toxic benzil and (*S*)-benzoin produced. Furthermore, the L-L IBR exhibited more excellent reducing activity than two-liquid-phase and S-L IBR systems, to afford 14.4 g/l of the organic phase of (*S*)-benzoin (99.0% ee). Thus the L-L IBR exhibited excellent performance in the asymmetric reduction of benzil to (*S*)-benzoin, in addition to the other bioconversion, the hydrolysis of 2-ethylhexyl acetate.[13] In addition to the high accumulation of hydrophobic products, easier fungal morphology control, a higher supply of nutrients, water, and oxygen, and a lower demand for energy are superior merits, as compared with traditional fungal cultivation systems, submerged, solid-state, and liquid-surface cultivation systems.

However, it was observed that excess (*S*)-benzoin produced accumulated as many needle-like crystals on a fungus-MS mat. This practically unfavorable phenomenon must be overcome by the construction of an *in situ* product removal system, such as the adsorption or crystallization of (*S*)-benzoin.[8]

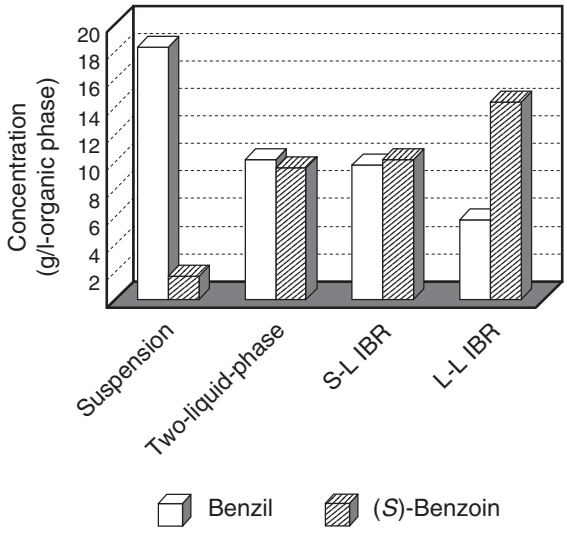

Fig. 2. Comparison of the Benzil-Reduction Activities among Suspension, Organic-Aqueous Two-Liquid-Phase, S-L, and L-L IBR Systems.

As for the suspension system, 5 ml of a 20% solution of benzil in DMSO and 250 µl of Tween-80 were added to 50 ml of 1-d broth. As for the two-liquid-phase system, 10 ml of a 2% solution of benzil in hexyl ether was added to 50 ml of 1-d broth. Both systems were applied to the reduction of benzil at 25 °C with shaking (220 rpm). As for the S-L and L-L IBRs, 2 ml of a 2% solution of benzil in hexyl ether was added onto a fungal and a fungus-MS mat (1 d of precultivation; surface area, 7.1 cm^2), respectively. The reduction was performed at 25 °C without shaking.

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