Novel, Non-aqueous Bioconversion Systems Using Fungal Spores
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Abstract: Two novel types of non-aqueous bioconversion systems using fungal spores, either adsorbed on the surface of a filter pad or entrapped in calcium alginate beads, were constructed and applied for a model reaction: reduction of benzil to benzoin by Aspergillus sojae NBRC 32074. The spores adsorbed on a filter pad catalyzed the reduction in some toxic organic solvents, such as methylecyclohexane (log P: 3.61) and di-n-butyl ether (3.21). For the relationship between the reduction activity and the log P value of the organic solvent, a highly positive correlation (R²: 0.815) was observed. Surprisingly, the reduction proceeded in the more hydrophilic and toxic tert-butyl acetate (log P: 1.76). Glycerol was selected as the best hydride source. The higher the glycerol content, the more the benzoin was produced. While the production of benzil by spores was lower than that by mycelia in harmless di-n-hexyl ether (log P: 5.12), mycelia could not catalyze the reduction in the toxic tert-butyl acetate. In contrast, the spores entrapped in the calcium alginate beads could catalyze the reduction. Although the reduction by alginate-entrapped spores could be stably repeated 5 times in di-n-hexyl ether without a decline in the reduction activity, it was observed that the reduction activity of the spores gradually decreased after repeated reduction in tert-butyl acetate.

Key words: non-aqueous bioreactor, fungal spore, immobilization, bioconversion, microbial reduction

1 Introduction
Fungal spores are anything but the inactive, dormant cells that many people think they are, because they have many active enzyme systems and metabolic pathways¹ - ². Indeed, the spores of Aspergillus niger³, A. ochraceus⁴, and Cunninghamella elegans⁵ have been used as biocatalysts for the bioconversion of steroids in an aqueous phase.

Concerning terpenoid conversion in an aqueous phase, the spores of A. niger spores converted nerol to α-terpineol⁶, while Penicillium digitatum spores converted geraniol, nerol, citral, and geranic acid to methylheptanone⁷. In other cases, some methylketones, such as 2-pentanone and 2-heptanone, have been produced from the corresponding fatty acids by the spores of Penicillium roquefortii⁸ - ⁹, and glucose has been converted to gluconic acid¹⁰ and D-mannitol¹¹ by A. niger and A. candidus spores, respectively. Thus, in all cases to date, the application of fungal spores for bioconversion has been limited to an aqueous reaction system.

Recently, the authors have developed two types of unique interface cultivation systems for fungi: extractive liquid-surface immobilization (Ext-LSI) and liquid-liquid interfacial bioreactor (L-L IBR) systems¹². The Ext-LSI and the L-L IBR systems consist of a hydrophobic organic solvent (upper phase), a fungal cell–microsphere mat (middle phase), and a liquid medium (lower phase). While the former system has been applied for the production of a hydrophobic fungicidal secondary metabolite, 6-pentyl-α-pyrone (6PP)¹³ - ¹⁵, and for the screening of antibiotic-producing fungi¹⁶, the latter has been applied for many microbial transformations, such as hydrolysis¹⁷ - ¹⁸, asymmetric reduction¹⁹, regio- and stereoselective hydroxylation²⁰, and epoxidation²¹.

On the other hand, it is well known that fungal spores exhibit the excellent tolerances to many kinds of stress, such as dryness, high temperatures, UV irradiation, and toxic and antifungal chemicals²² - ²⁴. The authors have also confirmed the excellent tolerance of fungal spores to various organic solvents²⁵ - ²⁶. This fact is very important for the production of pharmaceutical intermediates via bioconversion, because many pharmaceutical intermediates are slightly soluble in both water and hydrophobic organic solvents. Many intermediates can only be solubilized in some moderately polar organic solvents.
Therefore, this study aimed to apply the fungal spores for non-aqueous bioconversion with moderately polar organic solvents as a reaction solvents. The fungal spores were adsorbed on a filter pad to a model bioconversion of the reduction of benzil to benzoin, in relatively polar organic solvents, such as tert-butyl acetate (Fig. 1A). Furthermore, the spores entrapped in a calcium alginate beads were applied for the repeated batchwise production of benzoin in di-n-hexyl ether and tert-butyl acetate (Fig. 1B).

2 Experimental procedures

2.1 Microorganism used and its preservation and restoration

Spores of A. sojae NBRC32074 were used for the microbial reduction of benzil to benzoin, as a model reaction. For the preservation of the strain, a fungal mat was formed on the surface of a potato-dextrose agar (PDA, pH 6.0) plate, prepared in a polystyrene tube (8 mm i.d., 2 mL), and a highly branched hydrocarbon, 2,6,10,15,19,23-hexamethyl-tetracosane (squalane; C \(_{30}\)H\(_{62}\); Mw, 422.83), was added onto the fungal mat. The fungal mat was stored at 4°C. Squalane was used as an alternative organic solvent to liquid paraffin, because the highly branched solvent maybe have superior resistance against an attack by fungal enzymes, such as cytochrome P450 monoxygenase and dehydrogenase, excellent biocompatibility, high oxygen solubility, and very low volatility. The strain was restored by cultivation on a potato-dextrose agar at 25°C.

2.2 Relationship between the hydrophobicity of the reaction solvent and benzil reduction activity

A. sojae NBRC 32074 spores, formed on a PDA plate and diluted 2 times, were collected by centrifugation (4°C, 10,000 rpm, 5 min) and washed 3 times with sterilized water. The spores were re-suspended into a 1% glucose solution, containing FeSO\(_4\)-7H\(_2\)O, MnSO\(_4\)-5H\(_2\)O, and CaCl\(_2\) at 5, 20, and 10 mg/L, respectively. The concentration of the spores was adjusted to 1.2×10\(^4\) spores/mL. Three milliliters of the spore suspension was inoculated onto the surface of a filter pad (NA-100; diameter: 42 mm; thickness: 3 mm; Advantec Toyo, Ltd., Tokyo) set in a glass Petri dish (42 mm i.d.×18 mm). For the estimation of hydride sources besides glucose, while glycerol and 2-propanol were added into an aqueous phase, more hydrophobic sources, 2-octanol and cyclohexanol, were added into an organic phase.

2.3 Selection of the hydride source

For the estimation of hydride sources besides glucose, while glycerol and 2-propanol (1% w/v) were added into an aqueous phase on a filter pad, while cyclohexanol and 2-octanol (1% w/v) were added into an organic phase as a 1% solution of benzil in tert-butyl acetate–KF-96L-1CS (2:8 mixture). Three milliliters of the organic phase was added onto the spore layer that formed on the surface of the filter pad. The organic solvents tested were n-dodecane (log \(P\): 6.80), n-decane (5.98), di-n-hexyl ether (5.23), isooamyl ether (4.25), 2-ethylhexyl acetate (4.13), methylcyclohexane (3.88), di-n-butyl ether (3.11), styrene (2.95), 1-octanol (3.00), and tert-butyl acetate (1.76). The Log \(P\) value is defined as the logarithm of the partition coefficient in 1-octanol–water.

2.4 Comparison of the benzil reduction activity between mycelia and the spores of A. sojae adsorbed on a filter pad

Spores and mycelia of A. sojae NBRC 32074 were harvested from the fungal mat that formed on the diluted PDA plate and were separated by filtration via a Kimwipe\(^®\). One milliliter of the spore suspension (1×10\(^8\) spores/mL; 39 mg) or a mycelia suspension (wet mycelia, 23 mg) was
charged on a filter pad (20 x 20 x 3 nm). The filter pad was placed in 2 mL of a 1% solution of benzil in di-n-hexyl ether or a 10% solution in tert-butyl acetate. Four % of glycerol was added into the filter pad as a hydride source instead of glucose. Microbial reduction of benzil to benzoin was conducted at 25°C without shaking for 4 days.

2.5 Repeated reduction of benzil with A. sojae spores entrapped in calcium alginate beads

A spore suspension of A. sojae NBRC 32074 was prepared in the same manner as described above. The spore concentration was adjusted to 1.0 x 10^7 spores/mL. The spore suspension was mixed with an equal volume of 1% sodium alginate solution. The mixture was dropped into a 1% solution of CaCl_2 with a Pasteur pipette to form calcium alginate beads entrapped fungal spores. The bead diameter was approximately 3 mm. The alginate beads (1 mL, 37 pieces) were added into 2 mL of a 10% solution of benzil in di-n-hexyl ether or tert-butyl acetate–dimethylsilicone oil (KF-96L-1CS; Shin-Etsu Chemical Co., Ltd., Tokyo) 2:8. The reduction of benzil was performed at 25°C without shaking for 3 days and repeated 5 times.

2.6 Analytical methods

The concentrations of benzil and benzoin in the organic phase were determined directly by HPLC. A Shim-Pack VP-ODS column (4.6 mm i.d. x 150 mm; Shimadzu, Co., Ltd., Kyoto) was utilized with a column temperature of 40°C. The eluent was acetonitrile–H_2O–phosphoric acid (700:300:1), the flow rate was 1.0 mL/min, and detection was conducted by UV absorbance. The retention times of benzoin and benzil were 2.82 and 4.60 min, respectively. The maximum (λ_max) and minimum adsorption (λ_min) of benzil were 247.2 and 223.3 nm, respectively. The spore number was counted with a Thoma’s hemocytometer.

3 Results and Discussion

To date, fungal spores have been applied for some bioconversion reactions, such as the hydroxylation of steroids[1-5], cyclization of nerol and conversion of terpenoids to methylketones[6-11], and conversion of gluconic acid and D-mannitol[10, 11] in an aqueous phase to date. On the other hand, dried mycelia of some fungi such as Aspergillus oryzae[27, 28] and Rhizopus oryzae[29] have been used for esterification in some organic solvents. Thus, bioconversions with fungal cells, spores, and mycelia, have been limited to the usage of active cells in an aqueous phase or resting ones in an organic phase to date.

In contrast to the previous studies, the metabolically active fungal spores were used for a coenzyme-requiring bioconversion in an organic phase in this study. Two kinds of novel, non-aqueous bioconversion systems were used that featured fungal spores immobilized on a filter pad and in calcium alginate beads. In the former system, A. sojae spores, adsorbed on a filter pad containing water and the hydride sources, glucose or glycerol, catalyzed the reduction of benzil to benzoin in an organic phase. In the latter system, A. sojae spores entrapped in calcium alginate beads catalyzed the same reaction.

3.1 A novel, non-aqueous bioconversion system with spores adsorbed on a filter pad

First, reduction was performed with the A. sojae spores adsorbed on a filter pad in various organic solvents with the aim of estimating the solvent-tolerance of the spores. As shown in Fig. 2, the reduction of benzil proceeded efficiently in three types of solvents with log P values of more than 5: n-dodecane (log P: 6.80), n-decane (5.98), and di-n-hexyl ether (5.12). However, more hydrophilic solvents, whose log P values were less than 4.13, exhibited very strong inhibition of the reduction. For the relationship between the reduction activity and the log P value of the organic solvent, a highly positive correlation (R^2: 0.815) observed, as shown in Fig. 2. Inoue and Horikoshi reported the relationship between the log P value and the solvent tolerance for various bacteria, indicating that all of the bacteria tested were capable of growing on an interface between an agar plate and an organic phase with a log P > 7.0[27].

The authors have found that the toxicity of various hydrophobic solvents such as toluene and styrene, was remarkably reduced at an interface between the agar plate and a harmless hydrophobic organic solvent, such as n-decane[29]. Thus, the correlation between the microbial toxicity towards fungal spores and the hydrophobicity of the organic solvents was verified in this experiment, as shown in Fig. 2. Surprisingly, the A. sojae spores could catalyze the reduction of benzil in a relatively hydrophilic and more toxic solvent, tert-butyl acetate (log P: 1.76), to produce 10 mg/L of benzoin. The presence of benzoin was confirmed by HPLC-PDA analysis. The retention times, λ_max and λ_min of the product were 4.92 min, 247.2 nm, and 223.2 nm, respectively, which were identical to those of authentic benzoin.

Next, the optimal hydride source was determined, because of the requirement for coenzyme regeneration in the reduction of benzil. As shown in Fig. 3, all of the hydride sources tested were efficient. Specifically, glucose and glycerol were added in an aqueous phase on a filter pad, while 2-propanol, 2-octanol, and cyclohexanol were added in an aqueous phase. Glycerol was selected as the best hydride source. From 0.1 to 5.0% (v/v) of glycerol, the higher the glycerol content, the higher the observed reduction activity, as shown in Fig. 4.

Incidentally, the reduction was dependent upon the number of spores of the filter pad. However, the spore con-
Concentration of the inoculum was limited to the order of $10^9$ spores/mL, because of the increase in viscosity of the spore suspension. The accumulation of benzoin was $2.51 \pm 0.05$ and $5.28 \pm 0.18$ g/L after 5 and 11 days with $10^9$ spores/mL-di-n-hexyl ether, respectively. It has been reported that *Penicillium claviforme* IAM 7294 accumulated 14.4 g/L of \( \delta \)-benzoin (99.0% ee) in di-n-hexyl ether in a liquid–liquid interface bioreactor (L–L IBR) after 4 days. Thus, this system using an immobilized spore was found to be inferior to the present L–L IBR.

In addition, in order to confirm the activity of the spores in organic solvents, the reduction of benzil by *A. sojae* mycelia and spores adsorbed on a filter pad was compared in two kinds of organic solvents, di-n-hexyl ether and tert-

**Fig. 2** Relationship between the concentration of benzoin produced and log $P$ value of organic solvents in a filter pad type reactor. Three milliliters of a spore suspension (1.2 \times $10^8$ spores/mL) was charged onto the surface of a filter pad. Four milliliters of a 1% solution of benzil in each solvent was added onto a spore layer adsorbed on a filter pad containing 1% glucose as a hydride source. The reaction was performed at 25°C without shaking for 5 days. Four duplicates were prepared in each incubation system.

**Fig. 3** Selection of hydride source for the reduction of benzil with a filter pad type reactor. A cell suspension (8.0 \times $10^8$ spores/mL, 720 μL) was added onto a filter pad containing 1% glucose, glycerol or 2-propanol as a hydride source. Cyclohexanol or 2-octanol (1%) was added into 3 mL of di-n-hexyl ether layer. The reduction was done at 25°C without shaking for 10 days. Four duplicates were prepared in each incubation system.

**Fig. 4** Effect of glycerol content in a filter pad on the reduction activity of *A. sojae* spores adsorbed on a filter pad. A cell suspension (8.0 \times $10^8$ spores/mL, 720 μL) was charged onto a filter pad containing 0.1–5.0% (v/v) glycerol as a hydride source. Three milliliters of a 1% solution of benzil in tert-BuOAc–KF-96L-1CS was added, and incubation was done at 25°C without shaking for 6 days. Four duplicates were prepared in each incubation system.
butyl acetate. As shown in Fig. 5, although *A. sojae* mycelia could efficiently catalyze the reduction, compared with the spores in the harmless di-*n*-hexyl ether, the reduction barely proceeded in the toxic tert-butyl acetate. On the other hand, the spores adsorbed on the filter pad could catalyze the reduction both in di-*n*-hexyl ether and tert-butyl acetate to afford 1.81 ± 0.10 g/L (46.77 g/L/g-dry cells) and 1.01 ± 0.04 g/L (26.16 g/L/g-dry cells) after 4 days, respectively. Thus, it was confirmed that the *A. sojae* spores had the excellent tolerance against toxic organic solvents compared with mycelia.

### 3.2 A novel, non-aqueous bioconversion system with spores adsorbed in calcium alginate beads

Next, another type of novel, non-aqueous bioconversion system with fungal spores immobilized in calcium alginate beads was developed. It was expected that the entrapped biocatalyst could be used as an efficient packed-bed column bioreactor. It is well known that calcium alginate beads break down in an aqueous phase, due to leaking calcium ions. However, in our system, the collapse of the alginate beads was avoided, because calcium ions did not leak from the beads into the organic phase.

The reduction of benzil with the alginate-entrapped spores was partially dependent on the density of the cross-linkage of the alginate matrix. The concentrations of benzoin produced were 2.87, 2.59, and 2.54 g/L from an alginate content of 0.5, 1.0, and 2.0%, respectively. Thus, a higher the cross-linkage of the alginate matrix resulted in lower the benzoin production. The permeation and diffusion of benzil into the gel matrix was an obstacle for the production of benzoin. However, a gel strength of 0.5% calcium alginate beads was very low and was not worthy of real use. Therefore, the alginate content was optimized to 1.0%.

Finally, the stability of the *A. sojae* spores, entrapped in the alginate beads in the organic solvents was confirmed by the repeated batch reduction of benzil. As shown in Fig. 6, the reduction activity of the spores decreased when the reaction was repeated in tert-butyl acetate, because of the appearance of its toxicity or the removal of water from the gel matrix and spores. However, in the less toxic solvent, di-*n*-hexyl ether, the reduction activity of the entrapped *A. sojae* spores did not decline even after five repetitions over 15 days. Thus, although it was confirmed that the immobilized fungal spores could stably catalyze the reduction of benzil, which required a coenzyme in a harmless organic solvent, di-*n*-hexyl ether, the reduction activity of the immobilized *A. sojae* spores did not decline even after five repetitions over 15 days.

In our preliminary experiment, it was found that the reduction activity of the spores entrapped in the calcium alginate beads was partially recovered by the exchange of an aqueous phase with a gel matrix. Specifically, the production of benzoin by the entrapped spores improved by 30%, after the washing with hexane and an exchange of the aqueous phase with a gel matrix by soaking in it sterilized water for 1 min. The authors expect that the stability of the entrapped spores in tert-butyl acetate can be enhanced by optimizing the exchange of the aqueous phase with the gel matrix.

In this work, the absolute configuration and the enantio-
meric excess (ee) of benzoin produced were not identified, because the estimation of the stereochemistry after the reduction was not the purpose of this study. However, it has been observed that A. sojae NBRC 32074 yielded (S)-benzoin at 93.7% ee after submerged cultivation by use of HPLC (column: CHIRAL CEL OD-3R, Daisel Corporation, Tokyo). As a next stage, the authors are currently examining the stereochemistry of the reduction with A. sojae spores at present.

4 Conclusion
Two kinds of novel non-aqueous bioconversion systems using fungal spores, either adsorbed on a filter pad or entrapped in calcium alginate beads, were developed and applied for the coenzyme-requiring bioreduction of benzil to benzoin. The Aspergillus sojae spores adsorbed on the filter pad stably catalyzed the reduction in some hydrophobic organic solvents such as n-dodecane, n-decane, and di-n-hexyl ether, all of which had a log P > 5.12. The reduction activity of the A. sojae spores entrapped in calcium alginate beads was maintained even after five time repeated batchwise conversions in di-n-hexyl ether over 15 days. Although A. sojae mycelia could not catalyze the reduction in the toxic tert-butyl acetate, its spores immobilized on a filter pad could catalyze the reduction to yield 1.01 g/L of benzoin into tert-butyl acetate after 4 days. It is expected that immobilized fungal spores may be applicable for non-aqueous bioconversion with toxic tert-butyl acetate by dissolving many pharmaceutical intermediates in the future with some process modifications.

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