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Fusarium roseum and *Aspergillus oryzae*-mediated enantioselective reduction of benzils to benzoins

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ABSTRACT

Aspergillus oryzae OUT5048 and *Fusarium roseum* OUT4019 were found to be effective biocatalysts in the reduction of benzils to optically active benzoins. Easily available symmetrical benzil derivatives were reduced to the corresponding benzoins [(*S*)-2-hydroxy-1,2-diphenylethanones] by *A. oryzae* OUT5048 with up to 94% ee and by *F. roseum* OUT4019 with up to 98% ee, respectively. In this work, first general method for whole-cell-mediated selective reduction of benzils to benzoins is reported. It is also shown that this method is applicable for benzils with both electron-withdrawing and electron-donating groups.

1. Introduction

Whole-cell and enzyme-mediated biotransformations are tools of green chemistry. Via environmentally friendly biotransformations employing mild reaction conditions, side reactions such as isomerization, racemization, epimerization, etc. are also minimized. Furthermore these reactions are highly selective (chemo-, regio- and stereo-) [1a,b,2].

Optically pure α-hydroxy ketones have been used widely as chiral building blocks since they can be easily transformed into compounds with other mono- or multi-functionalities such as diols, diamines, aminoalcohols, epoxides, etc. There are several methods for the syntheses of chiral α-hydroxy ketones, namely; enzyme-mediated C—C bond formation reactions as in the case of benzaldehyde lyase (BAL, EC 4.1.2.38) [3a–d] or benzoylformate decarboxylase (BFD, E.C. 4.1.1.7) [4a,b]; enzymatic or whole-cell bioreduction of prochiral diketones [5a–d]; oxidation of the relevant diols [6a–f]; whole cell or chemoenzymatic α-hydroxylation of the ketones [7a–c] or kinetic resolution of racemic α-hydroxy and α-acetoxy ketones [8]. In the enzymatic oxidation and reduction reactions, a cofactor regeneration system such as coupled enzymatic assays is needed. Cheap whole-cell processes are favored for these reactions since cofactors are readily available in the cell and they are easier to use by avoiding tedious enzyme purification.

There are several examples in the literature for either enzyme or whole-cell-mediated syntheses of benzoins which are also important synthons for stereoselective synthesis [4b]. Saito et al. used *Bacillus cereus* whole cells for the reduction of only benzil to (*S*)-benzoin with a yield of 92% and enantiomeric excess (ee) of 94% [9]. In another study by Mahmoodi et al. Baker's yeast was used in the same bioreduction reaction. (*R*)-Benzoin was obtained with 50% ee while reduction of other derivatives 4-Me-Ph and furyl- were achieved with 36% and 82% ee [10a]. This study [10a] is different from the others [10b,c] in the perspective that Baker's yeast (either free or immobilized cells) were shown to perform this reaction in not an enantioselective manner previously. Demir et al. used *Rhizopus oryzae* and *Rhizomucor* sp. for obtaining both enantiomers of benzoin in whole-cell reactions. (*R*)-Benzoin was obtained with 99% ee while *Rhizomucor* sp. yielded (*S*)-benzoin with 73% ee [11]. Ohta et al. synthesized (*R*)-benzoin by the use of *Xanthomonas oryzae* with a yield of 86% and 99% ee [12]. In this study also unsymmetrical benzil derivatives were reduced to the relevant unsymmetrical benzoins, however ee values were not stated. Most probably, unsymmetrical benzoins formed were in racemic form. Konishi et al. used enzyme system of *X. oryzae* IAM 1657 in a whole-cell study and obtained (*R*)-benzoin with 86% ee [13]. In an enzymatic study by Maruyama et al. (*S*)-benzoin was synthesized by the use of *B. cereus* benzil reductase with 97% ee. Moreover homologous proteins yeast YIR036C and gerbil sepiapterin reductase (SPR) performed the same reaction with 94% and 97% ee,

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respectively [14]. A series of *para*-substituted symmetric (*p*-tolu and *p*-methoxy) and unsymmetric benzils were reduced by *C. macerans* to yield the *threo* (*R*,*R*) diols of high optical purity and the (*S*)-benzoins with enantiomeric excesses of 20–30% [15].

In this work we examined 21 different species of *Aspergillus* and *Fusarium* genus for enantioselective bioreduction of benzils to benzoins. Two candidates were selected from each genus: *Aspergillus oryzae* OUT5048 and *Fusarium roseum* OUT4019. Here we are reporting enantioselective reduction of a representative set of symmetrical benzil derivatives to the corresponding (*S*)-benzoin derivatives with high yield and ee.

2. Experimental

2.1. Microorganisms

21 different microorganisms were screened for the desired biotransformation. The sources of these microorganisms were as follows: 13 different *Aspergillus* sp., and 4 different *Fusarium* sp. were generous gifts of Osaka University Department of Biotechnology (OUT), 4 different *Aspergillus* sp. were purchased from TUBITAK Marmara Research Center (MRC culture collection).

2.2. Syntheses of benzil derivatives

Firstly, benzoins were synthesized according to the classical procedure, then they were oxidized with a common method given in the literature [16a–d].

2.3. Selection of the medium for cultivation of Aspergillus oryzae OUT5048

Potato dextrose broth (PDB) was prepared by dissolving 8 g potato extract and 20 g glucose in 1 L distilled water. Medium B, medium C, medium D were prepared according to the literature [17]. [B] = 30 g/L glucose (20 g/L in reference), 10 g/L peptone, [C] = 20 g/L glucose, 20 g/L yeast extract, 5 g/L peptone, 1 g/L KH_2PO_4, 2 g/L K_2HPO_4, 2 g/L $NaNO_3$, 0.5 g/L KCl, 0.5 g/L $MgSO_4{\cdot}7H_2O$, 0.02 g/L $FeSO_4{\cdot}7H_2O$; [D] = 4 g/L yeast extract, 15 g/L starch, 1 g/L K_2HPO_4, 0.5 g/L $MgSO_4{\cdot}7H_2O$.

Czapek Dox medium (modified) and malt extract broth (MEB) were purchased from Oxoid. Modified Czapek Dox medium: 30 g/L sucrose, 2 g/L $NaNO_3$, 0.5 g/L KCl, 0.5 g/L magnesium glycerophosphate ($C_3H_7MgO_6P$), 0.01 g/L $FeSO_4$, 0.35 g/L K_2SO_4. Malt extract broth: 17 g/L malt extract, 3 g/L mycological peptone.

2.4. Cultivation of Fusarium sp.

For the cultivation of *Fusarium* sp. (especially for *F. roseum*) glucose peptone medium (medium B) was used. For other *Fusarium* sp. potato sucrose broth (PSB) was preferred as growth media. PSB was prepared by dissolving 40 g of potato extract and 20 g of sucrose in 1 L distilled water.

2.5. Biotransformation procedure for Aspergillus oryzae OUT5048

A. oryzae OUT5048 was grown in potato dextrose agar (PDA) petri dishes. The streaked plates were incubated at 37 °C for 3–4 days for spore production and then stored at 4 °C until utilized. The surface of the petri plate containing spores was rubbed with a sterile inoculation loop and then transferred to a 250 mL sterile Erlenmeyer containing a 100 mL growth medium, in which the organism was grown in a rotary shaker at 37 °C for 60 h. After 2.5 days resting cells filtered from the growth medium were transferred to 250 mL sterile Erlenmeyer containing 100 mL 50 mM pH 5 potassium phosphate buffer, then benzil (21.0 mg, 0.1 mmol) dissolved in 1 mL DMSO was added and the reaction was started. pH adjustments were done with 1N NaOH and 1N HCl. Conversion was monitored by TLC. After the reaction was completed, microorganism was filtered off the supernatant, washed with distilled water and the combined aqueous phases were extracted three times with 50 mL ethyl acetate (3× 50 mL). The organic extract was dried over $MgSO_4$, filtered and concentrated and benzoin was purified by flash column chromatography (eluent: 1:3 ethyl acetate/hexane) and it was isolated as a colorless solid (20.1 mg, 95% yield); (94% ee); m.p. 135 °C [18a]; $[\alpha]_D^{20}$: +108 (+114.9 °C 1.5, acetone, for >97.7% ee) [18a]; HPLC (Chiralpak OD): R_t (*S*) = 17.0 min; R_t (*R*) = 25.0 min; ^{1}H NMR (400 MHz, $CDCl_3/CCl_4$): 7.92 (d, *J* = 7.9 Hz, 2H), 7.29–7.52 (m, 8H), 5.97 (d, *J* = 6.1 Hz, 1H), 4.58 (d, *J* = 6.1 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3/CCl_4$): 198.9, 139.6, 134.1, 134.0, 129.5, 129.4, 128.9, 128.8, 128.2, 76.5.

Enantiomeric excess was determined by the use of HPLC equipped with chiral column using authentic (*R*)- and (*S*)-benzoins, as references. Benzoin was obtained with 94% ee. All racemic benzoins are synthesized according to the literature procedure and used as reference [3a–d].

In *A. oryzae* OUT5048-mediated bioreduction of benzil derivatives same procedure was applied, i.e., resting cells were employed in potassium phosphate buffer with a pH value of 5, then the desired substrate was added (1 mmol dissolved in 1 mL DMSO).

2.6. Representative example for Fusarium roseum OUT4019-mediated bioconversion: reduction of 1,2-bis(4-florophenyl)ethane-1,2-dione (**2g**)

F. roseum OUT4019 was grown in glucose peptone agar (GPA) petri dishes. The streaked plates were incubated at 25 °C for 3–4 days for spore production and then stored at 4 °C until utilized. The surface of the petri plate containing spores was rubbed with a sterile inoculation loop and then transferred to a 250 mL sterile Erlenmeyer containing a 100 mL growing medium, in which the organism was grown in a rotary shaker at 25 °C for 4 days. After 4 days, 1,2-bis(4-florophenyl)ethane-1,2-dione (24.6 mg, 0.1 mmol) dissolved in 1 mL DMSO was added and the reaction was started. Conversion was monitored by TLC. After the reaction was completed, microorganism was filtered off the supernatant, washed with distilled water and the combined aqueous phases were extracted three times with 50 mL ethyl acetate (3× 50 mL). The organic extract was dried over $MgSO_4$, filtered and concentrated and 1,2-bis(4-florophenyl)-2-hydroxyethan-1-one was purified by flash column chromatography (eluent: 1:7 ethyl acetate/hexane) and it was isolated as a yellow solid (24 mg, 97% yield); (97% ee); m.p. 81–82 °C (m.p. 80 ± 82 °C for racemic compound) [18b]; $[\alpha]_D^{20}$: −88 (c 0.5, $CHCl_3$, for 97% ee) [18c]; HPLC (Chiralpak IA): R_t (*R*) = 16.4 min; R_t (*S*) = 18.0 min; ^{1}H NMR(400 MHz, $CDCl_3/CCl_4$): 7.84 (m, 2H), 7.25 (m, 2H), 7.12 (m, 2H), 7.09 (m, 2H), 5.86 (d, *J* = 5.4 Hz, 1H), 4.12 (d, *J* = 5.4 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3/CCl_4$): 197.2, 166.7 (d, *J* = 246 Hz), 165.7 (d, *J* = 232 Hz), 135.1, 134.9, 132.1 (d, *J* = 9.6 Hz), 130.2 (d, *J* = 9.4 Hz), 116.8 (d, *J* = 22 Hz), 116.1 (d, *J* = 20 Hz), 75.4.

In *F. roseum* OUT4019-mediated bioreduction of benzil derivatives a different procedure was applied compared to *A. oryzae* OUT5048-mediated biotransformation, growing cells were employed instead of resting cells where the substrate of interest was directly added to the growth medium.

2.7. HPLC analysis of other 2-hydroxy-1,2-diphenyl ethanone derivatives (**2a**–**g**)

2-Hydroxy-1,2-diphenyl ethanone (**2a**): Chiralpak OD column, UV detection at 254 nm, eluent: *n*-hexane/2-

propanol = 90:10, flow 1.0 mL/min, 20 °C; R_t (*S*) = 17.0 min; R_t (*R*) = 25.0 min.

2-Hydroxy-1,2-bis(2-methoxyphenyl)ethanone (**2b**): Chiralpak AD-H column, UV detection at 254 nm, eluent: *n*-hexane/2-propanol = 90:10, flow 1.0 mL/min, 20 °C; R_t (*S*) = 31.0 min; R_t (*R*) = 42.0 min.

2-Hydroxy-1,2-bis(3-methoxyphenyl)ethanone (**2c**): Chiralpak AD-H column, UV detection at 254 nm, eluent: *n*-hexane/2-propanol = 90:10, flow 1.0 mL/min, 20 °C; R_t (*R*) = 31.0 min; R_t (*S*) = 40.6 min.

2-Hydroxy-1,2-bis(4-methoxyphenyl)ethanone (**2d**): Chiralpak IA column, UV detection at 254 nm, eluent: *n*-hexane/2-propanol = 90:10, flow 1.0 mL/min, 20 °C; R_t (*R*) = 36.0 min; R_t (*S*) = 41.0 min.

1,2-Bis(2-florophenyl)-2-hydroxyethanone (**2e**): Chiralpak IA column, UV detection at 254 nm, eluent: *n*-hexane/2-propanol = 90:10, flow 1.0 mL/min, 20 °C; R_t (*S*) = 12.2 min; R_t (*R*) = 14.2 min.

1,2-Bis(3-florophenyl)-2-hydroxyethanone (**2f**): Chiralpak IA column, UV detection at 254 nm, eluent: *n*-hexane/2-propanol = 90:10, flow 1.0 mL/min, 20 °C; R_t (*R*) = 12.7 min; R_t (*S*) = 16.5 min.

3. Results and discussion

Several approaches are present for asymmetric biotransformation procedures. One can select a native organism performing the desired biotransformation with a high ee or an organism with a moderate ee can be improved via either site-directed mutagenesis approach or optimization of bioprocess parameters.

A more conventional strategy, i.e., enantioselectivity enhancement via optimization of physiological conditions of culture medium, presents a reliable alternative. Firstly, a screening among several organisms is performed, microorganism with the highest enantioselectivity is selected and the enantioselectivity of this microorganism is improved with bioprocess engineering means.

In this study *F. roseum* and *A. oryzae* were chosen for the conversion of benzils to benzoins as they showed highest enantioselectivity among *Fusarium* and *Aspergillus* genus. *F. roseum* showed really high enantioselectivity with an ee of 95% while ee of *Aspergillus oyzae* was a little bit lower (74% ee). The enantioselectivity of this strain was improved to 94% via bioreaction engineering means.

3.1. Optimization of fermentation conditions for A. oryzae-mediated bioreduction reaction

Several parameters such as growth medium, temperature, pH, and physiological state of the cell (resting, living (growing) or lyophilized) are effective on the enantioselectivity of a whole-cell bioreduction reaction. The *Aspergilllus* sp. used in this study were screened for reduction of benzil to benzoin in PDB medium and 37 °C. Among them *A. oryzae* gave, respectively, high and promising selectivity with 74% ee. Afterwards, enantioselectivity enhancement studies took place. Firstly, effect of growth medium on the enantioselectivity was examined by using six different mediums: PDB, medium B, medium C, medium D, modified Czapek Dox medium, and MEB. The highest enantioselectivity and yield were obtained with medium D. After selecting the growth medium, effect of initial pH was investigated by adjusting initial pH values from 4 to 8 and highest enantioselectivity was obtained at neutral pH at 37 °C with growing cells. pH profile was not changing over the biotransformation period. Another parameter, physiological state of the organism, was also shown to be effective on enantioselectivity. The organism grown in medium D (with an initial pH of 7) was transferred into potassium phosphate buffer solutions whose pH were arranged from 4 to 8 as wet cell and also lyophilized (crude) cell. Wet cells in pH 5 phosphate buffer achieved the desired biotransformation with 94% ee and 95% yield. pH profiles of the buffers with varying pH's were not changing over the course of biotransformation. At slightly alkaline condition (pH 8), although enantioselectivity was not high as acidic conditions (pH 5 and 6) a satisfactory ee value was obtained minimizing the possibility of partial racemization of (*S*)-benzoin at that pH. This situation can be explained with the short reaction time.

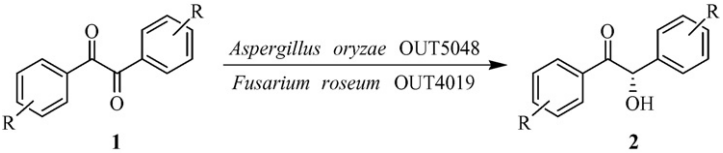
As a result of this optimization study, a microorganism achieving the desired bioconversion in moderate selectivity was improved to achieve the same conversion with high stereoselectivity.

3.2. Enantioselective bioreduction reactions of benzils to the corresponding benzoins

After designing the optimum biotransformation process for *A. oryzae*, symmetrical benzil derivatives were reduced to optically active benzoins with *F. roseum* and *A. oryzae* on this optimized system. The results are summarized in Table 1.

Both microorganisms were able to reduce a good range of benzil derivatives substituted with electron-donating as well as electron-withdrawing properties (Table 1). Absolute configuration of the stereocenters formed were assigned to be (*S*)- by comparing optical rotation (α) values with the literature. *F. roseum* seemed as a more efficient whole-cell catalyst compared to *A. oryzae* except for 1,2-bis(2-methoxyphenyl)ethane-1,2-dione (Table 1, entry b). *F. roseum*-mediated bioconversions were highly efficient and selective with a yield range of 80–99% and an

Table 1
Microbial bioreduction of benzil derivatives to (*S*)-2-hydroxy-1,2-diphenyl ethanones



1 2

Entry	Benzoin **2** R[a]	*Aspergillus oryzae* OUT5048			*Fusarium roseum* OUT4019		
		Reaction time (h)	ee (%)	Conversion (%)	Reaction time (h)	ee (%)	Conversion (%)
1	H **a**	3	94	95	24	95	99
2	*o*-MeO **b**	72	91	94	NR	–	–
3	*m*-MeO **c**	3	84	87	24	90	98
4	*p*-MeO **d**	72	47	15	96	86	85
5	*o*-F **e**	4	23	95	72	98	90
6	*m*-F **f**	2	36	99	24	93	92
7	*p*-f **g**	8	65	81	24	97	97

[a] All the spectral data of benzoins were identical with the literature values [3b].

ee range of 86–98%. *A. oryzae* achieved the bioconversion of all substrates at yields 81–99% (except 15% for 2-hydroxy-1,2-bis(4-methoxyphenyl)ethanone (Table 1, entry d)) while ee's were in between 23% and 94%. This microorganism showed high enantioselectivity with methoxy-substituted benzil derivatives (47–91% ee) while it showed low to moderate enantioselectivity (23–65% ee) with flor-substituted benzil derivatives. Best result among flor-substituted compounds was obtained with 1,2-bis(4-florophenyl)ethane-1,2-dione) (Table 1, entry g). *ortho*-Position was favored in the case of methoxy-substituted benzil derivatives [1,2-bis(2-methoxyphenyl)ethane-1,2-dione] (Table 1, entry b). Benzoins substituted at *ortho*-position are problematic and several chemical methods lack syntheses of those. So, whole-cell bioreductions presented herein propose another advantage in this aspect.

In the reduction of benzil, benzoin formed was not further reduced to hydrobenzoin neither by *A. oryzae* nor *F. roseum*. Reduction to hydrobenzoin was avoided by short reaction time in *A. oryzae*-mediated bioconversion, with prolonged reaction times hydrobenzoin was formed in the reaction medium. On the other hand with *F. roseum* did not perform further reduction to hydrobenzoin even at elevated reaction times. In the benzil derivatives with long reduction times trace amount of hydrobenzoin was formed: 1,2-bis(4-methoxyphenyl)ethane-1,2-dione with *A. oryzae* and 1,2-bis(2-florophenyl)-2-hydroxyethanone with *F. roseum*.

Enantioselectivity of the *A. oryzae* for the benzil derivatives was tried to be improved with the use of additives. They are molecules used to enhance enantioselectivities. They have several mode of action: they act as sources of hydrogen for cofactor regeneration (alcohols) or as enzyme inhibitors (allyl bromide, allyl alcohol); they increase availability of the substrate to the enzyme (surfactants) and they perform chemical modification of the enzyme increasing its activity (sulfur compounds) [19]. As *A. oryzae*-mediated bioreduction is a fast reaction, it is thought that hydrogen shortage might have occurred in the reaction medium. Therefore, two most popular alcohol derivatives; ethanol and isopropyl alcohol were tried out as additives for benzoin derivatives with low ee (**1e**, **1f**, **1g**), however positive effect of these chemicals on enantioselectivity was not observed. Hydrobenzoin formation was not common, so additives acting as enzyme inhibitors were not examined.

4. Conclusions

In this work whole cells of *A. oryzae* and *F. roseum* were used in the reduction of benzils. The reductions by these microorganisms proceed smoothly; hydrobenzoin formation was observed only for 1,2-bis(4-methoxyphenyl) ethane-1,2-dione with *A. oryzae*. *F. roseum* achieved further reduction to hydrobenzoin in 1,2-bis(2-florophenyl)-2-hydroxyethanone derivative, in all the cases hydrobenzoin derivatives formed were in trace amounts. *F. roseum* was shown to be better catalyst with an ee range of 86–98%. However, product formation was not observed in one derivative; 2-hydroxy-1,2-bis(2-methoxyphenyl)ethanone. *A. oryzae* was able to convert all the substrates to the desired products. Yields were in the range of 81–99% (except 15% for 2-hydroxy-1,2-bis(4-methoxyphenyl)ethanone while ee's were in between 23% and 94%. *A. oryzae* favored benzil derivatives substituted with electron-donating groups over the ones having electron-withdrawing groups. Methoxy-substituted benzil derivatives were reduced at a higher enantioselectivity (47–91% ee) compared to flor-substituted derivatives (23–65% ee). Additives tested to improve low-ee-reactions were inefficient.

Whole-cell reactions stated in this paper are highly efficient. *A. oryzae* and *F. roseum* have been shown to be effective catalysts for reduction of symmetrical benzils in the syntheses of (*S*)-benzoin derivatives. High enantioselectivities and yields were obtained; reduction proceeds with high specificity avoiding further reduction and by the use of whole cells the need for enzyme purification and cofactor regeneration is avoided.

To sum up bioreduction strategy described here is superior over biotechnological and chemical methodologies with short reaction times and applicability to derivatives. For the first time in literature symmetrical benzils were reduced enantioselectively to benzoins with high yields and ee's.

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