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Biocatalyzed asymmetric reduction of benzils to either benzoins or hydrobenzoins: pH dependent switch

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Enantiopure benzoins and hydrobenzoins are precursors of various pharmaceuticals and biologically active compounds. In addition, hydrobenzoins are precursors for chiral ligands and auxiliaries in stereoselective organic synthesis. Biocatalytic reduction of benzils is straightforward approach to prepare these molecules. However, known methods are not selective and lead to formation of a mixture of benzoin and hydrobenzoin, requiring expensive separation procedures. Here, we describe an enzyme system *Talaromyces flavus*, which exhibited excellent pH dependent selectivity for conversion of benzil to either benzoin or hydrobenzion in high ee. Thus, (*S*)-benzoin was the exclusive product at pH 5.0 (ee >99%), whereas at pH 7.0, (*S,S*)-hydrobenzoin (ee >99%, *dl/meso* 97:3) was the exclusive product. The observed pH dependent selectivity was shown to be due to presence of multiple enzymes in *Talaromyces flavus*, which specifically accepted either benzil or benzoin as substrate and exhibited different pH profile for their activity. The biocatalyst efficiently reduced a variety of symmetrical and unsymmetrical benzils. Moreover, a 36.4 kDa benzoin reductase was purified, the N-terminal sequence of which did not show significant similarity with any of the known reductase/dehydrogenase in database. The protein therefore appears to be a novel reductase.

Introduction

Enantiopure benzoins (α-hydroxyketones) are important intermediates for various pharmaceuticals, such as antifungal agents, antitumor antibiotics (Olivomycin A, Chromomycin A3, and epothilones),[1] selective inhibitors of amyloid-β-protein (for Alzheimer's disease treatment),[2] farnesyl transferase inhibitors Kurasoin A and B,[3] antidepressants (bupropion and its metabolites),[4] urease inhibitors[5] and heterocycles.[6] In addition, these are very interesting molecules from a synthetic chemistry point of view as these are precursors of amino alcohols and 1,2-diols, which in turn are precursors for various biologically active compounds as well as chiral ligands and auxiliaries in stereoselective organic synthesis. Because of the importance of α-hydroxyketones in organic synthesis and as intermediates of various bioactive molecules, many chemical approaches have been developed for their synthesis. These include α-hydroxylation of ketones and Sharpless asymmetric dihydroxylation of the silylenol ether of the corresponding ketone,[7] ketohydroxylation of olefins,[8] asymmetric mono-oxidation of 1,2-diols,[9] oxidative kinetic resolution of racemic α-hydroxyketones,[10, 11] organocatalytic strategies involving asymmetric α-oxygenation of ketones in the presence of proline or alanine[12, 13] and traditional benzoin condensation carried out stereoselectively by means of optically active catalysts such as chiral thiazolium and triazolium salts, in a biomimetic fashion.[14, 15] Reported chemical methods are successful for variety of molecules but suffer from drawbacks, such as large number of steps, rare high ee, low overall yields and high waste.

Biocatalytic ketone reduction combined with economic and environmentally friendly reaction conditions is effective strategy to overcome these limitations.[16-20] *Penicillium claviforme*,[21] *Pichia glucozyma*,[22] *Aspergillus oryzae*, *Fusarium roseum*,[23] *Bacillus cereus*[24] and *Xanthomonas oryzae*[25] have been used for preparation of benzoins by asymmetric reduction of benzils. *Cryptococcus macerans,*[26] yeast strains, *Saccharomyces uvarum*, *Saccharomyces montanus*[27] and *Rhizopus sp.*[28] and alcohol dehydrogenases from *Thermoanaerobacter* sp., *Lactobacillus brevis* and *Ralstonia* sp.[29] have been used for enantio- and diastereoselective synthesis of hydrobenzoins.

In principle, chiral benzoins as well as chiral hydrobenzoins are easily accessible via biocatalytic reduction of corresponding benzil. However, there is a dearth of biocatalysts which accept such bulky-bulky substrates.[29] Moreover, known biocatalysts

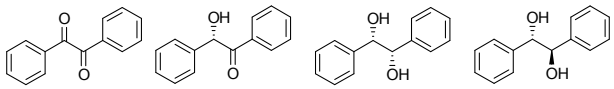
are not selective and lead to the formation of a mixture of benzoin and hydrobenzoin, requiring expensive separation procedures, which hampers the acceptability of these biocatalysts for application in technical scale preparations.

Herein, we describe an enzyme system *Talaromyces flavus*, which exhibited excellent pH dependent selectivity for conversion of benzil to either benzoin or hydrobenzion in high ee. Thus, (*S*)-benzoin was the exclusive product at pH 5.0 (ee >99%), whereas at pH 7.0, (*S,S*)-hydrobenzoin (ee >99%, *dl/meso* 97/3) was the exclusive product. We have demonstrated that the pH dependent selectivity of *T. flavus* is due to presence of multiple enzymes in the system, which specifically accepted either benzil or benzoin as substrate and exhibited different pH profile for their activity. The biocatalyst, *T. flavus* accepted a variety of symmetrical and unsymmetrical benzils as substrate. Moreover, a 36.4 kDa benzoin reductase was purified from *T. flavus*. N-terminal sequence obtained from the purified protein by Edman degradation did not show significant similarity with any of the known reductase/dehydrogenase in database. The protein therefore appears to be a novel reductase.

Results and discussion

Strain selection

Enantiopure benzoins and hydrobenzoins are intermediates for various bioactive molecules. These also serve as chiral ligands in stereoselective organic synthesis. In our previous report, we have described three biocatalysts *Penicillium sp.* MTCC 10832, *Alternaria alternata* MTCC 10833 and *Talaromyces flavus* MTCC 10834 for enantioselective reduction of methyl heteroaryl and aryl heteroaryl ketones to corresponding (*S*)-heteroaryl alcohols in >99% ee.[30] We tested these strains for enantioselective reduction of benzil **(1a)**. The fungal strains were grown as described in experimental section. The cells were isolated by centrifugation, washed with phosphate buffer (0.2 M, pH 7.0) and resuspended in same buffer at a concentration of 100 g L^{-1}. Benzil (**1a**; 50 mg, 0.24 mmol) was added to a suspension of 5g cells in 50 mL phosphate buffer and contents were shaken at 200 rpm and 30 ºC until complete consumption of substrate occurred. The progress of the reaction was followed by TLC, using commercially available standard sample of *rac*-benzoin (**2a)**. At 100% conversions, cells were removed from the reaction mixture by centrifugation and contents extracted in ethyl acetate. The organic layer was washed with saturated brine, dried over anhydrous sodium sulfate and solvents evaporated under the reduced pressure to yield a residue which was analysed by ^{1}H NMR. All the three strains were able to reduce **1a** to mixture of benzoin **(2a)** and hydrobenzoin (**3a**) in varying ratio (Table 1). The ratio of **2a**/**3a** was determined by ^{1}H NMR (Figure 1S, Supplementary Information). The single methine proton of pure *rac*-**2a** appeared as bs (broad singlet) at δ 5.9. Hydrobenzoin **3a** prepared by sodium borohydride reduction of **2a** showed two bs at δ 4.7 and 4.8 corresponding to two methine protons of *dl* and *meso* pair. The ratio of **2a**/**3a** in biocatalyzed product in the reaction mixture was determined from integral values of resonance peaks at δ 5.9, 4.7 and 4.8. The ee of **2a** was determined by chiral HPLC using Chiralcel OD-H column. The elution was done with hexane:isopropanol, 90/10 at flow rate of 1ml/min. The detection was done at $\lambda_{254.}$ Whereas, two enantiomers of *rac*-**2a** eluted at 11.9 and 17.4 min, the biocatalyzed product showed predominant presence of one enantiomer eluting at about 11.9 min (Figure 2S, Supplementary Information). Ee was calculated from relative area under the two peaks. *T. flavus* gave ee of >99%, whereas *Penicillium sp.* and *A. alternata* gave ee of 95% and 91%, respectively. The absolute configuration of *T. flavus* catalyzed product **2a** was assigned as (*S*) based on the comparison of sign of optical rotation with literature $[\alpha]_D^{25}$ = +115.2 (c 1.5, acetone) [lit.[31] $[\alpha]_D^{25}$ = +115.0 (c 1.5, acetone). Accordingly, the enantiomer eluting at 11.9 min in HPLC of (*S*)-**2a** correspond to (*S*)-enantiomer and that at 17.4 min to (*R*)-enantiomer.



1a **(*S*)-2a** **(*S,S*)-3a** **(meso)-3a**

Table 1 Biocatalyzed reduction of benzil

Entry	Strain	Conv (%)[b]	Time (h)	**2a:3a**[a]	(*S*)-**2a**[b,c]	(*S, S*)-**3a**[b,c]	
					Ee %	*dl/meso*[b]	Ee %
1	*Penicillium sp.*	100	24	1:0.21	95	65/35	93
2	*A. alternata*	100	18	1:1.53	91	94/6	85
3	*T. flavus*	100	37	1:0.94	>99	80/20	99

[a]Conversion, ratio of **2a:3a** and *dl/meso* ratio was determined by ^{1}H NMR based on integral of specified peaks. [b] Ee. of **2a** and **3a** was determined by chiral HPLC on Chiralcel OD-H and OJ (Diacel, Japan) column, respectively. [c]Absolute configuration was assigned based on comparison of sign of optical rotation with literature.

The *dl/meso* ratio of **3a** was determined by HPLC and ^{1}H NMR. A sample of *dl/meso*-**3a** prepared by sodium borohydride reduction of **2a** on chiral HPLC using Chiralcel OJ column resolved into three peaks with retention time of 16.2, 18.8 and 22.6 min (Figure 3S, Supplementary Information). The peaks at 16.2 and 18.8 min appeared in almost 1:1 ratio and were assigned to *dl* pair of **3a**. The peak eluting at 22.6 min was therefore assigned to *meso*-**3a**. The ratio of *dl/meso* in borohydride reaction was found to be 5:95, indicating the predominant formation of *meso* product in the reaction. Similar ratio was also obtained by comparison of integral values of resonance peaks at δ 4.84 and 4.72, corresponding to methine protons of *meso* and *dl* pair, respectively in ^{1}H NMR of the **3a** obtained from borohydride reduction of **2a** (Figure 1S, Supplementary Information). Thus, *dl/meso* ratio in all subsequent studies was calculated from ^{1}H NMR. In contrast to predominant formation of *meso* product in borohydride reduction, the biocatalyzed reduction produced *dl* as the major product. The ratio of *dl/meso* obtained from *Penicillium sp*., *A. alternata* and *T. flavus* was 65/35, 94/6 and 80/20 respectively. The ee of *dl* pair of **3a** was determined by chiral HPLC using Chiralcel OJ column (Figure 3S, Supplementary Information). *T. flavus* gave ee of 99% while the ee of 93 and 85% was obtained in case of *Penicillium sp. and A. alternata,* respectively. The absolute configuration of *T. flavus* catalyzed **3a** was assigned as 1*S*,2*S* based on sign of optical rotation $[\alpha]_D^{25}$ = -91.0 (c 1.05, ethanol)] [lit.[32, 33] $[\alpha]_D^{25}$ = +91.6 (c 1.05, ethanol), 99.9% ee (*R*,*R*)]. Thus, in *dl* pair the peak eluting at 16.2 min correspond to (1*S,2S*) configuration.

Exclusive preparation of (*S*)-benzoin and (*S,S*)-hydrobenzoin by *T. flavus*: pH dependent reduction of benzil

T. flavus was found to be the best biocatalyst for enantioselective preparation of (*S*)-benzoin and (*S,S*)-hydrobenzoin with >99% ee. However, it produced a mixture of **2a** and **3a**, which limts its scope for preparation of these molecules in high chemical purity. Demir *et al.* have reported an interesting effect of pH on *Rhizopus oryzae* catalyzed reduction of benzil/benzoin to give (*R*)-**2a** (>99% ee) at pH 6.5-8.5 and (*S*)-**2a** (80% ee) at pH 4.2-5.0 in 5-6 days. Continuation of the reaction for 21 days at pH 6.5-8.5 resulted in the formation of **3a** in *dl/meso* ratio of 99/1 and ee of >99%.[28, 34] Although high ee was observed, this method suffered from low yield and very long reaction time. Also, the reactions were done with growing culture, where media components and secondary metabolites are likely to contaminate the product. Moreover, no attempt was made to provide rationale for the effect of pH on the course of reaction.

Table 2 pH dependent asymmetric reduction of benzil by *Talaromyces flavus*

Entry	pH	Time (h)	Conv %[b]	**2a:3a**[a]	ee % (**2a**) (config)[b,c]	*dl:meso* (**3a**)[a]	ee % (**3a**) (conf)[b,c]
1	4.0	14	22	1:0	97 (*S*)	n.d.	n.d.
2		37	29	1:0	97 (*S*)	n.d.	n.d.
3		144	90	1:0.025	86 (*S*)	33/67	33.3 (*R,R*)
4		264	100	1:0.08	70 (*S*)	25/75	45.9 (*R,R*)
5	5.0	14	66	1:0	97 (*S*)	n.d.	n.d.
6		**37**	**100**	**1:0.035**	**99 (*S*)**	**43/57**	**91 (*S,S*)**
7		144	100	1:0.94	96 (*S*)	47/53	2.5 (*S,S*)
8		264	100	1:1.3	74 (*S*)	52/48	35 (*R,R*)
9	6.0	14	80	1:0.07	>99 (*S*)	56/44	**>99** (*S,S*)
10		37	100	1:0.393	>99 (*S*)	75/25	>99 (*S,S*)
11		144	100	1:1.2	96 (*S*)	88/12	>99 (*S,S*)
12		264	100	1:1.6	78 (*S*)	88/12	97.2 (*S,S*)
13	7.0	14	50	1:0.392	>99 (*S*)	80/20	>99 (*S,S*)
14		37	100	1:0.94	>99 (*S*)	80/20	>99 (*S,S*)
15		144	100	1:1.76	82 (*S*)	82/18	>99 (*S,S*)
16		264	100	1:2.14	81 (*S*)	84/16	97.6 (*S,S*)
17[d]		**18**	**100**	**0:1**	**-**	**97/3**	**>99 (*S,S*)**
18	8.0	14	65	1:0.174	>99 (*S*)	77/23	>99 (*S,S*)
19		37	100	1:0.441	>99 (*S*)	78/22	>99 (*S,S*)
20		144	100	1:1.07	80 (*S*)	83/17	96.2 (*S,S*)
21		264	100	1:1.26	76 (*S*)	88/12	63.7 (*S,S*)
22	9.0	14 h	65	1:0.25	>99 (*S*)	85/15	>99 (*S,S*)
23		37	98	1:0.51	>99 (*S*)	80/20	96 (*S,S*)
24		144	100	1:0.95	76 (*S*)	80/30	93.7 (*S,S*)
25		264	100	1:1	71 (*S*)	86/14	78.2 (*S,S*)

[a]Conversion %, ratio of **2a:3a** and *dl/meso* ratio was determined by ^{1}H NMR based on intergral of specified peaks.[b] Ee. of **2a** and **3a** was determined by chiral HPLC on Chiralcel OD-H and OJ (Diacel, Japan) column, respectively. [c]Absolute configuration was assigned based on comparison of sign of optical rotation with literature. [d]Substrate concentration was 2 mM in this case. n.d. = not detected

In view of these observations, we studied the effect of pH on *T. flavus* catalyzed asymmetric reduction of **1a**. Biocatalytic reduction of **1a** was carried out from pH 4.0-9.0 as described in experimental section using appropriate buffers. Aliquots were drawn at 14, 37, 144 and 264 h and analyzed for % conversion, product ratio and ee determination as described in Section 3.1. The results have been summarized in Table 2. It was observed that (*i*) at pH 4.0-6.0, (*S*)-**2a** was major product in high ee at 100% conversion (Entry 4, 6, 10; Table 2), (*ii*) the amount of **3a** increased with increasing pH. At pH 7.0, almost 1:1 ratio of **2a** to **3a** was observed at 100% conversion in 37 h. While ee of (*S*)-**2a** was >99%, **3a** was also produced in >99% ee (Entry 14; Table 2), (*iii*) decrease in ee of (*S*)-**2a** was observed at pH 5.0 to 9.0 when the reaction was continued for longer duration and (*iv*) at pH 4.0 and 5.0, not only diol was formed in poor ratio, it also gave poor *dl/meso* ratio. Also, the diol was formed in poor ee. The *dl* product had (*R,R*) configuration, whereas, at pH 6.0-8.0, diol **3a** was produced in high ee and had (*S,S*) configuration.

In summary, pH 5.0 was found to be optimal for production of (*S*)-**2a** (Entry 6, Table 2). Similarly, pH 7.0 was found to be optimal for production of (*S,S*)-**3a** (Entry 15, Table 2). Next, we reduced the substrate concentration from about 5 mM to 2 mM. We were pleased to note that at pH 7.0 and substrate concentration of 2 mM, (*S,S*)-**3a** was obtained as the exclusive product in *dl/meso* ratio of 97/3 and >99% ee (Entry 17; Table 2). Thus, depending on pH and concentration, either (*S*)-**2a** or (*S,S*)-**3a** can be selectively obtained in high ee and high *dl/meso* ratio. In contrast to *Rhizopus oryzae* we did not observe pH dependent complete reversal of enantioselectivity with *T. flavus*. However, we did observe drop in ee of (*S*)-**2a,** when the reaction was continued for longer period of 144-264 h. Optically pure (*S*)-**2a** did not show any racemization at pH 4.0 to 9.0 in absence of biocatalyst even after prolonged incubation for 264 h, indicating presence of a racemase activity in *T. flavus*.

We also studied the reduction of *rac*-**2a** with *T. flavus* as biocatalyst at various pH in the range of 4.0-9.0 in appropriate buffers. The results are summarized in Table 3. The following observation may be highlighted: (*i*) whereas no reaction occurred at pH 4.0, the reaction at pH 5.0 was very sluggish, (*ii*) at pH 6.0-9.0 and at about 50% conversion, the (*S*)-**2a** was recovered in ~30% ee and **3a** was obtained in *dl/meso* ratio of approximately 3/7 (Entry 5, 8, 11, 14; Table 3). These results suggest that both (*S*) and (*R*)-**2a** are substrates for diol reductase enzyme, but (*R*)-enantiomer reacts faster than (*S*)-enantiomer, which made partial resolution of *rac*-**2a** possible at about 50% conversion, (*iii*) it was interesting to note that after 24 h at pH 7.0-9.0, almost complete conversion of **2a** to **3a** occurred to give *dl/meso* ratio of 3:7 (Entry 9, 12, 15; Table 3). However, continuation of reaction for 96 h, the ratio of *dl/meso* changed to 1:1; the enanatiomeric excess of *dl* form increased to 96-98% (Entry 7, 10, 13, 16; Table 3). Continuation of the reaction for longer times did not result in any further change in *dl/meso* ratio of **3a**. These results may be explained by invoking presence of an oxidase activity, which converted meso **3a** to **2a**, which was re-reduced to (*S,S*)-hydrobenzoin.

pH dependent selectivity of *T. flavus* is due to presence of multiple dehydrogenases

T. flavus catalyzed bioreduction of **1a** resulted in formation of (*S*)-**2a** in >99 ee at pH 5.0 and (*S,S*)-**3a** in >99% ee at pH 7.0. In principle, these results may be explained by invoking a single enzyme, which has pH dependent substrate specificity or by invoking two enzymes system, each of which has a different pH profile for activity. In addition to above pH dependent selectivity, conversion of *meso*-**3a** to *dl*-**3a** was also observed, which could be explained based on the oxidation of a hydroxyl group to keto group to generate 2**a** as intermediate. Put together, these results indicate the presence of more than one

reductase in *T. flavus*, which are involved in the reduction of **1a**. Thus, we fractionated proteins of *T. flavus* in an attempt to isolate various reductase activities.

The cell free of extract *T. flavus*, designated as fraction F1 (see Figure 4S, Supplementary Information) was obtained by disruption of cells in Dyno Mill (KDL, Switzerland). *T. flavus* was mixed with glass beads (0.5-0.75 μm) in 1:1 ratio (wt/wt) and disruption was done at 4 °C for 15 min followed by centrifugation at 20,000g for 20 min at 4 ºC to remove cell debris. The cell free extract was subjected to ammonium sulfate fractionation, maintaining the pH and temperature constant at 7.0 and 4 °C, respectively. Whole of the activity was present in 40-80% ammonium sulphate pellet. The protein was redissolved in phosphate buffer (10 mM, pH 8.0) and desalted using sephadex G-25 column. The desalted protein was loaded on to reactive red column. The column was washed with same buffer and unbound protein fraction was assayed for activity. The bound protein were eluted with 0-1.5 M NaCl gradient and different fractions were assayed for activity. Both unbound and bound fractions of proteins were found to be active. The active fractions from eluent were combined and subjected to ion-exchange chromatography over Q-sepharose column followed by size-exclusion chromatography on superdex S-200 column. The active protein thus obtained exhibited only one homogenous band at ~40 kDa in SDS-PAGE, run under the reducing conditions (Figure 1b). It has been designated as fraction F3 (Figure 4S, Supplementary Information). Approximately 20-fold purification was achieved in 5-step purification procedure (see Table 1S and Figure 5S to 7S in Supplementary Information for summary of protein purification steps and purification profile on various columns).

Table 3 pH dependent asymmetric reduction of *rac*-benzoin **(2a)** by *Talaromyces flavus*

Entry	pH	Time h	Conv. %[a]	ee % residual (*S*)-**2a**[b,c]	*dl:meso* of **3a**[a]	ee % (*S,S*)- **3a**[b,c]
1	4	24	0	n.d.	n.d.	n.d.
2		96	0	n.d.	n.d.	n.d.
3	5	24	33	15	26/74	78.5
4		96	48	22	30/70	85
5	6	12	50	32	30/70	86
6		24	100	--	23/77	93.5
7		**96**	**100**	**--**	**50/50**	**97.9**
8	7	12	48	30	28/72	84.6
9		24	92	--	30/70	95.4
10		**96**	**100**	**--**	**50/50**	**98.5**
11	8	12	53	29	26/64	85.9
12		24	100	--	28/72	93.9
13		**96**	**100**	**--**	**47/53**	**96.7**
14	9	12	47	31	26/64	83.8
15		24	100	--	28/72	94.2
16		96	100	--	50/50	95.6

[a]Conversion %, *dl/meso* ratio was determined by ^{1}H NMR based on integral of specified peaks. [b]Ee of **2a** and **3a** was determined by chiral HPLC on Chiralcel OD-H and OJ (Diacel, Japan) column, respectively. [c]Absolute configuration was assigned based on comparison of sign of optical rotation with literature. n.d. = not detected

The pure protein fraction (F3) showed a specific activity of 4.75 μmolmin^{-1}mg^{-1} protein. The purified protein was subjected to MALDI analysis. The MALDI of the native protein gave molecular weight of ~36.4 kDa. Glycosylation is a key feature of posttranslational modification for protein stability, secretion and activity in fungi. Accordingly, the glycosylation of the *T. flavus* enzyme reported herein is possible, but its investigation was considered beyond the scope of this study.[35] The partial N-terminal amino acid sequence was determined by automated Edman degradation procedure. The N-terminal amino acid sequence was determined after blotting the enzyme onto a polyvinylidine difluoride (PVDF) membrane using a Trans-Blot Cell. The obtained N-terminal sequence, APTNQ _AYDHSV was then searched for homologous counterparts using the BLAST server at www//ncbi.nlm.nih.gov. The retrieved hits for the N-terminal sequence did not show significant homology with any of known benzil/benzoin reductase or dehydrogenase in the data base. Therefore, the protein appears to be a novel reductase.

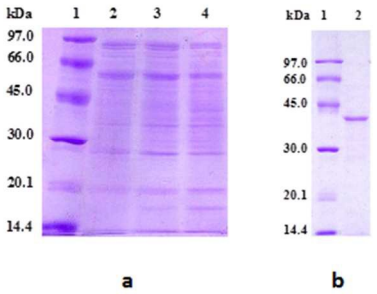


Figure 1. SDS-PAGE of Fractions F2 and F3 run under reducing conditions (a) Fraction 2; *Lane 1;* molecular wt. markers*, Lane 2, 3 and 4* are different fractions from size exclusion chromatography step (b) Fraction 3; *Lane 1:* molecular wt. markers; *Lane 2:* purified protein.

Similarly, the unbound protein fraction of reactive red step was also subjected to ion-exchange chromatography over Q-sepharose followed by size-exclusion chromatography on superdex S-200 column. The active fractions were pooled and designated as fraction F2. However, homogenous preparation could not be obtained in this case as revealed by SDS-PAGE of F2 run under reducing conditions (Figure 1a) Summary of fractionation of proteins of *T. flavus* is shown in Figure 4S, Supplementary Information.

Cell free extract (F1), partially purified protein (F2) and purified protein (F3) were studied for asymmetric reduction of **1a**, *rac*-**2a** and (*S*)-**2a** at pH 5.0 and 7.0 as described in experimental section. The results are summarized in Table 4. The presence of following enzymes activities were observed *(i) (S)-selective benzil reductase activity:* Fraction F2 converted **1a** to (*S*)-**2a** at pH 5.0 as well as pH 7.0 (Entry 6, 7; Table 4). No trace of hydrobenzoin **(3a)** could be detected in these reactions, which clearly shows that F2 is devoid of any benzoin reductase activity. However, lower ee of 91% was obtained with F2 compared to 99% obtained with whole cells at pH 5.0. The improved ee in whole cells could be due to the conversion of (*R*)-**2a** to *meso*-**3a** by benzoin reductase activity present in whole cells, but absent in Fraction F2, *(ii) (R,R)-selective benzoin reductase activity*: The pure protein (F3) showed (*R,R*)-selectivity for reduction of **1a** at pH 7.0 (Entry 9, 10; Table 4). F3 produced only (*R,R*)-**3a**, no trace of (*S,S*)-**3a** or *meso*-**3a** could be detected either by NMR or HPLC. However, the conversion rate for this enzyme was very poor compared to whole cells requiring 24 h for 25% conversion. Also, the (*R,R*)-selectivity was not strict, because when (*S*)-**2a** was used as substrate, (*S,S*)-**3a** was produced in 53% ee at 50% conversion

(Entry 11, Table 4). F3 did not show any appreciable activity at pH 5.0, *(iii) (S)-benzoin to (R)-benzoin epimerase activity*: When, the pure protein fraction F3 catalyzed conversion of (*S*)-**2a** (>99% ee) to **3a** was stopped at 50% conversion, the ee of remaining (*S*)**-2a** was found to be only 32% (Entry 11, Table 4). This clearly suggested that the pure protein in addition to benzoin reductase activity also has *S* to *R* epimerase activity. However, conversion rate for this activity was also very poor and *(iv) (S,S)-selective benzoin reductase activity:* Whole cell system of *T. flavus* contains a very strong (*S,S*)-selective benzoin reductase activity at pH 7.0, but not at pH 5.0. However this activity could not be recovered from any of the fractions except F1 (Entry 5; Table 4), which showed considerably lower activity compared to the whole cell system; indicating that enzyme responsible for this activity is unstable outside the cell. This is in spite of the presence of protease inhibitor in buffer at the time of preparation of cell free extract from whole cells. Several attempts to isolate protein corresponding to this activity were unsuccessful.

Table 4. Asymmetric reduction of benzil **(1a)** and benzoin **(2a)** with protein Fractions of *Talaromyces flavus*

Entry	Fraction	pH	Benzil (**1a**) as substrate				benzoin (**2a**) as substrate[a]			
			Conv %[b]	ee % (**2a**) (config)[c,d]	% **3a** (*dl/meso*)[b]	ee % *dl*-**3a** (config)[c,d]	Conv %[b]	ee % (**2a**) (config)[c,d]	% **3a** (*dl/meso*)[b]	ee % *dl*-**3a** (config)[c,d]
1	Whole cells	5.0	100	99 (*S*)	~3	--	48	28 (*S*)	26/74	75 (*S,S*)
2		7.0	100	--	100 (97/3)	>99 (*S.S*)	100	n.d.	50/50	97 (*S,S*)
3		7.0	-	-	-	-	80[e]	100 (*S*)	80/20	>99
4	F1	5.0	20	90 (*S*)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
5		7.0	40	86 (*S*)	7 (78/22)	80 (*S,S*)	15	11 (*S*)	16/84	86 (*S,S*)
6	F2	5.0	50	91 (*S*)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
7		7.0	50	96 (*S*)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
8	F3	5.0	<5	48 (*S*)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
9		7.0	16	75 (*S*)	20 (100:0)	>99 (*R,R*)	20	36 (*S*)	99/1	>99 (*R,R*)
10		7.0	25	6 (*R*)	33 (100:0)	>99 (*R,R*)	50	25 (*S*)	100:0	>99 (*R,R*)
11		7.0	-	-	-	-	50[e]	32 (*S*)	100/0	53 (*S,S*)

[a]Benzoin was racemic except in entries 3 and 11 where (*S*)-benzoin was used as substrate. [b]Conversion %, *dl/meso* ratio was determined by ^{1}H NMR based on integral of specified peaks. [c]Ee of **2a** and **3a** was determined by chiral HPLC on Chiralcel OD-H and OJ (Diacel, Japan) column, respectively. [d]Absolute configuration was assigned based on comparison of sign of optical rotation with literature. n.d. = not detected

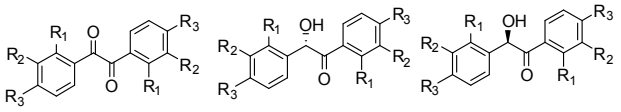
T. flavus catalyzed asymmetric monoreduction of various symmetrical benzils (1a-1g)

In order to study the scope of *T. flavus* catalyzed mono reduction of benzils in general, various symmetrical benzils **1a-1g** were synthesized and subjected to treatment with biocatalyst at pH 5.0 under the reaction conditions described in experimental section. The purification of products was done by flash chromatography over silica gel. The desired products **2a-2g** were obtained in 54-90% yield and 30-99% ee. The results have been summarized in Table 5 (Entries 1-7). These results suggested that (*i*) the substitution of phenyl ring with +I or –I substituent considerably slowed down the reaction. Whereas, benzil was reduced in 30 h, substituted benzils typically required about 96 h for the reaction to go to completion. No reduction occurred in case of 4-ethoxy substituent (Entry 6; Table 5), (*ii*) the substitution of electron donating methoxy group or electron withdrawing chloro group resulted in reduced enantioselectivity (Entry 2, 7; Table 5). The loss of enantioselectivity was considerably higher in case of chloro substitution compared to methoxyl substitution, (*iii*) the ee in case methoxyl substitution followed order 4-methoxy > 3-methoxy > 2-methoxy (Entry 2-4; Table 5). It indicates that in addition to electronic effects, steric effects in the vicinity of carbonyl group also play a role. Thus, ee increased as the methoxyl group was moved away from 2-position to 3-position of phenyl group from 42% to 77%. The ee was 82% when methoxyl group was moved to 4-position, (*iv*) an interesting observation was the reversal of stereoselectivity in case of 2-chloro substitution (Entry 7; Table 5). Whereas substituted benzil and methoxy or methyl substituted benzil produced corresponding benzoin predominantly in (*S*)-configuration, 2-chloro substituted benzil produced corresponding benzoins in (*R*)-configuration, (*v*) the substitution of methyl group at 4-position of phenyl group has minimal effect on the enantioselectivity of the biocatalyst and (*vi*) no trace of hydrobenzoin was observed at 100% conversion with any of the substrate under these reaction conditions.

T. flavus catalyzed enantio- and regioselective monoreduction of various unsymmetrical benzils (4a-4e)

In order to study the scope of *T. flavus* for regioselective reduction of various unsymmetrical benzil derivatives **4a-4e** were synthesized and subjected to treatment with biocatalyst at pH 5.0 under the reaction conditions described in experimental section. The purification of products was done by flash chromatography over silica gel. The desired products **5a-5e** were obtained in 80-91% yield. The results have been summarized in Table 5 (Entries 8-12). In summary the results indicate that (*i*) in all the examples studied, the carbonyl α to unsubstituted phenyl group was regioselectively reduced, (*ii*) there was no effect of

substitution on the enantioselectivity as ee of 96-99% was obtained in all examples studied except when methoxy group was present at 2-position of phenyl ring, which gave ee of 89% (Entry 8; Table 5). This is in contrast to symmetrical benzils, where substitution had detrimental effect on ee of product, (*iii*) *N*-substituent was well tolerated as dimethylamino group at 4-position of phenyl ring produced the desired product in >99% ee and 85% yield (Entry 12; Table 5), (*iv*) no hydrobenzoin was produced in any of the examples studied even when the reactions were carried out for longer times and (*v*) the ee and regioselectivity obtained in *T. flavus* catalyzed reaction for these examples is higher compared to any report in literature till date. In literature *Saccharomyces cerevisiae* catalyzed regioselective reduction of these substrates has been reported but ee of products was not calculated.[36] With *Xanthomonas oryzae* mixture of both regio isomers were obtained.[25]



a, R_1=R_2=R_3=H; **b**, R_1=OCH_3, R_2=R_3=H; **c**, R_1=R_3=H, R_2=OCH_3; **d**, R_1=R_2=H, R_3=OCH_3; **e**, R_1=R_2=H R_3=CH_3; **f**, R_1=R_2=H R_3=OEt; **g**, R_1=Cl, R_2=R_3=H

a, R_1=OCH_3, R_2=R_3=H; **b**, R_1=R_2=H, R_3=OCH_3; **c**, R_1=R_2=H R_3=OCH_2CH_3; **d**, R_1=R_2=OCH_3 R_3=H; **e**, R_1=R_2=H,R_3=NMe_2

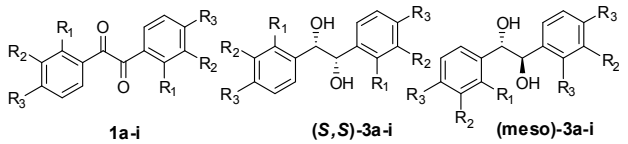
Table 5. Enantioseslective reduction of symmetrical and unsymmetrical benzils to benzoins by *Talaromyces* flavus at pH 5.0

Entry	Substrate	Product	ee %[a] (config)	Yield[d] %	Time (h)
1	1a	2a	99% (*S*)[b]	90	30
2	1b	2b	42 (*S*)[b]	90	96
3	1c	2c	77 (*S*)[b]	85	96
4	1d	2d	82 (*S*)[b]	54	96
5	1e	2e	97 (*S*)[b]	84	96
6	1f	2f	n.r.	n.r.	n.r.
7	1g	2g	30 (*R*)[b]	62	96
8	4a	5a	89 (*S*)[c]	91	17
9	4b	5b	96 (*S*)[c]	88	17
10	4c	5c	>99 (*S*)[c]	84	24
11	4d	5d	96 (*S*)[c]	80	96
12	4e	5e	>99 (*S*)[c]	85	48

[a] Ee. was determined by chiral HPLC on Chiralcel OD-H (Diacel, Japan) column. [b]Absolute configuration was assigned based on comparison of sign of optical rotation with literature. [c]Configuration tentative. n.r. = no reaction. [d]Yield of isolated product.

T. flavus catalyzed asymmetric reduction of various benzils (1a-1i) to corresponding 1,2-diols

We have already demonstrated that biocatalytic reduction of benzil by *Talaraomyces flavus* at pH 7.0 leads to predominant formation of chiral 1,2-diols. We extended this study to various derivatives of benzil in order to study the effect of substituent on conversion rate and ee of the product. *T. flavus* catalyzed reduction of benzil derivatives **(1a-1i)** at pH 7.0 produced corresponding 1,2-diol in 85-90% yield (Table 6). The following observations are worthy of note, (*i*) the presence of a substituent at 2 or 4 position of phenyl ring slowed down the reaction, whereas substituent at 3-position had much less effect on the conversion rate. No reaction occurred when a OCH_3 group was present at 2-position of benzil (Entry 2; Table 6), whereas 2-chloro derivative required 168 h for the reaction to go to completion (Entry 7; Table 6). 4-Methoxy and 4-methyl derivatives also required about 168 h for the reaction to go to completion (Entry 4, 5; Table 6). Surprisingly no reaction occurred with 4-ethoxy substituent (Entry 6; Table 6), (*ii*) the substitution of +I methoxy group or –I chloro group resulted in reduced *dl/meso* ratio. Whereas benzil gave *dl/meso* ratio of 97/3, 3-methoxy and 4-methoxy derivative gave *dl/meso* ratio of 89/11 and 38/62, respectively (Entry 3, 4; Table 6). Chloro substituent at 2-position gave *dl/meso* ratio of 10/90 (Entry 7; Table 6) and (*iii*) the presence of methoxy or methyl substituent at 4-position resulted in decreased ee of 78-84% (Entry 4, 5; Table 6), while with 2 -chloro substituent ee of >99% was obtained (Entry 7; Table 6).



a, R_1=R_2=R_3=H; **b**, R_1=OCH_3, R_2=R_3=H; **c**, R_1=R_3=H, R_2=OCH_3; **d**, R_1=R_2=H, R_3=OCH_3; **e**, R_1=R_2=H R_3=CH_3; **f**, R_1=R_2=H R_3=OEt; **g**, R_1=Cl, R_2=R_3=H; **h**, R_1=R_3=H R_2=Cl; **i**, R_1=R_2=H R_3=Cl

Table 6 Enantio and diastereoselective reduction of 1,2-diketones to chiral 1, 2-diols by *Talaromyces flavus* at pH 7.0

Entry	Substrate	Product	*dl/meso*[a]	ee% (config)[b,c]	Yield[d, e] %	Time (h)
1	1a	3a	97/3	>99 (*S,S*)	86	56
2	1b	3b	n.r.	n.r.	n.r.	n.r.
3	1c	3c	89/11	n.d.	88	48
4	1d	3d	38/62	84 (*S,S*)	89	168
5	1e	3e	67/33	78.5 (*S,S*)	87	168
6	1f	3f	n.r.	n.r.	n.r.	n.r.
7	1g	3g	10/90	>99 (*S,S*)	85	168
8	1h	3h	70/30	n.d.	88	48
9	1i	3i	67/33	>99%(*S,S*)	86	168

[a] *dl/meso* ratio was determined by ^{1}H NMR based on integral of specified peaks. [b] Ee of *dl* pair was determined by chiral HPLC on Chiralcel OJ (Diacel, Japan) column. [c]Absolute configuration was assigned based on comparison of sign of optical rotation with literature. [d] combined yield for *dl* and meso. [e]Yield of isolated product. n.r. = no reaction, n.d. = not determined.

Conclusions

We have shown that by modulating pH in *T. flavus* catalyzed reduction of benzil, the reaction can be controlled to obtain either (*S*)-benzoin (**2a**) or (*S,S*)-hydrobenzoin (**3a**) as the exclusive product in high ee. Two prominent enzymes involved in pH dependent selectivity are (i) (*S*)-selective benzil reductase, which is active at pH 5.0 and 7.0 and (ii) (*S,S*)-selective benzoin reductase, which is active at pH 7.0,

but has no activity at pH 5.0. In addition, a novel dual activity (*R*,*R*)-selective benzoin reductase and benzil epimerase protein was also purified. This enzyme had relaxed selectivity and also converted (*S*)-benzoin to (*S*,*S*)-hydrobenzoin. Benzoins were obtained in high ee when one or both phenyl groups of benzil were unsubstituted. Except for **3a**, all other hydrobenzoins were obtained in moderate de. However, considering the facts that (i) these bulky-bulky 1,2-diketones are very tough substrates for enzymes and (ii) ee and de obtained in benzil reduction with *T. flavus* compare quite favorably with any of the known biocatalysts in literature. Therefore, *T. flavus* expands and enriches the biocatalytic tool box for asymmetric reduction of benzils.

Experimental

General

Optical rotations were measured using Rudolph Autopol IV polarimeter. ^{1}H NMR spectra were obtained at 300 MHz and referenced to TMS (0.0 ppm) or the residual solvent peak ($CHCl_3$ 7.26 ppm). Chemical shifts are reported as parts per million (ppm) using the δ scale. ^{13}C NMR spectra were recorded at 75 MHz and referenced either to TMS (0.0 ppm) or internal solvent ($CDCl_3$ 77.0 ppm). Thin layer chromatography (TLC) was performed on Merck silica gel DC Alurolle Kieselgel 60F_{254} plates and were visualized under UV lamp and/or with 0.25% w/v $KMnO_4$ and 2% $NaHCO_3$ solution in water. Flash column chromatography was carried out using silica gel (200-400 mesh). Analytical HPLC analyses were performed on a system equipped with high pressure gradient dual pump, auto injector, variable temperature column compartment and PDA detector. Ee was determined by HPLC using one of the following systems. HPLC System 1 - Column: 250×4.6 mm Chiralcel OD-H (Diacel, Japan); detection UV at 254 nm; elution 10% 2-propanol in hexane at flow rate of 1 mL/min; temperature 25 °C. HPLC System 2 - Column: 250×4.6 mm Chiralcel OJ (Diacel, Japan) column; detection 254 nm; elution 10% 2-propanol in hexane at flow rate of 1 mL/min; temperature 25 °C.

General procedure for pH dependent asymmetric reduction of benzil and benzoin by *Talaromyces flavus*

A 100 mL round bottomed flask was charged with cells (5 g, wet weight) suspended in 50 ml of appropriate 0.2 M buffer (acetate buffer for pH 4.0 and 5.0; phosphate buffer for pH 6.0-8.0; Tris-HCl buffer for pH 9). Substrate (50 mg, 0.24 mmol) was added to the cell suspension and the mixture shaken on an orbital shaker at 30 °C and 200 rpm. During the course of reaction pH of the reaction mixture was maintained by addition of 1 N NaOH or 1 N HCl. 10 ml of sample from each reaction was taken after different time intervals intervals (14, 37, 144 and 264 h for benzil and 12, 24 and 96h for benzoin) and centrifuged (9000g, 4 °C, 15 min) to remove cells. The cell free broth was than extracted with ethyl acetate (3×10 ml), washed with brine (1×5 mL) and dried over anhydrous sodium sulfate. Solvents were removed under reduced pressure on a rotary evaporator to leave a residue. The conversion, ratio of benzoin to hydobenzoin and *dl/meso* ratio of hydrobenzoin was calculated by comparison of integral values of corresponding peaks from ^{1}H NMR and ee of products was deteremined by HPLC.

General procedure for enantioselective reduction of benzils and benzoin by with protein fractions of *T. flavus*

Enantioselective reduction of benzil, (*S*)-benzoin and *rac*-benzoin by protein fractions from *T. flavus* was carried out at pH 5.0 in 50 mM acetate buffer or pH 7.0 in 50 mM phosphate buffer. A solution of $NADP^+$ (784 nmol), glucose dehydrogenases (14 units), glucose (0.5 mmol) and protein fraction (8 units of dehydrogenase activity) and substrate (0.02 mmol) in 4 mL of appropriate buffer was energetically mixed on a magnetic stirrer. After 16 h the products were extracted in ethyl acetate (3×4 ml) and dried over anhydrous sodium sulfate. Solvents were removed under reduced pressure to leave a residue. The conversion, ratio of benzoin/hydobenzoin and *dl/meso* ratio of hydrobenzoin was calculated as described in section 2.4.2.

General procedure for microbial monoreduction of symmetrical and unsymmetrical benzils by *Talaromyces flavus*

A 100 mL round bottomed flask was charged with cells (5 g, wet weight) suspended in 50 mL acetate buffer (0.2 M, pH 5.0). Substrate (0.24 mmol, 50-72 mg depending upon molecular mass of the substrate) was added and the mixture was shaken on an orbital shaker at 30 °C and 200 rpm till complete consumption of starting material occurred. The reaction mixture was then centrifuged (9000g, 4 °C, 15 min) to remove cells. The supernatant was extracted with ethyl acetate (3x25 mL), washed with saturated brine (10 mL) and dried over anhydrous sodium sulfate. Solvents were removed under reduced pressure to leave a residue which was purified by flash chromatography (silica, methanol/chloroform; 10:90). Ee was determined by using HPLC (System 1).The yield, ee, NMR and HPLC data for products is given below.

(*S*)-2-hydroxy-1,2-diphenylethanone (2a): Yield: 45.2 mg (90%); 99%% ee; $[\alpha]_D^{25}$ = +115.2 (c 1.5, acetone) [lit.[31] $[\alpha]_D^{25}$ = +115.0 (c 1.5, acetone), >99% ee (*S,S*)]. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 4.54 (bs, 1H), 5.95 (s, 1H), 7.23-7.34 (m, 5H), 7.36-7.41 (m, 2H), 7.49-7.54 (m, 1H), 7.89-7.92 (m, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ = 76.3, 127.8, 128.7, 129.2, 133.6, 134.0, 139.0, 199.0; HPLC data: HPLC analysis (System 1)**:** retention time = 11.9 min (major) and 17.4 min (minor).

(*S*)-2-hydroxy-1,2-bis(2-methoxyphenyl)ethanone (2b): Yield: 58.3 mg (90%); 42 % ee; $[\alpha]_D^{25}$ = +52.0 (c 0.9, $CHCl_3$) [lit.[37] $[\alpha]_D^{25}$ = -125.0 (c 0.9, $CHCl_3$), >99% ee, (*R*)]; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 3.70 (s, 3H), 3.71 (s, 3H), 4.46 (bs, 1H), 6.09 (s, 1H), 6.73-6.77 (m, 2H), 6.80-6.85 (m, 1H), 6.89-6.94 (m, 1H), 7.12-7.20 (m, 2H), 7.32-7.38 (m, 1H), 7.65-7.69 (m, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ = 55.2, 55.3, 75.9, 110.9, 111.2, 120.5, 125.3, 127.6, 129.6, 130.0, 130.7, 133.9, 157.3, 158.2, 201.7; HPLC data: HPLC analysis (System 1)**:** retention time = 21.7 min (minor) and 31.2 min (major).

(*S*)-2-hydroxy-1,2-bis(3-methoxyphenyl)ethanone (2c): Yield: 55 mg (85%); 77% ee; $[\alpha]_D^{25}$ = +119.0 (c 1.0, CH_3OH) [lit.[37] $[\alpha]_D^{25}$ = -156.0 (c 1.0, CH_3OH), >99% ee, (*R*)]; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 3.71 (s, 3H), 3.75 (s,

3H), 5.90 (s, 1H), 6.77-6.80 (m, 1H), 6.86-6.87 (m, 1H), 6.91-6.93 (m, 1H), 7.02-7.05 (m, 1H), 7.18-7.28 (m, 2H), 7.45-7.49 (m, 2H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 55.3, 55.4, 76.3, 113.3, 113.5, 114.2, 120.2, 120.5, 121.8, 129.7, 130.2, 134.8, 140.5, 159.8, 160.2, 198.8; HPLC data: HPLC analysis (System 1)**:** retention time = 19.2 min (major) and 27.0 min (minor).

(*S*)-2-hydroxy-1,2-bis(4-methoxyphenyl)ethanone (2d): Yield: 35 mg (54%); 82% ee; $[\alpha]_D^{25}$ = +73.8 (c 1.0, CH_3OH) [lit.[37] $[\alpha]_D^{25}$ = -90.4 (c 1.0, CH_3OH), >99% ee, (*R*)]. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 3.74 (s, 3H), 3.81 (s, 3H), 5.84 (s, 1H), 6.82-6.86 (m, 4H), 7.24 (d, *J* = 8.6 Hz, 2H), 7.89 (d, *J* = 8.6 Hz, 2H);^{13}C NMR (75 MHz, $CDCl_3$): δ = 55.3, 55.5, 75.3, 114.0, 114.6, 126.4, 129.1, 131.6, 131.9, 159.7, 164.0, 197.4; HPLC data: HPLC analysis (System 1)**:** retention time = 42.0 min (major) and 44.4 min (minor).

(*S*)-2-hydroxy-1,2-dip-tolylethanone (2e): Yield: 48 mg (84%); 97% ee; $[\alpha]_D^{25}$ = +144.6 (c 0.7, CH_3OH) [lit.[37] $[\alpha]_D^{25}$ = -150 (c 0.7, CH_3OH), >99% ee, (*R*)]; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 2.28 (s, 3H), 2.34 (s, 3H), 5.89 (s, 1H), 7.11 (d, *J* = 8.2 Hz, 2H), 7.16-7.23 (m, 4H), 7.82 (d, *J* = 8.2 Hz, 2H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 21.2, 21.8, 76.8, 127.8, 129.4, 129.5, 129.9, 131.0, 136.4, 138.4, 145.0, 198.7; HPLC data: HPLC analysis (System 1)**:** retention time = 9.2 min (major) and 11.6 min (minor).

(*R*)-1,2-bis(2-chlorophenyl)-2-hydroxyethanone (2g): Yield: 41.5 mg (62%); 30% ee; $[\alpha]_D^{25}$ = -14.1 (c 1.0, $CHCl_3$) [lit.[37] $[\alpha]_D^{25}$ = -46.0 (c 1.0, $CHCl_3$), 97% ee, (*R*)]. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 4.53 (bs, 1H), 6.34 (s, 1H), 7.11-7.17 (m, 2H), 7.18-7.25 (m, 3H), 7.27-7.32 (m, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ = 75.5, 126.6, 127.4, 129.1, 129.4, 130.0, 130.1, 131.6, 132.4, 133.9, 134.9, 135.6, 200.9; HPLC data: HPLC analysis (System 1)**:** retention time = 11.9 min (minor) and 15.0 min (major).

(*S*)-2-hydroxy-1-(2-methoxyphenyl)-2-phenylethanone (5a): Yield: 52.4 mg (91%); 89% ee;^{1}H NMR (300 MHz, $CDCl_3$) δ = 3.79 (s, 3H), 4.64 (bs, 1H), 6.28 (s, 1H), 6.82-6.89 (m, 2H), 7.16-7.24 (m, 2H), 7.29-7.35 (m, 2H), 7.41-7.46 (m, 1H), 7.91-7.94 (m, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ = 55.6, 71.1, 111.6, 121.3, 127.7, 127.8, 128.6, 128.8, 128.9, 129.2, 130.1, 133.7, 156.6, 199.6; HPLC data: HPLC analysis (System 1): retention time = 19.4 min (major) and 21.7 min (minor). Configuration was assigned tentatively as *S* based on elution order.

(*S*)-2-hydroxy-1-(4-methoxyphenyl)-2-phenylethanone (5b): Yield: 51mg (88%); 96% ee; ^{1}H NMR (300 MHz, $CDCl_3$) δ = 3.81 (s, 3H), 4.64 (d, *J* = 5.16 Hz, 1H), 5.88 (d, *J* = 5.16 Hz, 1H), 6.86 (d, *J* = 8.9 Hz, 2H), 7.23-7.33(m, 5H), 7.90 (d, *J* = 8.9 Hz, 2H);^{13}C NMR (75 MHz, $CDCl_3$) δ = 55.8, 75.8, 114.0, 126.3, 127.8, 128.5, 129.2, 131.7, 139.6, 164.2, 197.2; HPLC data: HPLC analysis (System 1)**:** retention time = 19.1 min (major) and 21.2 min (minor). Configuration was assigned as tentatively as *S* based on elution order.

(*S*)-1-(4-ethoxyphenyl)-2-hydroxy-2-phenylethanone (5c): Yield: 52 mg (84%); >99% ee; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.36 (t, *J* = 6.87 Hz, 3H), 3.99 (q, *J* = 6.87 Hz, 2H), 5.90 (s, 1H), 6.82 (d, *J* = 8.9 Hz, 2H), 7.21-7.36 (m, 5H), 7.89 (d, *J* = 8.9 Hz, 2H). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 14.6, 63.9, 75.8, 114.4, 126.1, 127.8, 128.7, 129.1, 131.7, 139.7, 163.6, 197.3; HPLC data: HPLC analysis (System 1)**:** retention time = 15.7 min (major) and 16.3 min (minor). Configuration was assigned tentatively as *S* based on order of elution of HPLC.

(*S*)-2-hydroxy-1-(2,3-dimethoxyphenyl)-2-phenylethanone (5d): Yield: 52 mg (80%); 96% ee; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 3.77 (s, 3H), 3.78 (s, 3H), 4.71 (s, 1H), 5.86 (s, 1H), 6.70 (d, *J* = 8.2 Hz, 1H), 7.17-7.31 (m, 5H), 7.43-7.48 (m, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ = 55.9, 56.0, 75.8, 110.1, 111.2, 124.3, 126.4, 127.7, 128.5, 129.1, 139.9, 149.0, 153.9, 197.2; HPLC data: HPLC analysis (System 1)**:** retention time = 35.3 min (major) and 41.9 min (minor). Configuration was assigned tentatively as *S* based on order of elution of HPLC.

(*S*)-1-(4-(dimethylamino)phenyl)-2-hydroxy-2-phenylethanone (5e): Yield: 52 mg (85%); >99% ee; ^{1}H NMR (300 MHz, $CDCl_3$) δ = 3.01 (s, 6H), 4.80 (d, *J* = 5.85 Hz, 1H), 5.84 (d, *J* = 5.85 Hz, 1H), 6.56 (d, *J* = 8.9 Hz, 2H), 7.23-7.36 (m, 5H), 7.83 (d, *J* = 8.9 Hz, 2H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 40.1, 75.3, 110.7, 120.8, 127.7, 128.2, 129.0, 131.6, 140.6, 153.8, 196.0; HPLC data: HPLC analysis (System 1): retention time = 54.4 min (major) and 56.4 min (minor). Configuration was assigned tentatively as *S* based on order of elution of HPLC.

General procedure for microbial reduction of benzils to hydrobenzoins by Talaromyces flavus

The procedure was same as described in section 2.4.4 except that the phosphate buffer (0.2 M, pH 7.0) was used instead of acetate buffer. Ee was determined by using HPLC (System 2). Ee, NMR and HPLC data for products is given below.

(*1S,2S*)-1,2-diphenylethane-1,2-diol (*S,S*)-3a: >99% ee**;** $[\alpha]_D^{25}$ = -91.0 (c 1.05, ethanol)] [(lit.[32] $[\alpha]_D^{25}$ = +91.6 (c 1.05, ethanol), 99.9% ee (*R,R*)]; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 4.72 (s, 2H), 7.11-7.15 (m, 4H), 7.21-7.25 (m, 6H) ppm. HPLC data: HPLC analysis (System 2)**:** retention time = 16.2 min (*S,S*), 18.8 min (*R,R*) and 22.6 min (*meso*).

(*1S,2S*)-1,2-bis(3-methoxyphenyl)ethane-1,2-diol (*S,S*)-3c: ee; not determined ^{1}H NMR (300 MHz, $CDCl_3$): δ = 3.71 (s, 6H,), 4.68 (s, 2H), 6.71-6.80 (m, 5H), 7.12-7.26 (m, 3H) ppm.

(*1S,2S*)*-1,2-bis(4-methoxyphenyl)ethane-1,2-diol* (*S,S*)-**3d**: 84% ee; $[\alpha]_D^{25}$ = -99.12.0 (c 1.0, EtOH] [lit.[38] $[\alpha]_D^{25}$ = -118.3 (c 1.0, EtOH), 99% ee (*S,S*)]. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 3.75 (s, 6H,), 4.63 (s, 2H), 6.76 (d, *J* = 8.9 Hz, 4H), 7.0 (d, *J* = 8.9 Hz, 4H) ppm.

(*1S,2S*)-1,2-di-p-tolylethane-1,2-diol (*S,S*)-3e: 78.5% ee; $[\alpha]_D^{25}$ = -89 (c 1.0, ethanol)[lit.[38] $[\alpha]_D^{25}$ = -102.5 (c 1.0, ethanol), 99% ee (*S,S*)]; ^{1}H NMR **(**300 MHz, $CDCl_3$): δ = 2.29 (s, 6H,), 4.67 (s, 2H), 7.13 (d, *J* = 8.9 Hz, 4H), 7.19 (d, *J* = 8.9 Hz, 4H) ppm; HPLC data: HPLC analysis (System 2)**:** retention time = 12.2 min (*S,S*), 14.6 min (*R,R*) and 15.6 min (*meso*).

(*1S,2S*)-1,2-bis(2-chlorophenyl)ethane-1,2-diol (*S,S*)-3g: >99% ee; ^{1}H NMR (300 MHz, $CDCl_3$): δ =5.36 (s, 2H), 7.16-7.18 (m, 5H), 7.25-7.28 (m, 3H) ppm. HPLC data: HPLC analysis (System 2)**:** retention time = 26.3 min (*meso*), 50.9 min (*S,S*), 57.6 min (*R,R*).

(1S,2S)*-1,2-bis(3-chlorophenyl)ethane-1,2-diol *(S,S)*-3h*: ee: not determined ^{1}H NMR (300 MHz, $CDCl_3$): δ = 4.64 (s, 2H), 6.91 (d, *J* = 7.2 Hz, 2H), 7.12-7.26 (m, 6H) ppm.

(*1S,2S*)-1,2-bis(4-chlorophenyl)ethane-1,2-diol (*S,S*)-3i: >99% ee; $[\alpha]_D^{25}$ = -91.0 (c 1.0, $CHCl_3$) (lit.[38] $[\alpha]_D^{25}$ = +93 (c 1.0, $CHCl_3$), 99% e.e. (*R,R*)]. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 4.63 (s, 2H), 7.0 (d, *J* = 8.6 Hz, 4H), 7.21 (d, *J* = 8.6, Hz, 4H) ppm. HPLC data: HPLC analysis (System 2): retention time = 44.9 min (*S,S*), 47.0 min (*R,R*) and 55.0 min (*meso*).

Materials and Methods

Source of materials and microorganisms

Symmetrical and unsymmetrical benzils were synthesized by benzoin condensation followed by oxidation with pyridinium chlorochromate.[39] Culture media, TSB (tryptone soya broth) and PDB (potato dextrose broth) were purchased from Hi Media (Mumbai, India). Sephacryl S-200, bovine serum albumin (BSA), β-mercaptoethanol, sodium dodecyl sulfate (SDS), reactive red 120-agarose type 3000-CL, molecular weight standards were from Sigma Chemical Company, USA. Q-Sepharose fast-flow and Phenyl Sepharose CL-B4 were from Pharmacia (Freiburg, Germany). *Penicillium sp., Alternaria alternata* and *Talaromyces flavus* are our own isolates and have been described previously (Pal et al., 2012). These have been deposited with Microbial Type Culture Collection and GenBank (MTCC), IMTECH, Chandigarh (http://mtcc.imtech.res.in) with accession number MTCC 10832, MTCC 10833 and MTCC 10834, respectively.

Growth conditions

Fungal strains were maintained as 25% glycerol stocks at -78 ^{o}C. A 100 mL Erlenmeyer flask was charged with 20 mL sterile PDB media (24 g/L; pH 5.2-5.3). After inoculation with fungi, the flask was incubated with shaking at 200 rpm in an incubator-shaker at 30 ^{o}C for 24 h. This was used to inoculate a 2 L Erlenmeyer flask containing 400 mL of same media. The flask was then incubated at 30 ^{o}C with shaking at 200 rpm on an orbital shaker for 72 h. Microbial cells were isolated by centrifugation at 9000g for 15 min at 4 °C. Composition of PDB: potato infusions (200 g L^{-1}) and dextrose (3 g L^{-1}).

Preparation of cell free extract

T. flavus was grown in several 2L flasks, as described in section 2.2. Cells of *T. flavus* (75 g, wet weight) were suspended in 200 mL phosphate buffer (50 mM, pH 7.0), containing 1mM protease inhibitor cocktail (dithiothreitol, phenylmethylsulfonyl fluoride and 1, 10-phenanthroline) and cooled to 4 ^{o}C. The suspension was disrupted by glass beads (0.5-0.75 µm) in 1:1 (wt/wt) ratio at 4 ^{o}C for 15 min in Dyno Mill. The cell free extract was obtained by centrifugation at 20,000g for 20 min at 4 ºC

Ammonium sulfate fractionation

Solid ammonium sulfate was added in small portions to the cell free extract at 4 ºC to 40% saturation. The pellet was removed by centrifugation and supernatant was brought to 80% saturation with solid ammonium sulfate while maintaining the temperature at 4 ºC and pH at 7.0. The precipitated proteins were collected by centrifugation. The pellet was re-dissolved in phosphate buffer (10 mM, pH 8.0) and centrifuged to remove any precipitate.

Affinity chromatography

The supernatant from above step was desalted using Sephadex G-25 column. This solution was applied on a Reactive Red 120-agarose, type 3000-CL column (2×13 cm) pre-equilibrated with phosphate buffer (10 mM, pH 8.0). The column was washed with the same buffer until all unbound proteins were completely removed. An increasing linear gradient of NaCl (0-1.5 M) in phosphate buffer (10 mM, pH 6.5) at a flow rate of 60 mLh^{-1} was used to elute the proteins. The active fractions were pooled and concentrated by ultrafiltration (Millipore-Amicon Ultra-15 Centrifugal Filter Units with 10 kDa membrane). Desalting was done by repeated dilutions with Tris-HCl buffer (10 mM, pH 8.0) and sample was finally concentrated to 1 mL volume.

Ion exchange chromatography

The desalted and concentrated sample (1 mL) from Reactive Red chromatography was loaded onto a Q-Sepharose Fast flow column (2×5 cm) pre-equilibrated with Tris-HCl buffer (10 mM, pH 8.0). The column was then washed with the same buffer and the enzyme was eluted with thirty column volumes of a increasing linear gradient of NaCl (0-0.5 M) in the Tris-HCl buffer (10 mM, pH 8.0) at a flow rate of 60 mLh^{-1}. The active fractions were pooled and concentrated by ultrafiltration (Millipore-Amicon Ultra-15 Centrifugal Filter Units with 10 kDa membrane). Desalting was done by repeated dilutions with Tris-HCl buffer (10 mM, pH 8.0) and sample concentrated to a final volume of 0.5 mL.

Gel filtration chromatography

The desalted and concentrated sample (0.5 mL) from Q-Sepharose chromatography was loaded onto a Sephacryl S-200 column (2×60 cm, 43 mL bed volume) previously equilibrated with Tris-HCl buffer (50 mM, pH 7.5) containing 0.15 M NaCl at a flow rate of 30 mLh^{-1}. Active fractions were pooled and concentrated in an ultrafiltration cell using 10 kDa membrane.

Enzyme assay method

The reductase activity was determined by monitoring the decrease in absorbance of NADPH at 340 nm (molar absorption coefficient of 6800 $M^{-1}cm^{-1}$) as described previously.[40] The standard reaction mixture (1 mL) contained 0.1 mM NADPH, 50 µl cell-free extract and 2.5 mM substrate. One unit of enzyme activity was defined as the amount of enzyme that catalyzed the oxidation of 1 µmol NADPH per minute under specified conditions. Protein concentration was estimated by the coomassie brilliant blue-G method of Bradford using bovine serum albumin (BSA) as standard.[41]

Polyacrylamide gel electrophoresis (PAGE)

SDS-PAGE was performed in 12.5% (w/v) gels according to procedure of Laemmli.[42] Prior to loading to the gel, the samples were heated in a boiling water bath for 5 min. The discontinuous gel system usually had 5% stacking and 12.5% resolving gel. Electrophoresis was carried out using Laemmli buffer at constant current of 15 mA first, till the samples entered the gel and then at 20 mA till the completion. On completion of electrophoresis, gel was immersed in 0.05% Coomassie Blue R250 in methanol:acetic acid:water (4:1:5) with gentle shaking and was then destained in destaining solution (staining solution without dye) till the background was clear. The protein standards used were phosphorylase b, rabbit muscle (97,000 Da), albumin, bovine serum (66,000 Da), ovalbumin, chicken egg white (45,000 Da), carbonic anhydrase, bovine erythrocyte (30,000 Da), trypsin inhibitor, soybean (20,100 Da), and α-lactalbumin, bovine milk (14,400 Da).

N-terminal sequencing and bioinformatics studies

Purified protein was run on SDS-PAGE. The gel was washed once with water and equilibrated with 10 mM CAPS (3-[cyclohexylamino]-1-propanesulfonic acid) buffer, pH 11.0 containing 0.037% (w/v) SDS and 10% methanol for 15 min. A polyvinyl difluoride (PVDF) membrane of gel size was cut and wetted with methanol for 1 min, rinsed once with water and equilibrated with the above buffer for 15 min. Proteins were electrophoretically transferred onto PVDF membrane at 300-350 mA for 1 h at 4 °C. The extent of transfer was visualized by staining with 0.1% (w/v) amido black (in 1% acetic acid) for 5 min. Destaining was done in 50% (v/v) methanol. Transferred protein of interest was excised and washed once with 20% (v/v) methanol for 1 h at RT. Thereafter, cut-out PVDF bands were washed thoroughly (10 times) with double distilled water to remove traces of methanol. These PVDF bands were dried on Whatman sheets and stored at 4 °C until analyzed using Applied Biosystem's 476 A protein sequencer following Edman degradation protocol. The obtained N-terminal sequence, APTNQ_AYDHSV was then searched for homologous counterparts using the BLAST server at www//ncbi.nlm.nih.gov.

MALDI-TOF mass spectral analysis

Purified enzyme (10 μM) and fresh matrix (3,5-Dimethoxy-4-hydroxycinnamic acid, sinapinic acid, 10 mM) solutions were premixed in a small Eppendorf tube and applied directly to the sample support (i.e., sample plate). The sample was allowed to air evaporate and irradiated by a nanosecond laser pulse and analyzed using MALDI-TOF Voyager DE-STR instrument.

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Notes and references

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Electronic Supplementary Information (ESI) available: Figures 1S-3S showing determination of 2a/3a ratio, dl/meso ratio and ee, Figure 4S showing fractionation scheme for proteins of *T. flavus*, Figure 5S-7S showing protein purification profile on various columns and Table 1S showing summary of protein purification steps. Table 2S-4S showing HPLC traces of racemic and biocatalyzed benzoins and hydrobenzoins. NMR images of all relevant compounds. See DOI: 10.1039/b000000x/

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Graphical Abstract

Biocatalyzed asymmetric reduction of benzils to either benzoins or hydrobenzoins: pH dependent switch

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Different enzyme activities present in a whole-cell biocatalyst have been selectively harnessed to asymmetrically reduce bulky-bulky 1,2-diketones to either 2-hydroxyketones or 1,2-diols