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Efficient Enantioselective Biocatalytic Production of a Chiral Intermediate of Sitagliptin by a Newly Filamentous Fungus Isolate

Jia Sun[1] **· Jin Huang**[1] **· Xuzhong Ding**[1] **· Pu Wang**[1]

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Abstract (*S*)-3-Hydroxy-1-(3-(trifluoromethyl)-5,6-dihydro-[1,2,4]triazolo[4,3-a]pyrazin-7(8H)-yl)-4-(2,4,5-trifluorophenyl)butan-1-one ((*S*)-HTPP) is a crucial intermediate for the synthesis of Sitagliptin. A fungal strain ZJPH1308, capable of the biocatalysis of ketoamide 4-oxo-4-[3-(trifluoromethyl)-5,6-dihydro-[1,2,4]triazolo[4,3-a]pyrazin-7(8H)-yl]-1-(2,4,5-trifluorophenyl)butan-2-one (OTPP) to (*S*)-HTPP with excellent enantioselectivity, was isolated from a soil sample and identified as *Rhizopus microsporus* var. *rhizopodiformis* ZJPH1308 based on its morphological characteristics and internal transcribed spacer (ITS) sequence. Some key reaction parameters involved in the bioreduction catalyzed by isolate ZJPH1308 were then optimized. It demonstrated that the bioreduction of OTPP was effective conducted at relative high temperature (45 °C), along with distilled water as reaction medium and glycerol-coupling approach for cofactor regeneration. Under the optimal conditions, the preparative-scale bioreduction gave a 93.2 % yield (with >99.9 % of enantiomeric excess (ee)) at 15 mM of OTPP and 45 °C, reaction for 24 h. The results indicated that fungal isolate ZJPH1308 can afford a thermostable carbonyl reductase and is a promising biocatalyst for clean and efficient production of valuable chiral intermediate.

Keywords Bioreduction · *Rhizopus microsporus* var. *rhizopodiformis* · Thermophilic carbonyl reductase · Sitagliptin · Distilled water medium

Introduction

Biocatalysis has been considered as an attractive tool for organic synthesis, especially for the preparation of chiral compounds due to its excellent enantioselectivity [1, 2]. It has matured to a

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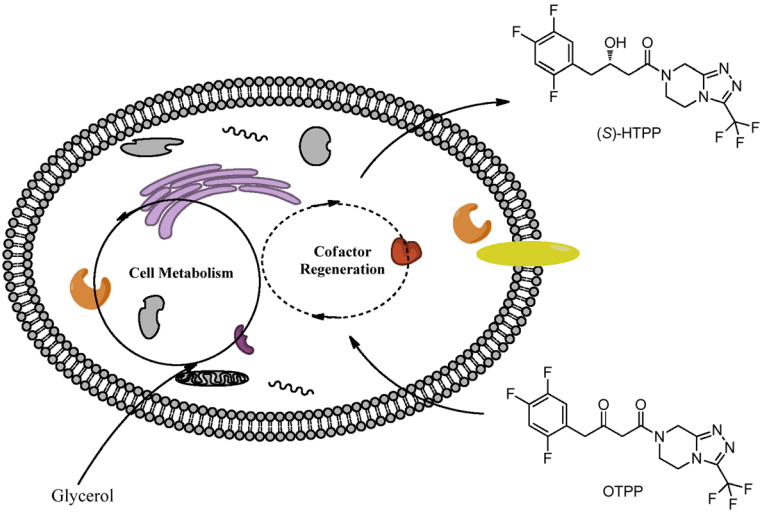
standard technology in the fine chemicals industry, which is reflected by the number of biotransformation processes running on a commercial scale for the production of pharmaceuticals, agrochemical precursors, and food additives [1, 3–5]. For biocatalytic methodologies on asymmetric synthesis, bioreduction has accounted for large portion of biocatalysis, which can theoretically lead to a 100 % conversion of compounds for optically pure product [1, 6]. Optically active alcohols have been proven to be versatile and valuable building blocks in organic synthesis [7, 8]. Bioreduction of prochiral ketones, ketoamides, and ketoesters to chiral alcohols has been investigated in depth, which can be conducted by enzymatic transformation and whole-cell bioconversion [5, 9].

(*S*)-3-Hydroxy-1-(3-(trifluoromethyl)-5,6-dihydro-[1,2,4]triazolo[4,3-a]pyrazin-7(8H)-yl)-4-(2,4,5-trifluorophenyl)butan-1-one ((*S*)-HTPP) is an important chiral intermediate for the synthesis of Sitagliptin [10], which is the active ingredient of type 2 diabetes treatment leading drug Januvia® [11, 12]. Up to date, two biocatalytic processes have been previously described to produce optically chiral intermediate (*S*)-HTPP from the original ketoamide 4-oxo-4-[3-(trifluoromethyl)-5,6-dihydro-[1,2,4]triazolo[4,3-a]pyrazin-7(8H)-yl]-1-(2,4,5-trifluorophenyl)butan-2-one (OTPP). Mendiratta et al. [10] presented an effective enzymatic reduction process to produce (*S*)-HTPP with a recombinant engineered oxidoreductase, which coupled glucose dehydrogenase for cofactor regeneration. Biocon Limited [13] reported that fungus *Penicillium breoicompactum* MTCC 5962 also displayed the synthesis of (*S*)-HTPP from OTPP. For industrial biocatalysis, novel biocatalysts with superior biochemical properties such as thermostability, pH stability, and operational stability have become increasingly attractive. Many strategies have been adopted to search for robust carbonyl reductases with better biocatalytic performances, such as screening for novel microorganisms from the environment, directed evolution of the known biocatalysts. In our present study, a fungal strain identified as *Rhizopus microsporus* var. *rhizopodiformis* ZJPH1308 was newly isolated from a soil sample for the preparation of (*S*)-HTPP, which exhibited excellent enantioselectivity and thermotolerance in chiral alcohol preparation. The asymmetric bioreduction of OTPP to (*S*)-HTPP was carried out in distilled water medium through a "coupled-glycerol" approach for cofactor regeneration (Scheme 1). The effects of some crucial reaction parameters on the yield



Scheme 1 The asymmetric reduction of OTPP to (*S*)-HTPP catalyzed by *R. microsporus* var. *Rhizopodiformis* ZJPH1308 cells

and stereoselectivity have also been investigated in detail. For industrial and biotechnological purpose, fungal oxidoreductase, especially thermotolerant carbonyl reductase, is a promising biocatalyst for chiral alcohol production.

Materials and Methods

Chemicals

Substrate OTPP (purity >99 %) was supplied by Arromax Pharmatecth Co. Ltd. (Suzhou, China). Racemate HTPP was synthesized by chemically reduction of OTPP with $NaBH_4$ and further purified in our laboratory (purity >98 %). All other chemicals were from commercial sources and were of analytical grade.

Screening and Cultivation of Microorganisms

One gram of each soil sample was suspended in 10 mL of saline (0.85 %, *w*/*v*) and allowed to vibrate for 10 min. After being diluted 10^3- or 10^4-fold, the diluents (200 μL) were respectively plated over the screening medium agar containing OTPP as the sole carbon source, and then incubated at 30 °C for 5–7 days. The screening medium was composed of the following (per liter): 3 g $(NH_4)_2SO_4$, 1 g KH_2PO_4, 0.3 g NaCl, 20 g agar, and 4 g OTPP. The initial pH of the medium was adjusted to 6.0. The pure colonies were obtained by continuous streaking of single colony on screening medium agar. The isolated pure colonies were then cultured on potato dextrose agar (PDA) medium and stored at 4 °C for further study.

The resulted isolates were inoculated individually into a liquid seed medium and grown aerobically at 30 °C for 22 h. Then, 7 % inoculum (*v*/*v*) was transferred into 250 mL Erlenmeyer flasks containing 100 mL of fermentation medium. After cultivation at 30 °C for 24 h, the incubated cells were harvested by centrifugation or filtration and washed twice with distilled water, and then applied in the bioreduction of OTPP to evaluate their catalytic abilities by TLC or HPLC analysis. All relevant culture mediums are as follows: seed culture medium (per liter), 25 g glucose, 27.5 g peptone, 3 g $(NH_4)_2SO_4$, 1 g KH_2PO_4, 0.3 g NaCl (pH 6.0); fermentation medium (per liter), 25 g dextrin, 30 g beef extract, 5 g $(NH_4)_2SO_4$, 0.3 g NaCl, 0.05 g $CoCl_2{\cdot}6H_2O$, 0.4 g $CaCl_2$ (pH 6.0).

Phenotypic Characterization of Isolate ZJPH1308

Morphological characterization of strain ZJPH1308 was investigated after the incubation on PDA plate or in liquid fermentation medium. Cell morphology of the filamentous fungus was observed with a light microscope (Olympus CX31, Japan).

ITS Sequence Determination and Phylogenetic Analysis

The taxonomic identification of isolate ZJPH1308 was examined with partial ITS sequence determination performed by Sangon Biotech Co. Ltd. (Shanghai, China). The extracted strain ITS genes were amplified by PCR with universal primer ITS1: (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4: (5′-TCCTCCGCTTATTGATATGC-3′). PCR

amplification was carried out by a following thermal cycler: 4 min at 94 °C, 30 cycles of 45 s at 94 °C, 45 s at 55 °C, 1 min at 72 °C, and a final extension step of 10 min at 72 °C. The PCR products were purified and sequenced by Sangon Biotech Co. Ltd. A 672-bp amplified fragment from the ITS gene was obtained. Related sequences with high similarities were then selected from the GenBank database (National Center for Biotechnology Information, NCBI) for homologous analysis. The determined ITS region sequences were aligned with the reference sequences obtained from GenBank databases using the multiple sequence alignment software CLUSTAL X ver. 1.83. A neighbor-joining phylogenetic tree with the nucleotide p-distance model and a bootstrap analysis for evolution of the phylogenetic topology was constructed with MEGA version 5.1.

Asymmetric Bioreduction Process

The bioreduction was performed in shaking flasks on an orbital shaker at 30 °C and 200 rpm. The harvested mycelia, 7 g (wet cell weight), were resuspended in 20 mL distilled water to form the reaction system. The reactions were initiated by the addition of 1 mM of OTPP, simultaneously employing 6 % (*v*/*v*) glycerol as cosubstrate for cofactor regeneration. The bioreduction was quenched by removing mycelia from the reaction system through centrifugation. The samples were withdrawn at regular time intervals and supernatant of samples was extracted with equal volume of ethyl acetate. The obtained ethyl acetate layer was dried overnight with hydrous Na_2SO_4 and then used for HPLC analysis. All experiments were carried out in triplicate.

Analytical Methods

TLC analysis was carried out on silica gel GF_{254} plates with a mixture of ethyl acetate/petroleum ether/acetic acid = 8/2/0.01(*v*/*v*/*v*) as developing agents. The point of product on silica gel was collected and redissolved in methanol for the preparation of pure product. The obtained purified product ((*S*)-HTPP) was then identified by the determination of specific optical rotation, as well as ^{1}H nuclear magnetic resonance (^{1}H-NMR) spectra and (+) electrospray ionization (ESI)-MS^n detection. ^{1}H-NMR (DMSO-D_6, 400 MHz): δ 2.46–2.50 (m, 1H), 2.68–2.78 (m, 3H), 4.02 (m, 2H), 4.09 (s, 2H), 4.22 (s, 1H), 4.83–5.02 (m, 3H), 7.46–7.41 (q, 2H); ESI-MS: 409 $(M+H)^+$.

The optical purity and quantity of substrate and product were determined by HPLC using a Daicel Chiralpak® ID column (250 mm × 4.6 mm i.d., particle size 5 μm, Daicel Chemical Ltd., Tokyo, Japan) with UV detection at 269 nm. A mixture of hexane and isopropanol (65:35, *v*/*v*) was used as the mobile phase at a flow rate of 0.8 mL/min. Retention times of OTPP, (*S*)-HTPP, and (*R*)-HTPP were 15.0, 12.5, and 10.0 min, respectively (see Fig. 1).

Results and Discussion

Isolation of Strains for the Production of (*S*)-HTPP

By substrate-directed isolation with OTPP as the sole carbon source in screening medium, several colonies exhibiting different morphologies appeared on the screening medium plates. Their enantioselective reductive capabilities of OTPP were further evaluated in terms of

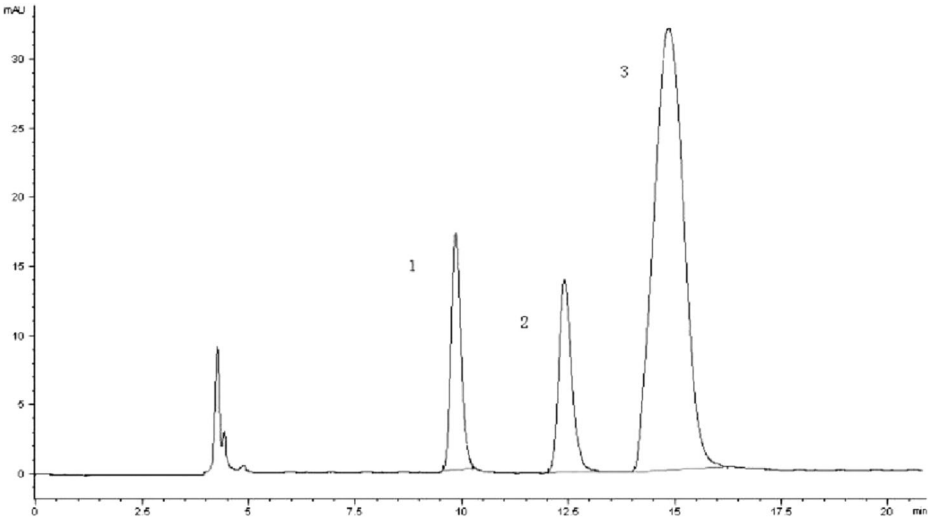


Fig. 1 HPLC analysis with chiral stationary phase. Retention time: (*1*) (*R*)-HTPP, 10.0 min; (*2*) (*S*)-HTPP, 12.5 min; (*3*) OTPP, 15.0 min

stereoselectivity and conversion. One fungal isolate ZJPH1308 turned out to be the most powerful strain with stricter *S*-enantioselectivity for (*S*)-HTPP production ($[a]_D^{25} = 21.68$), which had been deposited in the China Center for Type Culture Collection (Wuhan, China) with accession number CCTCC M 2014645. This isolate was selected for further experiments.

Identification and Characterization of Strain ZJPH1308

Morphological Characterization

After cultivation on PDA plates for 5 days at 30 °C, the isolate ZJPH1308 grew to a diameter of about 6 cm and showed dark, gray brownish colonies (Fig. 2a). The morphological characterization of hypha was observed by a light microscope (LM) (Fig. 2b). Rhizoids are simple, slightly. Sporangiophores on stolons are brownish, with one to four together, minutely spinulose. The sporangia are (sub-)globose, rather homogeneous. All characters of isolate ZJPH1308 are in accordance with species *R. microsporus* var. *rhizopodiformis*, which reported by Guimarães et al. [14] and Schipper et al. [15]. Mycelial pellets of this fungal isolate were formed by aerobic liquid fermentation culture (Fig. 2c).

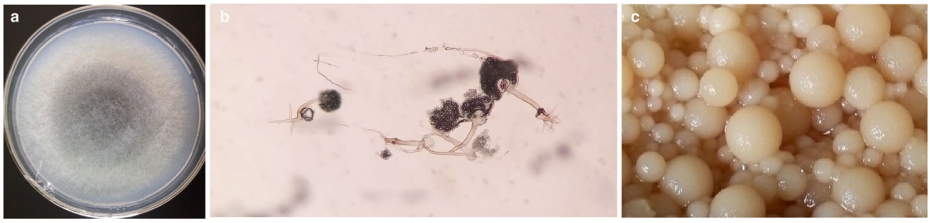


Fig. 2 Morphological characteristics of strain ZJPH1308. **a** Colonies on PDA plate. **b** Microscopic image (×1000) of hypha and sporangiospores. **c** Optical image of mycelial pellets after incubation in fermentation medium

ITS Sequencing and Phylogenetic Analysis

The partial ITS sequence of ZJPH1308 (672 bp, GenBank accession no. KR998045) was determined, and related species with similar sequence were used to conduct homologous analysis with strain ZJPH1308. A phylogenetic tree was constructed by a neighbor-joining method (Fig. 3). Strain ZJPH1308 was closely clustered with *R. microsporus* var. *rhizopodiformis* (GenBank accession no. KJ417564.1), sharing a high sequence identity of 100 %. Based on the results of morphological characterization and phylogenetic analysis, this isolate was designated as *R. microsporus* var. *rhizopodiformis* ZJPH1308.

R. microsporus var. *rhizopodiformis* belongs to class zygomycetes [16], which is thermotolerant and widely applied in food processing or feed industry for its ability of producing amylase, alkaline phosphatases, and pectinase [14, 17]. To our knowledge, this is the first reported case on the species *R. microsporus* var. *rhizopodiformis* capable of producing *S*-stereospecific carbonyl reductase and performing asymmetric reduction of ketoamide OTPP to its corresponding chiral alcohol (*S*)-HTPP, an intermediate of type 2 diabetes treatment drug Sitagliptin.

Effect of pH on the Asymmetric Reduction of OTPP

In general, the pH of reaction medium alters the ionic state of the substrates and the charging enzymes, thus affecting the biocatalytic efficiency in activity and enantioselectivity. In this study, the reduction of OTPP mediated by *R. microsporus* var. *rhizopodiformis* ZJPH1308 was investigated at different buffer pH levels in the range of 5.0–8.0. As illustrated in Table 1, within the assayed buffer pH range, the product ee remains intact (>99.9 %). However, buffer pH makes a remarkable impact on the yield. Strain ZJPH1308 shows a pH preference to 6.0. Furthermore, the yield was significantly improved with the decrease of ionic strength of phosphate buffer from 0.2 to 0.02 M at the same pH of 6.0, indicating that ionic strength of phosphate buffer could obviously inhibit the reaction in some way. It was observed that the fungal mycelium became incompact during the bioreduction with the enhancement of ionic strength of phosphate buffer. More interestingly, the bioreduction performed even the most powerful in distilled water medium (pH of 6.0), with 80.1 % yield and >99.9 % of product ee. Li et al. has previously reported that the bioreduction of arylalkyl ketone catalyzed by fungal strain *Trichoderma asperellum* ZJPH0810 also performed favorably in distilled water [18]. In comparison with conventional buffer reaction system, biocatalysis performed in distilled water medium exhibits a beneficial applicative advantage.

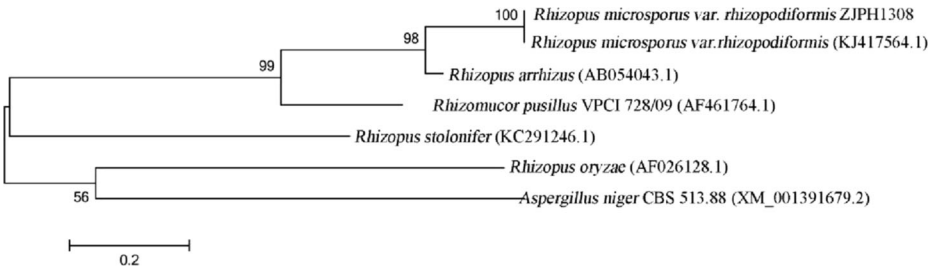


Fig. 3 The phylogenetic tree of *R. microsporus* var. *Rhizopodiformis* ZJPH1308 based on partial ITS sequences

Table 1 Effect of pH on the asymmetric reduction

	Buffer solution condition	Yield (%)	ee (%)
pH[a]	5.0 (0.2 M)	5.8	>99.9
	6.0 (0.2 M)	36.9	>99.9
	7.0 (0.2 M)	27.7	>99.9
	8.0 (0.2 M)	20.2	>99.9
pH 6.0	Phosphate buffer (0.02 M)	78.7	>99.9
	Distilled water	80.1	>99.9

Reaction conditions: 20 mL of buffer solution or distilled water, 1 mM of OTPP, 350 g/L cells (wet cell weight), 6 % (*v*/*v*) glycerol as cosubstrate, 30 °C, 200 rpm, reaction for 24 h

[a] pH 5.0 (NaAc-HAc), pH 6.0–8.0 (K_2HPO_4-KH_2PO_4)

Cosubstrate-Coupled System for Cofactor Regeneration

Considerable efforts have been devoted to the development of methods suitable for in situ regeneration of cofactors (particularly NAD(H) and NADP(H)) employed in asymmetric bioreduction, including enzyme-coupled, cosubstrate-coupled, electrochemical, and photochemical approaches [19]. Among above methods, cosubstrate-coupled strategy is cost-effective without external addition of expensive cofactors, and convenient to operate in whole-cell biocatalytic process. In this study, several sugars and alcohols such as glucose, sucrose, ethanol, and isopropanol were employed as cosubstrate for NAD(P)H regeneration. As shown in Table 2, glycerol gave the highest yield of 80.3 % among the tested cosubstrates. Moreover, all of the evaluated cosubstrates had no effect on the enantioselectivity (remained >99.9 %). Although several studies have reported the application of glycerol in biocatalysis, it was noted that the research on cofactor regeneration via glycerol-coupling approach is still limited. Glycerol can be employed in biocatalysis to play different roles: (i) Acting as a green solvent [20, 21], the homogeneous system created by the glycerol-water combination can increase the solubility of hydrophilic compounds in aqueous medium and improve the mass transfer efficiency, thus enhancing the enzyme-substrate interaction; (ii) Glycerol can stabilize the protein native structure and maintain the enzymatic activity [22, 23]; (iii) Within the whole-cell redox system, glycerol acts as hydrogen donor for regeneration of NAD(P)H [24, 25]. In

Table 2 Effect of different cosubstrates on the asymmetric reduction of OTPP

Cosubstrates	Yield (%)	ee (%)
Control	55.5	>99.9
Glucose	58.6	>99.9
Maltose	75.9	>99.9
Sucrose	76.9	>99.9
Methanol	75.8	>99.9
Ethanol	60.8	>99.9
Isopropanol	46.8	>99.9
Glycerol	80.3	>99.9

Reaction conditions: 350 g/L cells (wet cell weight), 1 mM of OTPP, in 20 mL distilled water, 30 °C, 200 rpm, reaction for 24 h; In this reaction, 6 % (*w*/*v* or *v*/*v*) cosubstrate was added, while control with no addition

the present study, we found that glycerol was superior to other candidates for cofactor regeneration in the bioreduction mediated by *R. microsporus* var. *rhizopodiformis* ZJPH1308.

In order to attain higher product yield, the effect of glycerol concentration was subsequently investigated (Fig. 4). The results demonstrated that the yield went up remarkably with increasing glycerol concentration up to 4 % (*v*/*v*), beyond which further rise in glycerol concentration decrease the yield obviously. It might be due to a comprehensive effect of glycerol on cofactor regeneration, cell membrane permeability [26], and mass transfer efficiency. Higher osmotic pressure resulting from relative higher glycerol concentration and mass transfer problem due to noticeable viscosity of glycerol-water solution might account for this decline. A similar result was also reported on the production of (*R*)-1-phenyl-1,2-ethanediol that the excess amount of glycerol as cosubstrate could decrease the conversion [24]. However, glycerol concentration showed no difference on the enantioselectivity (remained >99.9 %).

Effect of Cell Concentration on the Biocatalytic Reduction

Generally, in a limited reaction system, the amount of whole-cell biocatalysts makes significant difference on biocatalytic efficiency for both turnover number and mass transfer. Thus, the mycelia pellet concentrations varying from 50 to 600 g/L (wet cell weight) were evaluated in order to find the optimum concentration for the bioreduction of OTPP to (*S*)-HTPP. As shown in Fig. 5, the maximal yield for (*S*)-HTPP reached 88 % at 350 g/L (approximately 8.83 g/L dry cell concentration) mycelia pellet concentration. Further increase in mycelia pellet concentration decreased the yield slightly, probably resulted from the mass transfer limitation due to high viscosity of reaction system. All the tested mycelia pellet concentrations exhibited no appreciable impact on enantioselectivity (>99.9 %).

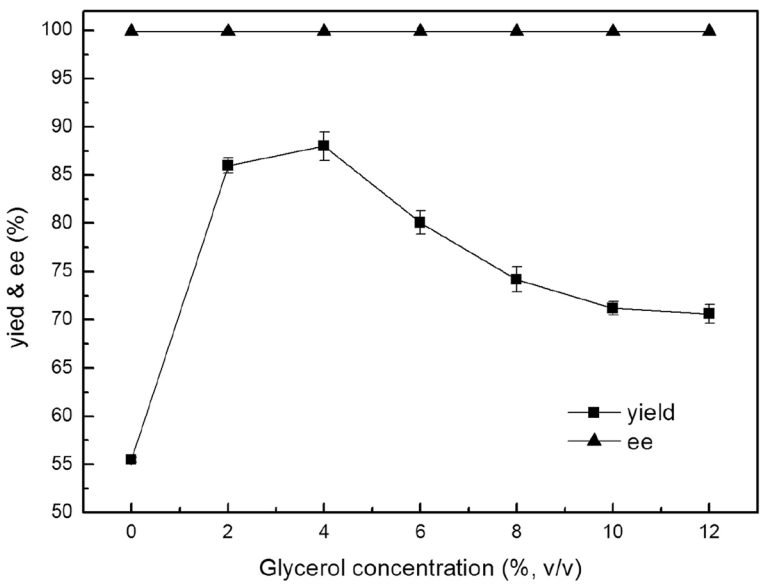


Fig. 4 Effect of glycerol concentration on the asymmetric reduction of OTPP. Reaction conditions: 20 mL distilled water, 350 g/L cells (wet cell weight), 1 mM of OTPP, different concentrations of glycerol were added, 30 °C, 200 rpm, reaction for 24 h

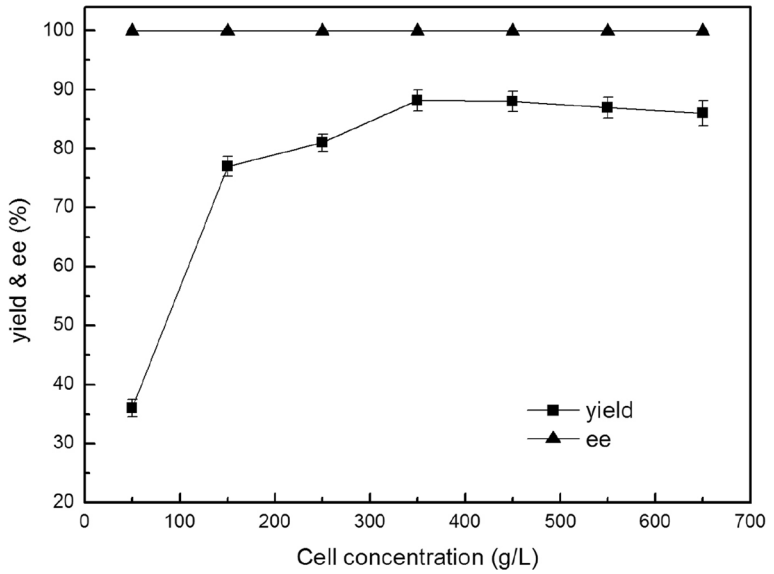


Fig. 5 Effects of cell concentration on yield and product ee. Reaction conditions: 20 mL distilled water, 50 g/L to 650 g/L of cells (wet cell weight), 1 mM of OTPP, 4 % (*v*/*v*) glycerol as cosubstrate, 30 °C, 200 rpm, reaction for 24 h

Effect of Temperature on the Asymmetric Reduction of OTPP

Enzyme can display accelerated activity at elevated temperature, showing a higher molecular catalytic efficiency K_{cat}/K_m in reaction [27]. Therefore, temperature acts as a significant parameter for enzyme or whole-cell catalysis. In recent years, there is a growing interest in thermophilic fungi for their potential of producing industry-relevant thermostable enzymes [28]. Strain *R. microsporus* var. *rhizopodiformis* ZJPH1308 shows good growth at 30 to 40 °C, belonging to a thermophilic eukaryotic organism [29]. Thus, we speculated the carbonyl reductase derived from ZJPH1308 might be a thermostable enzyme and give good performance in OTPP bioreduction. Therefore, the effect of different reaction temperatures ranged from 30 to 50 °C on yield and enantioselectivity was subsequently investigated and the results were shown in Fig. 6. As expected, higher reaction temperature (40–45 °C) clearly benefited reaction efficiency. The maximum yield of 98.6 % was obtained at 45 °C with above 99.9 % product ee at 10 mM of OTPP concentration. Generally, eubacteria and archaea are mostly reported for excellent thermophily on growth and biocatalysis [30], and previously reported fungus-mediated biocatalysis are usually conducted at mild temperature of 25–30 °C [18, 31–33]. Our results are remarkable and seem to show a beneficial applicative characteristic for filamentous fungi-mediated biocatalytic process.

Effect of Substrate Concentration on the Biocatalytic Reduction

To assess the applicability of bioreduction mediated by ZJPH1308 mycelia pellets, experiments with different OTPP concentrations from 10 to 30 mM were arranged to study the effect of substrate concentration on the reduction of OTPP. Figure 7 shows the variation of the yield and product ee with different substrate concentrations. Nearly 100 % conversion was achieved

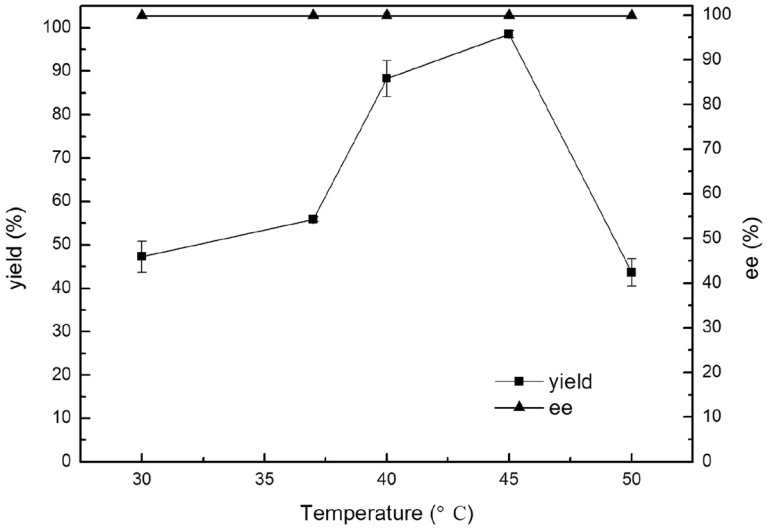


Fig. 6 Effect of temperature on the asymmetric reduction of OTPP. Reaction conditions: 20 mL distilled water, 350 g/L cells (wet cell weight), 10 mM of OTPP, 4 % (*v*/*v*) glycerol, 200 rpm, reaction for 24 h

at 10–15 mM of OTPP concentration within 24 h of reaction. A yield of 96.7 % was obtained at 20 mM of OTPP concentration after reaction for 48 h. Further increase of substrate concentration up to 30 mM, (*S*)-HTPP concentration reached 23.26 mM with a decreased yield of 77.5 % in 52 h, which possibly owing to the inhibition of substrate or product, and the mass transfer limitation as well. Substrate concentration showed no influence on the enantioselectivity (remained >99.9 %). Thus, 15 mM of substrate concentration was chosen

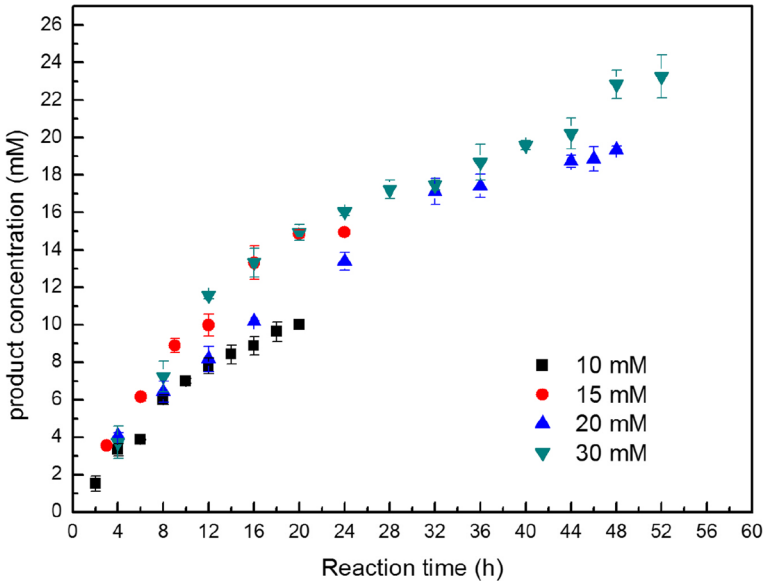


Fig. 7 Effect of substrate concentration on the asymmetric reduction of OTPP. Specified OTPP concentrations (10, 15, 20, and 30 mM) were added respectively into 20 mL distilled water containing 350 g/L cells (wet cell weight). The reaction was conducted at 45 °C, 200 rpm, with 4 % (*v*/*v*) glycerol as cosubstrate

as the optimal. Subsequently, a preparative-scale bioreduction of OTPP to (*S*)-HTPP at 15 mM of substrate concentration was carried out in 1 L Erlenmeyer flask (working volume of 200 mL) and a yield of 93.2 % was achieved after reaction for 24 h. Based on above results, the bioreduction process mediated by *R. microsporus* var. *rhizopodiformis* ZJPH1308 was shown to be feasible on a 1-L preparative scale.

Conclusion

In this study, we successfully demonstrated a whole-cell biocatalytic process for the production of (*S*)-HTPP, a crucial intermediate of Sitagliptin. The newly fungal isolate identified as *R. microsporus* var. *rhizopodiformis* ZJPH1308 showed excellent enantioselectivity (>99.9 % ee) in the bioreduction of OTPP. Distilled water manifested as the best reaction medium for the bioreduction, which is rarely reported and beneficial to industrial application. Strain ZJPH1308 could well conduct the whole-cell bioreduction at a relative high temperature of 45 °C, and particularly couple glycerol for efficient cofactor regeneration to execute the bioredcution process smoothly. The developed biocatalytic process for (*S*)-HTPP production is scalable. Our results demonstrated that the filamentous fungus *R. microsporus* var. *rhizopodiformis* ZJPH1308 harboring thermophilic carbonyl reductase is a promising biocatalyst for industrial production of some valuable chiral intermediates.

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References

1. Straathof, A. J. J., Panke, S., & Schmid, A. (2002). The production of fine chemicals by biotransformations. *Current Opinion in Biotechnology, 13*, 548–556.
2. Reetz, M. T. (2013). Biocatalysis in organic chemistry and biotechnology: past, present, and future. *Journal of the American Chemical Society, 135*, 12480–12496.
3. Wandrey, C., Liese, A., & Kihumbu, D. (2000). Industrial biocatalysis: past, present, and future. *Organic Process Research & Development, 4*, 286–290.
4. Patel, R. N. (2008). Synthesis of chiral pharmaceutical intermediates by biocatalysis. *Coordination Chemistry Reviews, 252*, 659–701.
5. Torrelo, G., Hanefeld, U., & Hollmann, F. (2015). Biocatalysis. *Catalysis Letters, 145*, 309–345.
6. Matsuda, T., Yamanaka, R., & Nakamura, K. (2009). Recent progress in biocatalysis for asymmetric oxidation and reduction. *Tetrahedron: Asymmetry, 20*, 513–557.
7. Ni, Y., & Xu, J. H. (2012). Biocatalytic ketone reduction: a green and efficient access to enantiopure alcohols. *Biotechnology Advances, 30*, 1279–1288.
8. Itoh, N. (2014). Use of the anti-Prelog stereospecific alcohol dehydrogenase from *Leifsonia* and *Pseudomonas* for producing chiral alcohols. *Applied Microbiology and Biotechnology, 98*, 3889–3904.
9. Kratzer, R., Woodley, J. M., & Nidetzky, B. (2015). Rules for biocatalyst and reaction engineering to implement effective, NAD(P)H-dependent, whole cell bioreductions. *Biotechnology Advances, 33*, 1641–1652.
10. Mendiratta, S. K., Pandey, B., Joshi, R., Trivedi, U., Dave, M. G., Kothari, H. M., & Shukla, B. (2012). Process for preparing an intermediate of sitagliptin via enzymatic conversion. WO 2012/046254 A2.
11. Thornberry, N. A., & Weber, A. E. (2007). Discovery of JANUVIA (Sitagliptin), a selective dipeptidyl peptidase IV inhibitor for the treatment of type 2 diabetes. *Current Topics in Medicinal Chemistry, 7*, 557–568.
12. Dhillon, S. (2010). Sitagliptin. *Drugs, 70*, 489–512.

13. Singh, S., Jothi, S., Pande, S., Lal, S., & Desai, S. (2015). Fungal mediated stereo-selective bioreduction of keto intermediates of pharmaceutically active compounds to their corresponding hydroxy compounds. WO 2015/071861 A2.
14. Guimarães, L. H. S., Peixoto-Nogueira, S. C., Michelin, M., Rizzatti, A. C. S., Sandrim, V. C., Zanoelo, F. F., Aquino, A. C. M. M., Junior, A. B., & Polizeli, M. L. T. M. (2006). Screening of filamentous fungi for production of enzymes of biotechnological interest. *Brazilian Journal of Microbiology, 37*, 474–480.
15. Schipper, M. A. A., & Stalpers, J. A. (1984). A revision of the genus *Rhizopus*. II. The *Rhizopus microsporus*-group. *Studies in Mycology, 25*, 20–34.
16. Ferreira, J. A., Lennartsson, P. R., Edebo, L., & Taherzadeh, M. J. (2013). Zygomycetes-based biorefinery: present status and future prospects. *Bioresource Technology, 135*, 523–532.
17. Peixoto-Nogueira, S. C., Sandrim, V. C., Guimarães, L. H. S., Jorge, J. A., Terenzi, H. F., & Polizeli, M. L. T. M. (2008). Evidence of thermostable amylolytic activity from *Rhizopus microsporus* var *rhizopodiformis* using wheat bran and corncob as alternative carbon source. *Bioprocess and Biosystems Engineering, 31*, 329–334.
18. Li, J., Wang, P., He, J. Y., Huang, J., & Tang, J. (2013). Efficient biocatalytic synthesis of (*R*)-[3,5-bis(trifluoromethyl) phenyl] ethanol by a newly isolated *Trichoderma asperellum* ZJPH0810 using dual cosubstrate: ethanol and glycerol. *Applied Microbiology and Biotechnology, 97*, 6685–6692.
19. Kara, S., Schrittwieser, J. H., Hollmann, F., & Ansorge-Schumacher, M. B. (2014). Recent trends and novel concepts in cofactor-dependent biotransformations. *Applied Microbiology and Biotechnology, 98*, 1517–1529.
20. Gu, Y. L., & Jérôme, F. (2010). Glycerol as a sustainable solvent for green chemistry. *Green Chemistry, 12*, 1127–1138.
21. Andrade, L. H., Piovan, L., & Pasquini, M. D. (2009). Improving the enantioselective bioreduction of aromatic ketones mediated by *Aspergillus terreus* and *Rhizopus oryzae*: the role of glycerol as a co-solvent. *Tetrahedron: Asymmetry, 20*, 1521–1525.
22. Feng, S., & Yan, Y. B. (2008). Effects of glycerol on the compaction and stability of the wild type and mutated rabbit muscle creatine kinase. *Proteins, 71*, 844–854.
23. Chen, S., Land, H., Berglund, P., & Humble, M. S. (2016). Stabilization of an amine transaminase for biocatalysis. *Journal of Molecular Catalysis B: Enzymatic, 124*, 20–28.
24. Wang, S. S., Xu, Y., Zhang, R. Z., Zhang, B. T., & Xiao, R. (2012). Improvement of (*R*)-carbonyl reductase-mediated biosynthesis of (*R*)-1-phenyl-1,2-ethanediol by a novel dual-cosubstrate-coupled system for NADH recycling. *Process Biochemistry, 47*, 1060–1065.
25. Taketomi, S., Asano, M., Higashi, T., Shoji, M., & Sugai, T. (2012). Chemo-enzymatic route for (*R*)-terbutaline hydrochloride based on microbial asymmetric reduction of a substituted α-chloroacetophenone derivative. *Journal of Molecular Catalysis B: Enzymatic, 84*, 83–88.
26. Ouyang, Q., Wang, P., Huang, J., Cai, J. B., & He, J. Y. (2013). Efficient enantioselective synthesis of (*R*)-[3,5-bis(trifluoromethyl)phenyl] ethanol by *Leifsonia xyli* CCTCC M 2010241 using isopropanol as co-substrate. *Journal of Microbiology and Biotechnology, 23*, 343–350.
27. Li, R., Perez, B., Jian, H., Jensen, M. M., Gao, R. J., Dong, M. D., Glasius, M., & Guo, Z. (2015). Characterization and mechanism insight of accelerated catalytic promiscuity of *Sulfolobus tokodaii* (ST0779) peptidase for aldol addition reaction. *Applied Microbiology and Biotechnology, 99*, 9625–9634.
28. Brink, J. V. D., Facun, K., Vries, M. D., & Stielow, J. B. (2015). Thermophilic growth and enzymatic thermostability are polyphyletic traits within *Chaetomiaceae. Fungal Biology, 119*, 1255–1266.
29. Maheshwari, R., Bharadwaj, G., & Bhat, M. K. (2000). Thermophilic fungi: their physiology and enzymes. *Microbiology and Molecular Biology Reviews, 64*, 461–488.
30. Morgenstern, I., Powlowski, J., Ishmael, N., Darmond, C., Marqueteau, S., Moisan, M. C., Quenneville, G., & Tsang, A. (2012). A molecular phylogeny of thermophilic fungi. *Fungal Biology, 116*, 489–502.
31. Kurbanoglu, E. B., Zilbeyaz, K., Taskin, M., & Kurbanoglu, N. I. (2009). Total production of (*R*)-3,5-bistrifluoromethylphenyl ethanol by asymmetric reduction of 3,5-bis(trifluoromethyl)- acetophenone in the submerged culture of *Penicillium expansum* isolate. *Tetrahedron: Asymmetry, 20*, 2759–2763.
32. Gong, J. S., Lu, Z. M., Shi, J. S., Dou, W. F., Xu, H. Y., Zhou, Z. M., & Xu, Z. H. (2011). Isolation, identification, and culture optimization of a novel glycinonitrile-hydrolyzing fungus—*Fusarium oxysporum* H3. *Applied Biochemistry and Biotechnology, 165*, 963–977.
33. Li, Q. Z., Shi, Y., He, L., & Zhao, H. (2016). Asymmetric bioconversion of acetophenone in nano-sized emulsion using *Rhizopus oryzae*. *Journal of Microbiology and Biotechnology, 26*, 72–79.