RSC Advances

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/advances

Highly enantioselective bioreduction of 1-(3,4-difluorophenyl)-3-nitropropan-1-one: Key intermediate of Ticagrelor

Manpreet Singh[1], Hare Krishnen[1], Uday Kumar Neelam[1], Kavitha Charugondla[1], Goverdhan Gilla[1], Karen Holt-Tiffin[2] and Rakeshwar Bandichhor[1*]

[1] *Integrated Product Development,Innovation Plaza, Dr. Reddy's Laboratories Ltd., Bachupalli, Qutubullapur, R. R. Dist. 500 072 Andhra Pradesh, India*

[2]*Chirotech Technology Ltd, 410 Cambridge Science Park, Milton Road, Cambridge, Cambridgeshire, U.K, CB4 OPE.*

*Corresponding author. Telephone: +91 40 44346430

Fax: +91 40 44346164

E-mail: rakeshwarb@drreddys.com

ABSTRACT

A simple, highly effective and economical whole-cell mediated process was developed for the biocatalytic reduction of a ketone, intermediate in the synthesis of the platelet inhibiting drug Ticagrelor. Sixteen different microorganisms were screened for the bioreduction of 1-(3,4-difluorophenyl)-3-nitropropan-1-one (**1**) to (*S*)-1-(3,4-difluorophenyl)-3-nitropropan-1-ol (**2**). Growing as well as resting cells of *Candida parapsilosis*104659 exhibited high conversion (>99.0%) and enantioselectivity (98.0%) for the *S*-enantiomer.

1. Introduction

Chiral alcohols are useful intermediates in the synthesis of active pharmaceutical ingredients.[1, 2] Biocatalytic reduction of prochiral ketones is finding foothold in industry and is replacing traditional chemocatalysis[3]. The use of wild type whole cells[4, 5], recombinant cells[6-8] and isolated enzymes[9] has been reported on a variety of substrates. In comparison to the isolated enzymes, whole cells offer the advantage of internal co-factor regeneration and are usually more stable and eliminate the cost of enzyme purification. However, the limiting factor in the case of whole cell processes is the low volumetric productivity. Limited solubility of the ketone starting material in water can also contribute to low productivity of the system. Medium engineering and modified substrate addition strategies lead to enhanced substrate utilization and play an important role in establishing whole cell processes at industrial scale. [10-12]

Ticagrelor (Scheme 1, (**5**)) is a novel P_2Y_{12} receptor antagonist which functions by blocking adenosine diphosphate-mediated platelet aggregation. There are several published reports on the synthesis of this carbocyclic nucleoside and analogs,[13-16] however insufficient research has been targeted on the key chiral intermediate diflurophenyl cyclopropylamine (**4**).[17] Zhang *et al* reported the synthesis of different derivatives of ticagrelor especially the prodrugs.[18] The chiral cyclopropylamine-**4** can be derived from (*S*)-1-(3,4-difluorophenyl)-3-nitropropan-1-ol (**2**). The (*S*)-nitro alcohol (**2**) can be chemically prepared by utilizing chiral catalysts such as CBS, however, the overall yields and enantioselectivity are often low and the catalyst is expensive (Scheme 1).[19] Biological synthesis of optically active compounds especially alcohols is more stereospecific, environmentally friendly and efficient in comparison to conventional chemical methods. Thus we envisaged a route to the (*S*)-nitro alcohol (**2**) *via* enantioselective bioreduction of the prochiral ketone (**1**).

In recent years, we have been investigating the microbial reduction of different prochiral ketones used as intermediates for the synthesis of active pharmaceutical ingredients.[20] In the present study, sixteen different microorganism strains obtained from Chirotech Technology Centre, Cambridge, UK were screened for their potential activity in the asymmetric reduction of 1-(3,4-difluorophenyl)-3-nitropropan-1-one (**1**). In the first stage of screening seven (*Candida tropicalis, Bacillus licheniformis, Saccharomyces cerevisiae, Rhodococcus sp., Pichia methanolica, Rhodotorulla sp.* and *Candida rugosa*) organisms of bacterial and fungal origin were investigated for their performance in the bioreduction reaction. The initial screening was carried out by supplementing the substrate in 24 h old fermentation media itself. *Candida* species demonstrated superior activity (>96.0%) and selectivity (≥82.0%) in comparison to other selected strains (Table 1). *Rhodotorula* sp. showed moderate conversion, whilst *S. cerevisiae* and *P. methanolica* showed preference for other enantiomer.

2. Results and discussions

2.1. Screening

Since the *Candida* species displayed better enantioselectivity and excellent conversion for the bioreduction of (**1**), eleven different types of *Candida sp.* were then screened for the biotransformation of (**1**) to (**2**). Table 2 shows the conversion and enantioselectivity obtained from the diverse species of *Candida*. All the species have different activity and selectivity for (**1**). *C. parapsilosis* and C. *rugosa* had the highest conversion (>98.0%) and enantioselectivity (>82.0%), while *C. tropicalis* showed excellent conversion (>99.0%) but poor selectivity (66.0%). *Candida etchellsii* showed good conversion but preference for the *R*-enantiomer (86.0%).

Based on the screening results, it was concluded that the *C. parapsilosis* and *C. rugosa* were the best organisms for the bioreduction of (**1**) to (**2**). *Candida parapsilosis* was chosen for further studies because of its better enantioselectivity.

2.2. Effect of co-solvents

To determine the effect of co-solvents, different solvents (methanol, ethanol, IPA, THF, and MTBE) were tested in the bioreduction reaction. The best bioreduction was in the presence of THF with >99.0% conversion and >94.0% enantioselectivity after 48h of incubation (Table 3). The reason behind the increased enantioselectivity in the presence of THF might be inhibition of other KREDs or a change in the conformation of the enzyme active site.[15] Evaluation of different concentrations of THF in the bioreduction revealed the optimum activity was at 1.0% THF concentration; further increase in concentration decreased the conversion rate.

2.3. Effect of substrate concentration

Substrate loading can have a substantial effect on the activity of enzymes; hence different concentrations of (**1**) were tested. As shown in Figure 1, the highest conversion was achieved at only 0.5 g/l of (**1**) concentration after 48 h of incubation. Further increase in (**1**) concentration decreased the conversion rate. We knew we would need to increase the substrate concentration to allow a cost effective process to be developed.

2.4. Reduction using resting cells of *C. parapsilosis*

The next strategy to increase the productivity of the bioreduction was to carry out the reaction using media free resting cells of *Candida parapsilosis*. Taking into account the effect of glucose as a co-substrate in bioreduction reactions, the experiments were conducted by charging resting

cells of *C. parapsilosis* in H_2O containing glucose and (**1**) dissolved in THF. The reactions were carried out at different concentrations of (**1**) (0.5 to 6.0 g/l). As illustrated in Figure 2, the resting cell reaction yielded excellent conversion (>99.0%) and enantioselectivity (98.0%) at a substrate concentration of 4.0 g/l. Further increase in substrate concentration however was not tolerated under these conditions.

2.5. Molasses as sole carbon source

We then turned our attention to optimize the fermentation media, since the cost of media components play a major role in any industrial scale fermentation process. Due to enormous utilization of sugar in India there are numerous sugar industries, therefore their byproduct, molasses is low cost in comparison to synthetic substrates. Several experiments were conducted by utilizing different concentrations of molasses as a sole fermentation media for the growth of *C. parapsilosis*. Pleasingly molasses as fermentation media yielded the same degree of growth, wet cell-mass and activity as seen in synthetic media made of potato infusion and dextrose.

2.6. Preparative-scale bioreduction using *C. parapsilosis*

With the aim of scale-up, the bioreduction of (**1)** was carried out at 10.0 g scale using the resting cells of *C. parapsilosis* produced in fermentation media containing molasses (8.0%) as the sole carbon source. After the completion of reaction the product was isolated at an overall yield of 85.0% with an ee >98.0% and >99% purity of (**2)**.

3. Conclusions

In conclusion, the *Candida sp.* mediated reduction of 1-(3,4-difluorophenyl)-3-nitropropan-1-one (**1**) is a convenient way to prepare a key chiral alcohol intermediate to Ticagrelor with high enantioselectivity. *C. parapsilosis* has shown complete conversion and excellent

enantioselectivity (>98.0%) at 4.0 g/l substrate concentration and the organism is robust enough to grow on natural media. Our whole-cell catalyzed system has the advantage of not requiring external cofactors or cofactor recycling systems, thus reducing costs.

4. Materials and methods

4.1. General method

All the microbial strains utilized in this study were obtained from Chirotech Technology Centre, Cambridge, UK. 1-(3,4-Difluorophenyl)-3-nitropropan-1-one, chirally pure standards and (*R,S*)-1-(3,4-difluorophenyl)-3-nitropropan-1-ol were synthesized and obtained from the chemistry department, IPDO, Dr. Reddy's Labs, Hyderabad, India following reported procedures.[19] The chemicals used were of analytical grade and procured from commercial sources. The synthetic medium used in fermentation process was procured from Himedia Laboratories Pvt. Ltd. Mumbai. The molasses used in the optimization study was obtained from the sugar industries in and around Hyderabad, Telengana, India. ^{1}H NMR and ^{13}C spectra were recorded in $CDCl_3$ solution on a Bruker AVANCE III HD NanoBay 400 MHz instrument. Infra-red spectra was recorded on Perkin Elmer Spectrum 100 instrument. HPLC analysis was performed on Agilent 1100 series instrument equipped with UV-detector. The conversion of (**1**) and (**2**) was determined using reverse phase Zorbex Eclipse plus C-18 column with flow rate of 1.0 ml min^{-1} using gradient flow consisting of 0.02 M potassium phosphate buffer pH 3.0 and acetonitrile water (90:10) as eluent). The chiral purity was determined by running the samples against chiral standards using Chiralcel OD-H column with flow rate of 0.5 ml min^{-1} using hexane, IPA and TFA in the ratio of 92:7.9:0.1 as eluent. All the samples were analyzed at 210 nm. LC mass spectrum was obtained on Agilent LC ABSciex 4000 with Q. Trap system.

4.2. Medium and cultivation

The microbial cultures preserved as glycerol stocks in -80°C were thawed and transferred to solid medium consisting of potato dextrose agar (PDA) for further use. The seed culture was prepared by inoculating single colony from agar medium to liquid medium (PDB, pH 7.0) in a 100 ml Erlenmeyer flask. Consequently, 5.0% of the seed culture was transferred into 100 ml of the production medium (PDB, pH 7.0) and incubated in an orbital shaker at 30°C and 200 rpm for 48 h. The media free cells were obtained by centrifugation (7000 rpm, 10 min) and the cell pellet was washed twice with de-ionized water and again separated by centrifugation.

4.3 Biotransformation of (1) to (2) using whole-cells of *Candida parapsilosis*104659

The keto substrate (**1**) (2.3 mmol, 500 mg) dissolved in THF (0.1 ml) was added to a 250 ml Erlenmeyer flask containing the cells prepared as above (1 g wet wt) in PDB medium (50 ml) and incubated in an orbital shaker at 200 rpm for 24 h at 30°C. The reactions were performed in triplicate. The reactions were monitored for 48 h by analyzing the crude reaction mass by reverse phase HPLC. After reaction completion, the reaction mass was extracted with ethyl acetate (3×5 ml) then the organic layers dried over anhydrous Na_2SO_4 and evaporated *in vacuo* to give concentrated product.

4.4 Preparative scale biotransformation using resting-cells of *Candida parapsilosis*104659

*Candida parapsilosis*104659 cells were grown in an 1000 ml Erlenmeyer flask containing 200 ml molasses (8.0%) incubated at 30°C, 200 rpm. The cells were harvested by centrifuging 48 h old culture broth at 7000 rpm for 10 min at 4°C and subsequently washed with distilled water to remove media components. Resting cells of *C. parapsilosis* (5 g) were dissolved in 2.0 L de-ionized H_2O and 1-(3,4-difluorophenyl)-3-nitropropan-1-one (10g, 50 mmol) in THF (2 ml) was added. The reaction mixture was incubated in an orbital shaker at 30°C, 200 rpm for 48 h. After complete conversion to (**2**)**,** the reaction mixture was extracted using ethyl acetate (3×300 ml)

and the combined organic layers were dried over anhydrous sodium sulfate. The solvent was removed by rota-vaporization and the samples were analyzed for purity and chiral purity by HPLC (section 4.3). Also the samples were submitted for recording FT-IR and LC-MS data.

4.5 1-(3,4-Difluorophenyl)-3-nitropropan-1-one

Dark brown colored liquid, ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.62 (2H, J = 5.92 Hz, t), 4.83-4.81 (2H, m), 7.17-7.15(1H, m) 7.3-7.82 (2H, m); ^{13}C NMR (100 MHz, $CDCl_3$): 196.6 (1C), 155.5 (1C), 153.0 (1C), 132.8 (1C), 125.3 (1C), 118.0 (1C), 117.7 (1C), 69.0 (1C), 34.7 (1C).

4.6 1-(3,4-difluorophenyl)-3-nitropropan-1-ol

Dark brown colored liquid ^{1}H NMR (400 MHz, CDCl3) δ 2.31-2.30 (2H, m), 4.50-4.49 (2H, m), 4.67-4.60 (2H, m), 4.83 (2H, J = 6.65Hz, 2.65Hz, dd), 7.07-7.19 (3H, m), EI-Mass: m/z 216 $[M-H]^{+l}$; IR (KBr): 877.2, 1283.3, 3435.0 cm^{-1}

5. Acknowledgements

The authors greatly appreciate K Lavanya and Subbaiah Arla for providing analytical support.

References and notes

1. G. W. Huisman, J. Liang, A. Krebber, *Curr. Opin. Chem. Biol.,* 2010, **14**, 122.
2. R. N. Patel, *Food Technol. Biotechnol.,* 2004, **42**, 305.
3. J. C. Moore, D. J. Pollard, B. Kosjek, P. N. Devine, *Acc. Chem. Res.,* 2007, **40**, 1412.
4. M. Kataoka, K. Kita, M. Wada, Y. Yasohara, J. Hasegawa, S. Shimizu, *Appl. Microbiol. Biotechnol.,* 2003, **62**, 437.
5. N. Kizaki, Y. Yasohara, J. Hasegawa, M. Wada, M. Kataoka, S. Shimizu, *Appl. Microbiol. Biotechnol.,* 2001, **55**, 590.
6. H. Groger, F. Chamouleau, N. Orologas, C. Rollmann, K. Drauz, W. Hummel, A. Weckbecker, O. May, *Angew Chem. Int. Ed. Engl.,* 2006, **45**, 5677.
7. Y. Ni, J. H. Xu, *Biotechnol. Adv.,* 2012, **30**, 1279.
8. K. Schroer, K. Peter Luef, F. Stefan Hartner, A. Glieder, B. Pscheidt, *Metab. Eng.,* 2010, **12**, 8.
9. Y. Ni, C.-X. Li, J. Zhang, N.-D. Shen, U. T. Bornscheuer, J.-H. Xu, *Adv. Synth. Cat.,* 2011, **353**, 1213.
10. C. Kohlmann, N. Robertz, S. Leuchs, L. Greiner, S. Na'amnieh, *Green Chem.,* 2011, **13**, 3093.
11. M. Miroliaei, M. Nemat-Gorgani, *Int. J. Biochem. Cell Biol.,* 2002, **34**, 169.
12. J. Schumacher, M. Eckstein, U. Kragl, *Biotechnol. J.,* 2006, **1**, 574.
13. J. Włodarczyk, A. Wolan, M. Rakowiecki, M, Jan Bosiak, M. Budny, *Tet. Lett.,* 2015, **56**, 6093.
14. H-J. Wen, Q. Chen, G-J. Zheng, *Chin. Chem. Lett.,* 2015, **26**, 1431.
15. W. Tu, J. Fan, H. Zhang, G. Xu, Z. Liu, J. Qu, F. Yang, L. Zhang, T. Luan, J. Yuan, A. Gong, J. Feng, P. Sun, Q. Dong, *Bioorg. Med. Chem. Lett.,* 2014, **24**, 141.
16. L. Hejtmankova, P. Lustig, *WO* 2012 142983 A1, 2012.
17. M. Mitsuda, T. Moroshima, K. Tsukuya, K. Watabe, M. Yamada, *US Pat.,* 8 026 396 B2*,* 2011.
18. H. Zhang, J. Liu, L. Zhang, L. Kong, H. Yao, H. Sun, *Bioorg. Med. Chem. Lett.,* 2012, **22**, 3598.

19. M. Rasparini, M. Taddei, E. Cini, C. Minelli, N. Turner, K.G. Hugentobler, EP 2 589 587 A1, 2013.
20. M. Singh, S. Singh, S. Deshaboina, H. Krishnen, R. Lloyd, K. Holt-Tiffin, A. Bhattacharya, R. Bandichhor, *Cat. Sci. Technol.,* 2012, **2**, 1602.

Scheme 1

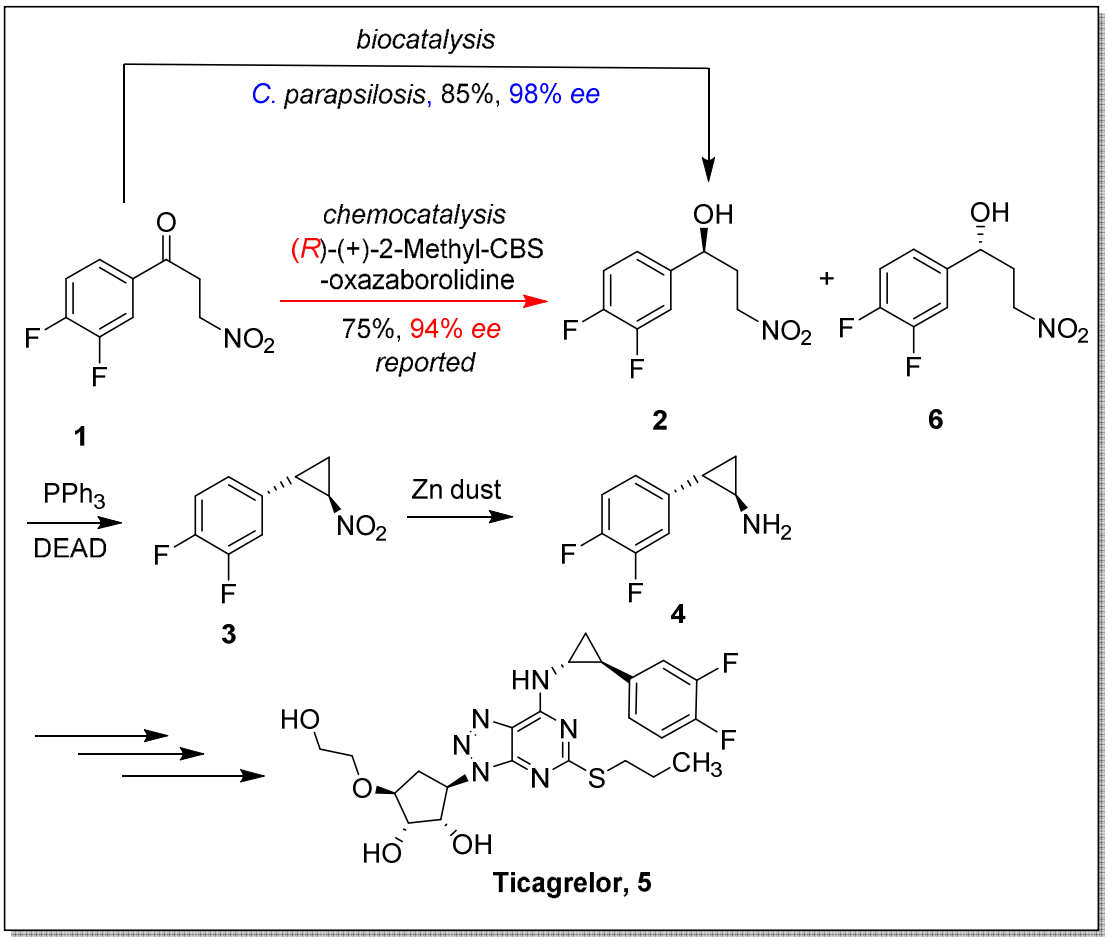
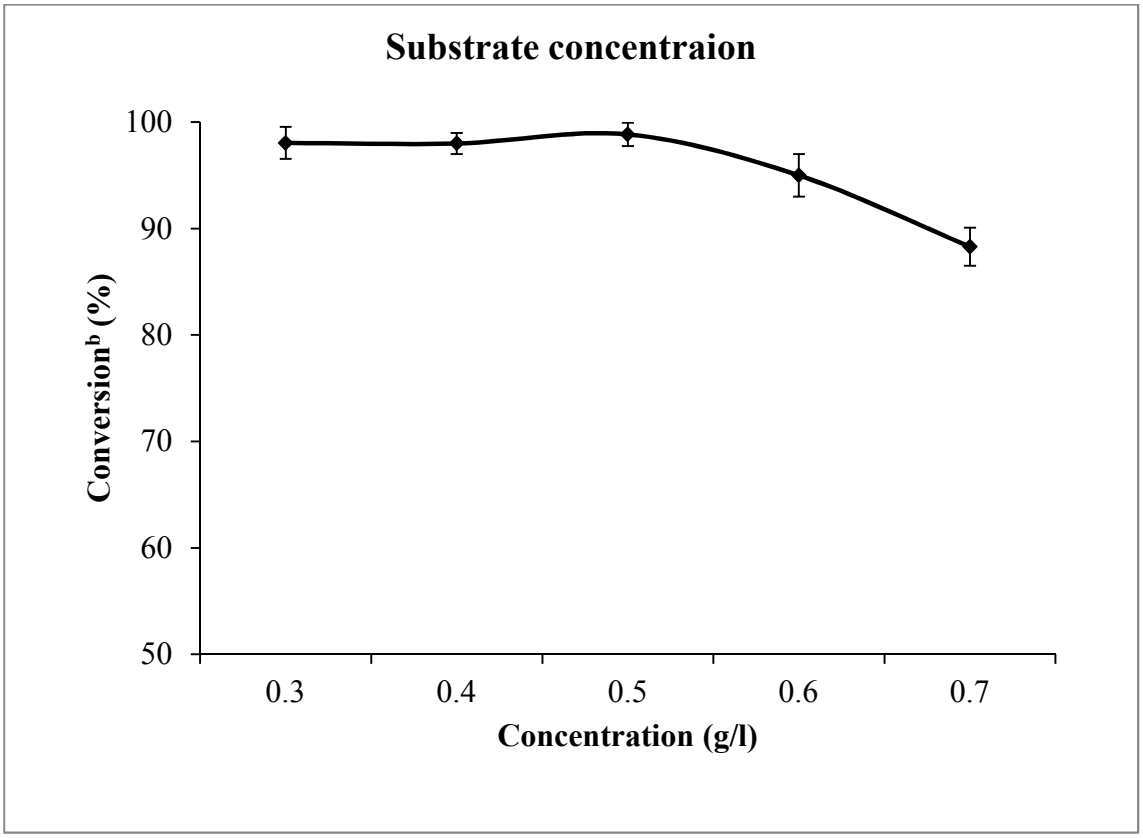
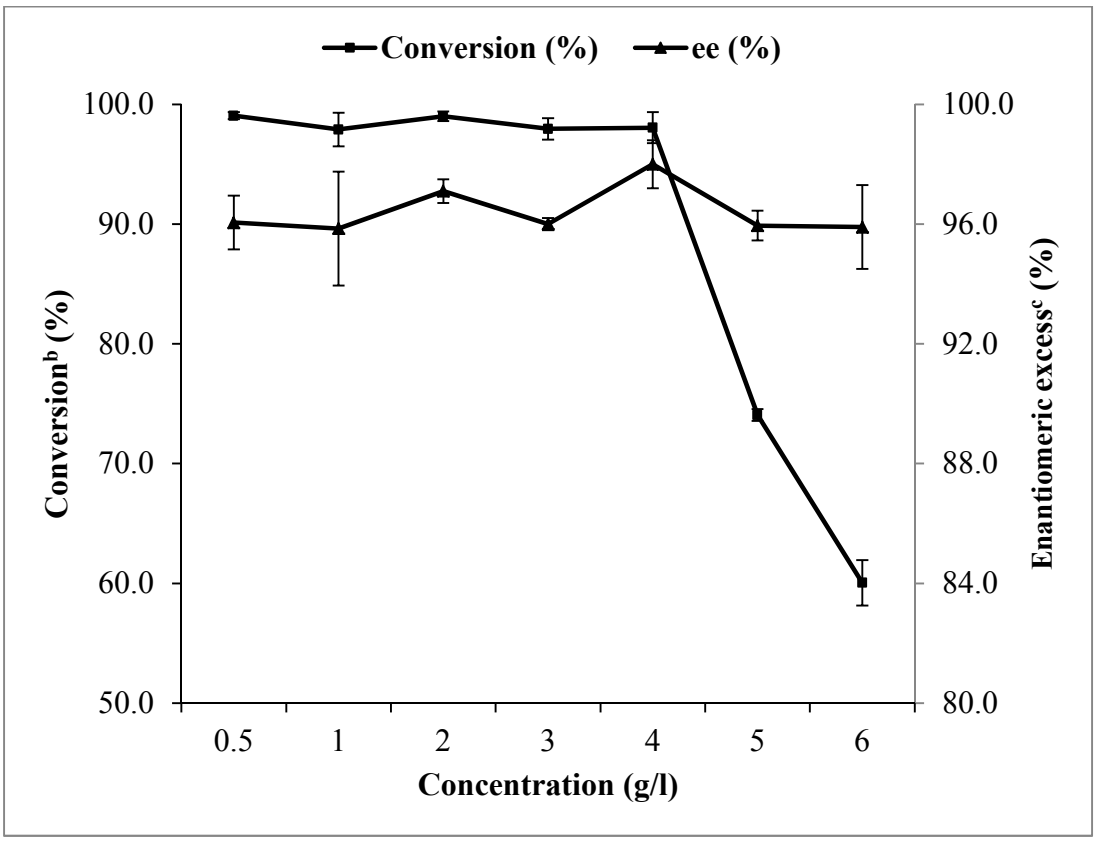


Figure 1: The effect of substrate concentration on the conversion of (1) to (2) using 24h old fermentation broth of *C. parapsilosis*.[a]



[a] Reaction conditions: 0.3-0.7 g/l of (**1**) was dissolved in 100 µl of THF and added to 24h old fermentation broth (50.0 ml) consisting of potato dextrose broth (24.0 g/l). Samples were taken after 48h and extracted with equal volume of ethyl acetate and dried using nitrogen.
[b] Determined by HPLC (Zorbex Eclipse Plus C-18, 210 nm); Mobile phase A: 0.02 M KH_2PO_4 buffer pH 3.0/ Mobile phase B: ACN:H_2O (90:10 v/v) - see S.I.

Figure 2: The effect of substrate concentration on the biotransformation of (1) using resting cells of *C. parapsilosis.*[a]



[a] Reaction conditions: Cells were harvested from 48h old culture by centrifugation (6000 rpm × 10 min). 1.0 g cells were taken, washed with de-ionized water and then re-dissolved in 5% glucose solution. Different concentrations (0.5-6.0 g/l) of (**1**) were dissolved in THF and then charged to the glucose solution containing cells. The reactions were incubated at 30°C and 200 rpm in an orbital shaker for 48h.

[b] Determined by HPLC (Zorbex Eclipse Plus C-18, 210 nm); Mobile phase A: 0.02 M KH_2PO_4 buffer pH 3.0/ Mobile phase B: ACN:H_2O (90:10 v/v) - see S.I.

[c] Determined by Chiral HPLC [Chiralcel OD-H column, Hexane/iPrOH/TFA (92:7.9:0.1)] - see S.I.

Table 1: Bioreduction of (1) to (2) using different strains of microorganisms.[a]

Microbial strains	Conv.[b] (%)	*R*[c]	*S*[c]
***Candida tropicalis*104656**	98.0	18.0	82.0
***Bacillus licheniformis*103023**	0.0	-	-
***Saccharomyces cerevisiae*103168**	6.0	100.0	-
***Rhodococcus sp.* 103102**	0.0	-	-
***Pichia methanolica*103660**	73.0	73.0	27.0
***Rhodotorula sp.* 103024**	41.0	20.0	80.0
***Candida rugosa*103332**	96.0	9.0	91.0

[a] Reaction conditions: 0.09 mol of (**1**) was dissolved in 100 µl of methanol and added to 24h old fermentation broth (50.0 ml) consisting of potato dextrose broth (24.0 g/l). Samples were taken after 48h and extracted with equal volume of ethyl acetate and dried using nitrogen.
[b] Determined by HPLC (Zorbex Eclipse Plus C-18, 210 nm); Mobile phase A: 0.02 M KH_2PO_4 buffer pH 3.0/ Mobile phase B: ACN:H_2O (90:10 v/v) - see S.I.
[c] Determined by Chiral HPLC [Chiralcel OD-H column, Hexane/iPrOH/TFA (92:7.9:0.1)]- see S.I.

Table 2: Bioreduction of (1) to (2) using several species of *Candida*.[a]

Microbial strains	Conv.[b] (%)	*R*[c]	*S*[c]
***C. glabrata*104523**	22.0	68.0	32.0
***C. boidinii*103655**	5.0	100.0	-
***C. sonorensis*104061**	7.0	100.0	-
***C. magnoliae*104517**	5.0	40.0	60.0
***C. apis*104519**	8.0	62.0	38.0
***C. maltosa*104532**	10.0	50.0	50.0
***C. parapsilosis*104659**	>98.0	7.0	93.0
***C. etchellsii*104521**	76.0	93.0	7.0
***C. tropicalis*104656**	>99.0	17.0	83.0
***C. utilis*104952**	50.0	60.0	40.0
***C. rugosa*103332**	>98.0	9.0	91.0

[a] Reaction conditions: 0.09 mol of (**1**) was dissolved in 100 µl of methanol and added to 24h old fermentation broth (50.0 ml) consisting of potato dextrose broth (24.0 g/l). Samples were taken after 48h and extracted with equal volume of ethyl acetate and dried using nitrogen.
[b] Determined by HPLC (Zorbex Eclipse Plus C-18, 210 nm); Mobile phase A: 0.02 M KH_2PO_4 buffer pH 3.0/ Mobile phase B: ACN:H_2O (90:10 v/v) - see S.I.
[c] Determined by Chiral HPLC [Chiralcel OD-H column, Hexane/iPrOH/TFA (92:7.9:0.1)] - see S.I.

Table 3: Effect of different co-solvents on the bioreduction of (1) using *C. parapsilosis*.[a]

Solvents	Conv.[b] (%)	ee[c](%)
Methanol	98.0	86.0
Ethanol	90.0	85.0
THF	>99.0	94.0
IPA	98.0	92.0
MTBE	76.0	80.0

[a] Reaction conditions: 0.09 mol of (**1**) was dissolved in 100 µl solvent and added in to 24h old fermentation broth (50.0 ml) consisting of potato dextrose broth (24.0 g/l). Samples were taken after 48h and extracted with equal volume of ethyl acetate and dried using nitrogen.
[b] Determined by HPLC (Zorbex Eclipse Plus C-18, 210 nm); Mobile phase A: 0.02 M KH_2PO_4 buffer pH 3.0/ Mobile phase B: ACN:H_2O (90:10 v/v) - see S.I.
[c] Determined by Chiral HPLC [Chiralcel OD-H column, Hexane/iPrOH/TFA (92:7.9:0.1)] - see S.I.

Scheme 1