An industrial perspective fermentative bioreduction of aromatic ketones by *Penicillium rubens* VIT SS1 and *Penicillium citrinum* VIT SS2

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ABSTRACT

Microbial mediated, especially the fungi mediated asymmetric reduction of the ketone is one of the most promising tools for the synthesis of chiral alcohols. Many fungal cultures were isolated from soil and screened for the stereo selective bioreduction of acetophenone. The potential isolates are characterised using molecular techniques and found to be *Penicillium rubens* VIT SS1 (Genbank ID: MK063869) and *Penicillium citrinum* VIT SS2 (Genbank ID: MW960208). Both the isolates were tested for the bioreduction of few aromatic ketones such as 4-fluoro acetophenone, 3-hydroxy acetophenone, and oxcarbazepine, which are the key chiral intermediates of various pharmaceutical drugs. The *P. rubens* VIT SS1 produced (S)-alcohol obeying Prelog’s rule, and *P. citrinum* was anti-Prelog configuration in nature. Preparatory scale reactions were conducted using the optimised bioreduction process, and the keto loading was significantly increased by 12-fold (from 0.5 to 6 g/L) with >99% conversion and >98% enantiomeric excess. The study discloses the vast prospective approach of exploring filamentous fungi for sustainable synthesis of chiral alcohols in an environment-friendly, novel, and cost competent way.

ARTICLE HISTORY

Received 16 April 2021
Revised 11 July 2021
Accepted 28 July 2021

KEYWORDS

Bioreduction; *Penicillium* sp; aromatic ketone; 3-hydroxy acetophenone; licarbazepine

Introduction

Asymmetric synthesis of chiral alcohol is a critical step in the production of many chiral pharmaceutical compounds, agrochemicals, flavors, and fine chemicals (Patel et al. 2004a; Koesoema et al. 2020; Şahin 2020a). The chiral alcohols can be synthesized via asymmetric reduction of ketones or the kinetic resolution of racemic mixtures, catalyzed by either metal/chemical catalysts or biocatalysts. The asymmetric synthesis is being preferred due to the 100% yield over the kinetic resolution processes (Bayḑaş et al. 2020). Likewise, the biocatalytic route is chosen over the synthetic methods, which involves the use of expensive chiral reagents, tedious synthetic processes, often utilise hazardous reagents and heavy metals (Nakamura et al. 2003; Şahin 2019). The key benefits of biocatalytic routes are: (i) They are highly chemo-, regio-, and enantio-selective in nature, (ii) Superior catalytic efficient with multiple recycling using immobilized enzymes, (iii) Milder reaction conditions and environment safer, and (iv) Sustainable and cost-effective process (Öksüz et al. 2018).

The biocatalytic reduction process using purified enzymes is considerably simple and easy to operate than the whole-cells/fermentation process (Chartrain et al. 2001). However, they have some constraints like the continuous supply of expensive cofactors (NAD/NADP), dependency on a second enzyme for cofactor recycling, and less stability in the biocatalytic process conditions (Goldberg et al. 2007). Additionally, the availability of versatile keto-reductase is a prime concern for commercial-scale synthesis. In this regard, the microbial-mediated processes are advantageous in producing chiral alcohols (Rocha et al. 2009). The whole-cell reduction during fermentation is better than resting cells and isolated enzymes. During fermentation, the co-factors can be effectively recycled and provide an ideal condition for the enzymes, thereby improves their operational life (Nakamura et al., 1990). The crucial benefit of fermentative biotransformation is the use of cheap raw materials and catalyze multistep conversions. The production cost for the industrial enzyme was reported as high as 100-fold than the generation of whole-cell catalyst (Hollmann et al. 2021). Therefore, the quest for novel microbial strains with improved selectivity becomes an
important task in the synthesis of chiral alcohols, wherein the microorganisms remain as the main source of KRED, with many advantages over free enzymes (An et al. 2019).

Among, the whole-cell processes, the fungal mediated bioreduction is viewed as a futuristic solution in overcoming substrate toxicity, complex metabolic pathways with highly stable enzymes, and ease in handling than other microbial flora. Due to the metabolic complexity of the fungal strains, undesired side reactions with the substrates are often possible (Kurbanoglu et al. 2009). The fungal cell wall protects and stabilizes the enzyme from the harsh reaction conditions, and reactants used in the bioreduction process. This phenomenon helps the enzyme to be functional for an extended period and thereby increasing the throughput and process yield. Hence, the screening of fungal strains with superior conversion and selectivity is mandatory for easy implementation (Jothi and Vuppu 2020). Moreover, the optimization of media such as carbon and nitrogen sources, process parameters such as pH, and temperature will overcome the other undesired reactions resulting in lower yields and low chiral purity of the product (Patil et al. 2013; Şahin 2020b).

In general, the bioreduction of pro-chiral ketone with fungi generates the (S)-alcohol as described by Prelog’s rule (Prelog 1964). Thus, fungal cultures with anti-Prelog activity will be more relevant to have a versatile library of whole cell ketoreductase.

Hence, this study focuses on the isolation of fungal cultures from soil and screening them for stereo-selective reduction of acetophenone, 4-fluoro acetophenone, 3-hydroxy acetophenone, and oxcarbazepine. Acetophenone is an extensively recognised model substrate and its derivatives are used in the synthesis of many chiral intermediates for pharmaceutical production (Patel et al. 2004b). These aromatic ketones were found to be vital chiral building blocks for the drugs belonging to the category of antidepressants, anti-bronchitis, and anti-asthmatic categories (Xie et al. 2006). (R)-1-(4-Fluorophenyl) ethanol is an intermediate of gamma-secretase modulator for Alzheimer’s disease treatment and an anti-malaria drug. The ketone, 3-hydroxy acetophenone is a key intermediate for Phenytoin, which is widely used as a decongestant as well as to relieve hemorrhoids, and Rivastigmine, a drug used in the treatment of Alzheimer’s disease, and Parkinson’s disease. The oxcarbazepine is a key intermediate used in the synthesis of eslicarbazepine, is an anticonvulsant drug used for the treatment of epilepsy.

Materials and methods

General

The dehydrated media components, general chemicals, and HPLC solvents were purchased from Himedia Laboratories, Sisco research laboratories, and Avantor, India. The aromatic ketones used in this study were acetophenone (A), 4-fluoro acetophenone (4FA), 3-hydroxy acetophenone (3HA), and oxcarbazepine. These ketones and alcohols standards were sourced locally from Sigma-Aldrich and TCI chemicals for the study. The ketones were reduced using 1–1.5 equivalents of sodium borohydride in methanol at 30°C to yield the corresponding racemic alcohols. The products were characterised after purification by a silica column. The product formation was analysed by reverse-phase high-performance liquid chromatography (Shimadzu HPLC) on a C-18 column (250 × 4.6 mm, 5-micron size) using 0.1% Trifluoroacetic acid in water and acetonitrile as the eluent in gradient mode. The flow rate was 1 mL/min, and the compounds were detected by a UV-Vis detector. The column temperature was set at 25°C and the sample cabinet was set at 15°C. The chiral purity of the product was determined by normal-phase HPLC on Chiralcel OD-H and Chiralpak IG chiral column (250 × 4.6 mm, 5-micron size). The mobile phase consists of hexane (90%) and isopropyl alcohol (10%) with 0.1% TFA as an additive (More et al. 2015; Şahin 2019). The flow rate was 0.5–1 mL/min, detection at UV detector and the column temperature was set at 25°C.

Isolation and cultivation condition for fungal cultures

The samples of enriched soil were collected from the coastal area, municipal waste dump yard, and effluent treatment area. The fungal cultures were purified by serial dilution and using standard microbial techniques in the laboratory. The purified cultures were streaked in potato dextrose agar (PDA) plates for further use. The fungal cultures were cultivated using defined complex media as mentioned in Table 1. The inoculum was generated using fungal spores from the PDA plates and sub-cultured to a production flask, which

| Media Components                | Concentration (g/L) |
|--------------------------------|--------------------|
| Dextrose monohydrate            | 50                 |
| Soya flour defatted and toasted | 20                 |
| Magnesium sulphate heptahydrate | 2                  |
| Sodium Nitrate                  | 1                  |
| Potassium dihydrogen phosphate  | 2.5                |
was incubated at 28 °C, 200 rpm in an incubator shaker for 24 h to conduct a bioreduction reaction.

**Biotransformation procedure**

The biotransformation reaction was carried out using the production flask. The keto intermediates were prepared as 10% w/v solution in dimethyl sulfoxide (DMSO). The substrate solution in DMSO was added at a concentration of 0.5 g/L to each flask and incubated at 28 °C, 200 rpm for 72 h. The compounds were extracted by adding one volume of ethyl acetate and separated by centrifugation at 4,000 rpm for 10 minutes. The upper organic layer was concentrated under vacuum. The crude compound was suitably diluted using diluents and analysed the percentage conversion of ketone and chiral purity of the alcohol produced.

**Identification by electron microscopic examination**

The potential isolate was visualized under a scanning electron microscope (SEM, ZEISS EVO 18 RESEARCH) using the dried mycelia from the production flask. The sample was prepared by fixation, washing, dehydration, critical point drying, mounting the sample on an SEM stub, followed by metal coating and sputter coating.

**Molecular characterization and gene sequencing**

The genomic DNA from the potential fungal isolates were extracted following a standard protocol (10). (Supplementary section 2). The amplified ITS gene product was sequenced by following the Big Dye terminator technique in an ABI 3500 sequencer instrument. The resulted gene sequence was blasted in NCBI for its identification.

**Gram scale biotransformation procedure**

The potential isolate *P. rubens* VIT SS1 was used for the gram scale bioreduction reaction. The fungal culture was grown in a 2 L conical flask containing 500 ml of sterilized defined media. The optimized process conditions reported earlier were used for the study (Jothi and Vuppu 2020). The growth conditions were pH (5 ± 0.5), temperature (25 ± 2 °C), and rpm (200 ± 10) in a shaker incubator. After 24 h of growth, the substrate stock in DMSO was fed to the fermentation broth in a fed-batch mode up to 6 g/L keto concentration. About 1 g/L of keto was added initially and followed by 1 g/L at every 6–12 h added to the flasks. The growth condition was maintained by adding 5 g/L dextrose feed (sterilized 50% stock) every 24 h. The samples were withdrawn at periodic intervals to analyse the conversion using reverse-phase HPLC. After 72 h, the product was extracted by adding one volume of ethyl acetate and distilled using a rotary evaporator under vacuum. The product was purified by silica column and characterised.

**1-Phenyl ethanol**

$^1$H NMR (500 MHz, DMSO-d6) $\delta$ 7.37 – 7.26 (m, 4 H), 4.89 (qdd, J = 6.3, 4.8, 0.7 Hz, 1 H), 1.46 (d, J = 6.4 Hz, 2 H). $^{13}$C NMR (125 MHz, DMSO-d6) $\delta$ 146.00, 128.50, 127.59, 125.62, 69.98, 25.11. HPLC conditions: Chiralcel OD-H column, 210 nm, flow rate: 1.0 mL/min, i-PrOH/n-hexane 5:95, tR ($R$) 7.8, (S) 9.2 min.

**4-Fluoro phenyl ethanol**

$^1$H NMR (500 MHz, DMSO-d6) $\delta$ 7.33 – 7.26 (m, 2 H), 7.13 – 7.05 (m, 2 H), 4.84 (ddt, J = 7.0, 6.1, 4.9 Hz, 1 H), 2.93 (s, 0 H), 1.46 (d, J = 6.4 Hz, 3 H). $^{13}$C NMR (125 MHz, DMSO-d6) $\delta$ 163.61, 161.64, 142.40, 142.38, 127.53, 127.46, 115.36, 115.18, 70.27, 25.33. HPLC conditions: Chiralcel OD-H column, 250 nm, flow rate: 1.0 mL/min, i-PrOH/n-hexane 5:95 with 0.1% TFA, tR ($R$) 36, (S) 33 min.

**3-Hydroxy phenyl ethanol**

$^1$H NMR (500 MHz, DMSO-d6) $\delta$ 8.57 (s, 1 H), 7.19 (t, J = 8.1 Hz, 1 H), 7.04 (ddt, J = 8.1, 2.0, 0.9 Hz, 1 H), 6.76 (td, J = 2.2, 0.6 Hz, 1 H), 6.69 (dd, J = 8.2, 2.2, 1.1 Hz, 1 H), 4.85 (ddt, J = 7.1, 6.2, 5.1 Hz, 1 H), 1.44 (d, J = 6.4 Hz, 3 H). $^{13}$C NMR (125 MHz, DMSO-d6) $\delta$ 157.19, 147.86, 129.86, 118.04, 115.49, 113.92, 70.22, 25.20. HPLC conditions: Chiralcel OD-H column, 210 nm, flow rate: 1.0 mL/min, i-PrOH/n-hexane 3:97 with 0.1% TFA, tR ($R$) 11.4, (S) 12.2 min.

**Licarbazepine**

$^1$H NMR (500 MHz, DMSO-d6) $\delta$ 7.36 (ddd, J = 13.5, 7.4, 1.2 Hz, 2 H), 7.36 – 7.20 (m, 4 H), 7.14 (td, J = 8.1, 1.5 Hz, 1 H), 7.01 (ddd, J = 8.8, 7.6, 1.4 Hz, 1 H), 6.45 (s, 2 H), 5.20 – 5.12 (m, 1 H), 4.16 (d, J = 5.7 Hz, 1 H), 3.19 – 3.11 (m, 1 H), 2.90 (ddd, J = 15.4, 7.6, 0.8 Hz, 1 H). $^{13}$C NMR (125 MHz, DMSO-d6) $\delta$ 157.29, 137.86, 137.05, 135.31, 129.74, 129.66, 128.90, 127.92, 127.16, 127.07, 126.61, 124.85, 124.05, 67.74, 40.24. HPLC conditions: Chiralpak IG column, 230 nm, flow rate: 1.0 mL/min, i-PrOH/n-hexane 20:80 with 0.1% TFA, tR ($R$) 19, (S) 20 min. (Supplementary section 3)
Results and discussion

Cultivation of fungi for asymmetric reduction

The proper cultivation of fungal culture is crucial for a biotransformation reaction to occur. The media selection is very essential for attaining thick mycelial growth and it acts as an inducer for the enzyme of interest. The commonly used thin media such as potato dextrose broth, malt extract broth, and Sabouraud dextrose broth will tend to form compact spherical masses of mycelium known as pellets or beads. This morphology provides lesser conversion due to the rigidity of the outer membrane and low diffusion of the substrate, oxygen, and nutrient to the inner mycelia. Hence, converting the pellets to propagated mycelia will be the better choice to increase the growth, metabolic activity, and microbial surface area to react with the substrates. The enzyme production was primarily dependent on the nature of the media components. It is important to identify the suitable media components to increase the enzyme production of interest. The media-rich in carbon sources will trigger the production of carbonyl reductase. Hence, the carbon sources such as soluble starch, raw starch, dextrose, sucrose, glycerol, sorbitol, and mannitol were studied. The results are shown in Figure 1. Dextrose (glucose monohydrate) was selected as it showed high mycelial growth than other carbon sources. The nitrogen sources such as soya peptone, soya flour, yeast extract, corn steak liquor, and cotton-seed flour were studied along with dextrose as a carbon source. The soya flour was found to be appropriate for attaining a thick mycelial growth with packed cell volume (PCV) of about 30-35% w/v.

Screening of fungal flora for the bio-reduction of acetophenone

The fungal cultures isolated from the soil were tested for the reduction of acetophenone to 1-phenyl ethanol. Most of them were provided conversion and shown varying stereo-selectivity for the 1-Phenyl ethanol. The bioreduction reactions were conducted directly in the fermentation medium without isolation of the cell mass. The substrate was dissolved in dimethyl sulfoxide and added to the flask in a concentration of 0.5 g/L. The flasks were harvested after 72 h by adding one volume of ethyl acetate to the fermentation broth. The solvent was allowed to mix for an hour and separated by centrifugation at 4,000 rpm for 10 minutes. The organic layer was separated, dried, and the conversion was determined by reverse-phase HPLC. The HPLC results for 40 fungal isolates screened are presented in Table 2, and Figure 2 represents the reaction scheme.

The fungal isolates predominantly reduced the pro-chiral acetophenone to (S)-Phenyl alcohol, comply

Table 2. Results for the bioreduction of acetophenone by soil fungal isolates.

| Fungal cultures | % HPLC Conversion | Alcohol chiral purity |
|-----------------|-------------------|----------------------|
| Isolate-01      | 5%                |                      |
| Isolate-02      | 33%               | 74% (R)              |
| Isolate-03      | 2%                | 74% (R)              |
| Isolate-04      | 12%               |                      |
| Isolate-05      | 2%                |                      |
| Isolate-06      | 62%               | 83% (R)              |
| Isolate-07      | 3%                | 86% (S)              |
| Isolate-08      | 17%               | 85% (S)              |
| Isolate-09      | 16%               | 85% (S)              |
| Isolate-10      | 18%               |                      |
| Isolate-11      | 72%               | 86% (S)              |
| Isolate-12      | 15%               | 65% (R)              |
| Isolate-13      | 86%               | 97% (S)              |
| Isolate-14      | 4%                |                      |
| Isolate-15      | 5%                |                      |
| Isolate-16      | 11%               |                      |
| Isolate-17      | 9%                |                      |
| Isolate-18      | 65%               | 64% (S)              |
| Isolate-19      | 15%               | 82% (S)              |
| Isolate-20      | 10%               |                      |
| Isolate-21      | 8%                |                      |
| Isolate-22      | 2%                |                      |
| Isolate-23      | 5%                |                      |
| Isolate-24      | 5%                | 70% (S)              |
| Isolate-25      | 25%               | 76% (S)              |
| Isolate-26      | 1%                |                      |
| Isolate-27      | 8%                |                      |
| Isolate-28      | 11%               |                      |
| Isolate-29      | 1%                |                      |
| Isolate-30      | 3%                |                      |
| Isolate-31      | 2%                |                      |
| Isolate-32      | 5%                |                      |
| Isolate-33      | 41%               | 75% (S)              |
| Isolate-34      | 30%               | 71% (S)              |
| Isolate-35      | 22%               | 55% (S)              |
| Isolate-36      | 2%                |                      |
| Isolate-37      | 11%               |                      |
| Isolate-38      | 1%                |                      |
| Isolate-39      | 3%                |                      |
| Isolate-40      | 24%               | 65% (R)              |

The conversion of keto to hydroxy is expressed in percentage (%) area of the product peak in the HPLC analysis. The chiral purity of the alcohol is expressed in % area of the enantiomers analysed in a normal phase HPLC.

Screening condition: Keto loading 0.5 g/L, Temp. 28°C, pH 6 and 200 rpm in a shaker for 72 h.

Figure 1. Effect of carbon sources on the fungal growth. The results are in the graphical format as bar diagram. The fungal growth was measured as packed cell volume and mentioned as % PCV.
with the Prelog rule as reported by (Decarlini et al., 2017; Quezada et al., 2012) towards the acetophenone. The fungal isolate-13 selectively converted the acetophenone to its corresponding (S)-alcohol with 97% chiral purity in accord with the Prelog rule and shown >95% conversion. Similarly, the fungal isolate-06 selectively produced (R)-alcohol with 83% chiral purity in accord with the anti-Prelog rule. Microbes having anti-Prelog configuration were rare and recently in demand. These two isolates were subjected to characterization using the widely reported methods (Wu et al. 2013).

The co-solvents for the bioreduction reaction were considered very critical, which helps in the dissolution of keto in the reaction medium. The solvents such as dimethyl sulfoxide (DMSO), methanol, ethanol, isopropyl alcohol (IPA), acetone, 1-butanol, 1,4-dioxane, tetrahydrofuran (THF), and dimethylformamide (DMF) were examined for the bio-reduction reaction using the fungal isolate-13. The co-solvent concentration was fixed at a maximum of 5%v/v to the reaction medium and acetophenone was dosed at 1 g/L. The reactions were conducted as per the conditions mentioned in the methods section. The results showed in Figure 3, wherein DMSO exhibited higher conversion and chiral purity for the product without affecting the fungal growth. Dimethyl Sulfoxide is a highly polar organic liquid and used widely as a chemical solvent. Moreover, it is a polar aprotic solvent with a log P value of −1.378. Hence, DMSO was selected as a co-solvent for further testing of aromatic keto compounds.

Identification and characterization

The fungal colonies were observed as flat, filamentous, velvety, and cottony in texture in PDA plates. The colonies were white during budding and become blue-green, green, olive, yellowish, or pinkish in time. The visualized image is shown in Figure 4 from the scanning electron microscope clearly showed the elongated hyphae with sausage-shaped cells that multiply by fission were identified as Penicillium sp.

The molecular characterization was carried out for the potential fungal isolate-13 and 06. The ribosomal internal transcribed spacer (ITS) region was amplified using suitable primers. Amplification of 530 bp gene was sequenced and blasted against the GenBank database, Ribosomal Database Project (RDP II). The results revealed that the soil fungal isolate-13 is closely related to Penicillium rubens with 98% similarity and the fungal isolate-06 was closely related to P. citrinum with 99% homology. The sequences were submitted to GenBank and the accession number of P. rubens is MK063869 and the accession number for P. citrinum is MW960208. The evolutionary history was inferred by using the Neighbor-Joining method. Evolutionary analyses were conducted in MEGA X (Kumar et al. 2018). These fungal isolates were named P. rubens VIT SS1 and P. citrinum VIT SS2.

Bioreduction of 4-fluoro acetophenone

Fluorinated 1-arylethanols are important building blocks in medicinal chemistry. They are used in the synthesis of kinase inhibitors for cancer therapy, NK1 receptor antagonists, and drugs applied in the treatment of osteoporosis (Hoff and Sundby 2013). The (R)-alcohol is an intermediate of an anti-malaria drug, and a gamma-secretase modulator useful for Alzheimer’s disease treatment (Huang et al. 2010). The potential bioactive compounds prepared using 1-(4-fluorophenyl) ethanol are mentioned in Figure 5.

The “Fluoro” group insertion in a molecule tends to affect the lipophilicity, electronic and steric effect as
well as its conformational properties of the molecule (O’Hagan 2008). ADH from Pyrococcus furiosus with NADP as cofactor provided (S)-(4-fluorophenyl) ethanol at 99% enantiomeric excess (Zhu et al. 2006). The P. rubens provides the (S)-(4-fluorophenyl) ethanol at chiral purity of 94%, and P. citrinum provided the (R)-(4-fluorophenyl) ethanol at chiral purity of 88% respectively (Table 3, entry 2). Figure 6 shows the reaction scheme.

The Penicillium sp. shown very high selectivity and conversion for the fluorinated aryl ethanol at the ambient condition with cheaper raw materials. The three fungi A. terreus CCT 3320, A. toreros CCT 4083, and A. terreus CCT 4964 were reported by ALM. Porto et.al. The reactions were conducted using resting cell bioreduction in buffer using 1 g/L loading of keto. The best conversions were achieved with A. terreus CCT 3320 and found to be 30% with 97% (S)-alcohol with 7 days of reaction time. The Penicillium species were shown a superior performance than Aspergillus cultures.

### Bioreduction of 3-hydroxy acetophenone

The 3-hydroxy acetophenone upon reduction yields 3-hydroxy phenyl ethanol is a key intermediate for Phenylephrine, a widely used decongestant drug, and Rivastigmine, a drug compound used for the treatment of Alzheimer’s disease and Parkinson’s disease. The chemo-enzymatic scheme is mentioned in Figure 7. Synthetic routes for the production of (R)-Phenylephrine consist of an asymmetric hydrogenation process in presence of an expensive metal catalyst, with multiple purification processes, high temperature, and use of several organic solvents (Peng et al. 2014). Similarly, (S)-Rivastigmine was synthesized by
resolution process, and by asymmetric reactions using transition metals and chiral organic ligands. The synthetic routes have drawbacks such as complex processes, poor yields, and multiple purification steps to remove impurities (Sethi et al. 2013). The enzymatic bioreduction reaction scheme is mentioned in Figure 8.

The \textit{P. rubens} provides the \((S)\) - alcohol at chiral purity of 95\% in accord with Prelog rule and \(P.\ citrinum\) provided the \((R)\) - alcohol at chiral purity of 82\% in line with the anti-Prelog rule, respectively (Table 3, entry 3). The microbes with anti-Prelog configuration are regarded as rare findings. The keto percentage conversion can be increased by optimization of substrate dosing strategy and nutrient feeding for microbial growth during the bioconversion process. Mainly, the carbon feed (Dextrose) performs a dual role as a suitable carbon source for the metabolism, and as the co-substrate for the recycling of the necessary co-factor used in the reduction process. The glucose was converted to gluconic acid by glucose dehydrogenase and regenerates NAD to NADH, an important co-factor for various ketoreductases. The preparatory scale study conducted using the optimized process by Taguchi method (Jothi and Vuppu 2020) resulted in >90\% conversion for 6 g/L loadings of keto compound and \((S)\)-alcohol chiral purity of as high as 99\% at pH 5 ± 0.5 at temperature 25 \(^\circ\)C.

**Bioreduction of oxcarbazepine**

\((S)\)-Licarbazepine is a key intermediate for the synthesis of eslicarbazepine acetate, which is used for the treatment of epileptic seizures in adults, psychosomatic diseases, trigeminal neuralgia, Parkinsonian syndromes, and AIDS-related neural disorders (Biton et al. 2017). Chemical reductions were reported using expensive chiral catalyst Ru or Rh and R-2-Me-CBS catalyst, which is a complex, hazardous, and expensive process (Ravinder et al. 2013). The whole cell-mediated ketoreduction of oxcarbazepine to \((S)\)-licarbazepine was reported in an aqueous and biphasic medium.

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Table 3. Bioreduction of aromatic ketones by \textit{Penicillium rubens} VIT SS1 and \textit{Penicillium citrinum} VIT SS2.

| Entry | Substrate Ketone | Product Alcohol | \textit{P. rubens} VIT SS1 | \textit{P. citrinum} VIT SS2 |
|-------|------------------|-----------------|-----------------------------|-----------------------------|
| 1     | Acetophenone(A)  | Phenyl ethanol(P) | 86\% 97% \((S)\)         | 62\% 83% \((R)\)           |
| 2     | 4-Fluoro A       | 4-Fluoro P       | 90\% 94% \((S)\)         | 68\% 88% \((R)\)           |
| 3     | 3-Hydroxy A      | 3-Hydroxy P      | 87\% 95% \((S)\)         | 45\% 82% \((R)\)           |
| 4     | Oxcarbazepine    | Licarbazepine    | 97\% 99% \((S)\)         | 85\% 92% \((S)\)           |

The conversion of keto to hydroxy is expressed in percentage (\%) area of the product peak in the HPLC analysis. The chiral purity of the alcohol is expressed in % area of the enantiomers analysed in a normal phase HPLC.

Screening condition: Keto loading - 0.5 g/L, Temp. 25 \(^\circ\)C, pH 5 and 200 rpm in a shaker for 72 h.
Figure 9. Bioreduction scheme of oxcarbazepine.

The aqueous-based bioreduction was conducted using *Saccharomyces cerevisiae* CGMCC No. 2266, in a whole-cell buffer reaction with a yield of 3.7 mmol/L/day after four days of continuous reaction (Ou et al. 2011). The titer was found to be poor due to the solubility of keto in the aqueous medium. Later, Singh et al. reported a reduction process in water and hexane biphasic solution using whole-cells of *Pichia methanolica* 103660 (Singh et al. 2012). The whole buffer reaction was conducted using 50% hexane: water medium and keto loading up to 1.25 g/L with >98% conversion and >98% chiral purity. Further, to improve the interfacial catalytic efficiency of ketoreduction, interface-assembled carbonyl reductase (IACR) was employed by Ou et al. The IACR was synthesized using the KRED from *Bacillus anthracis* CGMCC No. 12337 by polystyrene and Toluene mixture. The reaction was conducted in a buffer with 1 g/L of keto loading (Ou et al. 2018). The reported processes were insufficient to meet the commercial demands. An engineered enzyme was reported by Codexis inc. from *Lactobacillus kefir*. The wild-type culture provided only about 6% to 21% product formation when tested at 30°C and 40°C respectively in 24 h. The engineered KRED converted >100 g/L keto with >99% conversion and >99% chiral purity (Modukuru et al. 2014). Figure 9 shows the reaction scheme.

The biotransformation conducted using growing cells of *P. rubens* provided >99% chiral pure (S)-Licarbazepine. In the preparatory scale reaction using the substrate dosing strategy, the keto loading was increased up to 5 g/L, and >97% conversion was achieved without affecting the chiral purity. The fermentative bio-reduction using *P. rubens* was found to be a more superior process than the reported whole cell-mediated transformations. The co-solvent DMSO helped in the solubility of oxcarbazepine and makes it available to the culture. Interestingly, the *P. citrinum* provided (S) - alcohol at chiral purity of 92%, whereas the culture was identified as anti-Prelog configuration. The result (Table 3, entry 4) suggests that the chiral configuration was more influenced by the two bulkier groups attached to the functional group than the enzyme’s stereo-specificity. In general, ADH stereo-specificity will depend on the shape, and confirmation of pockets in the catalytic domain along with the type of substrate used such as aromatic ketones, aliphatic ketones, or keto esters (An et al. 2019). The bulkier side groups cause steric hindrance to the active site of the enzymes, and the enantio-selectivity was predominantly towards (S)-configuration (Koesoema et al. 2019). Similar phenomena were explained by Nie et al. the enantio-selectivity inversion of KRED was observed by a flip of the side-chain position in a keto compound. The mechanism for the enantio-selectivity preference was due to the side chain size differences, and the binding pose of the active site with the keto functional group decides the alcohol selectivity (Nie et al. 2018).

**Conclusion**

The fungal isolates studied were shown higher bioreduction ability towards acetophenone, 4-fluoroacetophenone, 3-hydroxy acetophenone, and oxcarbazepine. The fungal cultures provided both the chiral alcohols (R) and (S)-isomers upon reducing the aromatic ketones. The potential isolates characterised using molecular techniques and the sequence submitted to GenBank. The *Penicillium rubens* VIT SS1 reduced the keto compound to (S)-alcohol, and *Penicillium citrinum* VIT SS2 reduced the keto to (R)-alcohol. The aromatic ketones studied are key intermediates of pharmaceutical drugs. The enantio-selectivity and conversion were found to be highly influenced by the presence of substituents in the aromatic ring. The biotransformation process was optimized to produce (S)-alcohol using *Penicillium rubens* VIT SS1. The keto loading was significantly increased up to 6 g/L using fed-batch dosing of keto in a shake flask condition, which can be increased to multifold in a bioreactor. The co-solvent DMSO helps in the dissolution of keto compounds and has not shown any inhibitory effect on the culture. This study concludes that the fungal cultures are potential biocatalysts for fermentative bioreduction of keto-compounds from an industrial perspective. And the fermentative bioreduction process is a novel, eco-friendly and cost-effective route to producing chiral alcohols. In addition, testing of fungal cultures using various keto intermediates is ongoing in our laboratory.

**Acknowledgements**

The authors want to express their gratitude to DST, honorable Chancellor, Dr. G. Viswanathan, Dr. Sekar Viswanathan, Mr. Sankar Viswanathan, and Mr. G.V. Selvam of VIT University for their constant encouragement and laboratory
facilities from VIT University, Vellore, India, to carry out this valuable work. Also, we thank the management of IosynthLabs Private Limited, Bangalore, for their continuous encouragement.

**Disclosure statement**

The authors declare that they have no conflicts of interests.

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**References**

An J, Nie Y, Xu Y. 2019. Structural insights into alcohol dehydrogenases catalyzing asymmetric reductions. Crit Rev Biotechnol. 39(3):366–379.

Baydaş Y, Dertli E, Şahin E. 2020. Green synthesis of chiral aromatic alcohols with Lactobacillus kefiri P2 as a novel biocatalyst. Synth Commun. 50(7):1035–1045.

Biton V, Rogen JB, Krauss G, Abou-Khalil B, Rocha JF, Moreira J, Gama H, Trinka E, Elger CE, Cheng H, et al. 2017. Adjunctive eslicarbazepine acetate: a pooled analysis of three phase III trials. Epilepsy & Behavior. 72:127–134.

Chartrain M, Greasham R, Moore J, Reider P, Robinson D, Buckland B. 2001. Asymmetric bioreductions: application to the synthesis of pharmaceuticals. J Mol Catal B: Enzym. 1(1-4):503–512.

Decarlini MF, Aimar ML, Vázquez AM, Vero S, Rossi LI, Yang P. 2017. Fungi isolated from food samples for an efficient stereoselective production of phenylethanols. Biocatal. Agric. Biotechnol. 12:275–285. doi:10.1016/j.bcab.2017.10.014.

Goldberg K, Schroer K, Lütz S, Liese A. 2007. Biocatalytic ketone reduction-a powerful tool for the production of chiral alcohols-part II: whole-cell reductions. Appl Microbiol Biotechnol. 76(2):249–255.

Hoff BH, Sundby E. 2013. Preparation of pharmaceutical important fluorinated 1-arylthanol using isolated enzymes. Bioorg Chem. 51:31–47.

Hollmann F, Opperman DJ, Paul CE. 2021. Biocatalytic reduction reactions from a chemist’s perspective. Angew Chem Int Ed Engl. 60(11):5644–5665.

Huang X, Aslanian R, Zhou W, Zhu X, Qin J, Greenlee W, Zhu Z, Zhang L, Hyde L, Chu I, et al. 2010. The discovery of pyridone and pyridazone heterocycles as γ-secretase modulators. ACS Med Chem Lett. 1(4):184–187.

Jothi S, Vuppu S. 2020. Taguchi analysis and asymmetric keto-reduction of acetoephone and its derivatives by soil filamentous fungal isolate: *Penicillium rubens* VIT S51. Prep Biochem Biotechnol. 50(10):1042–1052.

Koesoema AA, Standley DM, Senda T, Matsuda T. 2020. Impact and relevance of alcohol dehydrogenase enantioselectivities on biotechnological applications. Appl Microbiol Biotechnol. 104(7):2897–2909.

Koesoema AA, Sugiyama Y, Xu Z, Standley DM, Senda M, Senda T, Matsuda T. 2019. Structural basis for a highly (S)-enantioselective reductase towards aliphatic ketones with only one carbon difference between side chain. Appl Microbiol Biotechnol. 103(23-24):9543–9553.

Kumar S, Stecher G, Li M, Knyaz C, Tamura K. 2018. MEGA X: molecular evolutionary genetics analysis across computing platforms. Mol Biol Evol. 35(6):1547–1549.

Kurbanoglu EB, Zilbeyaz K, Taskin M, Kurbanoglu NL. 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(23):2759–2763.

Modukuru NK, Sukumaran J, Collier SJ, Chan AS, Gohel A, Huisman GW, Keledjian R, Narayanswamy K, Novick SJ, Palanivel SM, et al. 2014. Development of a practical, biocatalytic reduction for the manufacture of (S)-licarbazepine using an evolved ketoreductase. Org Process Res Dev. 18(6):810–815.

More GV, Badgugar KC, Bhanage BM. 2015. Kinetic resolution of secondary alcohols with Burkholderia cepacia lipase immobilized on a biodegradable ternary blend polymer matrix as a highly efficient and heterogeneous recyclable biocatalyst. RSC Adv. 5(6):4592–4598.

Nakamura K, Kawai Y, Kitayama T, Miyai T, Ogawa M, Mikata Y, Higaki M, Ohno A. 1990. ChemInform Abstract: Asymmetric Reduction of Ketones with Microbes. ChemInform. 21(32):doi:10.1002/chin.199032318.

Nakamura K, Yamanaka R, Matsuda T, Harada T. 2003. Recent developments in asymmetric reduction of ketones with biocatalysts. Tetrahedron: Asymmetry. 14(18):2659–2681.

Nie Y, Wang S, Xu Y, Luo S, Zhao Y-L, Xiao R, Montelione GT, Hunt JF, Szyperski T. 2018. Enzyme engineering based on x-ray structures and kinetic profiling of substrate libraries: alcohol dehydrogenases for stereospecific synthesis of a broad range of chiral alcohols. ACS Catal. 8(6):5145–5152.

O’Hagan D. 2008. Understanding organofluorine chemistry. An introduction to the C–F bond. Chem. Soc. Rev. 37:308–319.

Öksüz S, Şahin E, Dertli E. 2018. Synthesis of enantiomerically enriched drug precursors by lactobacillus paracasei BD876 as a biocatalyst. Chem Biodivers. 15(6):e1800028.

Ou Z, Xu J, Du L, Tang L, Niu Y, Cui J. 2018. Preparation of interface-assembled carbonyl reductase and its application in the synthesis of S-licarbazepine in toluene/Tris-HCl buffer biphasic system. J Microbiol Biotechnol. 28(4):613–621.

Ou Z-M, Shi H-B, Sun X-Y, Shen W-H. 2011. Synthesis of S-licarbazepine by asymmetric reduction of oxcarbazepine with Saccharomyces cerevisiae CGMCC No. 2266. J Mol Catal B: Enzym. 72(3-4):294–297.

Patel RN, Goswami A, Chu L, Donovan MJ, Randuri V, Goldberg S, Johnston R, Siva PJ, Nielsen B, Fan J, et al. 2004a. Enantioselective microbial reduction of substituted acetoephones. Tetrahedron: Asymmetry. 15(8):1247–1258.

Patel RN, Goswami A, Chu L, Randuri V, Goldberg S, Johnston R, Donovan MJ. 2004b. Stereoselective reduction of substituted acetoephones.

Patil R, Banoth L, Singh A, Chisti Y, Banerjee UC. 2013. Enantioselective bioreduction of cyclic alkanones by whole cells of Candida Species. Biocatal Biotransform. 31(3):123–131.
Peng G-J, Kuan Y-C, Chou H-Y, Fu T-K, Lin J-S, Hsu W-H, Yang M-T. 2014. Stereoselective synthesis of (R)-phenyl-ephrine using recombinant Escherichia coli cells expressing a novel short-chain dehydrogenase/reductase gene from Serratia marcescens BCRC 10948. J Biotechnol. 170: 6–9.

Prelog V. 1964. Specification of the stereospecificity of some oxido-reductases by diamond lattice sections. Pure and Applied Chemistry. 9(1):119–130.

Quezada MA, Carballeira JD, Sinisterra JV. 2012. Diplogelasinospora grovesii IMI 171018 immobilized in polyurethane foam. An efficient biocatalyst for stereoselective reduction of ketones. Bioresour. Technol. 112: 18–27. doi:10.1016/j.biortech.2012.02.074.

Ravinder B, Rajeshwar Reddy S, Sridhar M, Murali Mohan M, Srinivas K, Panasa Reddy A, Bandichhor R. 2013. An efficient synthesis for eslicarbazepine acetate, oxcarbazepine, and carbamazepine. Tetrahedron Lett. 54(22):2841–2844.

Rocha LC, Ferreira HV, Pimenta EF, Berlinck RGS, Seleghim MHR, Javaroti DCD, Sette LD, Bonugli RC, Porto ALM. 2009. Bioreduction of alpha-chloroacetophenone by whole cells of marine fungi . Biotechnol Lett. 31(10): 1559–1563.

Şahin E. 2020a. Production of enantiopure chiral aryl heteroaryl carbinols using whole-cell Lactobacillus paracasei biotransformation. Synth Commun . 50(4):549–557.

Şahin E. 2020b. Candida zeylanoides as whole-cell biocatalyst to perform asymmetric bioreduction of benzophenone derivatives. Synth Commun . 50(4):612–619.

Şahin E. 2019. Green synthesis of enantiopure (S)-1-(benzofuran-2-yl)ethanol by whole-cell biocatalyst. Chirality. 31(10):892–897.

Sethi MK, Bhandya SR, Maddur N, Shukla R, Kumar A, Jayalakshmi Mittapalli VSN. 2013. Asymmetric synthesis of an enantiomerically pure rivastigmine intermediate using ketoreductase. Tetrahedron: Asymmetry. 24(7):374–379.

Singh M, Singh S, Deshaborina S, Krishnen H, Lloyd R, Holt-Tiffin K, Bhattacharya A, Bandichhor R. 2012. Asymmetric reduction of a key intermediate of eslicarbazepine acetate using whole cell biotransformation in a biphasic medium. Catal Sci Technol. 2(8):1602.

Wu Y, Guan K, Wang Z, Xu B, Zhao F. 2013. Isolation, identification and characterization of an electrogenic microalgae strain. PLoS One. 8(9):e73442.

Zhu D, Malik HT, Hua L. 2006. Asymmetric ketone reduction by a hyperthermophilic alcohol dehydrogenase. The substrate specificity, enantioselectivity and tolerance of organic solvents. Tetrahedron: Asymmetry. 17(21): 3010–3014.