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# Biocatalytic synthesis of *S*(−)-1-(1′-naphthyl) ethanol by a novel isolate of *Candida viswanathii*

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**Abstract**

A new yeast strain *Candida viswanathii* MTCC 5158, isolated from soil, is capable of carrying out the enantioselective reduction of 1-acetonaphthone to *S*(−)-1-(1′-naphthyl) ethanol which is an important synthetic intermediate of mevinic acid analogue, (potential inhibitor of 3-hydroxy methyl glutaryl coenzyme A reductase). Studies on the culture conditions and catalytic performance of this microorganism showed that the carbonyl reductase occurs constitutively in *C. viswanathii* and its production is enhanced by feeding acetonaphthone (2 mM) during the early period of cultivation. Mannitol (1%, w/v) was found to be beneficial both for growth and enzyme production. Supplementation of the media with yeast extract (0.5%) and $Ca^{2+}$ (2 mM) enhanced the enzyme production. The optimal temperature and pH for the growth and enzyme production were 25 °C and 10, respectively. The organism produced enantiopure (S)-alcohol with good conversion (>97%) and almost absolute enantioselectivity (ee >99%). Parameters of the bioreduction reaction were optimized and the optimal temperature and pH for the reduction were found to be 25 °C and 8, respectively. The optimized substrate as well as the resting cell concentration was 2 and 200 g/l, respectively. The preparative scale reaction using resting cells of *C. viswanathii* yielded (S)-alcohol with 97% conversion and >99% ee.
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*Keywords:* Asymmetric reduction; 1-Acetonaphthone; *S*(−)-1-(1′-naphthyl) ethanol; *Candida viswanathii*

## 1. Introduction

*Regio*- and *stereo*-selective oxidation and reduction are of paramount importance in the synthesis of pharmaceutically useful chiral building blocks [1] and their importance has been increasingly recognized in the pharmaceutical industry [2]. A range of biocatalysts is available to assist biotransformation [3,4]. Oxidoreductases are amongst the most useful of all the classes of enzymes for biotransformation [5,6]. Among the various chiral compounds, optically active alcohols have been increasingly recognized in the pharmaceutical field. Biocatalytic preparation of these chiral alcohols using microbial carbonyl reductase is one of the best methods of preparation of these chiral auxiliaries from their corresponding prochiral ketones. The inherent advantages of these microbial carbonyl reductases over chemo-catalysts in being highly *chemo*-, *enantio*- and *regio*-selective. Moreover, biocatalytic reduction can be carried out at ambient temperature and atmospheric pressure [7]. Carbonyl reductase can provide up to 100% theoretical yield along with an almost absolute enantioselectivity. The enzyme can be used in whole cell format to effect the reduction of substrate into desired product. The major advantage of whole cell over isolated NAD(P)H-dependent carbonyl reductase in the reduction processes is the regeneration of cofactors by the cells [8].

We have recently isolated a potent carbonyl reductase producing strain *Candida viswanathii* MTCC 5158 from the soil, using enrichment and isolation technique [9]. This paper describes the optimization of various physico-chemical parameters for the growth and enzyme production and the subsequent use of this microorganism in the whole cell format to reduce 1-acetonaphthone to *S*(−)-1-(1′-naphthyl) ethanol (Fig. 1). This alcohol is an important synthetic intermediate

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Fig. 1. Biocatalytic reduction of 1-acetonaphthone to *S*(−)-1-(1′-naphthyl) ethanol.

of mevinic acid analogue that is a potential inhibitor of 3-hydroxy methyl glutaryl coenzyme A reductase (HMGAR) [10].

To date, the biocatalytic reduction of 1-acetonaphthone has been reported using *Rhizopus arrhizus* [10], *Geotrichum candidum* [11], *Merulius tremellosus* ono991 [12] and immobilized *Daucus carota* cells [13]. However, all these processes required longer reaction time (>84 h in case of *Merulius* sp.) and gave relatively lower conversion. The present method with *C. viswanathii* offers advantages of shorter reaction time (12 h only), higher substrate tolerance (>2 g/l), use of lower cell concentration (200 g/l), excellent conversion (>97%) along with almost absolute enantioselectivity (ee >99%).

## 2. Results

### 2.1. Course of enzyme production

The course of growth and enzyme production by *C. viswanathii* (Fig. 2) was monitored by cultivating it in the production medium and performing the enzyme assays as mentioned in the materials and methods. A variety of inducers were used to choose the best one. Acetonaphthone, at a concentration of 2 mM, was found to be the best one (Table 1).

### 2.2. Optimization of physico-chemical parameters

These included initial pH, inoculum age, inoculum size and the production temperature. It is evident from Fig. 3 that the present strain was unique in the sense that it can produce carbonyl reductase in a broad pH range and the maximum enzyme activity was obtained in medium of pH 10. This unique trend of initial pH studies may be due to the presence of multiple carbonyl reductases in this organism, some of which might be acidic ketone reductases while others are basic carbonyl reductases. However, the production of carbonyl reductase was found to be maximum under alkaline condition (pH 10). The enzyme activity was maximum when the production temperature was 25 °C (Fig. 4). Inoculum age and size were also optimized and it was found that 3% (v/v) of 18-h old inoculum was optimum for achieving the maximum enzyme production in shake flask (data not shown).

Fig. 2. Course of growth and enzyme production by *C. viswanathii*.

Table 1
Effect of various inducers on cell growth and enzyme production by *C. viswanathii*

| Inducers | Growth (OD 600 nm) | Enzyme activity (U/ml) |
|---|---|---|
| Control | 0.47 | 4.6 |
| Acetyl pyridine | 0.55 | 4.3 |
| Acetophenone | 0.53 | 5.3 |
| Acetyl thiophene | 0.49 | 3.8 |
| Acetonaphthone | 0.52 | 6.5 |
| Acetaldehyde | 0.34 | 1.4 |

The cells were cultivated in growth medium consisted of different aliphatic and aromatic carbonyl compounds (2 mM). After 36 h of growth, the cells were harvested, washed and cell growth and enzyme activity were measured as monitored in Section 4.

Fig. 3. Optimization of initial pH for the growth and enzyme production by *C. viswanathii*.

### 2.3. Optimization of medium composition for *C. viswanathii*

As the media optimization is a relevant aspect to be considered [14], the composition of the medium was optimized

Fig. 4. Optimization of temperature for the growth and enzyme production by *C. viswanathii*.

Table 2
Effect of various carbon sources on cell growth and enzyme production by *C. viswanathii*

| Carbon source | Growth (OD 600 nm) | Enzyme activity (U/ml) |
|---|---|---|
| Control | 0.19 | 2.3 |
| Mannitol | 0.63 | 7.4 |
| Sucrose | 0.49 | 3.6 |
| Fructose | 0.46 | 2.9 |
| Glucose | 0.54 | 6.9 |
| Glycerol | 0.35 | 1.9 |
| Lactose | 0.14 | 2.1 |

The cells were cultivated individually in growth medium consisted of different carbon sources (1%). After 36 h of growth, the cells were harvested, washed and cell growth and enzyme activity were measured as monitored in Section 4.

for the maximum production of carbonyl reductase. Several carbon sources were investigated for their capacity to support growth and enzyme production. *C. viswanathii* grew poorly in media containing sucrose and fructose as sole carbon source. Lactose and glycerol were found to be inhibitory for both growth and enzyme production. As indicated in Table 2, mannitol and glucose both supported higher growth but maximum enzyme activity was obtained with mannitol (1%, w/v) as a carbon source. Different nitrogen sources such as yeast extract, beef extract, malt extract, peptone, tryptone and corn steep liquor were added at 0.5% (w/v) level into production medium. As shown in Table 3, both the cell growth and the enzyme activity were maximum when yeast extract was used in the medium. All other nitrogen sources had significant effect on cell growth, but resulted in relatively lower enzyme activity. The effect of several metal ions on growth and enzyme production was further investigated by supplementing different metal ions (magnesium, calcium, copper, potassium, ferrous and zinc) in the production media. It was found that carbonyl reductase production was increased when calcium (2 mM) was added to the media (data not shown). Various physico-chemical parameters were optimized with *C. viswanathii* and as a result of which a significant increase in the enzyme activity (from 4 to 10 U/ml) was achieved. Finally, we adopted a culture medium (pH 10) containing 1% mannitol, 0.5% yeast extract and 2 mM calcium chloride for subsequent experiments.

Table 3
Effect of various nitrogen sources on cell growth and enzyme production by *C. viswanathii*

| Nitrogen source | Growth (OD 600 nm) | Enzyme activity (U/ml) |
|---|---|---|
| Control | 0.62 | 7 |
| Malt extract | 0.68 | 5.4 |
| Corn steep liquor | 0.67 | 1.7 |
| Yeast extract | 0.61 | 9.2 |
| Peptone | 0.51 | 2.0 |
| Beef extract | 0.44 | 2.4 |
| Tryptone | 0.38 | 4.2 |

The medium consisted of mannitol (1%) with different nitrogen sources individually (0.5%), the assay methods were the same as in Table 1.

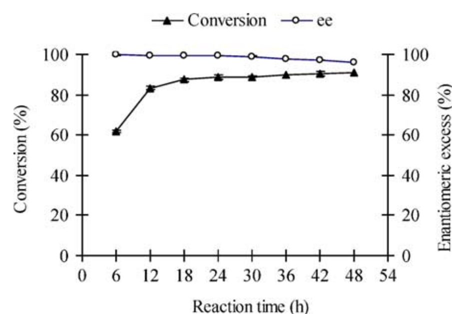


Fig. 5. Effect of reaction time on the bioreduction of 1-acetonaphthone by the resting cells of *C. viswanathii*.

### 2.4. Optimization of reaction conditions for reduction of 1-acetonaphthone by resting cells

The course of the bioreduction of 1-acetonaphthone for the production of $S(-)$-1-(1′-naphthyl) ethanol is shown in Fig. 5. The conversion of 1-acetonaphthone to $S(-)$-1-(1′-naphthyl) ethanol increased with time while the enantiomeric excess decreased as is commonly observed in such type of biotransformation. The reaction was allowed for 12 h to get an appreciable conversion of about 83% with an enantiomeric excess of >99%.

The pH optimum was determined in buffers of various pHs. As shown in Fig. 6, the maximum conversion (>90%) was achieved in phosphate buffer (0.2 M, pH 8). However, the enantiomeric excess was found to >99% always, proving that *C. viswanathii* contains prominently S-selective carbonyl reductase only. For the determination of optimum reaction temperature, the bioreduction was examined at different temperatures ranging from 20 to 45 °C. According to Fig. 7, maximum conversion took place at 25 °C and a significant decrease in conversion was observed when the temperature was increased above 30 °C. Based on these results, all further bioreduction experiments were carried out in phosphate buffer (0.2 M, pH 8) at 25 °C only.

In any enzymatic reaction, substrate concentration plays an important role, worthy of careful investigation, since it influences either sufficient expression of the enzyme activity, or may result in the substrate inhibition. The effect of different acetonaphthone concentration (1–6 g/l) on the conversion and enantioselectivity of the bioreduction reaction

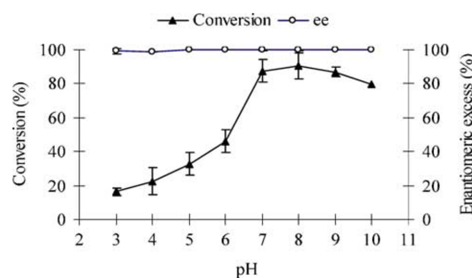


Fig. 6. Effect of reaction pH on the bioreduction of 1-acetonaphthone by the resting cells of *C. viswanathii*.

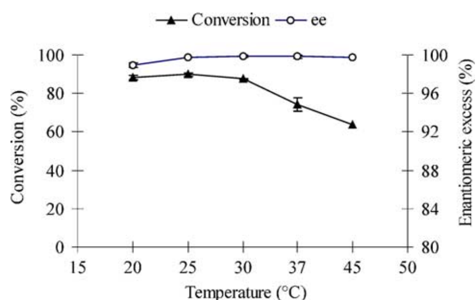


Fig. 7. Effect of reaction temperature on the bioreduction of 1-acetonaphthone by the resting cells of *C. viswanathii*.

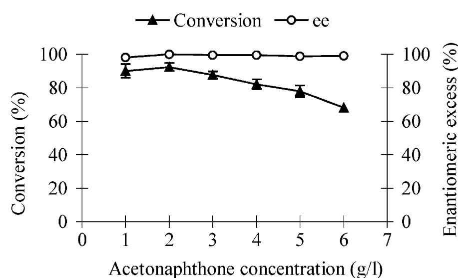


Fig. 8. Effect of substrate concentration on the bioreduction of 1-acetonaphthone by the resting cells of *C. viswanathii*.

was investigated (Fig. 8). The enantiomeric excess remained constant (>99%) at all the concentrations of substrate. However, the conversion was found to be maximum at an acetonaphthone concentration of 2 g/l, above that a decrease in conversion was observed indicating the toxic effect of acetonaphthone at higher substrate concentration. In order to scale-up the bioreduction process, determination of the resting cell concentration in the reaction medium was essential. Fig. 9 shows the effect of cell concentration on the conversion and enantiomeric excess. A high cell density (200 g/l) was found to give best results (conversion >97%, ee >99%).

Finally, preparative bioreduction was conducted with resting cells (200 g/l) in 500 ml of 0.2 M phosphate buffer (pH 8) containing 1 g 1-acetonaphthone. The reaction mixture was incubated at 25 °C for 12 h at 200 rpm. Under these conditions, *S*(−)-1-(1′-naphthyl) ethanol at a conversion of >97% and ee >99% (Chiracel OD-H) was obtained. MS (APcI): $m/z = 173$ $(M+1)^+$; $^1H$ NMR (300 MHz, $CDCl_3$): $\delta = 1.58$ (d, 3H, $J = 6.4$ Hz), 2.51 (bs, OH), 5.56 (q, 1H, $J = 6.4$), 7.47–7.37 (m, 3H), 7.59 (d, 1H, $J = 7.10$), 7.59 (d, 1H, $J = 8.16$), 7.82–7.78 (m, 1H), 8.01–7.98 (m, 1H); $^{13}C$ NMR (300 MHz, $CDCl_3$): $\delta = 24.23$, 66.78, 121.90, 123.07, 125.38, 125.41, 125.84, 127.68, 128.74, 130.12, 133.64, 141.31, IR (KBr): $\nu = 3040$, $[\alpha]_D^{20}$ –65.10 ($c = 1$, EtOH) [lit $[\alpha]_D^{20}$ –51.9 ($c = 1$, $CHCl_3$) [13]. These results demonstrate a promising prospect for the practical application of *C. viswanathii* MTCC 5158 in the production of *S*(−)-1-(1′-naphthyl) ethanol that is an important synthetic intermediate of mevinic acid analogue. Recently, we have filed a patent application for the same [15].

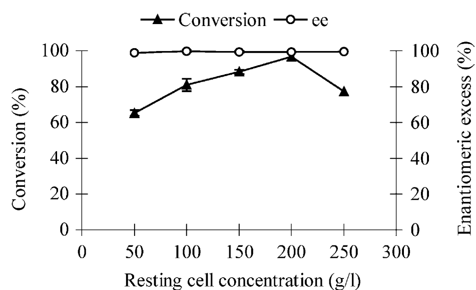


Fig. 9. Effect of resting cell concentration on the bioreduction of 1-acetonaphthone by the resting cells of *C. viswanathii*.

## 3. Discussion

In this study, enantioselective reduction of 1-acetonaphthone by the strain *C. viswanathii* MTCC 5158 was investigated. Carbonyl reductase reduces 1-acetonaphthone to *S*(−)-1-(1′-naphthyl) ethanol. This product alcohol is an important intermediate for the synthesis of mevinic acid analogue, which is a potential inhibitor of 3-hydroxy methyl glutaryl coenzyme A reductase (HMGAR). In order to increase the growth and carbonyl reductase production, the culture conditions have been optimized. Buffers of various pH ranges were used to determine the effect of pH on growth as well as enzyme activity. A marked decline in enzyme activity was found at acidic pH. Maximum activity of reductase was obtained when the pH of the medium was adjusted to alkaline region (pH 10). The optimal temperature for growth as well as enzyme production was found to be 25 °C. Acetonaphthone was found to be the best inducer at a concentration of 2 mM. Inoculum age and inoculum level for optimal production of reductase by *C. viswanathii* were found out. Enzyme production reached maximum (6.4 U/ml) when the inoculum level was kept at 3% (v/v). At lower inoculum concentration (<3%), the number of yeast cells might not be well enough to utilize essential amount of nutrients and at higher concentration, tremendous growth of yeast might have resulted in nutritional imbalance in the medium leading to relatively poor growth [16]. The present strain produced carbonyl reductase with a significant enzyme activity in the medium containing mannitol (1%, w/v) as a carbon source along with calcium (2 mM) as a metal supplement. Among the various nitrogen sources used, maximum enzyme activity was obtained with 0.5% (w/v) yeast extract, inferring that it may contain some essential factors for stimulating enzyme production [17]. As a ramification of these optimization studies, a significant increase in the enzyme activity (from 4 to 10 U/ml) was achieved. By using these optimized conditions, further biocatalytic studies have been carried out. The reaction occurred at a faster rate at pH 8 when the temperature was kept at 25 °C. Also maximum conversion was found at 2 g/l of substrate concentration with 200 g/l of resting cell concentration. Making use of these optimization studies, a

successful preparative scale reduction of 1-acetonaphthone has been carried out.

It may be concluded that the yeast strain *C. viswanathii* is a new and attractive biocatalyst, capable of carrying out the reduction of 1-acetonaphthone with remarkable stereoselectivity. This makes this specific yeast strain a highly attractive candidate for the biocatalytic preparation of chiral aryl alcohols. Further purification and characterization of the carbonyl reductase from *C. viswanathii* are in progress in our laboratory.

## 4. Materials and methods

### 4.1. Microorganism and chemicals

The microorganism used in the present work has been isolated from the soil of this institute after several days of enrichment using acetophenone as the sole source of carbon and energy. The organism has been identified by MTCC, Institute of Microbial Technology, Chandigarh, India as *Candida tropicalis* based on its morphological and physiological characters. However, accurate identification of the yeast strain using the sequence analysis of D1/D2 domain of large subunit ribosomal RNA gene (LSU rDNA) and Internal Transcribed Spacer (ITS) region of the ribosomal RNA gene cluster confirmed the identity of the yeast strain as *C. viswanathii.* The strain is deposited at MTCC with an accession number of 5158. A patent for the same has already been filed [18].

1-Acetonaphthone was obtained from Lancaster (Morecambe, England), growth media components were obtained from Hi-Media Inc. (Mumbai, India). Inorganic salts and other buffer salts were purchased from Qualigens Inc. (Mumbai, India). Solvents, mineral acids and other chemicals of analytical grade were procured from Ranbaxy fine chemicals Ltd. (Mohali, India) and S. D. fine Chemicals Ltd. (Boisar, India).

*C. viswanathii* MTCC 5158 was maintained on a nutrient agar medium (pH 7). Initially, seed culture was developed by inoculating single colony of *C. viswanathii* into 20 ml nutrient broth (pH 7) for 24 h at 30 °C in a rotary shaker (200 rpm). This inoculum (5%, v/v) was transferred to the production medium of same composition and grown for 48 h at 30 °C in a rotary shaker (200 rpm).

### 4.2. Preparation of crude extract

Cells were harvested by centrifugation at $7000 \times g$ for 20 min and washed with phosphate buffer (50 mM, pH 7) and centrifuged at $12,000 \times g$ for 10 min. The pellet was resuspended in the same buffer. From the cell suspension, 5 ml were used for disintegration using ultrasonicator. The cell debris was removed by centrifugation at $15,000 \times g$ for 60 min at 4 °C. The supernatant was used for activity measurement.

### 4.3. Optimization of culture conditions

Various physico-chemical parameters have been optimized to enhance the growth and enzyme production by *C. viswanathii*. These included initial pH, inoculum age and size, type of inducer and its concentration, production temperature and medium components. After achieving the maximum enzyme production by optimization of the various physico-chemical parameters, the resting cell suspension of *C. viswanathii* was used to reduce 1-acetonaphthone to obtain *S*(−)-1-(1′-naphthyl) ethanol. The biocatalytic reaction has been optimized in terms of reaction time, temperature, substrate and resting cell concentration.

### 4.4. Biocatalytic reaction optimization

To control the enantioselectivity of the reaction, optimization of the reaction conditions is necessary [19]. To ascertain the pH optima of the biocatalytic reaction, the reaction was carried out at different pHs using 0.2 M buffers; citrate for a pH range from 3 to 6, phosphate for a pH range from 7 to 8 and Tris–HCl for pH 9 and 10. Substrate was added to cell suspension at the different pHs and the bioreduction was monitored. To determine the optimum temperature for bioreduction, the resting cell suspensions were transferred to SPB (0.2 M, pH 8), substrate was added to this and conversion as well as enantioselectivity was measured. To find out the effect of substrate concentration on bioreduction, different substrate concentrations (1–6 g/l) were added to the resting cell suspension (166 g/l in buffer). Finally, in order to find out the cell concentration, varying concentrations of cell suspension (50–250 g/l) were subjected to 2 g/l substrate at 25 °C and monitored for conversion and enantioselectivity.

### 4.5. Analytical methods

Concentration of 1-(1′-naphthyl) ethanol was determined by HPLC using a reverse-phase column (4 mm diameter, 25 mm long, 5 μm film; Merck, Germany). The mobile phase consisted of acetonitrile and water (60:40) at a flow rate of 1 ml/min and detection was done at 233 nm. The enantiomers of 1-(1′-naphthyl) were analyzed by using a chiral column (0.46 mm diameter, 25 mm long, 5 μm film; Diacel) with hexane and isopropanol (90:10) as the mobile phase with flow rate of 0.5 ml/min and detected at 233 nm. The retention time of *S*(−)- and *R*(+)- 1-(1′-naphthyl) ethanol were 17.8 and 27.5 min in the Chiracel OD-H column, respectively. The ee was defined as the ratio of $[S] - [R]/[S] + [R] \times 100\%$, where $[S]$ and $[R]$ are the concentrations of the (*S*)- and (*R*)-enantiomers, respectively. The reductase activity was determined spectrophotometrically by measuring the decrease in the absorbance of NADH at 340 nm [20]. The reaction mixture contained 900 μl sodium phosphate buffer (SPB: 50 mM, pH 7), 50 μl cell free extract, 25 μl acetonaphthone (10 mM in dimethyl sulfoxide) and 25 μl NADH (4.5 mM). Ketone reduction was followed over a time period of 2 min. One unit

of the enzyme activity is defined as the amount of enzyme required to oxidize 1 μmol of NADH to $NAD^+$ per minute under standard reaction conditions. Cell mass was estimated by measuring optical density of the sample at 600 nm using Beckman spectrophotometer (Harbor Boulevard, U.S.A).

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