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# Optimization of culture conditions for enhanced asymmetric bioreduction of acetophenone and its derivatives by growing cells of *Pseudomonas* sp. AP1

Bhagwati Upadhyaya Joshi, Partapbir Singh, Harvinder Singh Saini *

*Department of Microbiology, Guru Nanak Dev University, Amritsar 143005, India*

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ABSTRACT

The microbial diversity of samples collected from a municipal solid waste disposal site located on outskirts of Amritsar (Punjab), India was isolated and screened for asymmetric transformation of acetophenone and its different derivatives to generate chiral synthons for commercially important drugs. A potential bacterial isolate AP1, identified as *Pseudomonas* sp., was selected for further studies as it transformed 1.0 g/L acetophenone to (*R*)-1-phenylethanol with an overall yield of 30.0% and enantiomeric excess (*ee*) $\leq$86% after 240 h. The activation of cells in the presence of acetophenone (2 mM) and optimization of nutrient supplements to minimal synthetic medium (MSM) significantly improved the transformation efficiency, as under optimized conditions cells of AP1 were able to transform 2.5 g/L acetophenone with an overall yield of 86% (*ee* $\geq$96%, *R*) in just 54 h. The overall yield of (*R*)-1-phenylethanol was dependent on available mannitol at the time of addition of acetophenone (2.5 g/L) to growing cells. The oxidation of mannitol to fructose by NADPH dependent mannitol dehydrogenase assured continuous supply of reduced cofactor to support the transformation. The cells of AP1 also transformed different halo, hydroxy and amino derivatives of acetophenone with an overall yield of 41.0–78.0% and *ee* $\geq$95%.



## 1. Introduction

Optically active compounds are important building blocks for the synthesis of enantiomeric pure pharmaceutical products. The use of active enantiomer of any drug is desirable to avoid harmful side effects of corresponding enantiomer as per guidelines issued by US Food and Drug Administration time to time (Agranat et al., 2012). The chiral building blocks required for the synthesis of various anti-depressants, anti-bronchitis and anti-asthmatic drugs are generally derived from chiral alcoholic product of acetophenone and its derivatives (Xie et al., 2006). The chiral product of chloro-acetophenone is building blocks of antidepressants such as (*S*)-fluoxetine and (*R*)-tomoxetine (Chenevert and Fortier, 1991). The optically active *R*-form of nitro and amino alcohols is useful for the synthesis of β-2 agonists such as (*R*)-albutamin, (*R*)-clenbuterol and (*R*)-sarmeterol (Shibasaki and Urata, 1999). In light of this, the researchers and pharmaceutical companies have always explored possibilities for efficient chemical and biological processes for production of desired chiral synthons.

The conventional chemical processes for synthesis of optically active compounds use environmentally hazardous components and involve tedious protocols for protection and deprotection of functional groups (Okumura et al., 1995; Sheldon, 2001). However, use of either microbial enzymes or cells as biocatalyst provides an environmentally benign "green" option. The pure enzyme based asymmetric reduction processes using efficient alcohol dehydrogenases and aldo-keto reductases purified from various microorganisms is one such approach (Moore et al., 2007; Huisman et al., 2010). However, the limitations like requirement of continuous supply of external cofactor (NADH/NADPH) or dependence on suitable enzyme based cofactor regeneration system and loss of enzyme activity over long term operation make these processes economically unfavorable (Wang et al., 2009). The use of actively growing cells is a preferred approach, as growing cells could generate reducing power using either carbon/energy source or a co-metabolite thus supporting desired level of transformation (Nakamura et al., 2003; Faber, 2004; Goldberg et al., 2007). However, the presence of multiple enzymes with opposite enantioselectivity might lead to low enantiomeric excess (*ee*) of the product. This could be overcome by selecting microbial isolates having higher activity of oxidoreductases, thus supporting desired levels of transformation of substrate used (Yoon et al., 2010; Araújo et al., 2011). For this, extensive screening of microbial

* Corresponding author. Tel.: +91 9463186886; fax: +91 18322258820.
*E-mail address:* sainihs@gmail.com (H.S. Saini).

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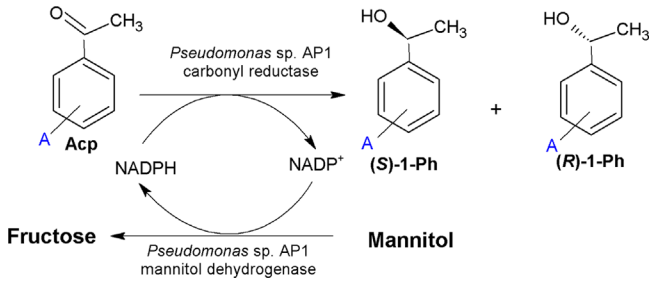


**Scheme 1.** Schematic diagram of co-factor regeneration by whole cells of *Pseudomonas* sp. AP1 during biotransformation process. Acp, acetophenone; (*S*)-1-Ph, (*S*)-1-phenylethanol; (*R*)-1-Ph, (*R*)-1-phenylethanol; *A*=*o*-F,OH; *m*-Br; *p*-F,Cl,I,$NH_2$.

diversity, especially from unexplored organic rich sites, is an effective way to obtain novel and efficient isolates showing specificity for substrate of interest and having ability to sustain supply of reduced cofactor for these enzymes. Further, optimization of nutrient supplement and growth conditions could help in achieving desired level of transformation which could make process economically viable and competitive with other processes for "*gram*" scale production of chiral synthons (Braun et al., 2012).

The present findings are related to screening of unexplored bacterial diversity of municipal solid waste dumping site located on outskirts of Amritsar (Punjab), India. An indigenous bacterial strain *Pseudomonas* sp. AP1 isolated from this site having potential to transform acetophenone was put through extensive optimization protocol which leads to significant increase in overall yield/*ee* in shorter incubation time. The cells grown in medium supplemented with mannitol supported higher transformation, as mannitol dehydrogenase mediated oxidation of mannitol ensured supply of $NADPH+H^+$ for asymmetric transformation of substrate. The versatility of the isolate is evident as it afforded efficient reduction of various derivatives of acetophenone.

## 2. Materials and methods

### 2.1. Chemicals

Analytical grade acetophenone (99% pure) was procured from S. D. Fine Chemicals Ltd., Mumbai, India. The derivatives of acetophenone viz., *p*-floroacetophenone, *p*-chloroacetophenone, *o*-floroacetophenone, *m*-bromoacetophenone, *o*-hydroxyacetophenone, *p*-nitroacetophenone and *p*-iodoacetophenone were procured from Hi Media Inc. Mumbai, India. The reduced products of acetophenone and its derivatives were chemically synthesized by using $NaBH_4$ as reducing agent. All the media components, chemicals and solvents used were of analytical/HPLC grade.

### 2.2. Media

Minimal synthetic medium (MSM, pH 7.5) as described by Khehra et al. (2006) was used for isolation and screening of potential bacterial isolates. Luria Bertani (LB) medium was used for activation and maintenance of *Pseudomonas* sp. AP1, unless specified otherwise.

### 2.3. Enrichment, isolation and screening for acetophenone transforming bacterial isolates

The microbial diversity of soil samples collected from municipal solid waste disposal site (Naraingarh, Amritsar, Punjab, India) was enriched in MSM (50 ml in 250 ml flask) supplemented with 1.0 g/L of acetophenone. The flasks were incubated at 100 rpm in an orbital shaker (Orbitek, Scigenics, India) at 30 °C. After incubation of seven days, 10% (v/v) inoculum was transferred to fresh flask containing 50 ml MSM supplemented with 1.0 g/L acetophenone and the procedure was repeated over a period of 21 days. The enriched microbial population was serially diluted and spread plated on MSM agar plates supplemented with 1.0 g/L acetophenone. The plates were incubated at 30 °C for 24 h and observed for appearance of colonies after every 24 h for 7 days. Morphologically distinct colonies were picked and purified by repeated streaking on LB agar medium.

The purified isolates (63) were screened for their ability to biotransform acetophenone to 1-phenylethanol as per following protocol. The single colony of respective culture, grown overnight on LB plate was inoculated to MSM supplemented with 0.5% (w/v) yeast extract and 1.0 g/L acetophenone. The flasks were incubated at 30 °C and after 240 h the contents of the flasks were extracted twice with equal volume of ethyl acetate and concentrated using rota-vapor (BUCHI R-114, Switzerland). The yield and *ee* of the product formed were determined by Gas Liquid Chromatography (GLC) as described in Section 2.6.

Isolate AP1 was selected for further studies as it afforded 30% conversion of acetophenone to (*R*)-1-phenylethanol with 86% *ee*. The morphological and biochemical characterization (oxidase test, catalase test, acid production, IMViC test and nitrate reduction test) were performed as per the Bergey's manual of systematic bacteriology (Sneath et al., 1986). The gene coding for 16s rRNA was amplified using 27F and 1492R universal primers. The amplified product (GenBank accession no. KJ513298) was compared with sequences available in GenBank using BLAST (Nogales et al., 2001).

### 2.4. Biotransformation of acetophenone by Pseudomonas sp. AP1

The following protocol was used for all the biotransformation reaction studies unless specified otherwise. The biotransformation reaction was carried in a 250 ml flask having 100 ml MSM (pH 7.5) supplemented with 0.5% (w/v) yeast extract and 0.5% (w/v) glucose. The medium was inoculated with overnight grown cells of AP1 ($OD_{540} \approx 0.5$) and incubated at 30 °C on an orbital shaker. After 8 h of incubation, 2.5 g/L acetophenone was added and flasks were further incubated at 30 °C. The content of respective flask was extracted with ethyl acetate at a regular interval of 24 h as per protocol mentioned above. The percentage conversion of acetophenone and enantiomeric excess of the product at each time interval was determined by GLC (Scheme 1).

#### 2.4.1. Effect of incubation time on biotransformation efficiency

The optimum reaction time for biotransformation was determined by withdrawing sample at regular interval of 24 h for 240 h. Based upon the observation, outlined in Section 3.2, further incubation was carried out upto 168 h, unless specified otherwise.

#### 2.4.2. Effect of induction of cells with acetophenone on biotransformation efficiency

The cells of AP1 were activated by growing overnight in MSM supplemented with different concentrations of acetophenone (2–10 mM) and their biotransformation efficiency was compared with cells grown in the absence of acetophenone. Further, the carbonyl reductase activity of cells activated in the presence or absence of acetophenone was evaluated as per method described by Vallee and Hoch (1955). The cell free extract was prepared by lysis of cells suspended in phosphate buffer (50 mM, pH 7.5) by ultrasonication (Misonix, USA) for 15 min (a pulse of 30 s followed by rest of 30 s). The suspension was centrifuged (3K30 Sigma, Germany) at 12,000 rpm for 30 min and the supernatant was used for assay. The assay reaction mixture (100 μL) contained 0.01 M