Introduction

Biological activity of a compound often depends on the absolute configuration of chiral centres in the molecules, because the receptor to which the molecule binds is made of an enantiomerically pure protein and its active site is chiral. The methods of obtaining the enantiomerically enriched compounds are classified into two broad categories: optical resolution of racemates and asymmetrization of prochiral or meso compounds. Biotransformations are widely utilized in both cases. Popularity of biotransformation route compared to chemical route has remained rather low, in spite of the economic and environmental benefits of the bio-route. Lack of the availability of stable strains or enzymes from the different enzyme classes ready for use in a screening for a new product has been a "burning issue", which has prevented widespread use of the biotransformation route in process chemistry. Although the advantages of the use of enzymes in organic synthesis have been fully illustrated, not much attention has been paid to the systematic exploitation of microorganisms producing new enzymes. This thesis describes the results of our effort at exploiting the rich microbial diversity of India for the isolation of new enzyme activities and their applications in the synthesis of enantiomerically enriched compounds. The thesis has been divided into two independent parts, Part 1 deals with hydrolytic enzymes and Part 2 with asymmetric reduction of carbonyl compounds. Each part is further divided into five sections. Section 4 and Section 5 of each part gives experimental details and bibliographic information, respectively.

Part 1, Section 1: Lipases and Their Applications in Organic Synthesis: Review of Literature

This section is a mini-review on the topic. No attempt has been made to exhaustively review the available literature, but adequate relevant information has been provided citing selected examples. Occurrence in microorganisms, mechanistic aspects and applications of lipases has been given more importance. In addition, a brief reference has been made to methods for improvement/modification of activity and also to the factors that affect activity and selectivity of lipases.

Part 1, Section 2: Characterization of a Surface-Bound Lipase from Stenotrophomonas maltophilia

This section describes the characterization of a novel surface-bound, enantioselective lipase from Stenotrophomonas maltophilia.
The presence of an enantioselective lipase on the surface of a cell is a highly desirable property from the practical point of view as it eliminates the need for expensive isolation, purification and stabilization of the protein. Moreover, the penetration of the substrate into the cell and excretion of the product into the media will not be an issue when lipase is present on the surface of the cell. Several expression systems have been developed to display lipases and other proteins on the surface of bacteria, fungi or mammalian cells by appropriately fusing them to surface anchoring motifs. The outer membrane proteins have been widely used as anchoring motifs, because they have unique membrane-spanning structures. Several membrane proteins including OmpA, OprF, OmpS, invasin, LamB, PhoE, OmpC and FadL have been used as anchoring motifs.

**Strain selection**

A three step screening procedure was followed for the screening of the microorganisms for the desired lipase activity. In the first step, purified cultures of several bacterial and fungal strains isolated from soil and water samples, collected from various niches in India were screened using tributyrin plates. To maximize the probability of finding a cell-surface displayed lipase, pure cultures of microorganisms in stationary phase were used for screening. The cultures which gave zone of clearance on the tributyrin plates were selected for second step of screening. In the second step, the selected cultures were screened for their ability to hydrolyze methyl 2-(4-isobutylphenyl)propanoate (1). In the third step, enantiomeric excess and configuration of the product, 2-(4-isobutylphenyl)propanoic acid (2), was determined by $^1$HNMR method using (1R,2R)-1,2-diphenylethane-1,2-diamine as chiral solvating agent according to a literature procedure.

![Chemical structures](image1)

**Strain identification**

The selected strain, RJ-8, which gave highest conversion and enantiomeric excess was aerobic, gram negative, non-spore forming, rod shaped bacterium. It was
identified as *Stenotrophomonas maltophilia* based on biochemical tests and full length 16S rDNA sequence.

**Isolation and purification of protein**

During preliminary investigations it was observed that the enzyme responsible for activity could be extracted from cells by incubation in buffer containing 1M NaCl. Thus, to extract protein, the cells were incubated in phosphate buffer, pH 7.0 containing 1 M sodium chloride at 37 °C for 2 h. The cell mass was removed by centrifugation at 7000 rpm. The supernatant enriched in enzyme activity was desalted and concentrated using 50 kDa centricon (Amicon, USA) ultrafiltration membrane. The concentrated protein was loaded on Sephacryl S-200 column pre-equilibrated with 50 mM tris-Cl buffer (pH 7.6) containing 150 mM NaCl. The elution was done with the same buffer at flow rate of 24 mL/h. Fractions of 1.0 mL were collected and tested for lipase activity. The enzyme activity was eluted near the void volume of the column. The protein, however, was electrophoretically pure and showed a single band at about 22 kDa on SDS-PAGE run under reducing conditions.

**Chain-length specificity of *Stenotrophomonas maltophilia* lipase**

To find the chain-length specificity of *Stenotrophomonas maltophilia* lipase, the standard lipase assay was performed using triacetin (C\(_{2:0}\)), tributyrin (C\(_{4:0}\)), trihexanoate (C\(_{6:0}\)), trioctanoate (C\(_{8:0}\)), tridecanoate (C\(_{10:0}\)), triolein (C\(_{18:0}\)) or olive oil as substrate. Maximum activity (1409 μmol min\(^{-1}\)mg\(^{-1}\) protein) was observed with olive oil, whereas pure triolein was less active and showed 62% activity compared to the maximal activity obtained with olive oil. The activity decreased with decrease in chain length of the lipid. The activity was in the range of 21-27% (compared to olive oil, 100%) for C\(_{2:0}\) to C\(_{8:0}\) lipids. Thus the lipase of *Stenotrophomonas maltophilia* was non-specific, but showed preference for longer chain lipids.

**Stenotrophomonas maltophilia** lipase is present on the surface of cells

The presence of lipase on the surface of *Stenotrophomonas maltophilia* was confirmed by EM immunogold labeling studies carried out with ultrathin sections of *Stenotrophomonas* cells. Polyclonal antibodies, AbLip raised against the purified lipase were assayed for their specificity by Western blotting. *Stenotrophomonas maltophilia* was grown to mid-exponential phase and after several dehydration steps,
embedded in LR white resin, which was then dehydrated in several steps. 0.2% glutaraldehyde was used as fixative. Thin sections cut using an ultramicrotome were incubated with AbLip followed by nanogold labelled secondary antibody and visualized under the transmission electron microscope. Different fields were observed and the gold particles were found to be exclusively present on the surface of the cells.

**Probing E. coli proteins for similarity with Stenotrophomonas maltophilia lipase**

*Stenotrophomonas maltophilia* lipase is naturally expressed on the surface of the cell. Although the study of the genetic mechanism involved in the expression of the lipase on the surface of the cell is way beyond the scope of the present study, the availability of lipase specific antibodies allowed us to probe *E. coli* proteins for possible similarities. *E. coli* proteins extracted from the cells by incubating with 1M NaCl at 37 °C for 2 h were desalted and run on SDS-PAGE, and after electroblotting on to nitrocellulose membrane were probed with AbLip. A single protein was labelled, the N-terminal sequence of which showed 100% similarity to Omp proteins of *E. coli*. This assumes significance in view of the facts that *Stenotrophomonas maltophilia* lipase is a surface bound protein and Omp have unique membrane-spanning structures. In literature, there are instances where Omp has been used as anchoring motif for non-natural expression of proteins on the surface of cells. It would be interesting to study if *Stenotrophomonas maltophilia* uses Omp as anchoring motif for display on the surface of cells.

In conclusion, we have purified a novel lipase from a bacterium, *Stenotrophomonas maltophilia*. The lipase has been purified to electrophoretic homogeneity and has been shown to be present on the surface of cells by immunogold electron microscopy. Polyclonal antibodies raised against *Stenotrophomonas maltophilia* lipase labelled a protein in *E. coli*, which was identified as outer membrane protein, Omp.

**Part 1, Section 3: Applications of Stenotrophomonas maltophilia Lipase in the Preparation of Enantiomerically Enriched Compounds**

This section describes the applications of *Stenotrophomonas maltophilia* in preparation of enantiomerically enriched compounds on preparative scale. We decided to explore biocatalysis by whole cells of *Stenotrophomonas maltophilia* for the following reasons: the lipase has been shown to be present on the surface of the
cells of *Stenotrophomonas maltophilia* (Section 2). The presence of an enantioselective lipase on the surface of a cell, as already stated is a highly desirable property from practical point of view as it eliminates the need for expensive isolation, purification and stabilization of the protein. Moreover, the penetration of the substrate into the cell and excretion of the product into the media is unlikely to be an issue when lipase is present on the surface of the cell and (ii) the disadvantages of using whole cell biocatalysis include reduction in e.e. because of the presence of other intracellular enzymes of opposite or relaxed specificity. But the lipase of *Stenotrophomonas maltophilia* has been shown to be present on the surface of the cells, we, therefore, envisaged that intracellular esterases, even if present, are unlikely to compete with the surface bound enzyme.

Traditional substrates, 2-methyl and 2-hydroxy substituted aromatic esters were selected for testing the substrate specificity of the enzyme. In addition, aliphatic ester, ethyl 2-chloropropanoate was also tested.

*Stenotrophomonas maltophilia* catalyzed kinetic resolution of 2-arylpropanoates: formation of enantiomerically pure (S)-acids

We started our investigation by studying the biocatalyzed hydrolysis of methyl 2-(4-isobutylphenyl)propanoate (ibuprofen methyl ester; 1). *Stenotrophomonas maltophilia* was grown in medium comprising of peptone (1.0%) and beef extract (0.5%) at 30 °C for 18-22 h. Cells were harvested by centrifugation and washed with phosphate buffer (50 mM, pH 7.0). The washed cells were resuspended in 100 mL of same buffer at a concentration of 0.1 g/mL. To the cell suspension, racemic methyl 2-(4-isobutylphenyl)propanoate (1) at 20 mM concentration was added and the contents incubated at 37 °C in an orbital shaker at 200 rpm. The reaction was stopped at 48% conversion (28 h; Scheme 1). Usual workup produced acid 2, in 45% yield. E.e. was determined by 1HNMR method using (1R,2R)-1,2-diphenylethane-1,2-diamine as chiral solvating agent. It is known in the literature, that in the presence of chiral solvating agent the doublet for the –CH3 protons at C-2 of (S)-enantiomer resonates consistently at lower frequency than the doublet of the corresponding (R)-enantiomer of 2-(4-isobutylphenyl)propanoic acid. Accordingly, the absolute configuration of the biocatalyzed product was assigned as (S). This was further confirmed by the +ve sign of optical rotation of the sample. The results are shown in Figure 1. Similar results were obtained during resolution of ethyl 2-(3-fluorobiphenyl-4-yl)propanoate (3) and
methyl 2-(3-benzoylphenyl)propanoate (5). The results are summarized in Schemes 2 and 3 and Figure 1. In both cases, (S)-enantiomer was obtained in >99% e.e.

Scheme 1

![Scheme 1 Diagram]

Scheme 2

![Scheme 2 Diagram]

Scheme 3

![Scheme 3 Diagram]

Figure 1: $^1$H NMR of (a) (R,S)-2-(4-isobutylphenyl)propanoic acid (2) (b) (S)-2-(4-isobutylphenyl)propanoic acid (2) obtained from biocatalyzed reaction, (c) (R,S)-2-(3-fluorobiphenyl-4-yl)propanoic acid (4) (d) (S)-2-(3-fluorobiphenyl-4-yl)propanoic acid (4) obtained from biocatalyzed reaction (e) (R,S)-2-(3-benzoylphenyl)propanoic acid (6) (f) (S)-2-(3-benzoylphenyl)propanoic acid (6) obtained from biocatalyzed reaction, in the presence of 1.5 equivalent of (1R,2R)-1,2-diphenylethane-1,2-diamine. Catalyst: untreated Stenotrophomonas maltophilia.
Stenotrophomonas maltophilia catalyzed kinetic resolution of 2-arylpropanoates: formation of enantiomerically pure (R)-acids - Kinetic and Kinetic-Dynamic Resolution

In the preceding section, we have described enantioselective formation of (S)-acids from the esters of 2-arylpropanoic acids. In this section, we describe the formation of corresponding (R)-acids using the cells of same organism, but after treatment with acetone. 10 g cells were suspended in 100 mL acetone and incubated at 30 °C for 30 min. Acetone was removed by filtration. Acetone treatment was repeated 4 times. Finally, cells were dried under vacuum, lyophilized and stored at 4 °C.

Resolution of 2-arylpropanoates 1, 3 and 5 was studied. In all cases, (R)-acid was produced in >99% e.e., when the reaction was stopped at 48% conversion; thus resulting in efficient kinetic resolution of esters. However, we were pleasantly surprised to note that even at 100% conversion, 2-arylpropanoates 1, 3 and 5 produced (R)-acids in >99% e.e. in 80-90% yield; thus resulting in efficient kinetic-dynamic resolution of 2-arylpropanoates. The results are summarized in Scheme 4 and Figure 2.

Scheme 4

**Figure 2:** $^1$H NMR of (a) (R,S)-2-(4-isobutylphenyl)propanoic acid (2), (b) (R)-2-(4-isobutylphenyl)propanoic acid (2) obtained from biocatalyzed reaction, (c) (R,S)-2-(3-fluorobiphenyl-4-yl)propanoic acid (4), (d) (R)-2-(3-fluorobiphenyl-4-yl)propanoic acid (4) obtained from biocatalyzed reaction, (e) (R,S)-2-(3-benzoylphenyl)propanoic acid (6) and (f) (R)-2-(3-benzoylphenyl)propanoic acid (6) obtained from biocatalyzed reaction, in the presence of 1.5 equivalent of (1R,2R)-1,2-diphenylethane-1,2-diamine. Catalyst: acetone treated *Stenotrophomonas maltophilia*. 

1. $R_1 = CH_3$; $R_2 = 4$-Isobutyl
2. $R_1 = CH_3CH_3$; $R_2 = 3$-Fluoro-4-phenyl
3. $R_1 = CH_3$; $R_2 = 3$-Benzoyl
Explanation of results

(i) Either enantiomer, one strain

Two possibilities exist: (i) the change in enantioselectivity is due to alteration of enantioselectivity of lipase on treatment with acetone and (ii) more than one enzyme with opposite selectivity are present but with greatly different rates of hydrolysis. Acetone treatment inactivates enzyme for major activity, but has very little or no effect on the enzyme for minor activity. But none of these appears to be operative in our case for following reasons (i) the lipase has been purified as described in Section 2. The pure lipase behaved in a manner similar to acetone untreated cells of *Stenotrophomonas maltophilia* and exhibited enantioselectivity for (9-enantiomer. Treatment of pure enzyme with acetone did not result in loss of activity or change in selectivity and (ii) the rate of reaction after treatment of *Stenotrophomonas maltophilia* cells with acetone remained almost similar, only the enantioselectivity was reversed.

The most likely explanation for the result is as follows. In Section 2, the presence of a 22 kDa lipase on the surface of the cells of *Stenotrophomonas maltophilia* was established by immunogold labelling studies, carried out with ultra thin sections of *Stenotrophomonas maltophilia*. We have also confirmed that the pure enzyme is enantioselective for the (S)-enantiomer of all the three esters of 2-arylpropanoic acids tested. Repeated treatment with acetone removes the surface bound (S)-selective lipase from the cells. The presence of 22 kDa lipase in acetone washing was confirmed by SDS-PAGE and activity assay. This observation is consistent with the fact that the lipase can be extracted from the cells with 1M NaCl. In addition, the acetone treatment increases the penetration of substrates into the cells by increasing the permeability of the outer membrane. This allows the substrate to experience the activity of other intracellular enzymes. The formation of (R)-enantiomer of 2-arylpropanonic acid, in all probability is due to the presence of an (R)-selective esterase in the cells of *Stenotrophomonas maltophilia*.

(ii) Dynamic-Kinetic Resolution

Kinetic resolution of racemates can proceed to a maximum of 50% conversion. In case of acetone treated cells high e.e. of acids was obtained at 100% conversion. The yield of isolated product was 80-90%, which rules out the selective degradation of one enantiomer. It is known that in rat liver (S)-ibuprofen undergoes
inversion to produce (R)-ibuprofen, but acetone treated cells of *Stenotrophomonas maltophilia* failed to deracemize ibuprofen even after 20 days, suggesting absence of this mechanism. Dynamic-kinetic resolution mechanism as shown in Scheme 5 is therefore proposed to explain the results.

Scheme 5

![Scheme 5](image)

*Stenotrophomonas maltophilia* catalyzed enantioselective resolution of 2-hydroxyarylacetates (mandelates): formation of enantiomerically pure (R)-2-hydroxyarylacetic acids (mandelic acids)

*Stenotrophomonas maltophilia* catalyzed hydrolysis of a series of ethyl mandelate derivatives was studied using intact cells and acetone treated cells (Table 1). In all the cases studied, hydrolysis of mandelates resulted in the formation of (R)-acids, except in the case of ethyl 4-bromomandelate (Entry 6, Table 1). This is in contrast to 2-arylpropanoates, where intact cells of *Stenotrophomonas maltophilia* gave (S)-acids and acetone treated cells gave (R)-acids. Two possibilities exist, (i) mandelates are not the substrates for surface bound lipase; all the activity for mandelates is because of intracellular esterase and (ii) surface bound lipase has opposite selectivity for mandelates. The pure lipase did not show any activity for ethyl mandelate indicating that the activity is due to intracellular esterase.

The e.e. of the product formed in most cases ranged between 60-70%, except in the case of mandelic acid, which was obtained in more than 99% e.e. The lower e.e. may be due to (i) relaxed selectivity of the enzyme or (ii) presence of a competing (S)-selective enzyme or (iii) partial conversion of (R)-mandelic acid to (S)-mandelic acid by the activity of deracemase. At least a part of (S)-enantiomer is formed by deracemase as shown below. Other two possibilities appear less likely as Entry 2 and 3 (Table 1) clearly show that (R)-mandelate is the preferred substrate; the hydrolysis of (S)-ester did not proceed to an appreciable level after 24 h.
Table 1: *Stenotrophomonas maltophilia* catalyzed hydrolysis of a series of 2-hydroxyaryl acetates using untreated and acetone treated cells

<table>
<thead>
<tr>
<th>Entry</th>
<th>Substrate</th>
<th>Time (h)</th>
<th>Untreated cells</th>
<th>Acetone washed cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>%conversion</td>
<td>e.e.</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>24</td>
<td>18</td>
<td>72</td>
</tr>
<tr>
<td></td>
<td></td>
<td>48</td>
<td>40</td>
<td>88</td>
</tr>
<tr>
<td></td>
<td></td>
<td>72</td>
<td>100</td>
<td>&gt;99</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>24</td>
<td>40</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>48</td>
<td>70</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>72</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>24</td>
<td>n.d.</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>48</td>
<td>n.d.</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>72</td>
<td>degradation</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>24</td>
<td>21</td>
<td>77</td>
</tr>
<tr>
<td></td>
<td></td>
<td>48</td>
<td>88</td>
<td>76</td>
</tr>
<tr>
<td></td>
<td></td>
<td>72</td>
<td>100</td>
<td>80</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>24</td>
<td>35</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>48</td>
<td>50</td>
<td>54</td>
</tr>
<tr>
<td></td>
<td></td>
<td>72</td>
<td>70</td>
<td>60</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>24</td>
<td>45</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td></td>
<td>48</td>
<td>100</td>
<td>85(S)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>72</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>24</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>48</td>
<td>28</td>
<td>82</td>
</tr>
<tr>
<td></td>
<td></td>
<td>72</td>
<td>35</td>
<td>72</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td>24</td>
<td>24</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>48</td>
<td>75</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>72</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

* unless stated otherwise, configuration of the acid produced is R.

The e.e. of the mandelic acid formed at 24 h (18% conversion) was 72% but increased to >99% after 72 h (100% conversion) (Entry1, Table 1). Thus, e.e. of the product increased with increase in conversion. This is possible if either (i) the (S)-acid formed is deracemized to (R)-acid or (ii) (S)-acid is selectively degraded. When (R,S)-mandelic acids were incubated with cells of *Stenotrophomonas maltophilia*, a slow degradation to benzoic acid and keto acid could be seen, when the crude product of the reaction was analyzed by NMR. However, (R)-acid showed no degradation product even after 100 h of incubation. This observation suggests that the increase in e.e. may have occurred due to selective oxidation and degradation of (S)-acid.
The presence of chloro group at ortho, meta or para position of aromatic ring of ethyl mandelate does not appear to have any significant effect on the rate of the reaction but a decreased enantioselectivity was observed. The substrate with 4-methoxy, however, resulted in greatly reduced rate of reaction (Entry 7, Table 1). Substitution by a bromo group at para position showed very interesting behaviour. Not only the rate of reaction was enhanced, the configuration of the product obtained at 100% conversion was (S) (Entry 6, Table 1).

The reaction with ethyl mandelate proceeded to 100% conversion, but the e.e. of product remained very high. This is possible if either (S)-ester or (S)-acid is selectively transformed to corresponding (R)-enantiomer. As described below deracemization of racemic acid occurs, but very slowly compared to rate of ester hydrolysis and produces (S) enriched acid. Therefore, deracemization must be occurring at the stage of ester. This was confirmed by HPLC analysis of the reaction on chiral column (Diacel chiraacel OB-H). As the reaction proceeded the (R)-ester started depleting till whole of it disappears, leaving behind only the (S)-ester. As the reaction is continued further, conversion of (S)-ester to (R)-ester could be clearly seen. The presence of a mandelate dehydrogenase in Stenotrophomonas maltophilia cells was demonstrated by peptide mass fingerprinting studies. A mechanism for deracemization of mandelates has been proposed based on these observations.

**Stenotrophomonas maltophilia** catalyzed deracemization of 2-hydroxyarylacetic acids

Racemic mandelic acid (7, R=H), 4-chloromandelic acid (7, R=Cl) and 4-methoxymandelic acid (7, R=OCH₃) were subjected to reaction with the acetone treated cells of Stenotrophomonas maltophilia. In all the cases, a conversion of (R)-enantiomer to (S)-enantiomer occurred (Scheme 6), but very slowly. In addition to (R)-acid, ¹HNMR showed the presence of corresponding keto acid and benzoic acid in all cases.

**Scheme 6**

![Scheme 6](https://example.com/scheme6.png)

7, R = H, Cl, OCH₃

(S)-8, e.e. 60-65%
*Stenotrophomonas maltophilia* catalyzed hydrolysis of 2-aryloxypropanoates and ethyl 2-chloropropanoate

The resolution of 2-aryloxypropanoates, (9) and (10) and ethyl 2-chloropropanoate (11) was also studied with intact cells as well as acetone washed cells of *Stenotrophomonas maltophilia*. Hydrolysis reaction occurred very fast but the e.e. obtained was poor in these cases.

![Chemical structures](image)

In conclusion, we have demonstrated the preparation of either (R) or (S)-enantiomer in >99% e.e. of 2-arylpropanoic acids by kinetic resolution of their esters using the same strain of microorganism, but used under different set of conditions. Detailed studies revealed that the result is due to presence of two different enzymes within the organism. We have also demonstrated Dynamic Kinetic Resolution of 2-arylpropanoates to (R)-acids in >99% e.e. Mandelates were also resolved by dynamic kinetic resolution process, but the e.e. of the acids produced was in the range of 62-99%. 2-Aryloxypropanoates and ethyl 2-chloropropanoate were hydrolyzed, but without any selectivity for enantiomers. In addition, we have demonstrated the deracemization of mandelic acids, in which biocatalyst converted racemic mandelic acids to (S)-mandelic acids in 60-65% e.e. Overall, the results achieve significance due to the fact that by using appropriate reaction conditions, we have been able to harness multiple enzyme activities of one organism for the selective production of either enantiomer of arylpropanoates and mandelates.

**Part 2, Section 1: Biocatalyzed Asymmetric Reduction of Carbonyl Compounds:**

**Literature Overview**

This section is a minireview on biocatalyzed asymmetric reduction of carbonyl compounds. The focus is on the applications of dehydrogenases in the enantioselective preparation of chiral alcohols using selected examples from literature. Methods for cofactor regeneration have been discussed, but very briefly.
Part 2, Section 2: Biological and Chemical Reduction of Hydantoin Derivatives

The objective of the work was to study the biological and chemical reduction of the carbonyl group of hydantoin derivatives. Hydantoin derivatives are important industrial intermediates especially for the preparation of natural and non-natural amino acids. Synthesis of hydantoin derivatives is well established. Conversion of hydantoins to corresponding carbamyl derivative or α-amino acids by enzymatic and chemo-enzymatic methods has been well documented. But, till date, no report has appeared in literature that documents the study of enzymatic reduction of carbonyl function of hydantoin derivatives. We envisaged that the reduction of carbonyl group of a suitably substituted hydantoin could lead to a quick entry into optically active cyclic, heterocyclic and alicyclic 1,2-diamines as described in Scheme 7.

Scheme 7

\[
\begin{array}{c}
\text{Reduction} \\
\text{12} \\
\text{Steps} \\
\text{13} \\
\text{14} \\
\text{15}
\end{array}
\]

\( R_1, R_2 = \text{protecting groups, } X = \text{CH}_2, n = 1 \text{ or } 2, Y = \text{CH}_2 \text{ or heteroatom} \)

Sodium borohydride reduction of 1,3-dibenzyl-5-methylhydantoin (16)

Initially sodium borohydride reduction of dibenzyl derivative, 16 was studied (i) to understand the behaviour of carbonyl group at position 4 and (ii) to obtain standard samples of alcohols, if reduction occurs. Moreover, the behaviour of 4-carbonyl group in borohydride reduction would have implications for the enzymatic reduction since borohydride and NAD(P)H reductions exhibit several similarities.

The reduction of 1,3-dibenzyl-5-methylhydantoin (16) was done with 0.5 eq. of sodium borohydride in ethanol at 0 °C (Scheme 8). Progress of the reaction was monitored by TLC and the the products were identified by \(^1\)HNMR spectroscopy. The chemical reduction resulted in the formation of two diastereomeric alcohols, 17 and 18, in approximately 1:1 ratio, structures of which were confirmed by \(^1\)HNMR of the crude product. Methyl protons at C-5 for the two diastereomers appeared as doublet at \(\delta 1.26 (J=6.6 \text{ Hz})\) and \(\delta 1.02 (J=6.6 \text{ Hz})\). Doublet of quartet for C-5 proton for the two diastereomers resonated at \(\delta 3.25 (J=6.6 \text{ and } 7.1 \text{ Hz})\) and \(\delta 3.18 (J=6.6 \text{ and } 8.0 \text{ Hz})\).
The doublet for the single proton at C-4 was observed at δ 4.28 (J=6.9 Hz) and δ 4.27 (J=7.5 Hz). The –OH protons appeared as broad singlet at δ 3.27 and δ 1.72. The doublets for the methylene protons at N-1 for the diastereomers appeared at δ 4.13 (J=6.9 Hz) and δ 4.62 (J=6.9 Hz) and δ 4.10 (J=7.1 Hz) and δ 4.60 (J=7.1 Hz) and the doublets for the methylene protons at N-3 were observed at δ 4.7 (J=6.9 Hz) and δ 4.87 (J=6.9 Hz) and δ 4.4 (J=7.2 Hz) and δ 4.54 (J=7.2 Hz). The aromatic protons were observed as multiplet at δ 7.0-7.35. The crude product also showed the presence of trace amount of starting material 16.

The diastereomeric alcohols were very labile and prone to undergo dehydration over silica-gel. Doping the silica-gel with triethylamine did not prevent dehydration. The compound was unstable even at 4 °C and dehydrated within 48 h of storage, presumably due to the presence of trace amounts of acetic acid left after ethyl acetate evaporation (Scheme 9).

Scheme 8

An interesting observation made during the course of this work was that when a solution of sample in CDCl₃ was scanned for ¹HNMR after standing for about two h, one of the diastereomers (slow moving compound on TLC) almost completely disappeared in favour of the olefin 19, leaving behind the second diastereomer (faster moving compound on TLC). Based on this observation, the faster moving compound on TLC was tentatively assigned cis-structure, 18. Its ¹HNMR spectral data (abstracted from the crude mixture, which contained starting material, olefin and the alcohol) is as given. Doublet for the –CH₃ appeared at δ 1.26 (J=6.6 Hz) and the doublet of quartet for C-5 proton was resonated at δ 3.25 (J=6.6 Hz and 7.1 Hz). The doublet for the proton at C-4 was observed at δ 4.28 (J=6.9 Hz). The –OH group at C-4 appeared at δ 3.27 as a broad singlet. The doublets for the methylene protons at N-1 appeared at δ 4.13 (J=6.9 Hz) and δ 4.62 (J=6.9 Hz) and the doublets for the methylene protons at N-3 were observed at δ 4.7 (J=6.9 Hz) and δ 4.87 (J=6.9 Hz). The aromatic protons appeared as multiplet at δ 7.21-7.35. Manual subtraction of the
peaks of tentatively assigned *cis*-alcohol from the mixture of diastereomeric alcohols produced the spectral data for the *trans*-alcohol, 17.

Scheme 9

Enzymatic reduction of 1,3-dibenzyl-5-methylhydantoin (16)

(i) Strain selection

Purified cultures of more than 300 bacterial and 200 fungal strains isolated from different soil samples were screened for their ability to reduce the carbonyl functionality of hydantoins and their benzyl derivatives. Progress of the reaction was monitored by TLC using samples of alcohols 17, 18 and olefin 19 obtained by the borohydride reduction of 16 as standards. Two strains, one bacterium and one fungal, were able to reduce the carbonyl of the substrate 16.

(ii) Strain identification

The fungal strain was a known strain, previously isolated in our laboratory for the enantioselective reduction of ethyl 4-chloro-3-oxobutanoate. The strain has been previously identified as *Penicillium funiculosum*. The bacterial strain, designated as B4W is a new isolate from the soil samples collected from Lothal, Gujrat. The strain has been identified as *Bacillus pumilis* based on morphological, biochemical characterization and full 16s rDNA sequence.

*Bacillus pumilis* catalyzed reduction of 1,3-dibenzyl-5-methylhydantoin (16)

The *Bacillus pumilis* catalyzed reduction of 1,3-dibenzyl-5-methylhydantoin (16) resulted in the exclusive formation of one diastereomer of 1,3-dibenzyl-4-hydroxy-5-methylhydantoin. TLC and $^1$H NMR spectral data of the product corresponded with the slower moving diastereomer obtained in sodium borohydride reduction and was therefore assigned *trans*-structure 17 (Scheme 10).
Sodium borohydride and *Bacillus pumilis* catalyzed reduction of 3-benzyl-5-methylhydantoin (20)

Next we investigated the reduction of 3-benzyl-5-methylhydantoin (20) with sodium borohydride. Desired alcohols could not be obtained even after many attempts, instead, complete reduction of the carbonyl functionality at C-4 occurred to form 3-benzyl-5-methylimidazolidine-2-one (21; Scheme 11). To our surprise, *Bacillus pumilis* catalyzed reduction of monobenzyl derivative, 20 also resulted in complete reduction of carbonyl group to yield 3-benzyl-5-methyl imidazolidine-2-one (21). Structure of the 21 was confirmed by $^1$HNMR spectroscopy in which doublet for the $-CH_3$ appeared at $\delta$ 1.01 ($J=6.6$ Hz) and the methine proton was seen as a multiplet at $\delta$ 3.75. The two methylene protons at C-4 appeared as doublet of a doublet at $\delta$ 3.45 ($J=10.8$ Hz, 3.9 Hz) and $\delta$ 3.33 ($J=10.8$ Hz, 6.9 Hz). The methylene protons of the benzyl group were observed as a singlet at $\delta$ 4.63. The aromatic protons appeared as multiplet at $\delta$ 7.18-7.28: The $-NH$ proton appeared as a broad singlet at $\delta$ 5.3.

Scheme 11

Sodium borohydride and *Bacillus pumilis* catalyzed reduction of methyl 2-{(1,3-dibenzyl-2,5-dioxoimidazolidin-4-yl)methylthio}acetate (22)

Substrate, 22 was selected as the next example because its reduction followed by cyclization would lead to the synthesis of an advanced intermediate of biotin. Borohydride reduction of 22 occurred with complete consumption of starting material. The products after flash chromatography over silica-gel were identified by
\textsuperscript{1}HNMR spectroscopy as racemic alcohols, 17/18 and olefin 19 (Scheme 12). The products were same as those obtained in borohydride reduction of 16. The proposed mechanism has been described in the thesis.

**Scheme 12**

Biocatalyzed reduction of 22 with \textit{Bacillus pumilis} resulted in the formation of alcohol 17 and olefin 19 (Scheme 13). The products were same as those obtained from the biocatalyzed reduction of 1,3-dibenzyl-5-methylhydantoin (16).

**Scheme 13**

Sodium borohydride and \textit{Bacillus pumilis} catalyzed reduction of 1,3-dibenzyl-5-phenylhydantoin

1,3-dibenzyl-5-phenylhydantoin failed to undergo reduction under both the conditions. A close analysis of the \textsuperscript{1}HNMR of 1,3-dibenzyl-5-phenylhydantoin revealed that this compound primarily exists in the enol form (23). The methylene protons of the benzyl group appeared as two singlets at $\delta$ 3.06 and $\delta$ 3.44. The singlet for C-5 proton could not be observed at its predicted place; instead a singlet corresponding to enol appeared at $\delta$ 8.24. The aromatic protons were observed between $\delta$ 7.17-7.59 as multiplet. The resistance of 1,3-dibenzyl-5-phenylhydantoin to undergo reduction with sodium borohydride or biocatalyst may thus be attributed to the existence of the compound in the enol form.
In conclusion, we have shown that borohydride and biocatalyzed reduction of hydantoin derivatives follows exactly the same path, only difference being that whereas biocatalyzed reduction is highly stereoselective and results in the production of only one diastereomer, the borohydride reduction is non stereoselective and produces a diastereomeric mixture of alcohols. Interesting results were obtained in borohydride or biocatalyzed reduction of 3-benzyl-5-methylhydantoin (20). Under both the conditions, carbonyl was completely reduced to methylene. 1,3-dibenzyl-5-phenylhydantoin failed to undergo reduction with borohydride or biocatalyst, presumably due to the occurrence of this compound in predominantly enol form.

Part 2, Section 3: Screening of Microbial Diversity for Preparation of Ethyl (S)-4-chloro-3-hydroxybutanoate (24), A High Value Intermediate for Statin Drugs

Statin drugs are an important class of cholesterol-lowering drugs. About half of statin drugs come from microbial sources, but others such as atorvastatin, fluvastatin and rosuvastatin are synthesized. These drugs command a multibillion dollar global market; atorvastatin, the active ingredient of Pfizer’s Lipitor, alone has sales value of over $12 billion per year. Statin drugs have a (3R,5S)-dihydroxyhexanoate (25) side chain, which accounts for above 25% of the compounds molecular weight, that should make it worth at least $3 billion on prorated basis. The annual requirement for intermediate 25 exceeds 220 tons.

Two major strategies are being followed for the synthesis of 25, making either a shorter-chain with only one chiral center or longer chain in which both chiral centres are targeted. Reduction of ethyl 4-chloro-3-oxobutanoate is particularly interesting because of easy availability of starting material at reasonably low price. Thus, ethyl (S)-4-chloro-3-hydroxybutanoate (24) has become an important intermediate that commands a market sales value of over $1 billion per year.

A key issue in the biocatalyzed reduction of ethyl 4-chloro-3-oxobutanoate is the requirement for NADPH-recycle system, which tends to not only increase the
cost but also complicates the process development. Whole cell systems offer advantage as co-factor recycling can be avoided. But, so far, e.e. and/or productivity of known microorganisms is less than optimal. Since different microorganisms contain different enzymes, screening of microorganisms is desired to find a microorganism having optimal properties. India is endowed with a rich microbial diversity and screening of microorganisms isolated from various niches in India is being currently pursued in our laboratory under a wider C.S.I.R. programme “Exploration and Exploitation of Microbial Diversity of India”.

Screening of microorganisms for enantioselective reduction of ethyl 4-chloro-3-oxobutanoate to ethyl (S)-4-chloro-3-hydroxybutanoate (24)

A large number of bacterial and fungal strains were screened. A number of them were able to reduce both ethyl 4-chloro-3-oxobutanoate and ethyl 3-oxobutanoate with low to high conversion rate. The conversion rate for most organisms was similar for both substrates except for few which showed some preference. Enantiomeric excess of the alcohol formed from ethyl 4-chloro-3-oxobutanoate and ethyl 3-oxobutanoate was tested for all positive strains, irrespective of the level of conversion. Either none or low e.e. (10-45%) was obtained for ethyl 3-hydroxybutanoate. However, the e.e. of ethyl 4-chloro-3-hydroxybutanoate was typically higher and ranged from 48-91%. Both (R)- and (S)-selectivity was observed. Unfortunately, the maximum e.e. obtained for the desired molecule, ethyl (S)-4-chloro-3-hydroxybutanoate was only about 91%. An organism, *Penicillium funiculosum*, MTCC 5246 has already been described from our laboratory, which gave 100% conversion and >97% e.e. Therefore, further efforts were directed towards cofactor regeneration and reaction conditions optimizations using this organism, increase in productivity being the main objective.

It has been reported previously, that *Penicillium funiculosum* reduced ethyl 3-oxobutanoate (EOB) to give ethyl 3-hydroxybutanoate with high conversion rate, but with poor e.e. of <10%. In contrast, ethyl 4-chloro-3-oxobutanoate (ECOB) was reduced at lower rate but e.e. of the product (S)-4-chloro-3-hydroxybutanoate was >97%. This suggested the presence of multiple dehydrogenases in *Penicillium funiculosum* with opposite selectivity. In all likelihood, EOB is a substrate for multiple dehydrogenases with opposite selectivity, but ECOB is a substrate for a single dehydrogenase or more than one dehydrogenase with similar selectivity.
Activity staining studies were performed to confirm these possibilities. Gel from NATIVE-PAGE was washed with Tris-Cl buffer and then immersed in a solution containing phenazine methosulfate (0.05 mg/mL), nitroblue tetrazolium (0.3 mg/mL), NADP (0.5 mM), glucose (20 mM), glucose dehydrogenase (15 units) and EOB (20 mM) in Tris-Cl buffer, pH 8.0. The contents were incubated overnight when four white bands appeared on purple background. When the procedure was repeated with ECOB as substrate, only one band appeared on purple background. This confirms that (a) EOB is a substrate for 4 different dehydrogenases and (b) ECOB is a substrate for only one dehydrogenase.

In order to find a co-substrate suitable for efficient cofactor regeneration in whole cell catalysis by *Penicillium funiculosum*, presence of glutamate dehydrogenase was tested using activity staining method. Native-PAGE was run and stained as described above except that glucose-glucose dehydrogenase was replaced with 20 mM glutamic acid in the staining solution with EOB as substrate and glutamate as co-substrate. Four white bands appeared on purple background, which corresponded with the bands that appeared in the presence of glucose-glucose dehydrogenase. When ECOB was used as substrate and glutamate as co-substrate, a single white band appeared on purple background, which corresponded with the band that appeared in the presence of glucose-glucose dehydrogenase. These results clearly show the presence of a glutamate-dehydrogenase in *Penicillium funiculosum*.

*Penicillium funiculosum* catalyzed reduction of ECOB was performed in the presence of 2-equivalent of glutamic acid. Approximately 3-fold increase in the productivity occurred. We were pleased to note that a similar 2.5-fold increase in the productivity occurred when glycine was used in place of glutamic acid.

Several solvents systems, such as hexane, ethyl acetate, n-butyl acetate and n-butyl ether were tested for studying the reaction in 2-phase system. No reaction occurred in the presence of ethyl acetate and n-butyl acetate. Enhancement in the productivity occurred with hexane, but the solubility of substrate and product was very low in this solvent. n-Butyl ether gave the best results. Thus, when a solution of ECOB in n-butyl ether was slowly added to a suspension of cells in buffer, a 10-fold increase in the productivity occurred compared to monophasic aqueous system. When the reaction was performed in the presence of 2 equivalents of glycine as co-substrate, a substantial 18-fold increase in the rate and amount of consumption of ECOB occurred.
In conclusion, screening of a large number of microorganisms failed to improve e.e. of (S)-ethyl 4-chloro-3-hydroxybutanoate (24) as compared to a strain of fungi, *Penicillium funiculosum* already described from our laboratory. By rational modification of the conditions, the productivity was increased from 2.46 mmolh$^{-1}$L$^{-1}$ to 43.90 mmolh$^{-1}$L$^{-1}$. An important feature to note is that no extraneous NAD(P) or cofactor regeneration system has been added, instead, glycine was used as cosubstrate, thereby significantly reducing the cost of production of 24. This became possible with the identification of the presence of an amino acid oxidase in *Penicillium funiculosum*, which was used for recycle of cofactor, NADPH, already present within the cells.