CHAPTER 1

Introduction
Chirality has become a major theme in the design, discovery, development and marketing of new drugs [1-3]. The role of chirality in efficacy and safety of drugs has been thoroughly identified and implicated globally by pharmaceutical industries as well as concerned regulatory agencies. Thus, in order to accomplish efficacious and safe medication and also compliance with the guidelines set by the regulatory agencies, pharmaceutical industries are compelled to move towards manufacturing and marketing of chiral drugs essentially in single-enantiomer dosage forms. The worldwide sales of chiral drugs in single-enantiomer dosage forms continued growing at about 13% annual rate over past few years [3]. To respond the rising industrial demand of enantiopure drugs, search of new efficient methods of asymmetric synthesis and the strategic development of the available methods have been the center-stage of academic as well as industrial pharmaceutical research over the recent years [4].

The biocatalytic enantioselective synthesis of highly versatile chiral entities having prospective as drugs or drug intermediates is attempted in the present thesis. This chapter outlines the basic concepts of chirality, mechanism of chiral pharmacology and toxicity, various methods of enantioselective synthesis and the importance of biocatalysis in enantioselective synthesis. Further, this chapter delineates the scope of thesis and elaborates the specific research objectives.

1.1. ROLE OF CHIRALITY IN PHARMACEUTICALS

1.1.1. Basic concepts of chirality

Chirality (also sometimes called stereoisomerism or dissymmetry) is a property of an object which is non-superimposable with its mirror image. The word chiral is derived from Greek word ‘cheir’, which means ‘handedness’. When a molecule cannot be superimposed on its mirror image, this molecule and its image are called chiral. It is like a pair of hands which are otherwise appear identical but in fact are non-superimposable on each other as demonstrated in Fig. 1.1 [5]. A chiral molecule contains at least one chiral center or asymmetric center, which is a central carbon atom to which four different atoms (or group of atoms) are attached. Carbon is not the only atom that can act as an asymmetric center. Sulfur, phosphorus and nitrogen can sometimes form chiral molecules such as omeprazole, cyclophosphamide and methaqualone, respectively [6].
The origin of the chirality lies in stereoisomerism. Stereoisomers are isomers which differ from each other only by way of orientation of atoms (or group of atoms) in a three dimensional space. The classification of stereoisomers is given in Fig. 1.2.

**Fig. 1.2: Classification of stereoisomers**

Stereoisomers which are non-superimposable mirror images of each others are called as enantiomers. Compounds which are chiral may exist as enantiomers while those which are achiral (non chiral) cannot exist as enantiomers. Thus chirality is the...
necessary and sufficient condition for the existence of enantiomers [9]. The ability of chiral molecules to rotate plane polarized light is termed as optical activity. Enantiomers are therefore also sometimes referred as optical isomers. Two enantiomers of the same compound rotate plane polarize light in opposite direction. Depending on whether they rotate the plane-polarized light towards right (+) or left (-), the two enantiomers of compound may be classified as dextrorotary (d-isomer) or levorotary (l-isomer) respectively.

Enantiomers have identical physical properties (e.g. melting point, boiling point, density etc.) except for the direction of rotation of plane polarized light and identical gross chemical properties (e.g. reactivity towards achiral acids and bases) except towards chiral (i.e. enantioselective) reagents/catalysts. An equimolar mixture (50:50) of the two enantiomers of a chiral compound is called a racemic mixture (racemate) that does not exhibit optical activity. Racemic mixture is denoted with sign (+) or (dl) or with prefix rac [5]. The arrangement of atoms (or group of atoms) that characterizes a particular stereoisomer is called its configuration. The configuration is indicated by use of prefixes R and S according to the Sequence Rule proposed by R.S. Cahn, C. Ingold and V. Prelog [9]. This system is based on a set of rules for ordering the priority to the substituents attached to the asymmetric atom. If the counting from the highest priority (highest atomic number or highest mass) to the lowest one, goes in a clockwise direction, the configuration is designated as R (Latin: Rectus means right); otherwise if counting goes in a counter clockwise direction, the configuration is designated as S (Latin: Sinister means left). A racemate is designated as RS. Depending upon the direction of plane polarized light towards right (+) or left (-), each R- and S-enantiomer is designated as R(+) or R(-) and S(+) or S(-) [5].

The stereoisomers that are not mirror images of each other are called as diastereomers. Unlike enantiomers, diastereomers exhibit different physical and chemical properties. Epimers are a special category of diastereoisomers. They are a pair of stereoisomers with more than one stereogenic center that differs in chirality at one and only one chiral center.

1.1.2. Chirality and biological activity

Although chirality is not a prerequisite for the biological activity, the chirality of bioactive compounds causes distinct differences in the biological activities of the
individual enantiomers. This is a general phenomenon and applies to all other bioactive substances, such as drugs, fragrances, flavours, insecticides and herbicides etc. [4]. For instance, (i) \( d \)-Propoxyphene (marketed as Darvon) has narcotic analgesic effects while \( l \)-propoxyphene (marketed as Novrad) has antitussive properties. (ii) \( (+) \)-Ascorbic acid has antiscorbic property while its optical isomer, \( (-) \)-ascorbic acid is inactive. (iii) \( S,S \)-Aspartame has sweet taste while its \( R,R \)-isomer is bitter. (iv) \( R(-) \)-Limonene has orange-like flavour while \( S(-) \)-limonene gives lemon-like flavour. (v) \( S \)-(+) -Carvone smells like caraway while \( R \)-(+) -carvone smells like spearmint. (vi) \( d \)-Bermethrine is potent insecticide while \( l \)-bermethrine is almost inactive. Thus, despite having identical chemical composition, the enantiomers of chiral bioactive compounds surprisingly exert dissimilar biological responses [6, 8].

1.1.3. Mechanism of chiral pharmacology and toxicity

Drugs work by binding to the specific biological sites (or drug binding sites), such as proteins (receptors, enzymes), nucleic acids (DNA and RNA) and biomembranes (phospholipids and glycolipids) present in the body. The pharmacological activity of drugs depends mainly on their interaction with these biological sites. All these sites are composed of homochiral biomolecules having complex three-dimensional structures, capable of recognizing the chirality of the drug molecule [5]. Whereas, only one enantiomer of a chiral drug preferentially interacts with the binding site, the other enantiomer has a weak interaction with that receptor or interacts with some other receptor(s) in body. The enantiomer which gives a desirable therapeutic effect is termed as ‘eutomer’ while its antipode is termed as ‘distomer’ which is either less potent, inactive, or even exhibits severe side effects or drug toxicity [10]. The molecular mechanism of chiral pharmacology and toxicity is illustrated in Fig. 1.3, using a hypothetical example of a chiral drug and its chiral binding site. For the drug to have its desirable pharmacological effect, the portions of the drug labelled as A, B, and C must interact with the corresponding regions of the binding site labelled a, b, and c respectively. As shown in the Fig. 1.3, among the two enantiomers, only one enantiomer of the drug has a 3-dimensional structure that can be aligned with the binding site to allow A to interact with a, B to interact with b, and C to interact with c. In contrast, another enantiomer of the same drug cannot bind in the same manner no matter how it is rotated in space. Thus, the difference in their 3-
dimensional structures allows the eutomer while prevents the distomer to exert a desirable pharmacological effect [6].

Fig. 1.3: Mechanism of chiral pharmacology and toxicity: the eutomer has a 3-dimensional structure that allows drug domain ‘A’ to interact with binding site domain ‘a’, ‘B’ to interact with ‘b’, and ‘C’ to interact with ‘c’ and therefore exerts a desirable biological effect (an active enantiomer). In contrast, the distomer cannot be aligned to bind these three binding sites simultaneously. As a result, it fails to exert a desirable effect (inactive or less active enantiomer or in few cases distomer interacts with an unusual binding site leading to undesirable biological effects i.e. drug toxicity). [Source: Adapted from ref. 6]

In rare cases, the chiral center(s) of a drug does not play an active role in drug-receptor interaction. In these instances, the individual enantiomers may display very similar or even identical pharmacological behaviour at their target site. However, the enantiomers may differ in their metabolic profiles as well as their affinities for other receptors, transporters, or enzymes. Hence, for a given chiral drug, it is appropriate to consider the two enantiomers as two separate drugs with different properties unless otherwise proven experimentally [6]. The examples of differential enantiomer potency and differential enantiomer toxicity of chiral drugs are enlisted in Table 1.1 and Table 1.2 respectively.
Table 1.1: Differential isomer potency of enantiomers of chiral drugs

<table>
<thead>
<tr>
<th>Specific examples</th>
<th>Therapeutic action and application</th>
<th>Isomer potency</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Case 1: One enantiomer is active while another is (almost) inactive</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Albuterol, Salmeterol</td>
<td>Bronchodilators used in the treatment of asthma</td>
<td><em>l</em>-Isomer is active whereas <em>d</em>-isomer is inactive [11,12]</td>
</tr>
<tr>
<td>Hexobarbital, Secobarbital</td>
<td>Hypnotics or sedative used in psychiatric treatment</td>
<td><em>l</em>-Isomer is active whereas <em>d</em>-isomer is inactive [13,14]</td>
</tr>
<tr>
<td>Ketamine, isoflurane</td>
<td>Anesthetic</td>
<td><em>d</em>-Isomer is active whereas <em>l</em>-isomer is almost inactive [15,16]</td>
</tr>
<tr>
<td><strong>Case 2: One enantiomer is therapeutically active while another is less active</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Verapamil, Nicardipine</td>
<td>Calcium channel antagonists used for cardiovascular therapy</td>
<td><em>l</em>-Isomer is 10-20 times more active than <em>d</em>-isomer [17, 18]</td>
</tr>
<tr>
<td>Captopril, Benazepril</td>
<td>ACE inhibitors used as antihypertensive [19]</td>
<td><em>l</em>-Isomer is more potent than <em>d</em>-isomer</td>
</tr>
<tr>
<td>Methadone</td>
<td>Analgesic for treatment of opiate dependence and cancer pain</td>
<td><em>l</em>-Isomer is 25-50 times more potent than <em>d</em>-isomer [20, 21]</td>
</tr>
<tr>
<td><strong>Case 3: Both enantiomers having equal therapeutic potency</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cyclophosphamide</td>
<td>Antineoplastic</td>
<td>Both enantiomers exhibit equal therapeutic activity [22]</td>
</tr>
<tr>
<td>Flecaïnide</td>
<td>Antiarrhythmic</td>
<td>Both enantiomers exhibit equal therapeutic activity [22]</td>
</tr>
<tr>
<td>Fluoxetine</td>
<td>Antidepressant</td>
<td>Both enantiomers exhibit equal therapeutic activity [22]</td>
</tr>
<tr>
<td><strong>Case 4: Both enantiomers having different pharmacological activities</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Propranolol</td>
<td><em>d</em>-isomer reduces plasma concentrations of T3 cells and used to treat hyperthyroidism, whereas <em>l</em>-isomer is β-blocking drug used as antihypertensive</td>
<td><em>d</em>-Isomer can inhibit the conversion of thyroxin (T4) to triiodothyronin (T3) which is opposite to the <em>l</em>-isomer [23, 24]</td>
</tr>
<tr>
<td>Propoxyphene</td>
<td><em>d</em>-Propoxyphene (Darvon) is a painkiller, whereas the <em>l</em>-propoxyphene (Novrad) is a cough suppressant.</td>
<td><em>d</em>- and <em>l</em>-Isomers of propoxyphene have independent pharmacological activities [25]</td>
</tr>
</tbody>
</table>
Table 1.2: Differential isomer toxicity of enantiomers of chiral drugs

<table>
<thead>
<tr>
<th>Specific examples</th>
<th>Therapeutic application of the eutomer</th>
<th>Isomer toxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Case 1: Eutomer is non-toxic while distomer shows toxic effect(s)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>l-DOPA</td>
<td>Treatment of parkinsonism</td>
<td>d-Isomer gives agranulocytosis (or grave toxicity) [26, 27]</td>
</tr>
<tr>
<td>S,S-Ethambutol</td>
<td>Treatment of tuberculosis</td>
<td>R,R-Isomer causes optical neuritis that can lead to blindness [5]</td>
</tr>
<tr>
<td><strong>Case 2: Eutomer is less toxic than distomer</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>l-Tetramisole (or Levamisole)</td>
<td>Nematocide</td>
<td>Toxicity (i.e. vertigo, vomiting, headache etc.) of l-isomer is less than d-isomer [27]</td>
</tr>
<tr>
<td>l-Bupivacaine</td>
<td>Local anaesthetic</td>
<td>l-Bupivacaine is less toxic than d-isomer [8]</td>
</tr>
<tr>
<td><strong>Case 3: Eutomer is more toxic than distomer</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>l-Secobarbital</td>
<td>Anaesthetic</td>
<td>l-Isomer is a more potent and also more toxic than d-isomer [28]</td>
</tr>
<tr>
<td><strong>Case 4: Eutomer and disomer are equally toxic</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cyclophosphamide</td>
<td>Antineoplastic</td>
<td>Both enantiomers exhibit same degree of toxicity [22]</td>
</tr>
</tbody>
</table>

1.1.4. Effect of chiral inversion

Some chiral drugs undergo *in vivo* and/or *in vitro* interconversion of enantiomers called as chiral inversion. This phenomenon greatly affects the differential potency and toxicity of such chiral drugs. Chiral inversion is categorized into two types: unidirectional inversion and bidirectional inversion [5].

2-Arylpropionic acids (ibuprofen, ketoprofen, fenprofen, benoxaprophen, etc. and collectively called as profens) are non-steroidal anti-inflammatory agents which undergo unidirectional *in vivo* chiral inversion. The S-enantiomer is therapeutically active (eutomer) while the R-enantiomer is weakly active or inactive (distomer). For example, (S)-ibuprofen is over 100-fold more potent as an inhibitor of cyclooxygenase I than (R)-ibuprofen. However, in the human body, only inactive R-enantiomer undergoes chiral inversion by hepatic enzymes into the active S-enantiomer and not vice-versa. This unique inversion process enhances the...
CHAPTER 1: Introduction

Effectiveness of racemic profen drugs. However, recent findings have revealed the undesirable biological effects of the $R$-enantiomer and thus emphasized the use of $S$-profens for a safe and effective treatment [26].

3-Hydroxy-benzodiazepines (oxazepam, lorazepam, temazepam) and thalidomide undergo bidirectional chiral inversion whereby the $R$ and $S$ enantiomers undergo racemization. 3-Hydroxy-benzodiazepines can racemize in vitro by aqueous solutions at elevated temperatures and thalidomide (a former sedative withdrawn from the market in the 1960s due to severe teratogenic effects) exhibits in vitro as well as in vivo bidirectional chiral inversion [5].

1.1.5. Potential advantages of using single enantiomer of drug [29]

- Improved therapeutic index – low (quantity and frequency of) dosage required
- Less complex pharmacokinetic profile
- Less complex pharmacodynamic profile
- Less complex relationship between plasma concentration and therapeutic effect
- Nil or less side effects/ drug toxicity
- Reduced potential for complex drug interactions

1.1.6. Emergence of chiral drugs

In view of heightened awareness regarding the differential biological and therapeutic behaviour of two enantiomers and the severe side effects associated with the non-functional enantiomer in the racemic drug, the drug stereochemistry has become an issue for the pharmaceutical industry as well as the regulatory authorities.

The first policy statement regarding the development of new stereoisomeric drugs was published by United States Food and Drug Administration in 1992 [30], which was closely followed by European guidelines in 1993 [31], which came into force in 1994. At present the decision regarding the stereoisomeric form, i.e. single enantiomer or racemic mixture, to be developed is left to the drug company. However, the decision taken requires scientific justification based on quality, safety and efficacy, together with the risk-benefit ratio and may be argued on a case-by-case basis [32, 33]. Further, the Food and Drug Administration (FDA) demands detailed documentation of pharmacological and pharmacokinetic behaviour of each enantiomer as well as their combined effects and permits only single-enantiomer
drugs to enter the market when the individual enantiomers show differences [34]. Indeed, the FDA guidelines had a major impact on the pharmaceutical industry resulting in the emergence of single-enantiomeric drugs from 1990’s (Fig. 1.4). However it is interesting to note that, well before the enforcement of FDA guidelines – roughly since 1980’s the single enantiomers were a significant component of approved drugs [1].

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Fig. 1.4: Distribution of worldwide-approved drugs according to chirality character in four-year ranges; *data including diastereomeric mixtures. [Source: Adapted from ref. 1]

Since 1990’s the chiral drugs have dominated the pharmaceutical business. More than 40% of all marketed drugs are currently sold in an enantiopure form [3]. The market share of chiral drugs is expected to rise sharply in the near future as approximately 80% of total drugs under development are chiral. About 70% of the new small-molecule drugs which received the approval by Food & Drug Administration in 2007 were chiral [35]. Past two decades have witnessed a significant expansion in the production of chiral drug molecules. A worldwide sinerio of emergence of single enantiomeric drugs over 20 years is given in Fig. 1.5.
Besides development of new pharmaceuticals in single enantiomeric form, chiral technology (i.e. chirotechnology) has a crucial role in re-evaluation and re-marketing of single enantiomeric forms of existing racemic drugs (called Racemic switching) which permits additional years of market exclusivity (patent protection) [29]. According to Frost & Sullivan, worldwide revenues from chiral compounds destined for the drug industry amounted to $4.8 billion in 1999 and will be triple to $14.9 billion by 2009, with the average annual growth of 12% [37]. The global growth in revenues of pharmaceutical sector driven by chirotechnology over last 10 years is given in Table 1.3.

Today chiral technology is mainly driven by pharmaceutical industries. Besides pharmaceuticals, the ‘chirality’ is receiving attention from several business
CHAPTER 1: Introduction

sectors such as biochemicals, agrochemicals, aroma and flavour compounds, dyes and pigments and polymers. The industrial demand of enantiopure chemicals is therefore expected to show explosive growth in the coming years [3].

Table 1.3: The global growth in revenues of pharmaceutical sector from chiral technology

<table>
<thead>
<tr>
<th>Year</th>
<th>Revenue ($ Billions)</th>
<th>Annual growth (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1999</td>
<td>4.80</td>
<td>-</td>
</tr>
<tr>
<td>2000</td>
<td>5.40</td>
<td>12.5</td>
</tr>
<tr>
<td>2001</td>
<td>6.10</td>
<td>13.0</td>
</tr>
<tr>
<td>2002</td>
<td>7.00</td>
<td>14.8</td>
</tr>
<tr>
<td>2003</td>
<td>7.74</td>
<td>10.6</td>
</tr>
<tr>
<td>2004</td>
<td>8.57</td>
<td>10.8</td>
</tr>
<tr>
<td>2005</td>
<td>9.53</td>
<td>11.1</td>
</tr>
<tr>
<td>2006</td>
<td>10.61</td>
<td>11.3</td>
</tr>
<tr>
<td>2007</td>
<td>11.85</td>
<td>11.7</td>
</tr>
<tr>
<td>2008</td>
<td>13.28</td>
<td>12.1</td>
</tr>
<tr>
<td>2009</td>
<td>14.94*</td>
<td>12.5</td>
</tr>
</tbody>
</table>

* predicted value; [Source: Adapted from ref 37]

1.2. METHODS OF ENANTIOSELECTIVE SYNTHESIS

The synthesis of enantiopure compound is generally initiated from (i) chiral pool; (ii) prochiral substrates; or (iii) racemates [34, 38]. The different methods of enantioselective synthesis from these three substrates are summarized in Fig. 1.6.

1.2.1. Chiral pool

The term ‘chiral pool’ refers to the many naturally available chiral molecules that exist in high enantiomeric purity. The most versatile chiral starting materials obtained from natural resources, in order of their industrial production per annum, are: carbohydrates, a-amino acids, terpenes, hydroxy acids and alkaloids. Other inexpensive chiral natural products are ascorbic acid, dextrose, ephedrine, limonene, quinidine and quinine, etc. [5, 34].

Further, these enantiopure compounds can be incorporated into the molecule to provide the desired chiral centre or to induce the desired chiral centre during
synthesis (chiral induction or diastereoselective synthesis). For example, naturally occurring enantiopure amino acids can be converted into antibacterials, cytotoxic agents and protease inhibitors (e.g. ritonavir) using this technology [39].

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**Fig. 1.6: Methods for synthesizing enantiopure compounds**

### 1.2.2. Starting from prochiral substrate - Asymmetric synthesis

The term asymmetric synthesis refers to stereoselective synthesis of chiral product staring from non-chiral (or prochiral) substrate(s). Early innovations in
asymmetric catalysis were contributed by both industry and academia. The Nobel Prize-winning work of W. S. Knowles, R. Noyori, and K. B. Sharpless fostered many subsequent innovations in this field. Knowles’s efforts led to the first industrial use of a chiral transition-metal complex to asymmetrically hydrogenate a prochiral substrate with high enantiomeric excess [35]. The asymmetric synthesis can be achieved either by means of a chiral auxiliary or using asymmetric catalyst [34].

(a) Chiral auxiliary

Chiral auxiliaries are a class of compounds which are used to modify the substrate molecule to introduce a stereogenic center. In the chiral auxiliary approach, the auxiliary is attached to the substrate, then stereoselective reaction is carried out and finally the auxiliary is removed or recycled.

(b) Asymmetric catalysis

An asymmetric catalyst as its name suggest catalyzes asymmetric reactions. Here no modification of the substrate is required (i.e. number of steps are reduced). Asymmetric catalysis can be achieved either by using a chemocatalyst or a biocatalyst.

1.2.3. Starting from the racemate – Resolutions

The separation of enantiomers of a compound from the racemic mixture is termed as resolution. It can be achieved by preferential crystallization, distereomeric salt formation, chromatographic resolution, kinetic resolution or inclusion resolution [34].

(a) Preferential crystallization

This method is based on the different crystallization pattern of two enantiomers. Only one enantiomer from the racemic mixture under specific conditions preferentially crystallizes in the form of a simple salt (e.g. hydrochloride). A mixture of crystals of individual enantiomers that can in principle be separated mechanically is termed as a conglomerate. Since approximately 7% of the racemic compounds form conglomerates and the manual ‘crystal picking’ is highly inconvenient and time consuming this method has poor industrial relevance. This method can be used for the
(b) Diastereomeric salt formation

The most practical and widely used technique via a ‘classical’ resolution of enantiomers is by diastereomeric salt formation. In this method, an acid-base reaction is involved between a racemate and a resolving agent, where two diastereomeric salts having dissimilar physical and chemical properties are formed. These two diastereomers obtained can be easily separated either by crystallization or by filtration if one is soluble and the other is insoluble. Finally, the salt is decomposed by treatment with either acid or base, when pure enantiomer is obtained [40]. The two diastereomers formed can also be separated by classical achiral liquid chromatography. This method has been used in the resolution of α-methyl-L-3,4-dihydroxy phenylalanine, aspargine and glutamic acid [8].

(c) Chromatographic resolution

The use of chromatographic techniques in the resolution of enantiomers to obtain large quantities of enantiomerically pure drugs and drug intermediates is a growing field. Chromatographic separation relies on a difference in affinity between a chiral stationary phase and a mobile phase. Simulated moving bed chromatography (SMBC) is a continuous chromatographic multi-column separation process wherein six to eight columns are run in series. In recent years, SMBC has become an alternative approach for the separation of enantiomers in quantities ranging from grams to several hundred kilograms [41,42]. A successful example of SMBC is in the commercial scale synthesis of enantiopure (R)-miconazole (used in treatment of skin diseases and tuberculosis) from a racemic intermediate.

(d) Kinetic resolution

Kinetic resolution can separate two enantiomers on the basis of their different reaction rates with a chiral entity. The chiral entity can be a chemocatalyst (e.g., a metal complex or an organic chiral catalyst) [43] or a biocatalyst (e.g., an enzyme or a microorganism) [44]. The main disadvantage of a kinetic resolution is the theoretical yield of the desired enantiomer can never exceed the limit of 50% of mole ratio. The Sharpless epoxidation and the lipase catalyzed kinetic resolution of racemic sec-
amines are the examples of chemocatalytic resolution and biocatalytic resolution respectively [45].

Kinetic resolution aided with in-situ racemization of slowly transformed enantiomer is called as ‘Dynamic Kinetic Resolution’ (DKR) which can, in principle, convert 100% of the racemate to the desired product. The in-situ racemization can be spontaneous or can be induced by using a racemization catalyst (transition metal catalyst or racemase enzymes) [34]. Industrial production of enantiopure D-amino acids currently practiced by DSM is based on a dynamic kinetic resolution process [45].

Enantioselective membranes can be employed for the kinetic resolution of enantiomers. These membranes are able to resolve optical isomers because of their chiral recognition sites (e.g., chiral side chains, chiral backbones, or chiral selectors). They act as selective barriers in the resolution process, and they selectively transport one enantiomer due to the stereospecific interaction between the enantiomer and chiral recognition sites, thereby producing a permeate solution enriched with one enantiomer. The separation of two enantiomers could result due to one or combination of following mechanisms viz. hydrogen bonding, hydrophobic, Coulombic forces, van der Waals interactions and steric effects with the chiral sites [46].

(c) Inclusion resolution

Inclusion resolution is a relatively novel method which is based on chiral discrimination and recognition in the crystalline phase [47]. A chiral host molecule forms an inclusion complex preferably with one of the enantiomers especially by means of hydrogen bonds. The most widely applied chiral host molecules are the derivatives of tartaric acid, succinamide and lactic acid. Recently, both enantiomers of 9,9’-spirobifluorene-1,1’-diol (used in synthesis of chiral ligand) were conveniently obtained by inclusion resolution with 2,3-dimethoxy-N,N,N’,N’-tetracyclohexylsuccinamide [48].

(f) Dutch resolution

The crucial step in the development of a resolution procedure is to find a suitable resolving agent. The choice of resolving agent has been done by trial-and-error based on the prior experience rather than any mechanistic approach. In 1998, a
new approach to classical resolution was reported whereby, instead of using one resolving agent, mixtures of structurally closely related resolving agents (called as families of resolving agents) were added to the racemic mixture. This method was coined “Dutch Resolution”- a name which has been widely adopted [49]. Structurally related enantiopure derivatives of sulphonic acid, mandelic acid, 1-phenylethylamine etc. have been known to constitute families of resolving agents. With these families many resolutions have been carried out readily whereas with single resolving agents resolutions were either poor or failed. Dutch Resolution certainly has something of combinatorial characteristics in it; upon the simultaneous addition of a family of resolving agents, higher de (diastereomeric excess) values of the first salts were obtained via this method.

DL-threo-(4-methylthiophenyl) serine amide could be successfully resolved by using 1 mol equivalent of the family of cyclic phosphoric acids. The resolved amide can be used as an intermediate in the synthesis of thiamphenicol [50], which is an antimicrobial substance used for the treatment of infectious diseases in cattle, pigs and poultry.

1.2.4. Comparison of chiral pool, asymmetric synthesis and resolution processes

Each method of enantioselective synthesis has specific advantages and disadvantages. The selection of appropriate method depends on several factors such as availability of substrate, scale of synthesis, cost of synthesis, desired enantiopurity and use of the chiral product.

The need of enantiopure compounds in bulk amount with considerably high optical purity at low cost can be accomplished easily via chiral pool. In the early 1990s, almost 80% of chiral drugs were derived from chiral-pool materials, whereas today, just 25% come from the chiral pool and rest use different chiral technologies viz. asymmetric synthesis or resolution procedures [51].

Asymmetric synthesis, either by chiral auxiliaries or by asymmetric catalysis, should be the most cost-effective method for producing single enantiomers since it has a theoretical yield of 100%. However use of chiral auxiliary is hampered by several factors viz. (i) unavailability of both enantiomers of the chiral auxiliary, (ii) additional steps required for attachment-detachment of an auxiliary to the substrate and (iii) difficulties in the removal of the auxiliary. Synthesis by asymmetric catalysis
often requires lengthy procedures such as selection of suitable catalyst and optimization of process parameters. Furthermore, the chemocatalytic process gives low enantiopurity. On the other hand, requirement of very low quantity of the catalyst and high efficiency of the process are some of the attractive features of asymmetric catalysis. Recently, the introduction of high-throughput experimentation has drastically accelerated the screening procedures for choosing the appropriate catalyst for certain process [52].

Despite the theoretical yield being 50%, resolution is one of the most common methods for laboratory or industrial scale synthesis of enantiopure compounds. Almost all resolution methods are simple, easy to scale up and therefore can be readily incorporated into an industrial process. If the unwanted isomer can find a profitable use or can be racemized in situ (e.g. dynamic kinetic resolution), the resolution method becomes even more advantageous.

1.3. BIOCATALYSTS FOR ENANTIOSELECTIVE SYNTHESIS

Enzymes are remarkable catalysts in terms of selectivity, specificity and efficiency. In the last decade, employing biocatalysts (enzymes) for organic synthesis has proved to be a valuable alternative to conventional chemical methods. Enzymes quite often catalyze reactions with exceptionally high chiral (enantio-) and positional (regio-) selectivities without formation of (unwanted) by-products. As a result, biocatalysts can be used in both simple and complex transformations without the need for the tedious blocking-deblocking steps that are common in enantio- and regioselective chemo-catalytic organic synthesis. The high selectivity and specificity of enzymes make them attractive catalysts especially for synthesis of pharmaceuticals, where the demand for enantiomerically pure molecules is continuously increasing [53].

Moreover, enzyme-catalyzed reactions are carried out generally under mild conditions (of pH temperature and pressure) that minimize problems like isomerization, racemization or epimerization of product. Enzymes are far more efficient than chemical catalysts. The initial reaction rates of enzymatic transformations are roughly $10^{10}$ to $10^{20}$ times higher than that of chemo-catalyzed reactions. In addition, biocatalytic processes are less hazardous, less polluting (environmentally benign) and consume less energy than conventional chemo-catalytic
processes, especially those which use heavy-metal catalysts [54].

Further, the recent advancements in the field of molecular biology is causing a revolution in the industrial utilization of biocatalysts due to following reasons: (i) availability of a wide range of expression hosts to produce biocatalysts cost effectively; (ii) rapid discovery of enzyme tool boxes through genome mining as a result of widespread availability of gene sequences; (iii) robust directed evolution (rational, semi-rational or random) and screening technologies to improve enzyme properties to meet process requirements [55].

In spite of the promising features, the industrial applications of many enzymes are hindered because of two inherent limitations (which are exhibited by almost all enzymes): (i) lack of (operational and storage) stability and (ii) high cost. The immobilization of enzyme is simple but equally effective method to overcome these limitations [56].

1.3.1. Immobilized enzyme

Immobilized enzymes are used as heterogeneous catalyst which can be easily recovered and reused. Immobilized enzymes are defined as enzymes that are physically confined or localized, with retention of their catalytic activity [57]. The physical confinement remarkably alters the physical, chemical, mechanical, catalytic and kinetic properties of an enzyme.

Immobilization is also useful in providing stability to the enzyme against denaturation by preventing conformational changes and protecting it in a confined microenvironment. Besides enhanced stability, immobilization of enzymes provides several advantages such as ease of separation from a reaction mixture, repeated or continuous use, possible modulation of the catalytic properties, prevention of microbial contaminations, easy handling and extended storage life, enabling greater control over catalytic processes and process economics [58].

1.3.2. Immobilization methods

A wide range of methods (e.g. non-covalent adsorption, covalent binding, entrapment, encapsulation etc.) and support materials (e.g. inorganic clays, polymer beads, membranes, nanoparticles etc.) has been proposed and used for the immobilization of enzymes [59, 60]. Immobilized enzymes can be broadly classified
as carrier-bound immobilized enzyme and carrier-free immobilized enzyme (Fig. 1.7).

Fig. 1.7: Methods of enzyme immobilization

In carrier-bound immobilized enzyme, the enzyme is immobilized on/inside (non-porous/porous) physical support or carriers. Carrier-bound immobilized enzymes can be obtained by two ways:

- Attachment of an enzyme to the pre-fabricated support by means of hydrophobic interactions and hydrogen bonds (adsorptive binding), electrostatic bonds (ionic binding), covalent binding or affinity binding.
- Encapsulation or inclusion of enzyme in microcapsules, membrane devices (e.g. hollow fibers) or in reverse micelles.

The cross-linking of enzyme molecules by bifunctional reagents (cross-linking agent) results in gelation/solidification of the soluble enzyme without losing their catalytic activity (provided binding does not affect the catalytic site). Owing to their
insoluble nature in both organic and aqueous media, cross-linked enzymes can be used as heterogeneous biocatalysts. Since the molecular weight of the cross-linking agent is negligible, compared with that of the enzyme, the resulting biocatalyst essentially comprises 100% wt of protein. Thus compared to carrier-bound biocatalyst, cross-linked enzymes express high catalytic activity per volume thereby maximizing volumetric productivity and space-time yields. [61].

Depending upon their method of preparation, the cross-liked enzymes can be classified as:
- Cross-linked enzyme (CLE) prepared by direct cross-linking of soluble enzyme
- Cross-linked enzyme crystals (CLEC) prepared by cross-linking of crystalline enzyme and
- Cross-linked enzyme aggregates (CLEAs) prepared by cross-linking of physically aggregated enzyme

In spite of this clear division, the procedure used in many cases requires a combination of two or more methods such as adsorption of the enzyme onto a suitable support followed by intermolecular cross-linking.

### 1.3.3. Enantioselectivity of an enzyme

When a racemic substrate is subjected to an enzymatic reaction (e.g. hydrolysis), a chiral discrimination occurs due to the difference in the rate of transformation of the two enantiomers. Owing to the three dimensional structure of the active site of the enzyme, one enantiomer fits better into the active site and is therefore transformed at higher rate than its counterpart. The selectivity of an enzyme towards chiral discrimination of two enantiomers is called as ‘enantioselectivity’.

Initially the enantioselectivity was expressed in terms of ‘stereoselectivity factor’ which was defined as ratio of rate of transformation of individual enantiomers [62-64].

For instance, assuming that the rate of transformation of $S$-substrate ($k_S$) is greatly higher than that of $R$-substrate ($k_R$) and the transformation proceeds with retention of configuration, $S$-substrate will be selectively transformed into $S$-product and transformation of $R$-substrate will begin after transformation of $S$-substrate. If the reaction is terminated at ~ 50% conversion then, the product formed gets enriched with $S$-enantiomer while the (un-transformed) substrate gets enriched with $R$-

---

Chiral Separation of Drugs and Drug intermediates by Immobilized Biocatalyst
enantiomer. On the other hand, if \( k_S \approx k_R \), distinct enantio-enrichment of product and substrate cannot occur [65].

Ideally, the ratio of the rates of transformation of two enantiomers should be infinite so that an enzymatic transformation will automatically stop at 50% conversion – where only one enantiomer of substrate will be transformed into enantiopure product while another enantiomer of substrate remained un-transformed. However, often in practice the ratio of the rates of transformation of two enantiomers is not infinite but measurable. In such cases, the enzymatic transformation needs to be (manually) stopped at certain point where maximum enantio-enrichment of product and unreacted substrate can be achieved.

Later, Chen et al. introduced a new dimensionless parameter, ‘enantiomeric ratio’ to describe the enantioselectivity of irreversible enzymatic resolution reaction [66]. It is an intrinsic property of the enzyme which remains constant through out the reaction. By definition, \( E \) can be expressed as ratio of specificity constants i.e. \( (V_{max}/K_m) \) of the two enantiomers (Eq. 1.1).

\[
E = \frac{(V_{max}^R / K_m^R)}{(V_{max}^S / K_m^S)}
\] 

– Eq. 1.1

Where, \( V_{max}^R \) and \( K_m^R \) are respectively the maximum reaction velocity and Michaelis constant for \( R \)-enantiomer. \( V_{max}^S \) and \( K_m^S \) are respectively the maximum reaction velocity and Michaelis constant for \( S \)-enantiomer.

The measurement of \( E \) using Eq. 1.1 needs pure enantiomers of substrate which are often not available. Hence the Eq. 1.1 is not commonly used in practice. Chen et al. have also derived useful mathematical expressions (Eq. 1.2, Eq. 1.3 and Eq. 1.4) for experimental determination of enantiomeric ratio [66]. These expressions are based on the dependence of \( ee_S \) and \( ee_S \) on the extent of conversion. During an irreversible chiral resolution process, the rate of transformation of each enantiomer varies with time, as the relative concentration of the two enantiomers is continuously changing. Hence, the enantiopurity of substrate (expressed as \( ee_S \)) and the enantiopurity of product (expressed as \( ee_P \)) becomes a function of the extent of conversion.

\[
E = \frac{\ln[1-C(1+ee_P)]}{\ln[1-C(1-ee_P)]}
\] 

– Eq. 1.2
CHAPTER 1: Introduction

Chiral Separation of Drugs and Drug intermediates by Immobilized Biocatalyst

\[
E = \frac{\ln [(1-C)(1-e_{eS})]}{\ln [(1-C)(1+e_{eS})]} \quad \text{– Eq. 1.3}
\]

\[
E = \frac{\ln [(1-e_{eS})/(1+e_{eS} / e_{eP})]}{\ln [(1+e_{eS})/(1+e_{eS} / e_{eP})]} \quad \text{– Eq. 1.4}
\]

Where, \( C \) is conversion ratio and, \( e_{eS} \) and \( e_{eP} \) are enantiomeric excess of substrate and that of product respectively. \( C \) can be determined by using Eq. 1.5 while \( e_{eS} \) and \( e_{eP} \) can be determined by using Eq. 1.6 and Eq. 1.7 respectively.

\[
C = 1 - \left( \frac{c_{S(t)}^S + c_{S(t)}^R}{c_{S(0)}^S + c_{S(0)}^R} \right) \quad \text{– Eq. 1.5}
\]

Where, \( c_{S(t)}^S \) and \( c_{S(t)}^R \) are concentration of \( S \)-enantiomer and concentration of \( R \)-enantiomer of substrate respectively at time, \( t \). \( c_{S(0)}^S \) and \( c_{S(0)}^R \) are concentration of \( S \)-enantiomer and concentration of \( R \)-enantiomer of substrate respectively at \( t = 0 \).

\[
e_{eS} = \frac{|c_{S}^S - c_{R}^R|}{c_{S}^S + c_{R}^R} \quad \text{– Eq. 1.6}
\]

\[
e_{eP} = \frac{|c_{P}^S - c_{P}^R|}{c_{P}^S + c_{P}^R} \quad \text{– Eq. 1.7}
\]

Where, \( c_{S}^S \) and \( c_{S}^R \) are concentration of \( S \)-enantiomer and \( R \)-enantiomer of substrate respectively while \( c_{P}^S \) and \( c_{P}^R \) are concentration of \( S \)-enantiomer and \( R \)-enantiomer of product respectively.

The interdependence of enantiomeric ratio and enantio-enrichment of product and unreacted substrate is illustrated in Fig. 1.8. Here, three hypothetical biotransformation reactions \( \text{viz.} \ A, B \text{ and } C \) having respective \( E \) values of 2 (indicating poor enantioselectivity), 20 (indicating good enantioselectivity) and 200 (indicating excellent enantioselectivity) are taken into consideration. The profiles of \( e_{eP} \) and \( e_{eS} \) for A, B and C as a function of extent of conversion were obtained from the ‘Selectivity’ software. (A free version of software was downloaded from the URL: http://borgc185.kfunigraz.ac.at/index.htm). At 50% conversion, values of \( e_{eS} \) and \( e_{eP} \) for the biotransformation were close to 22 while that for the biotransformation B were close 79 and that for the biotransformation C were close to 97. Thus, higher enantiomeric ratios indicate better enantio-enrichment of substrate and product.
The enantiomeric ratio is the prime parameter for describing the enantioselectivity of an enzyme. As a rule of thumb, $E$ below 15 is unacceptable for any practical purpose. $E$ in the range of 15 to 30 is regarded as acceptable, that in the range of 30-100 is regarded as good while above 100 is excellent [67].

It must be emphasized that determination of $E$ is very sensitive to values of ee$_S$ and ee$_P$. In general low $E$ values can be determined more accurately. In fact, when $E$ values are above 200 even a very small variation in ee$_S$ or ee$_P$ (arising from experimental errors in the analytical method) causes a large difference in numerical values of the former [67]. Hence, when experimental value of $E$ exceed 200, it is usually represented as $>200$ instead of the exact value. Furthermore, the accuracy of $E$ value estimates based on a single-point evaluation is inherently poor. Hence averaging of $E$ values from multiple data points must be performed [68].
1.4. SCOPE OF THE THESIS

The present thesis deals with enantioselective synthesis of commercially important chiral drug intermediates (unnatural amino acids and vicinal diols) using a suitable immobilized biocatalyst.

1.4.1. Unnatural amino acids - critical components of drugs and drug intermediates

Amino acids have always played a crucial role in drug discovery. Further, due to renewed interest in peptides as therapeutics, the amino acid market is growing steadily. This has led to the development of several marketed drugs composed of or derived from amino acids. The annual market of protein-based therapeutics is expected to surpass $50 billion by 2010 [69]. Besides pharmaceuticals, there is great demand for amino acids and amino acids derivatives in foods, agrochemicals and synthetic organic chemistry as a source of chiral materials. According to reports, about 20% of all new drugs launched during 1995-2001 have one or more amino acid residues incorporated into them. Most of these new drugs have an unnatural amino acid as a structural element [70-72].

Unnatural amino acids are non-genetically-coded amino acids that either occur naturally or are chemically synthesized. They are becoming very important tools for modern drug discovery led research. Due to their structural diversity and functional versatility, they are widely used as chiral building blocks and molecular scaffolds in constructing combinatorial libraries. While the total amino acid market is expanding at annual rate of roughly 10-15%, the market for natural amino acids is only growing at an annual rate of about 1-2% – evidently indicative of the increasing demand for unnatural amino acids [70].

Many of these unnatural amino acids are used as drugs or drug intermediates. Unnatural amino acids can be valuable pharmaceuticals. For example, L-DOPA (i.e. L-3,4-dihydroxy phenylalanine) is used in symptomatic treatment of Parkinson's disease, particularly to alleviate trembling, rigidity, and slow movements; D-penicillamine is used for symptomatic treatment of arthritis [71].

Numerous therapeutically relevant compounds with an unnatural amino acid moiety in their structures have been reported. In view of their dual functionality (carboxylic and amino), unnatural amino acids are recognized as highly versatile chiral synths (drug intermediates) that are widely used to introduce chirality in the
drug molecule. Unnatural amino acids are components of several known drug molecules as well as those being developed. For example, (R)-phenylglycine and (R)-4-hydroxy-phenylglycine are used in the semisynthetic broad-spectrum antibiotics Ampicillin and Amoxicillin. (R)-2-Naphthylalanine is found in peptide drug Nafarelin, a LHRH analogue used for the symptomatic treatment of endometriosis. A very important class of antihypertensive drugs (Benzpril, Enalapril, Lisinopril etc.) contains (S)-homophenylalanine [71].

Unnatural amino acids are vital in future peptide related drug discovery. If the use of peptide is associated with side effects arising from the conformational freedom of the flexible peptide, rendering a peptide more rigid would result in the selective peptide interactions with only one receptor, thereby producing fewer side effects. Incorporation of conformationally constrained unnatural amino acids has become a useful strategy in the design and development of selective peptide drugs [72]. Moreover, the possibility of protein engineering as a method of creating improved enzymes has infinitely widened the scope of enantioselective synthesis of diverse unnatural amino acids [73].

1.4.2. Vicinal diols - versatile chiral building blocks

The epoxides as well as their corresponding vicinal diols are highly versatile chiral building blocks used in synthesis of a variety of bioactive compounds, for example: β-3-adrenergic receptor agonists, anti-obesity drugs, N-methyl D-aspartate receptor antagonists, nematocides and anticancer agents [74-77]. In view of their commercial potential, the enantioselective synthesis of these compounds is of great interest in synthetic organic chemistry. In the past few years, different methods have been reported (mainly based on transition metal catalysis) for synthesis of chiral epoxides and vicinal diols. However, the environmental concerns and the regulatory constraints faced in the chemical and pharmaceutical industries have spurred the need of alternative biological methods that can offer cleaner and milder synthetic processes. One of the most promising methods to produce enantiopure vicinal diol involves kinetic resolution of racemic epoxide with epoxide hydrolases [78]. Epoxide hydrolases (EC 3.3.2.3) catalyze the addition of a water molecule to the oxirane ring of epoxides leading to formation of the corresponding 1,2-diols [75]. Epoxide hydrolases are widespread in nature. Recently, they have been obtained from
microbial sources and hence can be produced in bulk quantities as per demand for industrial applications. The enzyme does not require renewable co-factors and is therefore suitable for operation at large scale [79, 80].

Among the several types of biocatalytic reactions, kinetic resolution of racemates is dominant in the majority of applications. Despite their widespread applications, classic kinetic resolutions are impeded by several inherent limitations, the most crucial being – a restriction of the theoretical yield of each enantiomer to 50%. Enantioconvergent strategy is one of the most effective methods to overcome the 50% yield limitation [81].

An enantioconvergent process:

In an enantioconvergent process, two enantiomers of substrate are converted into single enantiomer of product and 100% theoretical yield is possible. As far as epoxide hydrolases-catalyzed reactions are concerned, ‘de-racemization’ can be achieved by making use of two independent reactions, which transform each of the enantiomers in an enantioconvergent manner. As demonstrated in Scheme 1.1, one enantiomer could be converted into product in an enantioselective way through retention of configuration whereas its antipode is transformed with inversion of configuration to give the same enantiopure vicinal diol.

**Scheme 1.1:** Principle of enantioconvergent process from racemic epoxides, leading to a theoretical yield of enantiopure diol to 100%
The different approaches to accomplish an enantioconvergent process are:

- Chemo-enzymatic approach;
- Bi-enzymatic approach;
- Mono-enzymatic approach

A ‘chemo-enzymatic approach’ is based on the consecutive use of an enantio- and regio-selective enzymatic hydrolysis followed by an opposite regio- and stereoselective chemical hydrolysis of the residual epoxide [82]. The chemo-enzymatic enantioconvergent process finds application in synthesis of (R)-p-nitro-phenylethane diol (a chiral intermediate of (R)-Nifenalol having β-blocker activity). Enzymatic hydrolysis was carried out using *Aspergillus niger* epoxide hydrolase and chemical hydrolysis was carried out using H₂SO₄.

‘Bi-enzymatic approach’ involves the use of two enantio-complementary epoxide hydrolases having an opposite regioselectivity towards the oxirane ring opening [83]. For example, the sequential use of *Solanum tuberosum* epoxide hydrolase and *Aspergillus niger* epoxide hydrolase enabled the enantioconvergent preparation of the corresponding (R)-p-chloro-phenylethane diol (a component of NMDA receptor antagonist: Eliprodil).

The most elegant enantioconvergent method reported used the ‘Mono-enzymatic approach’. It is based on the use of only one epoxide hydrolase having a characteristic opposite regioselectivity towards two enantiomers of the same substrate i.e. starting from racemic mixture of epoxide, 100% conversion to a single enantiomer of diol is possible. For example, enantioconvergent production of (R)-phenylethane diol is possible using *Solanum tuberosum* epoxide hydrolase [83, 84].

**1.5. RESEARCH OBJECTIVES**

**1.5.1.** Enantioselective synthesis of unnatural amino acids [namely: phenylglycine (PG), 3,4-dihydroxy phenylalanine (DOPA), homophenylalanine (HPA) and 2-naphthylalanine (NA)] using immobilized enzymes (viz. lipases, amidase and aminoacylase).

(R)-Phenylglycine is used in the semisynthetic broad-spectrum antibiotics Ampicillin and Amoxicillin. (S)-3,4-Dihydroxy phenylalanine is used as a drug in symptomatic treatment of Parkinson’s disease, particularly to alleviate trembling, rigidity, and slow movements. A very important class of antihypertensive drugs
(namely: Benzapril, Enalapril and Lisinopril) contains (S)-homophenylalanine (Scheme 1.2). (S)-2-Naphthylalanine is found in peptide drug Nafarelin, a LHRH analogue used for symptomatic treatment of endometriosis. Being components of high selling drugs, all these unnatural amino acid have high commercial potential especially in pharmaceutical and medicinal chemistry.

**Scheme 1.2:** L-Homophenylalanine as component of antihypertensive drugs (namely: benzapril, lisinopril and enalapril) [85, 86]

Prior art:

Specific details of previous reports regarding the enantioselective biocatalytic synthesis of phenylglycine, 3,4-dihydroxy phenylalanine, homophenylalanine and 2-naphthylalanine are summarized in Table 1.4, Table 1.5, Table 1.6 and Table 1.7
respectively. Most of the reports mention preliminary work that involves use of soluble enzyme. Detail reports on enantioselective biocatalytic synthesis of unnatural amino acids from the perspective of ‘industrial biocatalysis’ are scant.

Table 1.4: Reports on the enantioselective biocatalytic synthesis of phenylglycine (PG)

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>Process details</th>
</tr>
</thead>
<tbody>
<tr>
<td>Different lipases</td>
<td>PG-ethyl ester</td>
<td>Kinetic resolution, ester hydrolysis, porcine pancreatic lipase gave maximum enantioselectivity ($E = 1.9$) [87].</td>
</tr>
<tr>
<td><em>Candida antarctica</em> lipase B</td>
<td>PG-esters</td>
<td>Dynamic kinetic resolution, ester hydrolysis, ($E = 20$, for butyl ester) [88, 89].</td>
</tr>
<tr>
<td><em>Candida antarctica</em> lipase B</td>
<td>PG-esters</td>
<td>Kinetic resolution, ester hydrolysis, ($E = 43$, in ionic liquid) [90, 91].</td>
</tr>
<tr>
<td>Penicillin-G acylase</td>
<td>PG</td>
<td>Kinetic resolution, ester synthesis [92].</td>
</tr>
<tr>
<td><em>Rhodococcus</em> cells</td>
<td>PG-nitrile</td>
<td>Kinetic resolution, PG-nitrile is hydrolyzed to PG-amide (by nitrile hydratase) which was kinetically resolved by amidase [93-96].</td>
</tr>
</tbody>
</table>

Table 1.5: Reports on the enantioselective biocatalytic synthesis of 3,4-dihydroxy phenylalanine (DOPA)

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>Process details</th>
</tr>
</thead>
<tbody>
<tr>
<td>Different lipases</td>
<td>DOPA-ethyl ester</td>
<td>Kinetic resolution, ester hydrolysis, porcine pancreatic lipase gave maximum enantioselectivity ($E = 43.2$) [87].</td>
</tr>
<tr>
<td>Proteases (α-chymotrypsin, subtilisin)</td>
<td>DOPA-ethyl ester</td>
<td>Kinetic resolution in acetonitrile-water medium [97].</td>
</tr>
<tr>
<td>α-Chymotrypsin</td>
<td>N-protected DOPA</td>
<td>Transesterification, ester synthesis in organic solvents [98].</td>
</tr>
<tr>
<td>Tyrosinase and α-chymotrypsin</td>
<td>Tyrosine ester</td>
<td>Tyrosine ester was converted into DOPA ester by tyrosinase which was subsequently kinetically resolved by α-Chymotrypsin [99].</td>
</tr>
</tbody>
</table>
Table 1.6: Reports on the enantioselective biocatalytic synthesis of homophenylalanine (HPA)

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>Process details</th>
</tr>
</thead>
<tbody>
<tr>
<td>Different lipases</td>
<td>HPA-ethyl ester</td>
<td>Kinetic resolution, ester hydrolysis, <em>Rhizopus</em> lipase gave maximum enantioselectivity ($E = 5$) [87].</td>
</tr>
<tr>
<td>Alcalase</td>
<td>N-protected HPA-methyl ester</td>
<td>Kinetic resolution, ester hydrolysis (ee = 98%) [100].</td>
</tr>
<tr>
<td>Aminoacylase from (sheep, beef, hog) Kidney</td>
<td>N-acetyl HPA</td>
<td>Kinetic resolution, (ee = 94-99%) [101].</td>
</tr>
<tr>
<td><em>B. licheniforms</em> alcalase</td>
<td>HPA-ethyl ester</td>
<td>Kinetic resolution; reaction medium: organic solvents and ionic liquids; ionic liquids gave better results than organic solvents [102, 103].</td>
</tr>
<tr>
<td><em>B. caldolyticus</em> hydantoinase and <em>B. kaustophilus</em> L-N-carbamoylase</td>
<td>5-[2-phenylethyl]-imidazolidine-2,4-dione</td>
<td>Kinetic resolution, (RS)-5-[2-phenylethyl]-imidazolidine-2,4-dione is hydrolyzed to (S)-4-phenyl-2-ureidobutanoic acid by hydantoinase which was further transformed into (S)-HPA by L-N-carbamoylase [104].</td>
</tr>
<tr>
<td>Porcine pancreatic lipase</td>
<td>N-acylated HPA-ethyl ester</td>
<td>Kinetic Resolution, reaction medium: ionic liquids [105].</td>
</tr>
<tr>
<td>Different lipases and proteases</td>
<td>N-BOC-protected HPA methyl ester</td>
<td>Ammonolysis reaction wherein protected methyl ester is transformed into amide; <em>Thermomyces lanuginosus</em> lipase and <em>Bacillus licheniformis</em> protease gave maximum enantioselectivity ($E = 15$ and $&gt;100$ respectively) [106].</td>
</tr>
<tr>
<td>Aromatic amino acid transaminase</td>
<td>2-oxo-4-phenylbutyric acid</td>
<td>Asymmetric synthesis, (ee &gt;99) [107].</td>
</tr>
<tr>
<td>Aminoacylase</td>
<td>HPA-amide, N-acetyl HPA</td>
<td>Kinetic Resolution, ($E &gt;200$) [108].</td>
</tr>
</tbody>
</table>
Table 1.7: Reports on the enantioselective biocatalytic synthesis of 2-naphthylalanine (NA)

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>Process details</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Chymotrypsin and subtilisin</td>
<td>N-acetyl NA</td>
<td>Kinetic resolution, subtilisin offered high enantioselectivity than α-chymotrypsin [109].</td>
</tr>
<tr>
<td>Immobilized subtilisin</td>
<td>N-acetyl NA</td>
<td>Kinetic resolution, 92% yield of L-NA, subtilisin was immobilized on alumina support [110].</td>
</tr>
<tr>
<td>Pronase (a mixture of several proteolytic enzymes obtained from Streptomyces griseus)</td>
<td>DL-NA methyl ester</td>
<td>Kinetic resolution, the biotransformation gave optically pure L-NA (ee ≈ 100%) with 87% yield [111].</td>
</tr>
<tr>
<td>Thermitase (an alkaline serine protease from Thermoactinomycetes vulgaris)</td>
<td>N-Boc-NA methyl esters</td>
<td>Kinetic resolution, high yield (&gt; 90%) of L-NA was obtained [112].</td>
</tr>
</tbody>
</table>

1.5.2. Preparative scale enantioselective synthesis of (R)-phenylethane diol and (R)-m-chloro-phenylethane diol, based on a mono-enzymatic enantioconvergent strategy using immobilized Solanum tuberosum epoxide hydrolase.

Enantiopure phenylethane diols and their halo-substituted derivatives are useful for the synthesis of pharmaceutically active compounds [113-115]. (R)-Phenylethane diol, for example, is component of β-adrenoreceptor agonists (e.g. isoproterenol and its analogues used in treatment of cardiac arrest) [113]. In addition to this, enantiopure phenylethane diol is useful to synthesize (R)-1,3-amino alcohol and the latter is a chiral intermediate of (R)-norfluoxetine and (R)-fluoxetine (pharmaceuticals which are used in the treatment of psychiatric disorders like depression, anxiety and alcoholism) [114]. (R)-m-chloro-phenylethane diol is a key chiral intermediate for the synthesis of β-3 adrenalin receptor agonists (Scheme 1.3) namely SR 58611A or A-9677 (developmental drug compounds having potential to treat anxiety and depressive disorders) [116]. Enantiopure phenylethane diols and their halo-derivatives are also useful to synthesize chiral catalysts [117, 118], macrocyclic polyether–diester ligands [119] and liquid crystals [120].
Scheme 1.3: (R)-m-Chloro-phenylethane diol as chiral synthon for β-3 adrenalin receptor agonists namely SR-58611A, AJ-9677.

Prior art:

Limitations of substrate solubility and inherent complex nature of the enantioconvergent transformations present serious limitations for use of high substrate concentrations in reactions. This is why enantioconvergent processes are difficult to practice on commercial scale. The maximum reported substrate concentration in process of enantioconvergent production of (R)-phenylethane diol is 6 g/L [115] and that of (R)-m-chloro-phenylethane diol is 10 g/L [116]. In this context, the present work was undertaken to explore the preparative scale enantioconvergent production of (R)-phenylethane diol and (R)-m-chloro-phenylethane diol.

1.6. OUTLINE OF THE THESIS

Chapter 2: Enantioselective synthesis of unnatural amino acids using covalently immobilized lipase on porous beaded polymers

Five commercial lipases from different sources were screened for chiral resolution of unnatural amino acid esters. The Candida rugosa lipase (CRL) and porcine pancreatic lipase (PPL) were immobilized on epoxy activated functional
polymers. More than 50 functional polymers of different monomer-cross-linking agent compositions were screened for lipase immobilization. The effect of cross-link density and porogen on lipase immobilization was evaluated. The acrylic functional polymers containing allyl glycidyl ether (AGE) monomer units synthesized by using lauryl alcohol as a porogen, gave higher lipase binding and therefore thoroughly analyzed for their catalytic performance, stability and reusability. Under the optimum conditions, AGE-(L)-100 gave 96.64% activity recovery for CRL binding and 74.35% activity recovery for PPL binding. The immobilized CRL and immobilized PPL were employed for the kinetic resolution of unnatural amino acid ethyl esters.

Chapter 3: Chiral resolution of unnatural amino acid esters using immobilized lipase in membrane bioreactor

The Candida rugosa lipase (CRL) and porcine pancreatic lipase (PPL) were immobilized on poly(urethane methacrylate -co-glucidyl methacrylate)-supported-polypropylene biphasic membrane. A polypropylene membrane was hydrophilized by coating followed by UV curing of a blend of 2-hydroxyethyl methacrylate terminated polyurethane prepolymer and glucidyl methacrylate. This allows formation of a hydrophobic membrane with increased surface hydrophilicity, biocompatibility and stability. Immobilized membranes were treated with 5% glutaraldehyde as a cross-linking agent for post immobilization stabilization of enzyme on membrane. Under the optimum conditions, the biocatalytic membranes retained >90% of initial lipase activity. The biocatalytic membrane was characterized for its catalytic performance, stability and reusability. The immobilized membranes were placed in membrane reactor where enantioselective synthesis of unnatural amino acid esters was studied.

Chapter 4: Chiral resolution of unnatural amino acid amides using immobilized resting cells of Rhodococcus erythropolis MTCC 1526

Statistical experimental methodology was used to enhance the production of amidase from Rhodococcus erythropolis MTCC 1526. R. erythropolis MTCC 1526 was selected through screening of seven strains of Rhodococcus species. The Placket–Burman screening experiments suggested that carbon source (sorbitol), nitrogen sources (yeast extract and meat peptone) and amidase inducer (acetamide) are the most influential media components. The concentrations of these four media components were optimized using statistical methodology. The optimized conditions were used to study the kinetic resolution of unnatural amino acid amides.
components were optimized using face centered design of Response Surface Method (RSM). Use of RSM increased the production of amidase from *R. erythropolis* MTCC 1526 by 6.88 fold. The cells of *R. erythropolis* MTCC 1526 having enhanced amidase activity were immobilized by different entrapment methods. The immobilized cells of *R. erythropolis* MTCC 1526 were used for chiral resolution of unnatural amino acid amides.

**Chapter 5: Use of immobilized *Aspergillus melleus* aminoacylase for enantioselective synthesis of unnatural amino acids**

Macroporous functional polymers containing surface epoxy groups were synthesized for immobilization of *Aspergillus melleus* aminoacylase. The effect of cross-link density of polymer on enzyme immobilization was studied. The novel styrenated acrylic ter-polymers gave maximum aminoacylase activity recovery (75.47%). Immobilized polymers were characterized for pH, temperature and storage stability. Immobilization of aminoacylase on styrenated ter-polymers gave excellent thermal stability to the enzyme. A kinetic model of thermal inactivation was derived to quantify the extent of thermal stability conferred to aminoacylase by immobilization. Immobilized aminoacylase catalyzed enantioselective synthesis of unnatural amino acids was studied.

**Chapter 6: Preparation of cross-linked enzyme aggregates of *Aspergillus melleus* aminoacylase for enantioselective synthesis of unnatural amino acids**

The cross-linked enzyme aggregates (CLEA) of aminoacylase were prepared via co-aggregation of the enzyme with polyethylenimine (PEI). The PEI-enzyme co-aggregates were stabilized by cross-linking between primary amino groups of the PEI and the primary amino groups of enzyme using glutaraldehyde. The method described gave physically stable CLEAs and no release of enzyme was found upon prolonged storage. The process parameters such as PEI:enzyme ratio, glutaraldehyde concentration and time of glutaraldehyde treatment necessary to form stable CLEA were optimized. Under the optimum conditions, PEI-aminoacylase CLEA expressed 74.90% activity recovery with 81.20% aggregation yield. The thermal inactivation kinetics of soluble enzyme and PEI-aminoacylase CLEA was studied. The results suggest that the co-aggregation gave excellent thermal stability to the enzyme.
Finally, PEI-aminoacylase CLEA were employed for synthesis of enantiopure unnatural amino acids.

**Chapter 7: Preparative scale enantioselective synthesis of vicinal diols using immobilized Solanum tuberosum epoxide hydrolase**

The recombinant plasmid (pGEF-StEH) containing functional gene of Solanum tuberosum epoxide hydrolase was inserted in Escheria coli BL21(DE3) strain. The recombinant E. coli cells were grown in LB medium at 37°C for about 16 h in a 5L fermenter. The extracellular enzyme was isolated from the fermentation broth. The enzyme was immobilized by multipoint covalent attachment on glyoxyl-agarose support. Immobilized enzyme gave ~ 150 units epoxide hydrolase activity per gram of support. Further, the immobilized enzyme was characterized with respect to pH stability, temperature stability and miscible/immiscible solvent stability. The immobilized epoxide hydrolase was used for enantioselective production of two vicinal diols (namely: phenylethane diol and m-chloro-phenylethane diol) via hydrolysis of their corresponding epoxides (namely: styrene oxide and m-chloro-styrene oxide respectively). The effect of incorporation of ionic liquids and organic solvents in aqueous reaction medium on enzymatic hydrolysis of epoxides was studied. The ‘Regio-selectivity constants’ for enzymatic hydrolysis of styrene oxide and m-chloro-styrene oxide were calculated. The preparative scale production of (R)-phenylethane diol and (R)-m-chloro-phenylethane diol (on gram scale) was studied using stirred cell bioreactor. The bioreactor performance was evaluated over 10 repeated cycles for production of each diol.

**Chapter 8: Conclusions**

This chapter recapitulates the significant findings of the present work and delineates the concluding remarks.

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CHAPTER 1: Introduction


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