ASYMMETRIC SYNTHESIS OF DRUG INTERMEDIATES USING MICROORGANISMS

Thesis
Submitted in partial fulfillment of the requirement for the degree of
DOCTOR OF PHILOSOPHY

By

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DEPARTMENT OF CHEMICAL ENGINEERING
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DECLARATION

By the Ph.D Research Scholar

I hereby ***declare*** that the Research Thesis entitled **Asymmetric synthesis of drug intermediates using microorganisms** which is being submitted to the **National Institute of Technology Karnataka, Surathkal** in partial fulfillment of the requirements for the award of the degree of **Doctor of Philosophy** in **Chemical Engineering** is a ***bonafide report of the research work carried out by me***. The material contained in this research thesis has not been submitted to any University or Institution for the award of any degree.

Signature of the Research Scholar

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Date:

CERTIFICATE

This is to certify that the Research Thesis entitled **Asymmetric synthesis of drug intermediates using microorganisms** submitted by **Brahmani Priyadarshini S.R** (Register Number: CH06P02) as the record of the research work carried out by her is *accepted as the Research Thesis submission* in partial fulfillment of the requirements for the award of degree of **Doctor of Philosophy**

Research Guide

Dr. Gopal Mugeraya

Chairman, DRPC

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ABSTRACT

There has been an increasing awareness of the enormous potential of microorganisms and enzymes to transform synthetic chemicals, in a highly chemo, regio and enantioselective manner. In the present work, an attempt was made to evaluate the potential of some selected fungi species to effect reduction of selected ketones and oxidation of selected sulphides.

Saccharomyces cerevisae was selected for evaluating the effect of various physicochemical parameters on the (microbial induced) biotransformation, as it showed considerable reduction of the substrate, 3-[5-[(4-flurophenyl)-1, 5, dioxopentol]- yl] –4-(S) phenyl oxazolidin-2-one. The experimental results showed that maximum reduction occurred at pH of 7.6, temperature of 30° C, incubation period of 48 hours and biomass concentration of 6 g. High concentration of substrate had a negative effect on the rate of bioconversion. It was found that the organic solvents adversely affected the enzymatic reaction, while use of surfactant had a favorable effect on bioconversion. The interaction of various culture condition variables on reductase activity was also studied using *S. cerevisiae.* Central composite design and Response surface methodology were used in the design of the experiments and analysis of the results. The model could be successfully used for accurately predicting enzyme activity based on a new set of independent variables.

Rhizopus stolonifer was found to be effective in carrying out sulphoxidation of omeprazole intermediate and the various factors affecting the reaction were studied. The ideal reaction time for the conversion was found to be 48 hours. A substrate concentration of 0.04% was found to be well-tolerated by the microbial cell. The pH and temperature of the reaction mixture in the range selected, did not affect the enzyme activity significantly. Biosulphoxidation in water miscible solvents was significantly low while the reaction did not proceed in water immiscible solvents. Use of β-cyclodextrin and surfactants in the reaction did not increase the product yield. The study on variation in the culture conditions revealed that glucose as the carbon source was most suitable for sulphoxidation. The addition of organic nitrogen and divalent metal ion salts did not have

any significant effect on the ability of the organism in bringing about sulphoxidation. Change in initial media pH and temperature did not significantly affect bioconversion.

Key words: Bioreduction, Biosulphoxidation, Microorganisms, Ketones, Response surface methodology.

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NOMENCLATURE

MTCC	Microbial Type Culture Collection Center
ee	Enantiomeric excess
g	Gram
mg	Milligram
h	Hour
LC - MS	Liquid chromatography mass spectrometry
HPLC	High pressure liquid chromatography
min	Minute
SLS	Sodium Lauryl Sulphate

CTAB	Cetyltrimethyl ammonium bromide.
rev min^{-1}	Revolutions per minute
IR	Infra red spectroscopy
DMSO	Dimethyl sulphoxide
ml	Milli liter
TLC	Thin layer chromatography
µg	Microgram
µl	Microliter
MDC	Methylene di chloride
CNS	Central nervous system
AUC	Area under the curve
NADH	Nicotinamide adenine dinucleotide- Hydrogen

CHAPTER 1

INTRODUCTION

1.1 Chirality in drugs

Chirality in chemical compounds has been known since the eighteenth century when Louis Pasteur observed that he could separate the crystals of the different isomeric forms of tartaric acid. The synthesis of enantiomerically pure compound is becoming important for research and development especially in pharmaceutical industry, as chiral drugs now represent one-third of all pharmaceutical sales worldwide (Stinson 2000, Mc Conathy and Owens 2003, Shafatti 2007). Drug enantiomers should be considered as different chemical entities, because they often differ considerably in their potency, pharmacodynamic and pharmacokinetic profile (Hutf and Grady 1996, Federse 1994). This is because the modules with which they interact in the biological system are also stereospecific, i.e., proteins (receptors, enzymes), nucleic acids (DNA and RNA), and biomembranes (phospholipids and glycolipids) (Somogyi et al. 2004, Rouhi 2004, Caldwell 2001).

The interactions between a drug and the biopolymers in the body, that elicit therapeutic or adverse effects, require a specific three-dimensional configuration of drug and biopolymer. Since enantiomers have different three-dimensional configurations, the pharmacodynamics and pharmacokinetics of the two enantiomers, which make up a racemic drug, can be quite different (Williams 1989, Crom 1992, Eichelbaum 1988).

The differences often depend on the center of asymmetry of the drug in close proximity to the points of attachment to the biopolymer.

For example:

- (S)-Ibuprofen is over 100-fold more potent an inhibitor of cyclo-oxygenase-I than (R)-Ibuprofen
- (R)-Methadone has a 20-fold higher affinity for the Aμ opioid receptor than (S)-Methadone
- (S)-Citalopram is over 100-fold more potent an inhibitor of serotonin reuptake transporter than (R)-Citalopram

The inactive enantiomer or distomer (one that has much less affinity for the drug's target site) is not necessarily an inert substance with no effects *in vivo* (Caner et al. 2004)**.** For example, the cardiotoxicity of bupivacaine is mainly associated with the (R)-enantiomer, the psychomimetic effects of ketamine are more associated with the (R)-enantiomer, and (S)-baclofen antagonises the effects of (R)-baclofen. The beneficial effects of a drug can therefore reside in one enantiomer (eutomer), with its paired enantiomer having:

- No activity
- Some activity
- Antagonist activity against the active enantiomer
- Distinct therapeutic response or adverse activity from the active enantiomer.

Since absorption, metabolism, distribution and elimination of drugs in the body also involve their interaction with proteins, the pharmacokinetics of enantiomers can also be different.

For example:

- The bioavailability of (R)-verapamil is more than double that of (S)-verapamil due to reduced hepatic first-pass metabolism.

- The volume of distribution of (R)-methadone is double that of (S)-methadone due to lower plasma binding and increased tissue binding.
- The clearance of (R)-fluoxetine is about four times greater than (S)-fluoxetine due to a higher rate of enzyme metabolism.
- The renal clearance of (R)-pindolol is 25% less than (S)-pindolol due to reduced renal tubular secretion.

These differences in clearance and volume of distribution translate into differences in their half-lives. In addition, these pharmacokinetic properties can be modified in a stereoselective manner by disease, genetics, ethnicity, age and other drugs. Finally, the enantiomers of some drugs such as warfarin can be metabolized by different enzymes.

According to the Federal Drug Administration (FDA), USA, the physiological effects of both the enantiomers should be studied. FDA policy published in 1992 makes it mandatory for companies to evaluate racemates and individual enantiomers in detail before applying for new drugs. Even though in many cases a single-enantiomer drug is safer than a racemate, an effective racemate can still be marketed.

Advantages of enantiopure drug:

- An improved safety margin through increased receptor selectivity, potency, and reduced adverse effects.
- A longer or shorter duration of action due to pharmacokinetic considerations (e.g. half-life) resulting in a more appropriate dosing frequency.
- Decreased inter-individual variability in response commonly due to polymorphic metabolism.
- Decreased potential for drug-drug interaction.

To avoid adverse effects and optimize the therapeutic value of enantiomeric drugs, it is necessary that methods for the synthesis of one enantiomer be evolved. The production of chiral intermediate and ingredients have become increasingly important in the pharmaceutical industry.

1.2 Methods of synthesis of single enantiomer

There are three ways of synthesizing chiral compounds (Forman 2000). (Fig 1.2).

A. Chiral pool
B. Resolution of racemic mixtures
C. Asymmetric synthesis

A simpler method to obtain an enantiopure drug is to use molecule from the chiral pool. The chiral pool consists of naturally occurring chiral molecules, such as amino acids or sugars, which are modified to make useful intermediates, while keeping their chirality intact. Chiral pool synthesis is used to build a part of the epothilone molecule (an alternative to paclitaxel from readily available enantiopure (-)-pantolactone.

The other conventional method is to take a racemate and then separate the two enantiomers. The major disadvantage of this method is that the maximum yield is only 50 percent. The various methods of resolution of a racemic mixture are preferential crystallization, kinetic resolution, conversion to diastereoisomers and chromatographic separation.

In preferential crystallization, a supersaturated solution of the racemic mixture is treated with a crystal of preferred enantiomer, whereupon this form is precipitated. However, this method is not practical for all types of racemic mixtures.

Kinetic resolution is a process in which the differences in the rate of reaction of the enantiomer with a reagent, are used. Enzymes are particularly effective in making this distinction, so that a racemic mixture can often be easily resolved by reaction with some

simple substance in the presence of the chiral enzyme as catalyst. The enantiomer, which reacts faster, will be converted to new compound having an entirely different functional group, while the enantiomer which reacts more slowly will remain unchanged. As an example, if the racemic mixture is having an alcoholic functional group, it can be converted to an acetate ester by reaction with acetic acid in the presence of a suitable esterifying enzyme. The separation of the ester of one enantiomer from the alcohol of the other is then very easy. Ester can then be hydrolyzed to the alcohol, if desired, by either simple chemical means or by enzyme catalyzed reaction, to regain the enantiopure alcohol.

Separation by fractional crystallization, a common strategy for resolution of mixtures cannot be applied to racemic mixtures as enantiomers exhibit same solubility. This problem can be overcome by reacting a racemic mixture with a pure enantiomer of a chiral compound (Fig 1.1). This will form a compound with two chiral centers, and will give rise to two different diastereoisomers, having different solubilites and hence can be separated from each other easily. Following this separation, the chiral resolving agent can be removed through some chemical reaction to retrieve the two separated enantiomers. The chiral resolving agent can also be recovered for re-use. This method of converting enantiomers into diastereoisomers for resolution of racemic mixture is the most widely used.

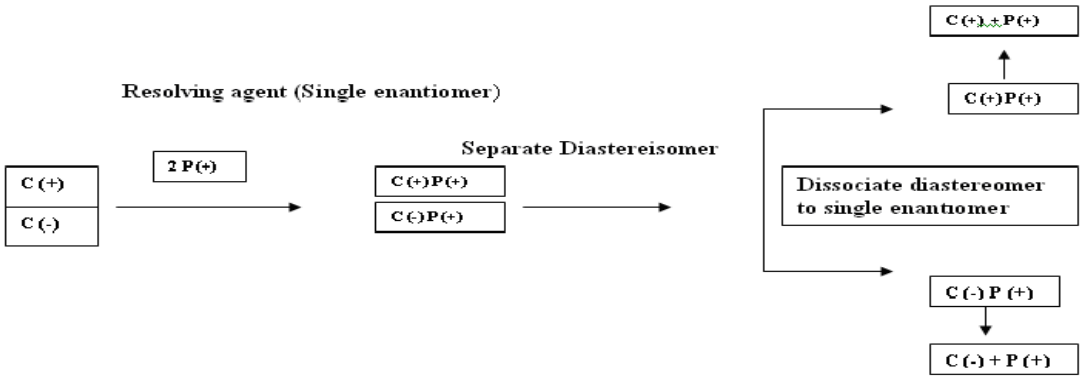


Fig. 1.1: Resolution of racemic mixture via conversion to diastereoisomers.

1.2.1 Asymmetric synthesis:

This is a reaction or reaction sequence that selectively creates one configuration of one or more stereogenic elements by the action of a chiral reagent or auxiliary, acting on heterotropic faces of atoms or groups of a substrate. The stereoselectivity is primarily influenced by the chiral catalyst, reagent or auxillary, despite any stereogenic elements that may be present in the substrate (Gawley and Aube 1996, Trost 2004)

In an asymmetric synthesis, the enantiomers (or diastereomers) of a chiral product are formed in different yields. In effect, the synthesis can be stereoselective if it displays a diastereomeric transition state, because the transition states for the formation of the different stereoisomeric products usually exhibit different energy levels. As a result, the activation energies differ from each other. Consequently, the stereoisomer whose formation requires lower activation energy is preferably formed. The stereo-selectivity increases with growing energy difference in the transition states. If a manufacturing process can be identified that selectively makes just one enantiomer, then this will almost certainly be cheaper and more effective in the long run. Almost 35 per cent of active pharmaceutical ingredients are made by chemo-catalysis, using some form of chiral catalyst or auxiliary to force the reaction to produce one enantiomer or the other. In the

recent years, the growing importance of chiral molecules in the pharmaceutical marketplace has driven the development of a vast toolbox of chiral synthesis techniques that can be applied on a production scale.

Many scientists working in chemical synthesis and drug discovery are striving to find new methods of asymmetric synthesis that would lead to the development of new and exciting chrial auxillaries. In addition, asymmetric catalysis is emerging as a new method for obtaining enantiomerically pure compounds and has fuelled rapid growth in the field of chemical synthesis.

Mostly three different kinds of chiral catalyst are employed,

1. Metal ligand complexes derived from chiral ligands.
2. Chiral organo catalyst
3. Biocatalysts

1.2.2 Asymmetric biocatalysis:

This method of asymmetric synthesis involves using enzymes or whole cells as biological catalysts. A major selectivity advantage of biocatalyst over traditional systems includes the former's ability to exhibit high stereoselectivity. While enzymes represent the most efficient catalytic systems known, their impact on the chemical industry relative to traditional catalyst is still meager.

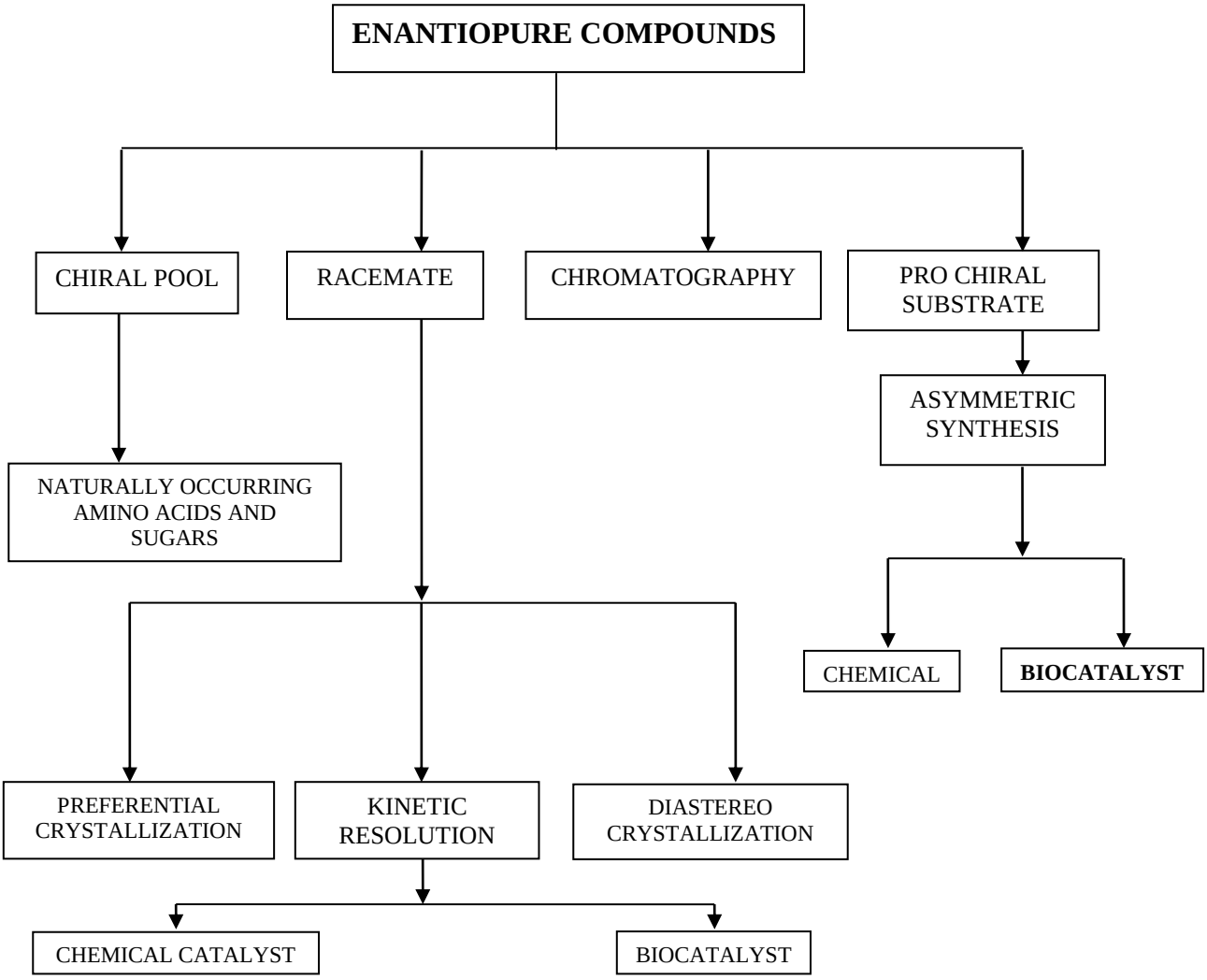


Fig. 1.2: Methods for synthesis of enantiopure compounds.

1.3 ADVANTAGES AND DISADVANTAGES OF BIOCATALYSIS OVER CHEMICAL SYNTHESIS (Faber 2004, Bommarius and Riebel 2004)

1.3.1 Advantages:

1. Biocatalytic reactions are often highly enantioselective and regioselective.

2. They can be carried out at ambient temperature and atmospheric pressure; thus, avoiding the use of more extreme conditions that can cause problems with isomerization, racemization, epimerization and rearrangement.

3. Microbial cells and enzymes derived from them can be immobilized and reused for many cycles.

4. Enzymes can be over-expressed to make biocatalytic processes economically efficient and enzymes with modified activity can be tailor made.

5. Preparation of thermo stable and pH stable enzymes by random and site directed mutagenesis has led to the production of novel biocatalysts.

1.3.2 Disadvantages:

1. *Narrow operation parameters:* Elevated temperature, extreme pH conditions, high pressure and high salt concentration can lead to deactivation of the proteins. Therefore, if the reaction proceeds too slowly under the given parameters of temperature and pH, there is a narrow operational window for alteration.

2. *Biocatalytic activity in organic phase:* Majority of organic compounds are poorly soluble in water and water is the less suitable solvent for most of the organic reactions. Thus, shifting of the enzymatic reaction from aqueous to an organic medium is preferred. But this can cause loss of enzymatic activity due to the toxic effects of organic solvents on enzymes.

3. *Biocatalysts are prone to inhibition:* Many biocatalytic reactions are prone to substrate/product inhibition. This factor also affects the efficiency of the process significantly.

1.4 FREE ENZYMES VERSUS WHOLE CELL SYSTEMS

Biocatalysis can be performed by using either isolated enzymes or whole cells of microorganisms in free / immobilized forms. The method employed will depend on the

1. Type of reaction.
2. Requirement of co-factors.
3. Scale of biocatalysis.

The advantages and disadvantages of using whole cell systems in comparison to free enzyme:

Table 1.1: Reactions using whole cells systems

Serial no.	Advantages	Disadvantages
1	Expensive co-factors not necessary.	The substrate may undergo more than one biochemical reaction as whole cells contain a complete metabolic complement of enzymes resulting in low product yield.
2	The system is more suited for multistep process requiring several enzymes.	Product purification is complicated due to the presence of other metabolic by-products, cell components and cell growth media components.
3	The process is cost effective compared to pure enzymes as it requires inexpensive co-substrates.	Maintenance of aseptic condition is critical as biological contamination may interfere with the product yield.
4	The whole cell systems are more stable compared to pure enzymes as they are not easily affected by environmental factors.	Semi-permeable nature of cell may complicate substrate entry and/or product release from the cell.

Table 1.2: Reactions using free enzyme

Serial no.	Advantages	Disadvantages
1	High activity resulting in better yield.	Expensive co-factor recycling is necessary.
2	Pure enzymes catalyze only a specific reaction therefore there is no risk of by-products or product breakdown.	Purified enzymes are expensive.
3	Downstream processing of the product is easier as compared to whole cell system as there are no interference of by products and cell components.	Some enzymes are unstable and sensitive to changes in environmental conditions.

1.5 BIOTRANSFORMATION PROCESS

In growing culture: In this method, the substrate is added to the fermentation medium at the time of inoculation or during a later phase of microbial growth. Later, it is incubated until maximum yield of transformation has been reached. The principle behind this method is that the expression of required enzyme for the desired biotransformation may be enhanced by induction, if the substrate is added during active growth of the microorganism. This process is suitable for inducible enzymes.

With resting cells: This method involves cultivation of microorganisms under conditions optimum for maximum growth, the actual biotransformation being performed in a second step. This method is applicable where substrate inhibits cell growth and the enzymes involved in the conversion are constitutive enzymes, production of which does not get affected by the chemical environment.

1.6 MECHANISM OF STEREOSELECTIVITY (Faber 2004)

Kinetic reasons for selectivity: As in other catalytic reactions an enzyme (E) accelerates the reaction by lowering the energy barrier between substrate (S) and product. The stereoselectivities of enzymes originate from the energy differences in the enzyme transition state complex [ES]. In an enantioselective reaction, either the enantiomeric substrates A and B or the two forms of mirror image orientation of a prochiral substrate involving its enantiotropic groups or faces, compete for the active site of the enzyme. Due to the chiral environment of the active site of the enzyme, diastereomeric enzyme-substrate complex (EA) and (EB) are formed, which possess different values of free energy (ΔG) for their respective transition states [EA]* and [EB]*. This brings in differences in activation energy (ΔΔ G*) for both of the enantiomeric substrates resulting in one of the enantiomer getting transformed faster than the other. This process is generally referred to as chiral recognition.

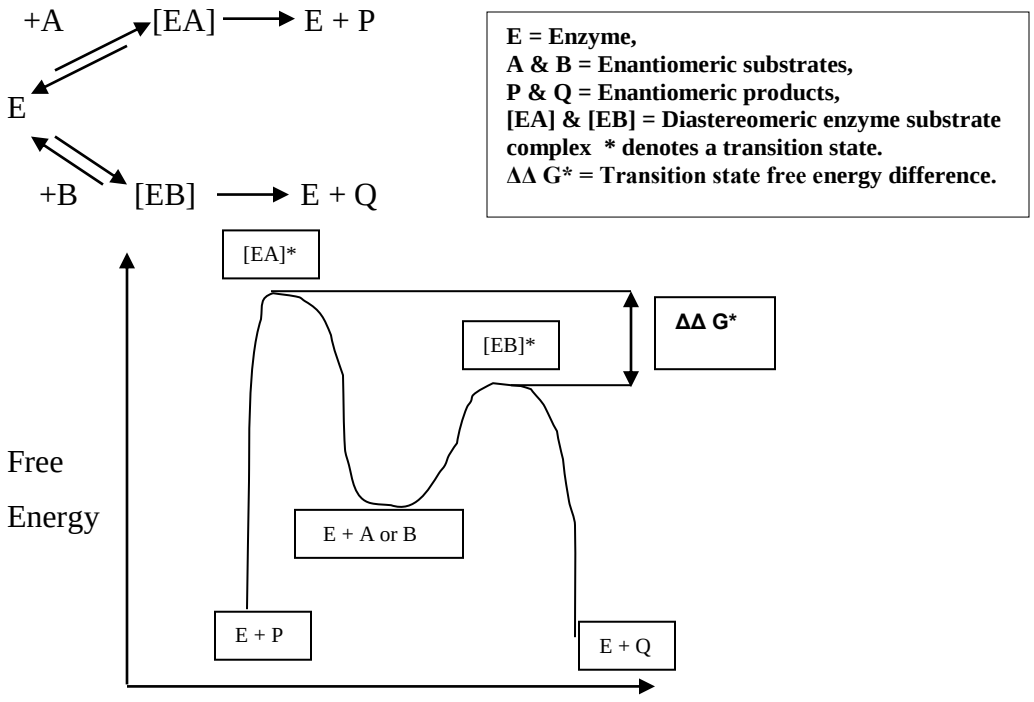


Fig. 1.3: Energy diagram of catalyzed versus uncatlyzed reaction.

The values of these differences in free energy, expressed as ΔΔ G*, is a direct measure of selectivity of the reaction which, in turn depends on the ratio of the individual reaction rates (V_A, V_B) of enantiomeric substrates A and B. The values of ΔΔ G* determine the optical purity of the product.

Table 1.3: Free energy values for optical purity of product

ΔΔG* (Kcal/mol)	**V_A/ V_B**	**ee%**
0.188	1.2	10
0.651	3.0	50
1.740	19.0	90
2.17	39.0	95
3.14	199.0	99
4.50	1999.0	99.9

From the above table it can be observed that even a remarkably small difference in free energy i.e. 1.74 Kcal/mol leads to a considerable enantiomeric excess of the product. For absolute selectivities, ΔΔ G* has to be ≥ 4.5 Kcal/mol.

Biocatalysts (as both isolated enzyme and whole-cell systems) are increasingly being used to assist in synthetic routes to make complex molecules of industrial interest. Currently, the biggest role for biocatalysis still remains in the pharmaceutical sector (Thayer 2006, Patel 2007), where its exquisite regioselective and stereoselective properties enable difficult synthesis (often requiring multiple protection and de-protection steps) to be circumvented. In the past few years, the number of processes that employ biocatalytic methods has grown rapidly as techniques that make the reactions more reproducible and give better yields, had been developed. A survey indicated that biological methods will account for almost a third of the total market for chirals, reaching revenues of USD 3.3 billion, having grown at 25 percent a year.

1.7 ENANTIOSELECTIVE BIOCATALYTIC REDUCTION OF KETONES

The enantioselective reduction of ketones to produce optically active alcohols is an important reaction as these form important building blocks for the production of various optically active pharmaceuticals. Therefore numerous efficient asymmetric catalytic routes based on various concepts have been identified, of which, asymmetric synthesis with chiral metal complexes as catalysts has been successfully used (Noyori 1994). However, some difficulties remain in attaining high optical purity and practical usage in comparison with the ones performed by enzymatic catalysis. In most cases, an enzyme as biocatalyst dramatically improves stereo chemical quality (Faber 2004). Biocatalysis has turned out more and more to be an alternative, highly competitive technology for the asymmetric ketone reductions (Nakamura et al. 2003). The recent increase in the number of industrial applications of the biocatalytic asymmetric ketone reductions underlines the tremendous potential for this type of white biotechnology in large scale manufacture of enantiomerically pure alcohols.

1.7.1 Concept of biocatalytic reduction: (Faber 2004, Patel 2002)

The enzymes employed in redox reactions are basically classified into three categories: 1. Dehydrogenases, 2. Oxygenases and 3. Oxidases. Among these, dehydrogenases have been widely used for the reduction of carbonyl groups of aldehydes or ketones and of carbon-carbon double bonds. As reduction generates a stereogenic center, the desymmetrization of prochiral carbonyl compounds is predominant. Redox reactions require redox co-factors, which donate or accept the chemical equivalents during reduction. For majority of redox enzymes, Nicotinamide adenine dinucleotide [NAD(H)] and its respective phosphate [NADP (H)] are required as co-factors. Reduction with NADH is as follows,

1. Co-enzyme and substrate bind to the enzyme.
2. The substrate is reduced, while the co-enzyme gets oxidized.
3. The co-enzyme and product dissociate from the enzyme.

The detailed study of the reaction mechanism of NAD(P)H dependent dehydrogenase (Matsuda et al. 2009) explains that there are four stereochemical patterns for transfer of a hydride from coenzyme, NAD(P)H, to substrate. The hydride attacks either si-face or re-face of the carbonyl group depending on the orientation of the binding of the substrate to the enzyme, which results in the formation of (R) and (S) alcohols respectively. On the other hand, enzyme transfers either pro-(R) or pro-(S) of the co-enzyme depending on the kind of enzyme.

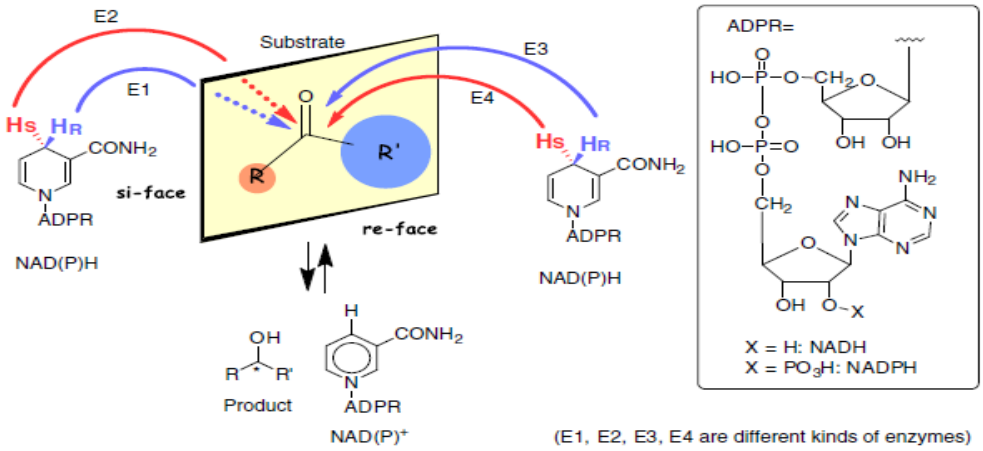


Fig. 1.4: Reaction mechanism of NAD(P)H dependent dehydrogenase.

1.7.2 Reduction of Aldehydes and ketones using whole cells:

In comparison with isolated enzymes, whole-cell bioconversion has distinctly different characteristics (Goldberg et al. 2007). Whole cell biocatalysis is usually more stable as the enzymes involved are in their natural environment. Since isolated dehydrogenases require sophisticated co-factor recycling, whole microbial cells can be employed in place of isolated enzymes. They contain multiple dehydrogenases which are able to accept a wide variety of unnatural substrates, all the necessary cofactors and the suitable metabolic pathways for their regeneration. Thus, co-factor recycling can be omitted as it is automatically done by the cell. Consequently, cheap carbon sources such as glucose or saccharose can be used as auxiliary substrates in asymmetric reduction reactions.

However, there are some drawbacks in the method:

- The productivity of microbial conversion is usually low as the majorities of unnatural substrates are toxic to living organisms and are therefore only tolerated at low concentrations.

- The large amount of biomass present in the reaction medium brings in inhibition factor resulting in low overall yields and makes product recovery troublesome.

- Chiral transport into and out of the cell may influence the specificity of the reaction particularly when racemic substrates are used.

Some of these problems can be overcome by screening of microorganisms to obtain most suitable strains with optimum properties and variation in the fermentation conditions.

1.7.3 Whole cell microbial biotransformations in organic solvents:

Nature has designed its biocatalysts to perform best in an aqueous surrounding, neutral pH and temperatures below 50ºC. However, these conditions are often contrary to the requirements of the process engineer or chemist to optimize a reaction with respect to volumetric productivity or any easy downstream processing when substrates and/or products are not easily soluble in water. To overcome these bottlenecks, addition of organic solvents is a common practice (Vermue and Tramper 1995, Silva et al. 2010). The solvent can either be water-miscible or water-immiscible, resulting in single or biphasic systems.

Depending on the solvent phase, biocatalytic transformations performed in organic media offer the following advantages: (Leon et al. 1998)

- The overall yields of processes performed in organic media are usually better due to the omission of extraction step during work-up. Thus, the loss due to formation of emulsion can be avoided and the recovery of products is facilitated by the use of low boiling organic solvents.

- Non polar substrates are transformed at better rates due to their increased solubility.

- Since an organic medium is a hostile environment for living cells, microbial contamination is negligible.

- Deactivation and/or inhibition of the enzymes caused by lipophilic substrates and/or products are minimized as their solubility in the organic medium leads to a reduced local concentration at the enzyme's surface.

- Many side reactions such as hydrolysis of labile groups like epoxides, acid anhydrides, polymerization of quinines, racemization of cyanohydrins or acyl-migration are water dependent and are therefore largely suppressed in an organic medium.

- Immobilization may not be necessary because they may be recovered by simple filtration after the reaction, due to their insolubility in organic solvents.

Choice of Solvent: The choice of the organic solvent is mainly influenced by enzyme behavior and by the partitioning behavior of the substrate and product. There is no general rule as to which solvent is enzyme friendly. To a certain extent log P concept can be used as guidelines in order to provide a measure for the compatibility of an organic solvent. Reliable results have been obtained by using the logarithm of the partition coefficient (log P) of a given solute between octanol and water (Faber 2004, Bie et al. 2008).

Table 1.4: Compatibility of solvents with enzymatic activity

Serial No.	Log P	Water Miscibility	Solvent – Effects on Enzyme Activity
1	-2.5 to 0	Completely miscible	May only be used to solubilize lipophilic substrates in concentrations of 10-50% v/v without deactivating the enzyme.
2	0 to 1.5	Partially miscible	Causes serious enzyme distortion; may only be used with unusually stable enzymes but deactivation is common.
3	1.5 to 2.0	Low miscibility	Causes some enzyme distortion; may be used with caution with many enzymes but activities are often unpredictable.
4	> 2.0	Immiscible	Causes negligible enzyme distortion and ensures high retention of activity.

1.7.4 Microbial transformations in presence of surfactants and a hydrophilic carrier:

Most of the pharmaceutical intermediates are hydrophobic in nature and this poses problem in biotransformation in aqueous media, where contact between substrate and enzyme is critical. This problem may lead to lower productivity which can be circumvented by using either a suitable surfactant (anionic, cationic or nonionic) (Goswami et al. 2000) or a hydrophilic carrier like beta cyclodextrin (Szejtli 1990).

Surfactants are known to play a vital role in many chemical and biochemical processes. One important property of the surfactant is the formation of colloidal size clusters in solution known as micelle. Surfactant micelles are capable of increasing the solubility of most organic molecules in water. The mechanism by which this solubilization occurs is by the incorporation of organic molecules into the hydrophobic interior part of the micelle.

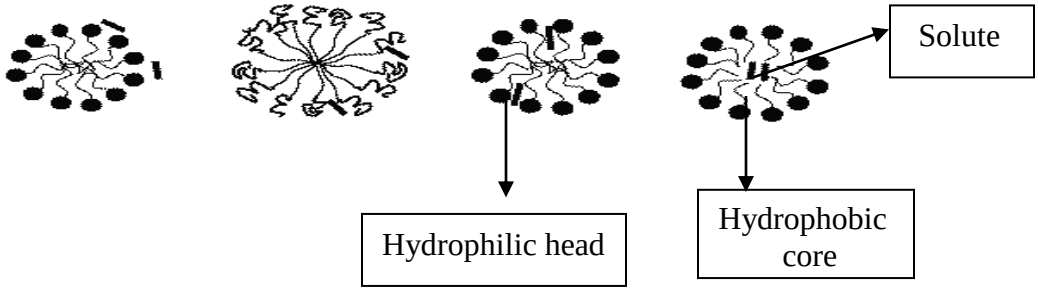


Fig. 1.5: Micelle with the hydrophobic solute.

Cyclodextrins are cyclic oligosaccharides with a hydrophilic outer surface and a hydrophobic central cavity. The hydrophilic exterior renders the cyclodextrin water soluble and the hydrophobic interior provides a microenvironment for relatively nonpolar molecules. In aqueous solutions, cyclodextrins can form inclusion complexes with hydrophobic compounds by entrapping either the entire molecule or a nonpolar part of it inside the hydrophobic cavity and thereby enhance the solubility of the solute.

1.8 Microbial Suphoxidation:

A number of sulphur compounds are known in which the sulphur is bonded to three substituents and also retain a lone pair of electrons. These types of compounds, in suitable cases, exhibit chirality. Sulphoxides also have a structure with three different substituents and a lone pair of electrons around a sulphur atom and hence can exhibit chirality. Several techniques are currently available to obtain enantiomerically pure sulphoxides. These include asymmetric oxidation of thioethers, asymmetric synthesis by nucleophilic substitution on chiral derivatives and kinetic resolution (Polyzos 2003, wojaczynska and wojaczynski 2010).

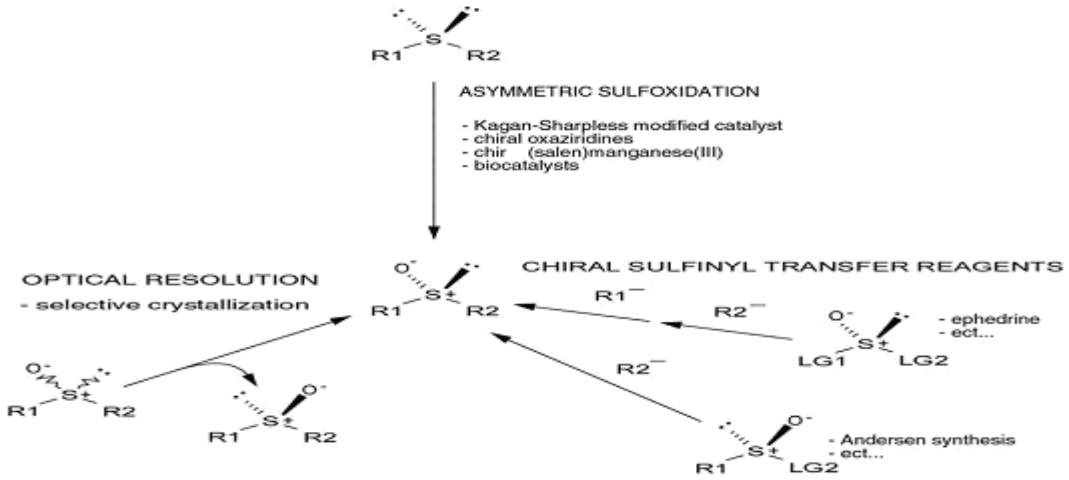


Fig. 1.6: Methods of preparation of enantiopure sulphoxide.

The most widely used method towards obtaining non-racemic sulphoxide is the oxidation of parent sulphide. Several oxidative methods have been applied to this task, with varying selectivity both in the stereochemistry of the reaction and the range of substrates that are accommodated. But, sulphoxidation in chiral environments is either limited to specific substituents or gives only moderate to low ee of product and use of chiral oxidants can be similarly unrewarding (Holland 1988). In contrast, one of the earliest methods of preparing chiral sulphoxides, microbial biotransformation, has not been extensively employed for synthetic purposes. The oxidative metabolism of many biomolecules containing sulphur provides a large assortment of enzymes for this chemistry. Despite the proven success of this method in performing stereospecific asymmetric oxidation of certain prochiral sulphides and the associated developments in the application of isolated oxidase enzyme methodology, studies on the synthesis of optically active sulfoxides (Holt et al. 1996) are relatively rare; partly due to the small number of pharmaceuticals containing sulfoxide groups and partly due to the fact that enzymes which react with the sulphur center are not available commercially. Therefore,

further work is still necessary to identify new substrates and microorganisms which will prove useful for the production of chiral sulphoxides.

The use of enzymes as reagents in organic synthesis provides exciting opportunities for the exercise of regio and stereoselectivity. In case of oxidative reactions catalyzed by oxygenase enzymes, whole microbial cells in an actively growing or resting stage are commonly employed. In general, organic sulfides, along with other xenobiotic organic compounds are oxidized both *in vivo* and *in vitro* by one or both of two types of monooxygenases enzymes, those dependent upon cytochrome P-450 for activation and transfer and those using a flavin molecule for the purpose (Holland et al. 1995). It has generally been assumed that the fungal enzymes responsible for oxidation of sulphide and sulphoxide are cytochrome P-450 containing monooxygenases. Whole microbial cells in an actively growing or resting state are commonly employed for the same purpose. Whole cell systems such as the fungi *Aspergillus niger*, *Mortierella isabellina*, *Helmenthosporium* sp. and bacterium *Rhodococcus equi* have been sussessfully employed in sulphoxidation.

Two important limitations hinder the biotechnological applications of oxidative enzymes in the synthesis of chiral sulphoxides. Many sulfides which are commonly used as substrates are sparingly soluble in water. This phenomenon can be responsible for the relatively low reaction rates observed for enzymatic sulfoxidations. Moreover, the enantiomeric purity of the desired product is sometimes lowered by the non-enzymatic reaction of substrate with the oxidant. These problems can be at least in part overcome by simply changing the reaction medium. It is now well established that many enzymes retain their catalytic properties while switching the solvent from water to an organic one. Such a change has a great impact on a structure and stability of a biocatalyst and on the catalytic action itself. The use of organic media instead of water leads to suppression of undesired side reactions and increases the solubility of hydrophobic reactants. Along with the ease of recovery of some products and of insoluble biocatalyst, these advantages

make nonaqueous systems an attractive alternative for water conducted biotransformations.

1.9 EFFECT OF MEDIA COMPOSITION ON BIOTRANSFORMATION

The basic nutrients for the growth of microorganisms include carbon, nitrogen and minerals. There is an array of carbon and nitrogen sources to choose. Once a culture media is developed, the next phase is to study the interactions of various media components to achieve best possible benefits. This is a tedious and labor intensive task. The usual, conventional "one variable at a time" approach is used; but it is time consuming and leads to confusion in understanding the process (Ye 2008). Response surface methodology (RSM), a collection of mathematical and statistical techniques for building empirical models, is gaining recognition and has proved to be useful for developing and improving the media composition for the production of industrially important products such as chemicals and enzymes (Khurana et al. 2007).

OBJECTIVES

An extensive review of literature has led to the understanding that there are still unexplored avenues for some of the pharmaceutical intermediates for their microbial conversion potential. Hence, the investigation of few fungi species for their bio-reductive ability on three ketone drug intermediates and bio-sulphoxidative property on two sulphide intermediates was designed.

The substrates selected for microbial conversion were,

1. 2 - Deoxy-2, 2 -difluro-D-erythro-pentofuranose-1-ulose 3,5 dibenzoate.
2. 3-[5-[(4-flurophenyl)-1, 5, dioxopentol]-yl] -4-(S) phenyl oxazolidin-2-one.
3. 4(3', 4'-dichlorophenyl)-2, 3-dihyrdronaphthone.
4. 5-methoxy-2-(((4-methoxy-3, 5-dimethyl-2-pyridinyl) methyl) sulfinlyl)-1H-benzimidazole.
5. 2[[[3-methyl-4-(2, 2, 2-trifluroethoxy)-2-pyridyl] methyl] sulfinlyl]-benzimidazole.

Ten fungal species were selected for screening, for their potential to effect bio-reduction and bio-sulphoxidation of selected substrates. The following species cultures were obtained from MTCC, Chandigarh.

1. *Saccharomyces cerevisiae* MTCC 174
2. *Pichia farinosa* MTCC 246
3. *Aspergillus niger* MTCC 961
4. *Candida viswanathii MTCC 1629*
5. *Rhizopus stolonifer* MTCC 2198

Few other fungi species, namely,

1. *Aspergillus niger*
2. *Aspergillus flavus*
3. *Aspergillus oryzae*
4. *Pencillium* species

which were isolated in the microbiology lab, were also roped in for screening, for their ability to effect reduction and sulphoxidation. In addition, dried baker's yeast, available locally, was also evaluated for its bio-conversion property.

It is an established attribute, that the biocatalytic reactions are affected by various parameters like incubation time, incubation temperature, pH of the reaction media, biomass concentration and substrate concentration.

Therefore, the objectives of the current research work were,

- To screen a few selected fungi species, for their bio-reductive ability on the selected ketone substrates.
- To screen a few selected fungi species, for their bio-sulphoxidative function on the selected sulphide substrates.
- To evaluate the effect of various physical parameters, namely, pH, incubation temperature, incubation time, biomass concentration, substrate concentration, organic solvents and surfactants on bioreduction and biosulphoxidation.
- To study the effect of culture conditions on production of carbonyl reductase and apply response surface methodology to evaluate the interactions of the variables.
- To examine the effect of culture conditions on sulphoxidation.

The following chapters provide a detailed account of the research plan and the methodical evaluation of the work to achieve these objectives.

Chapter one represents a general introduction to chiral drugs and biocatalysis. It emphasizes the need for the synthesis of an enantiopure drug. It also describes various aspects of biocatalysis and its advantages over chemical synthesis.

Chapter two is a comprehensive review of literature encompassing general biocatalysts, microbial reduction of ketones, biocatalysis in biphasic systems, biocatalysis in the presence of anionic and cationic surfactants, culture media optimization and microbial sulphoxidation.

Chapter three explains bioreduction in detail. It includes the materials used in the experiments carried out, and also provides a detailed description of the methodology employed. This chapter also discusses the results obtained.

Chapter four accounts for the study of culture conditions for the production of carbonyl reductase in *Saccharomyces cerevisiae.*

Chapter five discusses the process of microbial sulphoxidation.

Chapter six comprises of study of effect of culture media conditions on sulphoxidation by *Rhizopus stolonifer.*

Chapter seven summarizes the complete project and construes the results to provide conclusions.

CHAPTER 2

LITERATURE REVIEW

2.1 GENERAL BIOCATALYSIS

There has been an increasing awareness of the enormous potential of using microorganisms and enzymes for the transformation of synthetic chemicals in a highly chemo-, regio-, and enantioselective manner. This field is being explored exhaustively. Biocatalysis of enantiopure compounds for pharmaceutical intermediates has gained momentum due to the advances made in genomics, screening and evolution of technologies leading to the increased availability of new robust biocatalyst suited for industrial scale application.

Straathof et al. (2002) are of the opinion that biocatalysis is the standard technology for the production of chemicals. This has been proved by number of biotransformation processes running on a commercial scale. The review gives a quantitative analysis of the industrial biotransformations and it reveals overall applications of biocatalysis in the pharma sector.

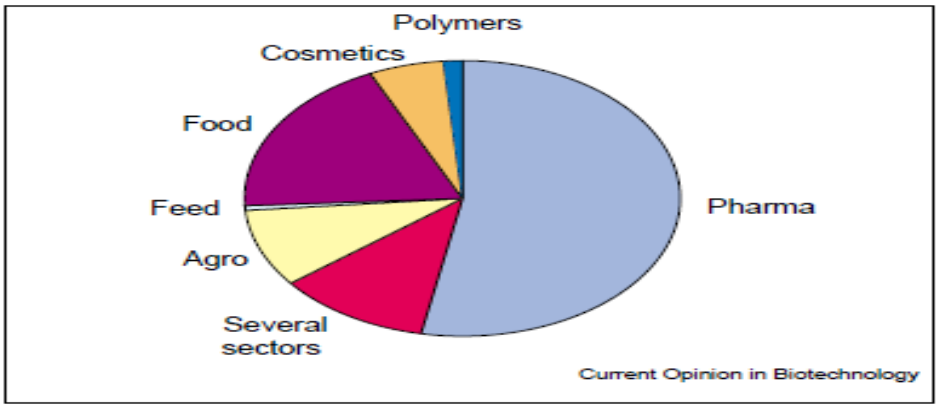


Fig. 2.1: Industrial sectors in which the products of industrial biotransformations are used.

Most of the biocatalytic research involves hydrolytic enzymes and only a quarter of the studies deal with oxidoreductases and around 15% with other categories. Whole cells are more popular than isolated enzymes.

Panke and Wubborts (2005) have reviewed the various biocatalytic synthesis of pharmaceutical intermediates with multiple chiral centers created by biocatalyst. Omapatrilat, an antihypertensive, is one such example and the scheme illustrates (Fig 2.2) the use of different enzymes to obtain enantiopure intermediate without affecting the sensitive functional groups.

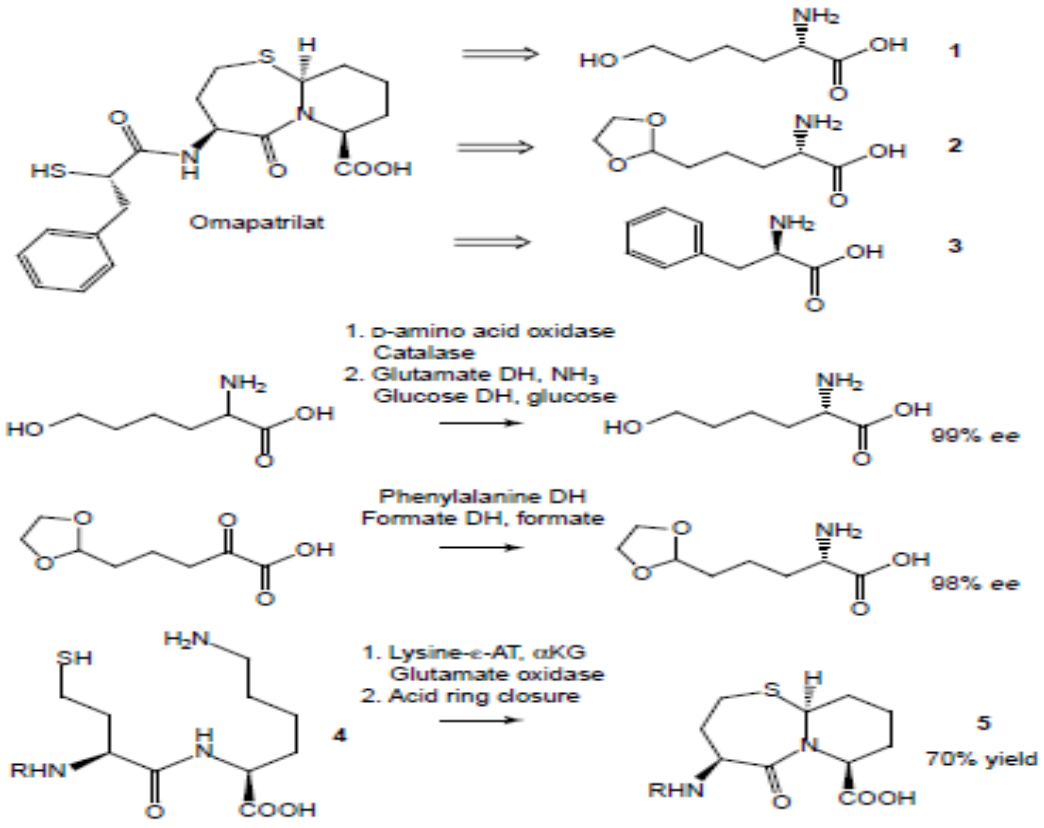


Fig. 2.2: Biocatalysis of omapatrilat intermediates.

The other example of biocatalytic synthesis is the manufacturing of a complex pharmaceutical ingredient, 3, 5 - dihydroxyhexanoate side chain of statin. Patel (2002) gives an exhaustive review of various microbial/ enzymatic processes for the synthesis of chiral intermediates for anticancer (Fig 2.3), antiviral, antihypertensive, anti cholesterol and calcium channel blockers. The use of different classes of enzymes for the catalysis of different types of chemical reactions enables generation of a wide variety of chiral compounds (Table 2.1).

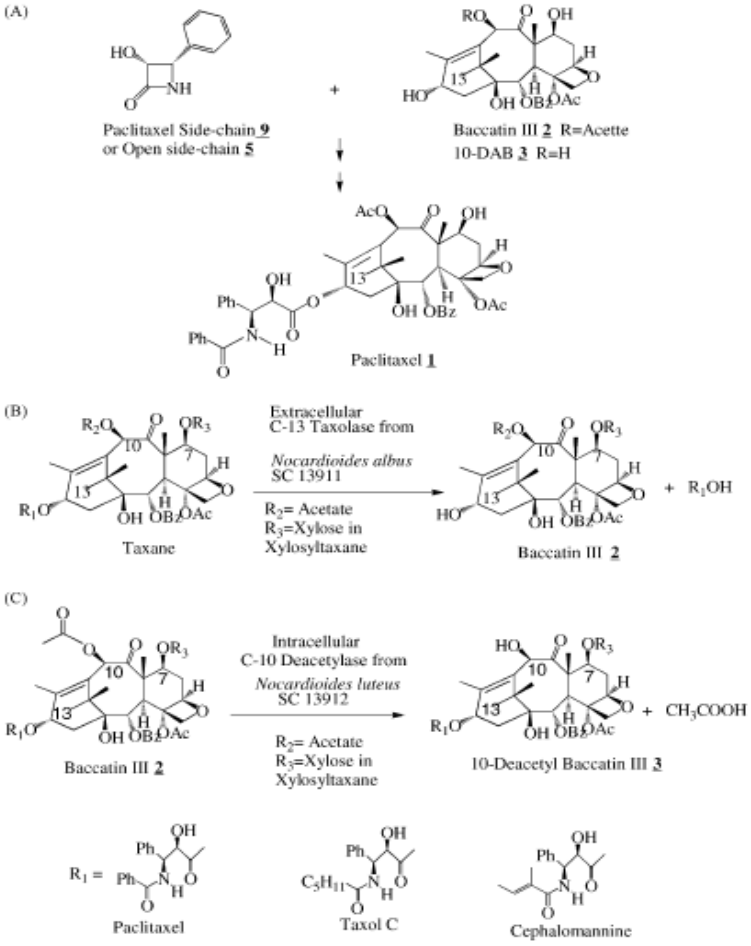


Fig. 2.3: Semi synthesis of paclitaxel.

Table 2.1: Biotransformation of drug intermediates

Drug intermediate	Microorganism/ Enzyme used	Reaction type	Product
2-keto-3(N benzoyl amino)-3 phenyl propionic acid ethyl ester	*H.polymorpha* SC 13865 and *H.fabianii* SC 13894	Reduction	(2R,3S)-N-benzoyl -3-phenyl isoserine ethyl ester
Racemic cis -3-(acetyloxy)-4-phenyl-2-azetidinone	Lipase PS 30 from *Ps. Cepacia*	Hydrolysis	S acetate
N-ε-Carboxybenzoxy L-Lysine	*Providencia alcalifaciens* SC 9036	Oxidative deamination	Keto acid
(exo,exo)-7-oxabicyclo[2.2.1] heptane 2,3 dimenthol	*Nocardia globerula* ATCC 21505 and *Rhodococcus sp* ATCC 15592	Oxidation	Corresponding lactase and lactol
Taxane	C-13 taxolase from *Nocardioides albus* SC 13911	Cleavage of C-13 side chain.	Baccalin III
2-keto-6-hydroxy hexanoic acid sodium salt	Phenylalanine dehydrogenase from *Sporosarcina* sp	Reductive amination	(S) – 6- Hydroxy norleucine
Racemic 2 methyl 4-methoxy phenyl alanine amide	Amidase from *Mycobacterium neoaurum*	Resolution	Chiral amino acids
Phenyl pyruvate condensation with aldehydes	*Achromobacter erydice*	Acyloin condensation	Optically active acyloins

This includes the use of hydrolytic enzymes like lipases, esterases, proteases, dehalogenases, acylases, amidases, nitrilases, lyases, epoxide hydrolases, decarboxylases and hydantoinase in the resolution of racemic compounds. Oxidoreductases and amino transferases have been used in the synthesis of chiral alcohols, amine alcohols, amino acids and amines. Aldolases and decarboxylases have been effectively used in asymmetric synthesis by aldol condensation and acyloin condensation reactions. In the course of last decade progress in biochemistry, protein chemistry, molecular cloning, random and site directed mutagenesis and fermentation technology has opened up unlimited access to a variety of enzymes and microbial cultures as tools in organic synthesis.

Faber and Patel (2000) in their editorial overview have opined that biocatalytic approach for the asymmetric synthesis of chiral drug intermediates is one of the alternative routes. Microbial cells and enzymes are now indispensable tools in the arsenal of synthetic organic chemist and biotechnologist. The techniques are increasingly being used for the asymmetric synthesis of pharmaceuticals, agro chemicals, vitamins and flavors. The authors are of the opinion that the potential of biocatalyst is far from being fully exploited.

Huisman and Gray (2002) wrote about the role of biocatalyst in the pharmaceutical and fine chemical industries. The article gives various technological innovations to develop an economical biocatalytic process like novel methods of cofactor regeneration concepts, enzyme manipulation, process guided catalyst development to name a few.

The development of novel biocatalytic methods is a continuously growing area of chemistry, microbiology, and genetic engineering due to the fact that biocatalysts are selective, easy-to-handle, and nature friendly (Borges et al. 2009). A wide range of reactions are catalyzed by microorganisms. Fungi can be considered as a promising source of new biocatalysts, mainly for chiral reactions. Chemo-, regio- and stereoselective processes are very important in the synthesis of many chemical, pharmaceuticals, and agrochemical intermediates; active pharmaceuticals; and food

ingredients. This report reviews stereoselective reactions mediated by fungi, such as stereoselective hydroxylation, sulfoxidation, epoxidation, Baeyer–Villiger oxidation, deracemization, and stereo- and enantioselective reduction of ketones, published between 2000 and 2007.

Wohlgemuth (2010) is of the view that microbial enzymes and cells continue to be important tools for preparing asymmetric molecules. Recent developments in the discovery, development and production of stable biocatalysts, in the design of new biocatalytic processes and in the product recovery and purification processes have made biocatalytic approaches using microbial cells and enzymes attractive choices for the synthesis of chiral compounds. Apart from use of hydrolytic enzymes, oxidoreductases, transferases and lyases have also found increasing applications in synthetic reactions. The production of a wide variety of industrial products by asymmetric biocatalysis has become the preferred method of synthesis.

Latest advances for asymmetric synthesis through reduction and oxidation including deracemization by biocatalysts has been reviewed (Matsuda et al. 2009). Newly developed methodologies as well as practical applications have been discussed in the article.

Drepper et al. (2006) in their review article discusses the uses of microorganisms and enzymes to manufacture a large variety of chemical products. The article also describes new methods for isolation of new enzyme genes, expression systems, isolation and characterization of biocatalyst relevant for the preparation of enantiopure compounds.

Biocatalysis is becoming a key component in the toolbox of the process chemist (Pollard and Woodley 2007). This has become all the more important because of the high demand for enantiopure compounds from the pharmaceutical industry. The integration of biology and engineering with chemistry has contributed to the success of biocatalysis.

2.2 Bioreduction

A review of asymmetric bioreduction of pharmaceutically relevant prochiral ketones has been done (Chartrain et al. 2001). From the data analyzed, it is clear that microbial screens lead to the identification of appropriate biocatalysts and also that there is a need to evaluate a relatively large and diverse microbial population. The authors have concluded that asymmetric bioreduction can be very valuable. Process development studies have demonstrated that optical purity can in some cases be controlled and the yields can be optimized to result in viable processes.

Set of microorganisms consisting of different strains belonging to bacteria and fungi were screened for the stereoselective reduction of carbonylic compounds (Carballeira et al. 2004). *Gongronella butleri*, *Diplogelasinospora grovesii* and *Schizosaccharomyces octosporus* were selected as the most interesting strains according to their productivity, their tolerance to high concentrations of ketones and the absence of secondry products in the reduction of cycloalkanones. The reduction of (4*R*) - or (4*S*)-carvones showed that the stereochemistry of the ketones determine the reduction pathway. *D. grovesii* and *S. octosporus* were the strains that displayed better selectivity. The use of resting cell conditions or the lyophilised whole cells reduced the catalytic activity.

A comparative study of enantioselective reduction of prochiral ketones by using whole cells of the white-rot fungus *Merulius tremellosus* ono991 and ruthenium(II) - amino alcohol and iridium(I) - amino sulfide complexes as metal catalysts has been carried out (Hage et al. 2001). Comparison of the results showed that the corresponding chiral alcohols could be obtained with moderate to high enantioselectivities (e.e.s of up to 98%). The biocatalytic approach was found to be most suitable for the enantioselective reduction of chloro-substituted aryl ketones, whereas in the reduction of α,β-unsaturated compounds excellent results were obtained using the metal complex catalysts.

The enantioselective bioreduction of a number of fluoroacetophenones was carried out with whole cells of *Rhizopus oryzae* CCT 4964, *Aspergillus terreus* CCT 3320 and *Aspergillus terreus* CCT 4083 giving the corresponding alcohols in good yield and high enantioselectivity (Comasseto et al. 2003). The results showed that these microorganisms had great potential to perform bioenzymatic reduction and deracemization reactions (Fig 2.4). The enantioselectivity was found to be anti-Prelog in some cases.

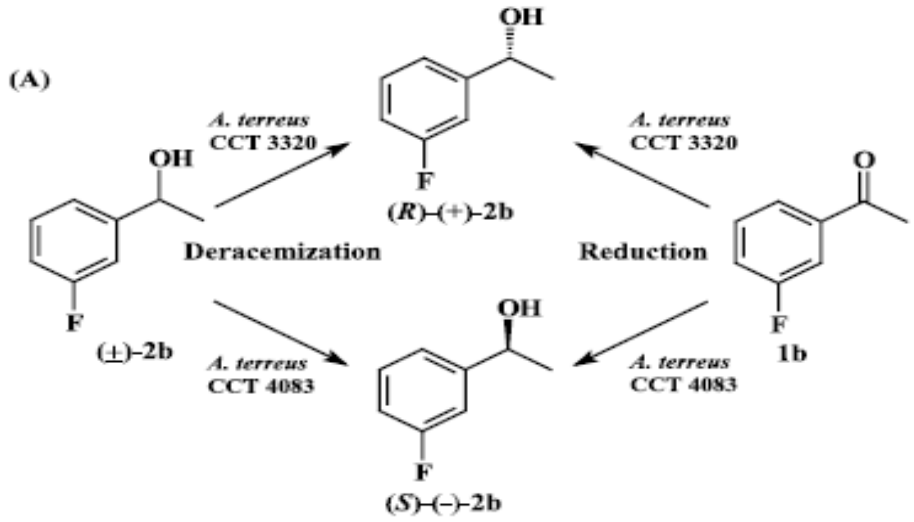


Fig. 2.4: Bioreduction of fluroacetophenones.

The favorable microbes for the asymmetric reduction of prochiral aromatic ketones was isolated from soil using acetophenone as the sole carbon source and the asymmetric reduction of acetophenone to chiral α-phenethyl alcohol was chosen as the model reaction (Yang et al. 2008). Two microbial strains with excellent catalytic activity were identified. They were *Geotrichum candidum* and *Pichia pastoris*. The product of the asymmetric reduction of ketone catalyzed by *Pichia pastoris* was mainly R- alcohol and that by *Geotrichum candidum* was mainly S-alcohol. The yield and enantiomeric

excesses of 75% and 90% respectively for *Pichia pastoris* could be achieved and 80% and 70% for *Geotrichum candidum* could be achieved.

A practical and highly efficient biocatalytic synthesis of optically active (R)-4-fluorophenylethan-1-ol has been developed based on reduction of the corresponding 4-fluoroacetophenone in the presence of a tailor-made recombinant whole-cell biocatalyst, containing an alcohol dehydrogenase and a glucose dehydrogenase (Groger et al. 2007). The reaction proceeded in a pure aqueous solvent media at a substrate concentration of 0.5 M, and gave the desired product with high conversion (>95 %), good yield (87 %) and with an excellent enantioselectivity of >99 %. In addition, activity tests further showed that the analogs 2- and 3- fluoroacetophenones were also promising substrates.

Production of a key chiral component for L-carnitine synthesis by the reduction of γ-chloro-β-ketobutyric acid ethyl esters using whole cells has been carried out (Popa et al. 2008). Several microbial strains of genera *Saccharomyces, Candida, Hansenula, Aspergillus* and *Lactobacilli* were screened for their ability to perform the reduction of the selected substrates. The optimal conditions for both stages of the bioprocess were established. Both stereoisomer of γ-chloro-β-hydroxybutyric acid ethyl ester were obtained under different biotransformation conditions. In aqueous medium, the S-isomer was obtained, while in organic media the product has been predominantly of R-configuration.

A practical method for producing S(-)-1-(1 napthyl) ethanol with high yields and excellent selectivity (>99%) has been reported (Roy et al. 2003) Resting cells of *Geotrichum candidum, Candida paraprilosis, Yarronia Lipolytica* and *Rhizopus arrhizus,* were employed for the bioreduction of 1- acetonapthone at 28°C. *Geotrichum candidum* and *Candida paraprilosis* have shown excellent results with respect to yield and selectivity of above 99 %. The pH of the medium, substrate concentration and time of incubation were optimized for the maximum conversion. A new technique of using resins as adsorbent has been employed to control the toxicity of product and substrate on the cell.

Diastereo and enantioselective bioreduction of 4-tert-butylcyclohexanone, 4-methyl cyclohexanone and 2-methyl cyclohexanone was attempted by using Brazilian strain of the bacteria *Serratia rubidaea* CCT 5742 (De Conti et al. 2001). The biotransformations were conducted with resting cells (biomass/substrate ratio being 30/1) in phosphate buffer solution of pH 7.0 at 30ºC and 110 rpm for 48 h. The substrates were reduced to corresponding alcohols with high distereo and enantio selectiveness.

Reduction of 1 - chloro-3-(1-napthyloxy) propan-2-one to (R) or (S) 1-chloro-3-(1-napthyloxy) propan-2-ol which is a 2-propranolol precursor has been achieved by using microbial cells (Lagos et al. 2002). Several yeast strains were screened for the bioreduction. The best strains were selected using the reduction of cyclohexanone as the model substrate. The stereoselective reduction of the substrate was performed using both actively fermenting cells and fresh resting cells. Among the strains screened, *Pichia menicana* 11015 resting cells and *Yarrowia lipolytica* 1240 resting cells gave good yield and ee ie 85% yield (ee 95%) and 87% (ee 99%) respectively.

Three new yeasts, *Saccharomyces bayanus* CECT 1317, *Yarrowia lipolytica* CECT 1240 and *Pichia mexicana* CECT 1015, were selected after a taxonomical screening, for the reduction of aryloxy-halo-2-propanones and 1-chloro-3(phthalimdyl)-propan-2-one (Lagos et al. 2004). *P.mexicana* (reduction of aryloxy-halo-2-propanones) and *S. bayanus* (the reduction of 1-chloro-3(phthalimdyl)-propan-2-one), gave ees greater than 90% and yields higher than 85% for the (R)-or (S)-halohydrins, respectively.

A total of 310 microorganisms (53 bacteria, 113 yeasts and 144 molds) have been evaluated for their ability to bioreduce the bisaryl ketone to its corresponding alcohol (Chartrain et al. 2000). Only eight strains were able to carry out the desired reaction (Fig 2.5). Evaluation of the optical purity of the alcohol produced by these eight microbial strains revealed that either enantiomer could be produced with elevated ee. *Rhodotorula pilimanae* ATCC 32762 which gave high ee of 98% was taken for scale up studies. The scale up process was performed in 23 L fermentor to get yield of 1.5 g of the (S) enantiomer.

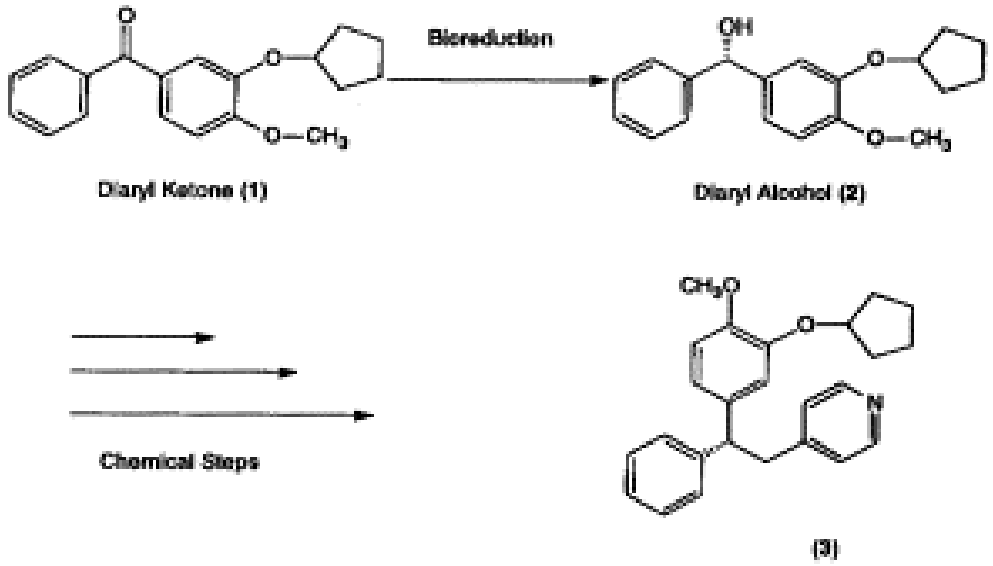


Fig. 2.5: Bioreduction of bisaryl ketone.

A new yeast strain, *Candida viswanathii* MTCC 5158, isolated from soil, was capable of carrying out enantioselective reduction of 1-acetonapthone to S(-)-(1-napthyl) ethanol which is an important synthetic intermediate of mevinic acid analogue (Kamble et al. 2005). The production of the carbonyl reduction enzyme was enhanced by addition of acetonapthone and mannitol to the growth media. The optimal temperature and pH for the bioreduction were 25ºC and 8 respectively. The organism produced enantiopure (S) - alcohol with good conversion (>97%) and ee of 99% (Fig 2.6). The optimized substrate and resting cell concentration was 2 and 200g/L respectively.

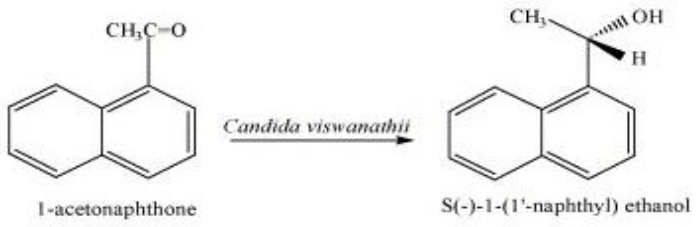


Fig. 2.6: Enantioselective bioreduction of 1-acetonapthone by *Candida viswanathii* MTCC 5158.

Aspergillus niger EBK-9 isolated from soil was tested for the production of (*R*)-1-(4-Bromo-phenyl)-ethanol from 1-(4-Bromo-phenyl)-ethanone (Zilbeyaz and Kurbanoglu 2008). The isolated strain was found to be an effective biocatalyst with a good enantiomeric excess and good conversion (Fig 2.7). Ram horn wastes which are organic wastes and rich in protein content and other basic nutrients was used for the cultivation of microorganism, thereby reducing the overall cost of the bioconversion.

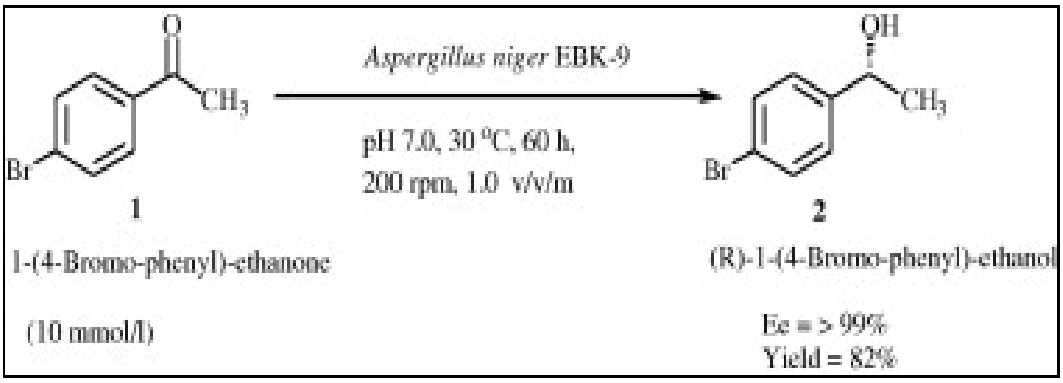


Fig. 2.7: Reduction of 1-(4-Bromo-phenyl)-ethanone by *Aspergillus niger* EBK-9.

The effect of pH on the bioconversion of (R) -1(4-bromo-phenyl)-ethanone by using *Aspergillus niger* has been studied. pH 7.0 gave the highest ee of 99.9% and conversion of 94.7% for the reduction time of 48 h (Abas et al. 2010).

Reduction of various ketones like benzaldehyde, ethyl-4-Chloro-3-oxybutyrate, 2-decalone, 4R carvone, 4S carvone and adamantavone has been carried out using *Monascus kaoliang* CBS 302.78 (Quezada et al. 2009). The reduction was carried out with various co-substrates like glucose (1%), saccharose (0.25%, 0.5% and 1%) and isopropanol (0.25%, 0.5% and 1%). Isopropanol was found to be the best co-substrate. The paper also describes the optimum growing conditions and also a novel immobilization technique by adsorption in polyurethane foam.

Asymmetric reduction of 4-(trimethylsilyl)-3-butyn-2-one to enantiopure (R)-4-(trimethylsilyl)-3-butyn-2-ol was conducted with high enantioselectivity using immobilized whole cells of a novel strain *Acetobacter* sp. CCTCC M209061 (Xiao et al. 2009). Compared with other microorganisms that were investigated, *Acetobacter* sp. CCTCC M209061 was shown to be more effective for the bioreduction reaction which afforded much higher yield and product enantiomeric excess (ee). The optimal buffer pH, co-substrate concentration, reaction temperature, substrate concentration and shaking rate were 5.0, 130.6 mM, 30ºC, 6.0 mM and 180 rpm respectively. Under the optimized conditions, the maximum yield and the product ee were 71% and >99% respectively, which are much higher than those reported previously. Additionally, the established biocatalytic system proved to be efficient for the bioreduction of acetyltrimethylsilane to (R)-1-trimethylsilylethanol with excellent yield and product ee.

Yamadazyma farinosa IFO 10896 was found to reduce 2-hydroxyketones bearing a phenyl ring to give optically active diols with anti-Prelog selectivity (Tsujigami et al. 2002). The reduction was performed using resting cells in a phosphate buffer under an atmosphere of argon. The distance between the carbonyl group and the phenyl ring was shown to have an effect on the reactivity and selectivity of the enzyme system. The enantioselectivity was anti-Prelog. The number of atoms between the carbonyl group and phenyl ring had a clear effect on the enantioselectivity of the enzyme, nearer the phenyl rings to the carbonyl group, the higher the ee of the product.

Different microorganisms were tested for the enantioselective reduction of ethyl 2-oxo-4 phenylbutyrate in aqueous medium for the preparation of ethyl(R) -2-hydroxy -4-phenylbutyrate, a key intermediate in the production of angiotensin converting enzyme (ACE) inhibitors (Lacerda et al. 2006). Among the organisms screened, *Pichia angusta* showed 100% conversion and 81% ee of the product.

Synthesis of ezetimibe requires enantiopure 3-[5-(4-fluorophenyl)- 5(S)- hydroxy pentanoyl]- 4(S)-4-phenyl-1,3-oxazolidin-2-one as a crucial intermediate which is produced by reduction of the corresponding prochiral ketone. *Burkholderia cenocepacia,*

a new biocatalyst was screened for bioreduction of the above prochiral ketone (Singh et al. 2009). Various physicochemical conditions were optimized to increase cell mass and enzyme activity. The overall increase in cell mass concentration and enzyme activity was 2.06 and 1.85 fold respectively. Various reaction conditions, for example pH, temperature, and agitation rate and cell mass concentration were optimized for maximum product yield with excellent enantioselectivity. Best reduction was achieved in phosphate buffer (50 mM, pH 8.0) at 40°C (200 rpm) and the yield of enantiopure alcohol from the corresponding prochiral ketone was 54%.

Asymmetric reduction of the prochiral aromatic ketone catalyzed by yeast cells is one of the most promising routes to produce its corresponding enantiopure aromatic alcohol. The toxicity of aromatic ketone and aromatic alcohol to the yeast cell was investigated and it has been found that the aromatic compounds are poisonous to the yeast cell (Yang et al. 2008). The activity of yeast cell decreased steeply when the concentration of acetophenone was higher than 30.0 mmol/L. Asymmetric reduction of acetophenone to chiral *S*-phenylethyl alcohol catalyzed by the yeast cell was chosen as the model reaction to study in detail the promotion effect of the introduction of the resin adsorption on the asymmetric reduction reaction. The resin acts as the substrate reservoir and product extraction agent *in situ*. It has been shown that the reaction could be remarkably improved with this technique when the appropriate kind of resin is applied. The enantioselectivity and yield are acceptable even though the initial ketone concentration reaches 72.2 mmol/L.

A highly stereoselective bioreduction of 2-alkyl-4,4,4-trichloro-2-butenals mediated by baker's yeast (*Saccharomyces cerevisiae*) has been described (Li et al. 2007). The E-isomers were regiospecifically converted into the saturated alcohols with high enantioselectivity, whereas the Z-isomers generated in situ by a competitive one-way isomerization were reduced only to the corresponding allyl alcohols during the reactions.

(1S, 2S)-Ephedrine, one of the four ephedrine isomers, is a common pharmacological agent; which is difficult to synthesize in a stereospecific manner using traditional organic

synthesis. A novel enzymatic process has been developed for the stereoselective synthesis of (1S, 2S)-ephedrine (Zhang et al. 2009). *Morganella morganii* CMCC (B) 49208 was found to asymmetrically reduce the prochiral carbonyl compound 1-phenyl-1-oxo-2-methylaminopropane (MAK), to optically pure (1S, 2S)-ephedrine which was identified and quantified by thin layer chromatography and high performance liquid chromatography technologies. In addition, a conversion process using air-dried *M. morganii* CMCC (B) 49208 cells was developed to produce (1S, 2S)-ephedrine at a final concentration of 0.852 g/L (>99% ee) and 84.4% molar yield.

An efficient whole cell biotransformation process using *Lactobacillus kefir* was developed for the asymmetric synthesis of tert-butyl (3R, 5S) 6-chloro-dihydroxy hexanoate, a chiral building block for the HMG-CoA reductase inhibitor (Amidjojo et al. 2005). The effects of buffer concentration, temperature, pH and oxygen on the asymmetric reduction were investigated in batch reactions. Improvements in final product concentration and yields of 153% and 79% with respect to the batch-process were achieved in an optimised fed-batch process.

Several strains of native microorganisms from Brazilian biomass like *Aspergillus terreus* CCT 3320, *Aspergillus terreus* UKM 3570, *Aspergillus niger* CCT 1078, *Rhizopus oryzae* CCT 4964 etc., were screened for their ability to enantioselectively reduce carbonyl groups present in propiophenones and mannich base hydrochlorides (Raminelli et al. 2007). Good results were obtained when the propiophenones were reduced to (S) alcohol. The biotransformation reactions of para-methoxy propiophenone using different microorganisms did not lead to desired alcohol in satisfactory enantiomeric excesses and conversion. The mannich bases, when subjected to biotransformation using whole cells of Brazilian microorganism, afforded the corresponding alcohol and the alcohol was obtained in high ee and conversion ie ee 99% and 100% respectively.

The enantioselective reductions of various heteroaryl methyl ketones, such as 2,3 and 4 acetyl pyridines, 2 acetyl thiophene, 2-acetyl furan and 2-acetyl pyrrole was carried out with the resting cells of novel yeast strain *Candida viswanathii* (Soni et al. 2005).

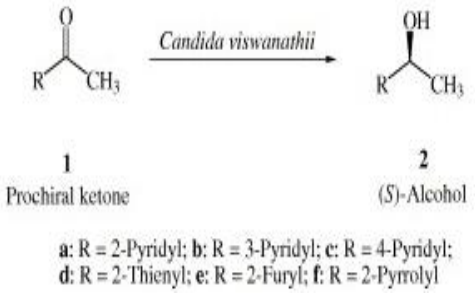


Fig. 2.8: Enantioselective bioreductions of various heteroaryl methyl ketones.

Excellent results have been reported with acetyl pyridines, moderate conversion with 2-acetyl thiophene and no significant reduction with 2-acetyl furan and 2-acetyl pyrrole. Preparative scale reduction of 3-acetyl pyridine has been carried out with excellent yield and almost absolute enantioselectivity (ee>99.9%).

The asymmetric hydrogenation of methyl acetoacetate was successfully performed with baker's yeast in pure glycerol and mixtures of glycerol and water (Wolfson et al. 2008). Though yeast viability was very low after exposure to glycerol, the enzymatic activity in pure glycerol was preserved for some days. In addition, a mixture of glycerol and water combined offered the advantages of each individual solvent and resulted in high catalytic performance and efficient product extraction.

United states patent no.6133001 claims the stereo selective microbial reduction for the preparation of 1-(4-flurophenyl)-3(R)-[3(S)- hydroxy-3- azetidione (Homann, and Previte 2000). The organisms screened for the reduction were *Rhodococcus fascians, Geotrichum candidum*, and different species of *Aspergillus, Mucor, Sacchromyces* and *Pichia. Rhodococcus* gave the best yield of 46%.

2.3 Biocatalysis in organic solvents

Reduction of keto esters with bakers yeast in organic solvents have been reported (Rotthaus et al. 1997). The ee of reductions of keto esters by *S.cerevisiae* has been altered in organic solvent such as diethyl ether, toluene, hexane and ethyl acetate compared to corresponding transformation in water.

The toxic effect of various solvents with different Log *P* values was studied on the whole cells of *Candida viswanathii* (Kansal and Banerjee 2009). Experiments with acetophenone as the model substrate showed that the lower concentrations of some solvent increased both the activity retention and enzymatic activity as compared to the control while this was not the case with higher concentrations of the same solvents. The percentage conversion improved from 76 to 94%.

Whole cells of *Saccharomyces uvarum* SW-58 were applied in an aqueous-organic solvent biphasic system for the asymmetric reduction of ethyl 4,4,4-trifluoroacetoacetate (1) to ethyl (*R*)-4,4,4-trifluoro-3-hydroxybutanoate [(*R*)-2] (He et al. 2007). The results of reduction in different aqueous-organic solvent biphasic systems showed that dibutylphthalate provided the best compromise between the biocompatibility and the partition of substrate and product among the solvents used. Reaction conditions were optimized by studying the effect of various factors like reaction pH, temperature, shaking speed, volume ratio of the aqueous phase to the organic phase and ratio of biomass to substrate. The bioconversion in the biphasic system was more efficient compared with that in the monophasic aqueous system, and product concentration as high as 54.6 g/L was reached in the organic phase without addition of co-enzyme.

The hydrophobic phenyl n-propyl ketone was used as a model compound to examine alcohol dehydrogenase activity in *S. cerevisiae* mediated cell culture (Cheng and Tsai 2008). Parameters such as pH, hexane to water volume percentage and the amount of co-factor Zn^{2+} ion for either cell growth or reduction were studied to see their effect on the enantio selectivity towards the production of alcohol. The pH for reduction in the

aqueous portion of the biphasic culture was 5.0. Without Zn^{2+} ion, the biphasic cultures of middle to high hexane-to –water volume percentage exhibited an R isomer with an ee of 53.7% to > 99%. Exclusively, the enantioselectivity for biphasic cultures containing Zn^{2+} ion was an S isomer of the alcohol with ee of 27.5% to > 99%. Therefore, the authors conclude that the enantioselectivity of *S. cerevisiae* mediated biphasic culture reduction of phenyl- n-propyl ketone can be manipulated thro the cofactor Zn^{2+} ion and hexane volume of the biphasic system

Asymmetric reduction of 2-octanone in water/organic solvent biphasic system has been carried out with Baker's yeast FD-12 (Li et al. 2007). Seven water/organic solvent biphasic systems were assayed and FD-12 showed the best tolerance, catalytic activity and enantioselectivity in the water/*n*-dodecane system among the biphasic systems assayed. The metabolic activity retention (MAR) and viability of FD-12 in the *n*-dodecane containing system were 98% and 91.6%, respectively. The reaction conditions were optimized and it was found that at Vaq/*V*org 20/5, pH 8.0, 32°C, 2-octanone concentration 500 mM, the final concentration of 2-octanol and the ee.(*S*) value reached 105.60 mM and 89.38% respectively, after 96 h reduction.

α,β-unsaturated carbonyl compounds were used as substrates in biotransformation reactions mediated by three industrial *Saccharomyces cerevisiae* strains in biphasic systems (Silva et al. 2010). Several reaction parameters were evaluated, such as yeast concentration, temperature, pH, substrate concentration, organic solvent, volume of aqueous and organic phases and the influence of substituent groups on chalcones were evaluated. The highest conversion (>99%) to the dihydrochalcone was obtained at 30 - 45°C and pH above 5.5. Organic solvents with log P >3.2 (hexane or heptane) were the most appropriate and 40–80% of aqueous phase allowed the highest conversions probably by maintaining the yeast enzymes catalytically active.

The effect of solvent nature and aqueous phase composition on the biocatalytic efficiency of *Arthrobacter simplex* in conversion of methyltestosterone to methandienone has been studied (Bie et al. 2008). A series of organic solvents with varying log P oct values,

together with five different aqueous media, were selected as organic and aqueous phase. The composition of the aqueous phase markedly influenced biocatalytic yields, particularly the addition of the menadione. In the system with carbon tetrachloride as the organic phase and phosphate buffer as aqueous phase, the volumetric phase ratio of 3org:7aq was found to be optimum.

An elaborate review has been made on asymmetric enzymatic redox reactions in organic solvents (Klibanov 2003). The article discusses the advantages of organic solvents in enzymatic reactions, the novel properties exhibited by enzymes in organic solvents and the other additional benefits like increased solubility of reactant, elimination of side reactions, and enhanced stability of enzymes.

Bioreduction of 2-oxo-4- phenylbutyrate to Ethyl (R)-2-hydroxy-4-phenylbutyrate, a key intermediate in the production of angiotensin converting enzyme (ACE) inhibitors, was carried out using microbial cells (Zhang et al. 2009). Over 63 microorganisms were screened and it was found that *Candida krusei* SW2026, was highly effective in this reduction process, leading to the (R)-enantiomer in 99.7% ee and 95.1% yield at 2.5 g/L of alcohol (under optimal conditions of 30ºC, pH 6.6 and in the presence of 5% glucose as co-substrate). In order to achieve higher product concentration with desired enantiopurity and yield for application in large-scale production, aqueous/organic biphasic system was used in the biotransformation reaction. The enantiomeric excess (ee), yield and product concentration were enhanced to 97.4% and 82.0% respectively, in water/dibutyl phthalate biphasic system, compared with 87.5% and 45.8% in aqueous medium.

The effect of organic co-solvents on ethyl -4- cyanobenzylformate reduction was also studied (Kratzer et al. 2008). Three solvents were selected viz., ethyl acetate, butyl acetate and hexane. Both the organisms selected for bioreduction (*E. coli* XR_FDH and *S. cerevisiae* XR2µ) displayed a significantly poorer biocatalytic performance as compared to reaction carried out in aqueous media.

Various strains of *Saccharomyces cerevisiae* yielding enantiomerically pure (R) - or (S)-1-(5-hydroxyhexyl)-3-methyl-7-propylxanthin by reduction of propentofylline has been reported (Pekala 2007). The reduction was carried both in aqueous and organic media. The results indicated that in aqueous media (S) alcohol with good enantiomeric excess of 78% was obtained. In case of reactions carried out in solvents like ethyl acetate and n-hexane, the yields were quite low.

Asymmetric bioreduction of Ethyl 4-chloro 3- oxobutanoate to (S)-4-chloro-3 hydro butanoate has been carried out using whole cells by of *Candida magnoliae*m JX 120-3 in biphasic system (Zhinan et al. 2006). Water to n-butyl acetate volume ratio of 1:1, 4 g of dry cell/ L, 50 g/L of glucose and 35ºC temperature were found to be optimum for maximum yield and enantiomeric excess.

2.4 BIOCATALYSIS WITH PERMEABILIZED CELLS

Ethyl(R)-4-chloro-3-hydroxybutanoate was obtained by cetyltrimetylammonium bromide (CTAB) permeabilized fresh brewer's yeast whole cells by bioconversion of ethyl 4-chloro-3-oxobutanoate in presence of allyl bromide (Yu et.al 2007). The results showed that the activities of alcohol dehydrogenase (ADH) and glucose-6-phosphate dehydrogenase (G6PDH) in CTAB permeabilized brewer's yeast cells increased 525 and 7.9 folds respectively, compared with that in the nonpermeabilized cells and had high enantioselectivity.

Anionic and catatonic surfactants were used for the microbial reduction of ω bromo acetophenone (Goswami et al. 2000). Several ω-bromoacetophenone derivatives were reduced to (R) - (-) 2 bromo -1- phenyl / substituted phenyl ethanol derivatives with whole cell biocatalyst in good yield. The ee were more than 95% using anionic surfactant under an inert atmosphere in an aqueous medium. Among the three organism's screened, *S. cerevisiae, R. rubra and C. tropicalies, R. rubra* gave the best results.

Biotransformation of hydrophobic sitosterol to androstenedione was reported with microemulsion prepared from nutrient broth and poly ethylene glycol 200 as aqueous phase, sitosterol dissolved in chloroform as organic phase and Triton X114 and Tween 80 as surfactant phase (Malaviya and Gomes 2008). The solubility of the substrate increased by 3 fold in the micoremulsion as compared to aqueous media and thereby increase in product formation was observed.

2.5 RESPONSE SURFACE METHODOLGY

Adinarayana and Ellaiah (2002) have optimized the fermentation media for maximum alkaline protease production using a new strain of *Bacillus* sp PE-11. Glucose as carbon source, peptone as nitrogen source and salt solution were selected to optimize the media conditions. Central composite design and response surface methodology were used in the design and analysis of the results. The optimum value for the tested variables for maximum enzyme production were glucose 7.798 g/L, peptone 9.548 g/L and salt solution 8.757%.

Response surface methodology has been used for optimization of media for pyruvic acid production by *Torulopsis glabrata* TP19 (Jian and Fa 2007). By screening experiments, ammonium sulphate, glucose and nicotinic acid were found to be important factors affecting pyruvic acid production. A 2^3 full factorial central composite design and response surface methodology were applied to determine the optimal concentration of each variable.

2.6 MICROBIAL SULPHOXIDATION

White-rot *basidiomycetes* promoted the oxidation of aromatic prochiral sulfides into sulfoxides with good enantioselectivity and conversion (Ricci et al. 2005). The reactions were carried out using whole cells of *Irpex lacteus*, *Pycnoporus sanguineus*, *Trichaptum byssogenum, Trametes rigida, Trametes Versicolor* and *Trametes villosa.* The enantioselectivity for all the aryl alkyl sulfoxides was in favour of (S)-enantiomers.

Oxidation of phenyl propyl sulfide produced (S) – phenyl propyl sulfoxide with high ee (ee>99%) by all the *Basidiomycetes* selected for the study.

Holland (1988) has reviewed the different methods for biotransformation of organic sulfides. The article discusses the different fungi, bacteria and isolated enzymes employed for sulphoxidation. The review discusses the mechanisms of enzymic sulphur oxidation and also the potential of enzymic sulfoxidation as a synthetic tool.

An European patent (Holt et al. 2003) claims an enantioselective preparation of pharmaceutically active sulfoxides by biooxidation. The enantiopure compounds obtained by this method include omeprazole, lansoprazole and pantoprazole. Biooxidation reactions have been performed using various bacterial and fungal species. The patent claims that while in a number of cases sulfoxide was produced, its concentration was too low to determine the enantiomeric excess. The species of *Mycobacterium* and *Pencillium frequentans* afforded sulfoxide of high enantiomeric excess but of opposite stereoselectivity

Biotransformation of organic sulphides has been carried out using the fungus *Helminthosporium* species (Holland et al 1995)**.** They have examined the formation of chiral para alkyl benzyl methyl sulphoxides and a range of phenyl and benzyl alkyl sulphoxides. They have reported yields ranging from 21% to 95% depending on the substrate.

The biotransformation of a series of substituted sulfides has been carried out with the filamentous fungi *Botrytis cinerea*, *Eutypa lata* and *Trichodermaviride* (Rivilla et al. 2007). Several products showed microbial oxidation of sulfide to sulfoxide with medium to high enantiomeric purity. With regard to sulfoxide enantioselectivity, the (*R*)-enantiomer was favoured in biotransformations by *T. viride* and *E. lata* while the (*S*)-enantiomer was favoured in those by *B. cinerea*. A minor amount of sulfone product was also detected.

A version of *Rhodococcus erythropolis* IGTS8 BKO-53, designed a model system for the biodesulfurization of crude oil with a high conversion activity of dibenzothiophenes to the corresponding sulfoxides which has been used for oxidation of a large number of simple sulfides (Holland et al. 2003). A large variety of sulfides were converted to chiral sulfoxides in good yield. Sulfoxide stereoisomers were generally formed with (*R*) configuration in moderate stereochemical purity, but the sulfoxide diastereomers of methionine amino acid derivatives were produced at >90% optical purity.

A new and efficient sulfide monooxygenase-producing strain, ECU0066, has been isolated and identified as a *Rhodococcus* sp. that transformed phenylmethyl sulfide to (*S*)-sulfoxide with 99% enantiomeric excess via two steps of enantioselective oxidations (Li et al. 2009). This bacterial strain has been reported to have displayed fairly good activity and enantioselectivity towards seven other sulfides, indicating a good potential for practical application in asymmetric synthesis of chiral sulfoxides.

The biocatalytic oxidation of various organic sulfides with whole cells of the commercially available topsoil bacterium *Pseudomonas frederiksbergensis* DSM 13022 (Fig 2.9) has been reported to afford the corresponding (*S*)-sulfoxides in high (up to >99% ee) enantiomeric excess upto > 99% (Adam et al. 2004).

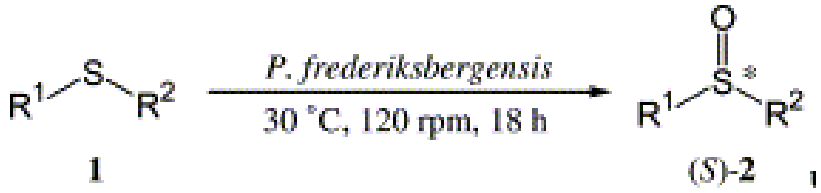


Fig. 2.9: Sulphoxidation of sulphides by *Pseudomonas frederiksbergensis* DSM 13022.

The enantiogenicity of biological and chemical oxidation at the sulfur atom was studied on a series of prochiral vinyl sulfides for the preparation of sulfoxides with (*S*) configuration (Rossi et al. 1992). Using fungal cultures, Sharpless-modified reagent or chiral oxaziridine, the enantiomeric excesses varied according to the substrate's steric and/or electronic structure; the three methods were complementary.

A variety of bacteria, yeasts and fungi were screened for the preparation of enantiomers of chiral sulfoxides from aryl-aryl or alkyl-aryl (E) and (Z) vinyl sulfides (Fauve et al 1991). The asymmetric oxidation was studied on a model substrate, (E)-methyl-(2-phenyl)-vinylsulfide. All the strains tested gave corresponding optically active sulfoxides with different chemical and optical yields.

2.7 EFFECT OF MEDIA COMPOSITION ON BIOCONVERSION

The culture conditions for glucose oxidase production by *Penicillium chrysogenum* SRT 19 strain have been optimized. The effects of different sugars and metal ions on enzyme production were studied (Bodade et al. 2010). The results revealed that presence of glucose and lactose resulted in maximum enzyme activity as compared to other sugars like sucrose, cellulose and mannitol. It was also observed that Ca and Fe had positive effect on enzyme production and presence of Mg, Cu, Zn and Ba gave fewer yields.

Effect of various parameters like time, temperature, carbon source, nitrogen source and mineral salts on production of amylase by *Aspergillus fumigatus* has been studied (Nwagu and Okolo 2011). Maximum growth and amylase production was recorded after 96 h incubation, at 30°C in a medium containing starch and a combination of inorganic and organic nitrogen source.

CHAPTER 3

BIOREDUCTION

3.1 INTRODUCTION

The asymmetric reduction of carbonyl compounds using microorganisms is now well recognized as an invaluable tool for the preparation of chiral alcohols. As there are number of oxidoreductases in a single microorganism, it can mediate the reduction of a variety of artificial ketones to produce chiral alcohols of remarkable optical purity. The three ketones which were selected for the bioreduction are key intermediates in the synthesis of pharmaceutically important molecules.

2-Deoxy-2, 2–difluoro-D-erythro pentofuranose-1-ulose-3, 5-dibenzoate, a lactone, is an intermediate in the synthesis of gemcitabine hydrochloride, an anti-cancer drug. A chiral center is generated when the dibenzoyl lactone is reduced to the corresponding hydroxy intermediate, affording a mixture of α and β anomers of which, the β anomer is more active biologically and hence preferred. Normally lactol is produced from lactone via chemical reduction using sodium bis (2-methoxyethoxy) aluminium hydride in an inert organic solvent at a temperature of -20 ºC to -30 ºC (Polturi et al. 2005). This established chemical method uses toxic organic solvent and expensive metal ion catalyst at very low temperatures which necessitates a simple, eco-friendly and cost effective synthetic method for the chiral product.

4-(3', 4'-dichlorophenyl)-2, 3-dihyrdronaphthone (Sertralone) is an intermediate in the synthesis of Sertraline, which is an active CNS depressant. The *cis* isomer of Sertraline is the preferred bioactive antidepressant than the *trans* isomer. Hence, stereoselective reduction of the racemic tetralone to yield only the preferred tetrol was attempted.

3-[5-[(4-fluorophenyl)-1,5-dioxopentol]-yl]-4-(S)-phenyl-oxazolidin-2-one is an intermediate in the synthesis of ezetimibe, a cholesterol lowering agent. The (S) alcohol of the reduced ketone, mentioned above, is the preferred biologically active isomer. Synthesis of ezetimibe involves reduction of 1-(4-fluorophenyl) -5-(2-oxo-4-phenyl-oxazolidin-3-yl)-pentane-1,5-dione to crucial homochiral intermediate, 3-[5-(4-fluorophenyl)-5-(S)-hydroxypentanoyl]-4(S)-4-phenyl- 1,3-oxazolidin-2-one, which is further processed to synthesize ezetimibe. Enantioselective reduction of the ketone intermediate to the chiral alcohol is achieved primarily by use of chemical catalysts like chiral boron catalyst. These boron based catalysts are hazardous being corrosive, flammable and unstable and/or water reactive towards hydrogen release. The stereopurity of the resulting alcohol is also inadequate (Grate 2007).

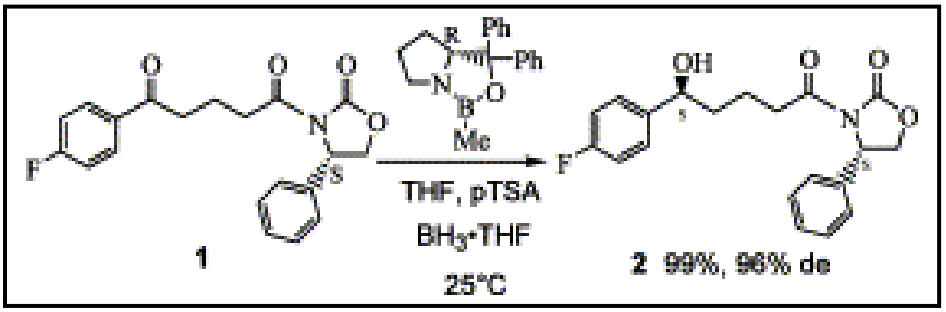


Fig. 3.1: Enantioselective reduction of ezetimibe intermediate (Fu et al. 2003).

The above mentioned ketones which are essential building blocks in the synthesis of pharmaceuticals were selected for bioreduction using microorganisms.

The microorganisms used in the study were selected based on the literature review. From the literature, it was found that fungi possess good enantioselective reduction power for ketones. Moreover, they are easy to culture and handle. As per the literature, the species of *Aspergillus*, *Rhizopus*, *Pichia*, *Penicillium*, *Candida* and *Saccharomyces* are well known for their ability to reduce a wide range of ketones to their corresponding alcohols. Ten microorganisms from these species were selected for the study.

The organism producing the maximum conversion of the ketone was selected for evaluation of effect of physicochemical parameters on bioreduction.

3. 2 MATERIALS AND METHODS

3.2.1 Chemicals:

The substrates selected for the study were,

1. 2-Deoxy-2, 2 –difluro-D-erythro pentofuranose-1-ulose 3,5 dibenzoate.
2. 3-[5-[(4-flurophenyl)-1, 5, dioxopentol] - yl] –4-(S) phenyl oxazolidin-2-one.

   The above mentioned ketones and chemically reduced alcohols of the ketones were kind gift samples from Cipla Pvt Limited, Bangalore.

3. 4(3', 4'-dichlorophenyl)-2,3-dihyrdronaphthone was gifted by Bio Organics And Applied Materials Pvt Limited, Bangalore.

Solvents used for HPLC were of HPLC grade. Inorganic salts and buffer salts were obtained from Qualigens. Growth media compounds were procured from Hi-Media (Mumbai, India).

All other chemicals used were of analytical grade.

3.2.2 Microorganisms: *Saccharomyces cerevisiae* MTCC 174, *Pichia farinosa* 246, *Aspergillus niger* MTCC 961, *Candida viswanathii* MTCC 1629 and *Rhizopus stolonifer* MTCC 2198 were obtained from MTCC, Chandigarh.

Aspergillus niger, Aspergillus flavus, Aspergillus oryzae and *Penicillium* species were cultured and isolated in the microbiology department of Dayananda Sagar College of Biological sciences, identified and authenticated at Bangalore University.

3.2.3 Media:

1. *Saccharomyces cerevisiae* MTCC 174: The organism was maintained on YEPD medium containing,

Ingredients	Quantity
Yeast extract	3.0 g
Peptone	10.0 g
Dextrose	20.0 g
Agar	20.0 g
Distilled water	1000 ml

2. *Pichia farinosa* MTCC 246: The organism was maintained on MYA medium containing,

Ingredients	Quantity
Malt extract	3.0 g
Yeast extract	3.0 g
Peptone	5.0 g
Dextrose	10.0 g
Agar	20.0 g
Distilled water	1000 ml

3. *Aspergillus niger*, *Aspergillus flavus*, *Aspergillus oryzae* and *Aspergillus niger* MTCC 961 were maintained on MRBA medium containing,

Ingredients	Quantity
Dextrose	10.0 g
Peptone	5.0 g
Potassium dihydrogen phosphate	1.0 g
Magnesium sulphate	0.5 g
Rose Bengal	0.0035 g
Agar	20.0 g
Distilled water	1000 ml
Streptomycin	0.03 g

4. *Candida viswanathii* MTCC 1629: The organism was maintained on YEPD medium.
5. *Pencillium species*:The organism was maintained on MRBA medium.
6. *Rhizopus stolonifer* MTCC 2198: The organism was maintained on MRBA medium.
7. Baker's yeast was purchased locally.

3.2.4 Methodology

The experimental work was divided into three phases:

1. Cultivation of the microorganism for obtaining substantial biomass.
2. Bioconversion of the substrate.
3. Extraction and analysis of the product.

3.2.4.1 Cultivation of microorganisms:

Cultivation of *Saccharomyces cerevisiae, Pichia farinosa and Candida viswanathii:*

The organisms from the slant culture were subcultured into 300ml sterile YEPD medium. The cultures were grown at 30 ºC and 160 rev min^{-1} for 24 h.

About 250ml of inoculum was inoculated into 2.5 L of fermentation medium. The inoculated medium was incubated at 30 ºC and 160 rev min^{-1} for 48 h. After 48 h of growth, the cells were separated by filtration using buchner funnel and the biomass was washed twice with phosphate buffer.

Cultivation of *Aspergillus niger, Rhizopus stolonifer, Aspergillus flavus, Aspergillus oryzae* and *Pencillium:*

The spores from the maintenance culture was inoculated onto 2 L of potato dextrose medium containing,

Ingredients	**Quantity**
Potato	400.0 g
Dextrose	20.0 g
Distilled water	2000 ml

The pH of the medium was adjusted to 6.0. The medium was sterilized at 121 ºC for 15 min. The inoculated medium was incubated at 25 ºC for 5 days to get sufficient biomass. The mycelial biomass was separated by filtration and washed twice with phosphate buffer.

Bakers yeast obtained from the local market was used as such.

3.2.4.2 Screening methodology:

Screening methodology for bioreduction of 2, Deoxy-2,2-difluro-D-erythro pentofuranose-1-ulose 3,5 dibenzoate:

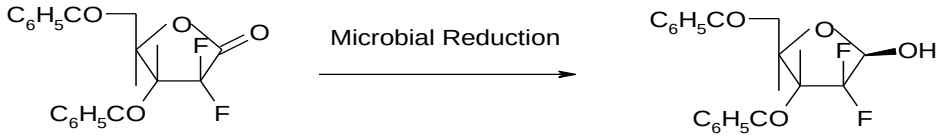


Fig. 3.2: Bioreduction of 2, Deoxy-2, 2 –difluro-D-erythro pentofuranose-1-ulose 3,5 dibenzoate.

About 10 g of wet biomass of the selected organism was taken in 20 ml of phosphate buffer pH 7.0 containing 5 g of glucose. 10 mg of the substrate dissolved in 1 ml acetone was added to the above suspension. The reaction mixture was incubated at 30 ºC and 160-rev min^{-1} for 48 h and the cells were separated by filtration at the end of incubation.

Screening methodology for bioreduction of Sertralone:

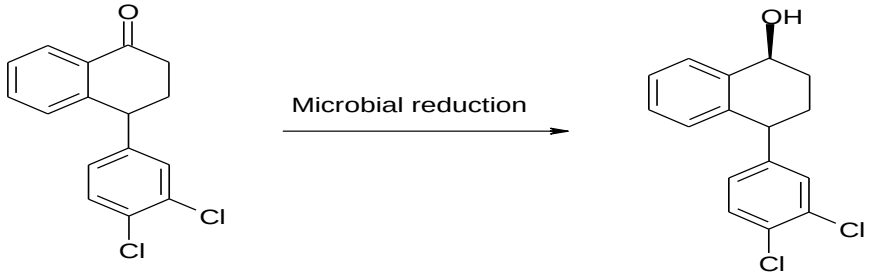


Fig. 3.3: Bioreduction of sertralone

About 10 g of wet biomass of the selected organisms was added to 20 ml of phosphate buffer of pH 7.0. 5 g of glucose and 10 mg of the substrate, dissolved in 1 ml acetone, was added to the aforesaid suspension. The reaction mixture was incubated at 30 ºC and 160-rev min^{-1} for 48 h and the cells were separated by filtration at the end of incubation.

Screening methodology for bioreduction of 3-[5-[(4-flurophenyl)-1, 5, dioxopentol]- yl] –4-(S) phenyl oxazolidin-2-one:

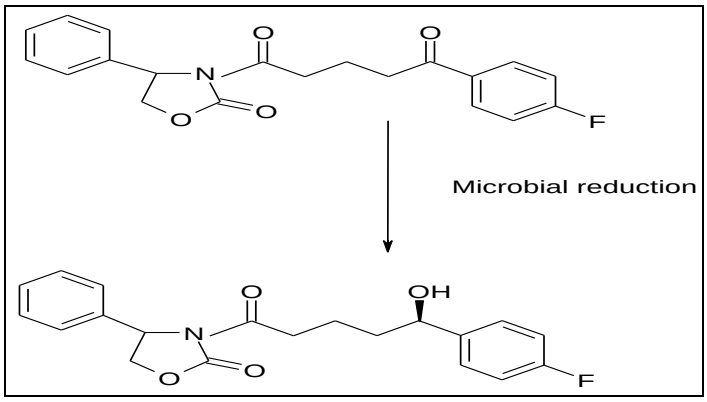


Fig. 3.4: Bioreduction of 3-[5-[(4-flurophenyl)-1,5, dioxopentol]- yl] –4-(S) phenyl oxazolidin-2-one.

About 10 g of the wet biomass was added to 20 ml of phosphate buffer of pH 7.0 containing 5 g of glucose. 20 mg of the substrate was dissolved in 1ml of dimethylsulphoxide (DMSO) and charged into the buffer. The resulting reaction mixture

was incubated at 30 ºC and 160-rev min^{-1} for 48 h. The cells were separated by filtration at the end of incubation.

3.2.4.3 Reduction of sertralone with sodium borohydride: (Sosale and Hota 2005)

About 500 mg of sertralone was dissolved in 10 ml of ethanol. The solution was stirred with a glass rod while in an ice bath and 1.0 g of sodium borohydride was added in small portions over 5 minutes to the solution. The solution was then stirred for 15 minutes using a magnetic stirrer. The solution was later transferred to a 100 ml beaker and 15 ml of water was added and the volume was reduced to about 15 ml by heating. The solution was then cooled to room temperature and was transferred to a separating funnel and extracted with 10 ml of ether twice. The ether extracts were washed twice with 10 ml brine solution and dried over anhydrous sodium sulphate. The dried ether extract was then evaporated to get the reduced product. The product was analyzed and confirmed by IR. This chemically reduced sample was used as standard for the detection of microbially reduced product.

3.2. 4.4 Extraction and analysis of the product:

The extraction procedure for all the three substrates was the same. The filtrate was extracted thrice with methylene dichloride (20 ml each). The combined extract was then washed with 20 ml of brine solution twice, dried over anhydrous sodium sulphate and evaporated to obtain the residue.

In case of lactone, TLC was carried out using methylene dichloride:methanol (9:1) solvent mixture. Chemically reduced pure lactol obtained from Cipla Pvt. Ltd., Bangalore, was used as standard.

The TLC for sertralone was performed with hexane**:**ethyl acetate (6:4) solvent system. Chemically reduced product was used as standard.

The reaction in case of 3-[5-[(4-fluorophenyl)-1, 5- dioxopentol]-yl]-4-(S) phenyl oxazolidin-2-one was monitored by using TLC with ethyl acetate: hexane: acetic acid (6:4:0.1) solvent system. The pure, chemically reduced alcohol (3-[5-(4-fluorophenyl)-

5(S)-hydroxypentanol]-yl-4(S)-phenyl 1,3-oxazolidin-2-one) from Cipla Pvt. Ltd., Bangalore was used as the standard for detection of product.

Of the ten strains of microorganisms screened for bioreduction of the selected substrates, only three strains *viz*., *S. cerevisae*, *C. vishwanathii* and soil isolated *A. niger* exhibited reduction of one of the substrate ie., 3-[5-[(4-fluorophenyl)-1,5-dioxopentol]yl] – 4-(S) phenyl oxazolidin-2-one.

Confirmation and quantification of the reduced product was done by HPLC and LCMS.

3.2.5 HPLC analysis

The reduced product of 3-[5-[(4-flurophenyl)-1, 5- di-oxopentol] yl]-4-(S) phenyl Oxazolidin-2-one was quantified by HPLC.

Methodology

The Mobile phase consisted of 250 ml of buffer 0.05 M Ammonium acetate adjusted to pH 5.0 with acetic acid and 500 ml methanol. The mobile phase was filtered through 0.45 membrane filter.

Chromatographic condition

Column	:	C18 phenomenex , 250 x 4.6 mm, .5 μm
Flow rate	:	1.5 ml/min
Wave length	:	215 nm
Injection volume	:	20 μl

Standard Preparation: 25 mg of the pure 3-[5-(4-fluorophenyl)-5(S)-hydroxypentanol]-yl-4(S)-phenyl 1,3-oxazolidin-2-one. Standard was dissolved in 25 ml of methanol and was diluted to obtain a series of concentrations from 5 μg/ml to 20.0 μg/ml.

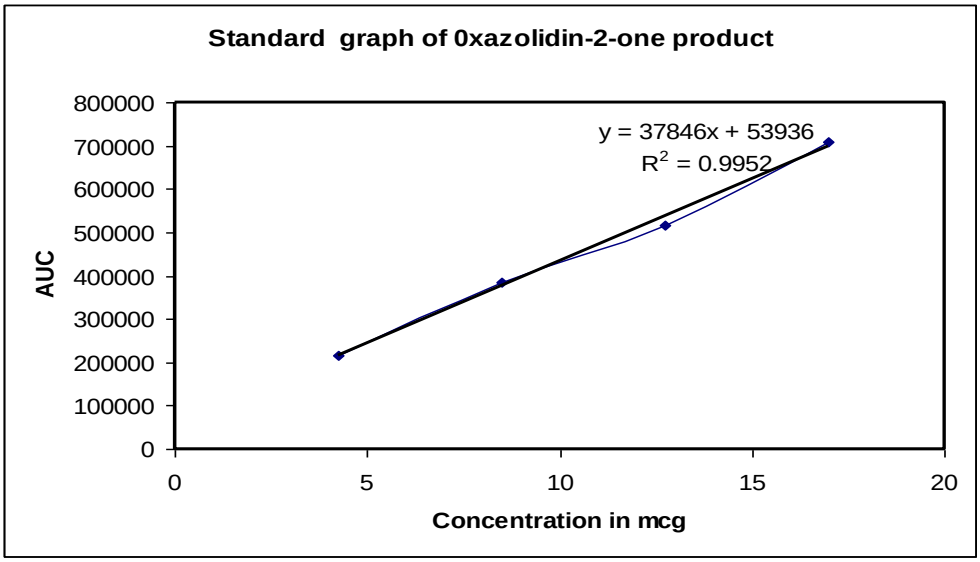


Fig. 3.5: Standard graph of 3-[5-(4-fluorophenyl)-5(S)-hydroxypentanol]-yl-4(S)-phenyl 1,3-oxazolidin-2-one.

Sample preparation: The sample obtained was lyophilized and diluted to 10 ml with methanol and injected.

3.2.6 Study of effect of various physical parameters on bioreduction of 3-[5-[(4-Fluorophenyl)-1, 5,dioxopentol]-yl] –4-(S) phenyl oxazolidin-2-one.

3.2.6.1 Effect of pH:

About 5 g of the wet biomass was added to 20 ml of buffer solution of pH ranging from 5.2-8.8 containing 2.5 g of glucose. 20 mg of the substrate was dissolved in 1 ml of dimethyl sulphoxide (DMSO) and added to the reaction buffer mixture and was incubated at 30 ˚C and 160-rev min^{-1} for 48 h.

The biomass was separated by filtration and the filtrate was extracted with 20 ml of methylene dichloride (MDC) thrice. The combined extract was washed with 20 ml of brine solution twice, dried over anhydrous sodium sulphate and evaporated to obtain the product.

3.2.6.2 Effect of temperature:

Bioconversion was carried out by the same procedure as detailed above with 2 g of biomass, at different incubation temperatures of 20, 25, 30, 35 and 40 ºC maintaining the pH constant at 7.6, which was found to be optimum from the earlier experiment.

3.2.6.3 Effect of reaction time:

The reaction was carried out as described above with 5 g of biomass at different time intervals of 12, 24, 36, 48, 72 and 96 h, maintaining the pH at 7.6 and temperature at 30 ºC, which were found to be suitable for the bio-reduction.

3.2.6.4 Effect of biomass concentration:

About 20 mg of the substrate was added to varied concentrations of biomass (1-10 g) in buffer solution of pH 7.6 containing glucose (50 % the weight of biomass), and incubated at 30°C and 160-rev min^{-1} for 48 h. The extraction was carried out in the same way as mentioned earlier.

3.2.6.5 Effect of substrate concentration:

A range of concentrations of the substrate (2, 4, 6, 8 and 10 mg) were added to 20 ml each of phosphate buffer of pH 7.6. 6 g of wet biomass and 3 g of glucose were added and the reaction mixtures were incubated at 30°C and 160-rev min^{-1} for 48 h. The spent biomass was later separated by filtration at pump and the filtrate was extracted in the same way as mentioned above.

3.2.6.6 Effect of organic solvents:

About 10 mg of substrate was added to 20 ml of reaction mixture containing 5 g of biomass, 2.5 g of glucose, a range of concentrations of hexane and buffer at pH 7.6. The volume of hexane was varied from 4, 8, 12, 16 to 20 ml. The mixtures were incubated at 30 °C and 160-rev min^{-1} for 48 h. After incubation, the hexane layer was separated and dried over anhydrous sodium sulphate. The aqueous layer of the biphasic culture was extracted with MDC thrice and washed with brine twice, dried over anhydrous sodium sulphate and was combined with hexane portion.

The reaction was repeated with water immiscible organic solvents such as benzene, toluene and water miscible solvents like acetone, dimethyl formamide and dimethyl sulphoxide.

3.2.6.7 Effect of surfactants:

About 4 mg of substrate dissolved in dimethyl sulphoxide (DMSO), was mixed with 12 mg of sodium lauryl sulphate (SLS) and stirred for 10 min (Goswami et al. 2000). The solvent was then removed under reduced pressure and the solid obtained was added to 20 ml of buffer at pH 7.6 with 5 g of wet biomass and 2.5 g of glucose. The reaction mixture was incubated at 30 °C and 160-rev min^{-1} for 48 h.

The reaction mixture was filtered and the filtrate was extracted with 20 ml MDC thrice and washed with brine twice and dried over anhydrous sodium sulphate.

The experiment was repeated using another surfactant, cetyltrimethylammonium bromide (CTAB) in the same manner as described above.

Since there was a significant increase in the yield in presence of SLS, the same experiment was carried out using different concentrations of SLS. 4 mg of substrate dissolved in DMSO was mixed with 4, 12 and 20 mg of SLS and the experiment was carried out as mentioned above.

Bioreduction in presence of SLS and hexane: The experiment was repeated with 40% hexane and SLS in pH 7.6 buffer.

3.2.7 Chiral analysis

The chiral purity of the product alcohol was determined by the use of a chiralcel OD-H column. The mobile phase was 75:25 hexane containing 0.1% trifluroacetic acid-ethyl alcohol at a flow rate of 0.5 ml $min.^{-1}$ Absorbance was monitored at 215 nm. The (S) and (R) alcohols were eluted at 24.27 and 27.34 min respectively (Singh et al. 2009).

3.3 RESULTS AND DISCUSSION

3.3.1 Screening of fungal strains for bioreduction of 3-[5-[(4-fluorophenyl) 1,5, dioxopentol]- yl] –4-(S) phenyl oxazolidin-2-one.

The reactions were carried out using resting cells of selected microorganisms. There were two reasons for selecting resting cells as biocatalyst.

1. The intermediates selected for the bioconversion arrested the growth of the cells when added to fresh inoculums, which confirmed their toxicity to the growing cells.
2. The process allows modification of bioconversion conditions as there is no interference from media components as well as accumulated metabolic products which are formed during growth (Grogan 2009).

Among the different strains of microorganisms screened for bioreduction of the selected substrate, only three strains viz., *S. cerevisae*, *C. vishwanathii* and soil-isolated *A. niger* were found to be effective in bringing about the reduction of ketone. The results are tabulated in Table 3.1.

Of the three microorganisms which showed ability to bring about ketone reduction, *S. cerevisae* showed considerable reduction when compared to *A. niger* and *C. vishwanathii*. Hence *S. cerevisae* was selected for further experiments.

Table 3.1: Screening of microorganisms for Bioreduction

Microorganism	Product yield mg/L
Saccharomyces cerevisiae MTCC 174	6.592
Candida vishwanathii MTCC 1629	4.166
Pichia farinose MTCC 246	0.00
Aspergillus niger	2.120
Aspergillus niger MTCC 961.	0.00
Rhizopus stolonifer MTCC 2198	0.00
Pencillium sp	0.00
Aspergillus flavus	0.00
Aspergillus oryzae	0.00
Baker's yeast	0.00

3.3.2 Study of effect of various parameters on bioreduction of 3-[5-[(4-flurophenyl) 1, 5,dioxopentol]-yl] –4-(S) phenyl oxazolidin-2-one

3.3.2.1 Effect of pH:

It is a well known fact that buffer pH plays a crucial role in enzymatic reactions (Lorraine et al. 1996, Kurbanoglu et al. 2008). Most of the enzymes possess optimum activity at a particular pH. Buffer pH not only influences the activity of the enzymes involved but also the regeneration of the co-enzyme present in microbial cells, which in turn affects the rate of reaction. As indicated in Fig. 3.6, the buffer pH exhibited a significant effect on the bioreduction. The concentration of the product increased with increase in pH and was found to be the maximum at pH 7.6. The result clearly indicated that the optimum pH for the bioreduction of the ketone using *S.cerevisiae* is 7.6. In most

of the bioreduction of ketones using fungi, the optimum pH for maximum conversion is around the neutral range. For example, the optimum pH for the reduction of 1-(-4-bromo –phenyl)-ethanone to (R) -1- (4-bromo phenyl)-ethanol by *Aspergillus niger* is in the pH range of 7.0 - 7.5 (Abas et al. 2010) and that for the conversion of 2-chloro-1-(3-chlorophenyl) ethanone to (R) -2-chloro-1-(3-chlorophenyl) ethanol by *Saccharomyces cerevisiae* CGMCC 2.396 is pH 6.5 (Lin et al. 2009).

Table 3.2 Effect of pH on bioreduction

pH	**Product concentration mg/L**
5.2	1.3
5.8	3.47
6.4	4.23
7.0	5.45
7.6	5.87
8.2	3.69
8.8	2.82

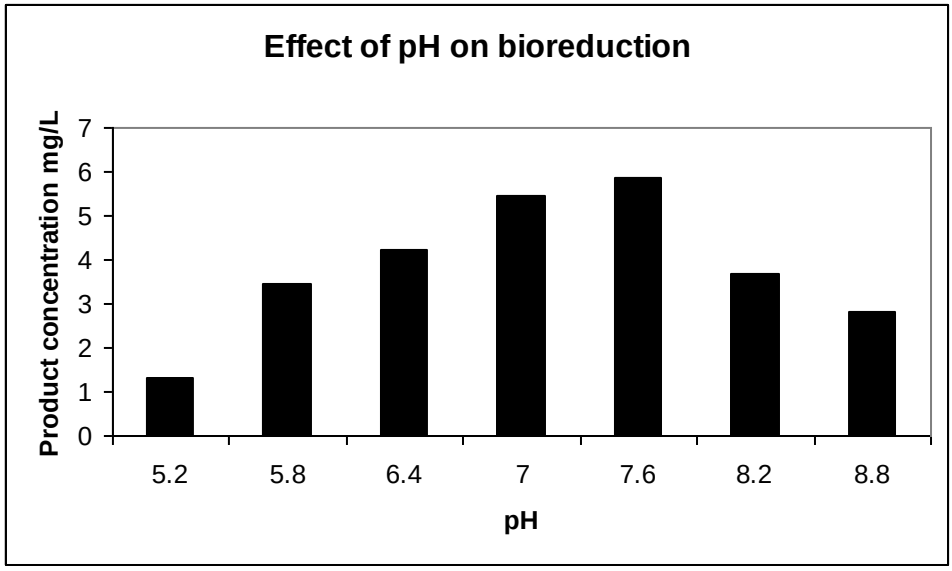


Fig. 3.6: Effect of pH on bioreduction of 3-[5-[(4- flurophenyl) 1, 5,dioxopentol]-yl] –4-(S) phenyl oxazolidin-2-one by *S.cerevisiae.*

3.3.2.2 Effect of temperature:

It is a well established fact that temperature affects the activity and stability of a biocatalyst and the equilibrium of a reaction as well (Singh et al. 2009). Therefore, the bioreduction was performed at various temperatures to study its impact on the reaction.

For the present study, different temperatures were selected (20, 25, 30, 35 and 40 ºC). As seen in Fig 3.7, the reaction was maximum at 30ºC. When the temperature was increased, there was a considerable drop in the concentration of product. This phenomenon had been observed even in biocatalytic synthesis of (S)-α-(3-pyridyl) ethanol (Soni et al. 2005) using *Candida viswanthii* and (R) -1- phenyl propanol by *Fusarium moniliforme* strain MS31 (Uzura et al. 2001). This decrease in bioconversion could be attributed to the partial inactivation of the enzyme or degradation of the substrates at higher temperatures. Inactivation of the enzyme seems to be a more appropriate explanation as the selected drug intermediate is stable in the temperature range tested.

Table 3.3: Effect of temperature on bioreduction

Temperature °C	Product concentration mg/L
20	0.52
25	2.09
30	3.30
35	2.16
40	1.20

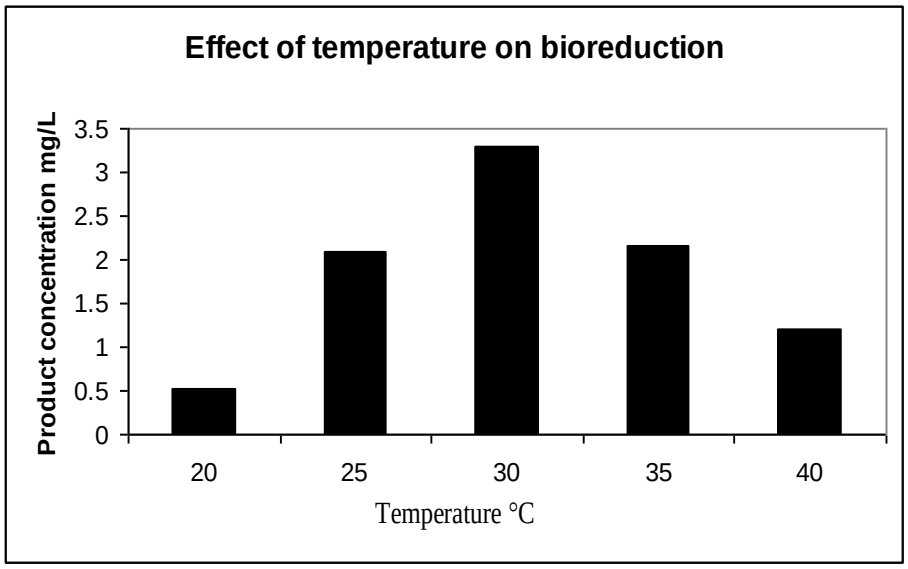


Fig. 3.7 Effect of temperature on bioreduction of 3-[5-[(4- flurophenyl) 1, dioxopentol]-yl] –4-(S) phenyl oxazolidin-2-one by *S.cerevisiae.*

3.3.2.3 Effect of incubation time

The study of reaction time in a biocatalytic process is an important parameter for optimization of the process. Unlike in chemical reactions, there are certain barriers in whole cell catalysis. The incubation time will depend on the time taken for the substrate

to permeate through the cell surface to come in contact with the specific enzyme and the product to permeate back into the medium. The time taken for these processes depends mainly on the nature of the substrate molecule and the specificity of the enzyme system (Soni et al. 2006). The bioconversion was carried out at different incubation time intervals such as 12, 24, 36, 48, 72 and 96 h and the optimum incubation time for the reduction of the ketone was identified as 48 h (Fig 3.8). Increase in reaction time showed decreased product concentration suggesting probable degradation of the reduced product over a period of time. This observation is contrary to the observations made by Soni et al. (2006) and Roy et al. (2003) where the microbial conversion of acetophenone by two different organisms produced maximum conversion at 12 and 24 h and further increase in the reaction time did not show any change in the percentage conversion; however, showed low ee.

Table 3.4: Effect of incubation time on bioreduction

Time h	**Product concentration mg/L**
12	0.707
24	0.77
36	2.58
48	2.9
72	1.24
96	1.01

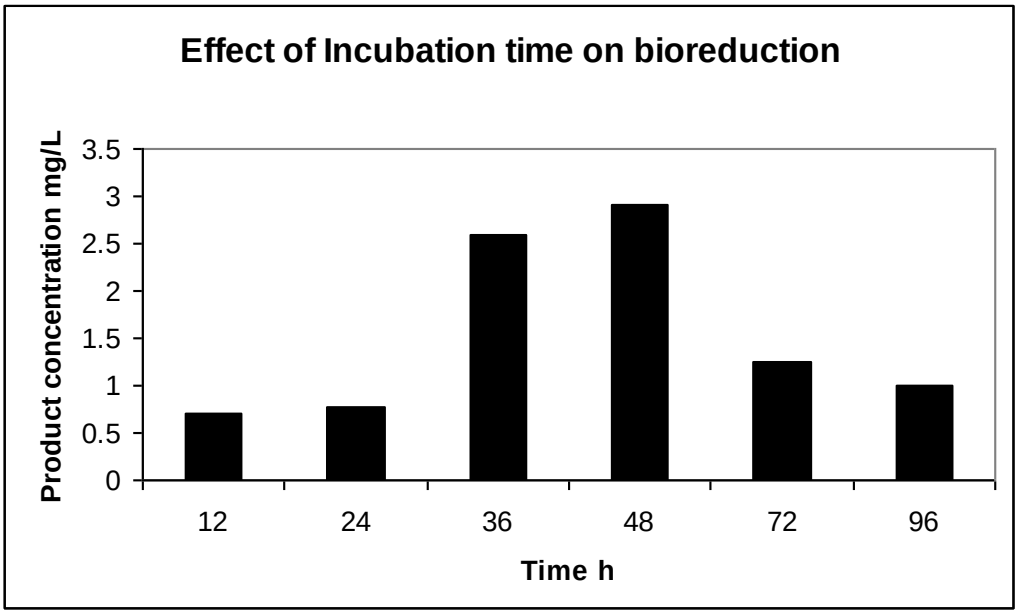


Fig. 3.8: Effect of incubation time on bioreduction 3-[5-[(4- flurophenyl) 1, 5,dioxopentol]-yl] –4-(S) phenyl oxazolidin-2-one by *S.cerevisiae.*

3.3.2.4 Effect of biomass concentration

The rate of reaction and the amount of conversion essentially depend on the quantity of enzymes available. Hence, the effect of weight of biomass utilized in the bioconversion was also investigated. For this study, a range of concentrations of 1 to 10 g of the biomass was used. The yield of the product increased with increase in cell mass and the maximum conversion was observed at a biomass concentration of 6 g. Further increase in biomass concentration did not have any effect on the product concentration. Similar results have been reported by Soni et.al (2005) in the reduction of heteroaryl methyl ketones by *Candida viswanathii* and Yang et al. (2007) in the reduction of acetophenone by *Pichia pastoris.* The results are shown in Fig- 3.9.

Table 3.5: Effect of concentration of biomass on bioreduction

Biomass g	Product concentration mg/L
1	1.32
2	3.92
3	4.14
4	5.16
5	6.58
6	9.02
7	8.2
8	8.16
9	7.8
10	7.5

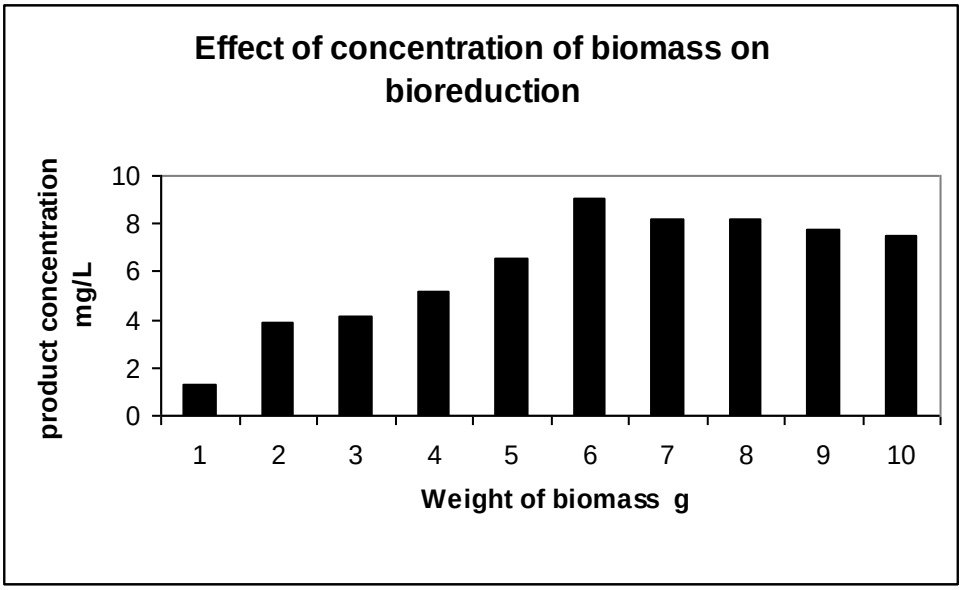


Fig.3.9: Effect of concentration of biomass on bioreduction.

3.3.2.5 Effect of substrate concentration

Most of the interesting substrates are non-natural and so, are toxic to living organisms and this is one of the drawbacks while employing whole cell systems. Therefore, substrates must be used in diluted systems at low concentrations. Normally in microbial reactions, the percentage yield of the product is dependent on the substrate concentration and this has been reported in various studies (Silva et al. 2010, Kamble et al. 2005). The variation is due to the fact that each enzyme in a microbe which is responsible for the reaction of interest has its own Michaelis constant Km, for a particular substrate. So change in substrate concentration brings about a change in the apparent activity of the enzyme. To find out the effect of substrate concentration on bioreduction, the experiment was performed using various concentrations of the substrate. From the data presented in Fig 3.10, it can be observed that, the product concentration was maximum at low substrate concentration and it showed a sharp decrease in the yield at higher substrate concentrations. The results indicate that the substrate probably has a detrimental effect on the microbial cells at higher concentrations. It is very clear from the literature that substrate concentration is one of the important parameters affecting the bioconversion of ketones. Concentration of more than 50mM of cyclohexanone was not tolerated by species of *Gongronell (Absidia) butleria, A.glauca* and *M. kaoliang* (Carballeira et al. 2004). Xiao et al. (2009) have reported inhibition of reductase enzyme of *Acetobacter sp*. CCTCC M209061 at high concentration of the substrate, 4-(trimethylsilyl)-3-butyn-2-one. Bioconversion of ethyl 4,4,4 trifluro acetoacetate with various initial substrate concentrations in aqueous-butylphthalate biphasic system has been performed by He (2007). Eventhough, the conversion was enhanced greatly in biphasic system compared to aqueous monophasic system, increase in substrate concentration caused reduction in product concentration in organic phase indicating the toxicity of the substrate.

Table 3.6: Effect of Substrate concentration on Bioreduction

Substrate concentration mg/L	% Product yield
100	4.6
200	3.7
300	0.62
400	0.76
500	0.703

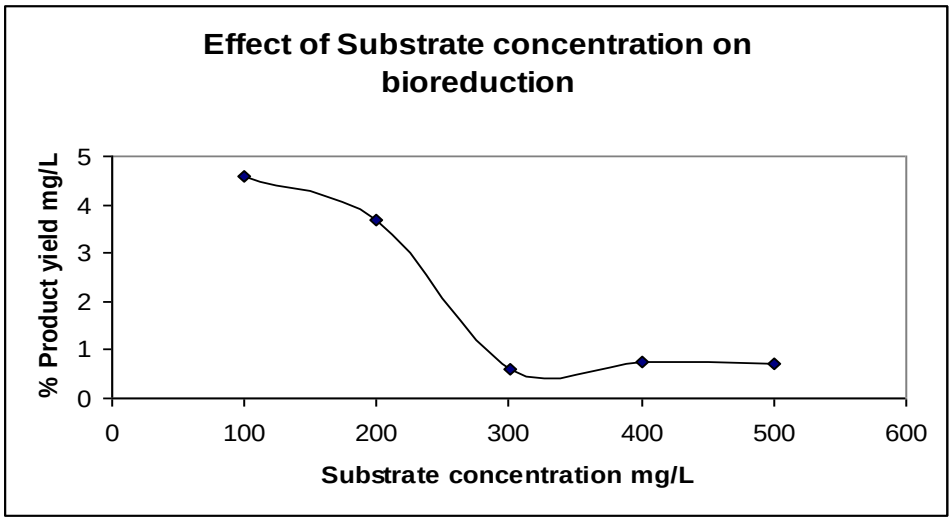


Fig. 3.10: Effect of Substrate concentration on Bioreduction 3-[5-[(4- flurophenyl) 1, 5,dioxopentol]-yl] –4-(S) phenyl oxazolidin-2-one by *S.cerevisiae.*

3.3.2.6 Effect of organic solvent

In general, biotransformation with microorganism and enzymes are performed in aqueous media. But many useful prochiral ketones are hydrophobic in nature making

biotransformation in aqueous medium difficult. In such cases, use of water immiscible organic solvents has been exploited to overcome the solubility problem (Yang et al. 2008). However, there is always a probability of deactivation of free cells or denaturation of enzymes which may occur in organic solvents. In order to provide a measure for the compatibility of an organic solvent with biocatalyst activity, many parameters describing the hydrophobicity of the solvent, such as Hildebrandt solubility parameters, dielectric constant and dipole moment have been proposed (Faber 2004). The most reliable results are obtained by using log P value of the solvent. Generally solvents with log P > 2.0 are biocompatible. Hexane with a log P value of 3.5 was taken as model solvent to analyze the effect of biphasic system on the reaction. Reactions in non-polar solvents have indicated that a polar solvent such as hexane will not distort the water coat of the biocatalyst present in the media thus maintaining its activity and the solvent tolerance of yeast for hexane has been shown to be the best (Cheng and Tsai 2008). Therefore, the effect of volume percentage of hexane in a biphasic reaction medium on bioconversion was studied. The results clearly indicated that the organic solvent had a toxic effect on the enzyme system as the yield was lower compared to the reaction carried out in aqueous medium (Table-3.7). The product yield was the highest when the concentration of hexane was 40%, which is probably due to solubilization of the substrate in hexane; however, the yield decreased significantly with further increase in hexane concentration which might be due to the toxic effect of the solvent.

The product could not be detected in reactions performed with water miscible and water immiscible solvents. The experimental results agree with the findings of Kansal and Banerjee (2009). The bioreduction of 1 acetophenone with *Candida viswanathii* in presence of different organic solvents has indicated that there was a sharp decrease in conversion with increase in the volume of organic solvents and authors claim that it may be due to the toxicity of the solvent system. The reduction of the ketone substrate by whole cells of *Saccharomyces uvarum* SW-58 in aqueous organic phase was influenced by the ratio of organic and aqueous phase (He et.al 2007). The conversion increased with

increase in organic phase initially similar to the present study and decreased at high concentration due to toxicity of the solvent.

Table 3.7: Effect of Hexane on Bioreduction

% of hexane	Product concentration mg/L
20	2.27
40	3.28
60	1.17
80	---
100	---
00	7.03

3.3.2.7 Effect of surfactants:

A possible process of enhancing the availability of the hydrophobic substrate to the microbial enzymes is the application of surfactants. Surfactants reduce surface tension and interfacial tension and lead to the formation of microemulsion in which the substrate can solubilize in water, increasing bioavailability. But, because of the amphipathic nature of surfactants, they are known to alter structure and function of cellular membrane, induce cell lyses and alter structure and function of microbial enzymes (Louvado et al. 2010)**.** Cationic surfactant is considered the most toxic compared to anionic and non ionic surfactants. There have been reports (Goswami et al. 2000, Xie et al. 2009) of studies on the effect of both anionic (sodium lauryl sulphate - SLS) and cationic (cetyl trimethyl ammonium bromide - CTAB) surfactants on bioreduction of ketone. Therefore, the effect of an anionic and a cationic surfactant on the process of bioreduction by *Saccharomyces cerevisiae* was tested. Cetyl trimethyl ammonium bromide was selected as the cationic surfactant and sodium lauryl sulphate, as anionic surfactant. There was a three-fold

increase in the concentration of the product in presence of SLS which is an anionic surfactant (Table-3.8). But, the presence of CTAB showed detrimental effect on the enzyme and on the yield of the product. Good yields were obtained when low concentration of SLS was used, compared to high concentration (Table- 3.9). This may probably be due to the toxic effect of the surfactant on the enzymatic activity.

Table 3.8: Effect of Surfactant on Bioreduction

Surfactant	**Product concentration mg/L**
SLS	11.078
CTAB	-----
pH 7.6 buffer	06.250

Table 3.9: Effect of concentration of Surfactant on Bioreduction

Substrate :SLS ratio	**Product concentration mg/L**
1:1	18.67
1:3	11.07
1:5	12.50
pH 7.6 buffer	06.25

3.3.2.8 Effect of surfactant on bioreduction in presence of hexane.

To know the combined effect of hexane and surfactant, the bioreduction was carried out in the presence of both hexane and SLS. The results indicated a marked decrease in the yield (Table 3.10). This finding confirmed that hexane has toxic effect on the biocatalyst.

Table 3.10: Effect of surfactant on bioreduction in presence of hexane

Substrate : SLS ratio in 40% hexane	Product concentration mg/L
1:1	3.28
1:3	1.45
1:5	-----
pH 7.6 buffer	6.25

3.3.3 Chiral analysis:

Asymmetric reduction of ketones to corresponding enantiopure alcohols has been performed using various biocatalysts. Alcohol dehdrogenases (ADHs) found in microbial strains catalyze the stereoselective transfer of a hydride from the cofactor NAD(P)H to the Si- or Re- face of the carbonyl group which results in the formation of the corresponding (S) or (R) alcohols. Most ADHs follow “prelog rule”, thus (S) – alcohols are usually obtained assuming that the smaller substituent of the ketone has the lower priority.

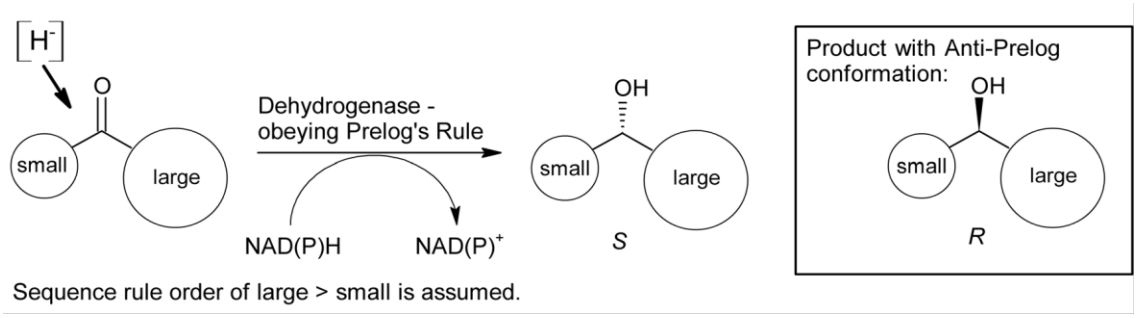


Fig. 3.11: Prelog rule (Faber 2004).

Zhang et.al (2011) reported that many carbonyl reductases were found to catalyze the stereoselective reduction of carbonyl group to the corresponding alcohol following prelog's rule and very few catalysts are found to possess anti-prelog selectivity.

The enantiopurity of all the analyzed samples were found to be 100% and of (R) isomer which indicated that the system followed anti-prelog conformation. The presence of organic solvents or surfactants did not affect the stereopreference of the enzyme. The result is interesting as the two studies (Homann and Previte 1997, Singh et al. 2009) available using different strains of microorganism, indicate the formation of (S) isomer in accordance with the Prelog rule.

3.4 SUMMARY

- Among the different strains of microorganisms screened for bioreduction of the selected substrates, only three strains viz *S. cerevisae*, *C. vishwanathii* and soil-isolated *A. niger* were found to be effective in bringing about the reduction of 3-[5-[(4-flurophenyl)- 1,5-dioxopentol]- yl] –4-(S) phenyl oxazolidin-2-one. As *S. cerevisae* showed considerable reduction, it was selected for evaluating the effect of various physicochemical parameters on the biotransformation.
- From the experimental results, it was found that the reduction was at its maximum at pH 7.6 and at a temperature of 30°C. The conversion was found to be the

maximum when the reaction mixture was incubated for 48h. The product yield increased with an increase in the biomass concentration up to a limit of 6 g. High concentration of substrate had a negative effect on the bioconversion.

- Reaction in organic solvents like hexane, toluene, dimethyl sulphoxide etc., did not improve the yield of the product and had a negative effect on the reaction indicating that the organic solvents had a detrimental effect on the enzyme involved in the reaction.
- The effect of both cationic and anionic surfactants on bioreduction of the selected ketone was studied. It was observed that the presence of SLS increased the product concentration by three-folds while cationic surfactant had a negative effect on the enzyme activity.
- The enantioselectivity of the system was specific to (R) isomer.

CHAPTER 4

INTERACTION OF CULTURE CONDITION VARIABLES ON CARBONYL REDUCTASE ACTIVITY OF *SACCHAROMYCES CEREVISIAE*

4.1 INTRODUCTION

It is well known that media components play a very important role in influencing microbial cell growth and enzyme production (Ibrahim and Elkhidir 2011). Carbonyl reductases are enzymes involved in the reduction of ketones. The productions of these enzymes are affected by several factors. Among them, the composition of the culture media plays a significant role in the expression of the enzyme (Soni et al. 2007). To achieve high product yields, it is a prerequisite to design an efficient medium that will help in rendering the process to be more economical. The source of carbon, nitrogen and addition of metal ions can affect the production of microbial enzymes along with the incubation pH and temperature.

Response surface methodology (RSM) is a powerful and efficient mathematical approach widely applied in the study of effect of different physicochemical parameters affecting various microbial processes (Adinarayana and Ellaiah 2002). It is also an important statistical technique employed for multiple regression analysis by using quantitative experimental data obtained from properly designed experiment using central composite design (CCD). It gives information about individual, interactive and cumulative effect of the variables (Manikandan and Viruthagiri 2010). RSM is a powerful technique for testing multiple process variables because fewer experimental trials are needed compared to the study of one variable at a time. Also, interactions between variables can be identified and quantified by such technique.

The effect of various components on production of reductase enzyme of *Saccharomyces cerevisiae* MTCC 174 involved in reduction of 3-[5-[(4-fluorophenyl)-1,5, dioxopentol]-yl] –4-(S) phenyl oxazolidin-2-one were studied.

4.2 MATERIALS AND METHODS

4.2.1 Chemicals

3-[5-[(4-fluorophenyl)-1,5, dioxopentol]-yl] –4-(S) phenyl oxazolidin-2-one was used as the substrate for media optimization for production of reductase enzyme.

NADH and growth media components were procured from HI-media (Mumbai, India). All other chemicals used were of analytical grade and obtained from standard companies.

4.2.2 Microorganism

Saccharomyces cerevisiae MTCC 174 was selected for the optimization experiment as it gave considerable conversion of the substrate. The organism was obtained from MTCC Chandigarh.

Saccharomyces cerevisiae MTCC 174 was maintained on YEPD media containing yeast extract 3 g, peptone 10 g, dextrose 20 g, agar 20 g and distilled water 1000 ml.

4.2.3 Methodology

4.2.3.1 Inoculum preparation

The organism from the slant culture was subcultured into 200 ml sterile YEPD liquid medium and incubated at 30ºC, 160 rev min^{-1} for 24 h.

4.2.3.2 Shake flask experiment

The effect of various carbon source, nitrogen source, metal ions, temperature and pH on enzyme activity was studied by inoculating 2 ml of the inoculum to each of 20 ml of different media as mentioned in Table 4.1. The flasks were incubated at the mentioned temperature and kept at 160 rev min^{-1} for 48 h.

4.2.3.3 Crude enzyme extract (Soni et al. 2006)

The cells were harvested by centrifugation at 10,000 rpm for 20 min. The pellet was washed with phosphate buffer pH 7.0 and centrifuged at 12000 rpm for 10 min. The pellet was resuspended in the same buffer and disintegrated using ultra sonicator. The cell debris was removed by centrifugation at 20000 rpm for 1h at 4ºC. The supernatant was used for activity measurement. Reductase activity was determined spectrophotometrically by measuring the decrease in the absorbance of NADH at 340 nm. The reaction mixture contained 4 ml of cell free extract, 20 µl of NADH (4.5 mM) and 20 µl of the ketone (10 mM in DMSO). Ketone reduction was followed over a time period of 5 min. One unit of enzyme activity is defined as the amount of enzyme required to oxidize 1 µl of NADH to NAD per minute.

Calculation of enzyme activity in micro moles/ min

Amount of product formed α ΔAU

ΔAU = ΔA (Change in absorbance) x Volume of assay reaction

= X AU/min

To convert absorbance units to mmole product divide AU by a_M (molar Absorbivity of NADH i.e. 6220.

4.2.3.4 Response surface methodology (RSM)

Based on the results obtained in the preliminary experiments glucose, peptone and copper sulphate were selected to analyze their effect on reductase production using central composite design (CCD).

The range and the level of the experimental variables investigated in the study are given in the Table 4.2. The central values (zero level) chosen for experimental design were glucose (2%), peptone (2%) and copper sulphate (2mM). CCD model was used for the

study. 2^3 factorial designs with six star points and six replicates at the centre point were employed to fit the model which indicates that 20 experiments were required for the prediction (Jian and Fa 2007). Coded levels for independents variables are presented in the Table 4.3.

Media Components → / Media ↓	Yeast Extract g	Peptone g	Carbon source g					Nitrogen source g			Metal ions mM				Dist. water L	Temp °C	pH
			Dextrose	Mannitol	Sucrose	lactose	Maltose	Malt Extract	Beef Extract	Tryptone	$ZnSO_4$	$CuSo_4$	$FeCl_3$	$CaCl_2$			
1	3	10	20	-											1	30	7.0
2	3	10		20											1	30	7.0
3	3	10			20										1	30	7.0
4	3	10				20									1	30	7.0
5	3	10					20								1	30	7.0
6	13		20												1	30	7.0
7		13	20												1	30	7.0
8			20					13							1	30	7.0
9			20						13						1	30	7.0
10			20							13					1	30	7.0
11	3	10	20								1				1	30	7.0
12	3	10	20									1			1	30	7.0
13	3	10	20										1		1	30	7.0
14	3	10	20											1	1	30	7.0
15	3	10	20												1	25	7.0
16	3	10	20												1	35	7.0
17	3	10	20												1	40	7.0
18	3	10	20												1	30	6.0
19	3	10	20												1	30	6.5
20	3	10	20												1	30	7.5
21	3	10	20												1	30	8.0

Table 4.1: Composition of various culture media

Table 4.2: Levels of variable tested in CCD

Variables	**Coded levels**				
	-1.682	-1	0	1	1.682
Glucose	0.318%	1%	2%	3%	3.682%
Peptone	0.318%	1%	2%	3%	3.682%
Copper Sulphate	0.318mM	1mM	2mM	3mM	3.682mM

Table 4.3 Experimental design matrix

Run no	**Glucose**	**Peptone**	**Copper Sulphate**
1	-1	-1	-1
2	-1	-1	1
3	-1	1	-1
4	-1	1	1
5	1	-1	-1
6	1	-1	1
7	1	1	1
8	1	1	1
9	-1.682	0	0
10	1.682	0	0
11	0	-1.682	0
12	0	1.682	0
13	0	0	-1.682
14	0	0	1.682
15	0	0	0
16	0	0	0
17	0	0	0
18	0	0	0
19	0	0	0
20	0	0	0

4.3 RESULTS AND DISCUSSION

4.3.1 Effect of media condition on enzyme activity

Effect of carbon source

Five different carbohydrates (glucose, mannitol, maltose, sucrose and lactose) were used in five different media and the results are tabulated in Table 4.4. Soni et al. (2006) have evaluated the effect of several carbon sources on carbonyl reductase activity of *C. vishwanathii.* They have found maximum activity when mannitol was used as the carbon source where as Singh et al. (2009), reported that glucose was the most suitable carbon source for maximum production of carbonyl reductase by *M. koreensis.* The results of the present study agree with both the reports as from the table it is clear that glucose is the preferred carbon source by the organism for production of the enzyme, followed by mannitol, which are simple sugars. Disaccharides had comparatively less effect on enzyme production

Table 4.4 Effect of carbon source on enzyme activity

Sl no	Carbon source	Enzyme activity $1x\ 10^{-2}$ μm/min
1	Glucose	0.302
2	Mannitol	0.250
3	Sucrose	0.100
4	Lactose	0.180
5	Maltose	0.120

Effect of nitrogen source

Source of nitrogen is one of the important factor that affect the growth and metabolites of microorganisms. Variation in the nitrogen source can affect the metabolic processes of the cell significantly. The effect of five different organic source of nitrogen was considered for the study (Table 4.5). The data collected during the study indicated that the addition of organic nitrogen did not have positive effect on the enzyme production. Similar results have been observed by Singh et al. (2011) in their study on effect of different nitrogen sources on carbonyl reductase activity of *M. koreenis.* The species of *B.cenocepacia* also exhibited similar results when 3-[5-[(4-fluorophenyl)-1,5, dioxopentol]-yl] –4-(S) phenyl oxazolidin-2-one was used as the ketone in the study (Singh et al. 2009). Among the different nitrogen sources used, peptone showed maximum effect compared to other nitrogenous compounds.

Table 4.5: Effect of nitrogen source on enzyme activity

Sl no	Nitrogen Source	Enzyme activity $1x\ 10^{-2}$ μm/min
1	Beef Extract	0.15
2	Peptone	0.17
3	Yeast Extract	0.14
4	Tryptone	0.12
5	Malt Extract	0.08

Effect of Metal ions

The presence of metal ions in the culture media greatly affects the metabolic activity of the microbial cell. The metal ions act as co-factors in various enzymatic reactions. They are important regulators of enzyme production, therefore the effect of some divalent ions was considered (Table 4.6). Among the various metal salts used, addition of copper

sulphate to the media had a moderate effect on enzyme activity, whereas addition of ferric ion had the least effect. Carbonyl reductase activity of *C. vishwanathii* (Soni et al. 2006) has been reported to have increased when Ca^{2+} was added to the growth media and considerably decreased when Fe^{2+} and Zn^{2+} were included individually to the medium. On the contrary the reductase activity of *B. cenocepacia* (Singh et al. 2009) is reported to have been enhanced on addition of Fe^{3+} salt to the media and addition of Ca^{2+} and Mg^{2+} had mild effect. Zn^{2+} was found to be the metal ion which promoted development of enzyme activity in case of *M. koreensis* (Singh et al. 2011). These findings clearly indicate that the effect of metal ions on reductase activity depend on the nature of microbial cell used as biocatalyst in bioconversion reactions.

Table 4.6: Effect of metal ions on enzyme activity

Sl no	Metal salt	Enzyme activity $1x\ 10^{-2}$ μm/min
1	Zinc Sulphate	0.24
2	Copper Sulphate	0.36
3	Calcium Chloride	0.18
4	Ferric Chloride	0.16
5	Magnesium Sulphate	0.26

Effect of pH

The optimum pH for growth of fungi usually falls in the acidic range. The effect of medium pH ranging from 6 to 8 on the enzyme induction was studied. The maximum enzymatic activity was found at pH 7 and there was no significant difference in enzyme activity in acidic pH but there was marked decrease when the pH was maintained at 8 (Table 4.7). The study on the effect of media pH on the activity of carbonyl reductase of *C. viswanthii* revealed sufficient enzyme activity over a broad range of pH which the authors explain may be due to the presence of multiple carbonyl reducases acting on the

substrate (Soni et al. 2006). In case of carbonyl reductase of *M. korneesis* involved in the reduction of 4-fluroacetophenone, the optimum pH was 7.0 (Singh et al. 2011), which is similar to the result obtained in the present study.

Table 4.7: Effect of pH on enzyme activity

Sl no	pH	Enzyme activity $1x\ 10^{-2}$ μm/min
1	6.0	0.257
2	6.5	0.280
3	7.0	0.302
4	7.5	0.160
5	8.0	0.096

Effect of Temperature

The incubation temperature of the microbial cells affects some of its metabolic activities. The cells grown at different temperatures did not show any marked difference in the activity of the enzyme involved. The maximum effect was observed at 30°C and the least was found at 40°C (Table 4.8). The optimum temperature for the production of enzyme involved in the reduction of the same molecule by *B. cenocepacia* (Singh et al. 2009) has been reported to be in the range of 35-37°C which is comparable to the result obtained in the present study for reduction of the same ketone using *S. cerevisiae.*

Table 4.8: Effect of incubation temperature on enzyme activity

Sl no	Temperature °C	Enzyme activity 1x 10^{-2} μm/min
1	25	0.240
2	30	0.302
3	35	0.280
4	40	0.170

4.3.2 Response surface regression

Table 4.10 gives the design and results of experiments carried out by CCD design. The results obtained were analyzed on MINITAB 15 package.

Enzyme activity represents dependent variable while glucose, peptone, copper sulphate and their corresponding cross products and 2nd order terms represent the independant variables. The response surface regression model could be represented as an empirical relationship between values of enzyme activity and test variables:

$$Y_{Enz} = A_0 + A_1 . X_{glu} + A_2 . X_{Pep} + A_3 . X_{Cuso4} + A_4 . X^2_{glu} + A_5 . X^2_{Pep} + A_6 . X^2_{Cuso4}$$
$$+ A_7 . X_{glu} . X_{Pep} + A_8 . X_{glu}. X_{Cuso4} + A_9 . X_{Pep} . X_{Cuso4} + E$$

Where,

Y_{Enz} (dependent variable) is the response, that is, Enzyme Activity

X_{glu}, X_{Pep} and X_{CuSo4} (basic indépendent variables) values of the test variables, glucose, peptone and copper sulphate, respectively and E represents the error in the model.

The above regression model was solved using least squares method and the unknown coefficients A_i were estimated as follows. For these estimated coefficients computation of Standard Error (SE) of the estimate was also done.

Table 4.9: Experimental design matrix and results of CCD

Run no	Glucose	Peptone	Copper Sulphate	Enzyme activity $1x\ 10^{-2}$ μm/min
1	-1	-1	-1	0.15
2	-1	-1	1	0.052
3	-1	1	-1	0.087
4	-1	1	1	0.090
5	1	-1	-1	0.098
6	1	-1	1	0.120
7	1	1	1	0.120
8	1	1	1	0.240
9	-1.682	0	0	0.064
10	1.682	0	0	0.139
11	0	-1.682	0	0.060
12	0	1.682	0	0.099
13	0	0	-1.682	0.21
14	0	0	1.682	0.230
15	0	0	0	0.084
16	0	0	0	0.098
17	0	0	0	0.098
18	0	0	0	0.088
19	0	0	0	0.096
20	0	0	0	0.064

Table 4.10: Estimated regression coefficients for enzyme activity

Term	Coef	SE Coef	T	P
A0 Intercept	0.094145	0.005870	16.039	0.0000*
FIRST ORDER				
A1 Glucose	0.014107	0.002519	5.601	0.0000*
A2 Peptone	0.005594	0.002506	2.233	0.0480*
A3Copper sulphate	0.001156	0.002506	0.461	0.6540
SECOND ORDER				
A4 Glucose*Glucose	0.000236	0.001355	0.174	0.865
A5 Peptone*Peptone	0.002513	0.001355	-1.854	0.093
A6 Copper sulphate*Copper sulphate	0.015041	0.001355	11.098	0.000*
QUADRATIC				
A7 Glucose*Peptone	0.007426	0.002061	3.602	0.005*
A8 Glucose*Copper sulphate	0.010519	0.002061	5.103	0.000*
A9 Peptone*Copper sulphate	0.011182	0.002045	5.469	0.000*

*** Significant at $p < 0.05$ $R^2 = 96.17\%$, R^2 (Adj) = 92.73%**

Correspondingly, the coefficient of determination (R^2) was computed to assess the performance of regression in terms of goodness of fit. The values obtained for both R^2 and R^2 (adj) indicates that the performance of regression is very good. In the present study, the value of the coefficient determination (R^2 = 96.17%) which indicates that 3.83% of the total variations are not explained by the model. The value of the adjusted coefficient determination (Adj. R^2 = 92.73%) is also on the higher side, which indicates a high significance of the model (Adinarayana and Ellaiah 2002)**.** The significance of each coefficient was determined by student's t-test and corresponding P values that are listed in Table 4.10. The larger the magnitude of the t-value and the smaller the P-value, the more significant is the corresponding coefficient. Accordingly the contribution of factors A1 (Glucose), A2 (Peptone), A6 (Copper sulphate*Copper sulphate), A7 (Glucose *Peptone), A8 (Glucose*Copper sulphate) and A9 (Peptone*Copper sulphate) have significant effect on enzyme activity. This implies that glucose and peptone have direct

relationship on the enzyme activity and copper sulphate alone does not show significant relationship with enzyme activity, however all quadratic coefficients are observed to be significant. The significance of A6 indicates that there is a curvature in the response surface of enzyme activity.

Analysis of variance for enzyme activity was also carried out with regard to various source of variation, namely regression and residual error "E" of the model above. Computation was done for sequential sum of squares, adjusted sum of squares and adjusted mean sum of squares. F-statics was calculated for regression model including that of linear, square and interaction terms in the model.

Table 4.11: Analysis of variance for enzyme activity

Source	**Degrees of freedom**	**Sequential sum of squares**	**Adjusted sum of squares**	**Adjusted mean sum of squares**	**F**	**P**
Regression	9	0.052065	0.052065	0.005785	27.94	0.000
Linear	3	0.017381	0.006879	0.002293	11.07	0.002
Square	3	0.025687	0.027781	0.009260	44.72	0.000
Interaction	3	0.008996	0.008996	0.002999	14.48	0.001
Residual Error	10	0.002071	0.002071	0.000207		
Lack-of – fit	4	0.000522	0.000522	0.000130	0.51	0.735
Pure Error	6	0.001549	0.001549	0.000258		
Total	19	0.054136				

The fisher F-test with a very low probability value "P" demonstrates a very high significance for the regression model.

The regression model was further used to predict the enzyme Activity Y_{Enz} for any given set of independent variables. Following Table 4.12 provides the predicted fit and corresponding Standard Error of the fit for all 20 observations with respective residual

error. It can be seen that except for observation number 1 and 5, rest of the observations showed very good predictions as compared to the actual values of enzyme activity.

Hence, this model could be successfully used for accurately predicting enzyme activity based on newer set of independent variables. All the above considerations indicate a good fit and adequacy of the regression model.

Table 4.12: Observed responses and predicted values

Observation	**Enzyme Activity**	**Fit**	**SE Fit**	**Residual**	**St Residual**
1	0.150	0.123	0.009	0.027	2.38 R
2	0.052	0.059	0.012	-0.007	-0.80
3	0.087	0.091	0.012	-0.004	-0.49
4	0.090	0.099	0.012	-0.009	-1.09
5	0.098	0.123	0.009	-0.025	-2.29 R
6	0.120	0.124	0.012	-0.004	-0.47
7	0.120	0.121	0.012	-0.001	-0.14
8	0.240	0.248	0.013	-0.008	-1.28
9	0.064	0.056	0.012	0.008	1.04
10	0.139	0.136	0.011	0.003	0.33
11	0.060	0.058	0.012	0.002	0.23
12	0.099	0.090	0.011	0.009	1.02
13	0.210	0.211	0.012	-0.001	-0.16
14	0.230	0.218	0.011	0.012	1.36
15	0.084	0.094	0.006	- 0.010	-0.77
16	0.098	0.094	0.006	0.004	0.29
17	0.098	0.094	0.006	0.004	0.29
18	0.088	0.094	0.006	-0.006	-0.47
19	0.096	0.094	0.006	0.002	0.14
20	0.099	0.094	0.006	0.005	0.37

R denotes an observation with a large standardized residual value

4.3.3 Analysis of interactions of variables

Contour plots gives better understanding about the influence of variables and their interactions on the response compared to other 3D plots (Ravikumar et al. 2007). Contour plot representing the combined effect of peptone and glucose on the enzyme activity keeping copper sulphate constant is shown in Fig 4.1. The enzyme activity increases with increase in concentration of both glucose and peptone. The contour plots (Fig 4.2 and Fig 4.3) indicate that increase in copper sulphate concentration alone does not have significant effect on enzyme production and the effect becomes significant only when the concentration of peptone and glucose are increased.

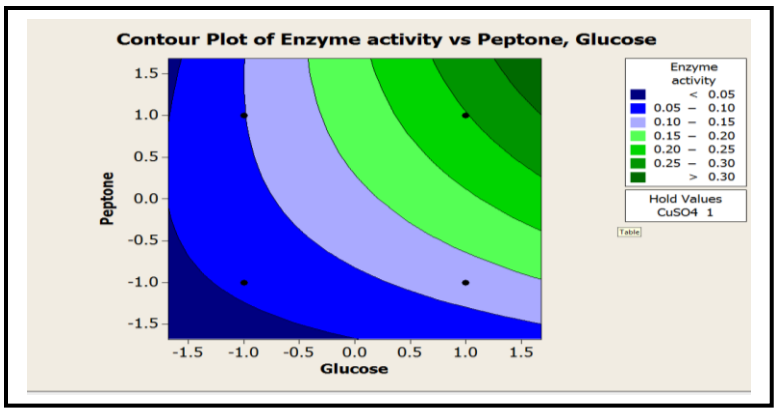


Fig 4.1 Contour plot of enzyme activity Vs peptone, glucose.

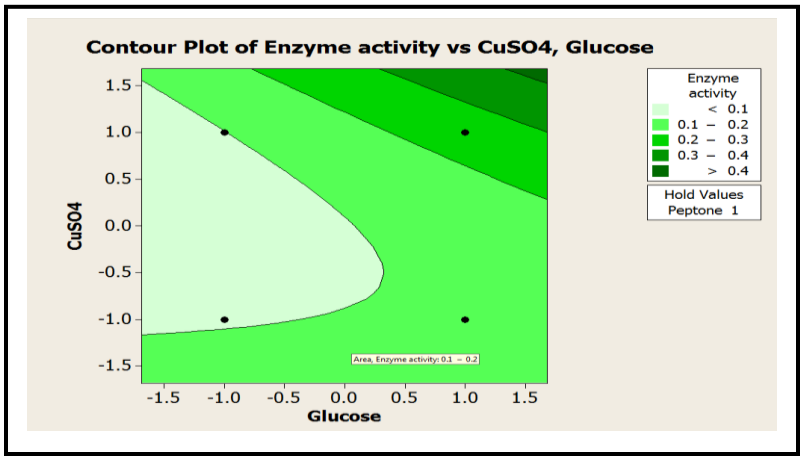


Fig. 4.2 Contour plot of enzyme activity Vs $CuSO_4$, glucose.

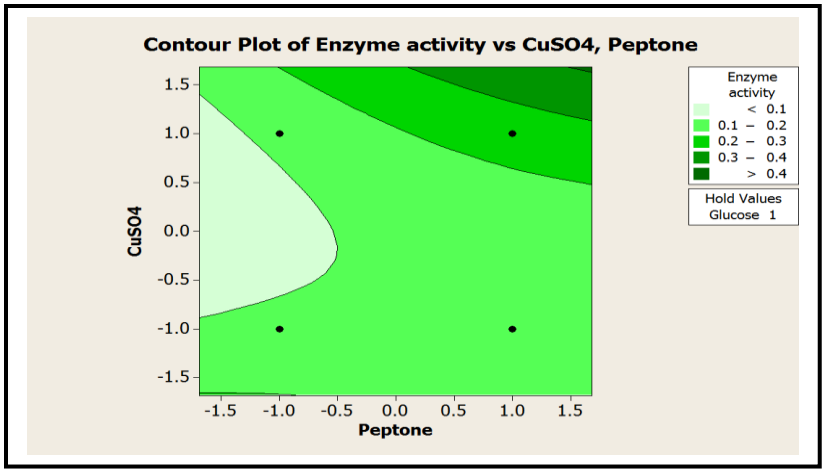


Fig. 4.3 Contour plot of enzyme activity Vs $CuSO_4$, peptone.

Optimization plot

A response optimization plot is useful in determining the optimal conditions that will result in desirable response (Jaiswal et al. 2011). The optimum conditions were analyzed for a set goal of enzyme activity of 0.3 x 10^{-2} µm/min. The best combination of factor setting for achieving the optimum response was found to be glucose-0.52%, peptone-0.5% and copper sulphate-0.53 mM, with a composite desirability value of 0.9999.

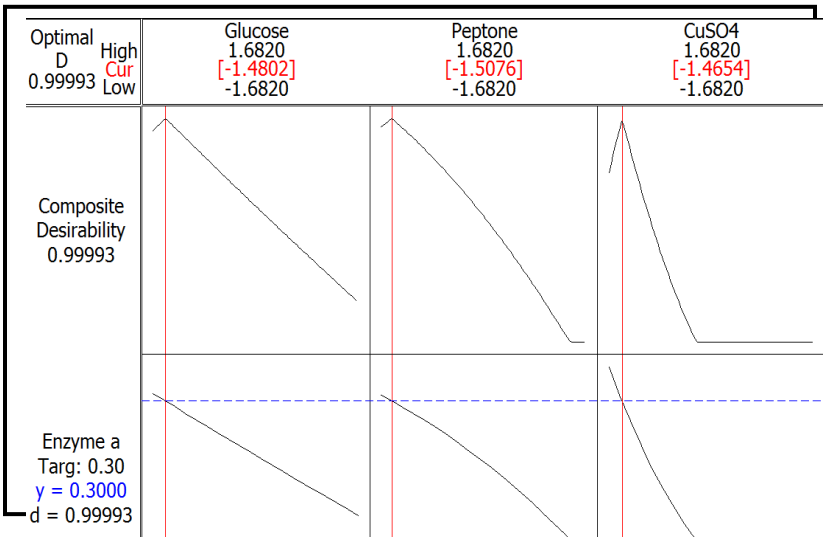


Fig. 4.4 Optimization plot.

4.4 SUMMARY

Study on effect of various carbon sources on enzyme production indicated that glucose in the media affected enzyme activity significantly.

Peptone was found to have maximum effect among the nitrogen sources tested for enzyme production. It was found that addition of nitrogen sources did not contribute significantly to the production of enzyme.

The culture media pH and incubation temperature were varied to know the effect of these parameters on enzyme activity. Media pH of 7.0 and incubation temperature of 30 °C was found to exert maximum enzyme activity

Various divalent metal salts were added to the culture media to study their effect on the enzyme activity. Addition of copper sulphate to the media had moderate effect on enzyme activity.

Response surface methodology was used to study the individual, interactive and cumulative effect of variables by selecting glucose, peptone and copper sulphate as the variables. The results obtained were analyzed using software MINITAB 15 and a second order polynomial equation was generated. Coefficients of determination (R2) were computed to assess the performance of regression in terms of goodness of fit. Both R^2 and R^2 (adj) indicated that the performance of regression is very good with R^2 value of 96.17% and adj. R^2 of 92.73%.

Analysis of variance for enzyme activity was also carried out with regard to various source of variation, namely, regression and residual error "E" of the model above. Computation was done for sequential sum of squares, adjusted sum of squares and adjusted mean sum of squares. F-statics was calculated for Regression model including for that of Linear, Square and Interaction terms in the model. The fisher F-test with a very low probability value "P" demonstrates a very high significance for the regression

model. Based on the model, optimum conditions for a set goal of enzyme activity was determined.

Interactions of the variables were analyzed with contour plots. The contour plots indicated that increase in glucose and peptone concentration enhanced the enzyme activity but increase in copper sulphate concentration alone did not significantly affect the enzyme production and the effect became significant only when the concentration of peptone and glucose increased simultaneously.

CHAPTER 5

MICROBIAL SULPHOXIDATION

5.1 INTRODUCTION

Non-racemic drugs have been of growing interest to the pharmaceutical industry in the recent years. Among them, sulphoxides are of special importance as they are extensively used as chiral auxiliaries and intermediates (Carreno 1995). Numerous methodologies have been reported for the transformation of a prochiral sulfide to a chiral sulfoxide. Most of these involve use of a chiral ligand with a transition metal such as titanium, vanadium or manganese, in the presence of hydrogen peroxide or a hydrogen peroxide adduct as the oxygen source (Gama et al. 2003). The chiral ligands that have been successfully used include: bidentate diethyl tartrate, diol, 1, 1-bis-2- naphthol (BINOL), tridentate Schiff base ligands and tetradentate Salen type ligands.

Esomeprazole, the (S) form of omeprazole, which is used to heal and relieve symptoms of gastric or duodenal ulcer, is considered amongst the world's most sold pharmaceuticals. The (S) isomer was originally obtained by the resolution of racemic omeprazole. In order to design an industrially scalable process of obtaining the required enantiopure product, several chemical catalysts have been employed (Khomenko 2008). One of the successful methodologies of asymmetric sulphoxidation was based on using titanium catalyzed asymmetric epoxidation of sulphides in presence of tartarate, water and N,N diisopropylethylamine.

Various aspects need to be taken into account when the asymmetric organic synthesis is planned. While the yield of the process and its stereoselectivity remain the most important criteria, safety, health and environmental aspects are of increasing interest. In this context, the application of biocatalysts to perform the desired chemical transformation has gained considerable attention. The use of enzymes allows reactions to

be conducted under mild conditions, typically with water as solvent so that, in many cases toxic organic solvents can be avoided. Moreover, the nonracemic product may be readily obtained due to the built-in chirality of the catalyst. However, biocatalysis has several drawbacks. Some of the substrates may not be accepted by the enzyme and the enzymes and cellular systems have limited stability.

Two sulphides were screened for asymmetric sulphoxidation using selected fungi strains, of which only the omeprazole intermediate was accepted by few of the species for conversion. Several physico-chemical parameters were modified to improve the efficiency of the bioconversion.

5.2 MATERIAL AND METHODS

5.2.1 Chemicals:

The sulphides selected for the sulphoxidation were:

1. 5- Methoxy-2-[[(4-methoxy –3, 5-dimethyl-2-pyridinyl) methyl]-sulphinyl]-1 H benzimidazole.

2. 2[[[3-methyl-4-(2,2,2-trifluroethoxy)-2-pyridyl] methyl] sulfinlyl] benzimidazole.

The above two sulphides and sulphoxide products, omeprazole and lanzoprazole were gift samples from Cipla Private Limited, Bangalore.

Solvents used for HPLC were of HPLC grade. Inorganic salts and buffer salts were obtained from Qualigens. Growth media compounds were procured from Hi-Media (Mumbai, India).

All other chemicals used were of analytical grade.

5.2.2 Microorganisms:

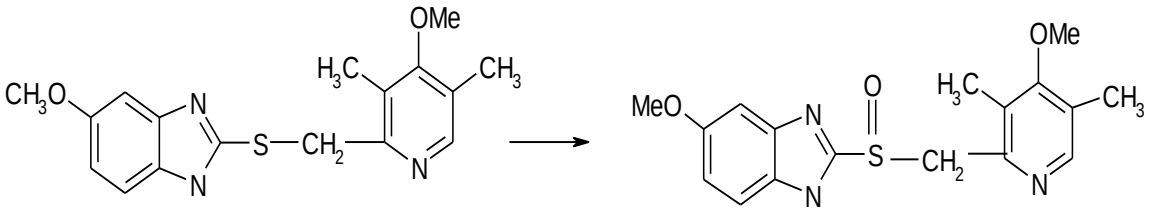
Saccharomyces cerevisiae MTCC 174, *Pichia farinosa* 246, *Aspergillus niger* MTCC 961, *Candida viswanathii* MTCC 1629 and *Rhizopus stolonifer* MTCC 2198 were obtained from MTCC, Chandigarh.

Aspergillus niger, Aspergillus flavus, Aspergillus oryzae and *Pencillium* species were isolated at Dayananda Sagar College of Biological sciences, identified and authenticated at Bangalore University.

Media and method of cultivation of the microorganisms are as mentioned in section 3.2.3 and 3.2.4.1 respectively.

5.2.3 Microbial sulphoxidation with selected fungi:

The ten fungal strains selected for bioreduction were also screened for their capacity to carry out sulphoxidation of 5- Methoxy-2-[[(4-methoxy –3, 5-dimethyl-2-pyridinyl) methyl]-sulphinyl]-1 H benzimidazole to omeprazole and 2[[[3-methyl-4-(2, 2, 2-trifluroethoxy)-2-pyridyl] methyl] sulfinlyl] benzimidazole to lanzoprazole.



About 10 g of the biomass was added to 20 ml phosphate buffer at pH 7.6 and the resultant suspension was added to 20 mg of omeprazole intermediate dissolved in 2ml alcohol and was incubated at 30˚ C and 160 r/min for 48 h.

5.2.4 Extraction and analysis of oxidized product:

The reaction mixture was filtered to remove the biomass. Filtrate was extracted with alkaline methylene dichloride (20 ml x 3), washed twice, each with 20 ml of brine and

dried over anhydrous sodium sulphate. The organic extract was then concentrated by evaporation.

The reaction was monitored by TLC using ethyl acetate as the mobile phase. The oxidized product, omeprazole and lanzoprozole, obtained from the industry, were used as the standard for comparison.

5.2.5 HPLC Analysis:

The oxidized product of omeprazole intermediate was identified and quantified by HPLC.

Chromatographic condition:

Mobile phase	:	Phosphate buffer (pH 7.6): methanol (25:75)
Column	:	C18 phenomenex, 250x4.6 mm, 5 μm
Flow rate	:	0.8 ml/min
Wave length	:	280 nm
Injection volume	:	20 μl

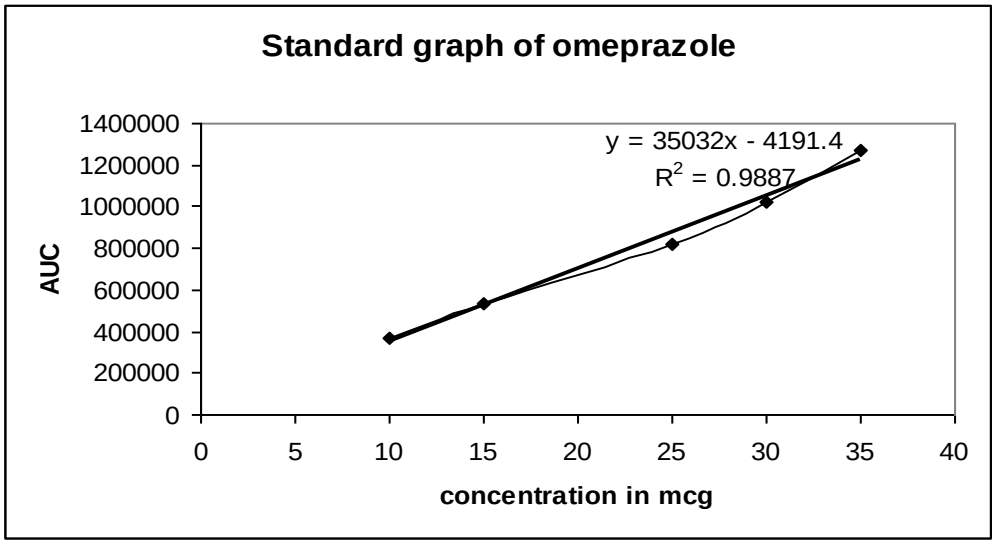


Fig. 5.1: Standard graph of omeprazole.

Standard Preparation: 25 mg of standard omeprazole was dissolved in 25 ml of methanol and was diluted to prepare a series of concentrations from 10 to 50 µg/ml. **Sample preparation:** The concentrated sample was made up to 10 ml with methanol.

5.2.6 Effect of various parameters on Microbial sulphoxidation

5.2.6.1 Effect of pH:

Sulphoxidation was carried out with 2 g of the wet biomass in 20 ml of buffer solution of pH ranging from 7.6 – 8.8. The extraction was carried out in the same way as described under 5.2.4.

5.2.6.2 Effect of Temperature:

Bioconversion was carried out with 5 g of biomass, by the same procedure at different temperatures such as 20 ºC, 25 ºC, 30 ºC and 35 ºC keeping pH constant at 7.6. The extraction was carried out in the same way as described earlier.

5.2.6.3 Effect of incubation time:

The reaction was carried out with 2 g of biomass as described above, at different time intervals of 24, 48, 72 and 96 h, maintaining the pH at 7.6 and the temperature at 30ºC which were found to be optimum for sulphoxidation. The extraction was carried out in the same way as described earlier.

5.2.6.4 Effect of biomass concentration:

About 20 mg of substrate was added to various concentrations of biomass (2 g, 4 g, 6 g, 8 g, 10 g and 12 g) in buffer solution of pH 7.6 and were incubated at 30˚C and 160 r/min for 48 h. The extraction was carried out in the same way as described earlier.

5.2.6.5 Effect of substrate concentration:

Bioconversion was carried out with 2 g of biomass, by the same procedure with different concentrations of the substrate (4, 8, 12, 16, 20 and 24 mg). The extraction was carried out in the same way as described earlier.

5.2.6.6 Effect organic solvents:

About 10 mg of the substrate dissolved in minimum volume of alcohol, was added to 20 ml of water immiscible solvents like toluene, benzene and hexane taken in separate 250 ml flaks. 5 g of the wet biomass was added to the above reaction mixture. Incubation was carried out as described earlier. After incubation the biomass was separated by filtration. The spent reaction mixture was washed with 20 ml of brine solution and dried over anhydrous sodium sulphate. The solvent was later evaporated and the residue was added to HPLC grade methanol for analysis.

Acuurately weighed 10 mg of substrate dissolved in minimum quantity of alcohol, and was added to a reaction mixture containing 10 % water miscible organic solvents like acetone, dimethyl formamide and dimethyl sulphoxide, at pH 7.6. 5 g of the wet biomass was added to the reaction mixtures. Incubation and extraction were carried out as described earlier.

5.2.6.7 Effect of surfactants:

4 mg of substrate dissolved in alcohol, was mixed with 12 mg of sodium lauryl sulphate (SLS) and was stirred for 10 min. The solvent was then removed under reduced pressure and the solid obtained was added to 20 ml of buffer at pH 7.6 with 5 g of wet biomass. The reaction mixture was incubated at 30˚C and 160 r/min for 48 h.

The reaction mixture was filtered and the filtrate was extracted thrice, each with 20 ml of alkaline MDC, washed twice with brine and was dried over anhydrous sodium sulphate.

The experiment was carried out following the same procedure, using tween 80 and cetyltrimethyl ammonium bromide (CTAB) in place of SLS.

5.2.6.8 Effect of presence of β-cyclodextrine:

Physical mixtures of the substrate and β-cyclodextrine were prepared in ratios of 1:1, 1:2 and 1:3 and the mixture was taken for the reaction. Procedure as mentioned in sections 3.3.2 and 3.3.3 was followed.

5.2.7 Chiral analysis:

The chiral purity of the sulphoxide obtained was determined by the use of chiral column. The mobile phase was phosphate buffer pH 6 and acetonitrile (25:75) at a flow rate of 0.6/minute. Absorbance was measured at 302 nm.

5.3 RESULTS AND DISCUSSION

5.3.1 Screening of microorganisms for Sulphoxidation

Totally ten micro organisms were selected for carrying out sulphoxidation of omeprazole and lanzoprozole e there was no conversion with respect to lanzoprozole intermediate.

Out of the ten micro organisms, one of the Rhizopus *stolonifer* strain was found to be effective in bringing about sulphoxidation of the selected substrate while one species of *Aspergillus niger* showed a negligible conversion (Table 5.1). Eventhough *Aspergillus* species have been reported to possess good sulphoxidation activity, the selected species in the present study were inactive in general, on the omeprazole intermediate.

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Table 5.1: Screening of micro-organisms for sulphoxidation

Microorganisms	Yield in mg/L
Aspergillus niger MTCC 961	00.00
Aspergillus niger	1.306
Aspergillus flavus	00.00
Aspergillus oryzae	00.00
Pencillium species	00.00
Pichia farinose MTCC 246	00.00
Candida viswanathii MTCC 1629	00.00
Saccharomyces cerevisiae MTCC 174	00.00
Bakers yeast	00.00
Rhizopus stolonifer MTCC 2198	13.802

As considerable bioconversion was observed in case of *R. stolonifer,* this organism was selected for further studies.

5.3.2 Effect of various physico-chemical parameters on microbial sulphoxidation

5.3.2.1 Effect of pH:

An important parameter which must be evaluated in biocatalyzed reactions is the pH of the reaction medium, as all enzymes have an optimal pH at which the reaction rate is maximum. Minor deviations in pH from the optimal value can lead to decreased enzymatic activity due to changes in the ionization of amino acid residues at the active site of the enzyme, while larger deviations in pH lead to denaturation of the enzyme protein itself. Therefore, pH is among the most significant factors affecting enzyme-catalyzed reactions (Adam et al. 2004).

Sulphoxidation was carried out in basic pH as the product; omeprazole is highly unstable in acidic pH. pH selected for the study were 7.6, 8.0, 8.4 and 8.8. The results revealed that the change of pH in the selected basic range did not have any significant effect on the

yield of omeprazole (Fig: 5.2). A pH range of 6-8 was found suitable for sulphoxidation of methyl phenyl sulphide with *P. frederiksbergensis* (Adam et al. 2004).

Table 5.2: Effect of pH on Sulphoxidation

pH	**Concentration of product mg/L**
7.6	1.96
8.0	1.6
8.4	1.51
8.8	1.67

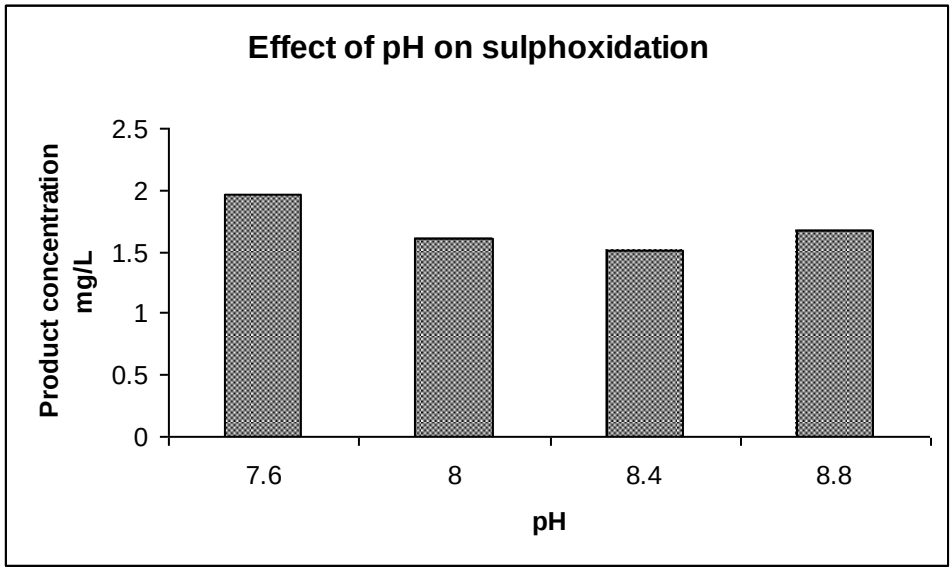

Fig. 5 2: Effect of pH on sulphoxidation.

5.3.2.2. Effect of Temperature:

The temperature in an enzyme-catalyzed process may alter the reaction rate because higher temperature accelerates molecular collisions between the enzyme and the substrate; On the contrary, inactivation of the enzyme at higher temperatures can also occur owing to thermal degradation. To evaluate the influence of temperature on microbial sulphoxidation, experiments were carried out at four different temperatures *viz.* 20 °C, 25 °C, 30 °C and 35 °C. The data obtained during the study clearly showed that bioconversion was not affected significantly when the temperature was varied between 25°C, 30°C and 35°C (Fig: 5.3); however, the conversion was considerably low at a temperature of 20°C. Li et al. (2009) have found that maximum activity of the biocatalyst in suphoxidation of phenyl methyl sulphide to phenyl methyl sulphoxide was at an incubation temperature of 35°C and at higher temperatures the activity significantly decreased. The effect of higher temperatures on sulphoxidation was not attempted in the present study as the product omperazole is sensitive to high temperature.

Table 5.3: Effect of temperature on sulphoxidation

Temperature °C	**Product concentration mg/L**
20	7.3
25	11.17
30	10.94
35	11.44

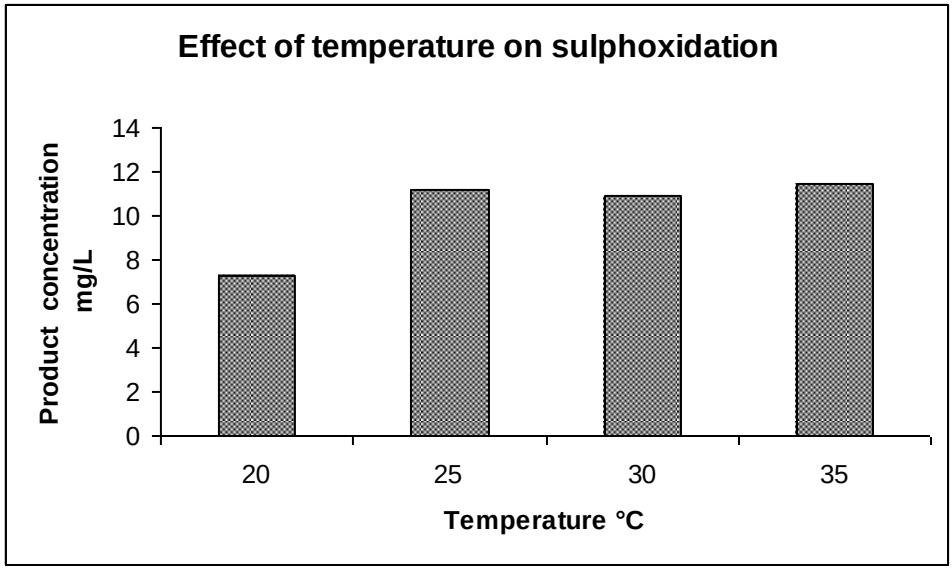

Fig. 5.3: Effect of temperature on sulphoxidation

5.3.2.3 Effect of incubation time:

One of the most important parameters that affect a chemical or biochemical reaction is the time of contact between the reactants. An ideal reaction time for a particular reaction to proceed depends on the type of reaction. Usually an increase in product formation is expected with increase in reaction time. In whole cell reactions, there are always chances of the product being acted upon by other enzymes present in the cell. Therefore, the effect of reaction time was studied by carrying out the reaction at various time intervals such as 24 h, 48 h, 72 h and 96 h. The observation made during the study showed that the product formation was maximum at an incubation time of 72 h (Table: 5.4) and no significant rise was observed with further increase in the reaction time.

Table 5.4: Effect of incubation time on sulphoxidation

Time h	Product concentration mg/L
24	0.57
48	2.20
72	2.31
96	2.11

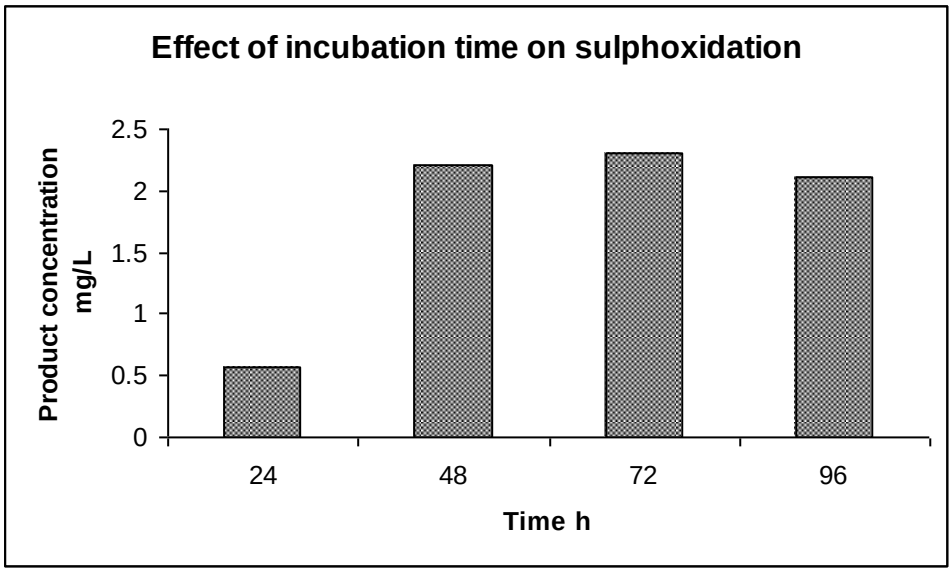

Fig. 5.4: Effect of incubation time on sulphoxidation.

5.3.2.4 Effect of biomass concentration:

In reactions involving whole cells, the concentration of enzymes contributing to the bioconversion is directly proportional to the biomass concentration. Therefore, when bioconversion was attempted by keeping the substrate concentration constant and varying the biomass concentration, increase in the product yield was expected. However, in some reactions where oxygen is involved, the increase in biomass may have a negative effect on the yield as the cells would compete for the molecular oxygen which is required for

the reaction (Holland 1988). So, the present study was aimed at finding out the effect of different concentrations of biomass (2, 4, 6, 8, 10 and 12 g in 20 ml reaction mixture) on the sulphoxidation of the selected substrate. The results recorded during the study indicated that the product concentration increased when the biomass concentration was increased up to 10 g. Further increase in biomass did not show any increase in the product concentration.

Table 5.5: Effect of biomass concentration on sulphoxidation

Weight of Biomass g	**Product concentration mg/L**
2	1.115
4	5.91
6	8.035
8	15.38
10	20.37
12	19.65

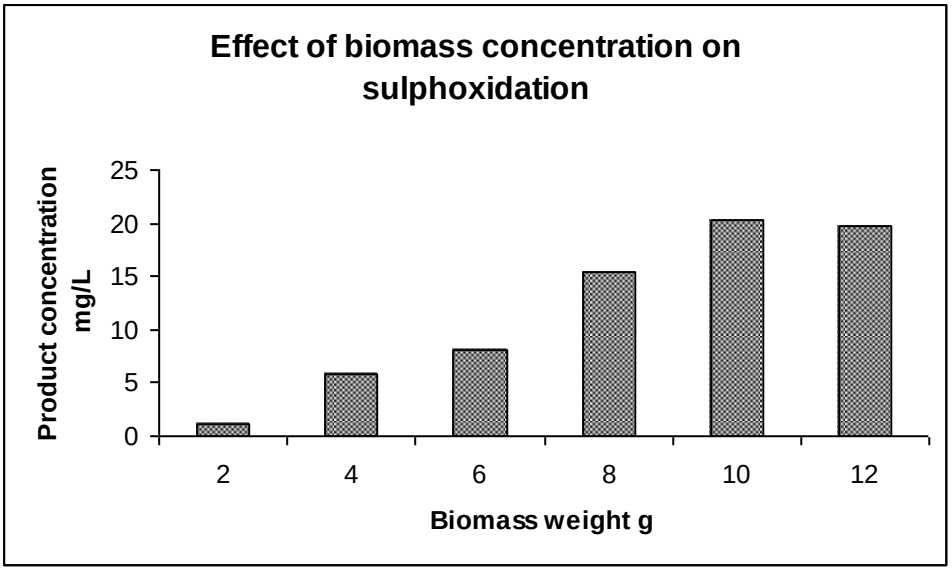

Fig. 5.5: Effect of Biomass concentration on sulphoxidation.

5.3.2.5 Effect of substrate concentration:

In a bioconversion process, substrate concentration is another important parameter worthy of investigation. Substrate concentration may affect reaction kinetics as substrate inhibition may occur at different concentrations for different enzymes present in the microbial cell. Thus, different concentrations of the substrate ranging from 0.02 % to 0.12 % were evaluated in the sulphoxidation reaction. Change in substrate concentration showed significant effect on the reaction kinetics. Maximum conversion was observed when the substrate concentration was 0.04 % (Fig: 5.6). Further increase in the concentration of the substrate showed a decline in sulphoxide concentration, which may be due to substrate inhibition of the enzyme involved in sulphoxidation. High concentration of thioanisole caused inhibition of the biotransformation process by *Rhodococcus* cells (El' Kin et al. 2010) which is similar to the phenomenon observed in the present study.

Table 5.6: Effect of substrate concentration on sulphoxidation

Substrate concentration %	**Product concentration Ppm**
0.02	1260
0.04	3520
0.06	2450
0.08	1770
0.10	1440
0.12	1160

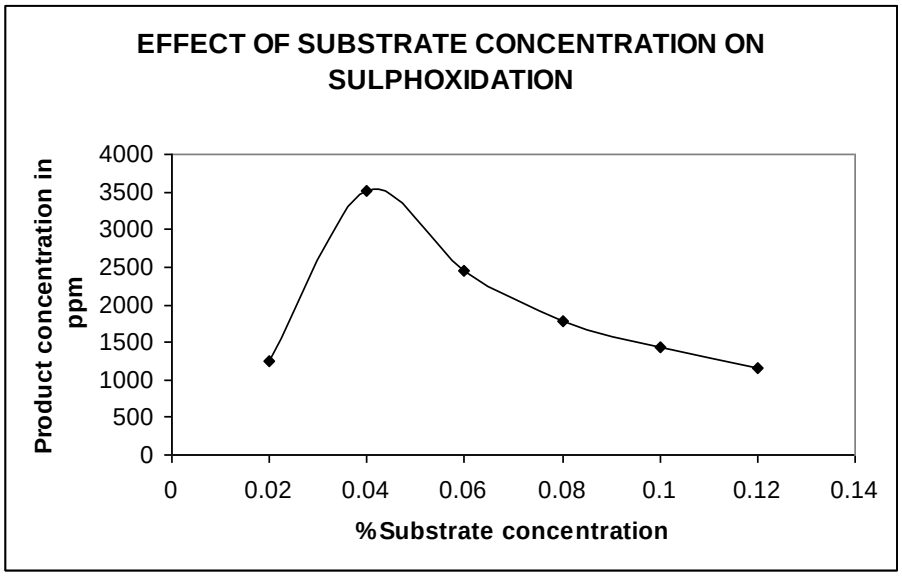

Fig. 5.6: Effect of substrate concentration on suphoxidation.

5.3.2.6. Effect of organic solvents

Most of the organic substrates used in bioconversion are hydrophobic in nature and their low water solubility limits the process productivity. One of the methods to overcome this problem is the application of organic solvents in biotransformation reactions. The organic phase acts as a reservoir and permits the storage of high concentration of substrate thereby, improving the process productivity. However, the disadvantage of organic–aqueous biphasic systems is the effect of solvent toxicity on the activity and stability of the biocatalyst (Leon et al. 1998). Li et al. (2011) have studied the influence of various organic solvents using different organic – aqueous biphasic systems on sulphoxidation of phenyl methyl sulphide by *Rhodococcus* sp. They observed that the yield decreased significantly in the presence of organic solvents with lower log P values like toluene and could find impressive results only with isooctane – aqueous biphasic system. In order to study this effect of organic solvent on sulphoxidation of the omeprazole intermediate by *Rhizopus stolonifer*, a series of organic solvents were chosen including both water miscible and water immiscible solvents with special emphasis on those which are hydrophobic and are often assumed to be more biocompatible in the biocatalytic process.

During the study, it was observed that there was no conversion in water immiscible solvents like hexane, toluene and benzene (Table 5.7). This indicated that probably the above organic solvents had a detrimental effect on the enzyme involved in biosulphoxidation. At the same time, biooxidation in presence of water miscible solvents such as acetone, dimethyl formamide and dimethyl sulphoxide was found to be possible but negligible as compared to the conversion in aqueous media.

5.3.2.7 Effect of surfactants:

Surfactants are amphiphilic compounds which can reduce surface and interfacial tension by accumulating at the interface of immiscible fluids and hence increase the solubility, mobility and subsequent biotransformation of hydrophobic or insoluble compounds. In the present study three different surfactants were used to enhance the microbial sulphoxidation. From the Table 5.8, it is clear that the anionic and cationic surfactants (SLS and CTAB) probably had a toxic effect on the biocatalyst as there was no bioconversion in both the cases while the nonionic surfactant, tween 80 showed very low conversion as compared to conversion in aqueous media in its absence.

Table 5.7: Effect of organic solvents on sulphoxidation

Solvent	**Product concentration ppm**
Aqueous (pH 7.6)	3313
Dimethyl sulphoxide (10%)	3085
Dimethyl fomamide (10%)	1469
Acetone (10%)	1276
Hexane (100%)	----
Benzene (100%)	----
Toulene (100%)	----

Table 5.8: Effect of surfactants on sulphoxidation

Surfactant	Product concentration Ppm
SLS	---
CTAB	---
Tween 80	1510
Aqueous buffer pH 7.6	3313

5.3.2.8 Effect of β cyclodextrin

Cyclodextrins are cyclic oligosaccharides with a hydrophilic outer surface and a hydrophobic central cavity. The hydrophilic exterior renders the cyclodextrin water soluble and the hydrophobic interior provides a microenvironment for accommodating relatively nonpolar molecules in it. In aqueous solutions, cyclodextrins can form inclusion complexes with hydrophobic compounds by entrapping either the entire molecule or a nonpolar part of it inside its hydrophobic cavity (Yaksh et al. 1991); based on this concept, physical mixtures of the substrate and cyclodextrin in three different ratios were prepared and were used in the sulphoxidation reaction. The results indicated that the product concentration increased with increase in concentration of cyclodextrin but overall, the yield was lower than the yield obtained without cyclodextrin (Table 5.9).

Table 5.9: Effect of β Cyclodextrin on sulphoxidation

Substrate: β Cyclodextrin	Product concentration ppm
1:1	----
1:2	1566
1:3	1672

5.3.3. Chiral analysis:

During chiral analysis to determine the stereo preference of the reaction, the enantiomeric excess of the R isomer was found to be 77% which is not very significant.

The existence of a multitude of cytochrome P-450 enzymes that function with different substrate specificity, and region and stereospecificities, and yet dependent on the same cofactors is well established in mammalian systems and close similarity may exist in the microbial world. The lack of absolute stereo selectivity observed for most enzymic sulphoxidations can be attributed to at least three alternative explanations (Holland 1988).

1. In the whole organism, there may be more than one sulphoxidizing enzyme; effecting simultaneous sulfide oxidation by two enzymes operating with different stereo specificities and rates.

2. There may be only a single enzyme, operating with non specific substrate binding followed by specific oxidation.

3. There may also be nonspecific direction of oxidation following specific binding of the substrate.

5.4 SUMMARY

- Totally 10 micro organisms were selected for carrying out sulphoxidation of omeprazole intermediate, out of which one *Rhizopus stolonifer* was found to be effective in bringing about sulphoxidation of the selected substrate, while one species of *Aspergillus niger* showed negligible conversion.

- While altering biomass concentration during bioconversion, it was found that the bioconversion was maximum at 10 g. The time taken for the conversion was 48 h and a 0.04 % substrate concentration was well tolerated by the microbial cell; however, further increase showed a decrease in bioconversion. The pH and

temperature of the reaction mixture chosen, did not affect the enzyme activity significantly.

- Biosulphoxidation in water miscible solvent showed insignificant conversion while the reaction did not proceed in water immiscible solvents. Use of β-cyclodextrin to enhance the solubility and thereby bioconversion of the substrate did not yield favorable results.

- The enantioselectivity of the enzyme system of the microorganism was found to be non-specific.

CHAPTER 6

STUDY ON EFFECT OF CULTURE CONDITIONS OF *RHIZOPUS STOLONIFER* ON SULPHOXIDATION

6.1 INTRODUCTION

Growth and potential of microbial enzyme production depend on several factors and media composition is one of the important factor (Singh et al. 2009). Microbial growth and enzyme production is dependent on growth conditions such as type and concentration of carbon, nitrogen, type of metal ions, pH and temperature of growth (Nwagu and Okolo 2011). Though microorganism can grow in media containing several types of carbon, nitrogen and metal ions, it is important to find the media in which maximum production occurs.

From the screening experiments it was found that *Rhizopus stolonifer* showed significant sulphoxidation ability of the sulphide compared to other organisms. Therefore the effect of media composition and growth conditions on the ability of the organism to bring about sulphoxidation was studied. The effect of different media constituents was studied by varying the media components of the growth media and carrying out sulphoxidation with the cultivated resting cells. Similarly, the effect of growth conditions i.e. pH and temperature were also studied.

6.2 MATERIAL AND METHODS

6.2.1 Cultivation of microorganism

As mentioned in Table 6.1, microorganism was cultured in different media and incubated at 30°C for 5 days. The mycelial biomass was separated by filtration and 5 g of the wet biomass was taken for bioconversion.

6.2.2 Bioconversion

Microbial sulphoxidation was carried out in 250 ml conical flask. About 5g of the wet biomass was taken in 20 ml phosphate buffer of pH 7.6, 20 mg of omeprazole intermediate dissolved in alcohol was added to the above suspension and incubated at 30 °C , 160rev min^{-1} for 48 h.

6.2.3 Analytical procedure

Assay of omeprazole was done by spectroflurimetry (Janna Shainsky et al. 2009).

Standard preparation: Stock solution of 1mg /ml concentration of standard omeprazole was prepared in phosphate buffer pH 7.0 and aliquots of 20, 40, 60, 80 and 100 µg/ml were prepared.

To 3 ml of each dilution, 1 ml of 0.1N HCl was added, incubated at room temperature for 5 min and the fluorescence signal was measured (excitation wavelength – 370 nm, emission wave length-560 nm) and the standard graph plotted.

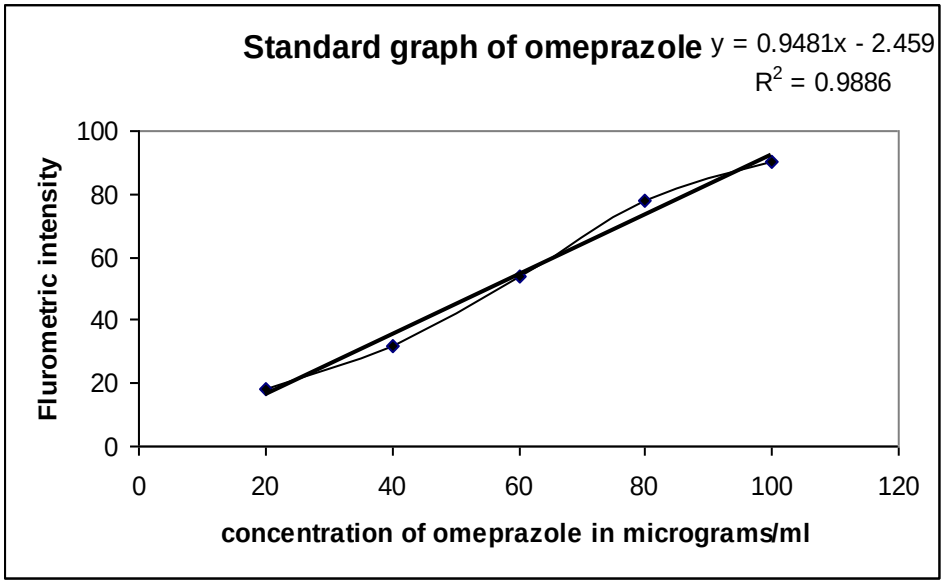

Fig. 6.1: Standard graph of omeprazole.

Sample preparation: The reaction mixture was centrifuged at 10000 rpm for 10 min and the supernatant was taken for estimation of sulphoxide. To 3 ml of the supernatant, 1ml of 0.1N HCl was added and incubated for 5 min and the fluorescence signal was measured as described under standard.

Media components → Media ↓	**Potato Extract**	**Carbon source g**				**Nitrogen source g**				**Metal ions mM**				**Dist. Water L**	**pH**	**Temp °C**
		Glucose	Lactose	Mannitol	Maltose	Peptone	Tryptone	Yeast Extract	Malt Extract	$CaCl_2$	$MgSO_4$	$FeCl_3$	$CuSO_4$			
1	20	0.4												1	6	30
2	20		.0.4											1	6	30
3	20			0.4										1	6	30
4	20				0.4									1	6	30
5	20	0.4				0.2								1	6	30
6	20	0.4					0.2							1	6	30
7	20	0.4						0.2						1	6	30
8	20	0.4							0.2					1	6	30
9	20	0.4								1				1	6	30
10	20	0.4									1			1	6	30
11	20	0.4										1		1	6	30
12	20	0.4											1	1	6	30
13	20	0.4												1	5	30
14	20	0.4												1	7	30
15	20	0.4												1	8	30
16	20	0.4												1	6	25
17	20	0.4												1	6	35
18	20	0.4												1	6	40

Table 6.1: Composition of media

6.3 RESULTS AND DISCUSSION

There was no marked effect on the capacity of the organism to bring about sulphoxidation when grown in different media and growth conditions. The addition of nitrogen source to the basal potato dextrose medium had a great impact on the mycelial growth but did not have significant effect on sulphoxidation.

6.3.1 Effect of carbon source

The carbohydrates in the media not only act as a major constituent for building the cellular material, but also influence the synthesis of various cellular compounds. The organism was cultivated in four different media containing different carbon sources viz., glucose, mannitol, maltose and lactose. Cells grown on glucose and maltose showed maximum conversion capacity as compared to mannitol and lactose grown cells. This may be due to the fact that the organism metabolizes glucose and disaccharide of glucose better as compared to the other two sugars.

Table 6.2: Effect of carbon source on sulphoxidation

Carbon source	**Omeprazole concentration μg/ml**	**Biomass g**
Glucose	52.05	1.76
Lactose	18.41	1.22
Mannitol	25.80	1.48
Maltose	52.00	2.04

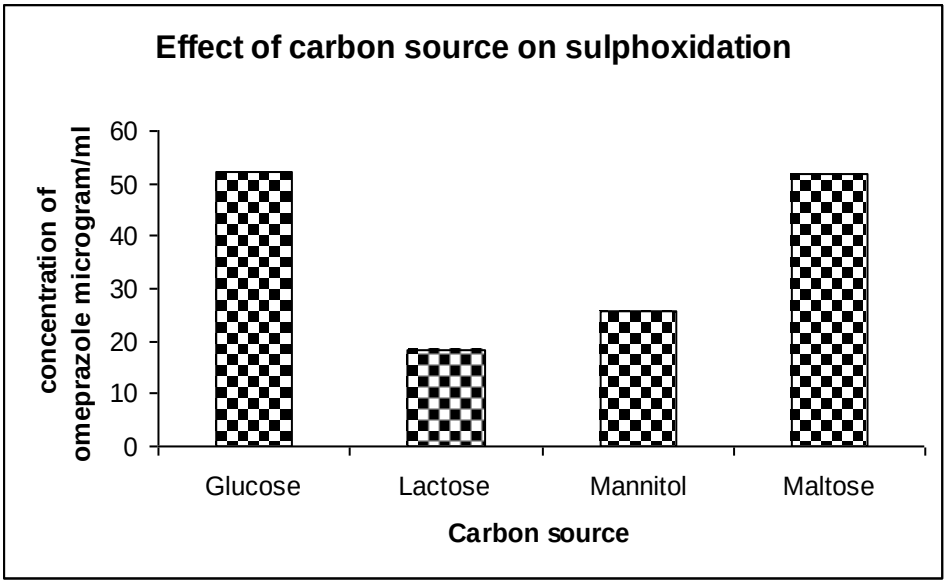

Fig. 6.2: Effect of carbon source on sulphoxidation.

6.3.2 Effect of Nitrogen source

It is well established that different nitrogen source affect the production of microbial enzymes differently. The effect of four different nitrogen sources on the microbial growth and subsequent sulphoxidation was studied. Addition of various nitrogen sources led to extensive growth of the mycelia but there was no significant effect on sulphoxidation by the cells grown on these media. However cells grown on media without nitrogen showed better sulphoxidation capability.

Table 6.3: Effect of nitrogen source on sulphoxidation

Nitrogen source	Omeprazole concentration μg/ml	Biomass g
Peptone	31.6	7.93
Tryptone	24.19	8.60
Yeast Extract	32.13	10.63
Malt Extract	28.54	4.80

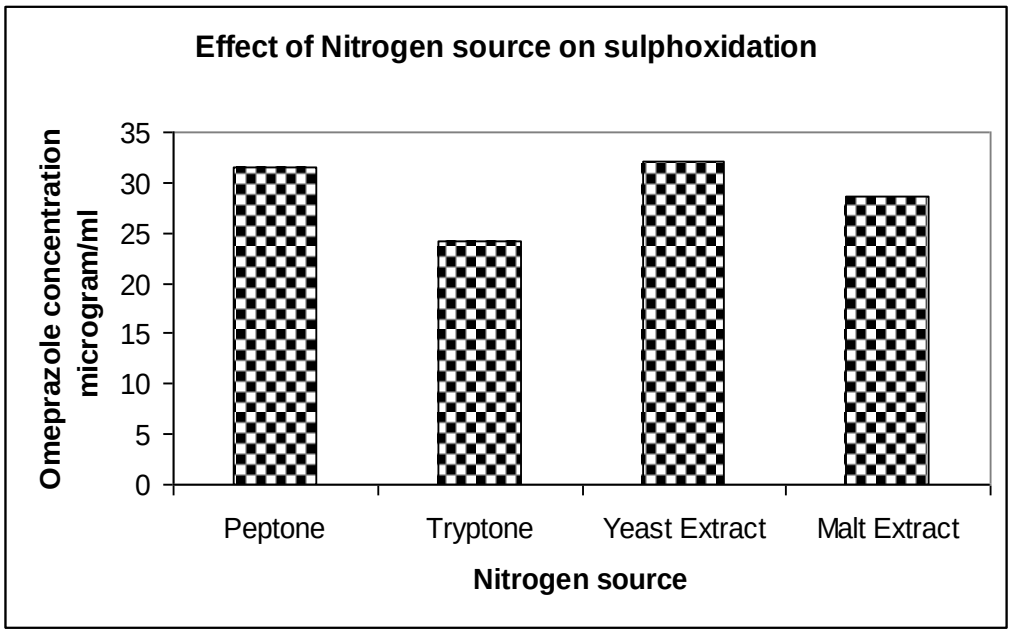

Fig. 6.3: Effect of nitrogen source on sulphoxidation.

6.3.3 Effect of Metal ions

Metal ions are known to regulate the synthesis of proteins in cells. Metal ions act as cofactors and affect the synthesis of certain enzymes. There are reports that addition of metal ions in medium affects cell wall permeability or buffered the media and hence affect enzyme production (Lee and Chen 1997). The effect of presence of some metal

ions in the media on production of enzyme involved in sulphoxidation was studied. The results indicated that there was no significant effect on enzyme production as the cells grown in presence of metal ions showed almost the same sulphoxidation capacity except for cells grown in presence of magnesium ion which showed slight increase in concentration of omeprazole.

Table 6.4: Effect of metal ions on sulphoxidation

Metal salt	**Omeprazole concentration μg/ml**	**Biomass g**
Calcium chloride	49.95	2.88
Magnesium sulphate	57.86	1.77
Ferric chloride	45.62	4.0
Copper sulphate	44.78	2.28

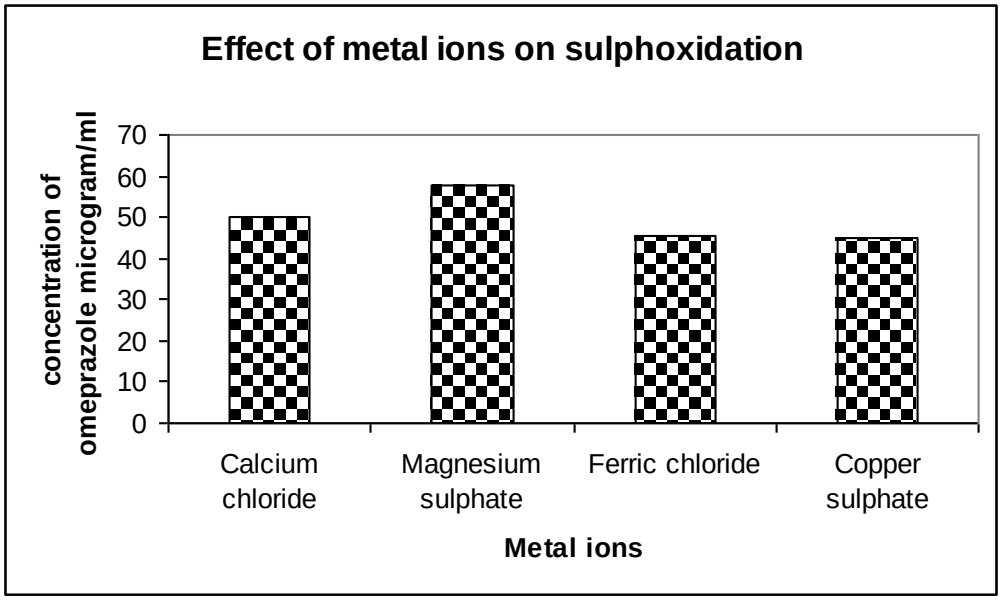

Fig. 6.4: Effect of metal ions on sulphoxidation.

6.3.4 Effect of pH

pH of the medium strongly affects the growth and activity of the microorganisms. Microbial enzymes are produced in higher yield at a pH near to the neutral. Fungal strains are noted for their best performance in the range of 3.5-7.0 and also low pH values. A change in pH affects the ionization of essential active sites of amino acid residue and thereby the enzyme catalysis. The effect of growth medium pH was analyzed. The changes in pH did not affect the sulphoxidation ability of the cells grown on different media. Cells grown at pH 8 showed slightly lesser ability for sulphoxidation.

Table 6.5: Effect of pH on sulphoxidation

pH	Omeprazole concentration μg/ml	Biomass g
5	41.3	3.0
6	52.05	1.76
7	45.62	3.0
8	39.93	1.8

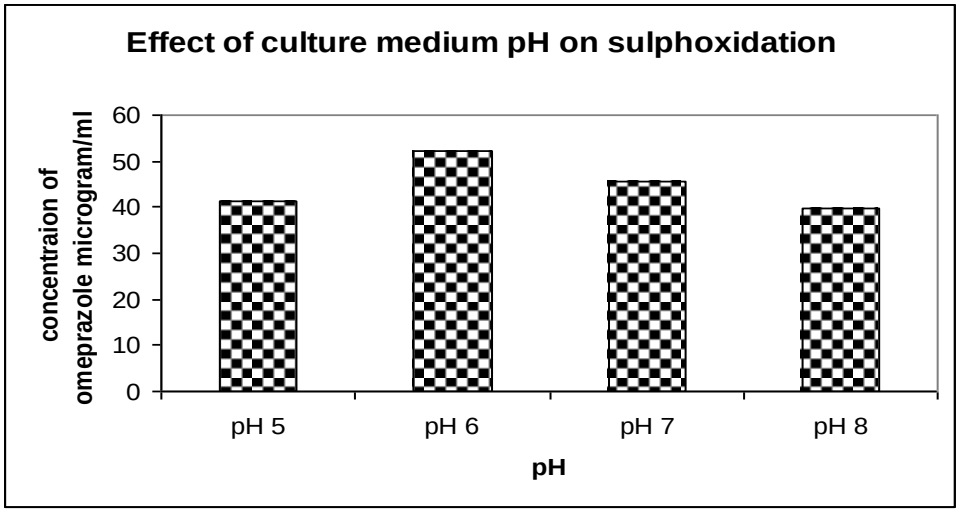

Fig. 6.5: Effect of media pH on sulphoxidation.

6.3.5 Effect of incubation temperature

The incubation temperature of culture media determines the effect of protein denaturation, enzyme inhibition, cell viability and death (Pallem et al. 2010). The effect of incubation temperature on the sulphoxidation ability was determined. The difference in the incubation temperature did not have much significant change in the sulphoxidation capability of the cultures.

Table 6.6: Effect of incubation temperature on sulphoxidation

Temperature °C	Omeprazole concentration µg/ml	Biomass g
25	41.5	1.57
30	52.05	1.76
35	39.72	1.98
40	23.29	1.02

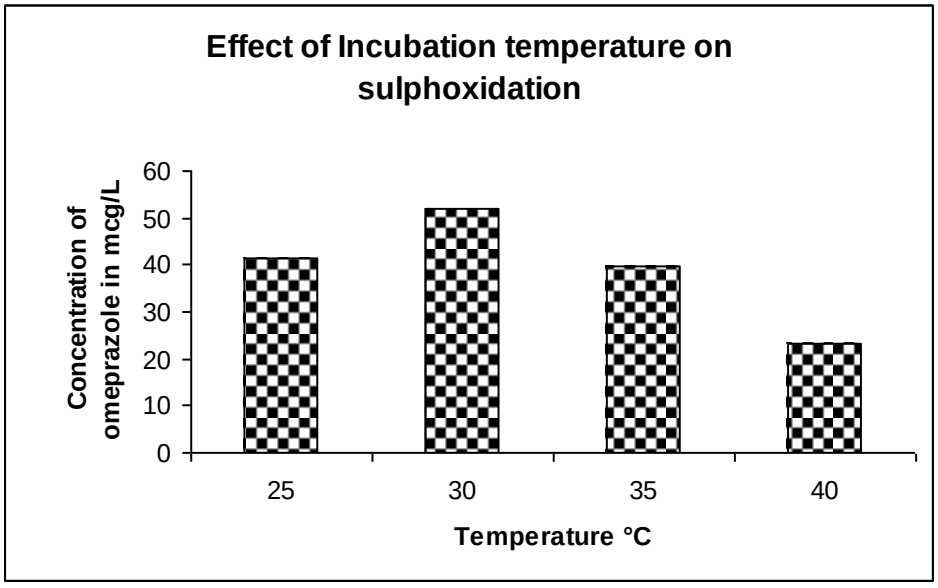

Fig. 6.6: Effect of incubation temperature on sulphoxidation.

6.4 SUMMARY

- Various media were designed to study the effect of different carbon and nitrogen sources and divalent metal ion salts.

- Different carbon sources like glucose, lactose, mannitol and maltose were tested to find the effect of these on the sulphoxidation process by *Rhizopus stolonifer*. It was found that glucose and maltose had maximum effect on the process as compared to other sugars.

- Addition of organic nitrogenous nutrients did not have positive effect on the sulphoxidation process. Among the various nitrogen sources analyzed media with yeast extract showed maximum effect.

- The effect of presence of some metal ions in the media on production of enzyme involved in sulphoxidation was studied. The results indicated that there was no significant effect on enzyme production as the cells grown in presence of metal ions showed almost the same sulphoxidation capacity except for cells grown in presence of magnesium ion which showed slight increase in concentration of omeprazole.

- The study on effect of initial pH of the media and incubation temperature revealed that they did not affect the sulphoxidation process significantly except that pH 8.0 had negative effect on the process.

CHAPTER 7

SUMMARY AND CONCLUSIONS

The present study was undertaken to carry out microbial reduction of three different ketone substrates and microbial sulphoxidation of two sulphides and can be summarized as follows.

- Different fungal strains were selected to carry out bioreduction of three different ketone drug intermediates. Among the strains of microorganisms screened for bioreduction of the selected substrates, only three strains viz *S. cerevisae, C. vishwanathii* and *A. niger* were found to be effective in bringing about the reduction of 3-[5-[(4-flurophenyl)- 1,5,dioxopentol]- yl] –4-(S) phenyl oxazolidin-2-one. As *S. cerevisae* showed considerable reduction, it was taken for evaluating the effect of various physical and chemical parameters on the biotransformation.

- The reaction was maximum at pH 7.6 of reaction media and temperature of 30°C was found to be the temperature for maximum conversion. Reaction time of 48 h was adequate and further increase in reaction time affected the product yield. Biomass concentration and product yield showed linear relationship. High concentration of substrate had a negative effect on the bioconversion.

- The effect of organic solvent on the bioconversion was studied by selecting hexane as the model solvent. The result showed that the solvent was not biocompatible with the selected biosystem as the yield decreased markedly with increase in concentration of the solvent. This may be due to the toxic effect of the solvent on the carbonyl reductases which are involved in the reaction.

- Reaction was tried in presence of surfactants with a view to increase the efficiency of conversion. Among the two surfactants tested, sodium lauryl sulphate increased the product yield by three folds. This proved that the solubility of the substrate was one of the important parameters to be considered in bioconversions.

- Chiral analysis revealed that the enzyme system involved in the reduction was 'R' specific.

- Culture conditions were varied to study the effect of various media constituents on enzyme production. Glucose as carbon source and peptone as the nitrogen source in combination had maximum effect on enzyme production. It was found that the addition of different nitrogen sources alone did not contribute significantly to the production of the enzyme.

- It is known that metal ions play an important role on enzyme activity, therefore effect of various metal ions on enzyme activity was analyzed. Addition of copper ion to the culture media showed moderate effect on the enzyme activity.

- The culture media pH and incubation temperature were varied to know the effect of these parameters on enzyme activity. Media pH of 7.0 and incubation temperature of 30°C was found to have maximum enzyme activity.

- Statistical tool like response surface methodology was used to study the individual, interactive and cumulative effect of variables on enzyme activity. Glucose as carbon source, peptone as nitrogen source and copper sulphate as the metal ion were selected as variable for the study. The result obtained was analyzed using software MINITAB 15 and a second order polynomial equation was generated. Coefficient of determination (R^2) was computed to assess the performance of regression in terms of goodness of fit. Both R^2 and R^2 (adj)

indicated that the performance of regression is very good with R^2 value of 96.17% and adj. R^2 of 92.73%.

- Analysis of variance for enzyme activity was also carried out with regard to various source of variation, namely, due to regression and residual error "E" of the model above. Computation was done for sequential sum of squares, adjusted sum of squares and adjusted mean sum of squares. F-statics were calculated for Regression model including that of Linear, Square and Interaction terms in the model. The fisher F-test with a very low probability value "P" demonstrates a very high significance for the regression model. Based on the model, optimum media was designed.

- Contour plots were used to analyze the interactions of the variables. The analysis revealed that increase in glucose and peptone concentration enhanced the enzyme activity but increase in copper sulphate concentration alone did not significantly affect the enzyme production and the effect became significant only when the concentration of peptone and glucose were increased simultaneously.

- Biosulphoxidation of benzimidazole derivative was carried out with different fungal species. Species of *Rhizopus stolonifer* was found to be effective in bringing about sulphoxidation of omeprazole intermediate.

- Various parameters that affect the biosulphoxidation process by *Rhizopus stolonifer* were studied. It was found that the bioconversion increased with increase in biomass concentration. The time taken for the conversion was 48 h and 0.04% substrate concentration was tolerated by the microbial cell, but further increase in substrate concentration showed decrease in bioconversion. The pH and temperature of the reaction mixture chosen in the study did not affect the enzyme activity significantly.

- Biosulphoxidation in water miscible solvent showed insignificant conversion while the reaction did not proceed in water immiscible solvents. Use of β-cyclodextrin and surfactants, to enhance the solubility and thereby bioconversion of the substrate did not yield favorable results.

- The enantioselectivity of sulphoxidation of the substrate was not specific and yielded 77% of (R) sulphoxide.

- Various media were designed to study the effect of different carbon and nitrogen sources and divalent metal ion salts. Different carbon sources like glucose, lactose, mannitol and maltose were tested to find the effect of these on the sulphoxidation process by *Rhizopus stolonifer*. It was found that glucose and maltose had maximum effect on the process as compared to other sugars.

- Effect of organic nitrogenous nutrients on the enzyme activity was found to be insignificant. Among the various nitrogen sources analyzed media with yeast extract showed maximum effect.

- The effect of presence of metal ions in the media on the production of enzyme involved in sulphoxidation was studied. The results indicated that the addition of metal ions to the media did not affect the production of enzymes involved in sulphoxidation, significantly.

- The study on effect of initial pH of the media and incubation temperature revealed that they did not affect the sulphoxidation process significantly except that pH 8.0 had negative effect on the process.

FUTURE OUTLOOK

Asymmetric reduction of ketones is an important key step in the synthesis of many enantiopure pharmaceuticals and there are not much literature available on the use of biocatalyst for the reduction of 3-[5-[(4-flurophenyl)- 1,5,dioxopentol]- yl] –4-(S) phenyl oxazolidin-2-one. More microbial species including bacterial strains can be screened for the reduction of this ketone.

Though whole cell method is cheaper with down stream processing, the method is time consuming and the yield is also very low. The efficiency of the process can be enhanced by isolating the enzyme responsible for the bioconversion, genetically engineered and further immobilized for efficient reuse.

Application of biocatalyst in synthesis of enantiopure sulphoxides is very limited. In depth study on screening of efficient microorganism, isolation of the enzyme and optimization of the process for the synthesis of enantiopure omeprazole can be carried out.

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LIST OF PUBLICATIONS BASED ON THIS WORK

Research papers published in international journals

1. **Brahmani Priyadarshini S.R**., Gopal Mugeraya and Sandhyavali M.S. (2011). "Bioreduction of a drug intermediate in presence of hexane and surfactants." *Asian Journal of Chemistry*, 23(1), 369-371. .
2. **Brahmani Priyadarshini S.R**., Gopal Mugeraya, Sandhyavali M.S. and Anupam Kumar Mishra. (2010). "Sulphoxidation of a drug intermediate using microorganisms." *Bioscience, Biotechnology Research Asia*, 7(1), 457-560.
3. **Brahmani Priyadarshini S.R**., Gopal Mugeraya and Sandhyavali M.S. (2009). "Screening and optimization of bioconversion parameters for the reduction of 3-[5-[(4-flurophenyl)-1,5,dioxopentol]-yl]–4-(S)phenyloxazolidin-2-one." *International journal of PharmTech. Research*, 1(4), 1601-1604.

Research papers presented in conferences

1. Evaluation of certain bioconversion parameters in the reduction of 3-[5-[(4-flurophenyl)- 1,5,dioxopentol]- yl] –4-(S) phenyl oxazolidin-2-one by *Saccharomyces cerevisiae.* National level seminar on stereochemical Adventures in synthesis of challenging drug molecules at PSG college of pharmacy, Coimbatore during July 3-4, 2009.
2. Sulphoxidation of a drug intermediate using microorganisms, National level seminar on stereochemical Adventures in synthesis of challenging drug molecules at PSG college of pharmacy, Coimbatore during July 3-4, 2009.
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