Biocatalytic Oxidation of Primary & Secondary alcohols by using *Candida Parapsilosis* ATCC 7330

A project Report

Submitted in partial fulfilment of the requirements

For the award of the degree of

MASTER OF TECHNOLOGY

In

CHEMICAL ENGINEERING

SPECIALISATION : CATALYSIS TECHNOLOGY

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MAY, 2011

CERTIFICATE

This is certify that the project report entitled "**Biocatalytic Oxidation of Primary & Secondary alcohol ”** submitted by **Sanjay Kumar Soni** to the Indian Institute of Technology, Madras for the award of the degree of Master of technology in Catalysis Technology,Chemical Engineering is a bonafide record of the work carried out by his under my supervision. The contents of the thesis, in full or parts have not been submitted to any other institute or university for the awards of any degree.

Guide

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Acknowledgment

It is with great pleasure I express my indebtedness to **Prof. Anju Chadha and Prof. R..Rravi** for providing me the rarest opportunity to pursue my M.Tech Project studies under their guidance in the prestigious institute of IIT, Madras. I am thankful to them for the forbearance, gentleness, humility and dignity they have shown towards me through out my association with them. I am indebted to them for the unfailing guidance, invaluable advice, fruitful discussions, steadfast support and the timely help they have provided all through my research. I deem it a unique blessing to be associated with them to whom I owe a great intellectual and inspirational debt. I am thankful to them for the innumerable helps they have rendered unto me. Mere words are not sufficient to express my gratefulness for their sincere efforts to help me in completing this task. With immense delight, I wish to place on record my deep sense of gratitude and indebtedness to **Prof. B. Viswanathan** for being an exemplary, virtuous and noble personality and a teacher to me. I am thankful to him for moulding me into an obedient, disciplined and humble student. It is a boon to me that I have come into acquaintance with such a teacher of vast wisdom, expertise and understanding. I am indebted to him for the enlightening discussions, illuminating and delightful ways of teaching science. But for his patient efforts I could not have realized my dream of pursuing M.tech. My stay at IITM was made pleasant and cherishable in his association. I shall remain ndebted to him through out my life for the innumerable helps he has rendered unto me. I am deficient of words to pay my tributes to him for the inspiration, motivation and encouragement he has provided me. I wish to express my sincerely thanks to the M.tech Committee members,for their kind help, suggestions and also for providing me the necessary infrastructure to carry out the research work .I am thankful to the Department of Biotechnology, **National Centre for Catalysis Research**,Department of chemical engineering for creating the where in I have carried out most of my research work. I am thankful to **Dr. S. Sivasanker** for his timely help and encouragement. I am thankful **to Dr. A. V. Ramasamy** and **Dr. K. R. Krishna Murthy** for their support. I am thankful to **Prof. P. Selvam** for his invaluable I

am thankful to **prof K.Krishnaiah ,Pushvanam S ,Ravi Krishna, Shirinivas Jayanti and Arun K Tangirala for** Suggestions.relative to core subject I express my sincere thanks to the staff and students of our lab member specially **Dr.Preeta**, Nidhi, Ritam, Pratap Sarwanan I owe a great debt to **IIT Madras** for providing the state-of-the-art facilities for carrying out my research work. I thank the non-teaching staff members of the Department of Biotechnology and Chemical engineering for their cooperation and enthusiastic support. And I thank SAF for the NMR spectroscopic analysis.

Sanjay Kumar Soni

Title - Whole cell ,*Candida parapsilosis* ATCC 7330 ,2,4-dichlorobenzaldehyde ,

Abstract

Oxidation of α-hydroxy esters and aromatic alcohols to the corresponding carbonyl compounds is an important and challenging transformation both at laboratory and industrial scales. It belongs to the most fundamental processes in organic chemistry. Recent efforts are dedicated to green catalytic oxidation methods. Conventionally, oxidation is brought about by utilizing stochiometric /superstochiometric amounts of inorganic oxidants like Ru,Rh and Ir or Oppenauer oxidation (with Al,Zr and lanthanides) which are incongruous to the principle of green chemistry.

In the present study, optimisation was done for non-enantioselective biocatalytic oxidation of α-hydroxy ester (racemic ethyl 2-hydroxy ,4- phenylbut-3-enoate) and aromatic alcohol (3-phenyl prop-2-enol (cinnamyl alcohol)). The oxidation was achived by using whole cells of *Candida parapsilosis* ATCC 7330 and the reaction conditions were optimized to get 61% oxidised product within 5 mins at 25°C.

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1. INTRODUCTION

1.1 Biotransformation or biocatalysis

In recent years, process chemists in the pharmaceutical industry have been turning attention to the application of biocatalysts using either isolated enzymes or more commonly, immobilized enzymes or whole-cell biocatalysts [1]. The use of biocatalyst or enzymes for chemical synthesis has several advantages which include elimination of the protection and deprotection steps in reactions (R). Secondly enzymes are highly selective and operate under mild conditions thereby reducing associated E-values. Therefore, the enzymes are of interest in their use as biocatalysts to create newer routes to high value chemicals. Moreover, biocatalyts still play a major role in the pharmaceutical sector due to its regioselective and stereoselective properties which enables them to carry out difficult chemical reactions. Also, most of the products are chiral (89%) and are used as fine chemicals [2]. For example, the reduction of asymmetric ketone for the production of (S)-3, 5-bistrifluromethyl phenyl ethanol, which serves as an important alcohol intermediate required for synthesis of NK-1 receptor antagonists [3]. The areas which use biocatalysts in chemical synthesis have been shown in figure 1 below. Figure 2, shows the cumulative number of biotransformation processes that have been started on an industrial scale.

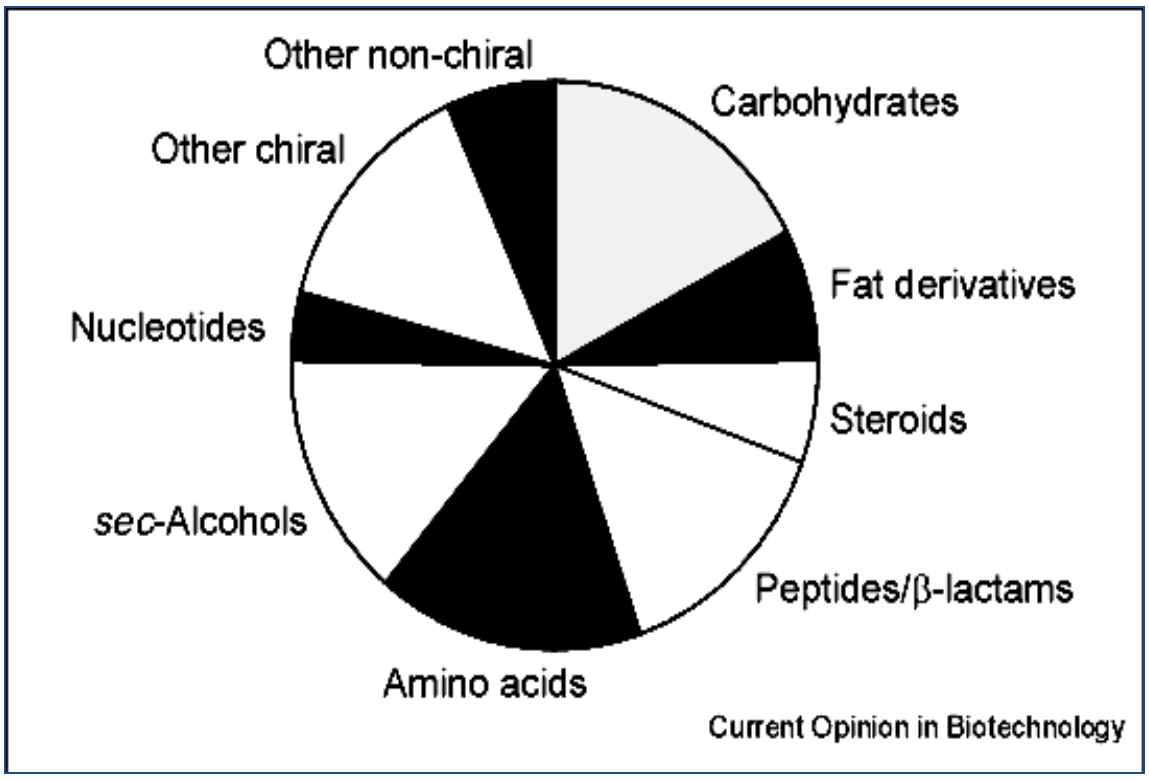


Fig.1.1 Biotransformation processes (based on 134 industrial processes) for the various types of compounds

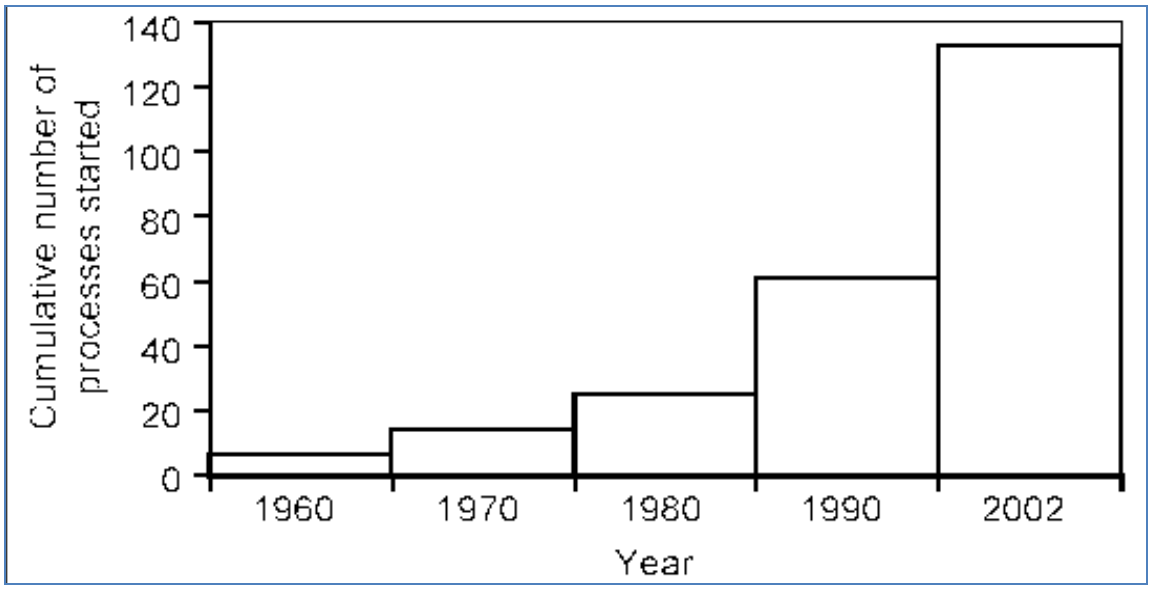


Figure 1.2: Cumulative number of biotransformation processes that have been started on an industrial scale.

Replacement of hazardous reagents and catalysts with less harmful alternatives is the major goal of green chemistry. Another example is the reduction of amides to amines. This reaction uses hydrides such as $LiAlH_4$ (Lithium aluminum hydride) or DIBAL (diisobutyaluminium hydride) which are corrosive or hazardous in nature. An anaerobic environment makes amide reduction possible and this strategy has been used recently with the cells of Clostridium *sporogenes,* which reduces benzamide to benzyl amine with yields of up to 73% [4].

Oxidation is also an important reaction in pharmaceutical synthesis which has resulted in the availability of greener regents. For example, processes have been developed which uses TEMPO (2,2,6,6,-tetramethylpiperidine-1-oxyl) , NaOCl (sodium hypochlorite) or Pd (palladium) instead of previously required high valent metal catalysts. These oxidizing regents are ideal in the presence of organic solvents, but incur significant safety concerns. Nevertheless, biocatalytic oxidation can be carried out by enzymes in an aqueous environment with oxygen that is usually supplied in the form of air [5]. For example, chloroperoxidases (CPOs) from *Cadariomyces fumago* has shown to be effective as an oxidizing agent with high yield and selectivity.

Organic solvents account for around 80% in typical pharmaceutical and fine chemical synthesis. Large number of steps in pharmaceutical synthesis means that the intermediate solvent extraction results in a large inventory of organic solvents. Therefore, replacement of organic solvents with environment friendly solvents has been seen as an alternative. As most of the biocatalytic reactions take place in water, some reactions with two phase biocatalytic system can partially replace solvents so as to facilitate substrate supply or product removal.

1.2 ADVANTAGES AND DISADVANTAGES OF BIOCATALYSTS

1.2.1 Advantage

- Enzymes are very efficient biological catalysts and are environmental friendly.

- They act under mild conditions of temperature [20-40 °C] and in the physiological pH range of 5-8. This helps to minimize undesired side reactions.
- As enzymes are compatible with one another, several biocatalytic reactions can be performed simultaneously in one flask. This is advantageous for sequential reactions using multi-enzyme systems, as the isolation of unstable intermediates can be omitted.
- In addition the enzymes exhibit high chemoselectivity, regioselectivity and enantioselectivity.

1.2 .2 Disadvantages

- Enzymes are provided by nature in only one enantiomeric form.
- Sometimes enzymes are extremely specific where they require narrow operation conditions. If a reaction proceeds slowly, under certain given parameters, there is only a narrow scope of alteration as it leads to deactivation of the enzyme catalyst.
- Enzymes display their highest catalytic activity in water, which is the least desired solvent for most of organic compounds and reactions. Therefore, shifting of enzymatic reactions from an aqueous to an organic medium reduces its activity as well as stability.
- Many enzymatic reactions are prone to substrate or product inhibition, which causes deactivation of the enzyme at higher substrate or product concentrations.

1.3 CLASSIFICATION OF BIOCATALYSTS

Biocatalysis presents a cross-section of recent advances in catalysis and biotechnology. Enzymes are known to catalyze a myriad of different chemical reaction types which are listed below in table 1.1.

Table 1.1 Function based classification of the enzyme groups

1.Oxidoreductase	Oxidation Reduction	Alcohols, Epoxides, Lactones, Amino acid sulphoxides
2.Transferase	Hydroxyl methyl transfer Amino group transfer	Hydroxyl amino acid Amino acid, amine
3. Hydrolase	Ester hydrolysis Trans-etherification Nitrile /amide hydrolysis Hydantoin hydrolysis Alkylhalide hydrolysis	Alcohol, Carboxylic acid, Carboxylic ester Alcohol, Carboxylic acid, Carboxylic ester, Carboxylic acid, Amino acid, Haloalkanoic acid, Alcohol, Epoxide
4 .Lyase	C-C bond formation C-O bond formation C-N bond formation	Amino acid, Acyloin, Cyanohydrin Alcohol, Amino acid Amino acid
5. Isomerase	Lactone formation	Lactone

1.4 ISOLATED ENZYMES VERSUS WHOLE CELL SYSTEMS AS BIOCATALYSTS

The physical state of biocatalysts that are used for biotransformation can be very diverse. Isolated enzyme systems or intact whole cells of microorganisms may be used for biotransformation depending on the factors like; type of reaction, cofactor recycling and the scale of biotransformation.

Each approach has its own advantages and disadvantages. Many isolated enzyme systems are now commercially available or are relatively easy to isolate and they can be stable and easy to use, often giving the desired product. However, for some reactions in which a cofactor has to be used, the need to regenerate the cofactor can be an added complication. Whole cells as biocatalysts do not have this disadvantage and, although, they do tend to give more than one product, they are often cheaper to use than isolated enzyme systems [6] and can take of cofactor regeneration and recycling in the reaction system.

1.5 Biocatalytic oxidation:

Oxidation of alcohols to corresponding carbonyl compounds is an important and challenging chemical reaction both in the laboratory and at industrial scales [7]. Conventionally, oxidation is carried out by utilizing stoichiometric amounts of inorganic oxidants [8] and organo-metallic catalysts which are unsuitable to the principles of Green Chemistry.

The biocatalytic oxidation chemistry can help shortening synthesis routes, avoiding protection group chemistry, and reducing byproduct formation. Further, biocatalysts generally operates under mild reaction conditions in terms of temperature and pH and do not require hazardous solvents. Overall, biocatalysis is an enabling technology with a tremendous potential of greening the way chemical synthesis is carried out.

1.6 Type of biocatalysts used in oxidation reactions:

A variety of different enzymes are involved in bio-oxidation reactions as follows;

1.6.1 Dehydrogenases

This class of enzymes catalyzes the reversible oxidation of alcohols and amines by hydrogen transfer mechanism. NADP is a classical example of hydride transfer reagent existing in an oxidized ($NADP^+$) and a reduced form ($NADPH^+$). $NADP^+$ is the active oxidant which abstracts a hydride from the alcohol –CH-group to pyridinium moiety as shown in figure 1.3 below.

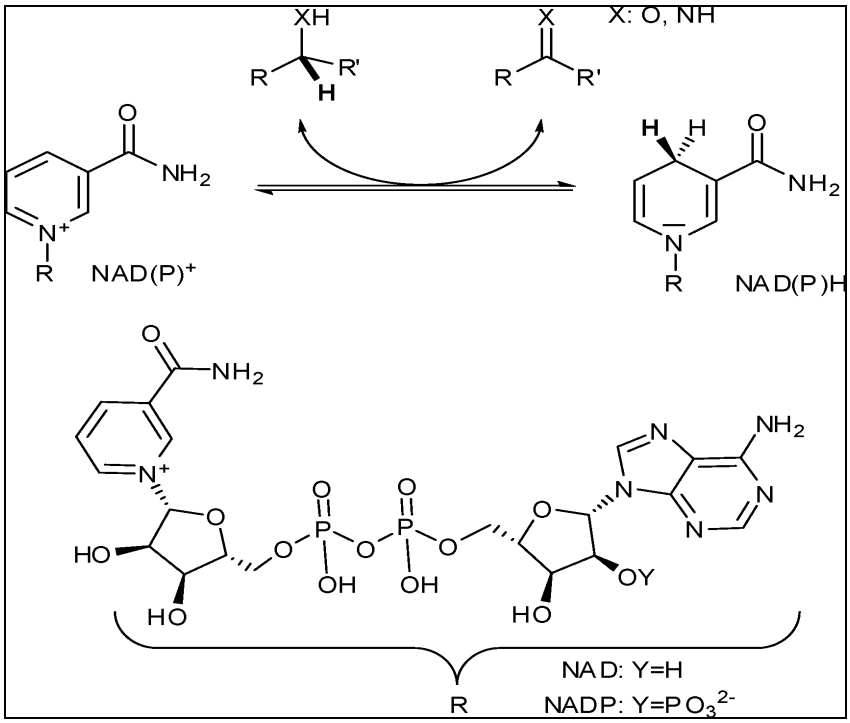


Figure 1.3: Structure and basic redox chemistry of the nicotinamide

Another example of alcohol dehydrogenase enzyme is the HLADH enzyme isolated from horse liver. HLADH exhibits a broad substrate tolerance towards primary and secondary alcohols. Aliphatic alcohol oxidase (AlcOx) enzymes which are isolated from

methylotrophic yeasts such as *Candida*, *Hansenula* and *Pichia* mostly accept primary alcohols [9].

1.6.2: Oxidases

An oxidase is an enzyme that catalyzes an oxidation-reduction reaction involving molecular oxygen (O_2) as the electron acceptor. In these reactions, oxygen is reduced to water (H_2O) or hydrogen peroxide (H_2O_2). The oxidases are a sub-class of the oxidoreductase enzymes. Oxidases are popular for the catalytic oxidation of alcohols and amines. A number of cupric Cu^{+2} dependent oxidases such a galactose oxidase are known. For example FAD or FMN function as primary hydride acceptors from which the excess reducing equivalents are eventually shuttled to molecular oxygen (Figure 1.4) [10].

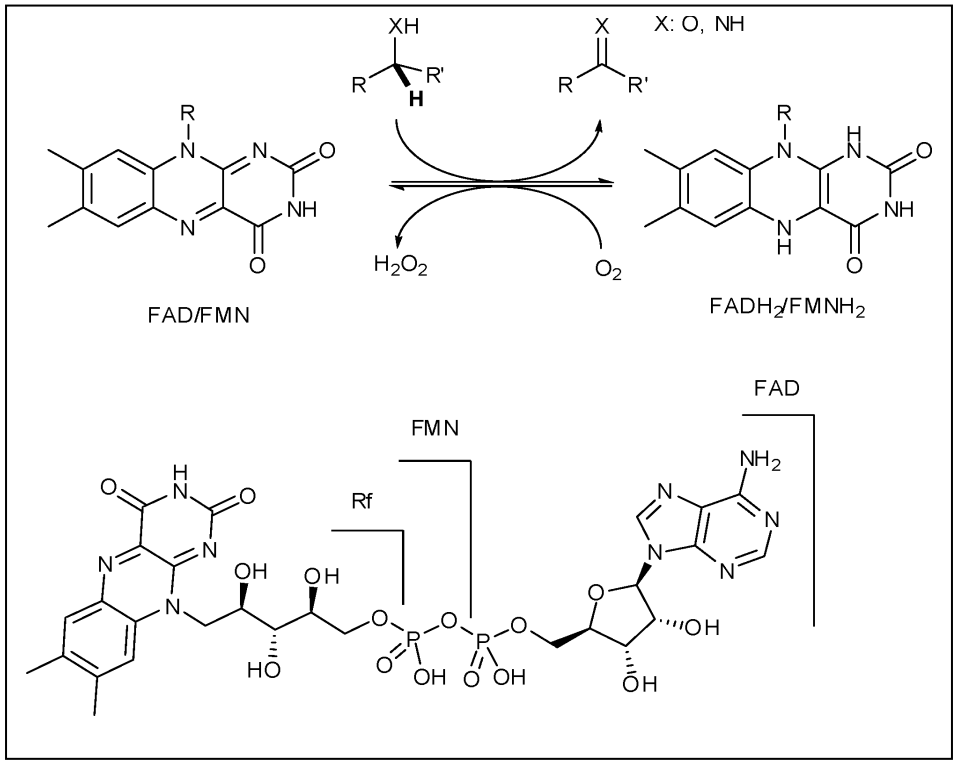


Figure 1.4: Structure and basis redox chemistry of the flavin group

1.6.3: Peroxidases

Peroxidases are a large family of enzymes that typically catalyze a reaction of the form;

$$ROOR' + \text{electron donor } (2\ e^-) + 2H^+ \rightarrow ROH + R'OH$$

The heme-thiolate peroxidases utilize H_2O_2 or organic peroxides to regenerate the catalytically active oxyferryl species. These enzymes are most active with organic hydroperoxides such as lipid peroxide and peroxidases containing a heme cofactor in their active sites or redox-active cystein or selenocysteine residue. Enantioselective sulfoxidation is carried out by cascade reaction of Pd (0) catalyzed formation of H_2O_2 and enzymatic oxidation using chloroperoxidase from *Caldariomyces fumago*. Supercritical carbon dioxide ($scCO_2$) is used as a medium for *in situ* generation of H_2O_2 directly from H_2 and O_2 using Pd-catalysts. Subsequently, H_2O_2 is utilized by the chloroperoxidase enzyme as an oxidant for the asymmetric sulfoxidation in the aqueous phase [11].

2. REVIEW OF LITERATURE

2.1: OXIDATION OF ALCOHOLS:

Bio-redox catalysis accounts for one third of all commercialized enzymatic processes. Out of these, approximately half comprises of oxidation processes. Interestingly, simpler alcohol and amine oxidations are only a minor fraction (22%) as compared to the enantiospecific oxyfunctionalizations such as hydroxylations, dihydroxylations, epoxidations and Baeyer–Villiger oxidations (Fig 1.5) [12].

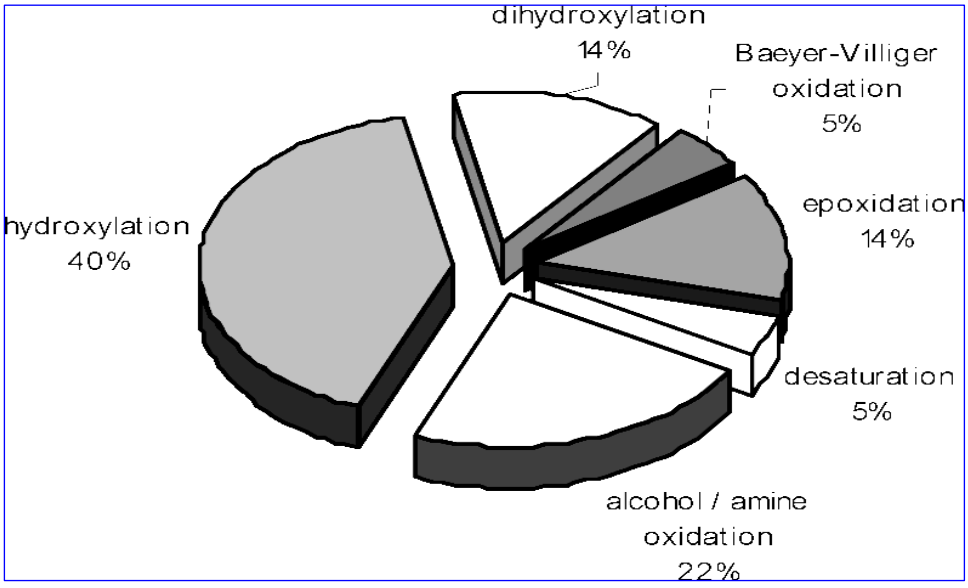


Figure 2.1: Distribution of reaction types amongst the industrial biocatalytic oxidation reaction

2.1.1: Synthesis of aldehydes from primary alcohols

The major problem in selective oxidation of primary alcohols to aldehydes is the over oxidation of the alcohol to acid which is the thermodynamically favored step. This is challenging particularly when applying whole cell biocatalysis where oxidation of aldehydes adds to the organism's energy balance by regenerating one equivalent of NADH. When isolated enzymes such as ADH or AlcOxs are used over oxidation may be observed. Therefore, one solution to this problem is to make use of *in situ* product removal from the reaction phase.

This concept was elegantly used by Buhler et al. (2002) for the selective oxidation of pseudocumene to 3,4-dimethyl benzaldehyde using recombinant *E. coli* [13]. By using dioctylphthalate as a second organic phase, both toxic effects of the reagents and undesired over-oxidation to the acid could be efficiently circumvented. Overall product titers increased up to 0.22 M and 70% isolated yields were achieved [14] as shown in figure 1.6.

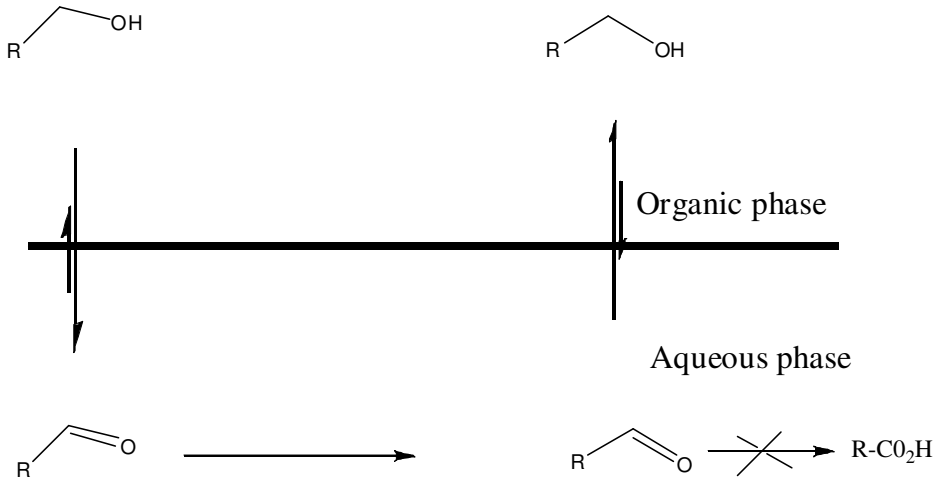


Fig2.2: Prevention of over oxidation by *in situ* removal of the intermediate aldehyde to an organic phase and the organic phase serves as substrate reservoir.

A similar application of the two liquid phase concept was also demonstrated by using acetic acid bacteria as biocatalysts. A range of primary alcohols were converted selectively to either aldehyde or acid, depending on the reaction medium used [15]. For

removal of the reactive aldehyde another alternative is a sequential chemical or enzymatic reaction. For example, undesired over-oxidation could be circumvented in case of conversion of glycolic acid into glyoxylic acid by *in situ* removal of glyoxylic acid as imine [16]. Another fine example of this was reported by Siebum et al. (2006). AlcOx from *Pichia pastoris* was used to generate the reactive aldehyde substrate for 2-deoxyribo-5-phosphate aldolase (DERA)-catalyzed synthesis of β-hydroxyketones (Fig.1.7) [17]. Similarly, Wong and coworkers (2008) reported the galactose oxidase catalyzed oxidation of glycerol to L-glyceraldehyde into a four-enzyme one-pot cascade reaction to form fructose from simple precursors [18].

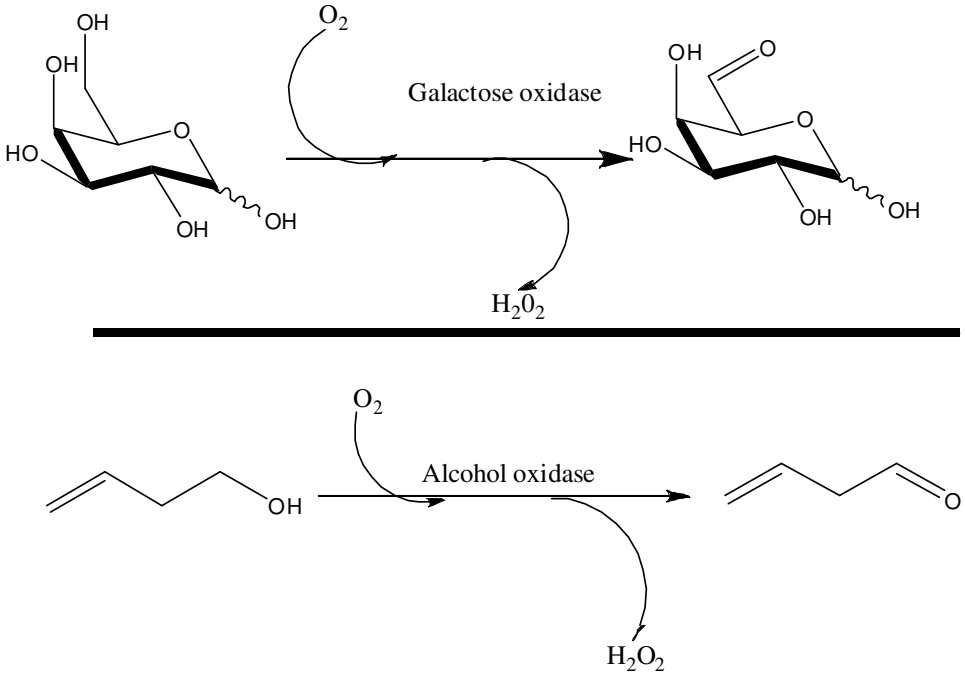


Figure2.3: The galactoses oxidase (GO) and alcohol oxidase (AO) catalytic systems.

2.1.2: Oxidation of secondary alcohols

Oxidation of alcohols play important roles in the preparation of enantiopure alcohols by means of kinetic resolution, deracemization and desymmetrization.

1. **Kinetic resolution**:

Kinetic resolution is one of the most popular approaches to obtain optically active compounds. In this approach oxidized compounds form as an intermediate and a chiral secondary alcohol goes hand in hand with the elimination of a chiral center. The α-oxidation of 7 to 16 carbon atom containing carboxylic acids and oleic acid was reported by using a crude homogenate of young pea leaves. These acids were transformed to the enantiomerically pure (*R*)-2-hydroxy acids **2** in the presence of molecular oxygen [19].

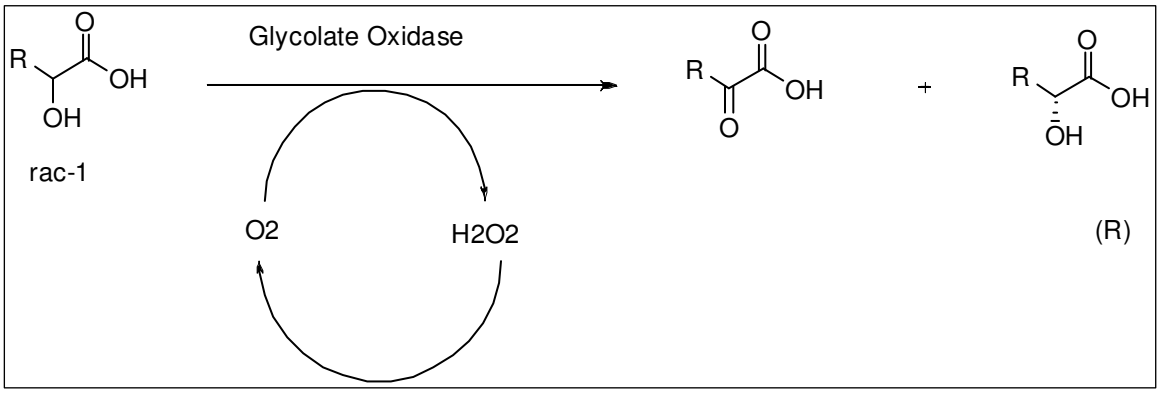


Fig 2.4: Oxidation of 2-Hydroxy Acids with Molecular Oxygen Catalyzed by Glycolate Oxidase from Spinach (*S. oleracea*)

2. **Desymmetrization:**

Optically active alcohol can also be made by desymmetrization of meso-alcohols which upon oxidation are transformed into chiral products. Desymmetrization of meso 2,5-hexandiole was reported by using ADH-A from *Rhodococcus ruber* yielding (R) -5-hydroxy -2-hexanone at 88% conversion within 2h C. V. Voss et al. 2008.

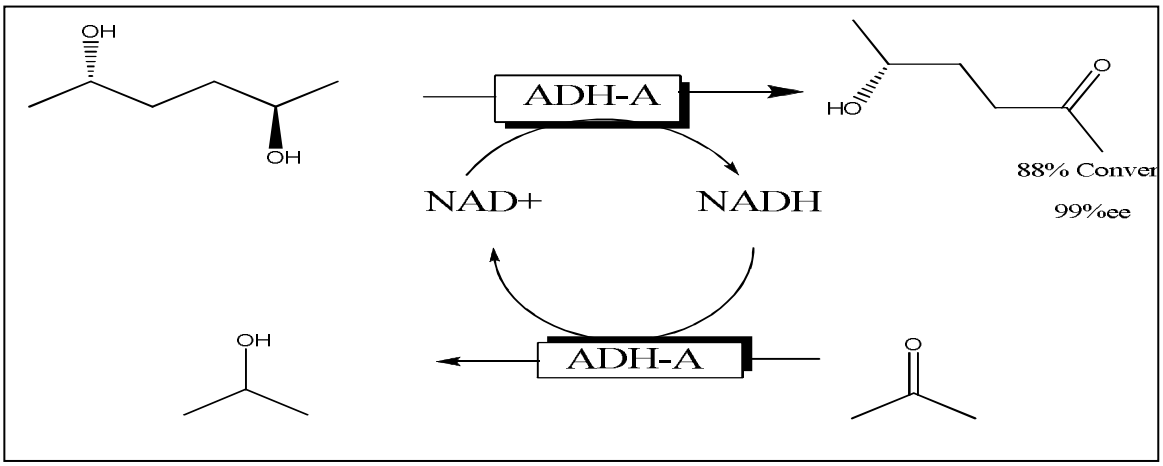
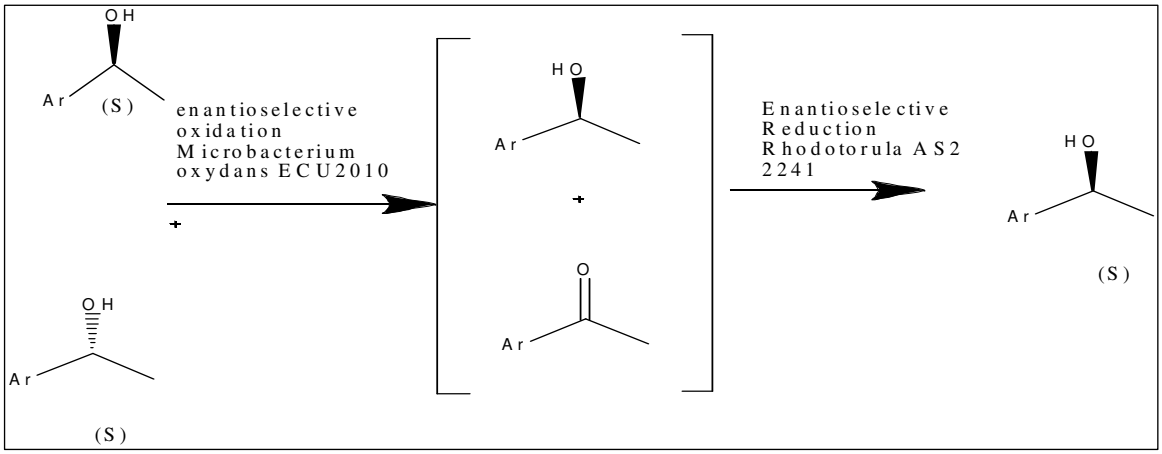


Figure 2.5: Dsymmetrization of meso-2,5-hexandiole using ADH-A from *Rhodococcus ruber*

3. Deracemization:

The basic requirement for redox-deracemization is that at least one of the steps, either oxidation or reduction, proceeds enantioselectively. Zilbeyaz et al. (2007) reported the deracemization of recemic 1-phenyl ethan-phenyl ethanol to (S) 1-phenyl ethanol with

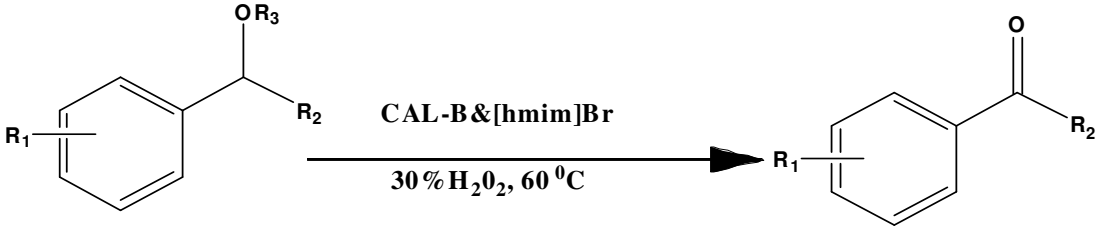


Two types of biocatalysts. *Microbacterium oxydans* ECU 2010 and *Rhodotorula sp.* AS2. 2241 were used as biocatalyst [20].

Figure-2.6: Deracemization of racemic 1-phenyl ethan-phenyl ethanol to (S) 1-phenyl ethanol with two types of biocatalysts. Microbacterium oxydans ECU 2010 was used as biocatalyst

2.1.3: Non-enantioselective oxidation:

Oxidation of both optical isomers of alcohols is called non-enantioselective oxidation. Non-enantioselective oxidation of rac 2-octanol within 24h via hydrogen transfer reaction using alcohol dehydrogenase from *Sphingobium yanoikuyae* was reported [21]. Non-enantioseletive but chemoselective oxidation of aryl alcohol and acetates was also reported [22] by using lipase and liquid [hmim] Br for metal free activation of H_2O_2.



R_1=H,OCH_3,OCH_2O,NO_2,Cl,et
R_2=H,-CH_3,-CH_2CH_3,-C_3H_7
R_3=H,-$COCH_3$

Figure-2.7: Non-enantioseletive but chemoselective oxidation of aryl alcohol and acetates also reported by using lipase and liquid [hmim]Br

2.2 OBJECTIVES OF THE PRESENT WORK

1 Synthesis of a variety of α-hydroxy esters.

2 Optimization of reaction conditions for biocatalytic oxidation of secondary alcohol (ethyl 2-hydroxy 4- phenylbut-3-enoate).

3 Optimization of reaction conditions for biocatalytic oxidation of the primary alcohol 3-phenyl prop-2-enol (cinamyl alcohol).

4 Enhancement of biocatalytic oxidation by using co-substrate and reductase inhibitors.

5 Standardization of growth conditions for *C.parapsilosis* ATCC 7330 for getting maximum yield using selected substrates.

3. MATERIALS AND METHODS

3.1 CULTURE CONDITIONS

3.1.1 Culture maintenance:

Candida parapsilosis ATCC 7330 was procured from ATCC, USA and maintained on yeast malt agar medium YMA [(g/l) containing yeast extract 3 g, peptone 5 g, dextrose 10 g, malt extract 3 g, agar 2.1%, pH of 6.5-6.8]. The culture could be maintained at 4^{o}C for one month without any loss of activity. Every month the culture was sub-cultured on fresh YMA plates to maintain viability.

3.1.2 Cell cultivation and harvest:

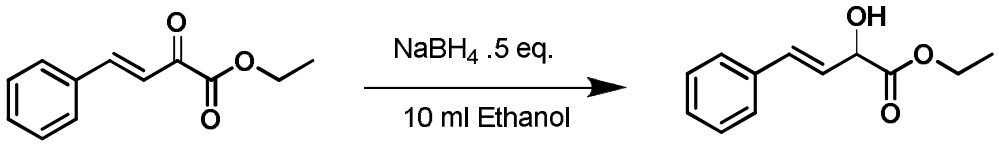
Cells were cultivated and harvested according to the method described by Baskar (2004). A single colony of *Candida parapsilosis* ATCC 7330 from YMA plate was used to inoculate 50 ml of autoclaved YMB (yeast malt broth) media in a 250 ml conical flask. The flask was incubated at 25°C on a rotary shaker at 200 rpm for 24h. This culture was used as inoculum for further cultivation of cells. The flasks were inoculated with 2% inoculum and incubated at 25°C on a rotary shaker at 200 rpm for 40h. The cells were then harvested by centrifugation at 4500 rpm at 4^{o}C for 15 min.

3.1.3 Procedure for biotransformation of 4-phenyl 2-hydroxy 3-butanoate and cinnamyl alcohol using free cells of *Candida parapsilosis* ATCC 7330:

4-phenyl 2-hydroxy 3-butenoate was synthesized by sodium borohydride reduction of 4-phenyl, 2-oxo, 3-butenoate. 4-phenyl, and cinnamyl alcohol was purchased from Lancaster chemical Co., U.K.

1. Procedure for synthesis of 4-phenyl 2-hydroxy 3-butenoate:

4-phenyl, 2-hydroxy, 3-butenoate (1gm) dissolved in ethanol (10ml) was charged into 50 ml round bottom flask fitted with nitrogen atmosphere and the reaction mass was then cooled to 0°C. Sodium borohydride (98gm) was then added to reaction mixture at 0°C and the reaction mass was stirred for 2h. After 2 hours TLC was checked and after reaction completion of the reaction, the mixture was evaporated under vacuum. Water was added to the residue, and the product was extracted with ethanol (3×15ml). Ethanol layer was dried over sodium sulphate and concentrated under vacuum. Purified product was obtained after column chromatography using hexane (85:15) as the eluent.



(*E*)-Ethyl 2-oxo-4-phenylbut-3-enoate **(*E*)-Ethyl 2-hydroxy-4-phenylbut-3-enoate**

Figure 3.1 Sodium borohydride reduction of 4-phenyl, 2-hydroxy, 3-butenoate by using candida parapsilosis ATCC 7330

2.Characterization of 4-phenyl, 2-hydroxy, 3-butenoate

^{1}H NMR (500 MHZ, $CDCl_3$):

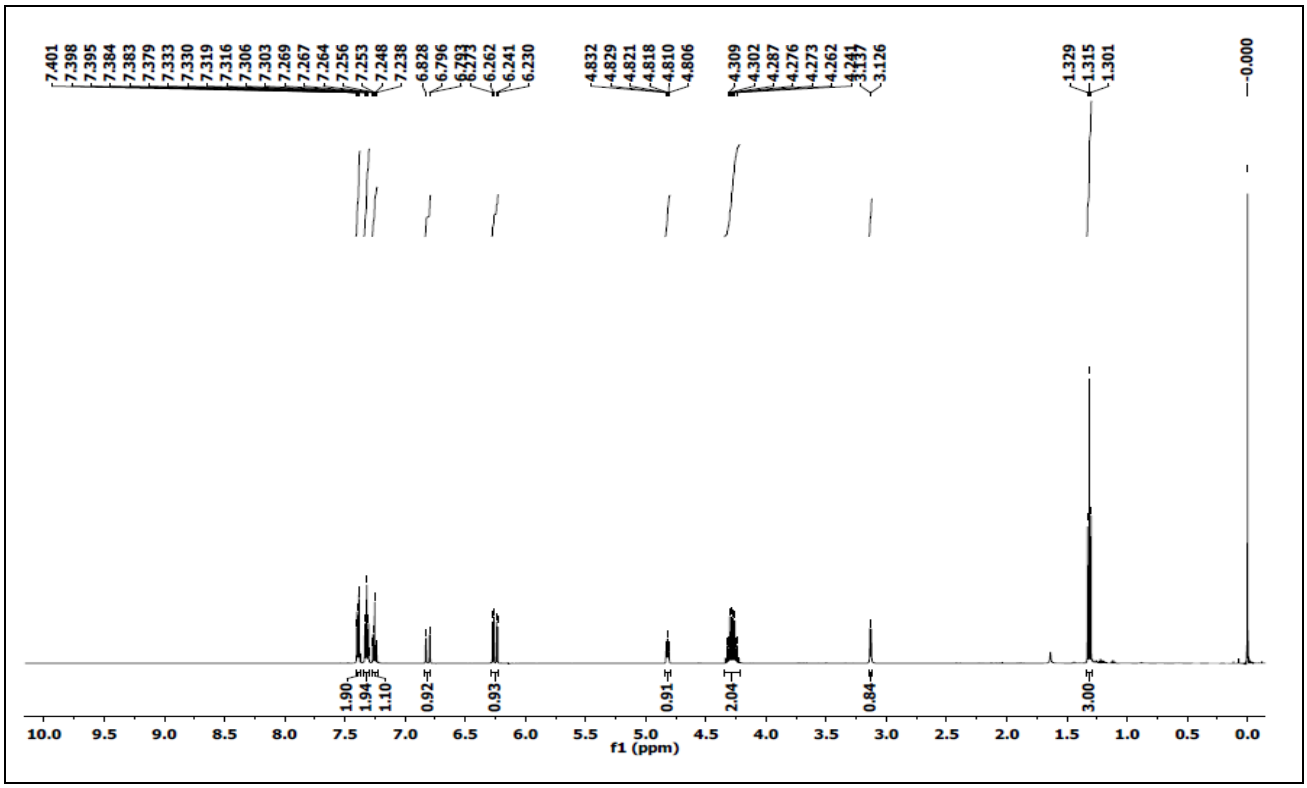
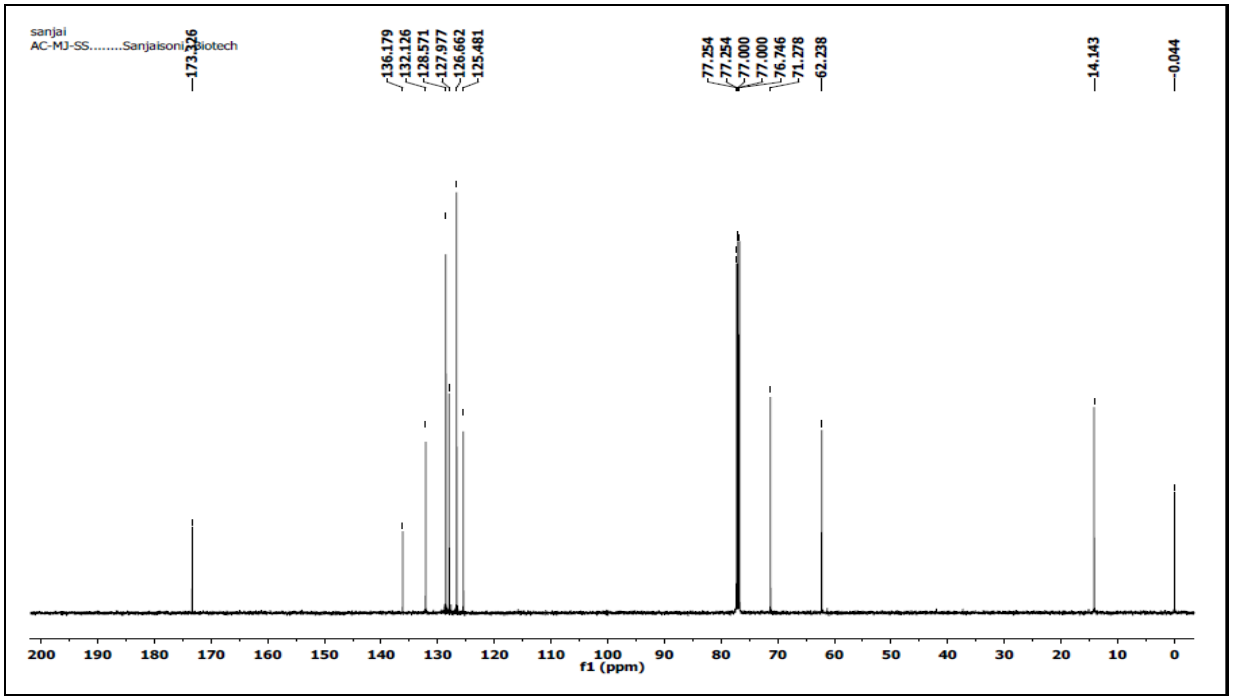


Figure-3.2 Characterization of 4-phenyl, 2-hydroxy, 3-butenoate by Proton NMR

^{13}C NMR (125 MHZ, $CDCl_3$):



Expansion for ^{1}H NMR:

Figure-3.3 Characterization of 4-phenyl, 2-hydroxy, 3-butenoate by ^{13}C NMR

3.1.4: Procedure for biotransformation of Ethyl 4-phenyl, 2-hydroxy, 3-butenoate and cinnamyl alcohol using free cells of *Candida parapsilosis* ATCC 7330:

To a 150 ml conical flask containing 5 g of pelleted *Candida parapsilosis* ATCC 7330 suspended in sterile distilled water, 15 mg of substrate dissolved in 375 µl of ethanol was added. The total volume of the reaction mixture was made upto 5 ml by adding sterile distilled water. Reaction was carried out in a water bath shaker at 150 rpm and 25°C for 5 min. The product formed was isolated using ethyl acetate (3×5ml) and the organic layer was dried over anhydrous sodium sulphate. The solvent was removed by evaporation and obtained product was analysed by using HPLC on a C-18 column [Acetonitrile : water]

(85:15). The yields of the products, ethyl (S)-4-phenyl, oxo, 3-butenoate and cinnamyldehyde were found to be 61% and 19% respectively.

4. Result and discussion

4.1 OXIDATION OF ETHYL 1,2-HYDROXY,4-PHENYLBUT-3-ENOATE

Anju Chadha et al. (2002) reported the deracemization of racemic ethyl 2-hydroxy ,4-phenylbut-3-enoate into the (S)- 2-hydroxy ,4-phenylbut 3-enoate by using *Candida parapsilosis* ATCC 7330. In this reaction etthyl 2-oxo 4-phenylbut-3-enoate [keto intermediate] is formed.

Upendra K.Sharma et al. (2009) reported the biocatalytic promiscuity of unique synergistic combination of lipase and liquid [hmim] Br for metal free activation of H_2O_2 which is used for chemoselective oxidation of aryl alcohol and acetates.

This clue lead us to explore the oxidation of ethyl 2-hydroxy ,4- phenylbut-3-enoate by using the biocatalyst *Candida parapsilosis* ATCC 7330 sourced from ATCC, USA. Ethyl 2-hydroxy ,4- phenylbut-3-enoate was converted into ethyl 2-oxo 4-phenylbut-3-enoate (61%). After extraction of the product using organic solvents, analysis by HPLC was carried out using a C-18 column with Acetonitrile : Water (85:15 ; v/v) as the mobile phase.

Optimization studies for oxidation of α-hydroxy ester were carried out with repect to;

a) Reaction conditions for biotransformation. In all these optimization reactions the chemical yeild of keto product obtained was monitored.

b) Growth conditions optimized and reported by Chadha et al. (2002) for the deracemisation of racemic ethyl 2-hydroxy 4- phenylbut-3-enoate was used for oxidation of secondary alcohol ethyl 2-hydroxy 4- phenylbut-3-enoate .

4.2 Optimisation of growth conditions of *Candida parapsilosis* ATCC 7330:

4.2.1 Optimization of harvesting time:

In this study *Candida parapsilosis* ATCC 7330 cells were harvested at time intervals of 12h, 18 h, 24h, 30h, 36h, and 40h and were used for oxidation. Cells harvested at 40h gave 5% coversion at 7.0 pH of ethyl 2-hydroxy 4- phenylbut-3-enoate. without reductase inhibitor 2,4-dichlorobenzaldehyde and 61% conversion with 2,4-dichlorobenzaldehyde at 7.5 pH of ethyl 2-hydroxy 4- phenylbut-3-enoate.

4.2.2. Effect of temperature:

In this study *Candida parapsilosis* ATCC 7330 cells harvested at different temperatures 25°C, 30^0C, 35°C, and 40°C were used for oxidation. Cells harvested at 25°C gave 61% conversion with 2,4-dichlorobenzaldehyde and 5% conversion without 2,4-dichlorobenzaldehyde at 7.0 pH of ethyl 2-hydroxy 4- phenylbut-3-enoate .

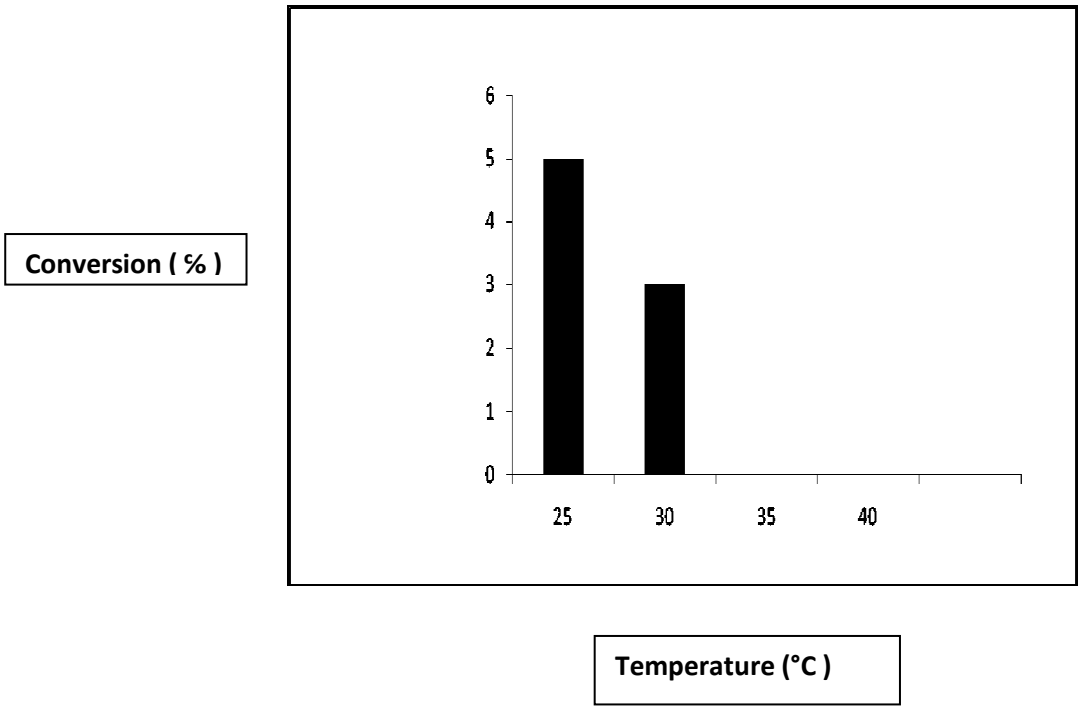


Figure 4.1 -Effect of Temperature on the oxidation reaction of (RS)-EHPB (ethyl 2-hydroxy 4- phenylbut-3-enoate) without reductase inhibitor 2,4-dichlorobenzaldehyde at 7,0 pH

4.3. Optimisation of conditions for biotransformation:

The reaction conditions (substrate concentration, reaction temperature, co-substrate, pH) for the biotransformation were optimized to get maximum chemical yield of the oxidised product ethyl 2-oxo 4-phenylbut-3-enoate keto compound.

4.3.1 Optimisation of time:

Lvan lavandera et al. (2008) achieved oxidation of rac 2-octanol within 24h via hydrogen transfer reaction using alcohol dehydrogenase from *Sphingobium yanoikuyae* and Upendra *K*. Sharma et al. (2009) reported the CAL-B and [hmin]Br catalysed oxidation of rac 4-methoxyphenylpropanol with 30% H_2O_2 was carried out for 16h. In this study the time of oxidation of ethyl 2-hydroxy 4- phenylbut-3-enoate was typically 5 min. At various time intervals 10 min, 20 min, 40 min and 50 min, the reaction mixture was analysed using C-18 column on HPLC. The maximum conversion of 61 % was achieved at 5 min.

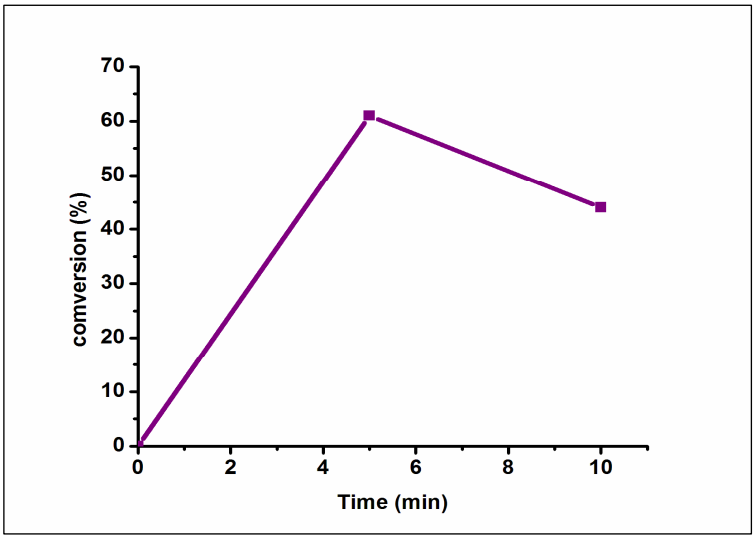


Figure:4.2: Time course of the oxidation reaction of (RS)-EHPB (ethyl 2-hydroxy ,4-phenylbut-3-enoate)

4.3.2 Effect of pH:

pH is also an important parameter which influences. In this study the biotransformation was carried out at various pH 7, 7.5-7.6, 8, 8.5. The reaction mixture was analysed using C-18 column HPLC for ethyl 2-hydroxy 4- phenylbut-3-enoate substrate. The maximum yield of 2-oxo 4- phenylbut-3-enoate product was obtained at pH 7.5-7.6.

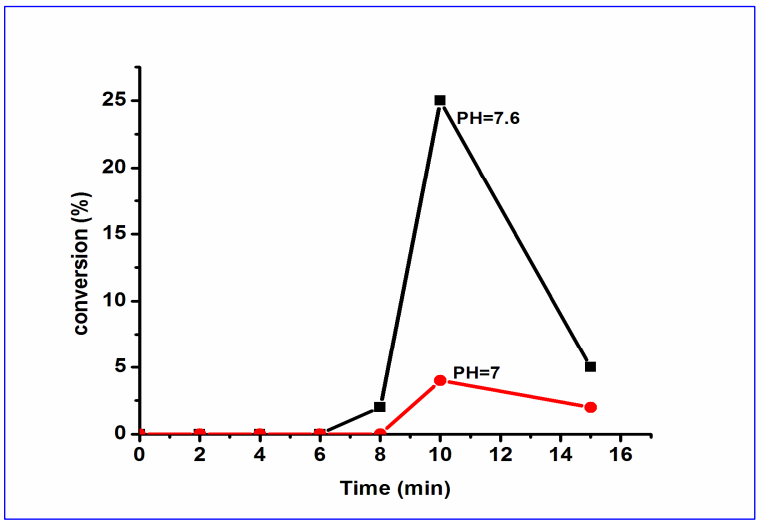


Figure:4.3: Effect of pH on the oxidation reaction of (RS)-EHPB (ethyl 2-hydroxy ,4- phenylbut-3-enoate) without reductase inhibitor DCB

4.3.3 Effect of buffer:

In two separate experiments under identical conditions (Wet cells: 0.3g/ml of reaction mixture, 25°C, reaction time 10 min), the reaction was carried out in deionised distilled water (pH 7.6) and in buffer (pH-7.6). The results showed that for oxidation using resting cells of *Candida parapsilosis* ATCC 7330 the biotransformation is more efficient in water.

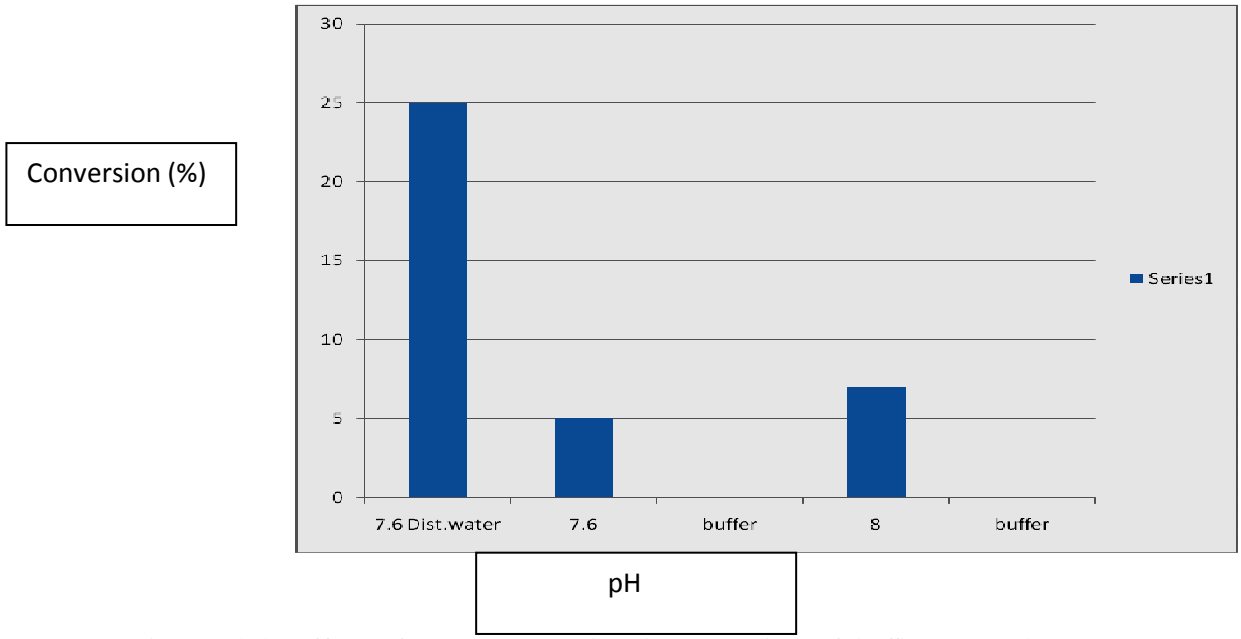


Figure:4.4: Effect of pH on the oxidation reaction of (RS)-EHPB (ethyl 2-hydroxy 4-phenylbut-3-enoate) without reductase inhibitor 2,4-dichlorobenzaldehyde

4.3.4 Effect of reductase inhibitor:

Biocatalytic methods are less employed for secondary alcohols via hydrogen tranfer with a single biocatalyst or whole cells. This apporoach would represent a very simple, green and elegant method. The Gibb's free energy for the hydrogenation of choro, bromo and fluoro substituted aldehydes and ketones are about 4-6 Kcal/mol which is more favoured than unsubstituted aldehydes and ketones which is calculated by *Calculation method used;MP2/cc-pVTZ/MP2/cc-pVDZ.* Lvan lavandera et al. (2008) reported that in presence of bromo and chloro substituted aldehydes and ketones as reductase inhibitors oxidation of rac secondary alcohol occurred. In this study various reductase inhibitors were used in the whole cell for oxidation of 2-hydroxy 4- phenylbut-3-enoate. The maximum yeild of 61 % was obtained with reductase inhibitor 2,4-dichloro benzaldehyde.

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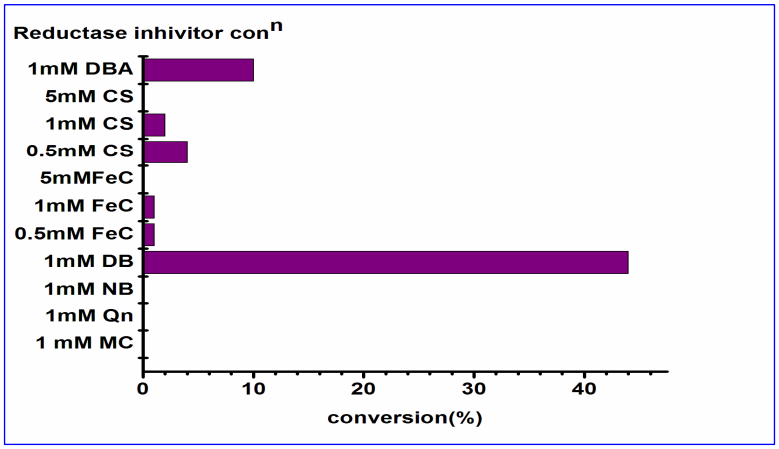


Figure:4.5: Effect of different reductase inhibitors 2,4-dichlorobenzaldehyde and its concentration on the oxidation reaction of (RS)-EHPB(ethyl 2-hydroxy ,4-phenylbut-3-enoate) and without reductase inhibitor. DB-2,4-dichlorobenzaldehyde, DBA-2,4-dibromo acetophenone,CS-copper sulphate,FeC-Ferric chloride ,DB-2,4-dichlorobenzaldehyde,NB-4-nitrobenzaldehyde,Qn-quarcetin,MC-merquric chloride.

4.3.5 Effect of reductase inhibitor concentration:

In this study various concentrations of reductase inhibitor 2,4-dichlorobenzaldehyde were used and 61% yield of 2-oxo 4-phenylbut-3-enoate product was observed with 1mM of 2,4-dichlorobenzaldehyde as a reductase inhibitor. When excess of reductase inhibitor was used the conversion decreased.

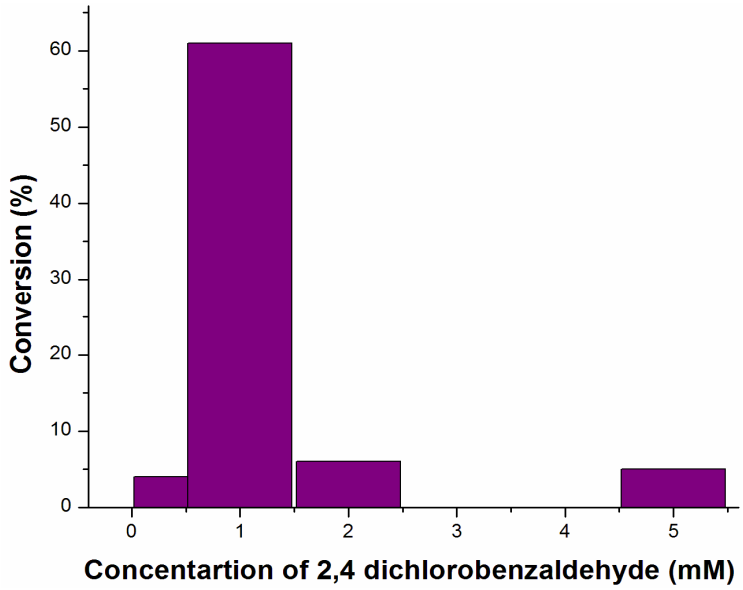


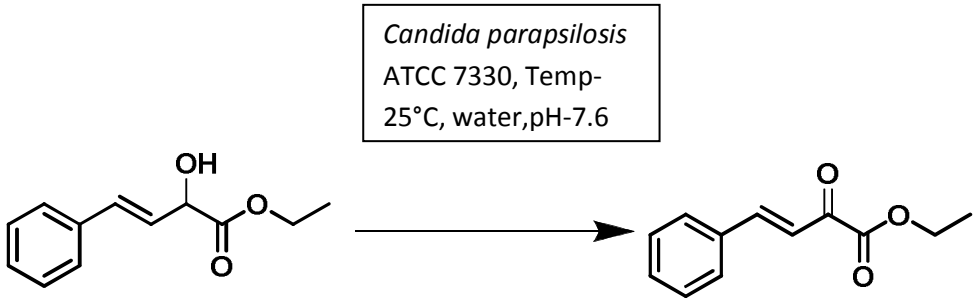
Figure:4.6: Effect of reductase inhibitor 2,4-dichlorobenzaldehyde on the oxidation reaction of (RS)-EHPB and without reductase inhibitor 2,4-dichlorobenzaldehyde

4.4 Conclusion

The optimized conditions for the Non enantioselective Oxidation of rac -2-hydroxy 4-phenylbut-3-enoate using *Candida parapsilosis* ATCC 7330 can be summarized as follows:

- Amount of substrate: 3 mg/ml
- Amount of solvent (ethyl alcohol): 75 µl for 3mg substrate (Reported)
- Temperature: 25°C

- Time: 5 min
- Wet weight of cells: 1 g/ml in a final volume of 1ml using deionised double distilled water.
- pH: 7.6
- Reductase inhibitor; 2,4-dichlorobenzaldehyde: 1mM



(*E*)-Ethyl 2-hydroxy-4-phenylbut-3-enoate **(*E*)-Ethyl 2-oxo-4-phenylbut-3-enoate**

Figure -4.7 biocatalytic oxidation of 4-phenyl, 2-hydroxy, 3-butenoate by using *Candida parapsilosis* ATCC 7330

4.5. OXIDATION OF CINAMYL ALCOHOL (3-PHENYL PROPENOL)

R.Gandolfi et al. (2001) reported oxidation of primary alcohol (cinamyl alcohol) by using acetic acid bacteria as a biocatalyst and the maximum yeild of less than 5% was obtained.

Upendra K.Sharma et al. (2009) reported the biocatalytic promiscuity of unique 00synergistic combination of lipase and liquid [hmim]Br for metal free activation of H_2O_2 used for chemoselective oxidation of aryl alcohol (cinamyl alcohol) and 55% maximum yield was obtained after 8h.

In the present study (sec-2.3) the chemoselective oxidation of rac -2-hydroxy 4-phenylbut-3-enoate using *Candida parapsilosis* ATCC 7330 was observed. This oxidation was not enantioselective oxidation so this method was used for oxidation of primary alcohol (cinamyl alcohol) and 19% mximum conversion was achieved.

4.5.1 Optimisation of time and reductase inhibitor concentration:

In this study the time of biotranformation of 3-phenyl prop-2-enol typically was 5 min. At various time intervals of 10 min, 20 min 40 min and 50 min the reaction mixture was analysed using C-18 column (HPLC). The maximum (19%) keto product of 3-phenyl prop-2-enal was obtained at 5 min.

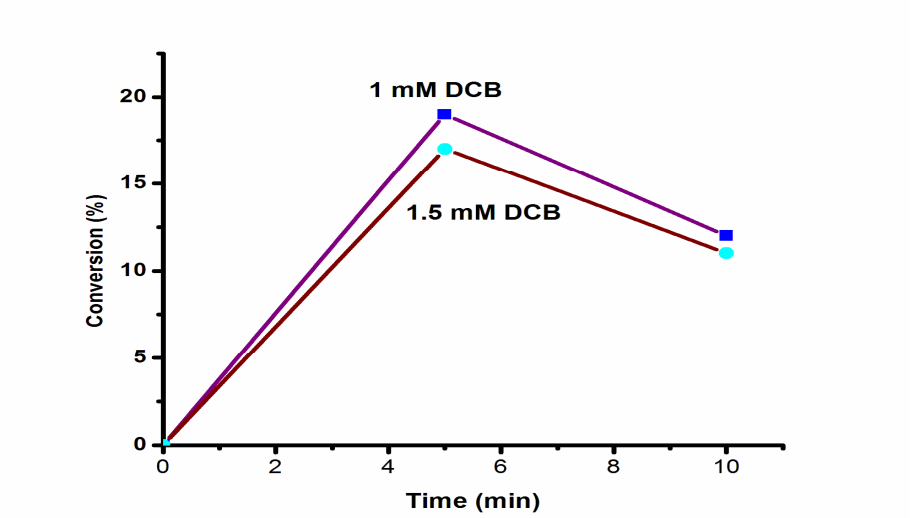
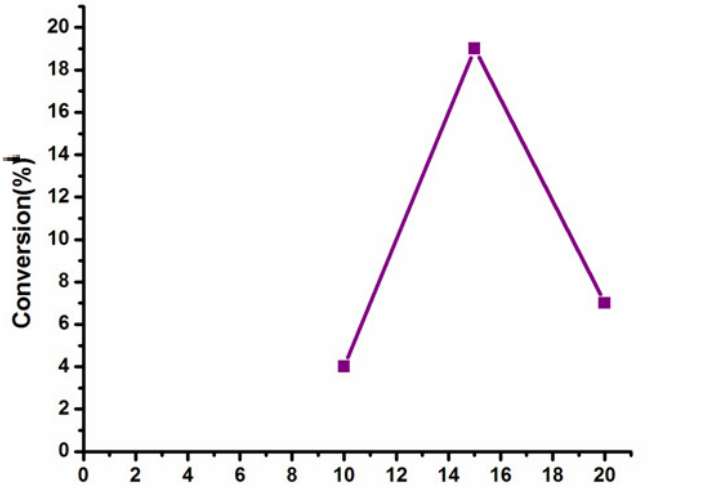


Figure:4.8: The course of the oxidation of Effect of defferent reductase inhibitor and ther concentration on the oxidation reaction of without reductase inhibitor 2,4-dichlorobenzaldehyd

4.5.2 Optimization of substrate concentration :

In this study various concentrations 10mg, 15mg, 20mg per 5ml of 3-phenyl prop-2-enol (cinamylalcohol) substrate were used. 19% yield of 3-phenyl prop-2-enal (cinamyladehyde) was observed with 1mM 2, 4-dichlorobenzaldehyde as a reductase inhibitor.



Concentration of substrtate 3-phenyl prop-2-enol (cinamyl alcohol)Figure

4.9: Effect of substrtate 3-phenyl prop-2-enol (cinamyl alcohol) concentration on oxidation .

4.6 Conclusion

The optimized conditions for the Non enantioselective Oxidation of 3-phenyl prop-2-enol (cinamyl alcohol) using *Candida parapsilosis* ATCC 7330 can be summarized as follows:

- Amount of substrate: 3 mg/ml
- Amount of solvent (ethyl alcohol): 75 µl for 3mg substrate (Reported)
- Temperature: 25°C
- Time: 5 min
- Wet weight of cells: 1g/ml in a final volume of 1ml using deionised double distilled water.
- pH: 7.6
- Reductase inhibitor; 2,4-dichlorobenzaldehyde: 1mM

In conclusion from both reaction we have developed a highly efficient green process for the chemoselective and Nonenatioselective oxidation of a of aryl alcohol 3-phenyl prop-2-enol (cinamyl alcohol) and racemic ethyl 2-hydroxy ,4- phenylbut-3-enoate using Whole cell biocatalyst candida parapsilosis ATCC 7330 whose significant feature are the following:

1).Waste free Reaction 2) .Efficiency under relatively mild condition 3)Metal free oxidation of alcohol.4) Cheaper process 5)Avoided inorganic oxidants which are incongruous .

HPLC PROFILE

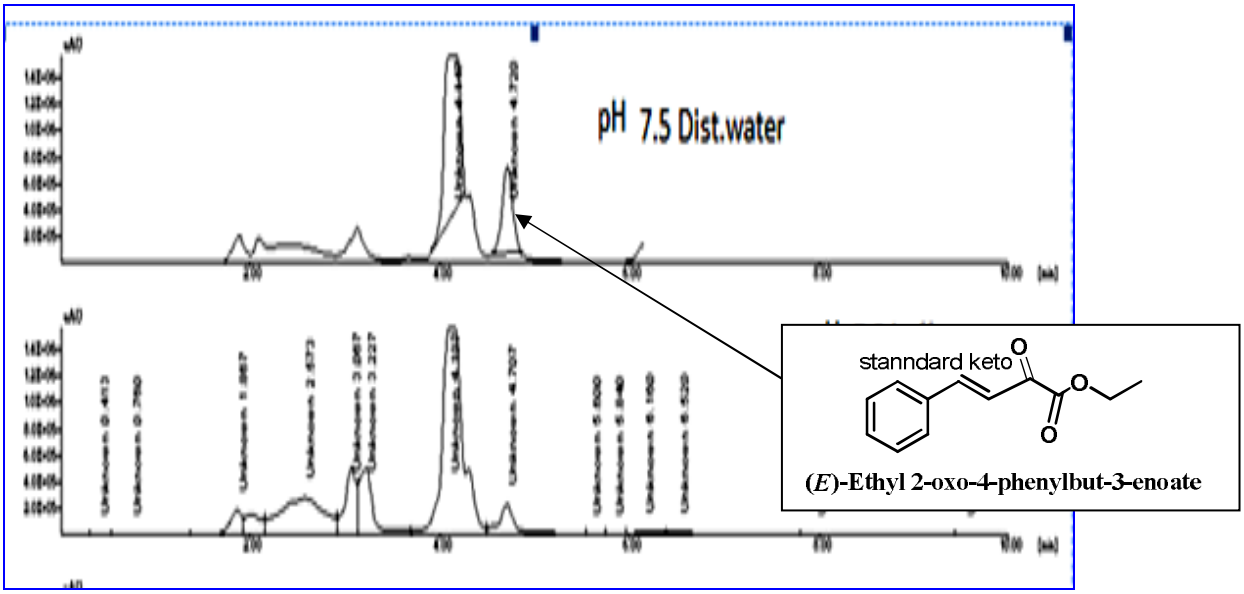


Figure 4.10 Effect of buffer on oxidation of rac -2-hydroxy ,4- phenylbut-3-enoate

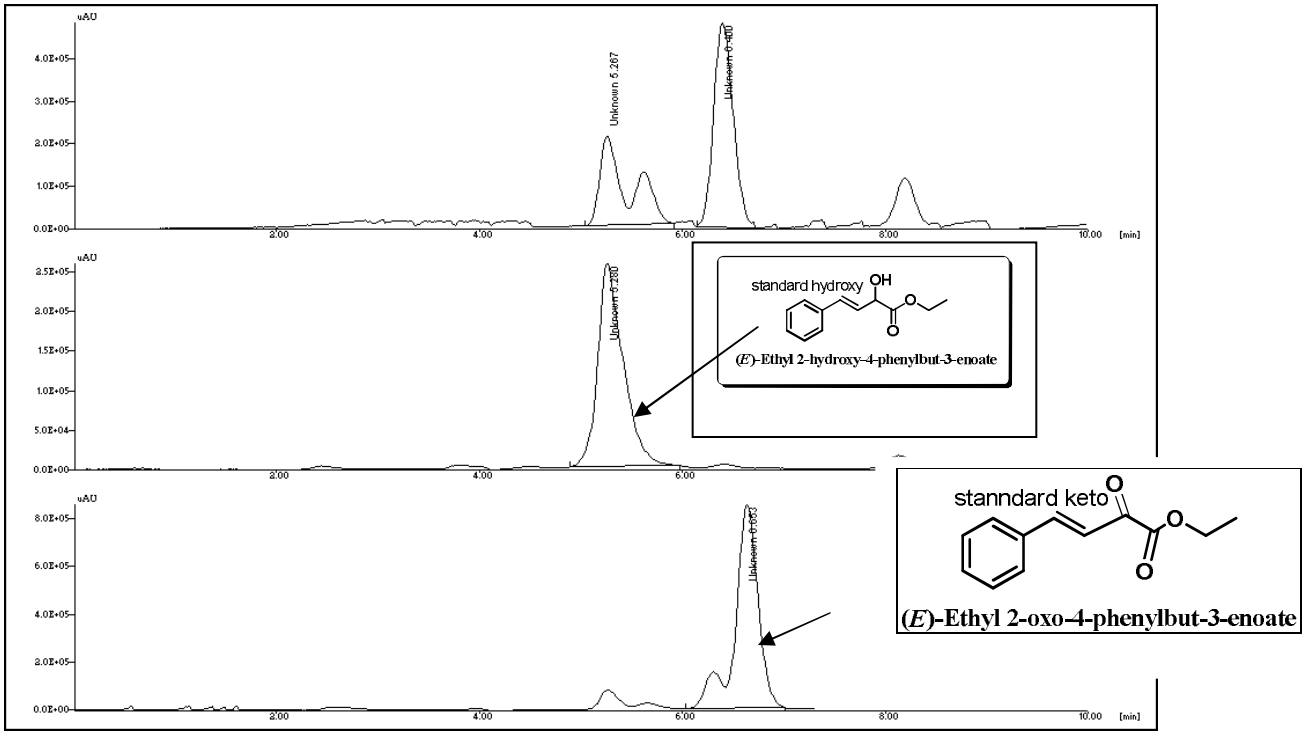


Figure :4.11 Analysis of candida parapsilosis ATCC 7330 mediate oxidation of of 2-hydroxy ,4- phenylbut-3-enoate on a C-18 column using 85:15 ACN : H_2O as the mobile phase showing the formation of 2-oxo ,4- phenylbut-3-enoat

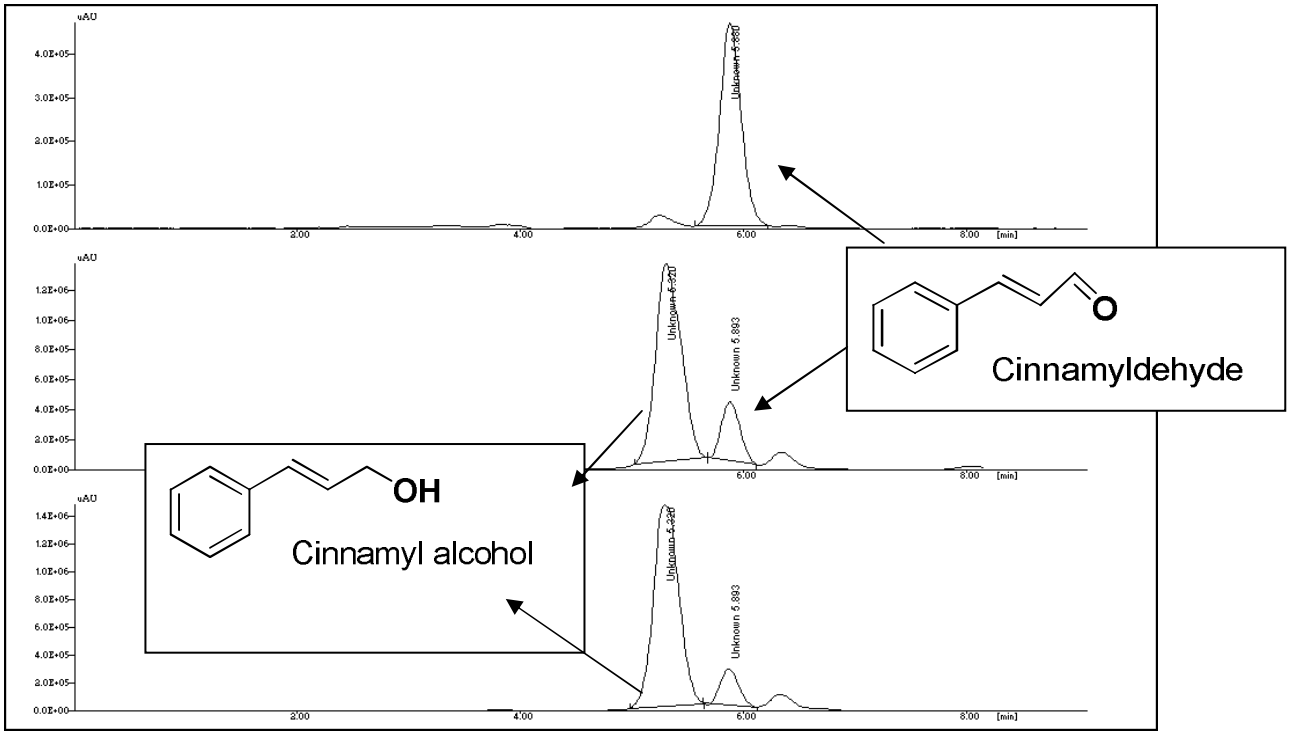


Figure :4..12 Analysis of candida parapsilosis ATCC 7330 mediate oxidation of of 3-phenyl prop-2-enol on a C-18 column using 85:15 ACN : H_2O as the mobile phase showing the formation of 3-phenyl prop-2-enal

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References

1. Pollard J.; Woodley M.; Biocatalysis for pharmaceutical intermediates: the future is now. *Trends Biotechnol*, 2007, 25, 66–73

2. Straathof A.; Panke S.; Schmid A.; The production of fine chemicals by biotransformations, *Current Opinion in Biotechnology*, 2002, 13,548–556

3. Adrie J.; Chemo-enzymatic synthesis of pharmaceutical intermediates X , *Expert Opinion on Drug Discovery*, 2008, 3, 187-245.

4. Dipeolu A.; Eruvbetine D.; Oguntona B.; Bankole O.; Sowunmi A.; Comparison of effects of antibiotics and enzyme inclusion in diets of laying birds, *Archivos De Zootecnia* ,(2005), 3–11.
5. Boshoff A.; Burton M.; Burton S.; *Biotechnology and Bioengineering*, 2003,83, 1–123
6. Fortin D.; Beveridge T.; *From biology to biotechnology and medical applications, in Biomineralization,* 2000
7. Wolfgang K.; herald M.; Edegger K.; Faber K.; *current opinion in chem.. Bio.; 2004, 8, 120 126*
8. Sharma K., Sharma N., Rakesh K,; Rajesh K.; Sinha K.; *Organic Letter*, 2009, 11, 4846 4848

9. Hummel W., in *Advances in Biochemical Engineering/ Biotechnology*, ed. T. Scheper, Springer, Berlin, 1997, 145–184

10. Whittaker J.;, *Arch. Biochem. Biophys.*, 2005, 433, 227–239

11. G. Mugesh, W. W. du Mont and H. Sies, *Chem. Rev.*, 2001, 101, 2125–2179

12. LieseA.; Seelbach K,; Wandrey C.; *Industrial Biotransformations*,Wiley-VCH, Weinheim, 2006

13. B. Buhler,A. Schmid, B. Hauer and B.Witholt, *J. Biol.Chem.*, 2000, 275, 10085–10092.

14. Buhler B.; Schmid A.;, Hauer B.; Witholt B.;, *J. Biol.Chem.*, 2000, 275, 10085–10092.

15. Molinari F.; Aragozzini F., Cabral J.; Prazeres D.; *Enzyme Microb. Technol.*, 1997, 20, 604 611
16. Gavagan J.; Fager s.; Seip j.; Clark D.; Payne Z.; Anton D; DiCosimo R.; *J. Org. Chem.*, 1997, 62, 5419–5427
17. Siebum A.; Wijk A.; Schoevaart R.; Kieboom T.; *J. Mol. Catal. B: Enzym.*, 2006, 41, 141- 145.
18. Siebum A.; Schoevaart R.; Kieboom T.; *J.Mol. Cat. B: Enzy.,,* 2008, 41, 141-145
19. K. Edegger, H. Mang, K. Faber, J. Gross and W. Kroutil, *J. Mol.Catal. A: Chem.*, 2006, 251, 66–70
20. Zilbeyaz K.; Taskin M.;Kurbanoglu E.; Namudari I.; Production of (*R)*-1-phenyletanols through Bioreduction of Acetophenones by a New Fungua Isolate *tricholthecdium roseum*, *Chirality*, 2010, 22, 543- 547
21. Voss C.; Gruber C.; Kroutil W.; *Angew. Chem*., Int. Ed., 2008, 47, 741–745