FUNGUS MEDIATED STEREOSELECTIVE ESTERIFICATION

OF

RACEMIC MANDELIC ACID

Thesis submitted

In partial fulfillment of requirement for the

Degree of Master of Science in Chemistry

Submitted by

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July 2011

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Acknowledgement

I would like to express my deep sense of gratitude to my research guide Dr. Manmohan Chhibber, Assistant Professor, School of Chemistry and Biochemistry, Thapar University, Patiala, Who helped me immensely at this golden stage of my carrier and provided all the facilities in the department during my work .I am extremely indebted to him for the scientific attitude and utmost patience he has installed in me which will definitely stand in all future endeavour.

My sincere thanks to Dr. Susheel Mittal, Head, School of Chemistry and Chemistry for his guidance and suggestions. I express my regards to all faculty members of School of Chemistry and Biochemistry for their help and moral support during my stay.

I also thankful to Dr. Ranjana Prakash, Assistant Professor, School of Chemistry and biochemistry, Thapar University, Patiala for providing me cultures to carry out the reactions.

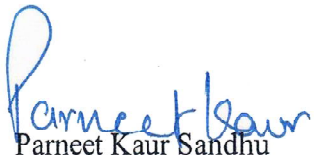
I also thank sophisticated instrument facility of Punjab University, Chandigarh.

I thank Miss. Ramandeep Kaur and Mr. Satnam Singh Research Scholars for their guidance and support to me.

I also thank the laboratory staff for their timely help.

I thank my labmates Gagandeep Bansal and Amrita to cooperate me in the laboratory.

Last but not least I owe my thesis to my parents who are the building pillars of my life.



Date: July 15, 2011.

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Candidate's Declaration

I hereby declare that the work being presented in the dissertation entitled **"Fungus Mediated Stereoselective Esterification of Racemic Mandelic Acid"**, in the partial fulfillment of the requirements for the award of the degree of Masters of Science (Chemistry), School of Chemistry and Biochemistry (SCBC), Thapar University, Patiala, is my own work during the period of January to June 2011, under the supervision of Dr.Manmohan Chhibber and Dr. Ranjana Prakash. I have not submitted the matter embodied in this disscrtation for the award of any other degree.

Patiala

Date: 15 July 2011

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This is to certify that the above statement made by candidate is correct and true to the best of our knowledge.

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Certificate

This is to certify that the project entitled "**Fungus Mediated Stereoselective Esterification of Racemic Mandelic Acid**", being submitted by Miss Parneet Kaur Sandhu in partial fulfillment of the requirement for the award of degree of Masters in Chemistry in the School of Chemistry and Biochemistry, Thapar University, Patiala, is a bonafide work carried out under the supervision of Dr. Manmohan Chhibber and Dr. Ranjana Prakash and that no part of this project has been submitted for the award of any other degree by me.

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List of Contents

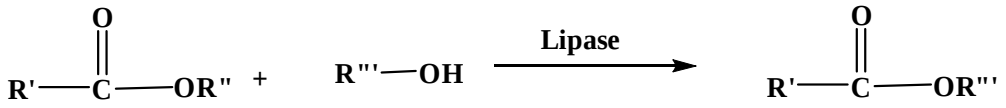
Introduction

Introduction

The biological world can be regarded as a chiral world in the chemical sense. Biological activity of a compound often depends upon the absolute configuration of chiral centres in molecule because the receptor, usually a protein, to which it binds has chiral active site[1].

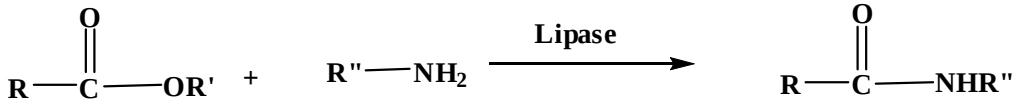
Therefore preparation of optically pure enantiomers of the compounds is extremely important from industrial point of view. The synthesis of such optically pure enantiomers can be carried out either chemically or biochemically. Among biochemical methods use of enzymes and microorganisms is of great potential. This is because of their stereoselectivity[2].

Stereoselective esterification[3] is popurlarly carried out using lipase enzymes and by chemical methods. Present work is an attempt to utilizes the enzyme generated bby fungus cultures for esterification reaction. In biological systems most of such reactions including transesterification is carried out by lipases.



R" = H (Esterification)
R = Alkyl or Aryl (Transesterification)

There are also reports where even amidation reaction have been carried out using lipase enzymes.



Such enzymatic enantioselective esterification is advantageous over chemical esterification for the preparation of lipophilic alkyl enantioselective ester, particularly for food use because no hazardous chemicals are used.

For these reactions, lipase is taken as such or immobilized on organic or inorganic carrier[4]. Lipases being robust class of enzymes can be immobilized to solve the problems of stability and recyclability for industrial and other uses.

Present work uses biomass immobilized fungus to carry out esterification of mandelic acid with short, medium and long chain alcohols without any organic solvent.

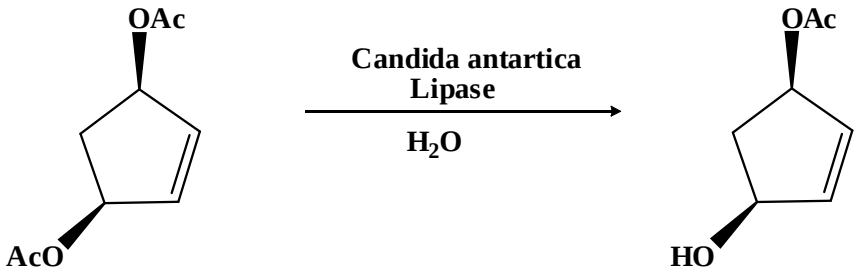
Review of Literature

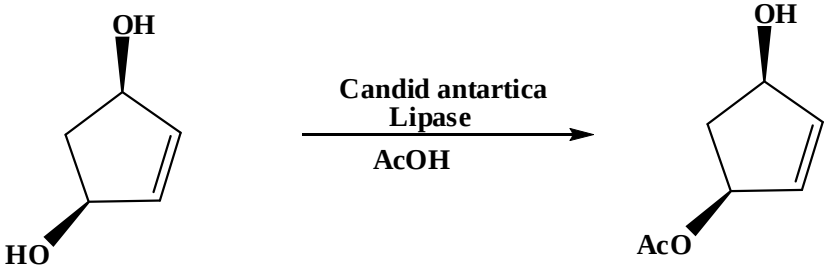
Literature Review

Catalytic asymmetric synthesis is an important and expansive area of organic chemistry[5]. Catalytic enantioselective organic reactions can be achieved either by chemocatalysis[6] or biocatalysis[7]. The former field is dominated by metal catalysis while biocatalysis involves enzymes.

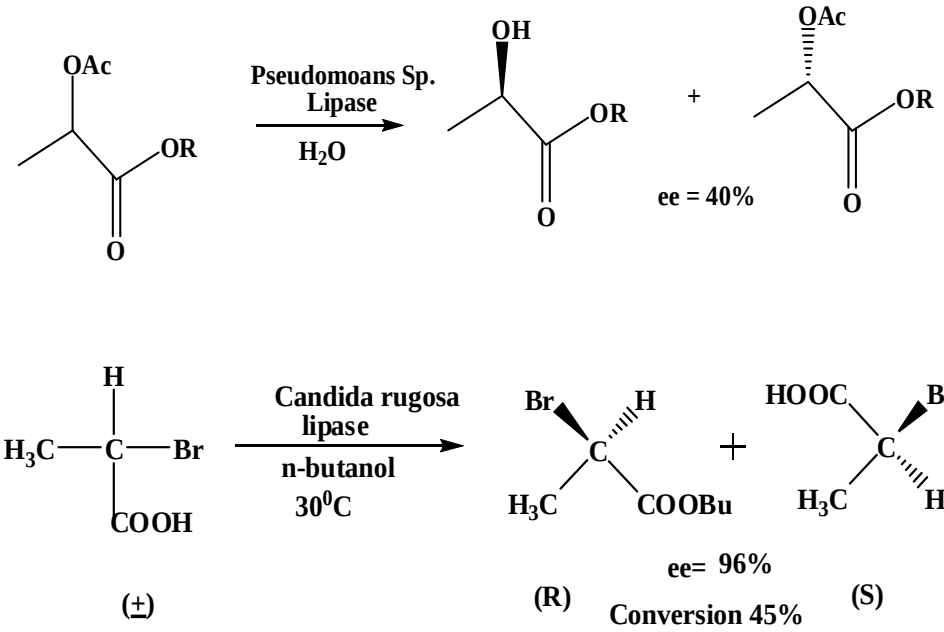
Among enzymes, lipase (triacylglycerol hydrolases, EC 3.1.1.3) catalyses hydrolysis of triglyceride to corresponding fatty acid and glycerol. In the past years, various lipase catalyzed reactions have attracted much interest due to their mild conditions and greener chemistry there by becoming very useful in chemical and pharmaceutical industries. In addition to this they also catalyse a plethora of other reactions such as esterification, transesterification, amidation and organic carbonates wherever the reactants are prochiral, lipase leads to the formation of enantioselective pure products[8] . A brief literature survey of lipase catalysed enantioselective organic reactions is presented in following lines.

Sanchez et.al.[9] have reported that lipase can catalyse either hydrolysis or synthesis of ester bonds depending upon the environment in which they carry out the reaction.

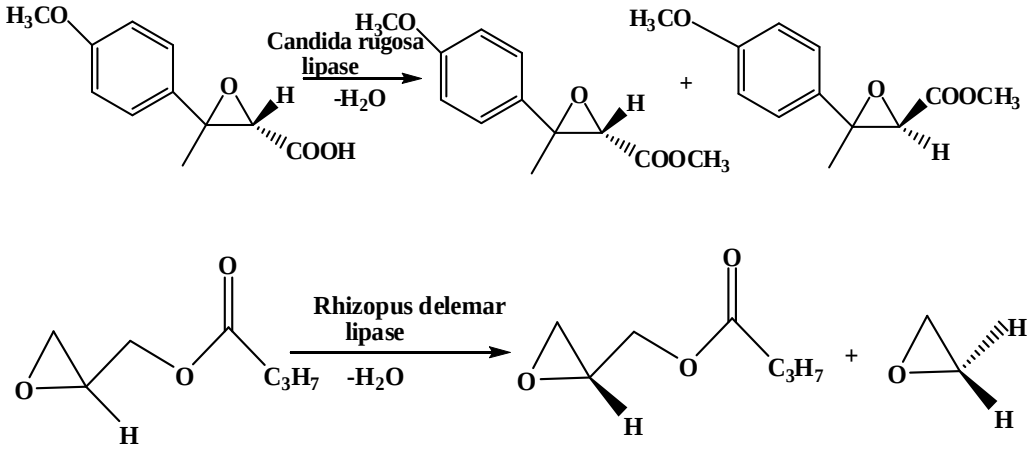




Chen and Sih et al.[10] and Tawaki and Klibanov et al.[11] have shown that in the esterification reactions enantioselectivity can be modulated and frequently improved when compared with hydrolytic reaction.

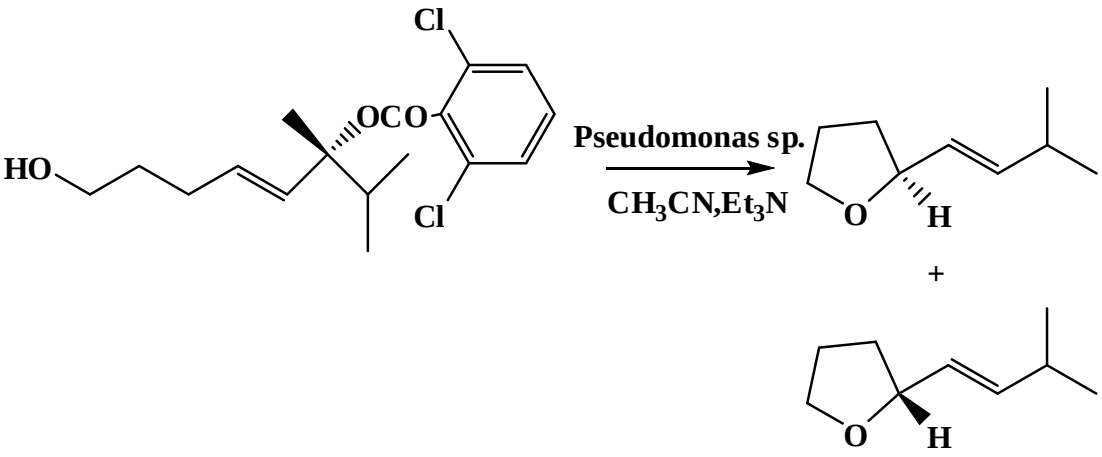


Santaniello et al.[12] and Schoffers et al.[13] demonstrated lipases have been used in resolution of racemic mixtures for the preparation of optically pure compounds, constituting an alternative method to chemical asymmetric synthesis.



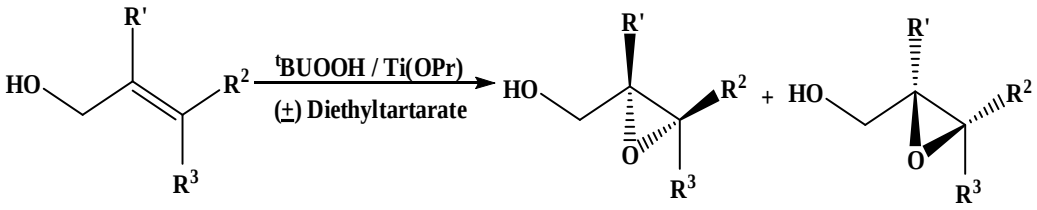
According to Izumi[14] asymmetric synthesis can be divided into two types i.e. enantioselective synthesis and diastereoselective synthesis. A reaction is described as

enantioselective if the reaction is carried out on an achiral molecule using an enantioselective reagent or catalyst **(Scheme-1).**



Scheme-1: Achiral substrate for enantioselective synthesis

In the case of diasteroselective synthesis, if a molecule contains a centre of chirality and a centre of prochirality, the molecule can be divided by a plane in such a way that the parts on either side of the plane are diastereotopically related to each other. If the reaction results in the conversion of centre of prochirality into a new centre of chirality, the reagent that may attack from either side of this diasterotopically reacting plane with the result that diastereoisomers are formed **(Scheme-2)**.



Scheme-2: Chiral substrate for enantioselective synthesis

Lipase is a wide class of enzymes produced by almost all types of microorganisms, including bacteria[15], yeast and fungi[16]. Lawson et al[17] had demonstrated that among lipases of plant, animal and microbial origin, microbial lipases have gained importance due to their easy cultivation, low cost of extraction, thermal and pH stability and substrate specificity. Still, the cost of pure lipase catalyzed organic reactions and their inability to reuse makes it necessary to look for alternative way to make its use cheaper. Therefore immobilization either on organic or inorganic surface has been in use. Because of the

wide variety of reaction systems in which lipases can be used, the preparation of immobilized derivatives has to be done according tothe requirement. Several approaches have been reported for the immbolisation of lipase[17]

a.) Adsorption on carrier

b.) Covalent binding to a solid matrix

c.) Entrapment in a solid support

d.) Membrane confinement

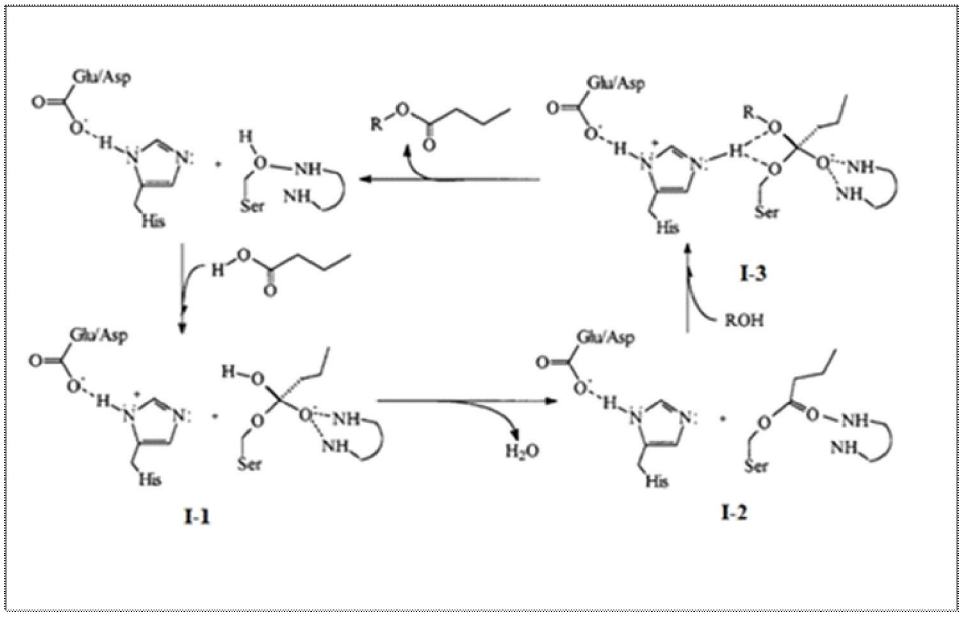
The selection of an immobilisation strategy is based on effectiveness of enzyme lipase utilization, cost of the immobilisation procedure, toxicity of immobilisation reagents and desired final properties of the immobilised biocatalyst[18].

Sarda and Desnuelle et al.[19] have reported the adsorption of lipase on hydrophilic surface. It involves the simple mixing of enzyme (lipase) with a suitable adsorbent like porous carbon, clay or resins. It involves incubation for sufficient time under appropriate conditions of pH and ionic strength followed by washing off loosely bound and unbound proteins to produce the immobilised lipase. The driving force causing this interaction is usually due to a combination of hydrophobic effects and the formation of several salt links per enzyme molecule. One of the drawbacks is the blocking of the active site of immobilized lipase due to matrix or bead, there by reducing the activity of lipase in some cases.

Immobilisation of lipase by their covalent bonding to an insoluble solid matrix is an extensively researched technique[20]. Only a small amount of lipase is immobilised by a chemical reaction in this method. While the binding is very strong and there is little leakage of lipase from support. The ease of formation of covalent bonding depends upon availability and nucleophilicity of various groups present in lipase. As the chemical reaction ensures that binding site does not cover the lipase's active site, the activity is only affected by immobility.

Entrapment of lipase within gels or fibres like cellulose acetate is another convenient method for lipase immobilisation[21]. It is suitable only for low molecular weight substrates. It may be a purely physical caging or involve covalent binding.

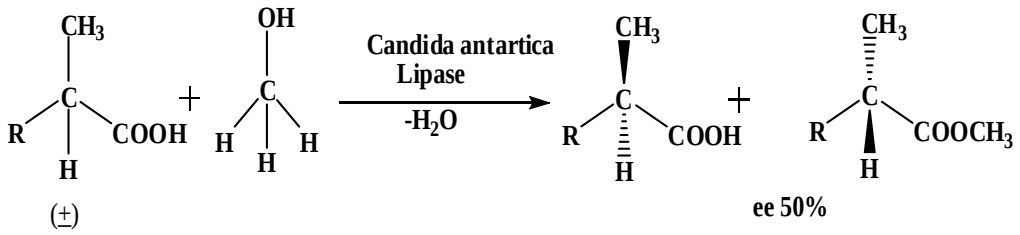
Membrane confinement involves the use of a semi-permeable[22] . On one side of this membrane lipase is present and on another side of membrane reactant and product stream is present. Hollow fibre membrane units are used for this purpose and this is permeable to only substrates of low molecular weight i.e. less than lipase's molecular weight. It is applicable to a wide variety of enzymes but it is very costly technique.



Scheme-3: Mechanism of lipase catalyzed transesterification

The mechanism of lipase catalysed esterification[23] involves two tetrahedral intermediate **(Scheme-3)**. The first tetrahedral intermediate is formed by nucleophilic attack of serine residue of the catalytic triad on the acid **(I-1).** The intermediate loses a water molecule to give an acyl-enzyme complex **(I-2).** An alcohol molecule attacks the complex (nucleophilic attack) to give another tetrahedral intermediate **(I-3),** which, finally, loses an ester molecule to give an enzyme in its native form. Both of the tetrahedral intermediates possess an oxyanion i.e. stabilised by hydrogen bonding.

Vander et al.[24] have reported that the enantioselective esterification catalysed by lipase was coupled with pervaporation (defined as transport of liquid through a non-porous membrane with simultaneous evaporation of the permeates) of water by-product produced by the reaction **(Scheme-4)** .



Scheme-4

This enantiselectivity is the degree to which one enantiomer of a chiral product is preferentially produced in a chemical reaction.

The mechanism of enantioselectivity has been probed by substrate mapping[25], and more recently by x-ray crystallographic structural determination[26]. Based on the observed enantioselectivities of lipases toward hundreds of secondary alcohols for hydrolysis reactions, where the substrate is an ester, and for esterification reactions, where the substrate is an alcohol, an empirical rule is formulated. It predicts which enantiomer of racemic acid mixture reacts faster in lipase-catalyzed reactions by comparing the sizes of the substituents at the stereocenter. This rule is represented schematically in **(Fig- 1)**. When the acid substrate is drawn with the carboxylic group pointing out of the plane of the paper, the favoured substrate enantiomer is an that bears a large substituent on the right (e.g., phenyl) and a medium substituent on the left (e.g., methyl). The attack for esterification and takes place from the left that is the side which bears medium substituent. The importance of substituent size was confirmed by studies, that showed that lipases resolve acids with two similarly sized substituents poorly, but they resolve these acids efficiently when the size of one substituent is increased. X-ray crystallographic structures of active site of lipases support the above hypothesis.

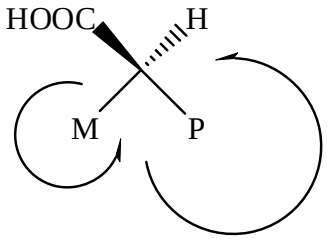


Fig-1: The schematic shows the favoured enantiomer for acid,

M and L represent medium and large substituents, respectively

The enantioselectivity can be calculated by measuring the enantiomeric excess (ee_s)[27] and enantiomeric factor (EF)[28]. The enantiomeric excess of the substrate, ee_s, at a given time was calculated using:

$$ee_s = [B] - [A] \setminus [A]+[B]$$

where [A] and [B] are the concentrations of the faster and slower reacting enantiomer respectively. The extent of conversion at any given time, c, was calculated using:

$$c = 1- [A]-[B] \setminus [A]_0+[B]_0$$

where the subscript $_0$ denotes initial concentrations.

The enantiomeric factor is defined as the correlation between the experimental enantiomeric excess and the theoretical enantiomeric excess of the reaction

$$EF = ee_s \setminus ee_t$$

In this esterification, ee_s is the one measured (for the remaining substrate) at a defined reaction time. The ee_t is the value which would be obtained at the same reaction time with the same measured yield, only if the fast reacting enantiomer is transformed. Therefore ee_t is calculated as

$$ee_t = [Yield] \setminus [100- Yield]\times 100$$

The maximum EF (1) value indicates that the enzyme is acting exclusively on one enantiomer. But when the reaction yield is less than 50% then EF value of 0.95 ensures a good resolution. And when reaction yield is higher than 50% then EF value must be

calculated considering ee_t = 100, a value which indicates maximum enantioselectivity. And this is measured by HPLC and capillary electrophoresis using a chiral stationary phase, chiral mobile phase or a ligand exchange and by fluorescence detector circular dichorism[29],[30].

Prakash et. al[31] have optimized the conditions of pH, growth medium, ambient temperature, source of nitrogen and carbon for the production of *Aspergillus* species. They have demonstrated that oil (50% v/v) acting as carbon source, peptone (0.5 w/v) as nitrogen source at 7.5pH and 35^0c gives maximum lipase activity for the production of biodiesel.

The group has also demonstrated that the fungus from *Aspergillus* species (RBD 01) acts as potential catalyst for the transesterification of used frying oil with an free fatty acid content upto 3.7%[32]. And they have also reported that extended frying of oil reduce the extent of initial biocatalyzed hydrolysis compared with final transesterification.

Present work has been initiated with an objective of developing an enzymatic method to obtain pure enantiomer of racemic mandelic acid mixture and to establish best conditions to achieve maximum activity. The lipase used is of *Aspergillus* sp. for which Prakash et. al. have optimized conditions as mentioned above.

Materials and Methods

MATERIALS AND METHODS

A. Source of reagents and analytical facilities

All the acids and alcohols used were procured from Aldrich. LR grade solvents were used for the synthesis and produced from S.D. Fine-Chem Limited, Mumbai, India. Powdered biomass of *Aspergillus* species was procured as such from Dr. R. Prakash, School of Chemistry and Biochemistry, Thapar University Patiala.^{1}H NMR spectral analysis were performed on BRUCKER ADVANCE Ï 400MHz spectrophotometer installed at SIAF, Panjab University Chandigarh.

Experimental

Ethyl 2-hydroxy 2-phenylacetate (Compound-1, Table -1): Lipase (2 gm, i.e. 3% by weight) was added to a round bottom flask containing racemic mandelic acid (5 gm) and dissolved in minimum quantity of water The reaction mixture was allowed to equilibrate by heating at a constant temperature (40^{0}C) water bath with continuous stirring for about one hour. The mild temperature and solvent conditions were unlikely to compromise the stability of the enzymes over the duration of the reaction. Ethanol (0.6 ml × 3, 1\3rd of total volume) was added to the reaction mixture to stirring at 40^{0}C every six hours. Progress of the reaction was monitored using TLC (Ethyl acetate : Pet ether, 25:75). A new spot was observed after 86 hours. The reaction mixture was brought to room temperature and ethyl acetate (10 ml) added and organic layer separated from aqueous one. The organic solvent is evaporated to get compound. (^{1}H NMR: δ 1.29 (t,3H), δ 4.22 (q,2H), δ 5.37 (s,1H), δ 7.28 (m,8H).

Oleyl 2-hydroxy 2-phenylacetate: Lipase (2 gm, i.e. 3% by weight) was added to a round bottom flask containing racemic mandelic acid (5 gm) and dissolved in minimum quantity of water. The reaction mixture was allowed to equilibrate by heating at a constant temperature (40^{0}C) water bath with continuous stirring for about one hour. The mild temperature and solvent conditions were unlikely to compromise the stability of the enzymes over duration of the reaction. Oleyl alcohol (0.6 ml × 3, 1\3rd of total volume) was added to the reaction mixture to stirring at

40^0C every six hours. Progress of the reaction was monitored using TLC (Ethyl acetate : Pet ether, 25:75). A new spot was observed after 86 hours. The reaction mixture was brought to room temperature and ethyl acetate (10 ml) added and organic layer separated from aqueous one. The organic solvent is evaporated to get compound.

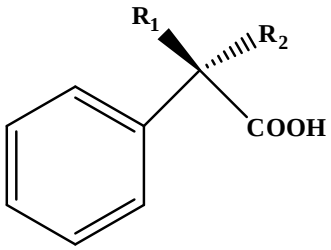
Butyl 2-hydroxy 2-phenylacetate: Lipase (2 gm, i.e. 3% by weight) was added to a round bottom flask containing racemic mandelic acid (5 gm) and dissolved in minimum quantity of water The reaction mixture was allowed to equilibrate by heating at a constant temperature (40^0C) water bath with continuous stirring for about one hour. The mild temperature and solvent conditions were unlikely to compromise the stability of the enzymes over duration of the reaction. Butyl alcohol (0.6 ml × 3, 1\3rd of total volume) was added to the reaction mixture to stirring at 40^0C every six hours. Progress of the reaction was monitored using TLC (Ethyl acetate : Pet ether, 25:75). A new spot was observed after 86 hours. The reaction mixture was brought to room temperature and ethyl acetate (10 ml) added and organic layer separated from aqueous one. The organic solvent is evaporated to get compound.

THIN LAYER CHROMATOGRAPHY (TLC): Aliquots were withdrawn from the reaction mixture and dissolved in minimum quantity of suitable solvent. The conversion by esterification was checked by TLC on 0.3mm layers of silicagel G as stationary phase and a mixture of ethyl acetate: pet ether (1:3) was used as a mobile phase. The plate was developed in an iodine chamber.

RESULTS AND DISCUSSION

RESULTS AND DISCUSSION

The use of *Aspergillus* sp. , isolated by Prakash et. al. for the production of highly pure samples of biodiesel is well known[32] . In this study the same species immbolized on its biomass was used to carry out esterification reaction of racemic acid **(A)** to study the



R_1 = H, R_2 = OH
R_1 = OH, R_2 = H
Racemic mandelic acid (A)

enantioselectivity of extracellular enzyme. Racemic Mandelic acid, an easily available chemical in any general laboratory, was chosen as substrate and made to react with different alcohols of short, medium and long chain length in aqueous medium.

The conditions used for the reaction were same as optimized by Prakash et. al. The initial attempt to carry out esterification by taking alcohol in excess and using limiting amount of acid was not successful. This may be due to the inactivation of the enzyme in the presence of excess alcohol. Many groups have[34] shown such inactivation.

Thus, alternative approach using limiting amount of alcohol and stiochometric amount of acid was used in the presence of 3% w/w biomass at 40^0C in aqueous medium. The alcohol addition was carried out over a period of 18 hours using one third of its total stiochometry every six hours. The progress of reaction as monitored by TLC showed a prominent spot of each ester after 86 hours though light esterification was observed after 40 hours also. The alcohols used were ethanol, butanol and oleyl alcohol. The TLC spot at R_f value lower than that of Mandelic acid confirmed by the formation of ester. This was validated with ^{1}H NMR in case of ethyl and oleyl ester with a signal corresponding to (-CO-C**H**$_2$) protons at 4.1- 4.3 ppm in both the cases.

The specific rotation of the reaction mixture was compared with that of racemic acid **(Table-1)**

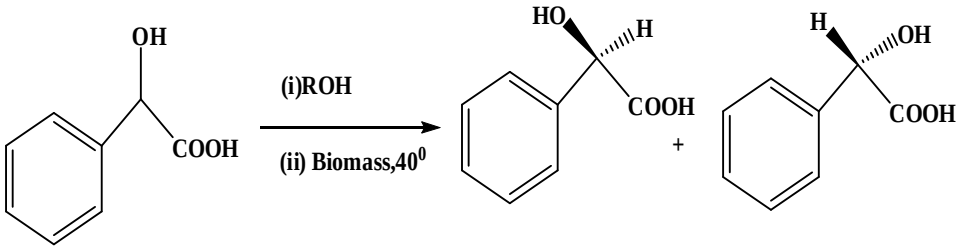


Table-1

Sr. No.	**R**	**%Yield**	**ee(%)**
1.	**CH_2CH_3**	50%	-
2.	**$CH_2CH_2CH_2CH_3$**	-	-
3.	**$CH_2(CH_2)_7CH=CH(CH_2)_7CH_3$**	30%	55.55 ($[\alpha]^{25}_{=66.66}{}^{0}$)
4.	**H**	-	0^0

The amount of ester formed was estimated by comparing the singlet due to α-H at 5.19 and 5.23 ppm due to mandelic acid and mandelic esters **(Fig-B).**

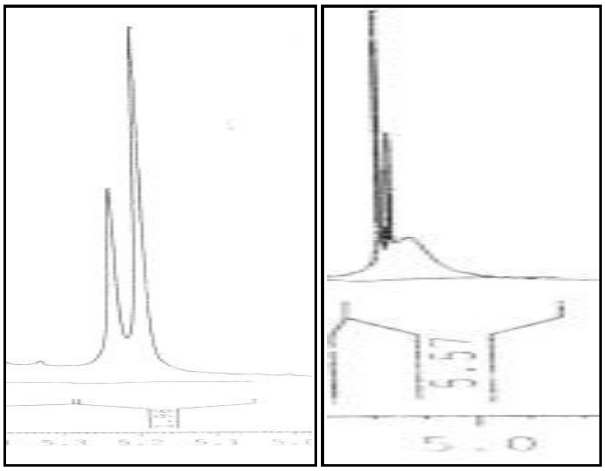


Fig-B

The ratio of protons due to (-COC**H**$_2$-) in the range of 4.14- 4.32 ppm was an indication of the enantioselectivity of the enzyme. In case of ethyl ester (compound-1) the intensity of quarters due to above mentioned protons was 1:1 there by indicating that both R and S ester are formed in equal amounts **(Fig-C)**

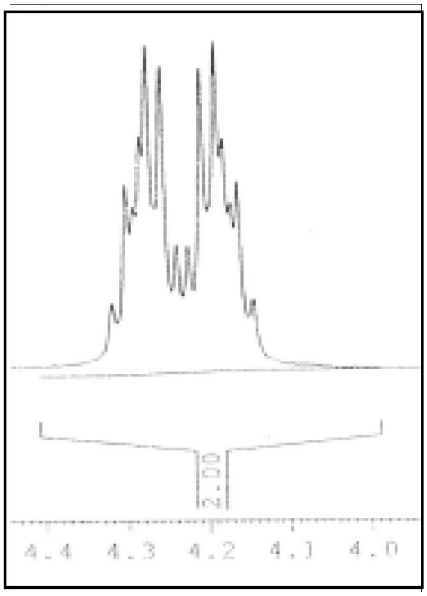


Fig-C

Intrestingly in case of oleyl ester the peaks in the region of 4.01-4.15 ppm indicated enantioselectivity due to varied intensity (-COC**H**$_2$-) protons in (**Fig-D)**

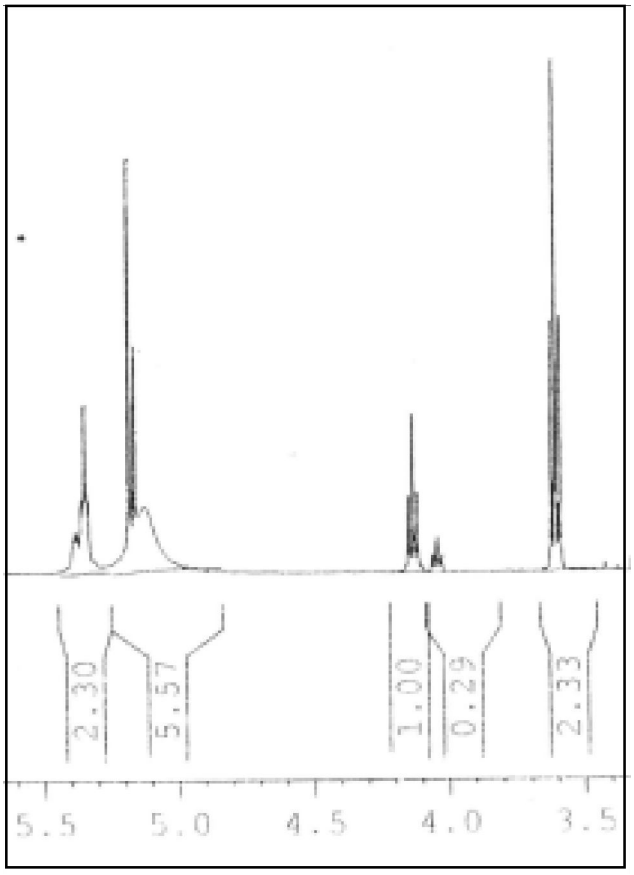


Fig-D

The triplet due to unreacted alcohol observed at its usual position 3.6 ppm.

The optical rotation observed in case of of reaction mixture is an indication that though reaction is enantioselective in all the cases, but ^{1}H NMR clearly indicates that as the chain length is increased selectivity of enzyme increases.

CONCLUSION

Conclusion

It can be concluded that biomass immobilized enzyme can carry out esterification for mandelic acid with short, medium and long chain alcohols. However the enzyme is more enantioselective in case of oleic ester, a long chain alcohol rather than in case of short chain alcohol.

REFERENCES

References

1. Rhys W.C., Carthy M.C., Bocker F., Hung J. ,Thomson Y.-F., Lloyd A. W., Hanlon G.W., *Enz. Microb. Technol.,* **1998,** 22, 281-287
2. J. Crosby, *Tetrahedron*, **1991**, 47, 4789
3. N. Kamiya, M. Goto, F. Nakashia, *Biotechnol. Prog*., **1995,** 11, 2
4. Weber N., Klein E., Vosman K., Meherjee K. D., *Biotechnol. Lett.*, **1998**, 20, 687
5. Oscar P., Backval J. E., *Chem. Rev*., **2003**, 103, 3247-3261
6. Ahrendt K.A., Borths C.J., Macmillan D.W.C., *J. Am. Chem. Soc.* , **2002**, 124, 7894
7. Schmid A., Dordick J.S., Hauer B., Kiener A., Wubbolts M., Witholt, *B. Nature*, ***2000***, 258, 409
8. Chen C.S., Sith C., *J. Angew. Chem. Int. Ed. Engl*., **1984,** 28, 695-707
9. Sanchez A., Rio J.L.D., Valero F., Laufente J., Faus I., Sola C., *J. Biotechnol*. , **2000**, 22-42
10. Sih C.J., Chen C.S., *J. Angew. Chem. Int. Ed. Engl*., **1989,** 23, 570-578
11. Tawaki S.H., Klibanov A.M., *Biocatalysis* ,**1993**, 3-8.,
12. Santaniello E., Ferraboschi P., Grisenti P., *Enz. Microbi. Technol* ,**1993,** 15, 367-382
13. Schoffers E., Golehiowski A., Johnson C.R., *Tetrahedron*, **1996**, 52, 3769-3826
14. Izumi Y., *J. Angew. Chem. Int, Ed. Engl*., **1971**, 10, 871
15. Jaeger K.E., Ransac S., Dijkstra B.W., Colson C., Heuvel V.M., Misset O., *FEMS Microbiol. Rev*., **1994,** 15, 29–63
16. Rapp P., Backhaus S., *Enzyme Microb. Technol.*, **1992,** 4, 938–943
17. Lawson D.M., Brozozwski A.M., Dodson G.G., Hubbard R.E., Huge Jensen B., Boel e., Delewenda Z.S., Wolley P., Peterson S.B., *U.K., Cambridge University Press,* **1994**, 79-94
18. Chaabouni M. K., Bouaziz I., Boufib S., RegocA. M. B. D., Gargouria Y., *Colloids and Surfaces B: Biointerfaces* , **2008,** 66, 168–177
19. Panzavolta F., Soro S., Amato R.D., Palocci C., Cernia E., Russo M.V., *J. Mol. Catal. Enz.*, **2005,** 32, 67

20. Surada L., Desnuelle P., *Biochem. Biophys. Acta*, **1958**, 30, 513-521

21. Bianco R.M., Guisan J.M., *Enz. Microbiol. Technol*., **1975**, 397, 412-417

22. Antczak T., Mrowiec J.B., Bielecki S., Jarzebski A.B., Malinowskim J.J., Lachowski A.I., Galas E., *Biotechnol. Technol.*, **1997**, 9,11

23. Bayramoglu G., Kacar Y., Denizli A., Arýca M. Y., *J. of Food Engi.*, **2002,** 367–374

24. Sugiura M., Isobe M., *Biochem. Biophys.* ,**1975,** 397,412-417

25. Vander P.A., Sewalt J.J.W., Riet K., *J. Mem. Sci*., **1993**, 80, 199-208

26. Kim M. J., Choi Y. K., *J. Org. Chem.*, **1992,** 57, 1605

27. Uppenberg J., Ohrner N., Noren M., Hult K., Kleyweqt G. J., Patkar S., Waapen V., Anthonsen T., Jones T. A., *Biochem. Biophys.*, **1997**, 54, 416

28. Klibanov A. M., *Acc. Chem. Research*, **1990**, 23, 114-120

29. Belmonte M. T.L., Alantara A. R., Sinistera J. V., *J. Org. Chem*., **1997,** 62, 1831-1840

30. Armstrong D. W., *J. Liq. Chromatogr.*, **1984**, 7, 353

31. Zukowski J., Tanq Y., Berthod A., Armstrong D. W., *Anal. Chem. Acta* , **1992,** 83, 25

32. Prakash R., Aulakh S.S., *J. Basic Microbiology*, **2010**, 37-42

33. Prakash R., Aukakh S.S., Kalra R., *Biocatalysis & Biotransformation*, **2010,** 403-407

34. Gandhi N.N., Sawant S.B., Joshi j.B., *Enzyme Microbial. Technol*., **1995,** 373-375