Isolation, Characterization and Utilization of Novel Lipases

Thesis submitted to the
Savitribai Phule Pune University
For the degree of

Doctor of Philosophy

In

Biotechnology

By

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CERTIFICATE

This is to certify that the work presented in this thesis entitled, "**Isolation, Characterization and Utilization of Novel Lipases**" by **Mr. Dipesh D. Jadhav**, for the degree of **Doctor of Philosophy**, was carried out by the candidate under my supervision in the Division of Organic Chemistry, CSIR-National Chemical Laboratory, Pune-411008, India. This work is original and has not been submitted for any other degree or diploma to this or any other university. Any material that has been obtained from other sources has been duly acknowledged in the thesis.

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Place: Pune

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DECLARATION

I, Dipesh D. Jadhav, hereby declare that the work incorporated in the thesis entitled "**Isolation, Characterization and Utilization of Novel Lipases**" submitted by me to **Savitribai Phule Pune University** for the degree of **Doctor of Philosophy** is original and has not been submitted to this or any other University or Institution for the award of Degree or Diploma. Such material, as has been obtained from other sources, has been duly acknowledged.

Date:

Place: Pune

Dipesh D. Jadhav,

Research Fellow,
Organic Chemistry
Division,
CSIR-NCL, Pune.

Dedicated to

My Family & Friends.....

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CONTENT

Thesis Abstract

Lipases/esterase's are of high commercial importance and thus there is always a need to find out novel biocatalysts with wide substrate specificity, high enantioselectivity and high stability. The thesis describes the isolation characterization and utilization of novel lipases/esterase's isolated from microbial origin. Two novel esterases SLC-6 and MLC-3 characterized were isolated using metagenomic techniques while another esterase pEST-1 was cloned from an isolated microorganism *Pseudomonas pseudoalcaligenes* NCIM 2864. These three esterases were able to carry out de-esterification/hydrolysis of racemic acetates in highly enantioselective manner. Results obtained in wet lab experiments were further confirmed using Molecular docking studies (AutoDock Vina tool) by docking individual (*R*) and (*S*) acetates with the respective enzyme. As an extension of this work, nine uncharacterized lipases were identified from psychrophillic yeast *Yarrowia lipoytica* NCIM 3639 using transcriptome sequencing. In last chapter, kinetic resolution of structural isomers of α/β-amyrins which are known to possess various biological activities was achieved using a commercial lipase. *Candida rugosa* lipase (CRL) was found to be more selective towards β-amyrin than α-amyrin compared to other fifteen lipases screened. Whole cell biocatalysts were screened for the enantioselective hydrolysis of racemic acetates to obtain corresponding enantiopure (*R)*-alcohols. Among the screened microorganisms, *Fusarium proliferatum* NCIM 1105 was found to be highly efficient in producing corresponding (*R)*-alcohols with more than 95 % enantiomeric excess (e.e) via two step de-esterification/oxidation of racemic acetates. (*R)*-alcohols were produced via selective oxidation of (*S)*-alcohol to corresponding ketone.

1) Chapter 1

Introduction

Lipases/Esterase's are the most important biocatalysts and are widely used in organic synthesis due to their ability to utilize a wide spectrum of substrates, high stability towards extremes of temperature, pH and organic solvents. These enzymes are cofactor/coenzyme independent and are highly chemo, regio and enantio selective in nature. Lipases/esterases are used in various industries such as dairy, food processing

and in production of biodiesel and biofuels. They are also used in the chemical industry for synthesis of fine chemicals/chiral intermediates via kinetic resolution of racemates which are further used in pharmaceutical, flavor and fragrance industry. The main aim of this thesis work is to isolate, characterize and study the application of novel lipases/esterases from microbial origin for the production of fine chemicals via kinetic resolution of racemates.

2) Chapter 2

Cloning, characterization and utilization of esterase/lipases isolated from non-culturable microorganism.

Section 2.1: Introduction

Metagenomics has emerged as a powerful tool that can be used to analyze microbial communities regardless of the ability of member organisms to be cultured in the laboratory. Less than 1 % of the microorganisms can actually be grown into the laboratory, so it becomes a very important field to mine the unknown diversity of the novel microbial enzymes/biocatalysts. Metagenomics is based on the genomic analysis of microbial DNA that is extracted directly from communities in environmental samples. A total of eight metagenomic clones showing high lipolytic activity were used to study the enantioselective hydrolysis of (±)-linalyl acetate, a model substrate. Among these, two clones SLC-6 and MLC-3 were found to be highly enantioselective to form (*S)*-linalool from (±)-linalyl acetate. These two clones were then further used for the cloning, expression and characterization of esterase gene responsible for the activity.

Section 2.2: Cloning, expression and characterization of novel enantioselective esterase SLC-6 obtained through sea sediment metagenomic library.

In present work, we have cloned a novel esterase from metagenomic library of a sea sediment sample from Goa, India. SLC-6 esterase gene (Gen Bank: GU331889.1.) is of size 1410 bp with ORF of 1374 bp and 36 bp 5'-UTR region. It shows maximum of 64% identity with existing gene database of microorganisms available in NCBI. It showed highest match with OsmC family protein (osmotically inducible protein) of *Alpha proteobacterium* species.

This esterase gene was cloned and expressed in pET 32 a vector using C41 (DE3) cells. Protein was purified using Ni-NTA His-tag agarose chromatography and biochemical characterization studies including optimum temperature, optimum pH, effect of various metal ions, *p*-nitrophenol ester substrate specificities, solvent stability study and effect of different inhibitors, detergent were carried out. Enzyme kinetic studies of the esterase were carried out using *p*-nitrophenol butyrate as a substrate at pH 7.5 and temperature 30ºC. This esterase was found to be (*S)* and (*R)*-enantioselective towards racemic acetates which are either flavour compounds or Active Pharmaceutical Intermediates (API's). SLC-6 esterase showed enantioselective hydrolysis of racemic acetates such as (±)-2-pentyl acetate, (±)-2-hexyl acetate, (±)-3-hexyl acetate, (±)-1-octen-3-yl acetate, (±)-1-phenyl ethyl acetate, (±)-3-methyl-1-phenyl ethyl acetate and (±)-1-phenylpropyl acetate. Based on percentage area of the each resolved (*R)* and (*S)* enantiomers on chiral GC, enantiomeric excess (e.e) was calculated for each reaction. SLC-6 esterase showed more than 90 % enantiomeric excess (e.e) to form (*R)* or *(S)*-alcohols from the racemic acetates in 48 h at 30 ºC and 200 rpm as shown in Fig. 1.

- **SLC-6 esterase (1410 bp)**

ATGCGAATAACGTCGGCGCCCATATCGGCCAACAGCATGGCACAGAATGGCCCGGGGCCGA
TGCCTGCGATTTCAATTACCTTGACCCCTTCGAGCGGTCCCATATCTGTCTCCCTTACCTGCG
GTTTGTCACTGTTTGGCGCTACCATAGGAACGCGGCCCCTCGAAACCAACAGGAATTCGCTG
ATGTCGATTTCAAGTGAGAAAACAGACTTTCGCGGCCATGACGGTCAGATGCTCGCTGCACG
GCTGGAACGAGCCTCCGGCGTGCCACGCGCATACGCGCTCTTTGCGCACTGTTTCACCTGCA
CAAAGGATATCTACGCGGCCAGGCGCATCTCCCAGGGCTTGGCAGAGCGCGGGATTGCTGTG
CTGCGCTTTGACTTCACCGGCCTTGGGGCGTCCGAAGGTGACTTCGGCAACACCGGCTTTAC
GTCCAATATCGACGACCTCATTGCCGCGGCAAGCTTTCTGCGGGAGGAGCACGAGGCACCGA
CCATTCTGATCGGCCATAGCCTGGGCGGCGCCGCTGTTCTACGTGCCGCTGAAGCGATCCCC
GAGGCTGCTGCTGTAGCAACCATCGGCGCGCCTGCCGACCCAGCCCACGTTGCTCACCTCCT
CGAAGACAAGGCTGATGAAATCCGTGATAAGGGCCGAGCTACCGTCAACATCGGTGGTCGC
CCGTTCGATATCCGGGCCGAATTCCTCGACGACATCACCGCAAACCGACCCAGGGATTACAT
TGGAGACCTGCGAAAAGCGCTGATTGTATTCCACGGGCCCCGCGATCAGATCGTCGGTATCG
AAAACGCTGCAGAGATATTCACAGCAGCCAAACACCCGAAGAGCTTCGTCAGCCTTGATGA
CGCGGACCATCTGCTGTCGCGACAGCAGGATGCGGACTATGTCGCCGATGTGCTTTCGGCCT
GGGCATCCCGTTATATCGGAGAGACAGAAAAGCGGACCACGCCTCAGCCGCCCGACGGAAT
CACCCGTGTCGCCGAATCCGGCACAGGAAGATTTACCCAGGATGTTTGGGCCGGCGGCCACT
TCCTTCAGGCCGATGAGCCCGCCAGCTTTGGCGGCGATAACGTTGGGCCCACACCCTACGAC
CTGTTGAGTGCCGCCTTAGGCGCCTGCACGACCATGACCATCCGCATGTATGCGGACCGCAA
AAAGCTGCCGCTCGAACAGGTTTCTGTCGATGTCTCGCATGAAAAGATTCACGCGAGCGACT
GCGCCGATTGCGAAACTGAATCTGGAAAGGTTGACCGGTTTTCGCGCGAGATCACCCTGTCA
GGCGATCTGGATGAAACTCAACGGGCACGACTTTTGGAGATCGCGGACAAATGCCCAGTTCA
CCGTACGCTCCTCAGCGAGGTGAAGGTCGAAACCCGAGAAGTAACCTGA.

➢ **Amino acid sequence of SLC-6 esterase (470 a.a)**

MRITSAPISANSMAQNGPGPMPAISITLTPSSGPISVSLTCGLSLFGATIGTRPLETNRNSLMSISSEK
TDFRGHDGQMLAARLERASGVPRAYALFAHCFTCTKDIYAARRISQGLAERGIAVLRFDFTGLG
ASEGDFGNTGFTSNIDDLIAAASFLREEHEAPTILIGHSLGGAAVLRAAEAIPEAAAVATIGAPADP
AHVAHLLEDKADEIRDKGRATVNIGGRPFDIRAEFLDDITANRPRDYIGDLRKALIVFHGPRDQIV
GIENAAEIFTAAKHPKSFVSLDDADHLLSRQQDADYVADVLSAWASRYIGETEKRTTPQPPDGIT
RVAESGTGRFTQDVWAGGHFLQADEPASFGGDNVGPTPYDLLSAALGACTTMTIRMYADRKKL
PLEQVSVDVSHEKIHASDCADCETESGKVDRFSREITLSGDLDETQRARLLEIADKCPVHRTLLSE
VKVETREVT

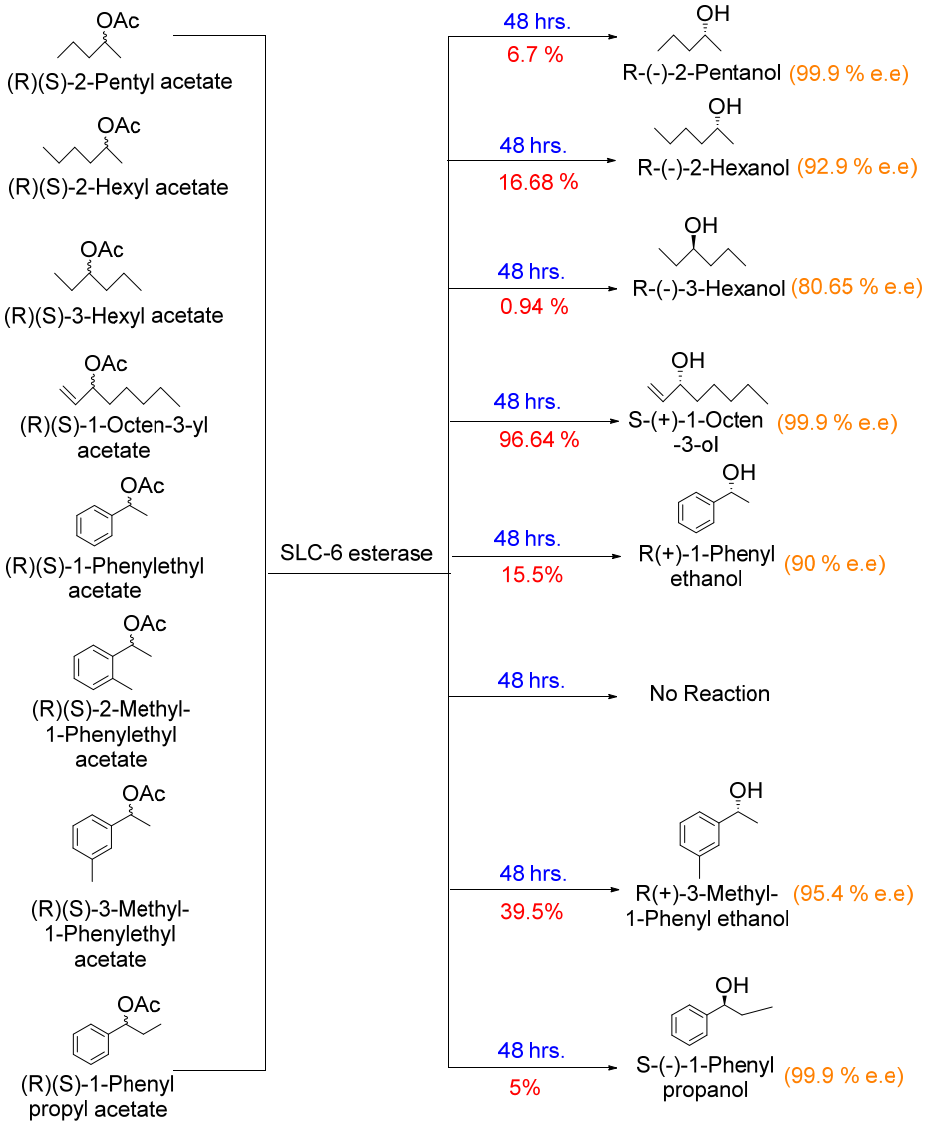


Fig. 1 Enantioselective hydrolysis of racemic acetates by SLC-6 esterase after 48 h.

Molecular docking studies were carried out to study the binding affinity of individual (*R*) and (*S*) acetates of each compound with SLC-6 esterase. Docking studies were found to be in corroboration with the wet lab experiments, where (*R*) acetate was

found to be strongly binding with the active site pocket of SLC-6 esterase enzyme compared to (*S*) acetate (Fig. 2) except in the case of compound 1-phenylpropyl acetate where reverse was observed as found in wet lab experiment.

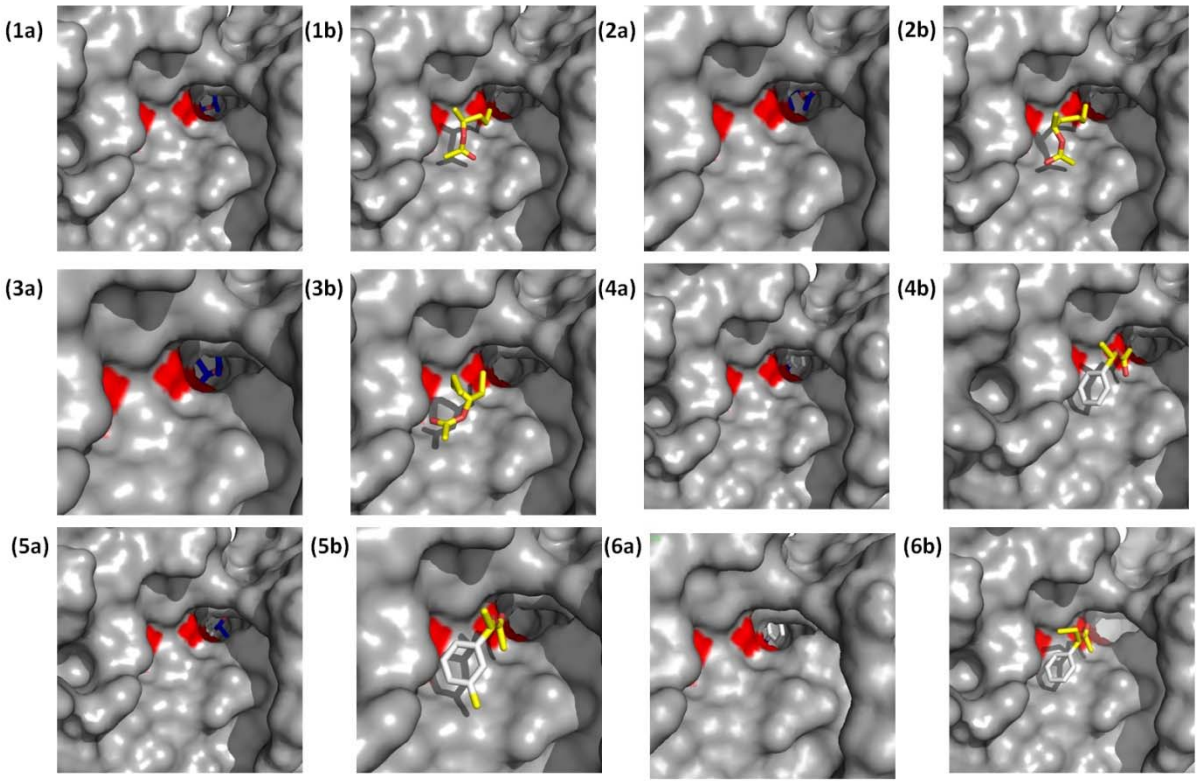


Fig. 2 Docking analysis of various substrates at the active site pocket of SLC-6 esterase protein model. (**1a**)(*R*)-2-pentyl acetate, (**1b**) (*S*)-2-pentyl acetate, (**2a**) (*R*)-2-hexyl acetate, (**2b**) (*S*)-2-hexyl acetate, (**3a**) (*R*)-3-hexyl acetate, (**3b**) (*S*)-3-hexyl acetate, (**4a**) (*R*)-1-phenylethyl acetate, (**4b**) (*S*)-1-phenylethyl acetate, (**5a**) (*R*)-3-methyl-1-phenylethyl acetate, (**5b**) (*S*)-3-methyl-1-phenylethyl acetate, (**6a**) (*R*)-1-phenylpropyl acetate, (**6b**) (*S*)-1-phenylpropyl acetate. Amino acid residues highlighted in red are active sight residues containing GHSLG motif.

Section 2.3 Cloning, expression & characterization of novel enantioselective esterase MLC-3 obtained through hot spring microbial mat metagenomic library.

In present work, we have cloned a novel esterase MLC-3 from metagenomic library of hot spring microbial mat from Khir Ganga region, Himachal Pradesh, India. MLC-3 esterase gene (Gen Bank: GU331883.1) is having ORF of size 843 bp showing maximum of 52% identity with α/β-hydrolase fold protein of *Halothiobacillus* species. This esterase gene was cloned and expressed in pET 32-a vector using Rosetta (DE3) cells. Protein was purified using affinity based Ni-NTA His-tag agarose chromatography. Biochemical characterization studies like optimum temperature,

optimum pH, effect of various metal ions, *p*-nitrophenol ester substrate specificities, solvent stability study and effect of different inhibitors, detergents were carried out with MLC-3 esterase enzyme. Enzyme kinetic studies of MLC-3 esterase were carried out using substrate *p*-nitrophenol butyrate at pH 8.0 and temperature 20 ºC.

- **MLC-3 esterase (843 bp)**

ATGAATCACCGTGATGGTATCCTTGCCCGCACCTTCCGCTCGGACACCCACGTGAAG
CTGCATTACGAAACCTTGGGCCGGGGCGAGCCGCTGATCATCCTGCACGGCCTGTT
CGGCTCGGGCGCGAACTGGCGAAGCATCGCCCAGCGCCTGGCCGATACCTGGCAGG
TCATCCTGCCCGACCTGCGCAATCACGGCGACTCCCCGCATGCGCCCACCAACCGTT
ACCAGGACATCGCGGGCGACACCCTGGCGCTGATGGATCGTCTGGGCCTTGCGCGT
GCCCACCTCCTGGGCCACTCGCTCGGCGGCAAGGCGGCAATGCTGCTCGCCAGCCG
CGCGCCCGAGCGCATCGATAGCCTGACCGTGGTCGACATCGCCCCTCGCGCCTACC
CGCCCCTGCACCTTGAGCTGTTCCGCGCCCTGCACGCCGTGCCGCTCGCCCGCATCA
CCTCACGCCGCGAGGCCAGCGAGGCGATGGCCGCGCACATCTCCAACCCGGCGGTT
CGCGACTTCCTTTTGACCAATCTCGCGCGGGATGGCAACGGACGGTTCCACTGGCG
GCTGAACCTGGCCGGGCTGGAGGAGGCGTACGAGGAGCTCAACGCCATGCCCTTCC
TCGATCGGTTGTATGAGGGGCCGGCCCTGTTCATCCGCGGCGGGCATTCGGACTAC
GTGCGCGACGCCGACCTCGGCCTGATCCACCAAAGCTTCCCGCGGGCCTGCGTGGT
CAGCCTGCCGCTGGCCCATCACTGGCCGCATGTCGAGACACCCAACGAGTTCCTGC
GCGCCCTGCGCGACTTCCTCGAGGCACGCTCCGAGCGCCTGCCCTGTGAGGCTTGA

- **Amino acid sequence of MLC-3 esterase (281 a.a)**

MNHRDGILARTFRSDTHVKLHYETLGRGEPLIILHGLFGSGANWRSIAQRLADTWQVIL
PDLRNHGDSPHAPTNRYQDIAGDTLALMDRLGLARAHLLGHSLGGKAAMLLASRAPE
RIDSLTVVDIAPRAYPPLHLELFRALHAVPLARITSRREASEAMAAHISNPAVRDFLLTN
LARDGNGRFHWRLNLAGLEEAYEELNAMPFLDRLYEGPALFIRGGHSDYVRDADLGLI
HQSFPRACVVSLPLAHHWPHVETPNEFLRALRDFLEARSERLPCEA-

MLC-3 esterase was found to be (*R)*-enantioselective to hydrolyse many racemic acetates of commercial importance. MLC-3 esterase showed (*R)*-selectivity towards racemic acetates including (±)-2-pentyl acetate, (±)-2-hexyl acetate, (±)-3-hexyl acetate, (±)-1-octen-3-yl acetate, (±)-1-phenyl ethyl acetate, (±)-3-methyl-1-phenyl ethyl acetate, (±)-1-phenylpropyl acetate. MLC-3 esterase showed more than 90 % e.e for (*R)*-alcohol in the hydrolysis of the above esters in 48 h at 20 ºC and 200 rpm as shown in Fig. 3.

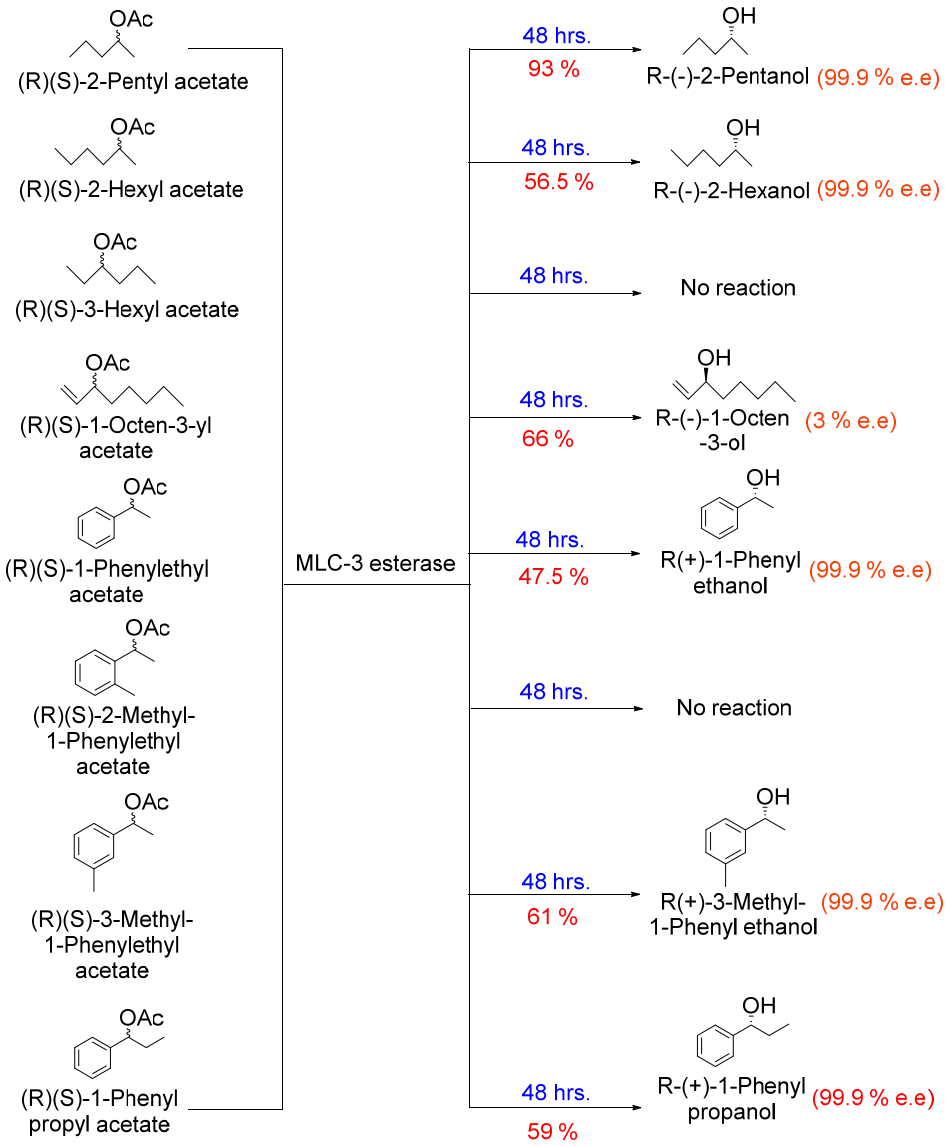


Fig. 3 Enatioselective hydrolysis of racemic acetates by MLC-3 esterase.

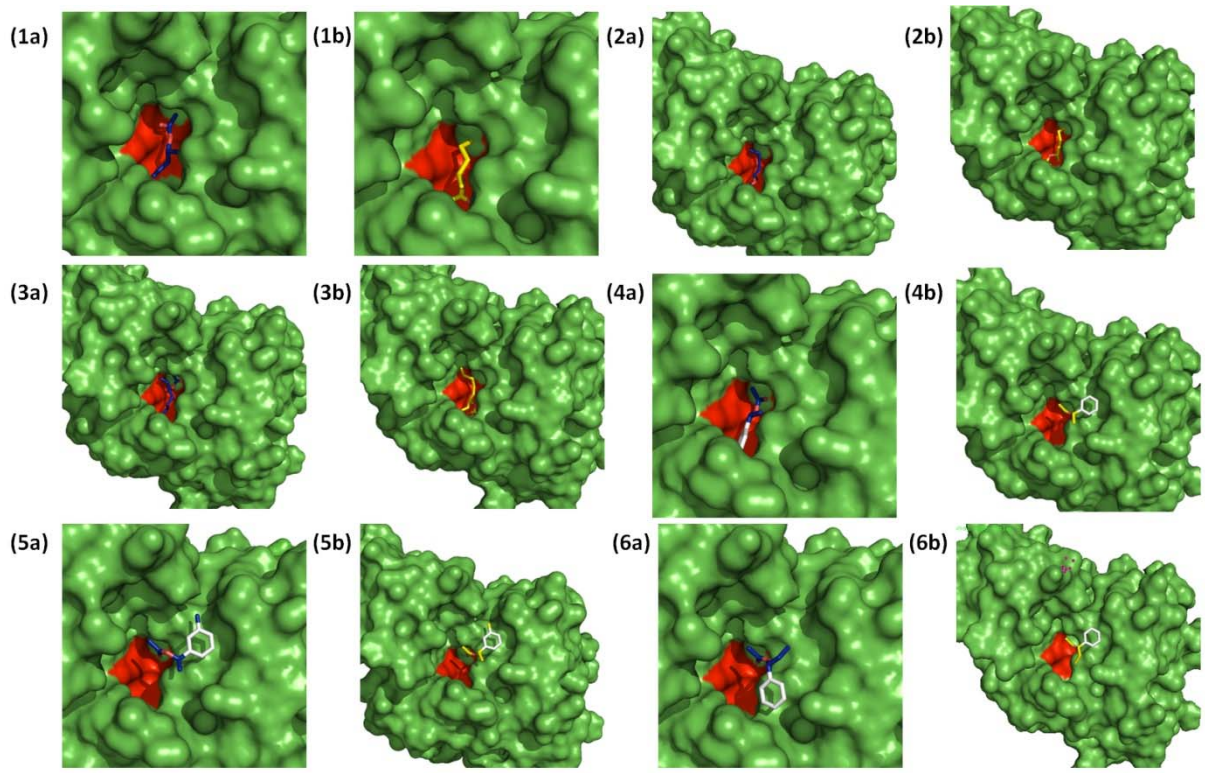


Fig. 4 Docking analysis of various substrates at the active site pocket of MLC-3 esterase protein model. (**1a**)(*R*)-2-pentyl acetate, (**1b**) (*S*)-2-pentyl acetate, (**2a**) (*R*)-2-hexyl acetate, (**2b**) (*S*)-2-hexyl acetate, (**3a**) (*R*)-1-octen-3-yl acetate, (**3b**) (*S*)-1-octen-3-yl acetate, (**4a**) (*R*)-1-phenylethyl acetate, (**4b**) (*S*)-1-phenylethyl acetate, (**5a**) (*R*)-3-methyl-1-phenylethyl acetate, (**5b**) (*S*)-3-methyl-1-phenylethyl acetate, (**6a**) (*R*)-1-phenylpropyl acetate, (**6b**) (*S*)-1-phenylpropyl acetate. Amino acid residues highlighted in red are active sight residues containing GHSLG motif.

Molecular docking studies were carried out to study the binding affinity of individual (*R*) and (*S*) acetates of each compound with MLC-3 esterase. Docking studies were found to be in corroboration with the wet lab experiments, where (*R*) acetate was found to be strongly binding with the active site pocket of SLC-6 esterase enzyme compared to (*S*) acetate (Fig. 4).

3) Chapter 3

Cloning, characterization and utilization of lipases/esterase isolated from culturable microorganism.

Section 3.1: Introduction

Lipase/esterase enzymes are commonly found in many microorganisms and thus can be a source for isolating a novel biocatalyst having desired properties.

Microorganisms were isolated from various environmental samples such as soil, lake water, sea water, hot spring water etc. and checked for esterase activity using tributyrin agar plates. The esterase positive microorganisms were then used for screening of enantioselective hydrolysis using (±)-linalyl acetate as a model substrate. Total of 62 bacterial and 32 yeast species were screened for the enantioselective hydrolysis of (±)-linalyl acetate out of which *Pseudomonas pseudoalcaligenes* NCIM 2864 showed highest e.e of ~84 % to form (*R)*-linalool from its corresponding (±)-linalyl acetate.

Section 3.2: Cloning &characterization of novel alkaline stable enantioselective esterase from *Pseudomonas pseudoalcaligenes* NCIM 2864.

In order to characterize the esterase responsible for the enantioselective activity of *P. pseudoalcaligenes,* molecular biology techniques were used. Cloning and over expression of the esterase gene (pEST-1) responsible for the activity using genomic DNA of *Pseudomonas pseudoalcaligenes* was carried out. pEST-1 esterase gene is having ORF of 612 bp (NCBI sequence: Gen bank: KX495763) was cloned, in pET 32-a vector and expressed using Rosetta gami (DE3) cells.

pEST-1 esterase (612 bp)

ATGACCGCATCCATCCTCTATATACACGGCCTCAACAGTTCGCCGGCCTCGCTCAAGGCCAGCCAGTTG
AGCCGTGCCATGGCTCATCTGGGCCTGGAAAACCAGTTGCGCATACCGGCCCTGCATCATCATCCGCGT
CAGGCCATCACGCAGTTGCAGGCGCTGATCAGCGAACTGGGCGCGCCGCTACTGGTGGGCAGCTCACT
GGGCGGCTACTACGCCACTTACCTGGCCGAGCAGCACGGGCTCAAGGCACTGCTGATCAATCCGGCCG
TGCAGCCGCACCTGCGTTTCGACGGCTACCTGGGCCCACAGAAGAATTACTACAGCGACGAGACCTGG
GACCTCACCGAGGATCACGTCCGCGCCCTGGCTGAACTCGACGTTGCAGCGCCGAACGACCCGGCGCG
TTACCAGGTGTGGCTGCAAACCGGCGACGAAACCCTCGACTACCGCGACGCCGAGCGTTACTACCGTG
CCTGCGCCCTGCGCATCCAGGCCGGTGGCGACCACGGTTTCCAGGGCTTTACCGAACACTTGCCGGCAC
TCTTCGCATTCGCCGACATTAGCGCCACACTCTGGCGTGATACCGACTTTTCCGCGTTCAATTGA.

pEST-1Amino acid sequence: (204 a.a)

MTASILYIHGLNSSPASLKASQLSRAMAHLGLENQLRIPALHHHPRQAITQLQALISELGAPLLVGSSLGGYY
ATYLAEQHGLKALLINPAVQPHLRFDGYLGPQKNYYSDETWDLTEDHVRALAELDVAAPNDPARYQVWL
QTGDETLDYRDAERYYRACALRIQAGGDHGFQGFTEHLPALFAFADISATLWRDTDFSAFN-

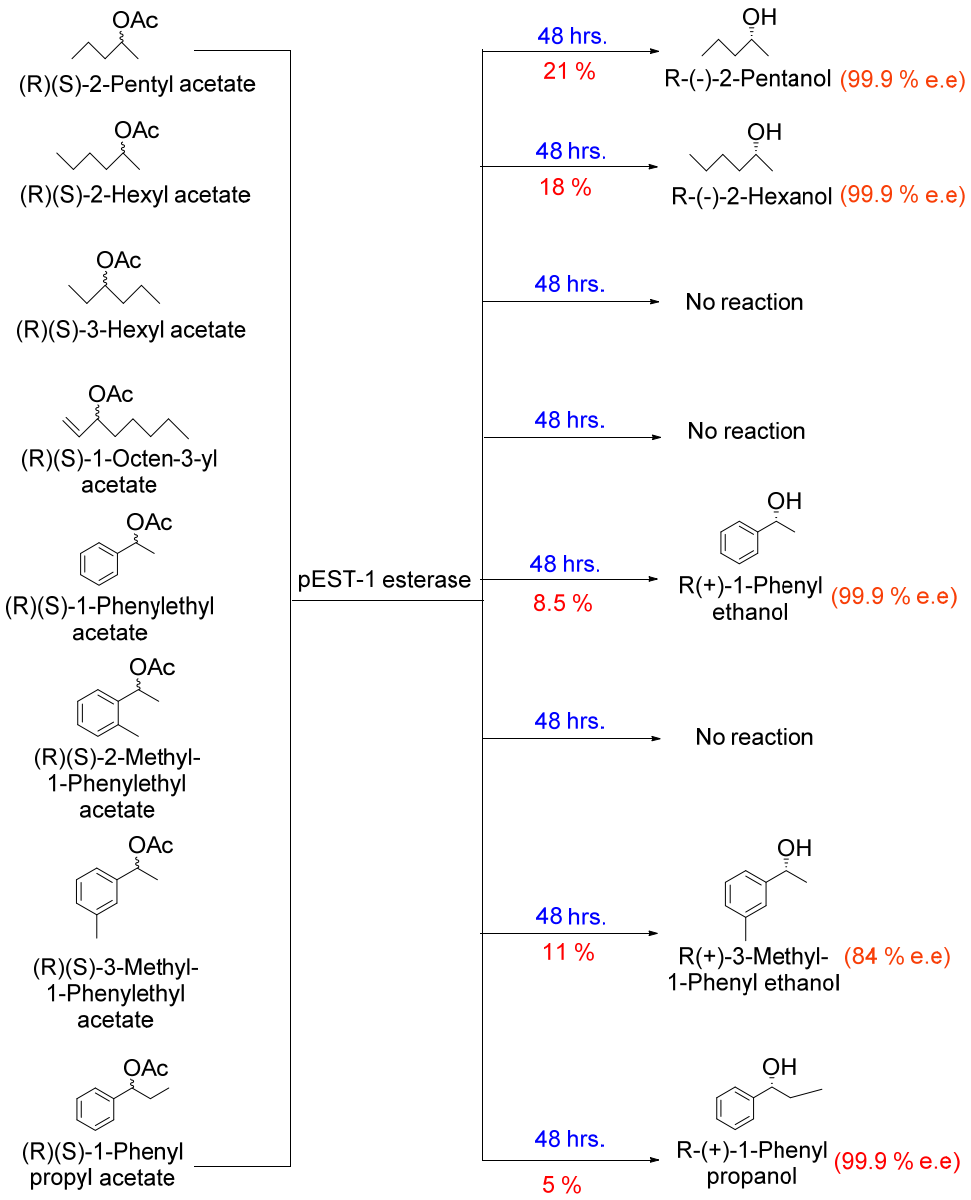


Fig. 5 Enantioselective hydrolysis of racemic acetates by pEST-1 esterase.

Protein was purified using affinity based Ni-NTA His-tag chromatography. Biochemical characterization studies including optimum temperature, optimum pH, effect of various metal ions, *p*-nitrophenol ester substrate specificities, solvent stability study and effect of different inhibitors, detergents were done. Enzyme kinetic studies of pEST-1 esterase were carried out using *p*-nitrophenol valerate as a substrate. pEST-1 esterase was found to be (*R*)-selective towards racemic acetates such as (±)-2-pentyl acetate, (±)-1-phenyl ethyl acetate, (±)-3-methyl-1-phenyl ethyl acetate and (±)-1-phenylpropyl acetate showing e.e value more than 99 % as shown in Fig. 5.

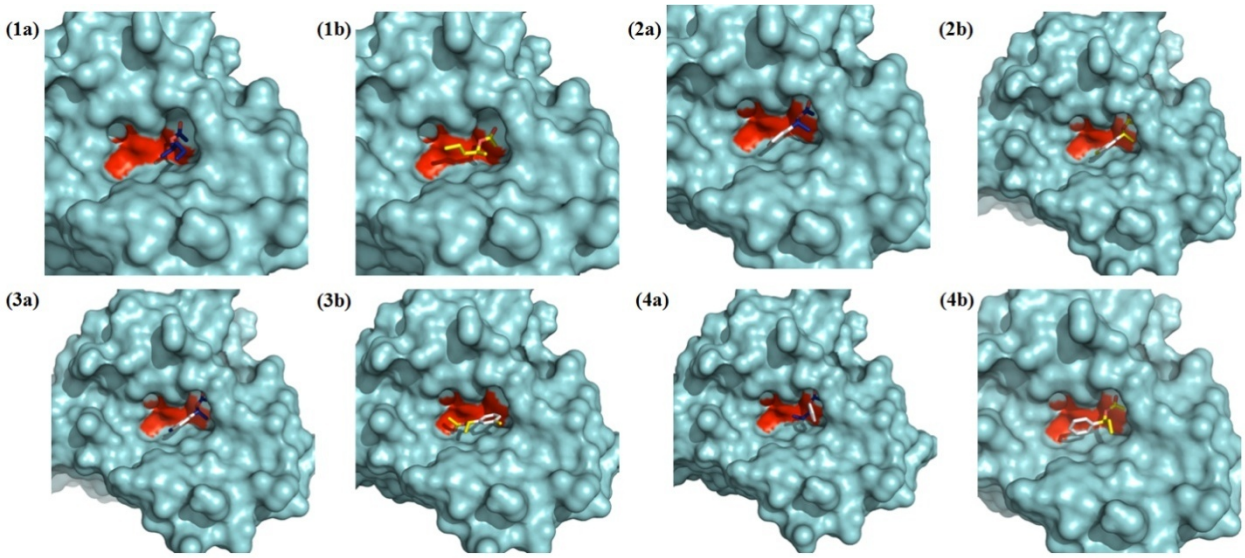


Fig. 6 Docking analysis of various substrates at the active site pocket of pEST-1 esterase protein model.

(**1a**)(*R*)-2-pentyl acetate, (**1b**) (*S*)-2-pentyl acetate, (**2a**) (*R*)-1-phenylethyl acetate, (**2b**) (*S*)-1-phenylethyl acetate, (**3a**) (*R*)-3-methyl-1-phenylethyl acetate, (**3b**) (*S*)-3-methyl-1-phenylethyl acetate, (**4a**) (*R*)-1-phenylpropyl acetate, (**4b**) (*S*)-1-phenylpropyl acetate. Amino acid residues highlighted in red are active sight residues containing GSSLG motif.

Molecular docking studies were carried out to study the binding affinity of individual (*R*) and (*S*) acetates of each compound with pEST-1 esterase. Docking studies were found to be in corroboration with the wet lab experiments, where (*R*) acetate was found to be strongly binding with the active site pocket of pEST-1 esterase enzyme compared to (*S*) acetate (Fig. 6).

Section 3.3: RNA isolation, transcriptome sequencing & identification of new uncharacterized lipases from *Yarrowia lipolytica* NCIM 3639.

Yarrowia lipolytica NCIM 3639 is psychrophillic yeast isolated from Tween 80 detergent. It produces two types of lipases namely cell bound /intracellular and extracellular lipase when induced with olive oil and Tween 80 detergents respectively**.** Extracellular lipase is a 400 kD homomeric protein. Transcriptome analysis was carried out in order to find out novel lipases from *Y. lipolytica.* In present work, total RNA was isolated from control and Tween 80 induced *Yarrowia lipolytica* NCIM 3639 strain and was subjected to transcriptome sequencing. The transcripts sequenced were then assembled using "Trinity" software and were functionally annotated for Pfam domains

to obtain novel Lipases. We have identified a total of 33 lipases/carboxyl esterases fromwith our transcriptome database.

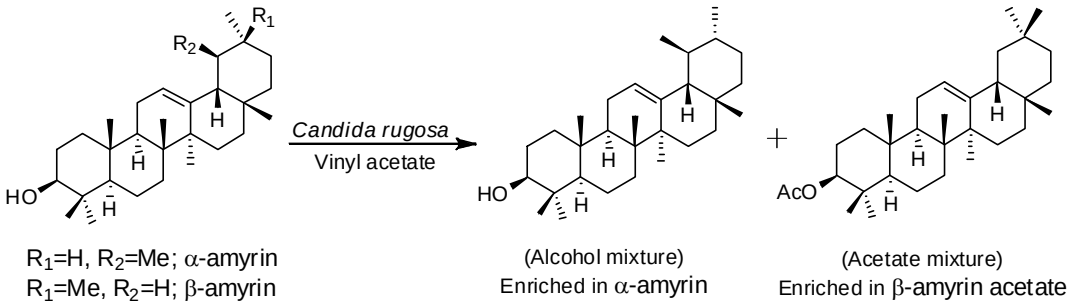
Among these 33 lipases/carboxyl esterases matched in database, total of nine were found to be new uncharacterized lipases with Pfam domains such as Abhydrolase-1, Abhydrolase-3, and Lipase-3 class. These nine lipases still remain to be functionally characterized.

4) Chapter 4

Biocatalysis: Enzyme and whole cell mediated kinetic resolution of α/β-amyrins and commercially important racemic esters.

Section 4.1 Kinetic resolution of structural isomers α and β amyrins using *Candida rugosa* lipase (*CRL*).

α/β-amyrin are structural isomers in relation and possess basic skeletons of two different subgroups of pentacyclic triterpenoids. α/β-amyrin and their derivatives shows broad spectrum activities including anti-inflammatory, insecticidal, anti-depressant, anti-arthritic, gastro protective, anti-hyperglycaemic, anti-microbial and cytotoxic activities. Chromatographic separation of these isomers is difficult so enzyme mediated kinetic resolution studies were carried out. Total of 16 commercial lipases were screened for the kinetic resolution α/β-amyrins. In present work, we have resolved these two structural isomers using *Candida rugosa* lipase (*CRL*) mediated transesterification reaction (scheme 1). In the mixture of α-amyrin: β-amyrin (78:22) β-amyrin was more preferred over α –amyrin using vinyl acetate as acyl donor in n-hexane solvent (Fig. 7).After the incubation period of 48 h, *CRL* converted 38 % of β-amyrin to β-amyrin acetate in the presence of vinyl acetate. However, less than 1% of α-amyrin acetate formation was observed when α-amyrin was used as the substrate with similar assay conditions.



Scheme 1 Kinetic separation of α and β amyrin mixture by *Candida rugosa* (*CRL*).

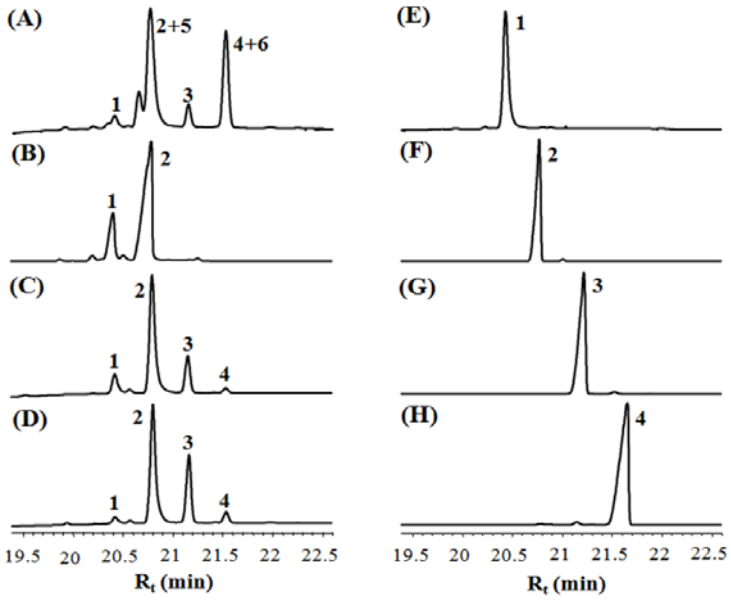


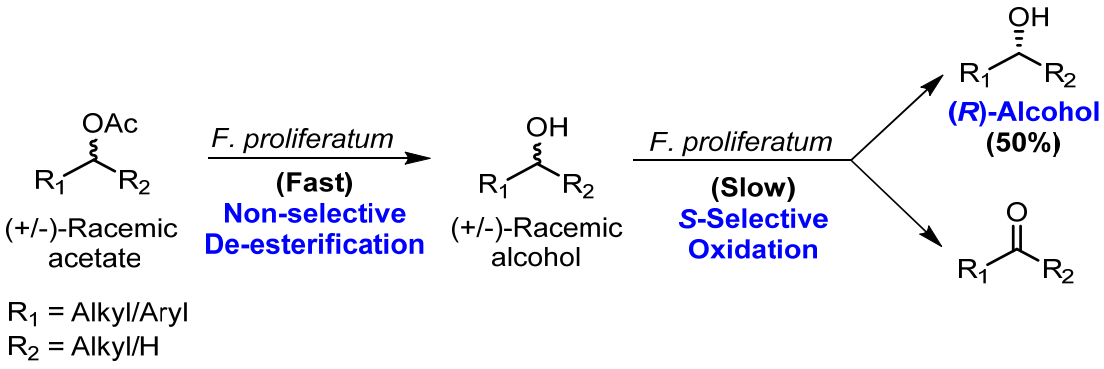
Fig. 7 GC-FID chromatograms of (A) *P. obtusa* extract, (B) α- and β-amyrin mixture obtained from *P. obtusa* extract, (C) CRL mediated transesterification reaction mixture after 1 day, (D) after 5 days, (E) Pure β-amyrin, (F) Pure α-amyrin, (G) Pure β-amyrin acetate, (H) Pure α-amyrin acetate. Labeling of compounds, 1: β-amyrin, 2: α-amyrin, 3: β-amyrin acetate, 4: α-amyrin acetate, 5: Lupeol, 6: Lupeol acetate.

Section 4.2: Fungal mediated kinetic resolution of racemic acetates to (*R*)-alcohols using *Fusarium proliferatum* NCIM 1105.

In the present work, we have studied fungus *F. proliferatum* as a whole cell biocatalyst to obtain enantiopure (*R*)-alcohols from racemic acyclic and aromatic acetates. (±)-lavandulyl acetate was used as a model substrate to screen for the

enantioselective de-esterification of racemic acetates using whole cell method. Among many fungi screened ten fungi showed good e.e value for the conversion of (±)-lavandulyl acetate to (*R*)-lavandulol. Among these fungi, *F. proliferatum* showed highest e.e of ≥ 99 % for the conversion of (±)-lavandulyl acetate to (*R*)-lavandulol, when incubated at the concentration of 0.6 gL^{-1} and 3 days of incubation at 200 rpm and 30 ºC.

Resting cell experiments were carried out to confirm the results obtained with (±)-lavandulyl acetate in whole cell method. Different racemic acetates such as (±)-2-hexyl acetate, (±)-2-heptyl acetate, (±)-3-hexyl acetate, (±)-1-phenyl ethyl acetate, (±)-3-methyl-1-phenyl ethyl acetate and (±)-1-phenylpropyl acetate were screened in the study. *Fusarium proliferatum* was able to convert these acetates to corresponding racemic alcohols in the first step and further (*S*)-alcohol was selectively oxidized to form corresponding ketone (scheme 2). (*R*)-alcohols obtained were with more than 95 % e.e and 100 % conversion as shown below in Fig. 8.



Scheme 2 Kinetic resolution of racemic acetates to (*R*)-alcohols by *F. proliferatum* NCIM 1105.

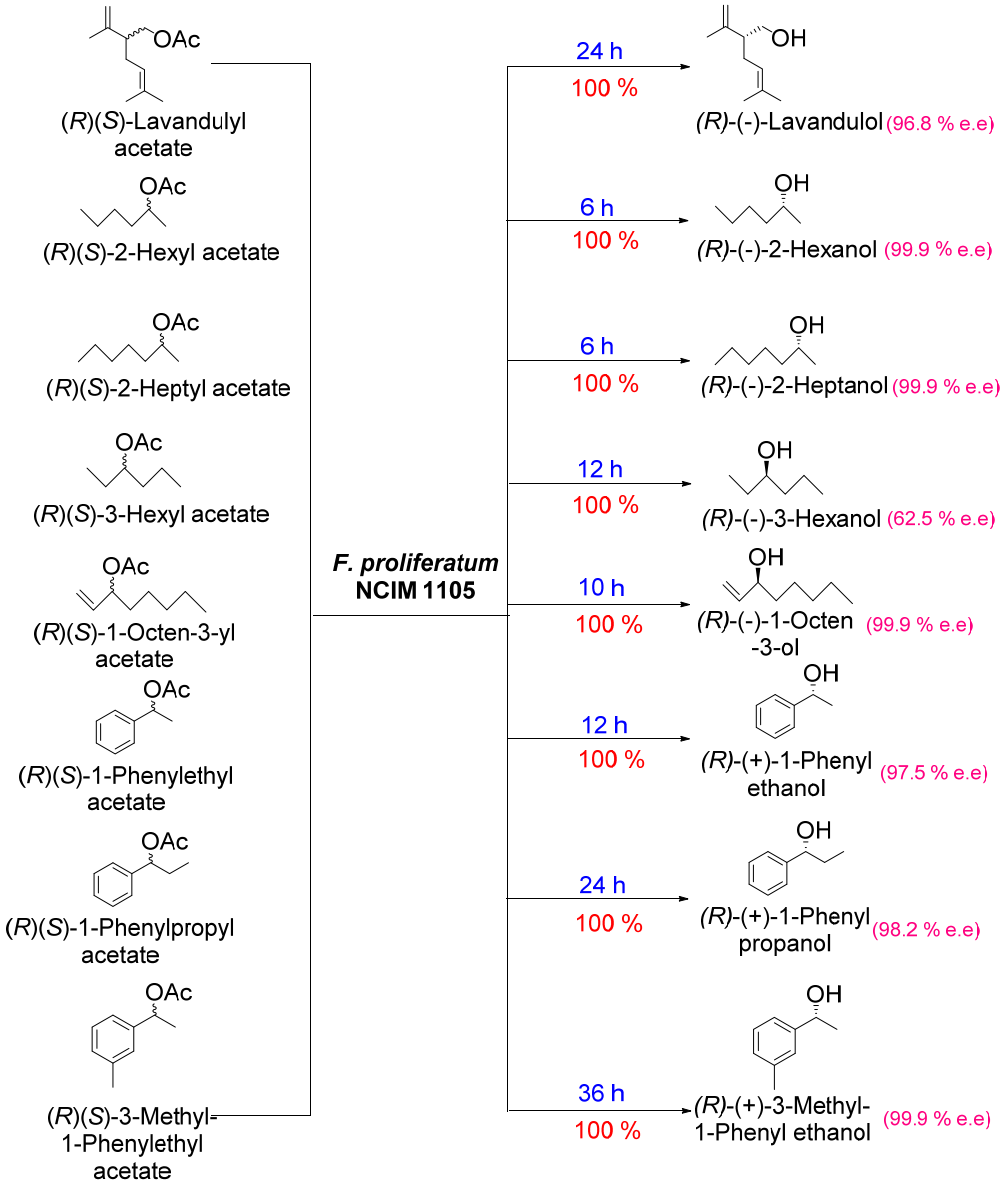


Fig. 8 Enantioselective hydrolysis of racemic acetates to (*R*)-alcohol by *F. proliferatum* NCIM 1105.

List of Abbrevations

A°	Angstrom
Amp	Ampicillin
AMV-RT	Avian Myeloblastosis Virus-Reverse Transcriptase
AA	Amino acid
BLAST	Basic Local Alignment Search Tool
bp	Base pair
BME	2-β-mercaptoethanol
CAM	Chloramphenicol
CHAPS	3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulphonate
DMSO	Dimethyl sulfoxide
DTT	DithioThreitol
DCM	Dichloromethane
EDTA	Ethylene diamine tetra acetic acid
GC	Gas chromatography
GC-MS	Gas chromatography Mass spectrometry
hr	Hours
IPTG	Isopropyl β-D-1-thiogalactopyranoside
kDa	kilo Dalton
K_m	Michaelis-Menten constant
K_{cat}	Catalytic turnover
L.B	Luria Bertani
min.	Minutes
mg	Milligram
mL	Millilitre
µg	Microgram
µL	Microlitre
µM	Micromolar

NCIM	National Collection of Industrial Microorganisms
O.D	Optical density
ORF	Open Reading Frame
PAGE	Poly-acrylamide gel electrophoresis
*p*NP	*p*-nitrophenol
*p*NPP	*p*-nitrophenol palmitate
rpm	revolution per minute
PCR	Polymerase Chain Reaction
PDB	Protein Data Bank
PMSF	Phenyl methyl sulphonyl fluoride
Pfam	Protein families
RNA	Ribonucleic acid
Rt	Retention time
Sec.	Second
SDS	Sodium dodecyl sulphate
SOB	Synthetic oil based medium
T.B	Terrific broth
THF	Terahydrofuran
UTR	Untranslated Region
X-gal	5-bromo-4-chloro-3-indolyl β-D-galactoside
[α] D	Optical rotation
° C	Degree celcius
%	Percentage

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List of Biocatalysts used

Sr. No	Microorganisms name	NCIM catalogue no.
1	*Pseudomomans pseudoalcaligenes*	2864
2	*Yarrowia lipolytica*	3639
3	*Fusarium proliferatum*	1105
4	*Aspergillus niger*	582
5	*Aspergillus niger*	589
6	*Neurospora crassa*	910
7	*Aspergillus gigantius*	568
8	*Cunnighamella irregularis*	1278
9	*Aspergillus niger*	612
10	*Aspergillus foetidus*	510
11	*Aspergillus niger*	572

Sr. No	Enzymes name	Source
1	SLC-6 esterase	Sea sediment metagenome library
2	MLC-3 esterase	Hot spring microbial mat metagenome library
3	pEST-1 esterase	*Pseudomomans pseudoalcaligenes* NCIM 2864
4	CAL-B (Novozyme 435)	*Candida antarctica*
5	CAL-A	*Candida antarctica*
6	Amano lipase M	*Mucor javanicus*
7	Amano lipase A	*Aspergillus niger*
8	Amano lipase PS	*Pseudomonas fluorescens*
9	Amano lipase G	*Penicillium camemberti*
10	Amano lipase AK	*Pseudomonas fluorescens*

11	lipase from *Thermomyces lanuginosa*	*Thermomyces lanuginosa*
12	lipase from *Penicillium camemberti*	*Penicillium camemberti*
13	lipase from wheat germ	Wheat germ
14	*Candida rugosa* lipase	*Candida rugosa*
15	Porcine pancreas type-II lipase	Porcine pancreas
16	lipase immobilized on immobead 150	*Candida rugosa*
17	lipase from *Rhizopus niveus*	*Rhizopus niveus*
18	lipase from *Rhizopus arrhizus*	*Rhizopus arrhizus*
19	Type-VII lipase	*Candida rugosa*

Chapter 1

Introduction

1.1 Biocatalysis

Biocatalysis involves chemical modification of organic compounds including natural products by means of nature's catalysts used in the form of whole cells, cell free extracts or purified enzymes. Early developments in biocatalysis date back to prehistoric times. Man has practised the controlled fermentation of fruit and grains for the preparation of alcohol and later in the bread making and in development of nourishing dietary factors from milk. The ability of yeast to make alcohol in the form of beer was known to the Sumarians and the Babylonians before 6000 B.C.[1] Egyptians discovered leaven bread in around 4000 B.C.[1]

With the development of modern natural science during the seventeenth and eighteenth centuries, biocatalytic processes began to take a form with more scientific basis. However, first breakthrough was when Paven and Persoz reported the production of dextrin and sugars in 1833 using diastase, now known as amylase.[2] In 1857, Wagner defined the nitrogen-containing organic substance as unorganized system and the living body as organized system are responsible for the fermentation processes.[3] Louis Pasteur who in 1858 placed a milestone in biocatalysis by treating an aqueous solution of racemic tartaric acid ammonium salt with a culture of the mold *Penicillium glaucum*, leading to the consumption of (+)-tartaric acid and successive enrichment of the (−)-tartaric acid. This can be considered as a pioneer example of enzyme-catalyzed kinetic resolution which is widely practiced today in academia as well as in industry. In 1876 Kunhe introduced the name "enzymes" for those substances that previously called "ferments" which carried out fermentation. In 1894, Emil Fischer proposed lock and key model to explain the mechanism of enzymatic catalysis and its substrate specificity for which he received Nobel Prize for chemistry in 1902. Another cornerstone of biocatalysis was laid by Eduard Buchner who in 1897 reported the successful fermentation of sugar by cell free yeast extracts, which constitutes indisputable proof that biological transformations do not necessarily require living cells.

In 1913, Leonard Michaelis and Maud Menten published an equation well known as "Michealis menten equation" by which the kinetics of enzyme catalyzed reactions can be described. In 1926, James Sumner reported the first crystallization of urease enzyme from jack beans followed by in 1944 Linus Carl Pauling developed the modern concept of enzymatic catalysis on the basis of transition-state complementarity. With discovery

of structure of crystallised DNA by Watson and crick in 1953 along with discovery of DNA sequencing technique in 1980 by Frederik sanger and Polymerase chain reaction (PCR) in 1985 by Kary mullis paved way for use of recombinant DNA technology in cloning and expression of enzymes into heterologous expression system.

In the past few decades, advancement in understanding protein structure–function relationship has increased the range of available biocatalytic applications.[4] Modern development in protein engineering such as rational design and directed evolution has enabled scientists to rapidly develop biocatalysts with desired properties for the particular chemical processes. Rational design involves rational alterations of selected amino acid residues in a protein to cause predicted changes in function, whereas directed evolution mimics the natural evolution process in the laboratory (*in vivo*) and involves in creating a library with variation at each amino acid level and then selecting the variants with the desired functions.[5] Enzyme properties such as temperature and pH stability, activity, enantio-selectivity and substrate specificity can now be routinely engineered in the laboratory. Presently, over 100 different bio catalytic processes are used in pharmaceutical, chemical, agricultural, and food industries.[6-8]

1.2 Scope of Biocatalysis

Biocatalysts increase the rate of reaction similar to chemical catalyst without changing the thermodynamics of the reaction. But, biocatalysis is preferred over the use of chemical catalysts in industry because it has certain advantages over conventional catalysts. Firstly, biocatalysts are highly selective i.e. have chiral selectivity (stereo selective), positional (regio-selective) and functional group specificity (chemo-selective). Such high selectivity has various advantages such as reduced/no use of protecting groups, minimized side reactions and fewer environmental problems.[9-14]Biocatalysts show various advantages and disadvantages over chemical catalyst thus favouring their use in industries (Table 1.1). [15]

Advantages	Disadvantages
Generally more efficient (lower concentration of enzyme needed)	Susceptible to substrate or product inhibition
Can be modified to increase selectivity, stability, and activity	Solvent usually water (high boiling point and heat of vaporization)
More selective (types of selectivity: chemo-selectivity, regio-selectivity, diastereo-selectivity and enantio-selectivity)	Enzymes found in nature in only one enantiomeric form
Milder reaction conditions (typically in a pH range of 5–8 and temperature range of 20–40ºC)	Limiting operating region (enzymes typically denatured at high temperature and pH)
Environment friendly	Enzymes can cause allergic reactions

Table.1.1 Advantages and disadvantages of biocatalysis in comparison with chemical catalysis.

1.3 Classes of enzymes

Enzymes (Biocatalysts) are categorised into several classes based on the reaction they catalyse in cellular environment. They are classified into six different categories as per rules prescribed by enzyme commission of International union of Biochemistry as follows:

1.3.1 Oxidoreductases (**EC 1**): These enzymes carry out oxidation-reduction in which oxygen and hydrogen are gained or lost. Examples of enzymes: alcohol dehydrogenase, cytochrome oxidase, lactate dehydrogenase, oxygenases, peroxidise.

1.3.2 Transferases (**EC 2**): These enzymes carry out transfer of functional groups, such as an amino group, acetyl group, or phosphate group. Examples of enzymes:Acetate kinase, alanine deaminase, glycosyltransferases, transketolases, methyltransferases, transaldolases, acyltransferases, transaminases.

1.3.3 Hydrolases (**EC 3**): These enzymes carry out hydrolysis of compounds in the presence of water. Hydrolases are classified as **EC 3** in the EC number, classification of enzymes. Hydrolases can be further classified into several subclasses, based upon the bonds they act upon:

- **EC 3.1**: ester bonds (esterases, lipases, nucleases, phosphodiesterases, phosphatase)
- **EC 3.2**: sugars (DNA glycosylases, glycoside hydrolase).
- **EC 3.3**: ether bonds.
- **EC 3.4**: peptide bonds (Proteases/peptidases).
- **EC 3.5**: carbon-nitrogen bonds, other than peptide bonds.
- **EC 3.6**: acid anhydrides (acid anhydride hydrolases, helicases and GTPase)
- **EC 3.7**: carbon-carbon bonds.
- **EC 3.8**: halide bonds.
- **EC 3.9**: phosphorus-nitrogen bonds.
- **EC 3.10**: sulphur-nitrogen bonds.
- **EC 3.11**: carbon-phosphorus bonds.
- **EC 3.12**: sulfur-sulfur bonds.
- **EC 3.13**: carbon-sulfur bonds.

1.3.4 Lyase (**EC 4**): These enzymes carry out removal of groups of atoms without hydrolysis. Examples of enzymes: Decarboxylases, aldolases, ketolases, hydratases, dehydratases.

1.3.5 Isomerase (**EC 5**): These enzymes carry out rearrangement of atoms within a molecule via epimerisation, racemisation and isomerisation reactions. Examples of enzymes: Glucose-phosphate isomerase, alanine racemase.

1.3.6 Ligase (**EC 6**): These enzymes carry out joining of two molecules by reactions like C-C, C-O, C-N, C-S bonds using energy usually derived from the breakdown of ATP. Examples of enzymes: acetyl-CoA synthetase, DNA ligase.

These six classes of enzymes work in all living things present on earth and are used for their metabolism. These six classes of enzymes have wide uses and are also used in industries for synthesis of fine chemicals, as shown in figure 1.1.Among them, hydrolases are widely used, estimating around 63 % among all industrial enzymes

followed by oxidoreductases (25 %), transferases (5 %), lyases (5 %), ligases (1 %) and isomerases (1 %).[15]

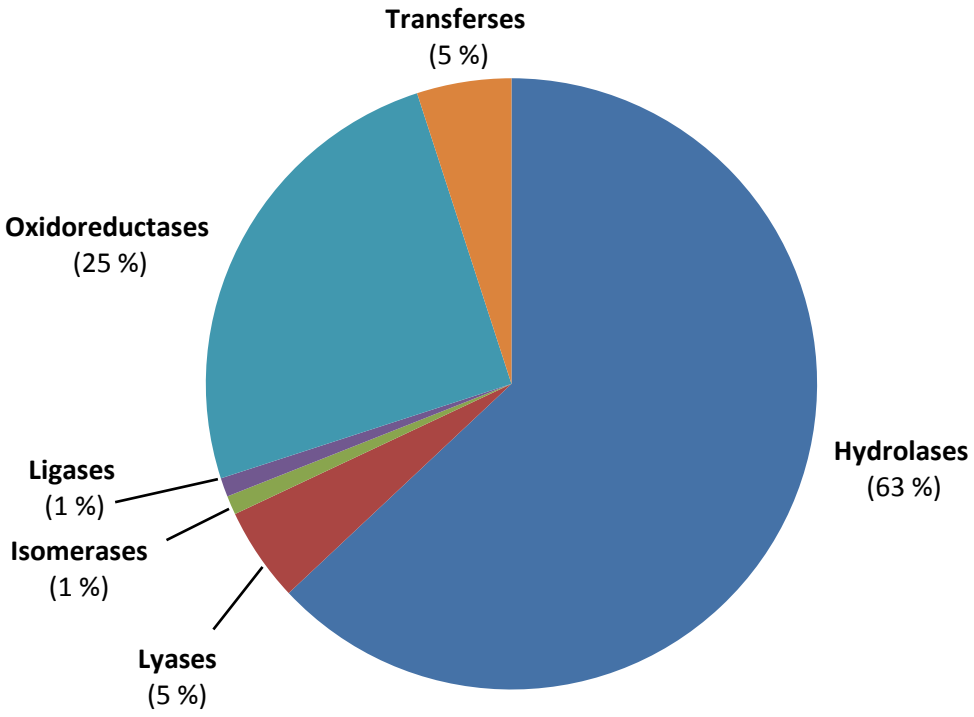


Fig.1.1 The relative use of enzyme classes in industry.[15]

1.4 Hydrolases (EC3)

Hydrolases (EC 3) are one of the biggest classes of enzymes working in any living system. Hydrolases are widely used in synthetic organic chemistry because of their remarkable properties such as broad substrate specificity, commercial availability, lack of cofactor dependency, and ability to work at high substrate concentrations in aqueous solutions, organic solvents and ionic liquids (Fig. 1.1). These properties make hydrolases a special class of enzymes used for the production of different enantiomerically pure form of compounds such as esters, carboxylic acids, alcohols, amines, carbonates andamides.[16,17]

Modern techniques in protein engineering such as directed evolution, site directed mutagenesis and other advanced techniques such as immobilization and medium engineering have mainly contributed to the success of hydrolaseenzymes.[18,19] Use of these techniques has shown improvements in enzyme stability leading to more stable and

selective catalysts. Hydrolases mainly lipases, esterases, epoxide hydrolase, amidases and proteases inside this class have allowed the development of extremely useful regio- and enantioselective transformations for the production of pharmaceuticals, agrochemicals and flavour/fragrance compounds.[20-43] In this thesis, we are dealing with novel lipases/esterases and their use in asymmetric synthetic reactions via kinetic resolution.

1.5 Esterase/lipases

Esterases (EC 3.1.1.x) represent a diverse group of hydrolases catalyzing the cleavage and formation of ester bonds. They include enzymes lipases (EC 3.1.1.1, triacylglycerol hydrolases) and esterases (EC 3.1.1.3, carboxyl ester hydrolases). These enzymes are widely distributed in plants, animals, fungi, bacteria and yeasts. These enzymes are highly stable in organic solvents and don't need any cofactor for their activity. Both the enzymes are stereo and regio selective in nature and thus are used in synthesis of fine chemicals in industries.[44]

1.6 Reactions catalysed by lipases/esterases

Lipases catalyze various types of reactions but mainly are known to catalyse hydrolysis of long chain esters such as triglycerides and forms di-, mono-acylglycerols, glycerol and free fatty acids. These enzymes are also known to catalyze the reverse reactions like esterification, transesterification (acidolysis, ester exchange, alcoholysis, aminolysis) in organic solvents (Fig 1.2). Because of versatility of the reactions catalysed by the lipases in aqueous and in organic solvents they have gained immense commercial importance in various industries. Various reactions catalysed by the lipases/esterases are as follows:

1.6.1 Transesterification reactions

The transesterification reaction is the process of interexchange of acyl groups between an ester and an acid (acidolysis), an ester and an alcohol (alcoholysis), an ester and another ester (interesterification) and an ester and amine (aminolysis) (Fig 1.2). For example, transesterification/acylation reactions of secondary alcohols [28,35,40], tertiary alcohols [33,45,46], phenolic acids [47], 1-phenyl-1-propanol [32] and enantiomers of lavandulol [48-50] are reported to be carried out by esterase/lipases. Trans-esterification of fats and oils

carried out by lipases offers great advantages because of mild conditions, reduced side reactions, and specificity.

1.6.2 Hydrolysis

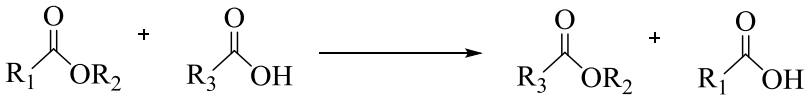
Lipases/esterases are mainly hydrolase enzymes, so are known to carry out simple hydrolysis or enantioselective hydrolysis of many chemical entities (Fig 1.2). In literature, there are reports on many hydrolytic reactions such as hydrolysis of triolein esters, rosmarinic acid, oleyl benzoate and enatio/regio-selective hydrolysis of esters such as esters of 1-phenylethanols [51], 2-hydroxyalkanoates [52], dissacharides [38] and tertiary alcohols [33,53].

1.6.3 Ester synthesis/esterification

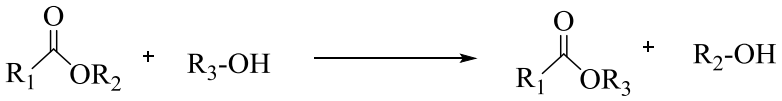
Esterification reactions are chemically carried out using acid or base catalysts. Lipases or esterases carry out these reactions either regio- enantioselectively under mild reaction conditions (Fig 1.2). Various acyl donors used in such reactions are succinic anhydride, acetic anhydride, vinyl donors (vinyl acetate, vinyl propionate, vinyl benzoate etc.). Various reports of esterification reactions have been demonstrated, such as esterification of phenyl carboxylic acids [39,54], 2-acetoxyphenyl acetic acid [55], 1-phenyl propanol [32], 1-phenyl ethanol [26,29,51,56] and lavandulol [48-50] using either esterases or lipases.

1) Transesterification reaction:-

i) Acidolysis:-

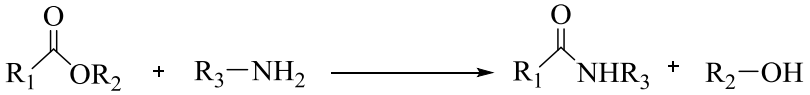


ii) Alcoholysis :-

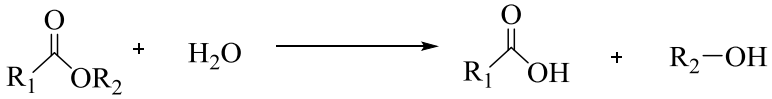


iii) Ester exchange :-

iv) Aminolysis :-



2) Hydrolysis :-



3) Ester synthesis :-

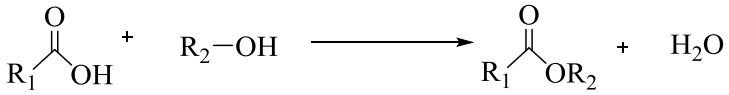


Fig. 1.2 Various reactions catalysed by lipase enzyme.

1.7 Structure and mechanisms

Lipases and esterases shows characteristic α/β hydrolase fold –a definite order of α-helices and β-sheets seen in all hydrolytic enzymes (Fig 1.3). The catalytic triad is composed of Ser-Asp-His (Glu instead of Asp for some lipases). They share the same G-X-S-X-G consensus sequence found at the active site of the enzyme.[44]

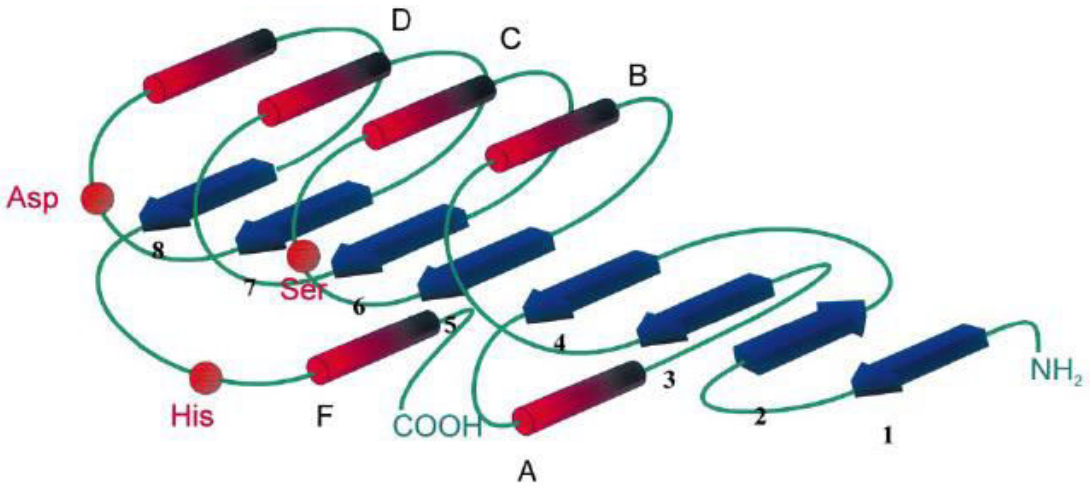


Fig. 1.3 Characteristic α/β-hydrolase fold seen in lipases/esterases.

The mechanism for esterification or hydrolysis remains same for lipases and esterases and consists of four steps (Figure 1.4): First, the acyl donor is bound to the active serine, yielding a stable intermediate (enzyme substrate complex) stabilized by the catalytic His and Asp residues. Next, the alcohol is released and an acyl enzyme complex is formed. Attack of a nucleophile/ acyl acceptor (water in hydrolysis, alcohol or ester in trans-esterification) forms again a tetrahedral intermediate (enzyme substrate complex), which after resolution yields the product (an acid or an ester) and freeenzyme.[25,57]

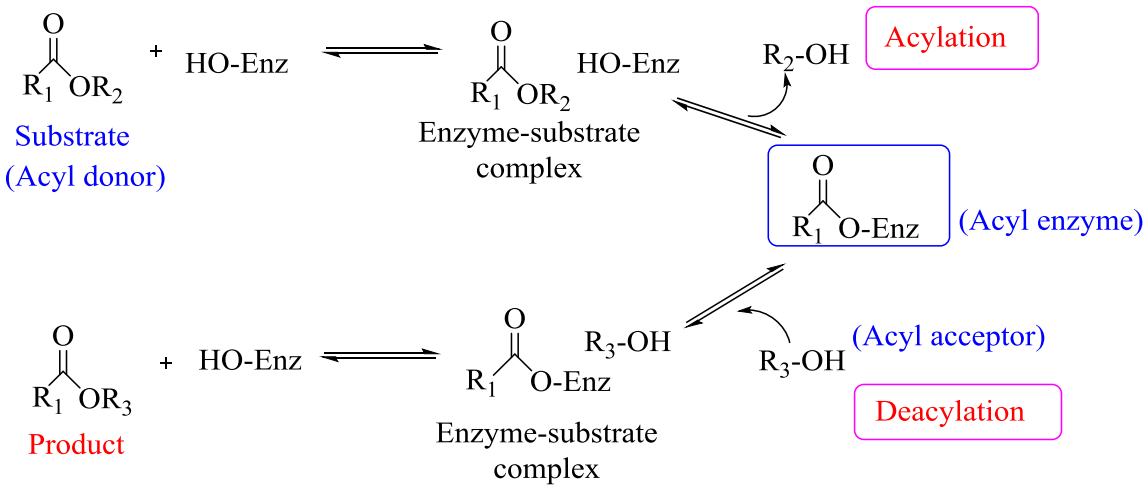


Fig. 1.4 Mechanism of esterification reaction occurring at the active site of lipase/esterase.

1.8 Sources of lipases/esterases

Lipases/esterase enzymes are ubiquitously found in nature. Their sources can be various living beings such as plants, animals, bacteria and fungi mentioned as below:

1.8.1 Plant lipases/esterases

Plant lipases have not been used as much as their counterpart i.e microbial lipases. But they certainly have many great advantages over microbial lipases in parameters such as their availability, their lower cost, their apparent ease of purification and their particularly unusual typo-selectivities. Plant lipases/esterases are mainly found in oil seeds and cereals. In most of the cases, lipase activity is not found in non-germinated seed butis present only in post-germination.[58] In oilseed, plant lipases are present in low levels which make them difficult for extraction and use in large scale. Lipases and esterases are reported from many plants such as rice, soybean, oat, rapeseed and castor bean.

The castor bean lipase is present in the active form in dormant seeds and active under acidic condition. This lipase does not show regio-selectivity but hydrolyzes ricinolic acid which is the major constituent of castor bean oil.[59] A 45 kDa alkaline stable esterase is purified from soybean (*Glycine max*) seed which was used for synthesis of low chain esters used in the food and chemical industries.[60] Lipases from oat seeds also have been reported in the literature.[61] Rice bran and wheat germ are very good sources for lipases.[62, 63] Lipases from wheat germ are commercially available and have many industrial applications.

Lipase activity of *Carica papaya* latex and its applications in oil and fat modifications has been studied in recent times.[64] Although plant lipases are not used as much as microbial lipases but certainly they have some advantages over them so are very potential biocatalysts which can be used in the industry.

1.8.2 Microbial lipases/esterases

Lipases/esterases are widely found from microbial sources. Lipases/esterases from bacterial and fungal sources have been exploited in industries. While those from yeast species have not been used much despite of lipases from *Candida* species have wide applications.

1.8.2.1 Bacterial lipases/esterases

Bacteria are one of the easiest sources for obtaining lipases or esterases. They are easy to cultivate, can be grown easily on large fermenter scale and enzyme purification is comparatively easier and cheaper. These properties of bacterial lipases/esterases make them industrially valuable enzymes. Various bacterial lipases/esterases are known till date as mentioned in table 1.2 and they have been exploited in various industries for commercial uses. Lipases/esterases from *Bacillus* and *Pseudomonas* species either in recombinant form or in pure form have been used extensively for kinetic resolution of racemic mixtures in pharmaceutical/flavour industry, dairy industry and food processing industries.

Microorganisms	References
Gram positive bacteria	
Bacillus subtilis DR8806	65
Bacillus sp VITL8	66
Bacillus sp THL027	67
Bacillus altitudinis AP-MSU	68
Bacillus sp BP-7	40
Bacillus cereus strain AGP-03	69
Bacillus sphaericus	52
Bacillus licheniformis	70-72
Bacillus sp W130-35	73
Bacillus gelatini KACC 12197	74
Bacillus sp SCSIO	56
Bacillus amyloliquefaciens	75
Bacillus thuringiensis	76
Bacillus pumilus	77-79
Bacillus thermocatenulatus	80

Lactobacillus johnsonii	81
Lactobacillus casei	82-85
Lactobacillus plantarum	86-89
Lactobacillus fermentum	90
Mycobacterium tuberculosis	91-95
Staphylococcus haemolyticus	96
Staphylococcus epidermidis	97
Gram negative bacteria	
Acinetobacter species	98-104
Acinetobacter calcoaceticus	105
Acinetobacter lwoffii	106
Acinetobacter venetianus	107
Acinetobacter baumannii	108
Arthrobacter sp.	51
Pseudomonas fluorescens	109-116
Pseudomonas alcaligenes	117
Pseudomonas aeruginosa	118-120
Pseudomonas sp.	121-129
Pseudomonas putida	46,130-133
Pseudomonas mandelii	134,135
Pseudomonas pseudoalcaligenes	136,137
Pseudomonas stutzeri	138
Pseudomonas cepacia	139,140
Pseudomonas vesicularis	141
Pseudomonas marginata	142

Table 1.2 Lipase/esterase producing bacterial strains.

1.8.2.2 Fungal lipases/esterases

Fungal lipases have been in study and used from mid 19th century as there are a number of reviews present on fungal lipases/esterase in literature. Fungi are well known as best for lipases and are used preferably in food industry. Among the fungi, lipases/esterases from *Mucor hiemalis, Mucor miehei, Rhizopus oryzae, Rhizopus chinesis* and from *Aspergillus* species such as *Aspergillus niger, Aspergillus nidulans*, *Aspergillus terreus, Aspergillus oryzae, Aspergillus awamori, Aspergillus carneus*are well studied. They have molecular weight ranging from 30-50 kDa and have been used for the conversion of triacylglycerols to their mono-glycerides and for inter-esterification of fats and oils. Few lipases/esterases from fungal species are reported in table 1.3.

Microorganisms	References
Aspergillus niger	38,143-146
Aspergillus nidulans	147-150
Aspergillus terreus	151
Aspergillus oryzae	152-154
Aspergillus awamori	155
Aspergillus carneus	156
Fusarium oxysporum	47,157-159
Fusarium heterosporum	160
Mucor miehei	161,162
Mucor hiemalis	163,164
Penicillium griseoroseum	165
Penicillium wortmanii	166
Penicillium frequentans	167
Rhizomucor miehei	53,168
Rhizopus oryzae	169-173
Rhizopus chinensis	174

Table 1.3 Lipase/esterase producing fungal strains.

1.8.2.3 Yeast lipases/esterases

Yeast lipases have attracted vast attention in recent times due to their vast industrial applications. Lipases from genus *Candida* and *Yarrowia* have been used widely in industries. *Candida antarctica* lipase B and A have been used for chiral resolution of aliphatic, aromatic secondary alcohols and tertiary alcohols respectively. *Candida rugosa* lipase is used for chiral resolution of α/β-amyrins. *Yarrowia lipolytica* lipases are used for the resolution of (±)-lavandulyl acetate and in dairy, food industries, etc. Few of the yeast lipases are mentioned in table 1.4 below:

Microorganisms	References
Candida antarctica	26,33,35,175
Candida rugosa	176-179
Saccharomyces cerevisiae	180-182
Saccharomyces carlsbergensis	183
Trichosporon asteroides	184
Yarrowia lipolytica	185-190

Table 1.4 Lipase/esterase producing yeast strains.

1.8.3 Metagenomic origin lipases/esterases

Lipases/esterases are being isolated from naturally isolated microorganisms from many decades. But screening of novel lipases/esterases from isolated microorganismsusing conventional cultivation techniques has limitsin exploring the vast genetic diversity of microorganisms. Since more than 99 % of microorganisms present in various environments cannot be cultured, access to this vast genome resource of uncultured microorganisms (Metagenomics) is very important. Metagenomics approach is very important to screen novel biocatalysts (lipases/esterases) from a "metagenome library".[191,192] Metagenomic library is constructed by isolation of genomic DNA from a pool of microbes present in a particular ecological niche such as soil, pond water, etc. and cloning them into various bacterial/ fosmid vectors. So, metagenomics is a culture-independent approach that can be used to uncover novel biocatalysts for various

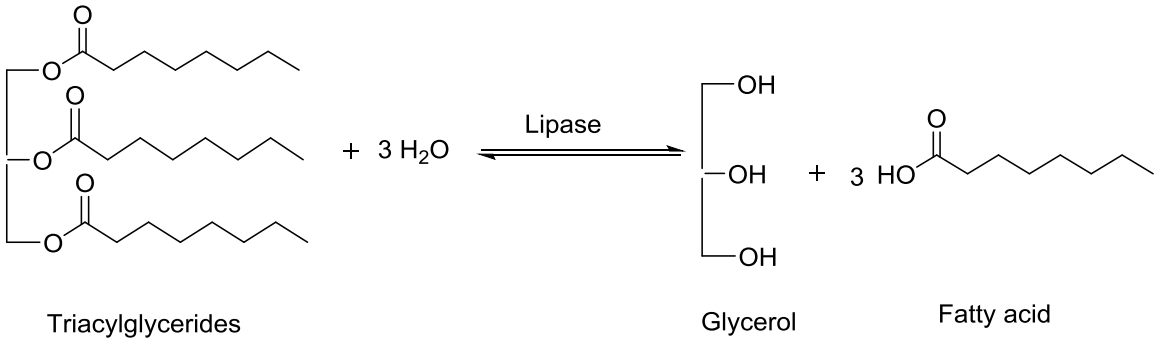
biotechnological and pharmaceutical applications. Table 1.5 shows few lipases/esterases isolated from various ecological niches.

Origin of the metagenomic library	References
Antarctic soil metagenomic library	193
Peat-Swamp Forest Soil metagenomic library	194
South China sea water metagenomic library	195
Arctic metagenomic library	196
Tibetan glacier metagenomic library	197
Marine metagenomic Library	41,192,195,198,199
Brazilian Atlantic Forest soil metagenomic library	200
Deep-sea metagenomic library	201
Marine mud metagenomic library	41
South China Sea marine sediment metagenomic library	202,203
Arctic Sediment metagenomic library	204
Deep-sea sediment metagenomic library	205-207
Tidal flat sediment metagenomic library	45,208
Colombian high Andean forest soil metagenomic library	209
Soil metagenomic library	210,211
Compost metagenomic library	212
Oil-Polluted Mud Flat Metagenomic library	213
Mountain soil metagenomic library	214
Rhizosphere soil metagenome	215
Permafrost metagenomic library	216
Arctic soil metagenome library	217

Table 1.5 Lipase/esterase isolated from various metagenomic sources.

1.9 Lipase/Esterase assay

Determination of lipase/esterase activity can be carried out using various physico-chemical methods where we monitor substrate depletion followed by gradual product formation in time course based study. Various methods are available for measuring the hydrolytic activity as well as the detection of lipase/esterase.[218-220] The numerous methods may be classified as: (1) Titrimetry (2) Spectroscopy (Photometry, Fluorimetry and Infrared) (3) Turbidimetry (4) Immunochemistry (5) Chromatography (6) Conductimetry (7) Radio activity, (8) Interfacial tensiometry and (9) microscopy. The general triacylglycerol hydrolysis reaction catalyzed by lipases is as follows:

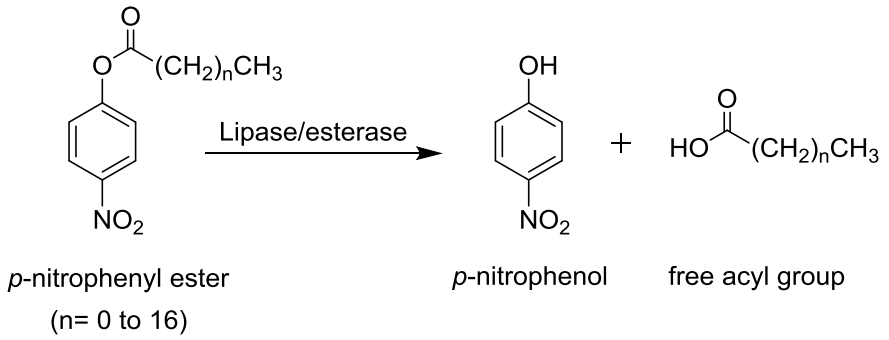


Scheme 1.1 General hydrolysis reaction of triacylglycerides catalysed by the lipases.

This reaction shows that the activity of lipases can be assayed by monitoring the release of either free fatty acids or glycerol from triacylglycerols (TAG) or fatty acid ester (Scheme 1.1). Presently, the most widely used lipase assay protocol is based on titrimetry assay using olive oil/ tributyrin as a substrate.[221, 222] Lipase activity is measured on a mechanically stirred emulsion of natural or synthetic TAGs in pH-stat meter by neutralising the free fatty acids (FFAs) released with time by adding titrated NaOH (0.1 M) in order to maintain thepH at a constant end point value. The pH-stat method is a quantitative method which is sensitive to within one μmole of released fatty acid per min at specified temperature and pH values.[223]

A very well known spectrophotometric assay is based on hydrolysis of esters of *p*-nitro phenol by pure lipases and esterase (Scheme 1.2.).[140,190] Alkyl chain of length ranging from 0 to 16 corresponds to different lengths of acyl group which are hydrolysed by lipases and esterase. *p*-nitro phenol released after hydrolysis gives yellow colour which is spectrophotometrically measured at 410 nm wavelength. One unit of enzyme

activity was expressed as the amount of enzyme that released 1µmoles of *p*-nitro phenol per min in the assay. Esterases prefer to hydrolyse low carbon chain length esters of *p*-nitro phenol while lipases prefer higher carbon length esters of *p*-nitro phenol.



Scheme 1.2 Hydrolysis of *p*-nitrophenol ester by lipases/esterase.

The chromatographic techniques, such as TLC, GC and HPLC are also used for determining fatty acids released by lipase hydrolysis of lipid substrates. Immunological methods include detection of lipases/esterases using ELISA (Enzyme Linked Immuno Sorbent Assay) techniques. Drawbacks with this technique is one has to purify the enzyme using routine techniques and raise the mono/polyclonal antibodies against the enzyme to be detected.

1.10 Production and purification of lipases/esterases

1.10.1 Production of lipases/esterase

Lipases and esterases are either secreted extracellular or intracellular in cell. Microorganisms secrete most of the lipases/esterases extracellularly. Extracellular lipases/esterases are easy to purify as they are secreted outside the cell and are produced in large quantity. These enzymes are produced in media containing proper carbon source, nitrogen source and other useful nutrients.[223] Their production is carried out in either fermenter or in shake flasks. These enzymes are produced at optimum pH and temperature on which microorganisms grow perfectly. Lipases/esterases are inducible enzymes so they are produced from well grown microbial culture at particular growth time using inducers such as olive oil, Tween 80, Tributyrin etc.[223] Inducing source can be any lipid oil, triacylglycerol, fatty acids etc. Non lipid sources such as higher chain alkanes are also used sometimes for lipase production. In recombinant lipase/esterase, IPTG is generally used for induction of these enzymes.

1.10.1.1 Carbon source

In lipid sources, edible oils such as cotton seed, corn, peanut, olive, sunflower, and sesame were used in the medium for production of high levels of lipases.[224] Vegetable oils such as corn oil, rapeseed oil are used in production of lipases from *Rhizopus oryzae*.[171] Babusa oil, mustard oil, palm oil is also used for production of lipases. Ground nut oil, sugar cane bagasse [225] was also used for production of lipases. Starch is also used as carbon source in media to produce lipases.[170]

Stearic acid, arachidonic acid have used as effective inducers of lipases. Oleic acid esters are used as effective carbon source for production of lipases from *Candida rugosa*. Various detergents and surfactants such as Tween, Triton, SDS, PEG and Gum Arabic have been used for induction of lipases. Triton X-100 is used for production of lipases from *Pseudomonas pseudoalkaligenes* F-111.[137]Tween 80 is used for production of a 400 kDa novel cold active lipase from *Yarrowia lipolytica* NCIM 3639.[190]

1.10.1.2 Nitrogen source

Along with carbon source, nitrogen source is important for the production of lipases in the media. Nitrogen sources can be of organic or non organic in origin. Tryptone, peptone and yeast extract can be organic source of nitrogen. NH_4Cl, NH_4NO_3, $(NH4)_2SO_4$, $(NH_4)_2HPO_4$salts can be a non organic sources of nitrogen which can be used in media for production of lipases/esterase.[223]

Production of lipase by *Yarrowia lipolytica* 681 was enhanced significantly when urea was used as the nitrogen source.[186] Solid-state production of esterase carried out using peptone as nitrogen source from *Bacillus altitudinis* AP-MSU.[68] Lipolytic enzymes such as esterases and lipases were produced on large scale from fungus *Ophiostoma piliferum* (Cartapip (TM)) when yeast extract was used as nitrogen source.[226] Extracellular lipase production was optimized from *Aspergillus terreus var. africanus* (CBS 130.55) when $COO(NH_4)(2)H_2O$ was used as nitrogen source.[151]N-Z-amine have been used as nitrogen source for production of esterases and lipases.[227]Urea, soy-peptone, yeast extract, a mixture of soy-peptone and yeast extract, cheese whey, and wheat mill bran were tested for the production of esterases from *Candida rugosa*.[179]

1.10.2 Purification of lipases/esterases

Protein/enzyme purification is one if the tedious process in biochemistry. Enzymes are purified upto homogeneity using various chromatographic techniques. There are mainly two ways to purify an enzyme from its crude mixture 1) Conventional method: used in purifying a non recombinant /wild type protein. This involves various steps such ultra filtration, affinity chromatography, gel filtration/permeation chromatography etc. 2) Affinity chromatography/recombinant protein purification: this technique is used to purify recombinant protein expressed in heterologous expression systems such as *E. coli*, *Saccharomyces cereviseae* and *Pichia pastoris*. These proteins are used by affinity chromatography technique which involves recombinant protein tagged with His-tag, GST-tag, GFP tag, thioredoxin-tag, Myc-tag etc.

Lipases/esterases are purified up to homogeneity using both such techniques. Few examples of such purification are given below in table:

Organism name	**Purification scheme**	**Protein mass**	**Reference**
Acinetobacter nov sp strain KM109	Sepharose CL-6B gel filtration, gel filtration and ion exchange chromatography	62 kDa	99
Aspergillus awamori	Ion-exchange, gel-filtration and hydrophobic-interaction chromatography	31 kDa	155
Aspergillus oryzae	Ammonium sulphate fractionation, acetone precipitation, anion-exchange chromatography, and gel filtration	29 kDa	154
Bacillus sp THL027	Ultra filtration, gel filtration chromatography	69 kDa	67
Bacillus licheniformis S-86	Ammonium sulphate precipitation, Ultra filtration, thermal treatment, HIC chromatography, ion exchange chromatography	38.4 kDa	71

Bacillus cereus strain AGP-03	Ultra filtration, gel filtration chromatography	41 kDa	69
Fusarium oxysporum	Affinity chromatography, HIC chromatography, gel filtration	27 kDa	159
Mucor hiemalis f. corticola	Ammonium sulphate precipitation, dialysis, gel filtration column chromatography and ion exchange chromatography	46 kDa	164
Pseudomonas sp TB11	Ammonium sulphate precipitation, ion exchange chromatography, gel filtration	65 kDa	121
Pseudomonas vesicularis PD	Ammonium sulphate fractionation, ion-exchange chromatography, gel filtration and hydroxyapetite column chromatography	80 kDa	141
Pseudomonas fluorescens	QAE-Sephadex A-50 and DEAE-Sepharose chromatography	26 kDa	114
Trichosporon asteroides	Ammonium sulphate precipitation, gel filtration (sepahdex G-200)	37 kDa	184
Yarrowia lipolytica NCIM 3639	Ultra filtration, ion exchange chromatography, gel filtration	400 kDa	190
Acinetobacter lwoffii 16C-1	Affinity chromatography (His-tag)	37.5 kDa	106
Bacillus pumilus	Affinity chromatography (His-tag)	28.4 kDa	79
Lactobacillus plantarum WCFS1	Ammonium sulphate and His tag affinity chromatography	28.7 kDa	89
Lactobacillus casei LILA	Affinity chromatography (His-tag)	36.7 kDa	85
Methylococcus capsulatus	Affinity chromatography (His-tag)	23-26	228

(bath)		kDa	
Pelagibacterium halotolerans B2(T)	Affinity chromatography (His-tag)	23.2 kDa	229
Photobacterium sp strain J15	Affinity chromatography (His-tag)	36 kDa	230
Saccharomyces cerevisiae	Affinity chromatography (His-tag), gel filtration chromatography	28 kDa	181
Staphylococcus epidermidis	Affinity chromatography (His-tag)	77 kDa	97

Table 1.6 Protein purification strategies employed for some microbial lipases/esterases.

1.11 Application of lipases/esterases

Lipases/esterases together constitute one third of the overall enzymes used industrially. Lipases/esterases have been exploited for a wide variety of applications which make them commercially highly important enzymes in the industry (Fig 1.5). Majority of the enzymes used in industry are of microbial origin. In addition to hydrolysis of ester bonds, lipases/esterases can also catalyze transesterification, esterification reactions in organic solvent/ non-aqueous media.

They can carry out enantioselective and regio-selective hydrolysis or esterification reactions of organic compounds. This versatility and applicability of lipases/esterase makes them potential biocatalysts for use in applications such as in food, flavour and fragrance industry, leather, detergent, pharmaceutical, textile, and paper industries [231,232] as mentioned below.

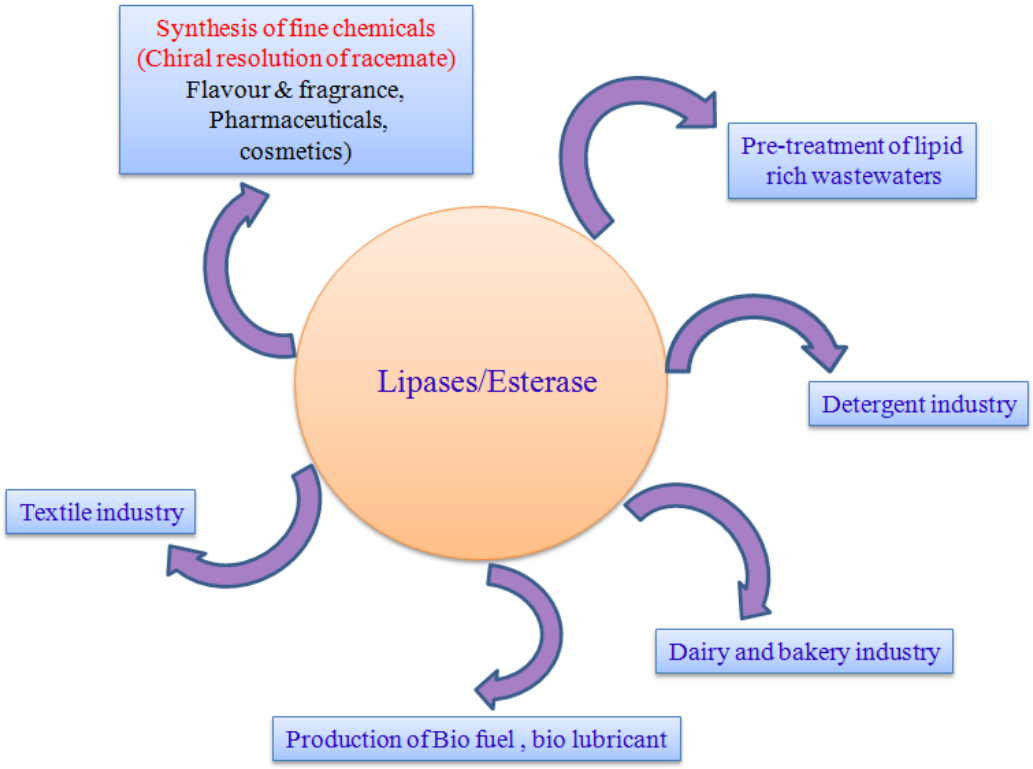


Fig. 1.5 Application of lipases/esterases.

1.11.1 Pre-treatment of lipid rich waste water

Lipid rich waste water is one the reason for contaminating the flowing water causing water pollution. Dairies and slaughterhouses are rich sources producing biodegradable lipid rich waste molecules especially fats and proteins.[233-235] These lipid rich waste causes land and water pollution increasing the BOD and COD levels of water. Hence treatment of effluent water coming out of these industries is very much necessary which includes leather and dairy industry. Very large pre-treatment systems are employed to remove these fatty substances prior to main biological treatment. However, these pre-treatments are costlier due to high cost of investment and reagents used. In addition, they are operating with low removal efficiency of dissolved or emulsified fats leading to accumulation of problematic sludge.

Pre-treatment involving dissolution/ hydrolysis of fats may significantly improve the degradation of waste water/effluents rich in high fats. Pre-treatment via hydrolysis have been mostly used on waste activated sludge and municipal waste which contain higher amounts of solids than waste water from dairy industry or slaughter house.[236]

Bacterial, plant and animal origin lipases have been used in the pre-treatment of slaughter house effluents.[234] Whole cells / lipases from *Pseudomonas aeruginosa* were used for the treatment of lipid rich waste waters.[118] *Candida rugosa* lipases is utilised inthe treatment of domestic waste water and sewage water.[232] Immobilized lipases have also been used to hydrolyze triglycerides in waste water treatment plants.[237,238]

1.11.2 Uses in detergent industry

Proteases enzymes have been used with a great commercial success in detergent industry. After the proteases, lipases are used as second enzyme group to be the biggest market for use in detergent industry.[232] Industrially used wash liquids contain non-ionic and ionic surfactants, oxidants having alkaline pH and works at high temperatures of about 50 ºC. These conditions are very harsh for any enzymes to work optimally. So development of such a highly stable lipase at alkaline pH and higher temperature is being carried out in industries. Several industries have produced such enzyme formulations with commercial name: (1) Lipolase (*Novo-Nordisk*, recombinant fungal lipase from *Humicola lanuginosa* expressed in host *Aspergillus oryzae, a*ctive at pH 10.0 and temperature of 40 ºC) [239] (2) Lumafast (*Genencore*, recombinant lipase from *Pseudomonas mendocina* cloned and expressed in *Bacillus* species, active at pH 10.5 and temperature 40 ºC) [239] (3) Lipomax (*Gist-Brocades*, recombinant *Pseudomonas pseudoalkaligenes*, cloned and expressed in the same organism, active at pH 11.0 and temperature 45 ºC).[239]

Importance of lipases in detergent industry is related to the removal of fattyresidues in laundry, dishwashers etc. These processes are performed under alkaline conditions and hence lipases active under such conditions are highly preferred. Thousands of tons of lipases are sold every year for use in detergent industry.[232] However due to limitations in having desired property of lipases they are not used as much as other enzymes used in industries. Protein engineering can be a solution to the problems faced in the use of lipases in detergents. Such lipases can be commercially very useful and thus can be used in detergents formulations in the industries.

1.11.3 Uses in food processing (Bakery and dairy) industry

Lipases have been extensively used in dairy industry for the hydrolysis of milk fat, flavour enhancement of cheeses, acceleration of cheese ripening, manufacturing of cheese like products, lipolysis of butterfat and cream. The hydrolysis of milk fat gives

specific flavour characteristics to many dairy products. The cheese preparation is done using rennet paste (containing chymosin) and also contains lipases and esterases which contribute to ripening of cheese.

Lipases/esterases play an important crucial role in having cheese with characteristics cheese flavour.[87,240] Cheese flavour is mainly developed by release of volatile free fatty acids (VFFA) and free fatty acids (FFA) by lipases/esterases of lactic acid bacteria. Release of short chain fatty acids (C4 – C6) develops a sharp tangy flavour while the release of medium chain length fatty acids (C12 – C14) gives a soapy taste to the cheese. Released free fatty acids (FFA) are further metabolized by the microorganisms found in cheese to produce flavour ingredients such as flavour esters, acetoacetate, β-keto acids, methyl ketones, and lactones.

Lipases also have been used to extend the shelf life of breads and improve the quality of breads. They also enhance and control the non enzymatic browning of bakery products.

1.11.4 Production of bio fuel/bio diesel

Depleting fossil fuel reserves and an increase in awareness of the impact of energy production on society and the environment has promoted us for the search of cleaner energy resources. Biodiesel and bio ethanol remains the primary replacement fuels for fossil-based diesel and petroleum respectively. Biodiesel is defined as a mixture of fatty acid methyl esters derived from edible and non edible oil such as vegetable oils (sunflower, soybean and rapeseed oil), animal fats and algae.[241] In India, jatropha and pongamia oil (non edible oil) are the sources for the production of biodiesel. Biodiesel can be a substitute for conventional diesel fuel as it produces slow sulphur dioxide and soot particulate which are the main causes of air pollution (reference).Biodiesel is meant to be used in standard diesel engines and is thus distinct from the vegetable and waste oils used to fuel converted diesel engines. Biodiesel can be used alone, or can be blended with diesel in distinct proportions.

Chemically catalysed reactions causes generation of toxic waste products and hence enzymatic methods have recently captured the attention of scientists. Various lipases catalysed reactions are reported in literature.[242] *Porcine* pancreatic lipase catalysed production of biodiesel using rapeseed oil is reported. While *Candida rugosa* mediated biodiesel production using woody oil, biodiesel production using micro-algal

lipids via lipase mediated transesterification is known to mankind.[243] Immobilised *Pseudomonas* lipase is used for transesterification of soya bean oil to produce biodiesel.[139] Fatty acid ethyl esters were prepared from two commercial lipases Novozym 435 and Lipozyme IM from castor oil using n-hexane as solvent.[244]The enzymatic production of biodiesel by methanolysis of cottonseed oil was carried out using immobilized *Candida antarctica* lipase as catalyst in *t*-butanol solvent.[245]Fatty acids alkyl esters were produced using palm kernel oil and coconut oil, by transesterification with methanol using PS30 lipase as a bio catalyst.[246]

1.11.5 Uses in textile industry

From last twenty years, the textile industry has become one of the main areas for industrial application of enzymes after detergent industry. Lipases are highly used in textile industry for degreasing and as a desizing auxiliary in the presence of natural fats or residual oils from the spinning process.[247] Combination of cellulases and lipases are used in industry for such applications. Various commercial companies provide lipases formulations which are used for desizing, stone washing of denim and jeans, enzymatic wash and bio-polishing. Esterases have been used for the treatment of polyester fabric which improves its ability to uptake dyes, antimicrobial compounds and anti-staining compounds.

1.11.6 Synthesis of fine chemicals (via kinetic resolution)

Kinetic resolution is meant as the separation of each enantiomer from its racemic mixtures by means of using either chemical catalysts or bio catalysts. Kinetic resolution of enantiomers gives out a specific enantiomer [(*R*) or (*S*)] in pure form which is of great commercial importance. Key intermediates used in fragrance/ flavour, pharmaceutical and agrochemical industry are complex or chiral compounds. One of the two drug enantiomers in its racemic mixture is biologically active while other is not useful in pharmaceutical preparations. Hence the synthesis of enantiomerically pure building blocks/chiral intermediate has become a crucial step in pharma industry.[248-252]

Lipases/esterases are known to be highly stereo and regio-selective enzymes which either works in aqueous and non aqueous solutions. They are known to be active in biphasic medium and in ionic liquids. *Candida antarctica* lipase B (CAL-B, Novozym 435) is a well known versatile lipase having wider substrate specificity and (*R*)-enantiomer selectivity. CAL-B is used for kinetic resolution of secondary alcohols such

as (±)-2-pentanol, (±)-2-hexanol, (±)-2-heptanol and (±)-2-octanol.[35] CAL-B is also used for the kinetic resolution of (±)-1-phenyl ethanol, (±)-1-phenyl propanol and their derivatives using vinyl acetate as acyl donor. [26]*Candida Antarctica* lipase A (CAL-A) is used for the resolution of important tertiary alcohols.[33] Various commercial lipases such as Amano lipases (A, AK, PS and M) have been used for the resolution of pharmaceutically important intermediates. *Porcine pancreatic* lipase [49,50]and *Yarrowia lipolytica*[190]lipases have been used for the resolution of enantiomers of (±)-lavandulol, a major ingredient present in lavender oil.

Esterases from *Pseudomonas* species are known for kinetic resolution of alpha-hydroxy acids, [132] 2-acetoxyphenylacetic acids,[55] racemic ketoprofen ethyl ester, [122,123,253] chiral intermediate of pregabalin [254] and tertiary alcohols [46]. Esterases from *Bacillus* species are known for kinetic resolution of esters from secondary and tertiary benzylic propargylic alcohols, [40] dl-menthyl acetate, [255] tertiary alcohols [40,256] and (±)-1-phenyl ethanol [56]. Esterases from *Aspergillus niger* carries out regioselective deacetylation of disaccharides.[38] Metagenomically isolated novel esterases are known for kinetic resolution of phenylalkyl carboxylic acid [39] and tertiary alcohols.[45]

1.12 Scope and significance of the present work

Lipases/esterases are among the widely used industrial enzymes as they possess various commercial applications. They are used in food and processing industry, dairy industry to breakdown the lipids present in oils to liberate free fatty acids and glycerol. They are used as catalysts for production of bio surfactant and biodiesel (bio fuel) required for the fuel industry. They are used in textile industry for removing size lubricants which provides a fabric with greater absorbency needed for improved levelness in dying. In detergent industry, alkaline stable lipases are used in formulation of anionic and cationic detergents. In bakery and dairy industry, lipase are used for flavour enhancement of cheese, the acceleration of cheese ripening, manufacturing of cheese like products and the lipolysis of butterfat and cream.

Along with all these applications, lipases/esterases are used in the synthesis of fine chemicals to synthesize chiral molecules which act as building blocks for synthesis of intermediates used in pharmaceutical, flavour-fragrance industries and agrochemical industries. Lipases/esterase are known to resolve mixture of enantiomers (racemic mixture) into individual (*R*) or (*S*) enantiomer via kinetic resolution. Since these intermediates are always required in large-scale at the industrial level, we are always in need of more efficient, faster, pH, temperature and organic solvent stable lipase or esterase.

In this thesis, we have shown cloning, characterisation and utilization of novel lipase/esterase enzymes from culturable and non- culturable microorganisms. We have isolated two novel esterases SLC-6 and MLC-3 from metagenomic clones which were shown to be (*R*) and (*S*) enantiomer selective towards various commercially important acyclic and aromatic racemic acetates such as (±)-2-pentyl acetate, (±)-2-hexyl acetate, (±)-3-hexyl acetate, (±)-1-octen-3-yl acetate, (±)-1-phenyl ethyl acetate,(±)-2-methyl-1-phenyl ethyl acetate and (±)-1-phenylpropyl acetate. SLC-6 and MLC-3 esterases hydrolysed the racemic acetates enantioselectively to their corresponding (*R*) or (*S*) alcohol with more than 90 % e.e value. An alkaline stable pEST-1 esterase was isolated from *Pseudomonas pseudoalcaligenes* NCIM 2864and was found to be (*R)*-selective towards racemic acetates such as (±)-2-pentyl acetate, (±)-2-hexyl acetate, (±)-1-phenyl ethyl acetate and (±)-1-phenylpropyl acetate giving e.e value of more than 99 %. Total of nine new and uncharacterized lipases were found out from the psychrophillic

Yarrowia lipolytica NCIM 3639 yeast through transcriptome mining. A commercial lipase, *Candida rugosa* lipase was shown for first time to be resolving the structural isomers of α/β-amyrins via selective acetylation using vinyl acetate as acyl donor. Whole cells of *Fusarium proliferatum* NCIM 1105 showed one pot two step de-esterification followed by selective (*S*) oxidation of numerous acyclic and aromatic acetates [(±)-2-hexyl acetate, (±)-2-heptyl acetate, (±)-1-octen-3-yl acetate, (±)-1-phenyl ethyl acetate,(±)-3-methyl-1-phenyl ethyl acetate and (±)-1-phenylpropyl acetate] to form corresponding (*R*) alcohols with > 95 % e.e.

Thesis is divided into four chapters as follows:

Chapter 1: Introduction

Chapter 2: Cloning, characterization & utilization of esterases/lipases isolated from non-culturable microorganisms: a Metagenomics approach.

Chapter 3: Cloning, characterization & utilization of esterases/ lipases isolated from culturable microorganisms.

Chapter 4: Biocatalysis: Enzyme and whole cell mediated kinetic resolution of α/β – amyrin and commercially important racemic esters.

1.13 References

(1) Oliver, S. G. In *Saccharomyces*; Tuite, M. F., Oliver, S. G., Eds.; Springer US: Boston, MA, 1991, p 213-248.

(2) Tipton, K.; Boyce, S. *Bioinformatics* **2000**, *16*, 34-40.

(3) Wagner, R. *O. Wiegand, Leipzig* **1857**.

(4) Li, Y.; Cirino, P. C. *Biotechnol. Bioeng.* **2014**, *111*, 1273-1287.

(5) Porter, J. L.; Rusli, R. A.; Ollis, D. L. *Chembiochem* **2016**, *17*, 197-203.

(6) Torrelo, G.; Hanefeld, U.; Hollmann, F. *Catal. Lett.* **2015**, *145*, 309-345.

(7) Turner, N. J. *Catal. Sci. Tech.* **2012**, *2*, 1523-1523.

(8) Wandrey, C.; Liese, A.; Kihumbu, D. *Org. Process Res. Dev.* **2000**, *4*, 286-290.

(9) Asano, Y.; Hollmann, F.; Stewart, J. D. *J. Mol. Catal. B-Enzym.* **2014**, *105*, 132-134.

(10) Bornscheuer, U. T. *Angew. Chem. Int. Ed.* **2016**, *55*, 4372-4373.

(11) Reetz, M. T. *J. Am. Chem. Soc.* **2013**, *135*, 12480-12496.

(12) Schmid, A.; Dordick, J. S.; Hauer, B.; Kiener, A.; Wubbolts, M.; Witholt, B. *Nature* **2001**, *409*, 258-268.

(13) Tawfik, D. S.; van der Donk, W. A. *Curr. Opin. Chem. Biol.* **2016**, *31*, V-VII.

(14) Wells, A.; Meyer, H.-P. *Chemcatchem* **2014**, *6*, 918-920.

(15) Faber, K. *Springer-Verlag:Berlin, Germany* **1997**.

(16) Mendez-Sanchez, D.; Lopez-Iglesias, M.; Gotor-Fernandez, V. *Curr. Org. Chem.* **2016**, *20*, 1186-1203.

(17) Siirola, E.; Frank, A.; Grogan, G.; Kroutil, W. *Adv. Synth. Catal.* **2013**, *355*, 1677-1691.

(18) Jochens, H.; Hesseler, M.; Stiba, K.; Padhi, S. K.; Kazlauskas, R. J.; Bornscheuer, U. T. *Chembiochem* **2011**, *12*, 1508-1517.

(19) Qian, Z.; Fields, C. J.; Yu, Y.; Lutz, S. *Biotechnol. J.* **2007**, *2*, 192-200.

(20) Adlercreutz, P. *Chem. Soc. Rev.* **2013**, *42*, 6406-6436.

(21) Ansorge-Schumacher, M. B.; Thum, O. *Chem. Soc. Rev.* **2013**, *42*, 6475-6490.

(22) Berglund, P. *Biomol. Eng.* **2001**, *18*, 13-22.

(23) Bornscheuer, U. T.; Bessler, C.; Srinivas, R.; Krishna, S. H. *Trends*

Biotechnol. **2002**, *20*, 433-437.

(24) Borrelli, G. M.; Trono, D. *Int. J. Mol. Sci.* **2015**, *16*, 20774-20840.

(25) Casas-Godoy, L.; Duquesne, S.; Bordes, F.; Sandoval, G.; Marty, A. *Methods mol. biol.* **2012**, *861*, 3-30.

(26) de los Rios, A. P.; van Rantwijk, F.; Sheldon, R. A. *Green Chem.* **2012**, *14*, 1584-1588.

(27) Dlugy, C.; Wolfson, A. *Bioproc. Biosyst. Eng.* **2007**, *30*, 327-330.

(28) Ferreira, H. V.; Rocha, L. C.; Severino, R. P.; Porto, A. L. M. *Molecules* **2012**, *17*, 8955-8967.

(29) Frings, K.; Koch, M.; Hartmeier, W. *Enzyme Microb. Tech.* **1999**, *25*, 303-309.

(30) Gotor-Fernandez, V.; Brieva, R.; Gotor, V. *J. Mol. Catal. B-Enzym.* **2006**, *40*, 111-120.

(31) Haldar, S.; Kale, B. S.; Jadhav, D. D.; Thulasiram, H. V. *Tetrahedron Lett.* **2014**, *55*, 3122-3125.

(32) Karadeniz, F.; Bayraktar, E.; Mehmetoglu, U. *Artif. Cell. Blood Sub.* **2010**, *38*, 288-293.

(33) Krishna, S. H.; Persson, M.; Bornscheuer, U. T. *Tetrahedron-Asymmetry* **2002**, *13*, 2693-2696.

(34) Kumar, A.; Dhar, K.; Kanwar, S. S.; Arora, P. K. *Biol. proced. online* **2016**, *18*, 2-2.

(35) Patel, R. N.; Banerjee, A.; Nanduri, V.; Goswami, A.; Comezoglu, F. T. *J. Am. Oil Chem. Soc.* **2000**, *77*, 1015-1019.

(36) Salameh, M. D.; Wiegel, J. In *Adv. Appl. Microbiol.* 2007; Vol. 61, p 253-283.

(37) Yang, Y.; Yu, Y.; Zhang, Y.; Liu, C.; Shi, W.; Li, Q. *Process Biochem.* **2011**, *46*, 1900-1908.

(38) Bavaro, T.; Torres-Salas, P.; Antonioli, N.; Morelli, C. F.; Speranza, G.; Terreni, M. *Chemcatchem* **2013**, *5*, 2925-2931.

(39) Fernandez-Alvaro, E.; Kourist, R.; Winter, J.; Boettcher, D.; Liebeton, K.; Naumer, C.; Eck, J.; Leggewie, C.; Jaeger, K.-E.; Streit, W.; Bornscheuer, U. T. *Microb. Biotechol.* **2009**, *3*, 59-64.

(40) Fillat, A.; Romea, P.; Pastor, F. I. J.; Urpi, F.; Diaz, P. *Catal. Today* **2015**,

255, 16-20.

(41) Gao, W.; Wu, K.; Chen, L.; Fan, H.; Zhao, Z.; Gao, B.; Wang, H.; Wei, D. *Microb. Cell Fact.* **2016**, *15*.

(42) Romano, D.; Bonomi, F.; de Mattos, M. C.; Fonseca, T. d. S.; Ferreira de Oliveira, M. d. C.; Molinari, F. *Biotechnol. Adv.* **2015**, *33*, 547-565.

(43) Rozeboom, H. J.; Godinho, L. F.; Nardini, M.; Quax, W. J.; Dijkstra, B. W. *Biochim. Biophys. Acta, Proteins Proteomics* **2014**, *1844*, 567-575.

(44) Bornscheuer, U. T. *FEMS Microbiol. Rev.* **2002**, *26*, 73-81.

(45) Oh, K. H.; Nguyen, G. S.; Kim, E. Y.; Kourist, R.; Bornscheuer, U.; Oh, T. K.; Yoon, J. H. *J. Mol. Catal. B-Enzym.* **2012**, *80*, 67-73.

(46) Rehdorf, J.; Behrens, G. A.; Giang-Son, N.; Kourist, R.; Bornscheuer, U. T. *Appl. Microbiol. Biot.* **2012**, *93*, 1119-1126.

(47) Topakas, E.; Stamatis, H.; Biely, P.; Kekos, D.; Macris, B. J.; Christakopoulos, P. *J. Biotechnol.* **2003**, *102*, 33-44.

(48) Cross, H.; Marriott, R.; Grogan, G. *Biotechnol. Lett.* **2004**, *26*, 457-460.

(49) Zada, A.; Dunkelblum, E. *Tetrahedron-Asymmetr.* **2006**, *17*, 230-233.

(50) Zada, A.; Harel, M. *Tetrahedron-Asymmetr.* **2004**, *15*, 2339-2343.

(51) Koul, S.; Taneja, S. C.; Parshad, R.; Qazi, G. N. *Tetrahedron-Asymmetr.* **1998**, *9*, 3395-3399.

(52) Jackson, M. A.; Labeda, D. P.; Becker, L. A. *Enzyme Microb. Tech.* **1995**, *17*, 175-179.

(53) Yan, Q.-j.; Yang, S.-q.; Duan, X.-j.; Xu, H.-b.; Liu, Y.; Jiang, Z.-q. *J. Mol. Catal. B-Enzym.* **2014**, *109*, 76-84.

(54) Palomo, J. M.; Fernandez-Lorente, G.; Mateo, C.; Fuentes, M.; Guisan, J. M.; Fernandez-Lafuente, R. *Tetrahedron-Asymmetr.* **2002**, *13*, 2653-2659.

(55) Ju, X.; Pan, J.; Yu, H.-L.; Li, C.-X.; Xu, J.-H. *Biochem. Eng. J.* **2011**, *57*, 63-68.

(56) Liang, J. Y.; Zhang, Y.; Sun, A. J.; Deng, D.; Hu, Y. F. *Appl. Microbiol. Biotechnol.* **2016**, *178*, 558-575.

(57) Brady, L.; Brzozowski, A. M.; Derewenda, Z. S.; Dodson, E.; Dodson, G.; Tolley, S.; Turkenburg, J. P.; Christiansen, L.; Hugejensen, B.; Norskov, L.; Thim, L.; Menge, U. *Nature* **1990**, *343*, 767-770.

(58) Villeneuve, P. *Eur. J. Lipid Sci. Technol.* **2003**, *105*, 308-317.

(59) Maeshima, M.; Beevers, H. *Plant Physiol.* **1985**, *79*, 489-493.

(60) de Barros, M.; Macedo, G. A. *J. Am. Oil Chem. Soc.* **2015**, *92*, 37-45.

(61) Jung, H.; Moon, S. *J. Korean Soc. Appl. Bi.* **2013**, *56*, 639-645.

(62) Bhardwaj, K.; Raju, A.; Rajasekharan, R. *Plant Physiol.* **2001**, *127*, 1728-1738.

(63) Rajeshwara, A. N.; Prakash, V. *Nahrung* **1995**, *39*, 406-418.

(64) Lomolino, G.; Zannoni, S.; Di Pierro, G. *Food Biotechnol.* **2015**, *29*, 297-316.

(65) Asoodeh, A.; Ghanbari, T. *J. Mol. Catal. B-Enzym.* **2013**, *85-86*, 49-55.

(66) Balaji, L.; Jayaraman, G. *Int. J. Biol. Macromol.* **2014**, *67*, 380-386.

(67) Dharmsthiti, S.; Luchai, S. *FEMS Microbiol. Lett.* **1999**, *179*, 241-246.

(68) Esakkiraj, P.; Usha, R.; Palavesam, A.; Immanuel, G. *Food Bioprod. Process.* **2012**, *90*, 370-376.

(69) Ghati, A.; Paul, G. *Process Biochem.* **2015**, *50*, 771-781.

(70) Ju, H.; Jang, E.; Ryu, B. H.; Kim, T. D. *Bioresource Technol.* **2013**, *128*, 81-86.

(71) Torres, S.; Baigori, M. D.; Pandey, A.; Castro, G. R. *Appl. Biochem. Biotechnol.* **2008**, *151*, 221-232.

(72) Torres, S.; Baigori, M. D.; Swathy, S. L.; Pandey, A.; Castro, G. R. *Food Res. Int.* **2009**, *42*, 454-460.

(73) Kim, H. J.; Jung, W. K.; Lee, H. W.; Yoo, W.; Kim, T. D.; Kim, H. *J. Microbiol. Biotechnol.* **2015**, *25*, 2024-2033.

(74) Kim, J.; Deng, L.; Hong, E.; Ryu, Y. *Protein Expres. Purif.* **2015**, *116*, 90-97.

(75) Liu, J.-Y.; Bian, H.-P.; Tang, Y.; Bai, Y.-P.; Xu, J.-H. *Appl. Microbiol. Biot.* **2015**, *99*, 1701-1708.

(76) Mazzucotelli, C. A.; Moreira, M. D.; Ansorena, M. R. *Can. J. Microbiol.* **2016**, *62*, 24-34.

(77) Moen, A. R.; Anthonsen, T. *Biocatal. Biotransfor.* **2009**, *27*, 226-236.

(78) Nair, S.; Kumar, P. *World J. Microbiol. Biotechnol.* **2007**, *23*, 1441-1449.

(79) Xue, Y. M.; Zhang, J. J.; Wu, X. X.; Sun, H. H.; Xu, C. Y. *Ann. Microbiol.* **2013**, *63*, 879-885.

(80) Schmidtdannert, C.; Sztajer, H.; Stocklein, W.; Menge, U.; Schmid, R. D.

BBA-Lipids.Lipid. Met. **1994**, *1214*, 43-53.

(81) Bel-Rhlid, R.; Crespy, V.; Page-Zoerkler, N.; Nagy, K.; Raab, T.; Hansen, C. E. *J. Agr. Food Chem.* **2009**, *57*, 7700-7705.

(82) Choi, Y. J.; Lee, B. H. *Bioprocess Biosyst. Eng*. **2001**, *24*, 59-63.

(83) Choi, Y. J.; Miguez, C. B.; Lee, B. H. *Appl. Environ. Microb.* **2004**, *70*, 3213-3221.

(84) Fenster, K. M.; Parkin, K. L.; Steele, J. L. *J. Dairy Sci.* **2003**, *86*, 2547-2557.

(85) Fenster, K. M.; Parkin, K. L.; Steele, J. L. *J. Dairy Sci.* **2003**, *86*, 1118-1129.

(86) Esteban-Torres, M.; Mancheno, J. M.; de las Rivas, B.; Munoz, R. *Lwt-Food Sci. Technol.* **2015**, *60*, 246-252.

(87) Esteban-Torres, M.; Mancheno, J. M.; Rivas, B. D.; Munoz, R. *J. Dairy Sci.* **2014**, *97*, 6737-6744.

(88) Esteban-Torres, M.; Reveron, I.; Santamaria, L.; Mancheno, J. M.; de las Rivas, B.; Munoz, R. *Front. Microbiol.* **2016**, *7*.

(89) Inmaculada, N. G.; Alvaro, S. F.; Francisco, G. C. *Mol. Biotechnol.* **2013**, *54*, 651-660.

(90) Gobbetti, M.; Smacchi, E.; Corsetti, A. *Int. Dairy J.* **1997**, *7*, 13-21.

(91) Cao, J.; Dang, G. H.; Li, H. F.; Li, T. T.; Yue, Z. G.; Li, N.; Liu, Y. J.; Liu, S. G.; Chen, L. P. *PLOS ONE* **2015**, *10*.

(92) Chen, L. P.; Dang, G. H.; Deng, X. X.; Cao, J.; Yu, S. Y.; Wu, D. F.; Pang, H.; Liu, S. G. *Protein Expres. Purif.* **2014**, *104*, 50-56.

(93) Dang, G. H.; Chen, L. P.; Li, Z. L.; Deng, X. X.; Cui, Y. Y.; Cao, J.; Yu, S. Y.; Pang, H.; Liu, S. G. *Appl. Biochem. Biotechnol.* **2015**, *176*, 1-12.

(94) Singh, G.; Kumar, A.; Arya, S.; Gupta, U. D.; Singh, K.; Kaur, J. *Enzyme Microb. Tech.* **2014**, *82*, 180-190.

(95) Zheng, X. D.; Guo, J. B.; Xu, L. P.; Li, H. L.; Zhang, D. W.; Zhang, K.; Sun, F.; Wen, T. Y.; Liu, S. G.; Pang, H. *PLOS ONE* **2011**, *6*.

(96) Oh, B. C.; Kim, H. K.; Kim, M. H.; Lee, J. K.; Oh, T. K. *J. Microbiol. Biotechnol.* **2000**, *10*, 656-662.

(97) Simons, J.; Van Kampen, M. D.; Riel, S.; Gotz, F.; Egmond, M. R.; Verheij, H. M. *Eur. J. Biochem.* **1998**, *253*, 675-683.

(98) Breuil, C.; Kushner, D. J. *Can. J. Microbiol.* **1975**, *21*, 423-433.

(99) Mitsuhashi, K.; Yamashita, M.; Hwan, Y. S.; Ihara, F.; Nihira, T.; Yamada, Y. *Biosci. Biotechnol. Biochem.* **1999**, *63*, 1959-1964.

(100) Qian, J. H.; Xu, J. H. *J. Mol. Catal. B-Enzym.* **2004**, *27*, 227-232.

(101) Suzuki, T.; Nakayama, T.; Kurihara, T.; Nishino, T.; Esaki, N. *Biosci. Biotechnol. Biochem.* **2002**, *66*, 1682-1690.

(102) Wei, Y. L.; Kurihara, T.; Suzuki, T.; Esaki, N. *J. Mol. Catal. B-Enzym.* **2003**, *23*, 357-365.

(103) Yin, J.-G.; Xu, G.-C.; Zheng, G.-W.; Xu, J.-H. *Appl. Biochem. Biotech.* **2015**, *176*, 1102-1113.

(104) Zheng, X. M.; Chu, X. Y.; Zhang, W.; Wu, N. F.; Fan, Y. L. *Appl. Microbiol. Biotechnol.* **2011**, *90*, 971-980.

(105) Dharmsthiti, S.; Pratuangdejkul, J.; Theeragool, G.; Luchai, S. *J. Gen. Appl. Microbiol.* **1998**, *44*, 139-145.

(106) Kim, H. E.; Lee, I. S.; Kim, J. H.; Hahn, K. W.; Park, U. J.; Han, H. S.; Park, K. R. *Curr. Microbiol.* **2003**, *46*, 291-295.

(107) Kim, Y. O.; Heo, Y. L.; Kim, H. K.; Nam, B. H.; Kong, H. J.; Kim, D. G.; Kim, W. J.; Kim, B. S.; Jee, Y. J.; Lee, S. J. *J. Microbiol. Biotechnol.* **2012**, *22*, 1245-1252.

(108) Park, I. H.; Kim, S. H.; Lee, Y. S.; Lee, S. C.; Zhou, Y.; Kim, C. M.; Ahn, S. C.; Choi, Y. L. *J. Microbiol. Biotechnol.* **2009**, *19*, 128-135.

(109) Cheeseman, J. D.; Tocilj, A.; Park, S.; Schrag, J. D.; Kazlauskas, R. J. *Acta Crystallogr. D.* **2004**, *60*, 1237-1243.

(110) Choi, K. D.; Jeohn, G. H.; Rhee, J. S.; Yoo, O. J. *Agr. Biol. Chem. Tokyo* **1990**, *54*, 2039-2045.

(111) Khalameyzer, V.; Fischer, I.; Bornscheuer, U. T.; Altenbuchner, J. *Appl. Environ. Microb.* **1999**, *65*, 477-482.

(112) Kim, S.; Tri Duc, N.; Kim, K. K.; Kim, T. D. *Acta Crystallogr. F.* **2012**, *68*, 1374-1377.

(113) Krebsfanger, N.; Schierholz, K.; Bornscheuer, U. T. *J. Biotechnol.* **1998**, *60*, 105-111.

(114) Krebsfanger, N.; Zocher, F.; Altenbuchner, J.; Bornscheuer, U. T. *Enzyme Microb. Tech.* **1998**, *22*, 641-646.

(115) Torres, L. L.; Schliessmann, A.; Schmidt, M.; Silva-Martin, N.; Hermoso, J. A.; Berenguer, J.; Bornscheuer, U. T.; Hidalgo, A. *Org. Biomol. Chem.* **2012**, *10*, 3388-3392.

(116) Zocher, F.; Krebsfanger, N.; Yoo, O. J.; Bornscheuer, U. T. *J. Mol. Catal. B-Enzym.* **1998**, *5*, 199-202.

(117) Chen, H.; Wu, J.; Yang, L.; Xu, G. *J. Mol. Catal. B-Enzym.* **2014**, *102*, 81-87.

(118) Dharmsthiti, S.; Kuhasuntisuk, B. *J. Ind. Microbiol. Biotechnol.* **1998**, *21*, 75-80.

(119) Kovacic, F.; Bleffert, F.; Caliskan, M.; Wilhelm, S.; Granzin, J.; Batra-Safferling, R.; Jaeger, K.-E. *FEBS Open. Bio.* **2016**, *6*, 484-493.

(120) Lee, J. H.; Rhee, S. K.; Kim, C. H. *Enzyme Microb. Tech.* **2004**, *35*, 563-567.

(121) Dong, J.; Zhao, W.; Gasmalla, M. A. A.; Sun, J.; Hua, X.; Zhang, W.; Han, L.; Fan, Y.; Feng, Y.; Shen, Q.; Yang, R. *J. Mol. Catal. B-Enzym.* **2015**, *121*, 53-63.

(122) Kim, G. J.; Choi, G. S.; Kim, J. Y.; Lee, J. B.; Jo, D. H.; Ryu, Y. W. *J. Mol. Catal. B-Enzym.* **2002**, *17*, 29-38.

(123) Kim, G. J.; Lee, E. G.; Gokul, B.; Hahm, M. S.; Prerna, D.; Choi, G. S.; Ryu, Y. W.; Ro, H. S.; Chung, B. H. *J. Mol. Catal. B-Enzym.* **2003**, *22*, 29-35.

(124) Lee, E. G.; Won, H. S.; Ro, H. S.; Ryu, Y. W.; Chung, B. H. *J. Mol. Catal. B-Enzym.* **2003**, *26*, 149-156.

(125) Prim, N.; Bofill, C.; Pastor, F. I. J.; Diaz, P. *Biochimie* **2006**, *88*, 859-867.

(126) Reetz, M. T.; Jaeger, K. E. *Chem. Phys. Lipids* **1998**, *93*, 3-14.

(127) Shimada, Y.; Nagao, T.; Sugihara, A.; Iizumi, T.; Yui, T.; Nakamura, K.; Fukase, T.; Tominaga, Y. *Biochim. Biophys. Acta* **1993**, *1174*, 79-82.

(128) Svendsen, A.; Borch, K.; Barfoed, M.; Nielsen, T. B.; Gormsen, E.; Patkar, S. A. *BBA-Lipids Lipid. Met.* **1995**, *1259*, 9-17.

(129) Wicka, M.; Wanarska, M.; Krajewska, E.; Pawlak-Szukalska, A.; Kur, J.; Cieslinski, H. *Acta Biochim. Pol.* **2016**, *63*, 117-125.

(130) Dou, S.; Kong, X.-D.; Ma, B.-D.; Chen, Q.; Zhang, J.; Zhou, J.; Xu, J.-H. *Biochem. Bioph. Res. Co.* **2014**, *446*, 1145-1150.

(131) Hasona, A.; York, S. W.; Yomano, L. P.; Ingram, L. O.; Shanmugam, K. T. *Appl. Environ. Microb.* **2002**, *68*, 2651-2659.

(132) Ma, B.-D.; Yu, H.-L.; Pan, J.; Liu, J.-Y.; Ju, X.; Xu, J.-H. *Bioresource Technol.* **2013**, *133*, 354-360.

(133) Ozaki, E.; Sakimae, A. *J. Ferment. Bioeng.* **1997**, *83*, 535-539.

(134) Hong, S.; Lee, C.; Jang, S.-H. *Biotechnol. Lett.* **2012**, *34*, 1051-1055.

(135) Lee, C.; Kim, J.; Hong, S.; Goo, B.; Lee, S.; Jang, S.-H. *Appl. Biochem. Biotech.* **2012**, *169*, 29-40.

(136) Inglis, G. D.; Yanke, L. J.; Selinger, L. B. *Can. J. Microbiol.* **2011**, *57*, 902-913.

(137) Lin, S. F. *J. Ferment. Bioeng.* **1996**, *82*, 448-451.

(138) Nicolay, T.; Devleeschouwer, K.; Vanderleyden, J.; Spaepen, S. *Appl. Environ. Microb.* **2012**, *78*, 2533-2542.

(139) Noureddini, H.; Gao, X.; Philkana, R. S. *Bioresource Technol.* **2005**, *96*, 769-777.

(140) Pencreach, G.; Baratti, J. C. *Enzyme Microb. Tech.* **1996**, *18*, 417-422.

(141) Sakai, K.; Fukuba, M.; Hasui, Y.; Moriyoshi, K.; Ohmoto, T.; Fujita, T.; Ohe, T. *Biosci. Biotech. Bioch.* **1998**, *62*, 2000-2007.

(142) Wagner, U. G.; Solkner, B.; Petersen, E. I.; Schlacher, A.; Schwab, H.; Kratky, C. *Acta Crystallogr. D.* **1997**, *53*, 596-598.

(143) Bourne, Y.; Hasper, A. A.; Chahinian, H.; Juin, M.; de Graaff, L. H.; Marchot, P. *Structure* **2004**, *12*, 677-687.

(144) Fernandez-Lorente, G.; Ortiz, C.; Segura, R. L.; Fernandez-Lafuente, R.; Guisan, J. M.; Palomo, J. M. *Biotechnol. Bioeng.* **2005**, *92*, 773-779.

(145) Shu, Z. Y.; Wu, J. G.; Xue, L. Y.; Lin, R. F.; Jiang, Y. M.; Tang, L. H.; Li, X.; Huang, J. Z. *Enzyme Microb. Tech.* **2011**, *48*, 129-133.

(146) Wang, G. L.; Liu, Z. M.; Xu, L.; Zhang, H. J.; Yan, Y. J. *Microbiol. Res.* **2015**, *178*, 27-34.

(147) Brito-Madurro, A. G.; Cuadros-Orellana, S.; Madurro, J. M.; Martinez-Rossi, N.; Rossi, A. *Ann. Microbiol.* **2005**, *55*, 291-294.

(148) Pena-Montes, C.; Gonzalez, A.; Castro-Ochoa, D.; Farres, A. *Appl. Microbiol. Biotechnol.* **2008**, *78*, 603-612.

(149) Pena-Montes, C.; Lange, S.; Flores, I.; Castro-Ochoa, D.; Schmid, R.;

Cruz-Garcia, F.; Farres, A. *Appl. Microbiol. Biotechnol.* **2009**, *84*, 917-926.

(150) Pena-Montes, C.; Mondragon-Tintor, M. E.; Castro-Rodriguez, J. A.; Bustos-Jaimes, I.; Navarro-Ocana, A.; Farres, A. *Enzyme res.* **2013**, *2013*, 928913-928913.

(151) Hamdy, H. S.; Abo-Tahon, M. A. *Ann. Microbiol.* **2012**, *62*, 1723-1736.

(152) Koseki, T.; Asai, S.; Saito, N.; Mori, M.; Sakaguchi, Y.; Ikeda, K.; Shiono, Y. *Appl. Microbiol. Biotechnol.* **2013**, *97*, 5351-5357.

(153) Molinari, F.; Gandolfi, R.; Converti, A.; Zilli, M. *Enzyme Microb. Tech.* **2000**, *27*, 626-630.

(154) Toida, J.; Arikawa, Y.; Kondou, K.; Fukuzawa, M.; Sekiguchi, J. *Biosci. Biotechnol. Biochem.* **1998**, *62*, 759-763.

(155) Koseki, T.; Furuse, S.; Iwano, K.; Sakai, H.; Matsuzawa, H. *Biochem. J.* **1997**, *326*, 485-490.

(156) Saxena, R. K.; Isar, J.; Saran, S.; Kaushik, R.; Davidson, W. S. *Curr. Sci. India* **2005**, *89*, 1000-1003.

(157) Christakopoulos, P.; Tzalas, B.; Mamma, D.; Stamatis, H.; Liadakis, G. N.; Tzia, C.; Kekos, D.; Kolisis, F. N.; Macris, B. J. *Process Biochem.* **1998**, *33*, 729-733.

(158) Stamatis, H.; Christakopoulos, P.; Kekos, D.; Macris, B. J.; Kolisis, F. N. *J. Mol. Catal. B-Enzym.* **1998**, *4*, 229-236.

(159) Tamerler, C.; Keshavarz, T. *J. Chem. Technol. Biotechnol.* **2000**, *75*, 785-790.

(160) Nagao, T.; Shimada, Y.; Sugihara, A.; Tominaga, Y. *J. Biochem.* **1994**, *116*, 536-540.

(161) Bayramoglu, G.; Arica, M. Y. *Fiber. Polym.* **2014**, *15*, 2051-2060.

(162) Karra-Chaabouni, M.; Pulvin, S.; Touraud, D.; Thomas, D. *J. Am. Oil Chem. Soc.* **1998**, *75*, 1201-1206.

(163) Hiol, A.; Jonzo, M. D.; Druet, D.; Comeau, L. *Enzyme Microb. Tech.* **1999**, *25*, 80-87.

(164) Ulker, S.; Karaoglu, S. A. *J. Biosci. Bioeng.* **2012**, *114*, 385-390.

(165) Correa, T. L. R.; Zubieta, M. P.; Teixeira, J. A.; de Queiroz, M. V.; Araujo, E. F. *J. Appl. Microbiol.* **2013**, *115*, 114-124.

(166) Costa, M. A. F.; Peralta, R. M. *J. Basic Microb.* **1999**, *39*, 11-15.

(167) Woroniecki, S. R.; Armitage, P. A.; Elson, S. W.; Ford, B. D.; Sime, J. T. *Biocatalysis* **1994**, *8*, 309-320.

(168) Liu, Y.; Xu, H.; Yan, Q.; Yang, S.; Duan, X.; Jiang, Z. *PLOS ONE* **2013**, *8*.

(169) Balasubramaniam, B.; Perumal, A. S.; Jayaraman, J.; Mani, J.; Ramanujam, P. *Waste Manage.* **2012**, *32*, 1539-1547.

(170) Bapiraju, K.; Sujatha, P.; Ellaiah, P.; Ramana, T. *J. Basic Microb.* **2005**, *45*, 257-273.

(171) Essamri, M.; Deyris, V.; Comeau, L. *J. Biotechnol.* **1998**, *60*, 97-103.

(172) Guillen, M.; Benaiges, M. D.; Valero, F. *Biochem. Eng. J.* **2011**, *54*, 117-123.

(173) Takahashi, S.; Ueda, M.; Tanaka, A. *Appl. Microbiol. Biotechnol.* **1999**, *52*, 534-540.

(174) Yu, X. W.; Wang, L. L.; Xu, Y. *J. Mol. Catal. B-Enzym.* **2009**, *57*, 304-311.

(175) Uppenberg, J.; Patkar, S.; Bergfors, T.; Jones, T. A. *J. Mol. Biol.* **1994**, *235*, 790-792.

(176) de Maria, P. D.; Alcantara, A. R.; Carballeira, J. D.; de la Casa, R. M.; Garcia-Burgos, C. A.; Hernaiz, M. J.; Sanchez-Montero, J. M.; Sinisterra, J. V. *Curr. Org. Chem.* **2006**, *10*, 1053-1066.

(177) de Maria, P. D.; Sanchez-Montero, J. M.; Sinisterra, J. V.; Alcantara, A. R. *Biotechnol. Adv.* **2006**, *24*, 180-196.

(178) Lee, L. C.; Chen, Y. T.; Yen, C. C.; Chiang, T. C. Y.; Tang, S. J.; Lee, G. C.; Shaw, J. F. *J. Agr. Food Chem.* **2007**, *55*, 5103-5108.

(179) Takac, S.; Erdem, B. *Prep. Biochem. Biotechnol.* **2009**, *39*, 323-341.

(180) Fukuda, K.; Kiyokawa, Y.; Yanagiuchi, T.; Wakai, Y.; Kitamoto, K.; Inoue, Y.; Kimura, A. *Appl. Microbiol. Biotechnol.* **2000**, *53*, 596-600.

(181) Fukuda, K.; Kuwahata, O.; Kiyokawa, Y.; Yanagiuchi, T.; Wakai, Y.; Kitamoto, K.; Inoue, Y.; Kimura, A. *J. Ferment. Bioeng.* **1996**, *82*, 8-15.

(182) Ploier, B.; Scharwey, M.; Koch, B.; Schmidt, C.; Schatte, J.; Rechberger, G.; Kollroser, M.; Hermetter, A.; Daum, G. *J. Biol. Chem.* **2013**, *288*, 36061-36072.

(183) Horsted, M. W.; Dey, E. S.; Holmberg, S.; Kielland-Brandt, M. C. *Yeast* **1998**, *14*, 793-803.

(184) Dharmsthiti, S.; Ammaranond, P. *Biotechnol. Appl. Biochem.* **1997**, *26*, 111-116.

(185) Bankar, A. V.; Kumar, A. R.; Zinjarde, S. S. *Appl. Microbiol. Biotechnol.* **2009**, *84*, 847-865.

(186) Corzo, G.; Revah, S. *Bioresource Technol.* **1999**, *70*, 173-180.

(187) Fickers, P.; Fudalej, F.; Le Dall, M. T.; Casaregola, S.; Gaillardin, C.; Thonart, P.; Nicaud, J. M. *Fungal Genet. Biol.* **2005**, *42*, 264-274.

(188) Jolivet, P.; Bordes, F.; Fudalej, F.; Cancino, M.; Vignaud, C.; Dossat, V.; Burghoffer, C.; Marty, A.; Chardot, T.; Nicaud, J. M. *FEMS Yeast Res.* **2007**, *7*, 1317-1327.

(189) Kim, J.-T.; Kang, S. G.; Woo, J.-H.; Lee, J.-H.; Jeong, B. C.; Kim, S.-J. *Appl. Microbiol. Biot.* **2007**, *74*, 820-828.

(190) Yadav, K. N. S.; Adsul, M. G.; Bastawde, K. B.; Jadhav, D. D.; Thulasiram, H. V.; Gokhale, D. V. *Bioresource Technol.* **2011**, *102*, 10663-10670.

(191) Lopez-Lopez, O.; Cerdan, M. E.; Siso, M. I. G. *Curr. Prot. Pept. Sci.* **2014**, *15*, 445-455.

(192) Popovic, A.; Tchigvintsev, A.; Tran, H.; Chernikova, T. N.; Golyshina, O. V.; Yakimov, M. M.; Golyshin, P. N.; Yakunin, A. F. *Adv. exp. med. biol.* **2015**, *883*, 1-20.

(193) Berlemont, R.; Pipers, D.; Delsaute, M.; Angiono, F.; Feller, G.; Galleni, M.; Power, P. *Rev. Argent. Microbiol.* **2011**, *43*, 94-103.

(194) Bunterngsook, B.; Kanokratana, P.; Thongaram, T.; Tanapongpipat, S.; Uengwetwanit, T.; Rachdawong, S.; Vichitsoonthonkul, T.; Eurwilaichitr, L. *Biosci. Biotechnol. Biochem.* **2010**, *74*, 1848-1854.

(195) Chu, X. M.; He, H. Z.; Guo, C. Q.; Sun, B. L. *Appl. Microbiol. Biotechnol.* **2008**, *80*, 615-625.

(196) De Santi, C.; Altermark, B.; Pierechod, M. M.; Ambrosino, L.; de Pascale, D.; Willassen, N. P. *BMC Biochem.* **2016**, *17*.

(197) De Santi, C.; Ambrosino, L.; Tedesco, P.; de Pascale, D.; Zhai, L.; Zhou, C.; Xue, Y. F.; Ma, Y. H. *Biotechnol. Progr.* **2015**, *31*, 890-899.

(198) Fang, Z. M.; Li, J. J.; Wang, Q.; Fang, W.; Peng, H.; Zhang, X. C.; Xiao, Y. Z. *J. Microbiol. Biotechnol.* **2014**, *24*, 771-780.

(199) Kennedy, J.; O'Leary, N. D.; Kiran, G. S.; Morrissey, J. P.; O'Gara, F.; Selvin, J.; Dobson, A. D. W. *J. Appl. Microbiol.* **2011**, *111*, 787-799.

(200) Faoro, H.; Glogauer, A.; Couto, G. H.; de Souza, E. M.; Rigo, L. U.; Cruz, L. M.; Monteiro, R. A.; Pedrosa, F. D. *FEMS Microbiol. Ecol.* **2012**, *81*, 386-394.

(201) Fu, C. Z.; Hu, Y. F.; Xie, F.; Guo, H.; Ashforth, E. J.; Polyak, S. W.; Zhu, B. L.; Zhang, L. X. *Appl. Microbiol. Biotechnol.* **2011**, *90*, 961-970.

(202) Hu, Y.; Liu, Y.; Li, J.; Feng, Y.; Lu, N.; Zhu, B.; Xue, S. *J. Ind. Microbiol. Biotechnol.* **2015**, *42*, 1449-1461.

(203) Zhang, H.; Li, F. C.; Chen, H. X.; Zhao, J.; Yan, J. F.; Jiang, P.; Li, R. G.; Zhu, B. L. *Chinese J. Oceanol. Limnol.* **2015**, *33*, 819-827.

(204) Jeon, J. H.; Kim, J. T.; Kang, S. G.; Lee, J. H.; Kim, S. J. *Mar. Biotechnol.* **2009**, *11*, 307-316.

(205) Jeon, J. H.; Kim, J. T.; Kim, Y. J.; Kim, H. K.; Lee, H. S.; Kang, S. G.; Kim, S. J.; Lee, J. H. *Appl. Microbiol. Biotechnol.* **2009**, *81*, 865-874.

(206) Jiang, X. W.; Xu, X. W.; Huo, Y. Y.; Wu, Y. H.; Zhu, X. F.; Zhang, X. Q.; Wu, M. *Arch. Microbiol.* **2012**, *194*, 207-214.

(207) Park, H.-J.; Jeon, J. H.; Kang, S. G.; Lee, J.-H.; Lee, S.-A.; Kim, H.-K. *Protein Expres. Purif.* **2007**, *52*, 340-347.

(208) Jeon, J. H.; Lee, H. S.; Kim, J. T.; Kim, S. J.; Choi, S. H.; Kang, S. G.; Lee, J. H. *Appl. Microbiol. Biotechnol.* **2012**, *93*, 623-631.

(209) Jimenez, D. J.; Montana, J. S.; Alvarez, D.; Baena, S. *World J. Microbiol. Biotechnol.* **2012**, *28*, 361-370.

(210) Kim, N. H.; Park, J. H.; Chung, E.; So, H. A.; Lee, M. H.; Kim, J. C.; Hwang, E. C.; Lee, S. W. *J. Microbiol. Biotechnol.* **2016**, *26*, 248-254.

(211) Wang, S. D.; Wang, K.; Li, L.; Liu, Y. H. *J. Mol. Catal. B-Enzym.* **2013**, *95*, 1-8.

(212) Kang, C.-H.; Oh, K.-H.; Lee, M.-H.; Oh, T.-K.; Kim, B. H.; Yoon, J.-H. *Microb. Cell Fact.* **2011**, *10*.

(213) Kim, H. J.; Jeong, Y. S.; Jung, W. K.; Kim, S. K.; Lee, H. W.; Kahng, H.-Y.; Kim, J.; Kim, H. *Mol. Biotechnol.* **2015**, *57*, 781-792.

(214) Ko, K. C.; Rim, S. O.; Han, Y.; Shin, B. S.; Kim, G. J.; Choi, J. H.; Song, J. J. *J. Ind. Microbiol. Biotechnol.* **2012**, *39*, 681-689.

(215) Lee, M. H.; Hong, K. S.; Malhotra, S.; Park, J. H.; Hwang, E. C.; Choi, H. K.; Kim, Y. S.; Tao, W. X.; Lee, S. W. *Appl. Microbiol. Biotechnol.* **2010**, *88*, 1125-1134.

(216) Petrovskaya, L. E.; Novototskaya-Vlasova, K. A.; Spirina, E. V.; Durdenko, E. V.; Lomakina, G. Y.; Zavialova, M. G.; Nikolaev, E. N.; Rivkina, E. M. *FEMS Microbiol. Ecol.* **2016**, *92*.

(217) Yu, E. Y.; Kwon, M.-A.; Lee, M.; Oh, J. Y.; Choi, J.-E.; Lee, J. Y.; Song, B.-K.; Hahm, D.-H.; Song, J. K. *Appl. Microbiol. Biotechnol.* **2011**, *90*, 573-581.

(218) Beisson, F.; Tiss, A.; Riviere, C.; Verger, R. *Eur. J. Lip. Sci. Tech.* **2000**, *102*, 133-153.

(219) Gilham, D.; Lehner, R. *Methods* **2005**, *36*, 139-147.

(220) Gupta, R.; Rathi, P.; Gupta, N.; Bradoo, S. *Biotechnol. Appl. Biochem.* **2003**, *37*, 63-71.

(221) Ferrato, F.; Carriere, F.; Sarda, L.; Verger, R. In *Lipases, Pt B: Enzyme Characterization and Utilization* 1997; Vol. 286, p 327-347.

(222) Jaeger, K. E.; Ransac, S.; Dijkstra, B. W.; Colson, C.; Vanheuvel, M.; Misset, O. *Fems Microbiology Reviews* **1994**, *15*, 29-63.

(223) Rathi, P.; Saxena, R. K.; Gupta, R. *Process Biochem.* **2001**, *37*, 187-192.

(224) Gao, Y.; Breuil, C. *World J. Microbiol. Biotechnol.* **1995**, *11*, 638-642.

(225) Cordova, J.; Nemmaoui, M.; Ismaili-Alaoui, M.; Morin, A.; Roussos, S.; Raimbault, M.; Benjilali, B. *J. Mol. Catal. B-Enzym.* **1998**, *5*, 75-78.

(226) George, E.; Tamerler, C.; Martinez, A.; Martinez, M. J.; Keshavarz, T. *J. Chem. Technol. Biotechnol.* **1999**, *74*, 137-140.

(227) Junker, B.; Moore, J.; Sturr, M.; McLoughlin, K.; Leporati, J.; Yamazaki, S.; Chartrain, M.; Greasham, R. *Bioprocess Biosyst. Eng.* **2001**, *24*, 39-49.

(228) Soni, S.; Odaneth, A. A.; Lali, A. M.; Chandrayan, S. K. *Protein Expres. Purif.* **2016**, *122*, 31-37.

(229) Wei, X.; Jiang, X.; Ye, L.; Yuan, S.; Chen, Z.; Wu, M.; Yu, H. *J. Mol. Catal. B-Enzym.* **2013**, *97*, 270-277.

(230) Shakiba, M. H.; Ali, M. S. M.; Rahman, R. N. Z. R. A.; Salleh, A. B.;

Leow, T. C. *Extremophiles* **2016**, *20*, 45-55.

(231) Jaeger, K. E.; Dijkstra, B. W.; Reetz, M. T. *Ann. Rev. Microbiol.* **1999**, *53*, 315-+.

(232) Jaeger, K. E.; Reetz, M. T. *Trends Biotechnol.* **1998**, *16*, 396-403.

(233) Jung, F.; Cammarota, M. C.; Freire, D. M. G. *Biotechnol. Lett.* **2002**, *24*, 1797-1802.

(234) Masse, L.; Kennedy, K. J.; Chou, S. *Bioresource Technol.* **2001**, *77*, 145-155.

(235) Omil, F.; Garrido, J. M.; Arrojo, B.; Mendez, R. *Water Res.* **2003**, *37*, 4099-4108.

(236) Knezevic, Z.; Mavinic, D. S.; Anderson, B. C. *Water Environ. Res.* **1995**, *67*, 835-841.

(237) DiCosimo, R.; McAuliffe, J.; Poulose, A. J.; Bohlmann, G. *Chem. Soc. Rev.* **2013**, *42*, 6437-6474.

(238) Liese, A.; Hilterhaus, L. *Chem. Soc. Rev.* **2013**, *42*, 6236-6249.

(239) Antrim, R. L.; Buchert, J.; Burrows, H.; Herbots, I.; Kottwitz, B.; Lenting, H. B. M.; Niku-Paavola, M.-L.; Reilly, P. J.; Suurnäkki, A.; Viikari, L. In *Enzymes in Industry*; Wiley-VCH Verlag GmbH & Co. KGaA: 2004, p 155-244.

(240) Kinsella, J. E.; Hwang, D. H.; Dwivedi, B. *Crit. Rev. Food Sci.* **1976**, *8*, 191-228.

(241) Fukuda, H.; Kondo, A.; Noda, H. *J. Biosci. Bioeng.* **2001**, *92*, 405-416.

(242) Kia-Kojouri, M.; Darzi, G. N.; Rupani, B.; Mohammadpour, M. *Indian J. Chem. Techn.* **2016**, *23*, 216-220.

(243) Su, F.; Peng, C.; Li, G. L.; Xu, L.; Yan, Y. J. *Renewable Energy* **2016**, *90*, 329-335.

(244) De Oliveira, D.; Di Luccio, M.; Faccio, C.; Dalla Rosa, C.; Bender, J. P.; Lipke, N.; Menoncin, S.; Amroginski, C.; De Oliveira, J. V. *Appl. Biochem. Biotechnol.* **2004**, *113*, 771-780.

(245) Royon, D.; Daz, M.; Ellenrieder, G.; Locatelli, S. *Bioresource Technol.* **2007**, *98*, 648-653.

(246) Zhang, Y.; Dube, M. A.; McLean, D. D.; Kates, M. *Bioresource Technol.* **2003**, *89*, 1-16.

(247) Galante, Y. A.; Formantici, C. *Curr. Org. Chem.* **2003**, *7*, 1399-1422.

(248) Patel, R. N. *Curr. Opin. Biotechnol.* **2001**, *12*, 587-604.

(249) Patel, R. N. *Adv. Synth. Catal.* **2001**, *343*, 527-546.

(250) Patel, R. N. *Curr. Opin. Drug Discov. Devel.* **2006**, *9*, 741-764.

(251) Patel, R. N. *Coordin. Chem. Rev.* **2008**, *252*, 659-701.

(252) Patel, R. N. *ACS Catal.* **2011**, *1*, 1056-1074.

(253) Choi, G. S.; Kim, J. Y.; Kim, J. H.; Ryu, Y. W.; Kim, G. J. *Protein Expres. Purif.* **2003**, *29*, 85-93.

(254) Xu, F.; Chen, S.; Xu, G.; Wu, J.; Yang, L. *Biotechnol. Biop. Eng.* **2015**, *20*, 473-487.

(255) Gong, Y.; Xu, G.-C.; Zheng, G.-W.; Li, C.-X.; Xu, J.-H. *J. Mol. Catal. B-Enzym.* **2014**, *109*, 1-8.

(256) Heinze, B.; Kourist, R.; Fransson, L.; Hult, K.; Bornscheuer, U. T. *Protein. Eng. Des. Sel.* **2007**, *20*, 125-131.

Chapter 2

Cloning, characterization & utilization of esterases/lipases isolated from non-culturable microorganisms: a Metagenomics approach.

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Cloning, characterization & utilization of esterases/lipases isolated from non-culturable microorganism: a metagenomics approach.

A metagenomic pUC based library was created from sea sediment and hot spring microbial mat sample from Goa and Khir Ganga region, Himachal Pradesh, India respectively. Among many esterase positives clones showing greater zone of clearance on tributyrin agar plates, two clones SLC-6 and MLC-3 shown to be highly enantioselective in hydrolysis of various acyclic and aromatic acetates. SLC-6 esterase gene posses open reading frame (ORF) region of 1374 bp and 36 bp 5'-UTR region with signal sequence while MLC-3 esterase gene shows ORF region of 843 bp. Both esterase shows pentapeptide region GHSLG common among many esterases/lipases and α/β-hydrolase domain seen in various serine hydrolase family proteins. SLC-6 and MLC-3 esterase gene were cloned, expressed in pET 32-a vector with *Escherichia coli* as host and were purified to carry out biochemical characterization. SLC-6 esterase showed optimum pH and temperature 7.5 and 30 ºC while MLC-3 showed optimum pH of 8.0 and temperature of 20 ºC. Both esterase were highly active towards *p*-nitrophenol butyrate and showed enhancement in activity with the addition of Mg^{+2}, Na^{+1} and K^{+1} ions. SLC-6 and MLC-3 esterase showed enhanced stability in polar solvents such as DMSO and ethanol respectively. SLC-6 esterase hydrolysed various racemic acetates to give corresponding (*R*) and (*S*) alcohols with > 90 % e.e value while MLC-3 hydrolysed to give selectively (*R*) alcohols with > 99 % e.e value. Molecular docking studies were carried out which were very well *in corroboration* with the experimental results obtained.

2.1 Introduction

Lipolytic enzymes belong to family of serine hydrolases and do possess a distinctive α/β hydrolase fold. Enzymes such as esterases (EC 3.1.1.1) and lipases (EC 3.1.1.3) are industrially valuable enzymes belong to this family. Both the enzymes catalyse hydrolysis of an ester to carboxylic acid and alcohol in aqueous media while catalyses reverse reaction such as esterification in organic solvents/ non aqueous media. These enzymes show a pentapeptide sequence GXSXG found conserved in all lipases/esterases family proteins and posses aspartate, glutamate and serine in their active sites. These enzymes are highly stereo and regio-selective in nature and don't require any cofactors for their activity.[1-5] Lipases/esterases are used in manufacturing and processing of detergents, drugs, paper, textiles, leathers, foods and fine chemicals, showing their versatility for biotechnological applications.[6-8] Due to cofactor independence and greater hydrophobicity found in tertiary structure, these are highly stable in non aqueous media and so are used in organic solvents/biphasic media to carry out different asymmetric synthesis reactions. Requirement of novel, highly enantioselective and efficient biocatalyst is always there when we look for getting industrially important chiral synthons at large scale.[9-13]

Various enantioselective esterases and lipases are reported from microorganisms isolated from the natural habitats. Metagenomics branch deals with the non-culturable microorganisms as there are more than 99 % of the microorganisms which are non-culturable at laboratory level. Thus metagenome of such microorganisms remains a potential source for isolating a novel biocatalyst with desired properties.[14-17] Till now there are reports on isolation of novel lipases/esterases from metagenomic libraries isolated from ecological niches such as antarctic soil, [18] peat-swamp forest soil, [19] south china sea water, [20] tibetan glacier, [21] deep-sea water [22] and sediment, [23-25] rhizosphere soil, [26] tidal flat sediment, [27,28] oil-polluted mud [29] and permafrost. [30]

In this study, we have investigated enantioselective hydrolytic properties of various esterase clones isolated from the metagenomic library of sea sediment and hot spring microbial mat sample (Dr. Ravi Ranjan's PhD thesis titled, "Functional Metagenomics to identify novel genes for biocatalysts. Supervisors: Prof. Rup Lal and Dr. Rakesh Sharma, Th-15607, Delhi University). pUC libraries were created from metagenomic DNA isolated from sea sediment sample (Goa, India) and hot spring microbial mat sample (Khir Ganga region, Himachal Pradesh, India). These libraries were screened for esterase/lipase activity using tributyrin agar plate method. Among hundreds of clones studied, two clones were showing

enantioselective hydrolysis of commercially valuable racemic acetates. Two esterase genes SLC-6 and MLC-3 were cloned, over expressed in *E. coli* as host and recombinant proteins were purified. Biochemical characterization, enzyme kinetics studies of these two esterases were carried out. SLC-6 and MLC-3 esterases showed enantioselective hydrolysis of eight commercially valuable racemic acetates with greater than 90 % enantiomeric excess (e.e) showing the applicability and efficiency of the enzymes for the use in synthesis of fine chemicals in industry.

2.2 Cloning, expression & characterization of novel enantioselective esterase SLC-6 obtained through sea sediment metagenomic library.

2.2.1 Materials and method

2.2.1.1 Chemicals

Peptone, yeast extract, malt extract, glucose, agar, Luria Bertani broth (L.B broth) used for bacterial culture maintenance and protein over expression was purchased from Himedia Laboratories Limited, Mumbai. Kit used for metagenomic DNA isolation and library preparations were purchased from Epicentre Company, USA. All the recombinant DNA technology experiment kits were purchased from Invitrogen, USA.

All the *p*-nitrophenol esters *p*-nitrophenol acetate (C2), butyrate (C4), valerate (C5), caprylate (C8), decanoate (C10), laurate (C12), myristate (C14), palmitate (C16) and stearate (C18) were purchased from Sigma Aldrich, USA. Racemic alcohols (±)-2-pentanol, (±)-2-hexanol, (±)-3-hexanol, (±)-1-octen-3-ol, (±)-1-phenylethanol, (±)-2-methyl-1-phenylethanol, (±)-3-methyl-1-phenylethanol and (±)-1-phenylpropanol were purchased from Sigma Aldrich/Fluka, USA.

Racemic esters (±)-2-pentyl acetate (**1**), (±)-2-hexyl acetate (**2**), (±)-3-hexyl acetate (**3**), (±)-1-octen-3-yl acetate (**4**), (±)-1-phenylethyl acetate (**5**), (±)-2-methyl-1-phenylethyl acetate (**6**), (±)-3-methyl-1-phenylethyl acetate (**7**) and (±)-1-phenylpropyl acetate (**8**) were purchased from Sigma Aldrich/Fluka, USA. All the pure enantiomeric (*R*) and (*S*) standards of alcohols were purchased from Sigma Aldrich USA.

2.2.1.2 Bacterial strain, plasmid used

pUC-19 and pET 32-a vector used for library preparation and over expression of proteins were purchased from Thermo Fischer scientific, USA and Novagen, USA company respectively. *E. coli* DH5α, C41 (DE3) cells (Novagen) were used for the transformation and over expression of SLC-6 esterase recombinant protein respectively.

2.2.1.3 Cloning of SLC-6 esterase gene

SLC-6 esterase gene was selected from the pUC-19 metagenomic libraries prepared from sea sediment sample collected from Goa, India (Dr. Ravi Ranjan's PhD thesis titled, "Functional Metagenomics to identify novel genes for biocatalysts. Supervisors: Prof. Rup Lal and Dr. Rakesh Sharma, Th-15607, Delhi University). SLC-6 esterase gene is a 1410 bp gene

encoding 1374 bp of open reading frame (ORF) region and 36 bp 5'-ÚTR region containing a signal sequence 5'-ATGCGAATAACGTCGGCGCCCATATCGGCCAACAGC-3' as identified using SignalP 3.1 server tool. ORF region of 1374 bp was cloned into pET 32-a vector using following primers: Forward primer: 5' –CAT GCC ATG GCA CAG AAT GGC CCG GGG -3' and Reverse primer: 5'- CCG CTC GAG GGT TAC TTC TCG GGT TTC GAC -3' (Underlined are the restriction sites for Nco-I and Xho-I restriction enzymes). PCR was performed using high fidelity "Vent polymerase" under following conditions: One cycle: 95 ºC for 5 min. (Initial denaturation), 35 cycles: denaturation: 95 ºC for 30 sec., 65 ºC for 30 sec. (Primer annealing) and 72 ºC for 1 min. 30 sec. (DNA amplification), One cycle: 10 min at 72 ºC (final hold) and then at 4 ºC for infinite time. PCR amplified SLC-6 esterase gene and pET 32-a vector were digested with Nco-I and Xho-I restriction enzymes and loaded onto 1 % agarose gel and purified using Gel purification kit (Invitrogen). Double digested vector and digested PCR product was ligated using T4 DNA ligase enzyme (Invitrogen) as per standard procedure to get a SLC-6-pET 32-a plasmid construct.

2.2.1.4 Over expression and protein purification of SLC-6 esterase

C41 (DE3) cells harbouring construct SLC-6-pET 32-a was inoculated into 1 litre Terrific Broth (T.B) media [tryptone (12 g), yeast extract (24 g), potassium phosphate, dibasic (9.4 g), potassium phosphate ,monobasic-2.2 g, glyceol-4 mL, pH-7.2]. When cell reached O.D (600 nm) ~ 0.6-0.8 ,expression was induced with 1mM IPTG at 16 ºC for 12 hrs with shaking condition (200 rpm). After induction, cells were kept 15 min on ice and then pelleted down at 4000 X g and 4 ºC for 20 min. Cell pellet thus obtained was lysed using lysis buffer [Tris-HCl buffer (50 mM) pH 8.0, 300 mM NaCl, 1 mM EDTA,0.1 % Triton X-100, 2 mM β-ME, lysozyme (1mg/mL), 10 % glycerol] with lysozyme concentration of 1mg/mL using ultrasonic cell disruptor (Sonics vibracell ultrasonic cell disruptor) at 6 pulses each with 30 sec. on and 30 sec. off cycles at 80 % amplitude for 10 min. Lysed cells extract was clarified at 10,000 X g for 15 min and was kept for binding with Ni-NTA agarose resin (1 mL Ni-NTA resin for 5 mL of clarified cell extract) for 1 hour at 4 ºC. Protein bound Ni-NTA resin was then washed with two gradient wash buffer [Tris-HCl buffer (50 mM) pH 8.0, 300 mM NaCl, 10 % glycerol] containing 20 mM and 60 mM imidazole. Final elution was done with elution buffer [Tris-HCl buffer (50 mM) pH 8.0, 300 mM NaCl and 10 % glycerol] containing 100 mM imidazole to get well purified protein. Protein thus obtained checked for purity over SDS PAGE gel and protein concentration was determined by Bradford's method using bovine serum albumin (BSA) as a standard.[31]

2.2.1.5 Enzyme activity assay and molar extinction coefficient of *p*-nitrophenol

Esterase activity was assayed using *p*-nitrophenol palmitate (*p*-nitrophenol palmitate) as a substrate for purified enzyme as reported earlier.[32] The assay conditions were as follows: *Solution A*: 37 mg of *p*-nitrophenol palmitate in 10 mL isopropanol. *Solution B*: 0.1 g Gum Arabic and 0.4 g Triton X-100 in 90 mL distilled water. 1 mL of solution A was mixed with 9 mL of solution B to make substrate solution. In the final reaction mixture, 0.9 mL of substrate solution was added into 0.1 mL buffer (potassium phosphate buffer, 10 mM, pH 7.0) and 0.1 mL (100 µg) esterase enzyme. Assay mixture was incubated at 37ºC for 30 min under shaking condition to quantitate release of *p*-nitrophenol in the reaction. Blank reaction was kept under similar conditions without enzyme. The release of *p*-nitrophenol from *p*-nitrophenol palmitate was spectrophotometrically determined at 410 nm using Cary 300 UV visible spectrophotometer (Varian). One unit of esterase activity was defined as the amount of enzyme releasing 1 µmol *p*-nitrophenol in 1 min under the assay conditions used. Experiments were conducted in triplicates and average values were calculated.

Molar extinction coefficient of *p*-nitrophenol was calculated at pH 7.5 in potassium phosphate buffer (10 mM). Three sets of each concentrations ranging from 20-200 µM of *p*-nitrophenol (99.9 % pure) were made and optical density was measured at 410 nm using UV visible spectrophotometer. Graph was plotted to calculate molar extinction coefficient. Molar extinction coefficient values for *p*-nitrophenol in potassium phosphate buffer (10 mM) at pH 7.5 were determined to be 12,000 $mol^{-1}.cm^{-1}$.

2.2.1.6 Effect of pH and temperature on the enzyme activity

To investigate effect of pH on the SLC-6 esterase activity, buffers of various pH were prepared. For pH range of 4-4.5 (acetate buffer, 100 mM), 6-8 (phosphate buffer, 100 mM), 8.5- 10.5 (Glycine-sodium hydroxide buffer, 100 mM) and 11-12 (Na_2HPO_4-Sodium hydroxide buffer, 100 mM) were used. Enzymatic reaction was done under standard assay condition as mentioned earlier.

To study effect of temperature on the enzyme activity, initial reaction velocity (V_0) of the enzyme was calculated at various temperatures (10 ºC, 15 ºC, 20 ºC, 25 ºC, 30 ºC, 35 ºC, 40 ºC, 45 ºC, 50 ºC). In 0.9 mL of substrate solution containing 0.1 mL buffer of optimum pH, 0.1 mL of purified enzyme (100 ng) was added and initial reaction velocity (V_0) was calculated for the enzyme using UV-visible spectrophotometer (Cary 300, Varian).

2.2.1.7 Determination of the substrate specificity of the enzyme

To study the substrate specificity of the purified SLC-6 esterase enzyme, *p*-nitrophenol esters of various acyl chain lengths (C2, C4, C5, C8, C10, C12, C14, C16 and C18) were used. The enzymatic reaction was carried out under standard assay condition as mentioned earlier at the optimum pH of 7.5 and temperature of 30 ºC. *p*-nitrophenol esters (C2-C18) used were at the final concentration of 100 µM in the reaction mixture.

2.2.1.8 Effect of metal ions on the activity of the enzyme

To investigate effect of metal ions on the activity of purified SLC-6 esterase, various divalent and monovalent salt such as $CaCl_2$, $MgCl_2$, $NiCl_2$, NaCl, $CoCl_2$, $CuCl_2$, KCl, $FeSO_4$, $ZnCl_2$, $HgCl_2$, NH_4Cl and $Ba(OH)_2$ were used. Enzymatic reaction was done under standard assay condition and the final concentration of salts used in the reaction was 5 mM.

2.2.1.9 Effect of various polar and non polar organic solvents on the activity of the enzyme

To study the stability of the SLC-6 esterase in different organic solvents, various polar and non polar solvents such as toluene, ethanol, isopropanol, isoamyl alcohol, dimethyl formamide (DMF), acetonitrile, dimethyl sulfoxide (DMSO), n-hexane, carbon tetrachloride (CCl_4), dichloromethane (DCM), tetrahydrofuran (THF) and chloroform were used. The enzyme was preincubated with organic solvent for 1 hour at RT with final concentration of 25 % v/v of solvent is to enzyme solution. This pre incubated enzyme then used for activity under standard assay conditions at and release of *p*-nitrophenol was quantitated at 410 nm using UV-visible spectrophotometer.

2.2.1.10 Effect of chelating agents, reducing agents and detergents on enzyme activity

Effect of chelating agent such as EDTA, reducing agents such as dithiothreitol (DTT), 2-β-mercapto ethanol (2-βME) and sodium dodecyl sulphate (SDS) and detergents like Triton X-100, Tween 80 and CHAPS were studied on SLC-6 esterase enzyme. EDTA, DTT, 2-βME and CHAPS were used at the final concentration of 10 mM in the reaction while Triton X-100, Tween-80 and SDS were used at the final concentration of 10 %, 10 % and 1 % respectively in the reaction mixture. All other assay conditions were as per standard conditions.

2.2.1.11 Enzyme kinetics study

The kinetic constants (K_m, V_{max}, K_{cat}, K_{cat} /K_m) of SLC-6 esterase were calculated based on the Michaelis menten graph. The initial rate of hydrolysis was determined by using

various concentration range of the substrate *p*-nitrophenol butyrate (50 µM to 2800 µM) under standard conditions with purified SLC-6 esterase enzyme (50 nM). Optimum pH of 7.5 and optimum temperature of 30 ºC were used for determining the kinetic parameters for the pure esterase enzymes.

2.2.1.12 Enantioselective hydrolysis of racemic esters by SLC-6 esterase

Time course studies of enantioselective hydrolysis of eight commercially important racemic acyclic and aromatic acetates were performed using both esterase enzymes. 620 µL of SLC-6 esterase enzyme (2 mg, 1.3 IU/mg) was dissolved in 1380 µL of potassium phosphate buffer (pH-7.5, 100 mM) to make final volume of assay to 2 mL. 4 mg of racemic acetate was added to each assay reaction and was incubated at 30 ºC and 200 rpm in an incubator shaker. Aliquots of 200 µL were taken out at the interval of 6, 12, 18, 24, 30, 36, 42, 48 h and each was extracted twice with 400 µL of ethyl acetate (EtOAc). The upper extracted solvent layer was collected and passed over anhydrous sodium sulphate to inject into GC-FID for chiral resolution analysis having injector with split ratio of 1:10.

2.2.1.13 Chiral GC Analysis

Gas chromatographic analysis (Agilent GC-FID, 7890 A with auto sampler, Chemstation software, Agilent, USA) was performed using three chiral stationary phases. Astec CHIRALDEX G-TA (2,6-di-O-pentyl-3-trifluoroacetyl derivative of γ-cyclodextrin, length: 30 m, I.D: 0.25 mm x film thickness: 0.12 µm, supelco, sigma aldrich, USA), Astec CHIRALDEX B-DP (2, 3-di-O-propionyl-6-t-butyl silyl derivative of β-cyclodextrin, length: 30 m, I.D: 0.25 mm x film thickness: 0.12 µm, supelco, sigma aldrich, USA) and Astec CHIRALDEX B-DM (2,3-di-O-methyl-6-t-butyl silyl derivative of β-cyclodextrin, length: 30 m, I.D: 0.25 mm x film thickness: 0.12 µm, supelco, sigma aldrich, USA), a flame ionisation detector (FID) temperature: 200 ºC, nitrogen as carrier gas (flow rate: 1mL/min) and front inlet (temperature: 200 ºC, split ratio:1:10). All the enantiomers were well separated and enantiomeric excess, conversion values were calculated. Absolute configurations for all compounds were determined by co injecting racemic mixture of alcohols with pure standards of (*R*) or (*S*) enantiomers into chiral GC.

2.2.1.13.1 GC/GC-MS conditions

A) While for (±)-2-pentanol and (±)-2-hexanol chiral GC separation was done on GC column "Astec CHIRALDEX G–TA" capillary column (30 m X 0.25 mm X 0.12 µm, Supelco) with following temperature programme-

Oven temperature programme: Initial temperature maintained at 35 ºC for 1 min, followed by a temperature gradient from 35 ºC to 65 ºC at 1 ºC min^{-1} and then raised to a final temperature of 180 ºC with a 10 ºC min^{-1} rise.

Retention times (R_t): (*S*)-2-pentanol, (*R*)-2-pentanol, (*S*)-2-pentyl acetate & (*R*)-2-pentyl acetate were eluted at retention times of 13.2, 13.6, 17.8 and 18.6 min respectively**.**

Retention times (R_t): (*S*)-2-hexanol, (*R*)-2-hexanol, (*S*)-2-hexyl acetate & (*R*)-2-hexyl acetate eluted at retention times of 20.4, 21.25, 28.17 & 29.99 min respectively.

B) Chiral GC separation of (±)-3-hexanol, (±)-1-octen-3-ol, (±)-1-phenyl ethanol, (±)-3-methyl-1-phenyl ethanol and (±)-1-phenylpropanol was done on "Astec CHIRALDEX B-DP" capillary column (30 m X 0.25 mm X 0.12 µm, Supelco) with following oven temperature programme-

1) **For (±)-3-hexanol and (±)-3-hexyl acetate:**

Oven temperature programme: Initial temperature maintained at 40 ºC for 1 min, followed by a temperature gradient from 40 ºC to 60 ºC at 1 ºC min^{-1} and then raised to final temperature of 200 ºC at the rate of 10 ºC $min^{-1}$and kept on hold for 3 min.

Retention times (R_t): (±)-3-hexyl acetate, (*R*)-(-)-3-hexanol and (*S*)-(+)-3-hexanol were eluted at retention times of 14.9 min, 15.3 min, 17.9 min and 18.7 min respectively.

2) For (±)-1-octen-3-ol and (±)-1-octen-3-yl acetate:

Oven temperature programme: Initial temperature maintained at 40 ºC for 1 min, followed by a temperature gradient from 40 ºC to 80 ºC at 5 ºC min^{-1} and from 80 ºC to 105 ºC at 1 ºC min^{-1}.It was then raised to final temperature of 200 ºC at the rate of 10 ºC $min^{-1}$and kept on hold for 5 min.

Retention times (R_t): (±)-1-octen-3-yl acetate, (*R*)-(-)-1-octen-3-ol and (*S*)-(+)-1-octen-3-ol were eluted at retention times of 16.47 min, 16.6 min and 17.3 min respectively.

3) For (±)-1-phenyl ethanol and (±)-1-phenyl ethyl acetate:

Oven temperature programme: Initial temperature maintained at 40 ºC for 1 min, followed by a temperature gradient from 40 ºC to 90 ºC at 5 ºC min^{-1} and from 90 ºC to 110 ºC at 1 ºC min^{-1}.It was then raised to final temperature of 200 ºC at the rate of 10 ºC $min^{-1}$and kept on hold for 3 min.

Retention times (R_t): (±)-1-phenylethyl acetate, (*R*)-(+)-1-phenyl ethanol and (*S*)-(-)-1-phenyl ethanol were eluted at retention times of 21.2 min, 23.4 min and 24.4 min respectively.

4) For (±)-3-methyl-phenyl ethanol and (±)-3-methyl-phenyl ethyl acetate:

Oven temperature programme: Initial temperature maintained at 50 ºC, followed by a temperature gradient from 50 ºC to 115 ºC at 3 ºC min^{-1} and from 115 ºC to 135 ºC at 1 ºC min^{-1}.It was then raised to final temperature of 200 ºC at the rate of 10 ºC $min^{-1}$and kept on hold for 5 min.

Retention times (R_t): (±)-3-methyl-1-phenylethyl acetate, (*R*)-(+)-3-methyl-1-phenyl ethanol and (*S*)-(-)-3-methyl-1-phenyl ethanol were eluted at retention times of 24.2 min, 24.9 min and 25.33 min respectively.

5) For (±)-1-phenylpropanol and (±)-1-phenylpropyl acetate:

Oven temperature programme: Initial temperature maintained at 40 ºC for 1 min, followed by a temperature gradient from 40 ºC to 70 ºC at 5 ºC min^{-1} and from 70 ºC to 105 ºC at 1 ºC min^{-1}.It was then raised to final temperature of 200 ºC at the rate of 10 ºC $min^{-1}$and kept on hold for 3 min.

Retention times (R_t): (±)-1-phenylpropyl acetate, (*R*)-(+)-1-phenylpropanol and (*S*)-(-)-1-phenylpropanol were at retention times of 35.2 min, 37.8 min and 38.6 min respectively.

C) Chiral GC separation of (±)-2-Methyl-1-phenyl ethanol was done on "Astec CHIRALDEX B-DM" capillary column (30 m X 0.25 mm X 0.12 µm, Supelco) with following oven temperature programme:-

1) For (±)-2-Methyl-1-phenyl ethanol and (±)-2-Methyl-1-phenyl ethyl acetate:

Oven temperature programme: Initial temperature maintained at 50 ºC, followed by a temperature gradient from 50 ºC to 115 ºC at 3 ºC min^{-1} and from 115 ºC to 135 ºC at 1 ºC min^{-1}

[1].It was then raised to final temperature of 200 ºC at the rate of 10 ºC $min^{-1}$and kept on hold for 5 min.

Retention times (R_t): (±)-2-methyl-1-phenylethyl acetate, (*R*)-(+)-2-methyl-1-phenyl ethanol and (*S*)-(-)-2-methyl-1-phenyl ethanol were eluted at retention times of 19.7 min, 25.2 min and 26.53 min respectively.

2.2.1.14 Nucleotide Sequence Accession Number

The nucleotide sequence of SLC-6 esterase gene is available at Gen Bank database as Gen Bank: GU331889.1.

2.2.1.15 Molecular docking studies

Homology modeling was performed using SwissModel server to predict three dimensional structure of SLC-6 esterase. X ray crystal structure of bacterial esterases (PDB ID: 3PF8) were used as templates for homology modeling.[33] Predicted models were energy minimized using GROMOS 43BI force field [34] and the assessed for its quality using ProSA and Ramchandran plot analysis. Docking studies were performed using AutoDock 4.2 version.[35] Ligands and receptor files were optimized for docking studies and converted from .pdb to .pdbqt format. The grid was set around active site residues of SLC-6 esterase with dimensions of 20 x 20 x 20 Å. The docking parameters were configured on a LGA calculation of 10,000 runs. Energy evaluations were set to 1,500,000 and 27,000 generations. Population size was set to 150 and the rate of gene mutation and the rate of gene crossover were set to 0.02 and 0.8 respectively.[36] The obtained conformations were later summarized, collected and extracted by using Autodock Tool. Geometry of resulting complexes was studied using the PyMol molecular viewer utility (The PyMOL Molecular Graphics System, Version 1.5.0.4 Schrödinger, LLC).

2.2.2 Results and Discussion

2.2.2.1 Cloning and sequence analysis of SLC-6 esterase gene

Nucleotide sequence of the SLC-6 esterase gene of consists of 1410 bp region having 1374 bp open reading frame region (ORF) and 36 bp 5'-UTR region with signal sequence. SLC-6 esterase gene was amplified using "Vent polymerase" (Fig.2.1) and was cloned into pET 32-a vector.

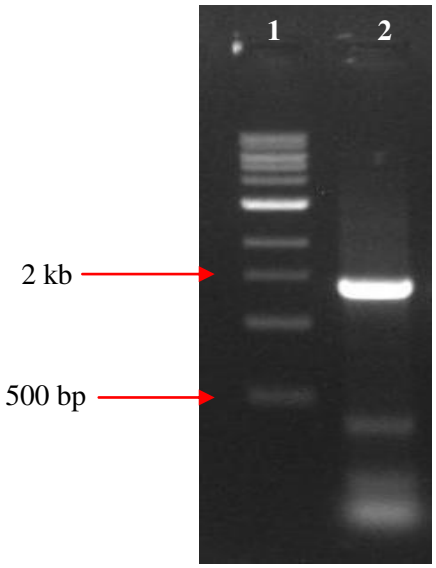


Fig. 2.1 Agarose gel for PCR amplification of SLC-6 esterase. **Lane 1**:- 1kb DNA ladder **Lane 2**:- PCR amplified SLC-6 esterase gene (1374 bp)

Positive colonies identified from colony PCR (Fig. 2.2) (using T7 forward and reverse primers) were picked and sent for sequencing to check the frame and for any unwanted mutations.

- **Agarose gel for colony PCR:**

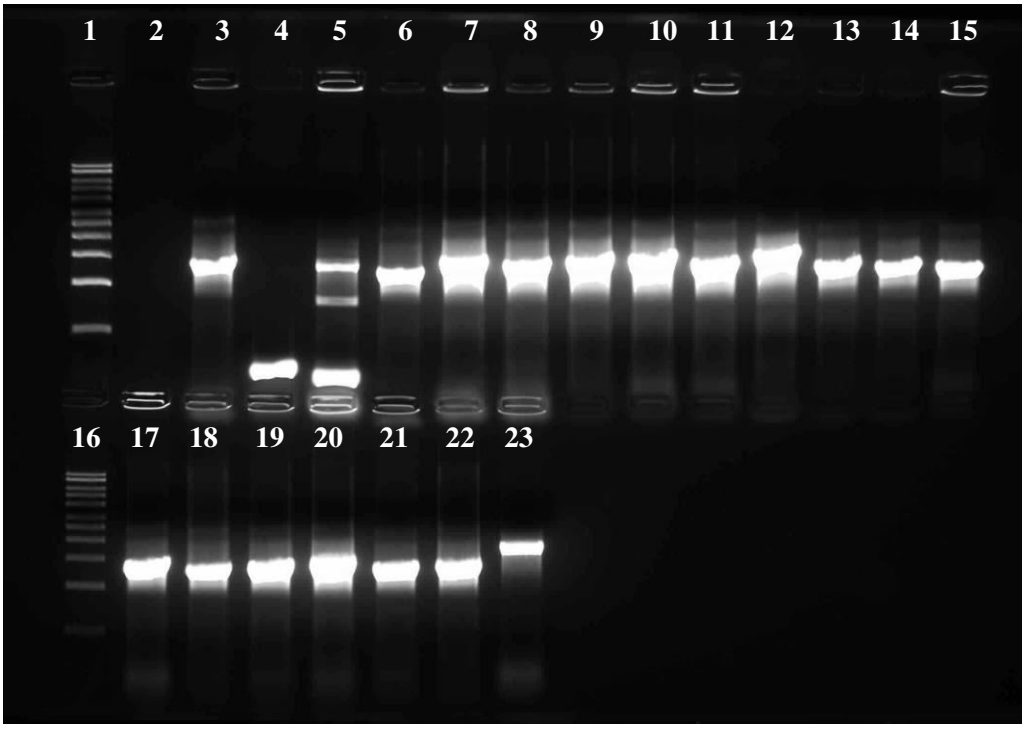


Fig.2.2 Agarose gel for colony PCR of SLC-6 esterase cloning into pET 32-a vector. **Lane 1, 16**:- 1kb DNA ladder, **Lane 2**:- Negative control, **Lane 3 to 23**:- colony PCR product of colonies 1 to 20.

Nucleotide and amino acid sequence of SLC-6 esterase:

- **SLC-6 esterase (1410 bp)**

ATGCGAATAACGTCGGCGCCCATATCGGCCAACAGCATGGCACAGAATGGCCCGGG
GCCGATGCCTGCGATTTCAATTACCTTGACCCCTTCGAGCGGTCCCATATCTGTCTCC
CTTACCTGCGGTTTGTCACTGTTTGGCGCTACCATAGGAACGCGGCCCCTCGAAACC
AACAGGAATTCGCTGATGTCGATTTCAAGTGAGAAAACAGACTTTCGCGGCCATGA
CGGTCAGATGCTCGCTGCACGGCTGGAACGAGCCTCCGGCGTGCCACGCGCATACG
CGCTCTTTGCGCACTGTTTCACCTGCACAAAGGATATCTACGCGGCCAGGCGCATCT
CCCAGGGCTTGGCAGAGCGCGGGATTGCTGTGCTGCGCTTTGACTTCACCGGCCTTG
GGGCGTCCGAAGGTGACTTCGGCAACACCGGCTTTACGTCCAATATCGACGACCTC
ATTGCCGCGGCAAGCTTTCTGCGGGAGGAGCACGAGGCACCGACCATTCTGATCGG
CCATAGCCTGGGCGGCGCCGCTGTTCTACGTGCCGCTGAAGCGATCCCCGAGGCTGC
TGCTGTAGCAACCATCGGCGCGCCTGCCGACCCAGCCCACGTTGCTCACCTCCTCGA
AGACAAGGCTGATGAAATCCGTGATAAGGGCCGAGCTACCGTCAACATCGGTGGTC
GCCCGTTCGATATCCGGGCCGAATTCCTCGACGACATCACCGCAAACCGACCCAGG
GATTACATTGGAGACCTGCGAAAAGCGCTGATTGTATTCCACGGGCCCCGCGATCA
GATCGTCGGTATCGAAAACGCTGCAGAGATATTCACAGCAGCCAAACACCCGAAGA

GCTTCGTCAGCCTTGATGACGCGGACCATCTGCTGTCGCGACAGCAGGATGCGGACT
ATGTCGCCGATGTGCTTTCGGCCTGGGCATCCCGTTATATCGGAGAGACAGAAAAG
CGGACCACGCCTCAGCCGCCCGACGGAATCACCCGTGTCGCCGAATCCGGCACAGG
AAGATTTACCCAGGATGTTTGGGCCGGCGGCCACTTCCTTCAGGCCGATGAGCCCGC
CAGCTTTGGCGGCGATAACGTTGGGCCCACACCCTACGACCTGTTGAGTGCCGCCTT
AGGCGCCTGCACGACCATGACCATCCGCATGTATGCGGACCGCAAAAAGCTGCCGC
TCGAACAGGTTTCTGTCGATGTCTCGCATGAAAAGATTCACGCGAGCGACTGCGCCG
ATTGCGAAACTGAATCTGGAAAGGTTGACCGGTTTTCGCGCGAGATCACCCTGTCAG
GCGATCTGGATGAAACTCAACGGGCACGACTTTTGGAGATCGCGGACAAATGCCCA
GTTCACCGTACGCTCCTCAGCGAGGTGAAGGTCGAAACCCGAGAAGTAACCTGA

Amino acid sequence of SLC-6 esterase (470 a.a)

MRITSAPISANSMAQNGPGPMPAISITLTPSSGPISVSLTCGLSLFGATIGTRPLETNRN
SLMSISSEKTDFRGHDGQMLAARLERASGVPRAYALFAHCFTCTKDIYAARRISQGLAE
RGIAVLRFDFTGLGASEGDFGNTGFTSNIDDLIAAASFLREEHEAPTILIGHSLGGAAVLR
AAEAIPEAAAVATIGAPADPAHVAHLLEDKADEIRDKGRATVNIGGRPFDIRAEFLDDIT
ANRPRDYIGDLRKALIVFHGPRDQIVGIENAAEIFTAAKHPKSFVSLDDADHLLSRQQDA
DYVADVLSAWASRYIGETEKRTTPQPPDGITRVAESGTGRFTQDVWAGGHFLQADEPA
SFGGDNVGPTPYDLLSAALGACTTMTIRMYADRKKLPLEQVSVDVSHEKIHASDCADC
ETESGKVDRFSREITLSGDLDETQRARLLEIADKCPVHRTLLSEVKVETREVT -

SLC-6 esterase gene encodes a 470 amino acid protein with molecular size of 50 kDa. SLC-6 esterase contains a pentapeptide GHSLG which corresponds to GXSXG motif which is commonly found in serine hydrolases family mainly lipases, esterases, proteases etc (Fig.2.3). SLC-6 esterase shows two domains, commonly found α/β hydrolase fold domain of family Abhydrolase_5 and OsmC superfamily domain found in many osmotically inducible proteins suggesting it to be induced due to high salt concentration found in sea environment. A putative signal sequence was detected in SLC-6 esterase using SignalIP server 3.0 which encompasses first 25 amino acids. A putative cleavage site was predicted between amino acid 34 and 35. This suggests that SLC-6 esterase can probably be a secretory protein.

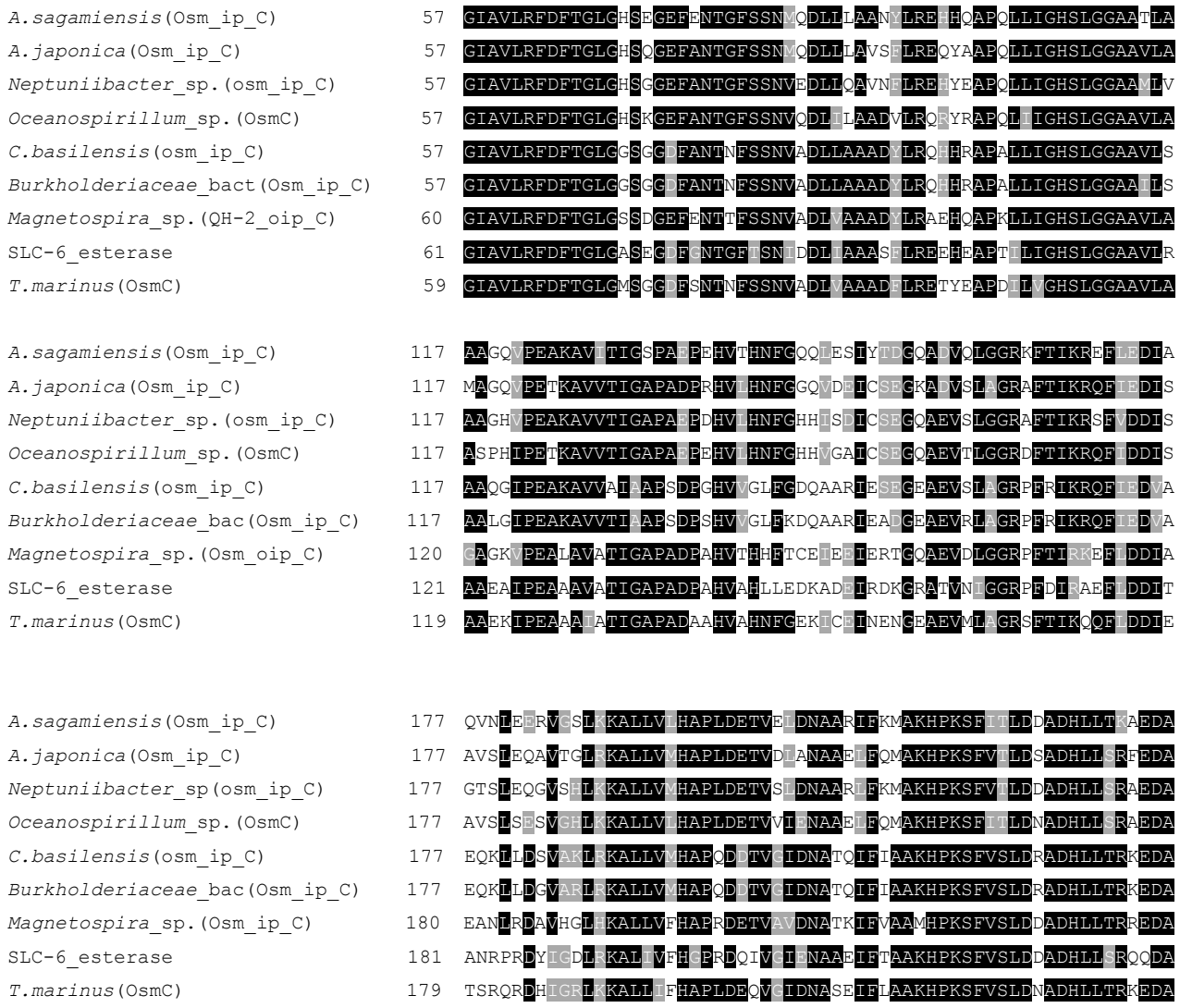


Fig. 2.3 Multiple sequence alignment of SLC-6 esterase with closely related sequences. The amino acid sequence corresponds to SLC-6 esterase (GI accession no. ADM63085.1), *A. sagemiensis* osmolytically inducible protein (WP_018691762.1 ,58 % identity with SLC-6 esterase), *A.japonica* osmolytically inducible protein (WP_019622077.1, 61 % identity), *Neptunibacter* species osmolytically inducible protein (KXJ50790.1, 58 % identity), *Oceanospirillum* species osmolytically inducible protein (WP_028301466.1,56 %% identity), *C. basilensis* osmolytically inducible protein (EHP38162.1, 59 % identity), *Burkholderiaceae bacterium* osmolytically inducible protein (WP_045240539.1, 58 % identity), *Magnetospira* species osmolytically inducible protein (WP_046021068.1, 62 % identity), *T. marinus* Osm C family protein (WP_045445689.1, 64 % identity). Residues that are 100% conserved are shadowed in black, and those between 75% and 100% are shadowed grey.

2.2.2.2 Phylogenetic analysis

BlastP results shows homology between SLC-6 esterase with similar Osm C family proteins from various proteobacterias. Based on phylogenetic analysis, SLC-6 esterase is

closely related to *Nesterenkonia* species Osm C family protein along with *Magenetospira* and *Halomonas* species Osmolytically inducible proteins on evolutionary scale. SLC-6 esterase is distantly related to Osm C family proteins from bacterial species such as *Alpha proteobacterium*, *Amphritea japonica*, *Neptunibacter*, *Oceanospirrulum, Algicola sagamiensis, Pseudorhodobacter* species etc. (Fig. 2.4).

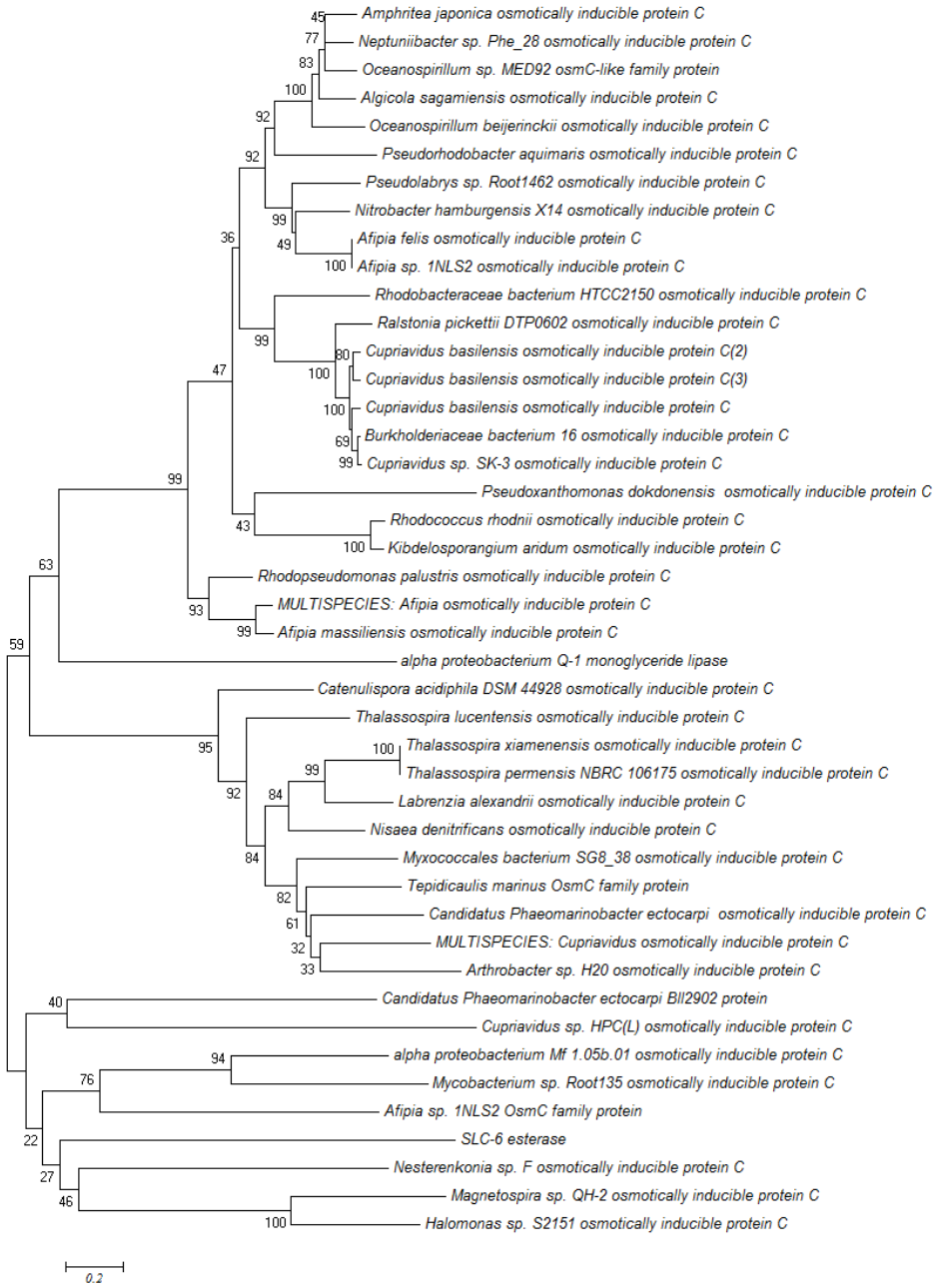


Fig. 2.4 Phylogenetic tree of SLC-6 esterase and other closely related enzymes. The phylogenetic analysis was performed by the neighbour joining method using MEGA 6.0 software. The values at nodes indicate the bootstrap percentage of 1,000 replicates.

2.2.2.3 Expression and purification of the recombinant SLC-6 esterase

SLC-6 esterase gene was over expressed in C41 (DE3) cells to get His-tagged pure protein. SLC-6 esterase was purified from the cell lysate using Ni-NTA agarose affinity chromatography. Purified protein was analyzed on 12 % sodium dodecyl sulphate polyacryl amide gel electrophoresis (SDS-PAGE) gel and proteins were corresponded well with their expected mass of ~70 kD due to additional *Trx* solubilisation domain and His-tag present in pET 32-a vector (Fig. 2.5).

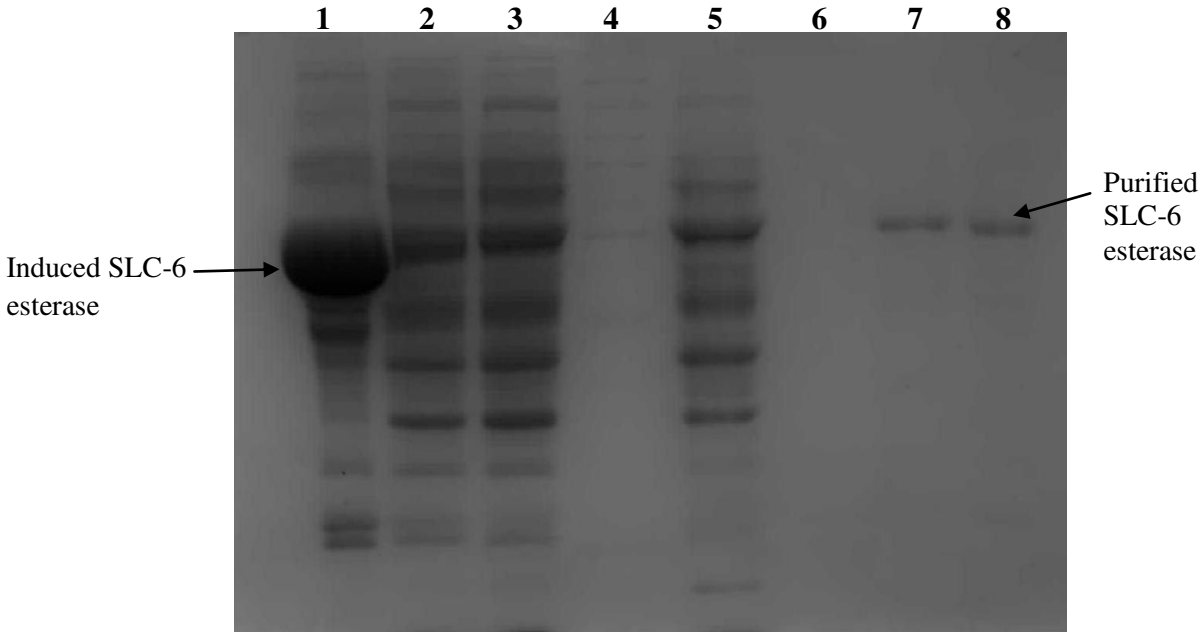


Fig. 2.5 SDS gel for purification of SLC-6 esterase using Ni-NTA agarose chromatography. **Legends: Lane 1**: Induced pellet of SLC-6 esterase expressed in pET 32-a vector. **Lane 2**: Induced supernatant of SLC-6 esterase expressed in pET 32-a vector. **Lane 3**: Unbound fraction **Lane 4**: 20 mM imidazole wash fraction **Lane 5**: Protein marker **Lane 6**: 60 mM imidazole wash fraction. **Lane 7**: Elution fraction 1 (100 mM imidazole).**Lane 8**: Elution fraction 2 (100 mM imidazole).

2.2.2.4 Effect of pH and temperature on the activity of SLC-6 esterase

Optimum pH was SLC-6 esterase was determined using pH range from 4 to 11 using *p*-nitrophenol butyrate as a substrate. SLC-6 esterase displayed to be highly active at pH of 7.5 which is commonly observed in case of many enzymes [Fig. 2.6 (A)]. Optimum temperature of SLC-6 esterase was determined by calculating initial reaction velocity of both the enzymes at

various temperature ranges. SLC-6 esterase was exhibiting highest reaction velocity at temperature of 30 ºC suggesting its origin to be from mesophillic microorganisms [(Fig. 2.6 (B)].

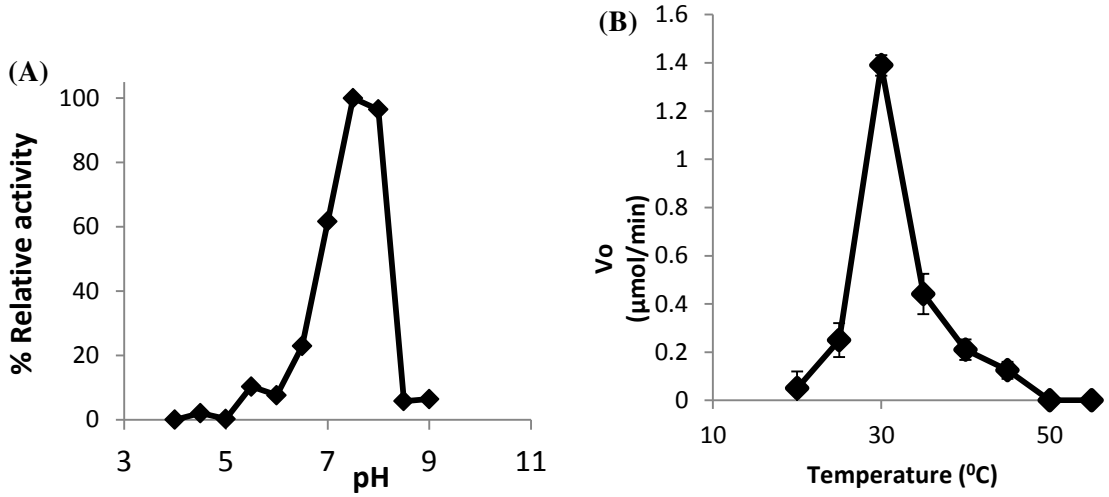


Fig. 2.6 Study of optimum pH and temperature of SLC-6 esterase enzyme (**A**) Optimum pH study of SLC- 6 esterase (**B**) Optimum temperature study with SLC-6 esterase. All the experiments were carried out in triplicates and error bars shows the standard deviation.

2.2.2.5 Determination of substrate specificity and effect of metal ions on the activity of SLC-6 esterase enzyme

Different fatty acid esters of *p*-nitrophenol were tested for determining the substrate specificity of SLC-6 esterase enzymes. A SLC-6 esterase enzyme was found to be highly active towards *p*-nitrophenol butyrate [*p*-nitrophenol butyrate (C4)] and activity was decreasing towards higher carbon number esters (C6-C18). Higher activity towards short chain esters of *p*-nitrophenol confirms that enzymes to be esterases than lipases. [(Fig. 2.7 (A)].

Various metal ions either show inhibition or enhancement in the activity of esterases. SLC-6 esterase was completely inhibited by the salt $NiCl_2$ to 0 %, while salts such as $CoCl_2$, $CuCl_2$, $ZnCl_2$ and $HgCl_2$ decrease enzyme activity drastically to 20.9 5, 25.7 %, 25.5 % and 27.9 % respectively. SLC-6 esterase was partially inhibited by use of $CaCl_2$, $FeSO_4$ and NH_4Cl to 62.3 %, 68 % and 98.2 % respectively. Salts such as $MgCl_2$, KCl and NaCl increased enzyme activity drastically to 108.9 %, 145.1 % and 110.3 % respectively [Fig. 2.7 (B)].

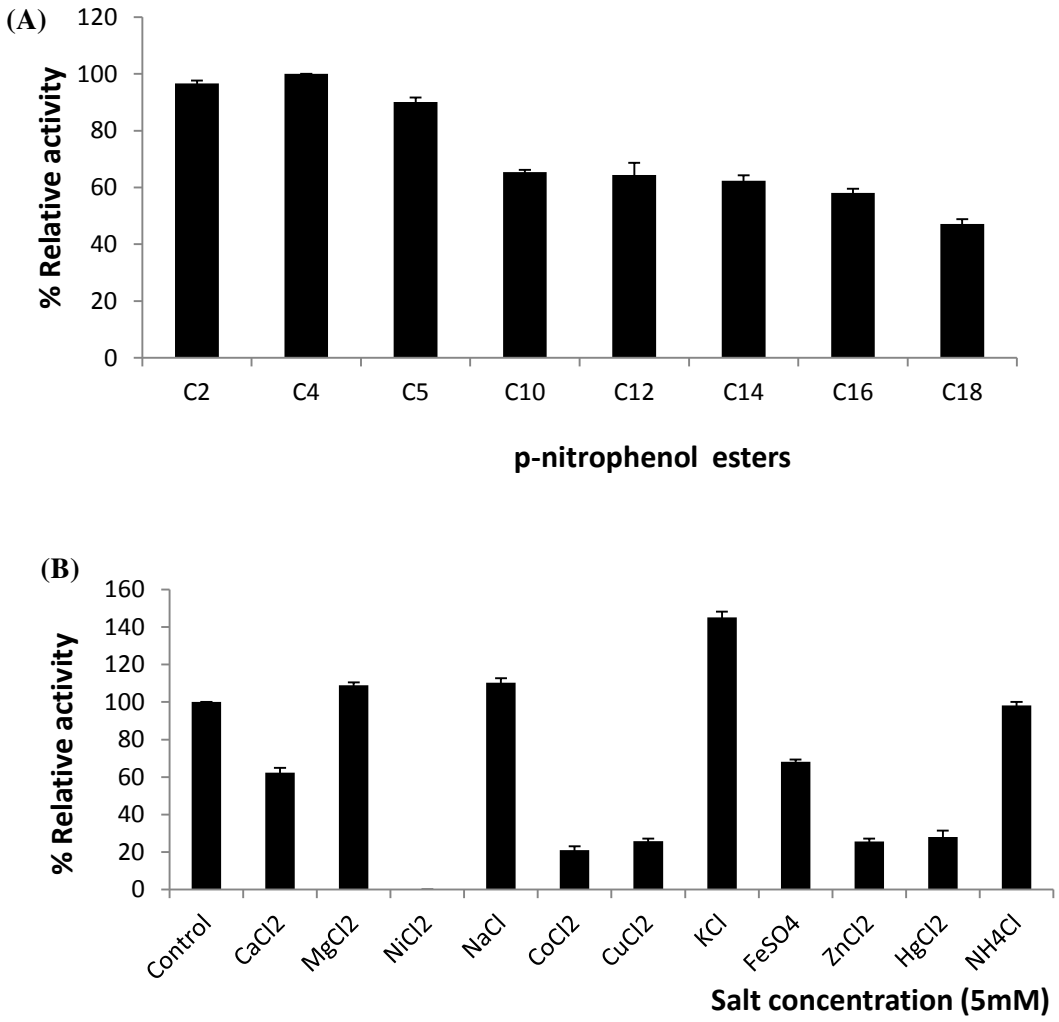


Fig. 2.7 Substrate specificity study and effect of various metal ions on the activity of SLC-6 esterase enzyme (**A**) Determination of chain length specificity with *p*-nitrophenol esters of fatty acid with SLC- 6 esterase (**B**) Study of different salts at the final concentration of 5 mM with SLC-6 esterase enzyme. All the experiments were carried out in triplicates and error bars shows the standard deviation.

2.2.2.6 Effect of organic solvents, chelating agents, inhibitors and detergents on the activity of SLC-6 esterase enzyme

SLC-6 esterase was studied for its stability in organic solvents. Organic solvents such as isopropanol, isoamyl alcohol, acetonitrile and carbon tetrachloride decreased SLC-6 enzyme activity drastically to 16.9 %, 12 %, 17.5 % and 26.4 % respectively. Solvent tetrahydrofuran completely inhibited the enzyme activity. Solvents such as toluene, ethanol, n-hexane and dichloromethane partially decreased enzyme activity to 62.3 %, 44.7 %, 39.4 % and 73.2 % respectively. Dimethyl sulfoxide (DMSO) increased enzyme activity drastically to 170 % when compared to the control activity. Enhancement or maintenance of the activity was

observed in case of polar/partially polar solvents such as dimethyl sulfoxide, ethanol, toluene and non polar solvents such as n-hexane and dichloromethane while inhibition was seen in case of isopropanol, isoamyl alcohol, acetonitrile and carbon tetrachloride [Fig. 2.8 (A)].

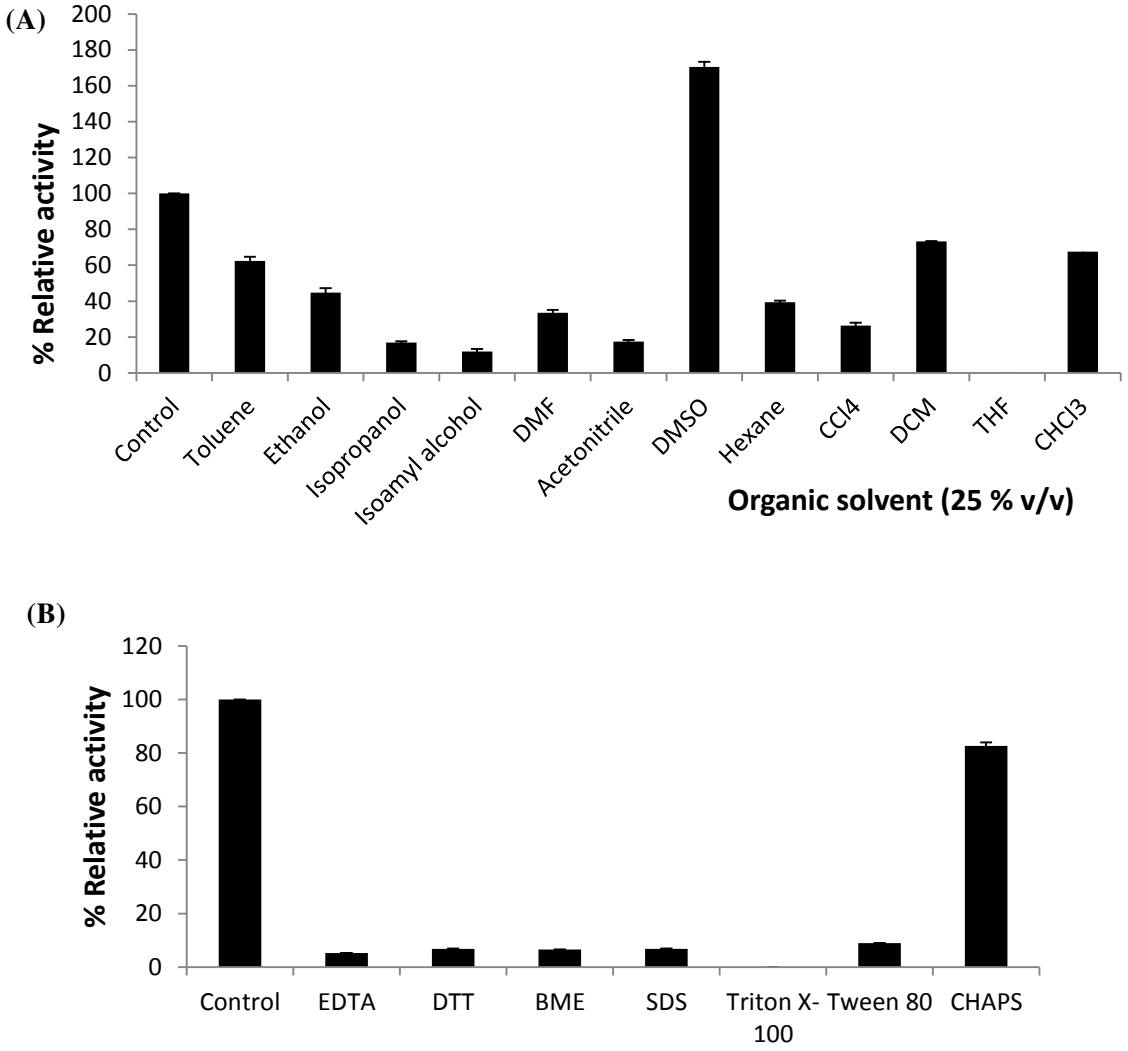


Fig. 2.8 Study of stability of enzymes in different organic solvents and effect of chelators, inhibitors and detergents on SLC-6 esterase enzyme (**A**) Determination of stability of SLC- 6 esterase in different polar and non polar solvents (**B**) Determination of stability of SLC- 6 esterase in presence of chelator (EDTA), inhibitors (DTT, BME, SDS) and detergents (Triton X-100,Tween 80, CHAPS). All the experiments were carried out in triplicates and error bars shows the standard deviation.

Chelator EDTA (10 mM) decreased enzyme activity of SLC-6 esterase to 5 % suggesting a strong inhibition of the enzyme [Fig.2.8 (B)]. This suggests that the SLC-6 esterase enzymes do requires some metal ions for their optimal activity which is in accordance with literature. Inhibitors such as DTT and BME reduce the enzyme activity to 6.8 and 6.5 % respectively.

Non ionic detergents such as Triton x-100 and Tween 80 have strong inhibitory activity on SLC-6 esterase reducing its activity to 0 % and 8.8 % respectively. Triton X-100 completely nullifies the activity of SLC-6 esterase. Zwitter ionic detergent CHAPS, partially reduces activity to 82.6 % while ionic detergent sodium deodecyl sulphate (SDS) completely reduces the activity of enzyme to 6.8 %. In conclusion, SLC-6 esterase is strongly inhibited by non ionic detergents such as Triton x-100, Tween 80 and ionic detergents such as SDS. Zwitterionic detergent CHAPS found to be retaining the activity of SLC-6 esterase enzyme [(Fig. 2.8 (B)].

2.2.2.7 Determination of enzyme kinetic parameters

Enzyme kinetic parameters were studied SLC-6 esterase enzyme using *p*-nitrophenol butyrate as a substrate at their optimum pH and temperatures. Kinetic parameters were calculated using Michaelis menten graph plotted for both the enzymes [Fig 2.9].

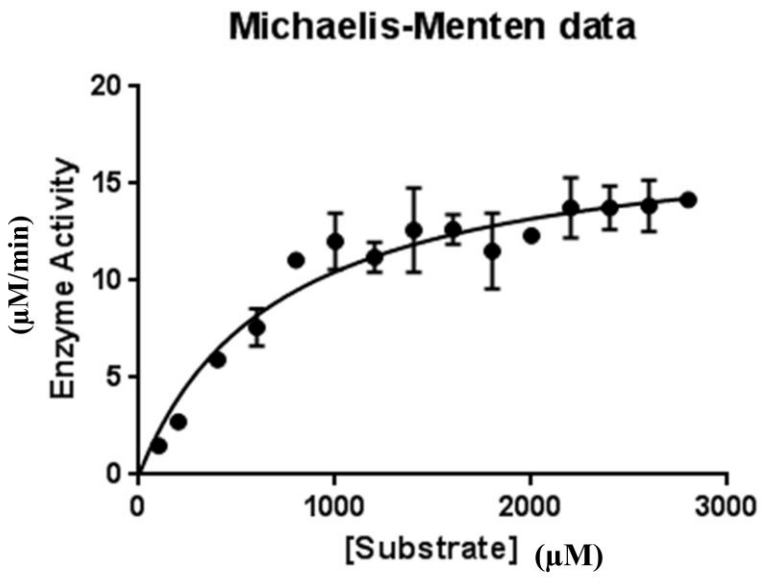


Fig. 2.9 Michealis menten graph for SLC-6 esterase enzyme. (Kinetics study was carried out in at each substrate concentration in triplicates. Graph was plotted using GraphPad Prism software).

SLC-6 esterase enzyme (50 nM) kinetics was studied with *p*-nitrophenol butyrate (C4) range from 50 µM to 2800 µM and it showed V_{max}, K_m, K_{cat} and K_{cat}/K_m value of 17.8 µM min^{-1}, 705 µM, 5.9 S^{-1} value 8425 $M^{-1}.S^{-1}$ respectively (Table 2.1). Kinetic parameters of this enzyme are comparable with many carboxyl esterases present in BRENDA enzyme database.[37]

Enzyme	Substrate	V_{max} ($\mu M\ min^{-1}$)	K_m (μM)	K_{cat} (S^{-1})	K_{cat}/K_m ($M^{-1}.S^{-1}$)
SLC-6 esterase	C4	17.84	705	5.94	8425.5

Table 2.1 Enzyme kinetic parameters of SLC-6 esterase enzyme

2.2.2.8 Enantioselective hydrolysis of racemic acyclic/aromatic acetates by SLC-6 esterase

Time course study of hydrolysis of various racemic acetates (**1**), (**2**), (**3**), (**4**), (**5**), (**6**), (**7**) and (**8**) were carried out using purified SLC-6 esterase enzyme (Fig 2.15). Enantioselective hydrolysis of acetates was carried out at 30 ºC and 200 rpm in incubator shaker (Table 2.2).

Acyclic racemic acetates such as (**1**) and (**2**) were hydrolysed to (*R*)-(-)-2-pentanol (**1a**) [(Fig. 2.15 (**A**)] and (*R*)-(-)-2-hexanol (**2a**) (Fig. 2.10) [(Fig. 2.15 (**B**)] respectively with 99.9 % e.e and 92.9 % e.e with 6.7 % and 16.7 % conversion in 48 h by SLC-6 esterase.

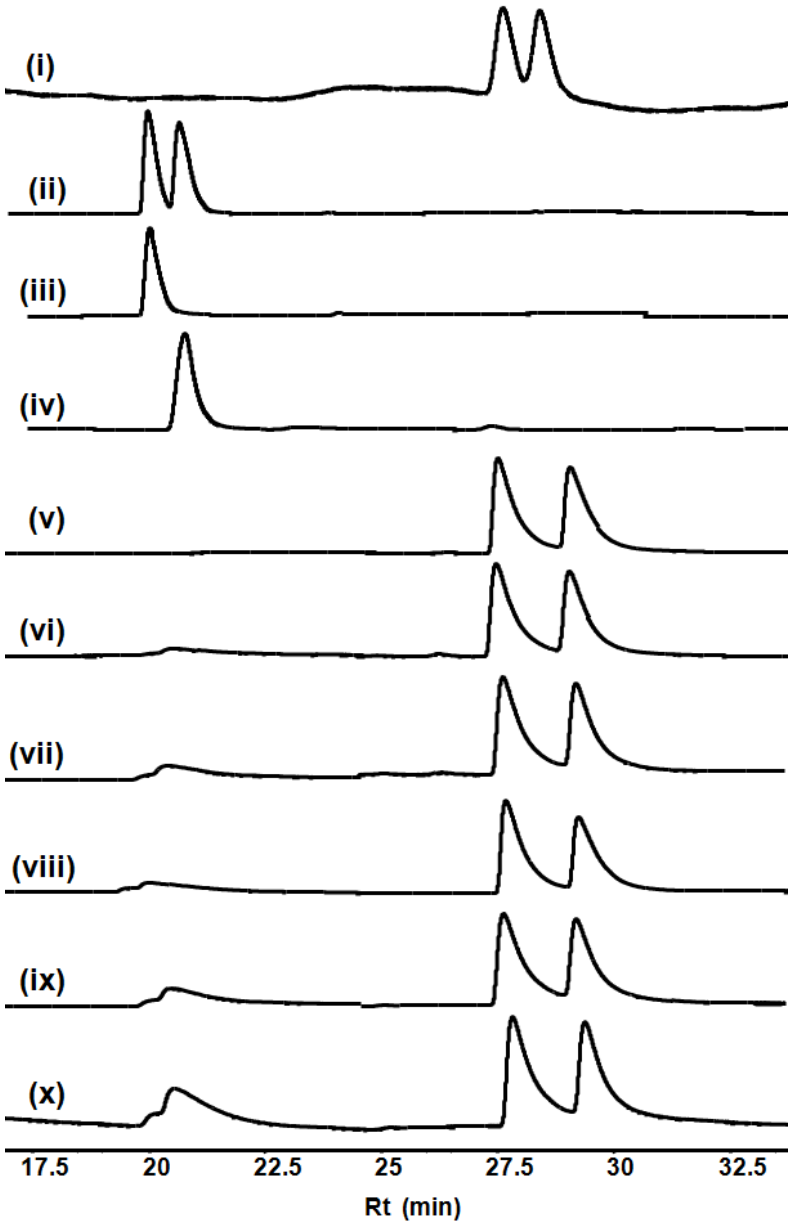


Fig. 2.10 Time Course study for the conversion of (±)-2-Hexyl acetate by SLC-6 esterase.
(i) Standard (±)-2-Hexyl acetate (ii) Standard (±)-2-Hexanol (iii) Standard (*S*)-(+)-2-Hexanol (iv) Standard (*R*)-(-)-2-Hexanol (v) Conversion of (±)-2-Hexyl acetate after 6 h incubation (vi) Conversion of (±)-2-Hexyl acetate after 18 h incubation (vii) Conversion of (±)-2-Hexyl acetate after 24 h incubation (viii) Conversion of (±)-2-Hexyl acetate after 30 h incubation (ix) Conversion of (±)-2-Hexyl acetate after 42 h incubation (x) Conversion of (±)-2-Hexyl acetate after 48 h incubation .

Compounds (**3**) and (**4**) hydrolysed to (*R*)-(-)-3-hexanol (**3a**) (Fig. 2.11) [Fig. 2.15 (**C**)] and (*S*)-(+)-1-octen-3-ol (**4b**) [Fig. 2.15 (**D**)] respectively with 99.9 %, 33 % e.e and 0.9 %, 96.6 % conversions.

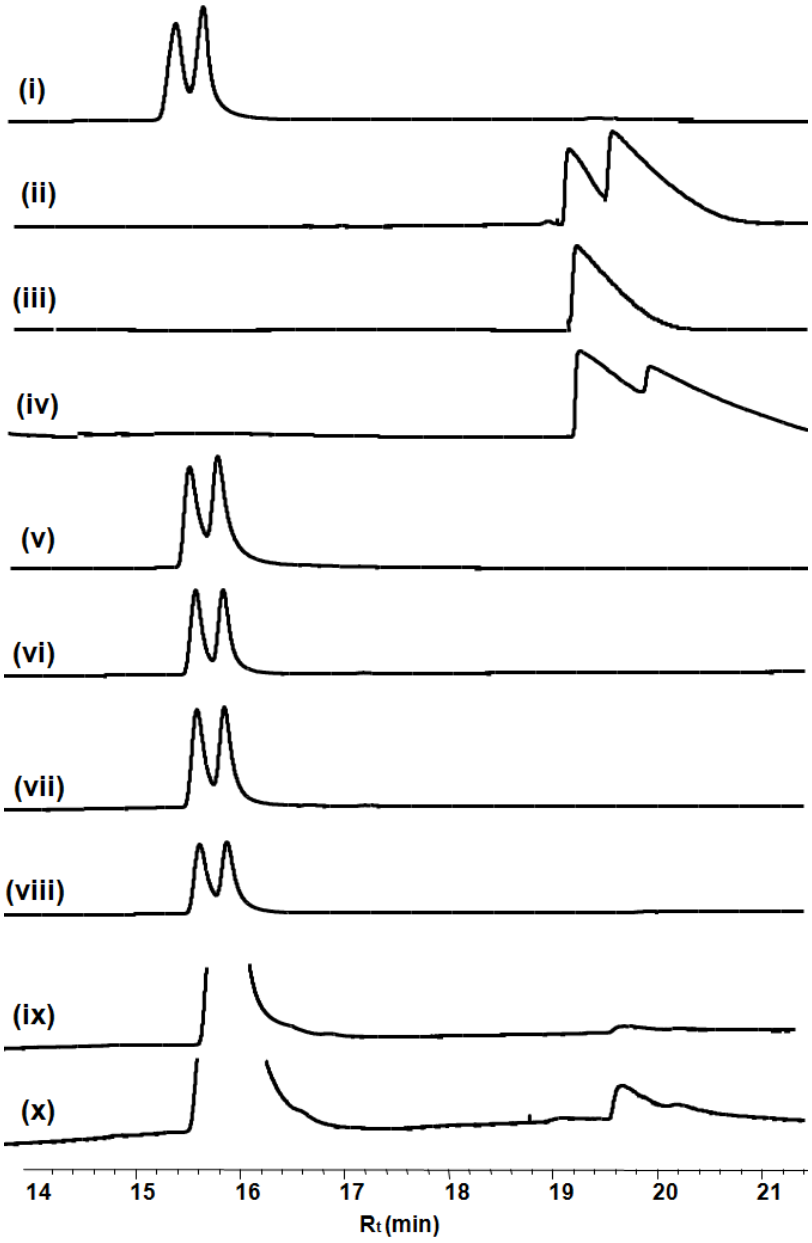


Fig.2.11 Time Course study for the conversion of (±)-3-Hexyl acetate by SLC-6 esterase.
(i) standard (±)-3-Hexyl acetate (ii) standard (±)-3-Hexanol (iii) standard (*R*)-3-Hexanol (iv) Co injection of (±)-3-Hexanol and (*R*)-3-Hexanol (v) Conversion of (±)-3-Hexyl acetate after 6 h incubation (vi) Conversion of (±)-3-Hexyl acetate after 18 h incubation (vii) Conversion of (±)-3-Hexyl acetate after 24 h incubation (viii) Conversion of (±)-3-Hexyl acetate after 30 h incubation (ix) Conversion of (±)-3-Hexyl acetate after 42 h incubation (x) Conversion of (±)-3-Hexyl acetate after 48 h incubation.

SLC-6 esterase hydrolysed aromatic acetates such as (**5**) and (**7**) to form (*R*)-(+)-1-phenyl ethanol (**5a**) (Fig.2.12) [Fig. 2.15 (**E**)] and (*R*)-(+)-3-methyl-1-phenyl ethanol (**7a**) (Fig. 2.13) [Fig. 2.15 (**F**)] with e.e of 90 %, 95.4 % and 15.5 and 39.5 % conversion respectively.

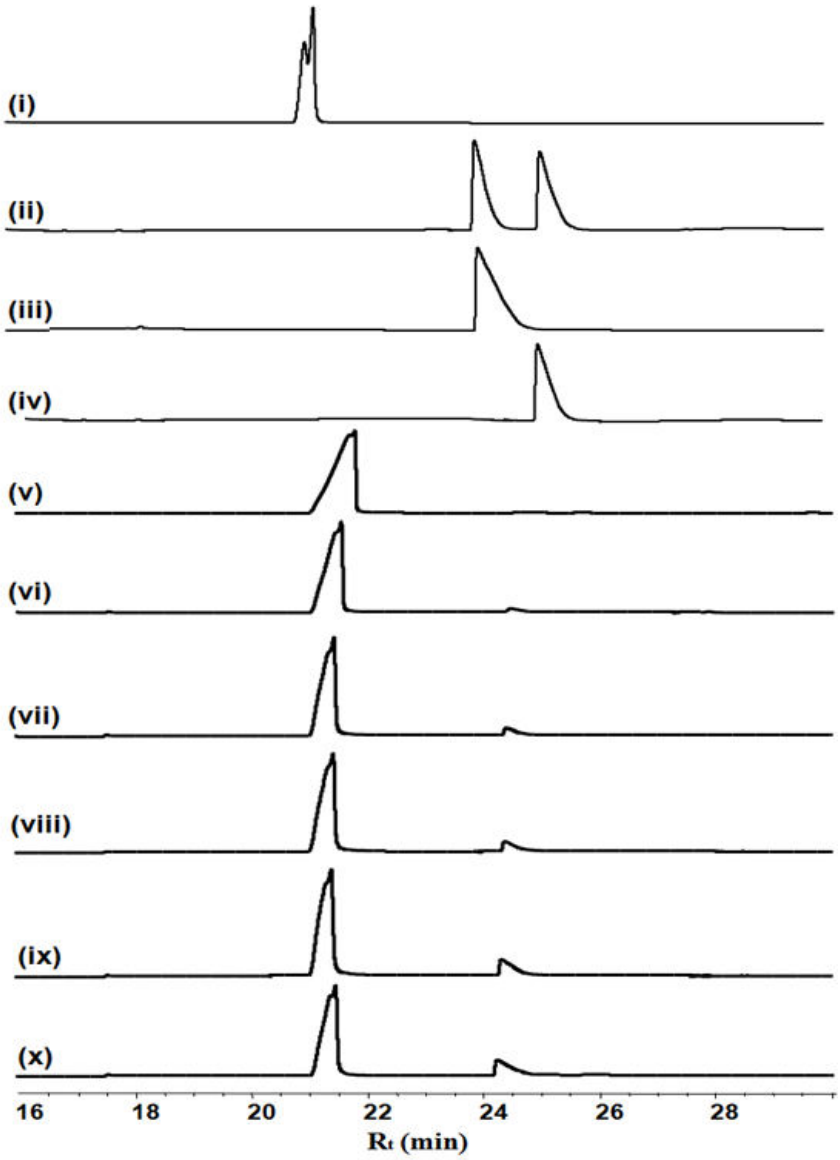


Fig. 2.12 Time Course study for the conversion of (±)-1-Phenyl ethyl acetate by SLC-6 esterase. (i) standard (±)-1-Phenyl ethyl acetate (ii) standard (±)-1-Phenyl ethanol (iii) standard (*R*)-1-Phenyl ethanol (iv) standard (*S*)-1-Phenyl ethanol (v) Conversion of (±)-1-Phenyl ethyl acetate after 6 h incubation (vi) Conversion of (±)-1-Phenyl ethyl acetate after 18 h incubation (vii) Conversion of (±)-1-Phenyl ethyl acetate after 24 h incubation (viii) Conversion of (±)-1-Phenyl ethyl acetate after 30 h incubation (ix) Conversion of (±)-1-Phenyl ethyl acetate after 42 h incubation (x) Conversion of (±)-1-Phenyl ethyl acetate after 48 h incubation .

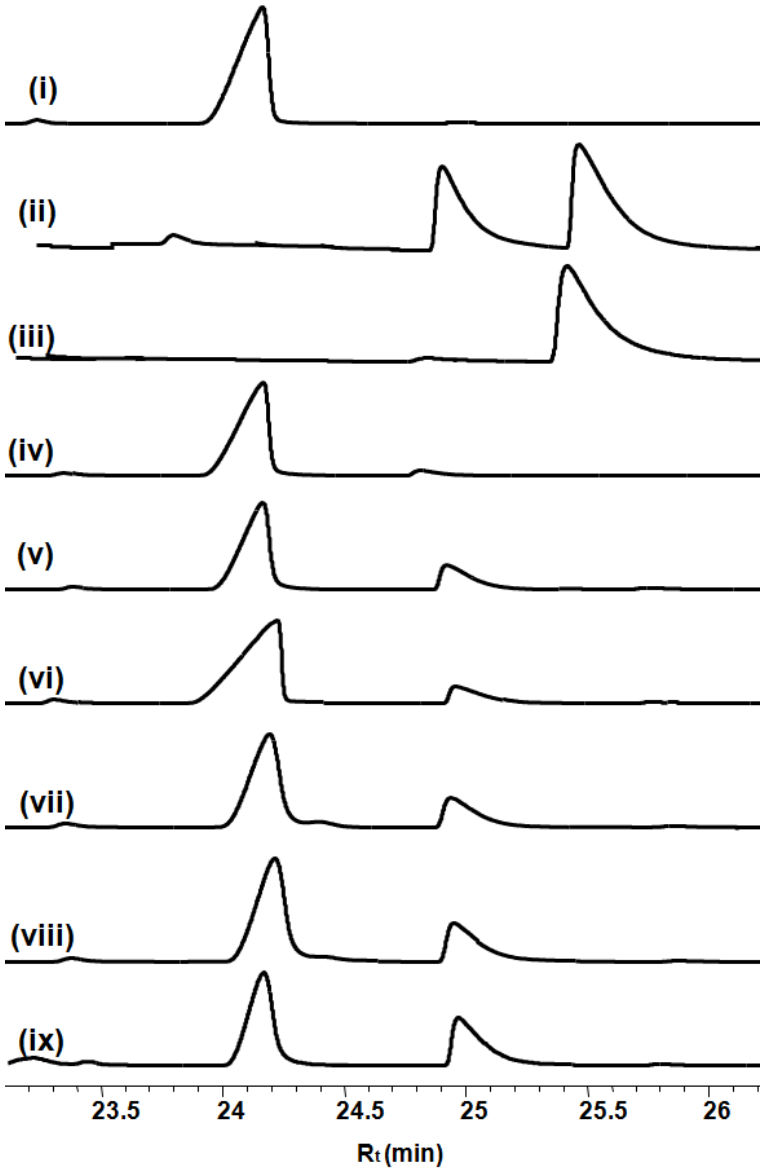


Fig. 2.13 Time Course study for the conversion of (±)-3-methyl-1-Phenyl ethyl acetate by SLC-6 esterase.

(i) standard (±)-3-methyl-1-Phenyl ethyl acetate (ii) standard (±)-3-methyl-1-Phenyl ethanol (iii) standard (*S*)-3-methyl-1-Phenyl ethanol (iv) Conversion of (±)-3-methyl-1-Phenyl ethyl acetate after 6 h incubation (v) Conversion of (±)-3-methyl-1-Phenyl ethyl acetate after 18 h incubation (vi) Conversion of (±)-3-methyl-1-Phenyl ethyl acetate after 24 h incubation (vii) Conversion of (±)-3-methyl-1-Phenyl ethyl acetate after 30 h incubation (viii) Conversion of (±)-3-methyl-1-Phenyl ethyl acetate after 42 h incubation (ix) Conversion of (±)-3-methyl-1-Phenyl ethyl acetate after 48 h incubation .

Compound (**6**) was not hydrolysed by the enzyme suggesting interference of methyl group at C2 of the phenyl ring with the active site pocket. Compound (**8**) was hydrolysed to (*R*)-(+)-1-phenylpropanol (**8a**) with 99.9 % e.e and 5 % conversion (Fig. 2.14) [Fig. 2.15 (**G**)].

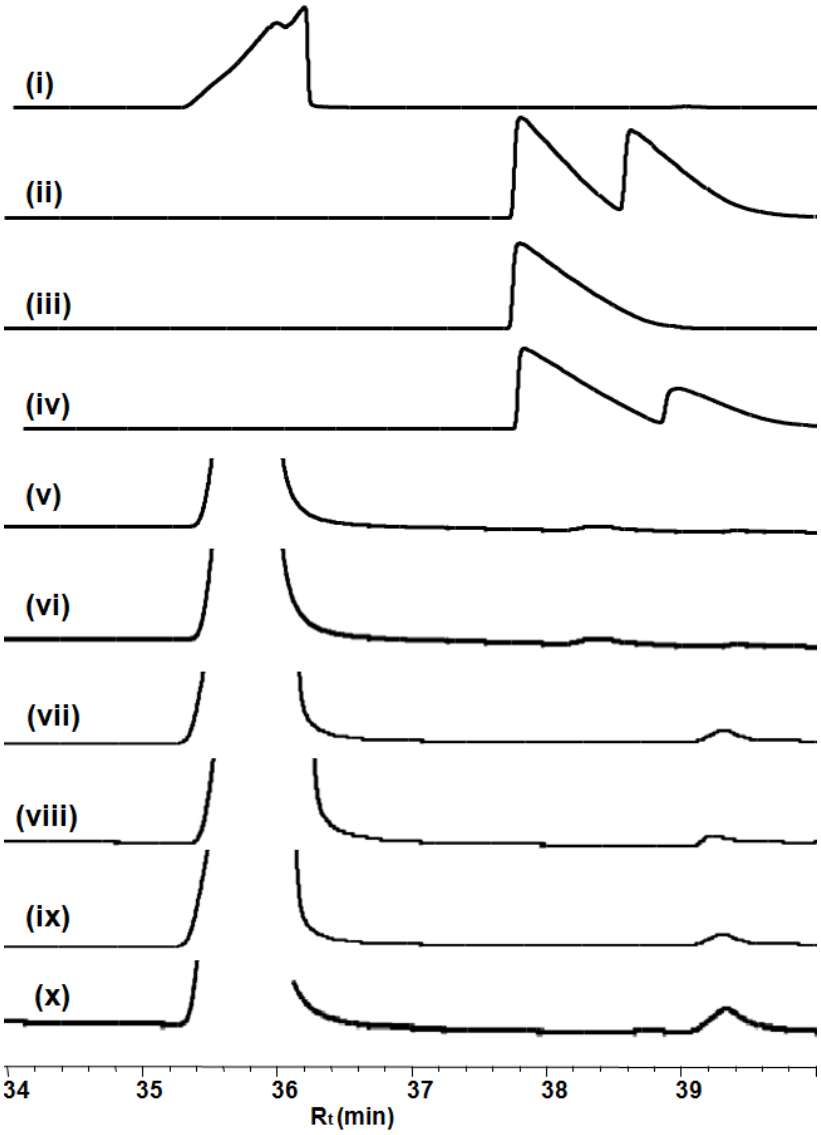
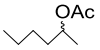
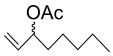
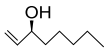


Fig.2.14 Time Course study for the conversion of (±)-1-Phenylpropyl acetate by SLC-6 esterase. (i) standard (±)-1-Phenylpropyl acetate (ii) standard (±)-1-Phenylpropanol (iii) standard (*R*)-1-Phenylpropanol (iv) Co injection of (±)-1-Phenylpropanol and (*R*)-1-Phenylpropanol (v) Conversion of (±)-1-Phenylpropyl acetate after 6 h incubation (vi) Conversion of (±)-1-Phenylpropyl acetate after 18 h incubation (vii) Conversion of (±)-1-Phenylpropyl acetate after 24 h incubation (viii) Conversion of (±)-1-Phenylpropyl acetate after 30 h incubation (ix) Conversion of (±)-1-Phenylpropyl acetate after 42 h incubation (x) Conversion of (±)-1-Phenylpropyl acetate after 48 h incubation .

From above results, it can be observed that increase in carbon length from C5 to C8 increased the conversion rate but decreased the overall e.e of the resulting alcohols. These results suggest that SLC-6 esterase is a low carbon chain specific thus confirming to be an esterase.

Entry	Compounds	Conversion (%)	Product	Time (h)	Configuration and % e.e*
1	 **(1)**	6.7	 **(1a)**	48	*R*,99.9
2	 **(2)**	16.7	 (**2a**)	48	*R*,92.9
3	 **(3)**	0.9	 **(3a)**	48	*R*,99.9
4	 **(4)**	96.6	 **(4b)**	42	*S*,33
5	 **(5)**	15.5	 **(5a)**	48	*R*,90
6	 **(6)**	--	**NR**[#]	48	**NR**[#]
7	 **(7)**	39.5	 **(7a)**	48	*R*,95.4
8	 (**8**)	5	 (**8b**)	48	*S*,99.9

*e.e = enantiomeric excess,# NR= No Reaction

Table 2.2 Enantioselective hydrolysis of racemic acetates using SLC-6 esterase [Racemic acetate concentration= 4 mg, SLC-6 esterase enzyme concentration and specific activity = 2 mg, 1.3 IU/mg. All incubations were done at 30 ºC and 200 rpm in incubator shaker for 48 h.

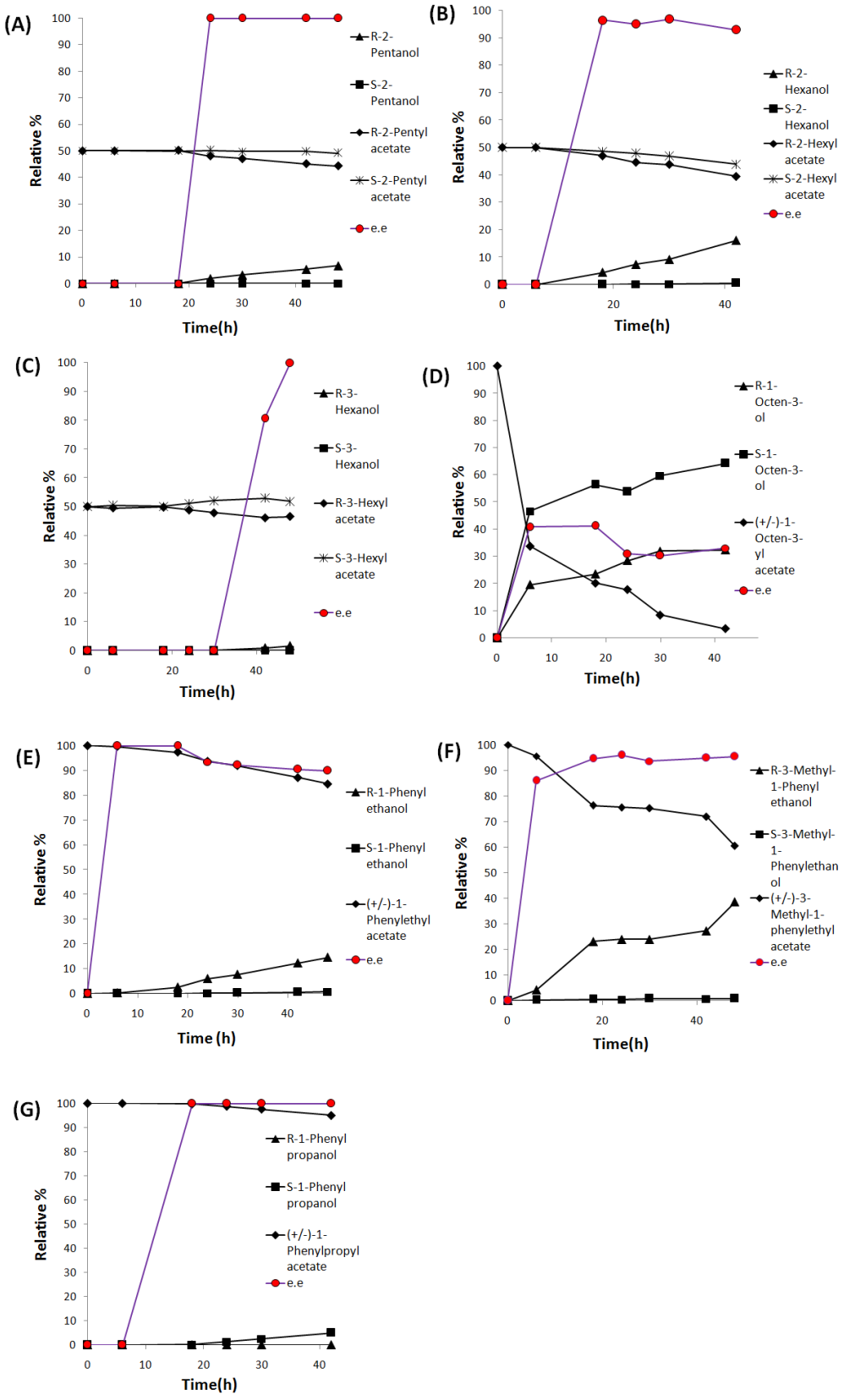


Fig. 2.15 Time-course experiment of kinetic resolution achieved using SLC-6 esterase in graphical representation with **(A)** (±)-2-pentyl acetate **(B)** (±)-2-hexyl acetate, **(C)** (±)-3-hexyl

acetate, **(D)** (±)-1-octen-3-yl acetate, **(E)** (±)-1-phenylethyl acetate, (**F**) (±)-3-methyl-1-phenylethyl acetate, **(G)** (±)-1-phenylpropyl acetate respectively.

2.2.2.9 Molecular docking analysis

The molecular docking studies were carried out between (*R*) and (*S*)-acetates of the substrates and SLC-6 esterase enzyme. (*R*) and (*S*)-acetates of six compounds [(**1**),(**2**),(**3**),(**5**),(**7**) and (**8**)] were individually docked with the protein model of SLC-6 esterase. Docked complexes provide the information about binding pose and atomic interaction between active site of SLC-6 esterase and substrates. Interaction in active site residues and reactive centers of the substrates are stearically well positioned in the case of favorable interactions. Binding affinity with docking of ligands in the active site of enzyme displayed that (*R*)-acetates of the compounds bind fit inside the active site, while substrate (*S*)-acetates showed stearic hindrance in the binding pocket (Fig. 2.16) (Table 2.3). Except in the case of substrate (**8**) where (*S*) acetate binds strongly compared to (*R*) acetate in the active site.

In case of short chain acyclic esters, (*R*)-2-pentyl acetate and (*R*)-2-hexyl acetate found to be interacting strongly compared to (*S*)-2-pentyl acetate and (*S*)-2-hexyl acetate with the active site residue of SLC-6 esterase [Fig. 2.16 (1a) and (1b)] [Fig.2.16 (2a) and (2b)]. Ligand (*R*)-3-hexyl acetate was also seems to be strongly binding with the stereo selective pocket of SLC-6 esterase enzyme compared to (*S*)-2-hexyl acetate as the difference seen in the binding affinity (Table 2.3) [Fig.2.16 (3a) and (3b)].

In case of cyclic/aromatic acetates, (*R*)-1-phenylethyl acetate [Fig.2.16 (4a)] and (*R*)-3-methyl-1-phenylethyl acetate [Fig. 2.16 (5a)] were binding strongly deep inside the stereo selective pocket with minimum binding affinity as compared to counter substrates such as (*S*)-acetates such as (*S*)-1-phenylethyl acetate [Fig.2.16 (5b)], (*S*)-3-methyl-1-phenylethyl acetate [Fig. 2.16 (6b)] respectively. Aromatic acetate, (*S*)-1-phenylpropyl acetate was showing high binding affinity as compared to its corresponding (*R*)-1-phenylpropyl acetate with the active site of enzyme which in reality is also observed in wet lab experiments [Fig. 2.16 (6a) and (6b)].

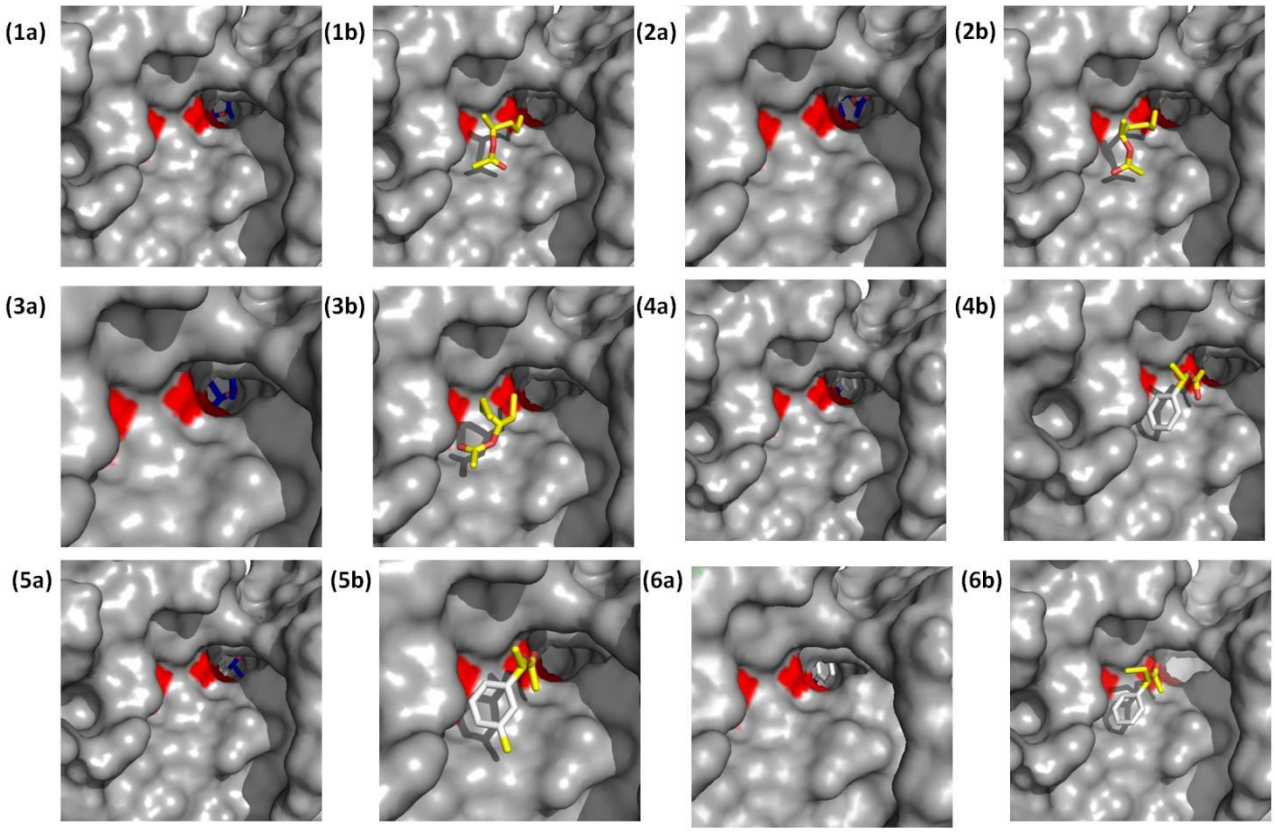


Fig. 2.16 Docking analysis of various substrates at the active site pocket of SLC-6 esterase protein model. (**1a**)(*R*)-2-pentyl acetate, (**1b**) (*S*)-2-pentyl acetate, (**2a**) (*R*)-2-hexyl acetate, (**2b**) (*S*)-2-hexyl acetate, (**3a**) (*R*)-3-hexyl acetate, (**3b**) (*S*)-3-hexyl acetate, (**4a**) (*R*)-1-phenylethyl acetate, (**4b**) (*S*)-1-phenylethyl acetate, (**5a**) (*R*)-3-methyl-1-phenylethyl acetate, (**5b**) (*S*)-3-methyl-1-phenylethyl acetate, (**6a**) (*R*)-1-phenylpropyl acetate, (**6b**) (*S*)-1-phenylpropyl acetate. Amino acid residues highlighted in red are active sight residues containing GHSLG motif.

Sr. no.	Substrates	Binding affinity (Kcal/mol)	
		(*R*)-acetate	(*S*)-acetate
1	2-Pentyl acetate	-3.4	-2.7
2	2-Hexyl acetate	-3.8	-3.6
3	3-Hexyl acetate	-3.4	-3.3
4	1-Phenylethyl acetate	-4.0	-3.4
5	3-Methyl-1-Phenylethyl acetate	-3.8	-3.4
6	1-Phenylpropyl acetate	-2.8	-3.0

Table 2.3 Binding affinity in Kcal/mol of (*R*) and (*S*) acetates with SLC-6 esterase after molecular docking studies.

Binding poses and energy suggest that SLC-6 esterase utilizes (*R*)-acetates preferentially over (*S*) acetate to convert them into corresponding (*R*)-alcohols. Docking studies were found to be in well corroboration with *in vitro* studies depicting that SLC-6 esterase stereo selective pocket mostly preferred (*R*)-acetates over the (*S*)-acetates except in case of compound (**8**) where reverse is observed.

2.3 Cloning, expression & characterization of novel enantioselective esterase MLC-3 obtained through hot spring microbial mat metagenomic library.

2.3.1 Materials and method

2.3.1.1 Chemicals

Peptone, yeast extract, malt extract, glucose, agar, Luria Bertani broth (L.B broth) used for bacterial culture maintenance and protein over expression were purchased from Himedia Laboratories limited, Mumbai. Kit used for metagenomic DNA isolation and library preparations were purchased from Epicentre Company, USA. All the recombinant DNA technology experiment kits were purchased from Invitrogen, USA.

All the *p*-nitrophenol (*p*-nitrophenol) esters *p*-nitrophenol acetate (C2), butyrate (C4), valerate (C5), caprylate (C8), decanoate (C10), laurate (C12), myristate (C14), palmitate (C16) and stearate (C18) were purchased from Sigma Aldrich, USA. Racemic alcohols (±)-2-pentanol, (±)-2-hexanol, (±)-3-hexanol, (±)-1-octen-3-ol, (±)-1-phenylethanol, (±)-2-methyl-1-phenylethanol, (±)-3-methyl-1-phenylethanol and (±)-1-phenylpropanol were purchased from Sigma Aldrich/Fluka, USA.

Racemic esters (±)-2-pentyl acetate (**1**), (±)-2-hexyl acetate (**2**), (±)-3-hexyl acetate (**3**), (±)-1-octen-3-yl acetate (**4**), (±)-1-phenylethyl acetate (**5**), (±)-2-methyl-1-phenylethyl acetate (**6**), (±)-3-methyl-1-phenylethyl acetate (**7**) and (±)-1-phenylpropyl acetate (**8**) were purchased from Sigma Aldrich/Fluka, USA. All the pure enantiomeric (*R*) and (*S*) standards of alcohols were purchased from Sigma Aldrich USA.

2.3.1.2 Bacterial strain, plasmid used

pUC-19 and pET 32-a vector used for library preparation and over expression of proteins were purchased from Thermo Fischer scientific, USA and Novagen, USA company respectively. *E. coli* DH5α, Rosetta (DE3) cells (Novagen) were used for the transformation and over expression of MLC-3 esterase recombinant protein.

2.3.1.3 Cloning of MLC-3 esterase gene

MLC-3 esterase gene was selected from the pUC-19 metagenomic libraries prepared from hot spring microbial mat sample collected from Khir Ganga region, Himachal Pradesh, India. (Dr. Ravi Ranjan's PhD thesis titled, "Functional Metagenomics to identify novel genes for biocatalysts. Supervisors: Prof. Rup Lal and Dr. Rakesh Sharma, Th-15607, Delhi University). MLC-3 esterase gene encodes a 843 bp of open reading frame (ORF) region which was further cloned in pET 32-a vector using following primers: Forward primer: 5'-CAT GCC ATG GGC ATG AAT CAC CGT GAT GGT ATC -3' and Reverse primer: 5'-CGC GC GGA TCC AGC CTC ACA GGG CAG GCG CTC -3' . (Underlined are the restriction sites for Nco-I and Bam-HI restriction enzymes).PCR was performed using high fidelity "Accuprime *pfx* polymerase" using following PCR conditions: One cycle: 95 ºC for 5 min. (Initial denaturation), 35 cycles: denaturation: 95 ºC for 30 sec., 65 ºC for 30 sec. (Primer annealing) and 68 ºC for 1 min (DNA amplification), One cycle: 10 min. at 68 ºC (final hold) and then at 4 ºC for infinite time. PCR amplified MLC-3 gene and pET 32-a vector were digested with Nco-I and Bam-HI restriction enzymes was then loaded and purified from the agarose gel using Gel purification kit (Invitrogen). Digested vector and MLC-3 gene were then ligated using T4 DNA ligase (Invitrogen) enzyme as per standard protocol to get MLC-3-pET 32-a plasmid construct.

2.3.1.4 Over expression and protein purification of MLC-3 esterase

Rosetta (DE3) cells containing construct MLC-3-pET 32-a was inoculated into 1 litre Terrific Broth (T.B) media [tryptone (12 g), yeast extract (24 g), potassium phosphate dibasic-(9.4 g), potassium phosphate monobasic (2.2 g), glycerol (4 mL), pH-7.2]. When cell reached O.D (600 nm) ~ 0.6-0.8, expression was induced with 1mM IPTG at 16 ºC for 12 hrs with shaking condition (200 rpm).After induction, cells were kept 15 min on ice and then pelleted down at 4000 X g and 4 ºC for 20 min. Cell pellet thus obtained was lysed using lysis buffer [Tris-HCl buffer (50 mM) pH 8.0, 300 mM NaCl, 0.5 % CHAPS, lysozyme (1mg/mL),1 mM PMSF,10 % glycerol] using ultrasonic cell disruptor (Sonics vibracell ultrasonic cell disruptor) at 6 pulses each with 30 sec on off cycles at 80 % amplitude for 10 min. Lysed cells extract was clarified at 10,000 X g for 15 min and was kept for binding with Ni-NTA agarose resin (1 mL Ni-NTA resin for 5 mL of clarified cell extract) for 1 hour at 4 ºC. Protein bound Ni-NTA resin was then washed with wash buffer [Tris-HCl buffer (50 mM) pH 8.0, 300 mM NaCl, 20 mM imidazole, 10 % glycerol]. Final elution was done with elution buffer [Tris-HCl buffer (50 mM) pH 8.0, 300 mM NaCl, 250 mM imidazole, 10 % glycerol] to get well purified protein.

Protein thus obtained checked for purity over SDS PAGE gel and protein concentration was determined by Bradford's method using bovine serum albumin (BSA) as a standard.[31]

2.3.1.5 Enzyme activity assay and Molar extinction coefficient of *p*-nitrophenol

Esterase activity was assayed using *p*-nitrophenol palmitate (*p*-nitrophenol palmitate) as a substrate for purified enzyme as reported earlier.[32] The assay conditions were as follows: *Solution A*: 37 mg of *p*-nitrophenol palmitate in 10 mL isopropanol. *Solution B*: 0.1 g Gum Arabic and 0.4 g Triton X-100 in 90 mL distilled water. 1 mL of solution A was mixed with 9 mL of solution B to make substrate solution. In the final reaction mixture, 0.9 mL of substrate solution was added into 0.1 mL buffer (potassium phosphate buffer, 10 mM, pH 7.0) and 0.1 mL (100 µg) esterase enzyme. Assay mixture was incubated at 37ºC for 30 min under shaking condition to quantitate release of *p*-nitrophenol in the reaction. Blank reaction was kept under similar conditions without enzyme. The release of *p*-nitrophenol from *p*-nitrophenol palmitate was spectrophotometrically determined at 410 nm using Cary 300 UV visible spectrophotometer (Varian). One unit of esterase activity was defined as the amount of enzyme releasing 1 µmol *p*-nitrophenol in 1 min under the assay conditions used. Experiments were conducted in triplicates and average values were calculated.

Molar extinction coefficient of *p*-nitrophenol was calculated at pH 8.0 in potassium phosphate buffer (10 mM). Three sets of each concentrations ranging from 20-200 µM of *p*-nitrophenol (99.9 % pure) were made and optical density was measured at 410 nm using UV visible spectrophotometer. Graph was plotted to calculate molar extinction coefficient. Molar extinction coefficient values for *p*-nitrophenol in potassium phosphate buffer (10 mM) at pH 8.0 were determined to be 15,000 $mol^{-1}.cm^{-1}$.

2.3.1.6 Effect of pH and temperature on the enzyme activity

To investigate effect of pH on the MLC-3 esterase activity, buffers of various pH were prepared. For pH range of 4-4.5 (acetate buffer, 100 mM), 6-8 (phosphate buffer, 100 mM), 8.5- 10.5 (Glycine-Sodium hydroxide buffer, 100 mM) and 11-12 (Na_2HPO_4-Sodium hydroxide buffer, 100 mM) were used. Enzymatic reaction was done under standard assay condition as mentioned earlier.

To study effect of temperature on the enzyme activity, initial reaction velocity (V_0) of the enzyme was calculated at various temperatures (10 ºC, 15 ºC, 20 ºC, 25 ºC, 30 ºC, 35 ºC, 40 ºC, 45 ºC, 50 ºC). In 0.9 mL of substrate solution containing 0.1 mL buffer of optimum pH, 0.1 mL of purified enzyme (100 ng) was added and initial reaction velocity (V_0) was calculated for the enzyme using UV-visible spectrophotometer (Cary 300, Varian).

2.3.1.7 Determination of the substrate specificity of the enzyme

To study the substrate specificity of the purified MLC-3 esterase, *p*-nitrophenol esters of various acyl chain lengths (C2, C4, C5, C8, C10, C12, C14, C16 and C18) were used for the study. The enzymatic reaction was carried out under standard assay condition as mentioned earlier at the optimum pH and temperature of the respective enzymes. *p*-nitrophenol esters (C2-C18) used were at the final concentration of 100 µM in the reaction mixture.

2.3.1.8 Effect of metal ions on the activity of the enzyme

To investigate effect of metal ions on the activity of purified MLC-3 esterase, various divalent and monovalent salt such as $CaCl_2$, $MgCl_2$, $NiCl_2$, NaCl, $CoCl_2$, $CuCl_2$, KCl, $FeSO_4$, $ZnCl_2$, $HgCl_2$, NH_4Cl and $Ba(OH)_2$ were used. Enzymatic reaction was done under standard assay condition and the final concentration of salts used in the reaction was 5 mM.

2.3.1.9 Effect of various polar and non polar organic solvents on the activity of the enzyme

To study the stability of the MLC-3 esterase in different organic solvents, various polar and non polar solvents such as toluene, ethanol, isopropanol, isoamyl alcohol, dimethyl formamide (DMF), acetonitrile, dimethyl sulfoxide (DMSO), n-hexane, carbon tetrachloride (CCl_4), dichloromethane (DCM), tetrahydrofuran (THF) and chloroform were used. The enzyme was preincubated with organic solvent for 1 hour at RT with final concentration of 25 % v/v of solvent is to enzyme solution. This preincubated enzyme then used for activity under standard assay conditions at and was quantitated at 410 nm using UV-visible spectrophotometer.

2.3.1.10 Effect of chelating agents, reducing agents and detergents on enzyme activity

Effect of chelating agent such as EDTA, reducing agents such as dithiothreitol (DTT), 2-β-mercapto ethanol (2-βME) and sodium dodecyl sulphate (SDS) and detergents like Triton X-100, Tween 80 and CHAPS were studied on enzyme MLC-3 esterase. EDTA, DTT, 2-βME and CHAPS were used at the final concentration of 10 mM in the reaction while Triton X-100,

Tween 80 and SDS were used at the final concentration of 10 %, 10 % and 1 % respectively in the reaction mixture. All other assay conditions were as per standard conditions.

2.3.1.11 Enzyme kinetics study

The kinetic constants (K_m,V_{max}, K_{cat}, K_{cat} /K_m) of MLC-3 esterase were calculated based on the Michaelis Menten graph. The initial rate of hydrolysis of *p*-nitrophenol butyrate was determined by using various concentration range of *p*-nitrophenol butyrate (50 µM to 2200 µM) under standard conditions with purified MLC-3 esterase (100 nM). Optimum pH of 8.0 and optimum temperature of 20 ºC were used for determining the kinetic parameters for the pure esterase enzymes.

2.3.1.12 Enantioselective hydrolysis of racemic esterase by MLC-3 esterase

Time course studies of enantioselective hydrolysis of eight commercially important racemic acyclic and aromatic acetates were performed using both esterase enzymes. 320 µL of MLC-3 esterase enzyme (2 mg, 5.2 IU/mg) was dissolved in 1680 µL of potassium phosphate buffer (pH-8.0, 100 mM) to make final volume of assay to 2 mL .4 mg of racemic acetate was added to each assay reaction and incubated at 20 ºC and 200 rpm in an incubator shaker. 200 µL of aliquots were taken out at the interval of 6, 12, 18, 24, 30, 36, 42, 48 h and was extracted twice with 400 µL of ethyl acetate (EtOAc). The upper extracted solvent layer was collected and passed over anhydrous sodium sulphate to inject into GC-FID. 1µL of the extracted solvent was injected into GC-FID each time having injector with split ratio of 1:10.

2.3.1.13 Chiral GC Analysis

Gas chromatographic analysis (Agilent GC-FID, 7890 A with auto sampler, Chemstation software, Agilent, USA) was performed using three chiral stationary phases. Astec CHIRALDEX G-TA (2,6-di-O-pentyl-3-trifluoroacetyl derivative of γ-cyclodextrin, length: 30 m, I.D: 0.25 mm x film thickness: 0.12 µm, supelco, sigma aldrich, USA), Astec CHIRALDEX B-DP (2, 3-di-O-propionyl-6-t-butyl silyl derivative of β-cyclodextrin, length: 30 m, I.D: 0.25 mm x film thickness: 0.12 µm, supelco, sigma aldrich, USA) and Astec CHIRALDEX B-DM (2,3-di-O-methyl-6-t-butyl silyl derivative of β-cyclodextrin, length: 30 m, I.D: 0.25 mm x film thickness: 0.12 µm, supelco, sigma aldrich, USA), a flame ionisation detector (FID) temperature: 200 ºC, nitrogen as carrier gas (flow rate: 1mL/min) and front inlet (temperature: 200 ºC, split ratio:1:10). All the enantiomers were well separated and enantiomeric excess, conversion values were calculated. Absolute configurations for all

compounds were determined by co injecting racemic mixture of alcohols with pure standards of (*R*) or (*S*) enantiomers into chiral GC.

2.3.1.13.1 GC/GC-MS conditions

A) While for (±)-2-pentanol and (±)-2-hexanol chiral GC separation was done on GC column "Astec CHIRALDEX G–TA" capillary column (30 m X 0.25 mm X 0.12 µm, Supelco) with following temperature programme-

Oven temperature programme: Initial temperature maintained at 35 ºC for 1 min, followed by a temperature gradient from 35 ºC to 65 ºC at 1 ºC min^{-1} and then raised to a final temperature of 180 ºC with a 10 ºC min^{-1} rise.

Retention times (R_t): (*S*)-2-pentanol, (*R*)-2-pentanol, (*S*)-2-pentyl acetate & (*R*)-2-pentyl acetate were eluted at retention times of 13.2, 13.6, 17.8 and 18.6 min respectively**.**

Retention times (R_t): (*S*)-2-hexanol, (*R*)-2-hexanol, (*S*)-2-hexyl acetate & (*R*)-2-hexyl acetate eluted at retention times of 20.4, 21.25, 28.17 and 29.99 min respectively.

B) Chiral GC separation of (±)-3-hexanol, (±)-1-octen-3-ol, (±)-1-phenyl ethanol, (±)-3-methyl-1-phenyl ethanol and (±)-1-phenylpropanol was done on "Astec CHIRALDEX B-DP" capillary column (30 m X 0.25 mm X 0.12 µm, Supelco) with following oven temperature programme-

1) **For (±)-3-hexanol and (±)-3-hexyl acetate:**

Oven temperature programme: Initial temperature maintained at 40 ºC for 1 min, followed by a temperature gradient from 40 ºC to 60 ºC at 1 ºC min^{-1} and then raised to final temperature of 200 ºC at the rate of 10 ºC $min^{-1}$and kept on hold for 3 min.

Retention times (R_t): (±)-3-hexyl acetate, (*R*)-(-)-3-hexanol and (*S*)-(+)-3-hexanol were eluted at retention times of 14.9 min, 15.3 min, 17.9 min and 18.7 min respectively.

2) For (±)-1-octen-3-ol and (±)-1-octen-3-yl acetate:

Oven temperature programme: Initial temperature maintained at 40 ºC for 1 min, followed by a temperature gradient from 40 ºC to 80 ºC at 5 ºC min^{-1} and from 80 ºC to 105 ºC at 1 ºC min^{-1}.It was then raised to final temperature of 200 ºC at the rate of 10 ºC $min^{-1}$and kept on hold for 5 min.

Retention times (R_t): (±)-1-octen-3-yl acetate, (*R*)-(-)-1-octen-3-ol and (*S*)-(+)-1-octen-3-ol were eluted at retention times of 16.47 min, 16.6 min and 17.3 min respectively.

3) For (±)-1-phenyl ethanol and (±)-1-phenyl ethyl acetate:

Oven temperature programme: Initial temperature maintained at 40 ºC for 1 min, followed by a temperature gradient from 40 ºC to 90 ºC at 5 ºC min^{-1} and from 90 ºC to 110 ºC at 1 ºC min^{-1}.It was then raised to final temperature of 200 ºC at the rate of 10 ºC $min^{-1}$and kept on hold for 3 min.

Retention times (R_t): (±)-1-phenylethyl acetate, (*R*)-(+)-1-phenyl ethanol and (*S*)-(-)-1-phenyl ethanol were eluted at retention times of 21.2 min, 23.4 min and 24.4 min respectively.

4) For (±)-3-methyl-phenyl ethanol and (±)-3-methyl-phenyl ethyl acetate:

Oven temperature programme: Initial temperature maintained at 50 ºC, followed by a temperature gradient from 50 ºC to 115 ºC at 3 ºC min^{-1} and from 115 ºC to 135 ºC at 1 ºC min^{-1}.It was then raised to final temperature of 200 ºC at the rate of 10 ºC $min^{-1}$and kept on hold for 5 min.

Retention times (R_t): (±)-3-methyl-1-phenylethyl acetate, (*R*)-(+)-3-methyl-1-phenyl ethanol and (*S*)-(-)-3-methyl-1-phenyl ethanol were eluted at retention times of 24.2 min, 24.9 min and 25.33 min respectively.

5) For (±)-1-phenylpropanol and (±)-1-phenylpropyl acetate:

Oven temperature programme: Initial temperature maintained at 40 ºC for 1 min, followed by a temperature gradient from 40 ºC to 70 ºC at 5 ºC min^{-1} and from 70 ºC to 105 ºC at 1 ºC min^{-1}.It was then raised to final temperature of 200 ºC at the rate of 10 ºC $min^{-1}$and kept on hold for 3 min.

Retention times (R_t): (±)-1-phenylpropyl acetate, (*R*)-(+)-1-phenylpropanol and (*S*)-(-)-1-phenylpropanol were at retention times of 35.2 min, 37.8 min and 38.6 min respectively.

C) Chiral GC separation of (±)-2-Methyl-1-phenyl ethanol was done on "Astec CHIRALDEX B-DM" capillary column (30 m X 0.25 mm X 0.12 µm, Supelco) with following oven temperature programme:-

1) For (±)-2-Methyl-1-phenyl ethanol and (±)-2-Methyl-1-phenyl ethyl acetate:

Oven temperature programme: Initial temperature maintained at 50 ºC, followed by a temperature gradient from 50 ºC to 115 ºC at 3 ºC min^{-1} and from 115 ºC to 135 ºC at 1 ºC min^{-1}. It was then raised to final temperature of 200 ºC at the rate of 10 ºC $min^{-1}$and kept on hold for 5 min.

Retention times (R_t): (±)-2-methyl-1-phenylethyl acetate, (*R*)-(+)-2-methyl-1-phenyl ethanol and (*S*)-(-)-2-methyl-1-phenyl ethanol were eluted at retention times of 19.7 min, 25.2 min and 26.53 min respectively.

2.3.1.14 Nucleotide Sequence Accession Number

The nucleotide sequence of MLC-3 esterase gene is available at Gen Bank database as Gen Bank: GU331883.1.

2.3.1.15 Molecular docking studies

Homology modeling was performed using SwissModel server to predict three dimensional structure of MLC-3 esterase. X ray crystal structure of bacterial esterases (PDB ID: 3BF8) were used as templates for homology modeling.[33] Predicted models were energy minimized using GROMOS 43BI force field [34] and the assessed for its quality using ProSA and Ramchandran plot analysis. Docking studies were performed using AutoDock 4.2 version.[35] Ligands and receptor files were optimized for docking studies and converted from .pdb to .pdbqt format. The grid was set around active site residues of MLC-3 esterase with dimensions of 20 x 20 x 20 Å. The docking parameters were configured on a LGA calculation of 10,000 runs. Energy evaluations were set to 1,500,000 and 27,000 generations. Population size was set to 150 and the rate of gene mutation and the rate of gene crossover were set to 0.02 and 0.8 respectively.[36] The obtained conformations were later summarized, collected and extracted by using Autodock Tool. Geometry of resulting complexes was studied using the PyMol molecular viewer utility (The PyMOL Molecular Graphics System, Version 1.5.0.4 Schrödinger, LLC).

2.3.2 Results and Discussion

2.3.2.1 Cloning and sequence analysis of the MLC-3 esterase genes

Nucleotide sequence of the MLC-3 esterase gene of consists of 843 bp open reading frame (ORF) region. MLC-3 esterase gene was amplified using "Accuprime *pfx* polymerase" (Fig.2.17) and was cloned into pET 32-a vector.

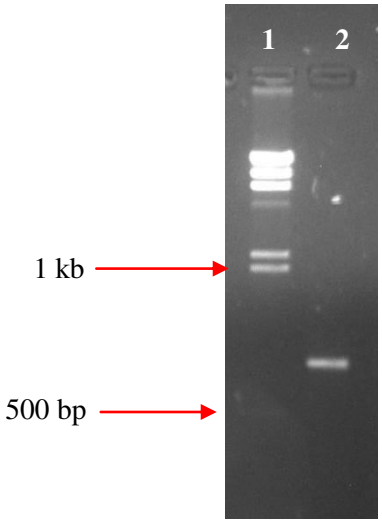


Fig. 2.17 Agarose gel for PCR amplification of MLC-3 esterase. **Lane 1**:- Hind III DNA ladder, **Lane 2**:- PCR amplified MLC-3 esterase gene (843 bp)

Positive colonies identified from colony PCR (Fig. 2.18) using T7 forward and reverse primers were picked and sent for sequencing to check the frame and for any unwanted mutations.

- **Agarose gel for colony PCR**

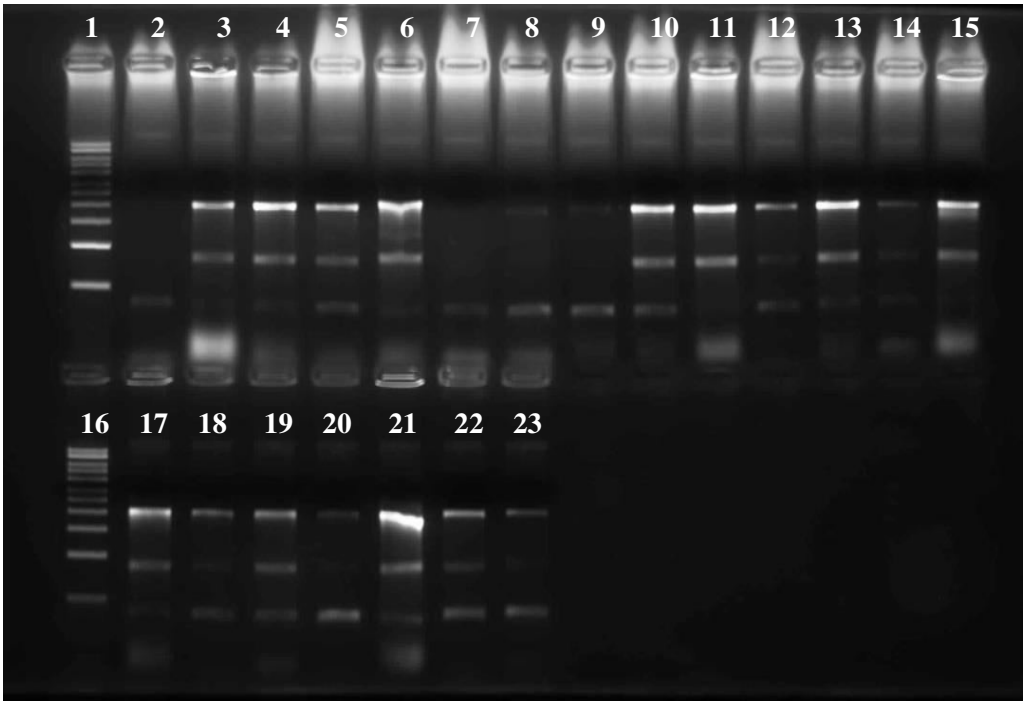


Fig.2.18 Agarose gel for colony PCR of MLC-3 esterase cloning into pET 32-a vector. **Lane 1, 16**:- 1kb DNA ladder, **Lane 2**:- Negative control, **Lane 3 to 23**:- colony PCR product of colonies 1 to 20.

Nucleotide and amino acid sequence of MLC-3 esterase:

- **MLC-3 esterase (843 bp)**

ATGAATCACCGTGATGGTATCCTTGCCCGCACCTTCCGCTCGGACACCCACGTGAAG
CTGCATTACGAAACCTTGGGCCGGGGCGAGCCGCTGATCATCCTGCACGGCCTGTTC
GGCTCGGGCGCGAACTGGCGAAGCATCGCCCAGCGCCTGGCCGATACCTGGCAGGT
CATCCTGCCCGACCTGCGCAATCACGGCGACTCCCCGCATGCGCCCACCAACCGTTA
CCAGGACATCGCGGGCGACACCCTGGCGCTGATGGATCGTCTGGGCCTTGCGCGTG
CCCACCTCCTGGGCCACTCGCTCGGCGGCAAGGCGGCAATGCTGCTCGCCAGCCGC
GCGCCCGAGCGCATCGATAGCCTGACCGTGGTCGACATCGCCCCTCGCGCCTACCCG
CCCCTGCACCTTGAGCTGTTCCGCGCCCTGCACGCCGTGCCGCTCGCCCGCATCACC
TCACGCCGCGAGGCCAGCGAGGCGATGGCCGCGCACATCTCCAACCCGGCGGTTCG
CGACTTCCTTTTGACCAATCTCGCGCGGGATGGCAACGGACGGTTCCACTGGCGGCT
GAACCTGGCCGGGCTGGAGGAGGCGTACGAGGAGCTCAACGCCATGCCCTTCCTCG
ATCGGTTGTATGAGGGGCCGGCCCTGTTCATCCGCGGCGGGCATTCGGACTACGTGC
GCGACGCCGACCTCGGCCTGATCCACCAAAGCTTCCCGCGGGCCTGCGTGGTCAGC
CTGCCGCTGGCCCATCACTGGCCGCATGTCGAGACACCCAACGAGTTCCTGCGCGCC
CTGCGCGACTTCCTCGAGGCACGCTCCGAGCGCCTGCCCTGTGAGGCTTGA

- **Amino acid sequence of MLC-3 esterase (281 a.a)**

MNHRDGILARTFRSDTHVKLHYETLGRGEPLIILHGLFGSGANWRSIAQRLADTWQVILP
DLRNHGDSPHAPTNRYQDIAGDTLALMDRLGLARAHLLGHSLGGKAAMLLASRAPERI
DSLTVVDIAPRAYPPLHLELFRALHAVPLARITSRREASEAMAAHISNPAVRDFLLTNLA
RDGNGRFHWRLNLAGLEEAYEELNAMPFLDRLYEGPALFIRGGHSDYVRDADLGLIHQ
SFPR ACVVSLPLAHHWPHVETPNEFLRALRDFLEARSERLPCEA-

MLC-3 esterase also contains a conserved pentapeptide GHSLG corresponding to GXSXG motif commonly found in serine hydrolases family proteins. MLC-3 esterase shows α/β hydrolase fold domain belonging to an esterase /lipase super family (Fig.2.19).

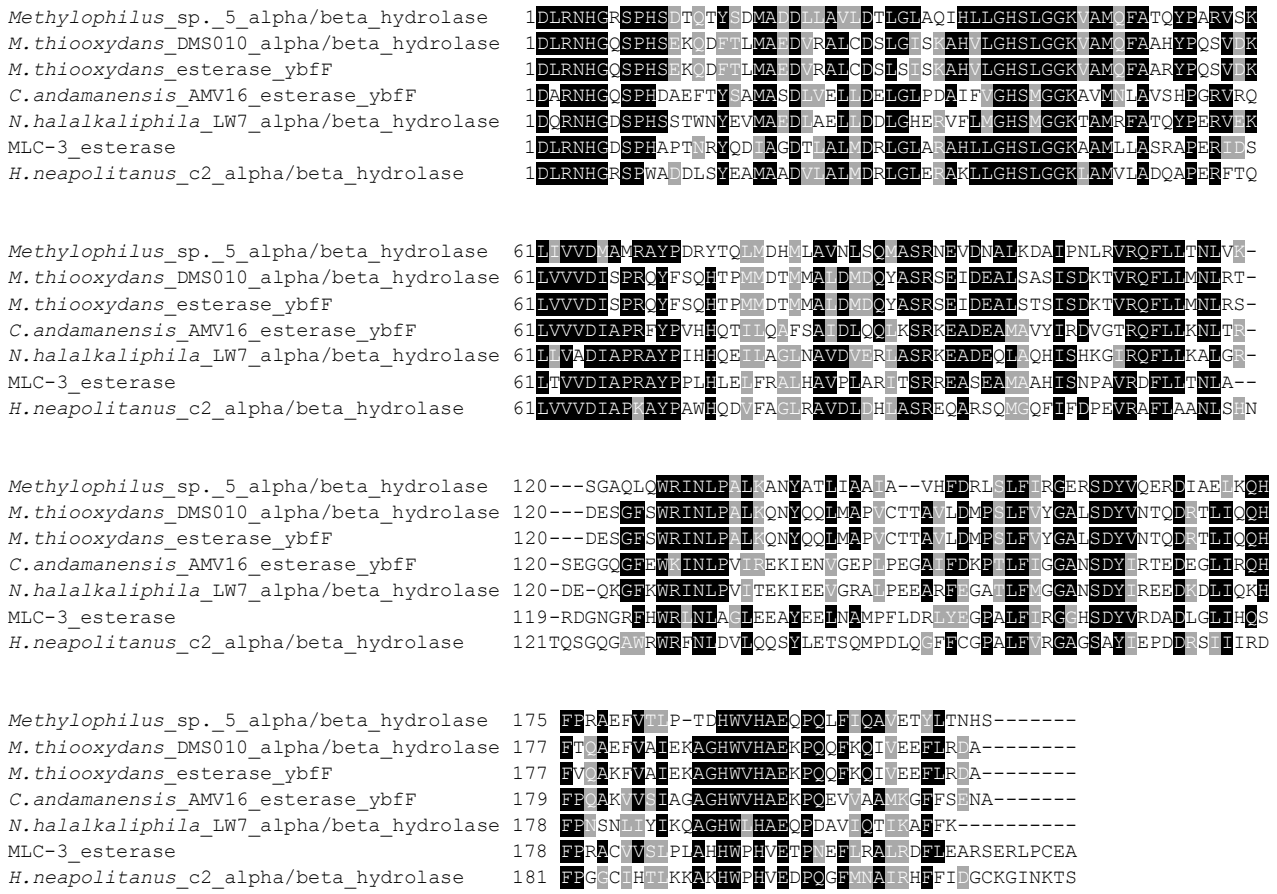


Fig. 2.19 Multiple sequence alignment of MLC-3 esterase with close homologue sequences. The amino acid sequence corresponds to MLC-3 esterase (GI accession no.ADM63071.1), *Methylophillus species alpha beta hydrolase protein* (WP_029148270.1, 48 % identity with SLC-6 esterase), *M. thiooxydans DMS010 alpha/beta hydrolase protein* (WP_008290677.1, 46 % identity), *M. thiooxydans* esterase (KGM06527.1, 45 % identity), *C. andamanensis AMV16 esterase* (WP_009194495.1, 47 % identity), *N. halalkaliphila* LW7 alpha/beta hydrolase protein (WP_009056845.1, 46 % identity), *H. neapolitanus* c2 alpha/beta hydrolase (ACX96995.1, 52 % identity). Residues that are 100% conserved are shadowed in black, and those between 75% and 100% are shadowed grey.

2.3.2.2 Phylogenetic analysis

BlastP results shows homology between MLC-3 esterase with similar alpha beta hydrolase family proteins from various proteobacterias. Based on phylogenetic analysis MLC-3 esterase is closely related to *Methylotenera* species α/β-hydrolase protein along with *Halothiobacillus neapolitanus* α/β-hydrolase protein and *Leucothrix mucor* acyl coA esterase proteins on evolutionary scale. MLC-3 esterase is distantly related to α/β-hydrolase fold proteins from proteobacterial species such as *Methylophillus*, *Methylophaga*, *Nitrosococcus*, *Pontibacter* etc. (Fig.2.20)

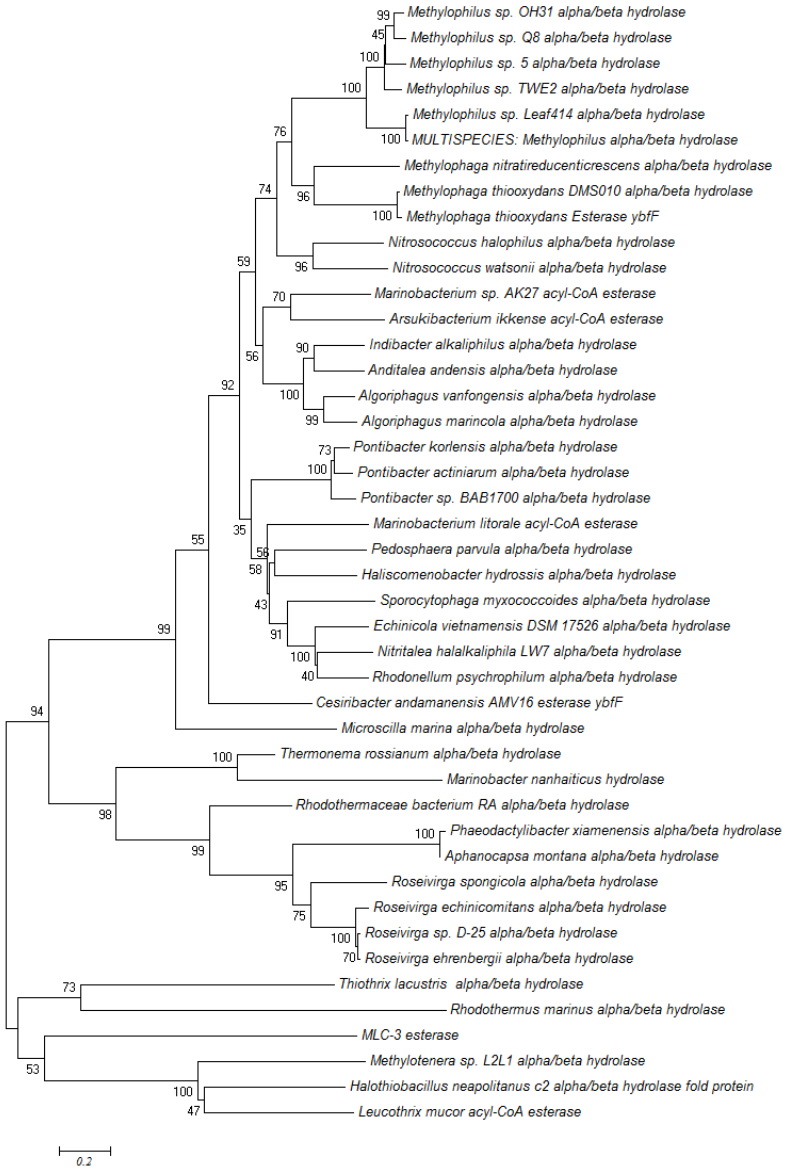


Fig. 2.20 Phylogenetic tree of MLC-3 esterase and other closely related alpha/beta hydrolase fold proteins. The phylogenetic analysis was performed by the neighbour joining method using MEGA 6.0 software. The values at nodes indicate the bootstrap percentage of 1,000 replicates.

2.3.2.3 Expression and purification of the recombinant MLC-3 esterase

MLC-3 esterase gene was expressed in C41 (DE3) and rosetta (DE3) cells respectively. MLC-3 esterase was purified from the cell lysate using Ni-NTA agarose affinity chromatography. Purified protein was analyzed on 12 % sodium dodecyl sulphate polyacryl amide gel electrophoresis (SDS-PAGE) gel and both the proteins were corresponded well with their expected mass of ~50 kD respectively due to additional *Trx* solubilisation domain and His-tag present in pET 32-a vector (Fig. 2.21).

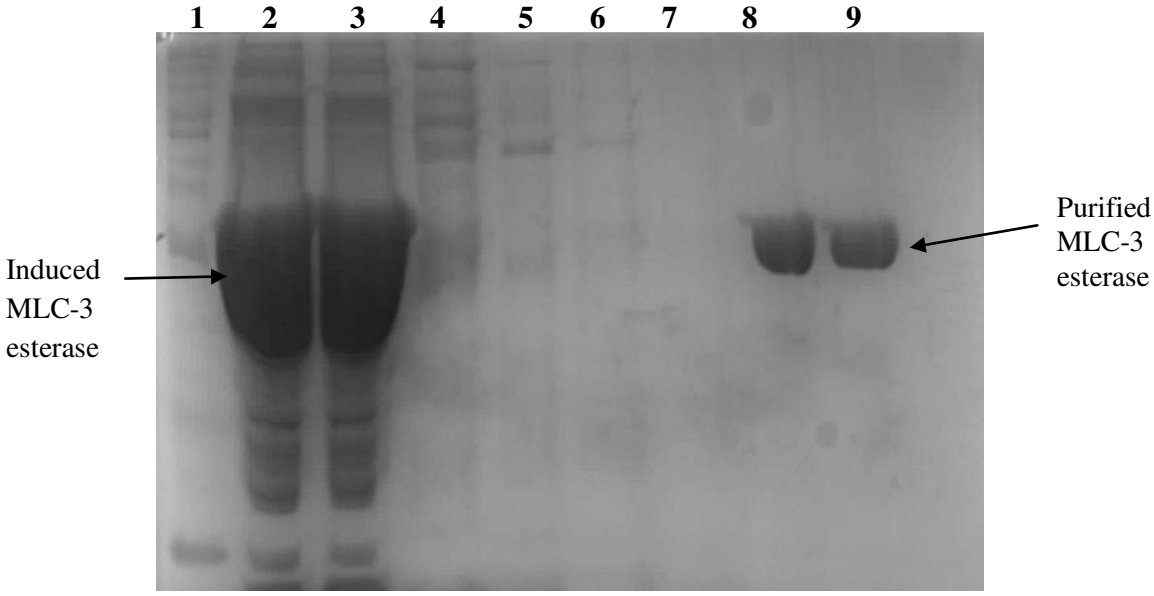


Fig. 2.21 SDS gel for purification of MLC-3 esterase using Ni-NTA agarose chromatography. **Legends: Lane 1**: Protein marker. **Lane 2:** Induced pellet of pEST-1 esterase expressed in pET 32-a vector. **Lane 3**: Induced supernatant of pEST-1 esterase expressed in pET 32-a vector. **Lane 4**: Unbound fraction. **Lane 5, 6, 7**: 20 mM imidazole wash fraction. **Lane 8**: Elution fraction 1 (250 mM imidazole). **Lane 9**: Elution fraction 2 (250 mM imidazole).

2.3.2.4 Effect of pH and temperature on the activity of MLC-3 esterase

Optimum pH study was performed with MLC-3 esterase using pH range from 4 to 11 using *p*-nitrophenol butyrate as a substrate. MLC-3 esterase was highly active at slightly alkaline pH of 8.0 suggesting being an alkaline stable esterase. [Fig. 2.22 (A)]

Optimum temperature of MLC-3 esterase was determined by calculating initial reaction velocity of both the enzymes at various temperature ranges. MLC-3 esterase showed optimum

temperature of 20 ºC suggesting having a origin from psychrophillic microorganism. [Fig. 2.22 (B)]

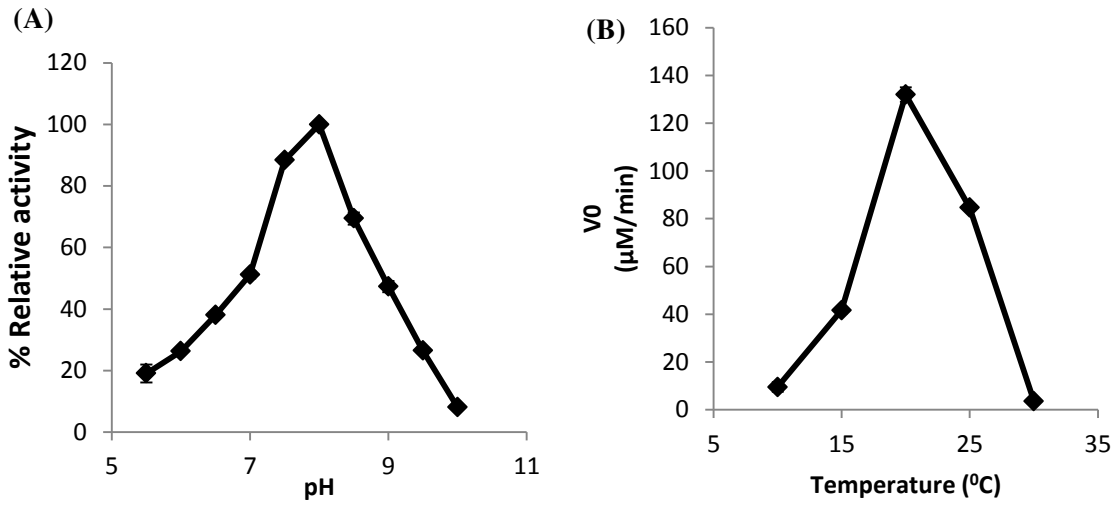


Fig. 2.22 Study of optimum pH and temperature of MLC-3 esterase enzyme (**A**) Optimum pH study of MLC-3 esterase (**B**) Optimum temperature study with MLC-3 esterase enzyme. All the experiments were carried out in triplicates and error bars shows the standard deviation.

2.3.2.5 Determination of substrate specificity and effect of metal ions on the activity of enzymes

Different fatty acid esters of *p*-nitrophenol were tested for checking the substrate specificity of both the enzymes. MLC-3 enzymes were found to be highly active towards *p*-nitrophenol butyrate [*p*-nitrophenol butyrate (C4)] and activity was decreasing towards higher carbon number esters (C6-C18). Higher activity towards short chain esters of *p*-nitrophenol confirms the enzyme to be an esterase than lipase. [Fig. 2.23 (**A**)]

Various metal ions either show inhibition or enhancement in the activity of esterases. MLC-3 esterase activity was drastically inhibited by $FeSO_4$, $CuCl_2$, $NiCl_2$, NH_4Cl and $HgCl_2$ to 6.9 %, 16 %, 46.2 %, 63.4 % and 29.7 % respectively. Salts such as $CaCl_2$, $CoCl_2$, $ZnCl_2$ partially decreased enzyme activity to 91.5 %, 80.2 % and 88.9 % respectively. Divalent salts such as $MgCl_2$ and monovalent salts such as NaCl and KCl enhanced the MLC-3 esterase activity to 108.7 %, 106.9 % and 103.3 % respectively. Though esterase doesn't need cofactor for their activity, increase in the activity of enzymes by addition of salts such as $MgCl_2$, KCl and NaCl. Probable reason for increase in the activity may be due to binding of these ions to allosteric sites of the enzyme and positively modulating the enzyme conformation to enhance the activity. . [Fig. 2.23 (**B**)]

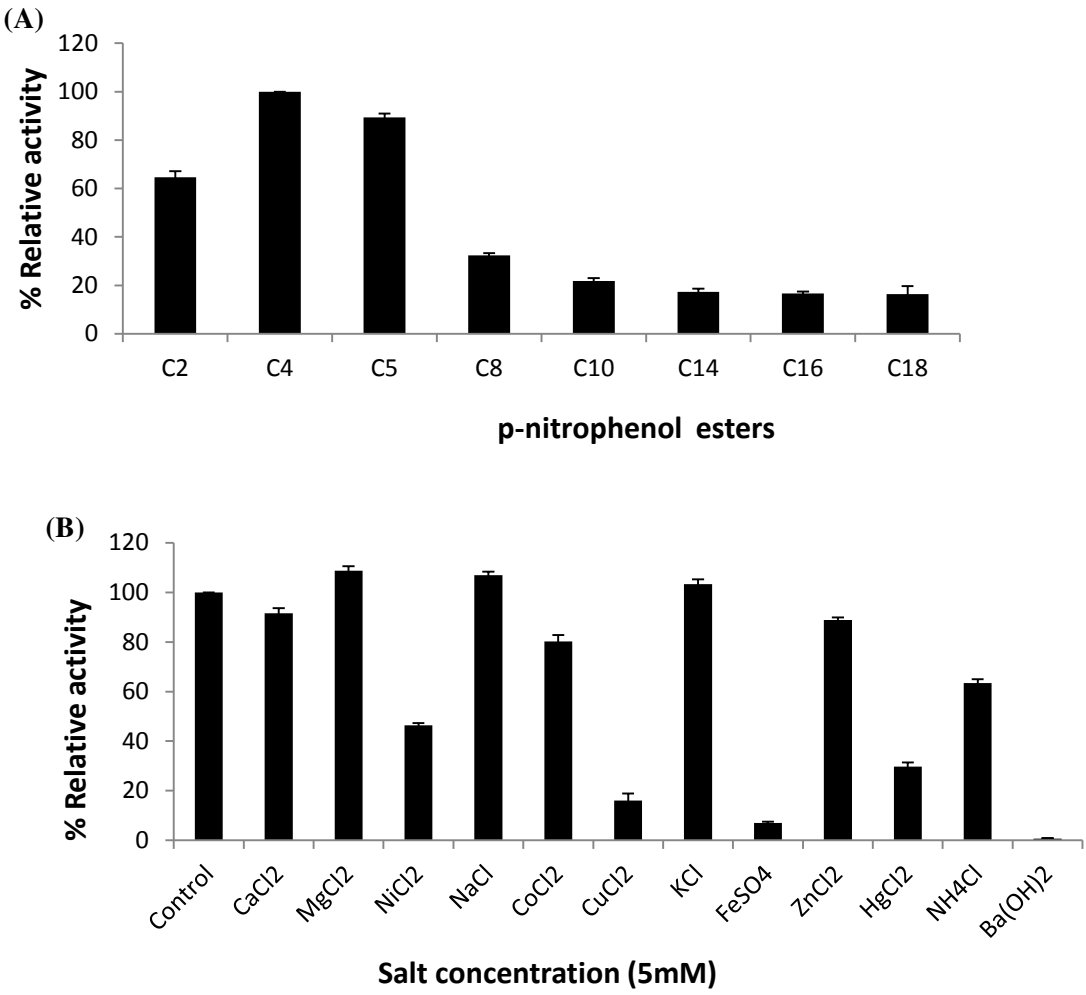


Fig. 2.23 Substrate specificity study and effect of various metal ions on the activity of MLC-3 esterase enzyme (**A**) Determination of chain length specificity with *p*-nitrophenol esters of fatty acid with MLC-3 esterase (**B**) Study of different salts at the final concentration of 5 mM with MLC-3 esterase enzyme. All the experiments were carried out in triplicates and error bars shows the standard deviation.

2.3.2.6 Effect of organic solvents, chelating agents, inhibitors and detergents of the activity of enzyme

MLC-3 esterase was studied for its stability in organic solvents. MLC-3 esterase activity was drastically inhibited by solvents such as toluene, isoamyl alcohol, tetrahydrofuran, dichloromethane, chloroform and isopropanol to 11.6 %, 10.1 %, 13.2 %, 34 %, 35 % and 50.3 % respectively. Enzyme activity was partially inhibited by acetonitrile, dimethyl sulfoxide, carbon tetrachloride, dimethyl formamide to 72.7 %, 81.7 %, 70.8 %, 52 % respectively. Ethanol showed drastic increase in the activity of MLC-3 esterase to 104.2 % when compared to control activity. [Fig. 2.24]

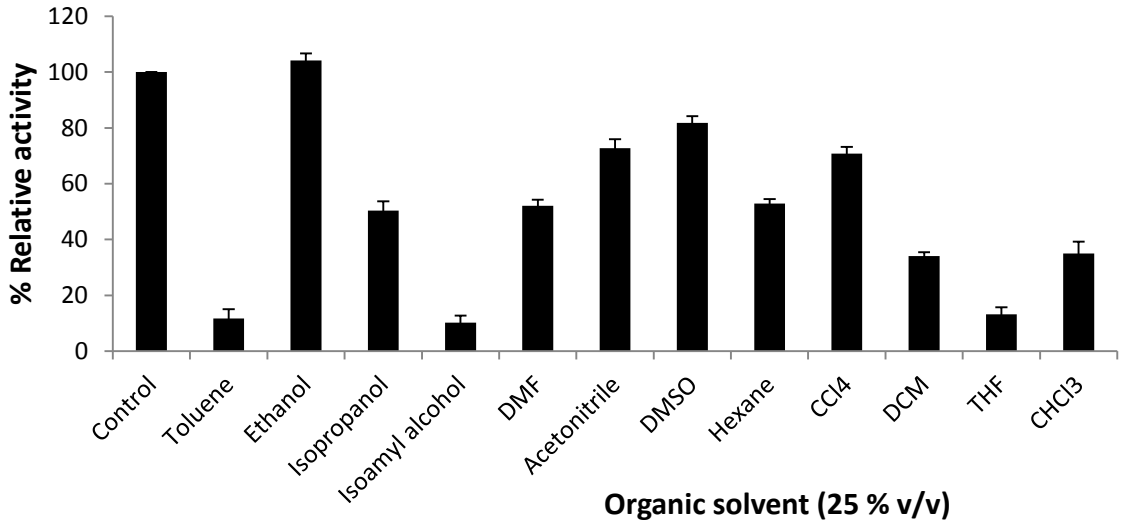


Fig. 2.24 Determination of stability of MLC-3 esterase in different polar and non polar solvents. All the experiments were carried out in triplicates and error bars shows the standard deviation.

Chelator EDTA (10 mM) decreased enzyme activity of MLC-3 to 3 % suggesting a strong inhibition of the enzymes [Fig. 2.25]. This suggests that enzyme do requires some metal ions for their optimal activity. Inhibitor such as DTT and βME reduces enzyme activity to 3 % and 0.1 % respectively.

Tween 80 and Triton X-100 reduces the activity of MLC-3 esterase to 90 % and 31.3 % respectively. Zwitterionic detergent CHAPS, partially reduces activity to 83.7 % while ionic detergent sodium deodecyl sulphate (SDS) completely reduces the activity of MLC-3 esterase enzyme to 2 %. MLC-3 esterase is strongly inhibited by detergents Triton x-100 and SDS. Zwitterionic detergent CHAPS found to be retaining the activity of enzyme. [Fig. 2.25].

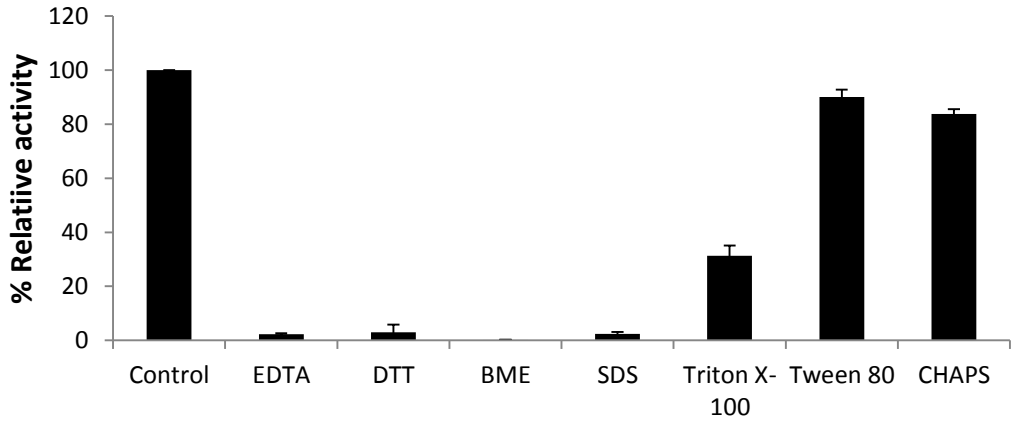


Fig. 2.25 Study of different chelators, inhibitors and detergents on MLC-3 esterase enzyme. Determination of stability of MLC-3 esterase in presence of chelator (EDTA),inhibitors (DTT, BME,

SDS) and detergents (Triton X-100,Tween 80, CHAPS). All the experiments were carried out in triplicates and error bars shows the standard deviation.

2.3.2.7 Determination of enzyme kinetic parameters

Enzyme kinetic parameters were studied for both the enzymes using *p*-nitrophenol butyrate as a substrate at their optimum pH and temperatures. Kinetic parameters were calculated using Michaelis menten graph plotted for both the enzymes.

MLC-3 esterase showed values of V_{max}, K_m, K_{cat} and K_{cat}/K_m value of 1.43 µM min^{-1}, 399 µM, 0.23 S^{-1} value 576 $M^{-1}.S^{-1}$ respectively (Table 2.4). Kinetic parameters of these enzymes are comparable with many carboxyl esterases present in BRENDA enzyme database.[37]

Enzyme	Substrate	V_{max} (µM min^{-1})	K_m (µM)	K_{cat} (S^{-1})	K_{cat}/K_m ($M^{-1}.S^{-1}$)
MLC-3 esterase	C4	1.43	399	0.23	576.5

Table 2.4 Enzyme kinetic parameters of MLC-3 esterase enzyme.

2.3.2.8 Enantioselective hydrolysis of racemic acyclic/aromatic acetates by MLC-3 esterase

Time course study of enantioselective hydrolysis of racemic acetates was carried out using MLC-3 esterase to study the applicability of the enzyme [Table 2.5] [Fig.2.32]. MLC-3 esterase hydrolysed (**1**) and (**2**) to (*R*)-(-)-2-pentanol (**1a**) (Fig. 2.26) [Fig. 2.32 (**A**)] and (*R*)-(-)-2-hexanol (**2a**) (Fig. 2.27) [(Fig. 2.32 (**B**)] with 99.9 % e.e and 93 % and 56.5 % conversion respectively in 48 h.

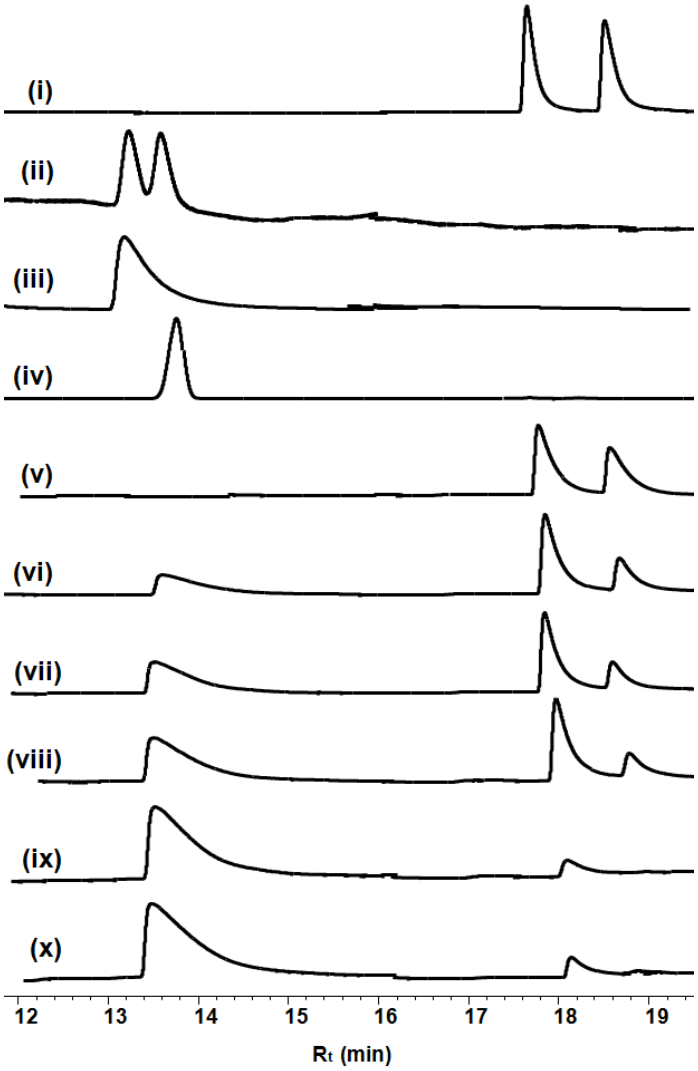


Fig.2.26 Time Course study for the conversion of (±)-2-Pentyl acetate by MLC-3 esterase.
(i) Standard (±)-2-pentyl acetate (ii) Standard (±)-2-pentanol (iii) Standard (*S*)-(+)-2-pentanol (iv) Standard (*R*)-(-)-2-pentanol (v) Conversion of (±)-2-pentyl acetate after 6 h incubation (vi) Conversion of (±)-2-pentyl acetate after 18 h incubation (vii) Conversion of (±)-2-pentyl acetate after 24 h incubation (viii) Conversion of (±)-2-pentyl acetate after 30 h incubation (ix) Conversion of (±)-2-pentyl acetate after 42 h incubation (x) Conversion of (±)-2-pentyl acetate after 48 h incubation .

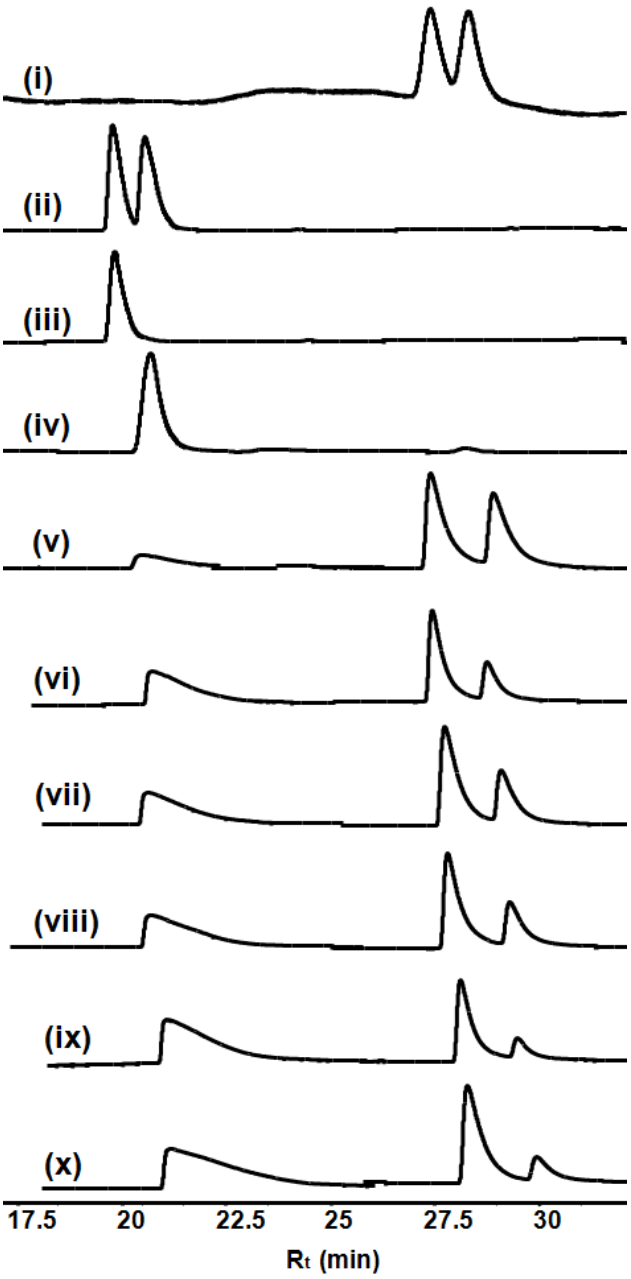


Fig.2.27 Time Course study for the conversion of (±)-2-Hexyl acetate by MLC-3 esterase.
(i) Standard (±)-2-Hexyl acetate (ii) Standard (±)-2-Hexanol (iii) Standard (*S*)-(+)-2-Hexanol (iv) Standard (*R*)-(-)-2-Hexanol (v) Conversion of (±)-2-Hexyl acetate after 6 h incubation (vi) Conversion of (±)-2-Hexyl acetate after 12 h incubation (vii) Conversion of (±)-2-Hexyl acetate after 18 h incubation (viii) Conversion of (±)-2-Hexyl acetate after 30 h incubation (ix) Conversion of (±)-2-Hexyl acetate after 42 h incubation (x) Conversion of (±)-2-Hexyl acetate after 48 h incubation .

MLC-3 esterase did not show any hydrolysis activity on compounds (**3**) to form corresponding alcohols. Such results may be observed due to no recognition of substrate by the enzyme. Compound (**4**) was hydrolysed to (*R*)-(-)-1-octen-3-ol (**4a**) with very less e.e of 3 % and 66 % conversion in 48 h (Fig.2.28) [Fig. 2.32 (**C**)].

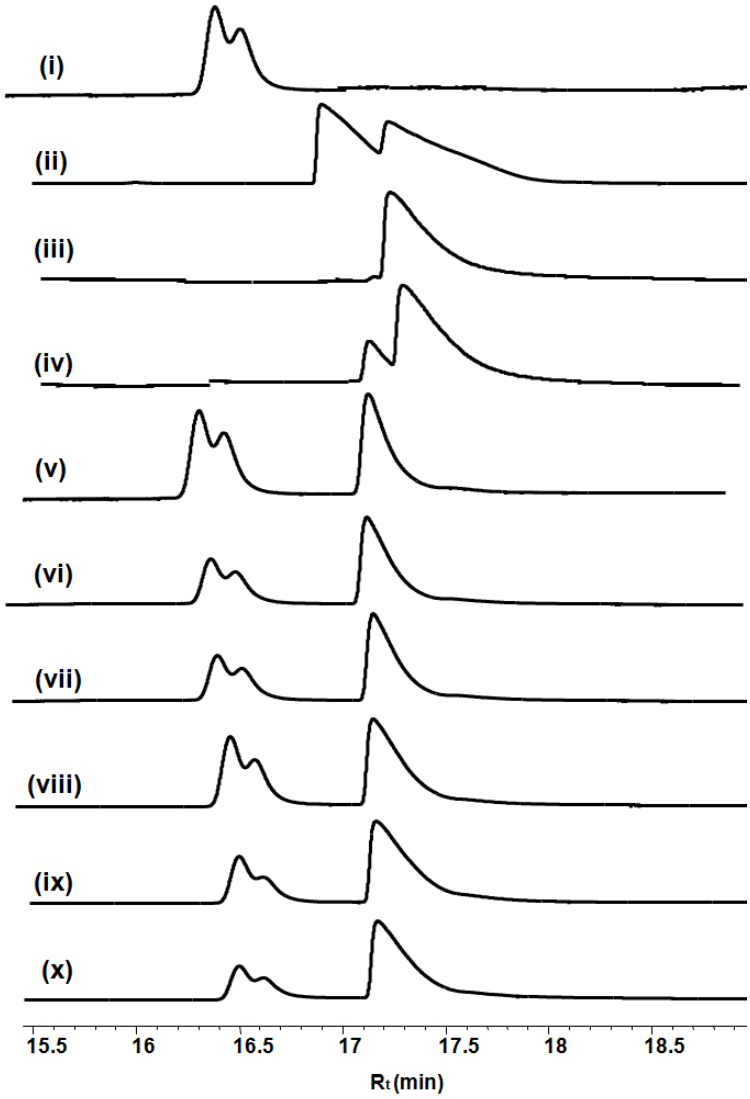


Fig. 2.28 Time Course study for the conversion of (±)-1-octen-3-yl acetate by MLC-3 esterase. (i) standard (±)-1-Octen3-yl acetate (ii) standard (±)-1-Octen3-ol (iii) standard (*S*)-1-Octen3-ol (iv) Co injection of (±)-1-Octen3-ol and (*S*)-1-Octen3-ol (v) Conversion of (±)-1-Octen3-yl acetate after 6 h incubation (vi) Conversion of (±)-1-Octen3-yl acetate after 18 h incubation (vii) Conversion of (±)-1-Octen3-yl acetate after 24 h incubation (viii) Conversion of (±)-1-Octen3-yl acetate after 30 h incubation (ix) Conversion of (±)-1-Octen3-yl acetate after 42 h incubation (x) Conversion of (±)-1-Octen3-yl acetate after 48 h incubation.

Compounds (**5**) and (**7**) were hydrolysed to corresponding (*R*)-(+)-1-phenyl ethanol (**5a**) (Fig. 2.29) [Fig. 2.32 (**D**)] and (*R*)-(+)-3-methyl-1-phenyl ethanol (**7a**) (Fig. 2.30) [Fig. 2.32 (**E**)] with high enantiomeric excess value (e.e) of 99.9 % and 47.5 and 61 % conversion respectively in 48 h.

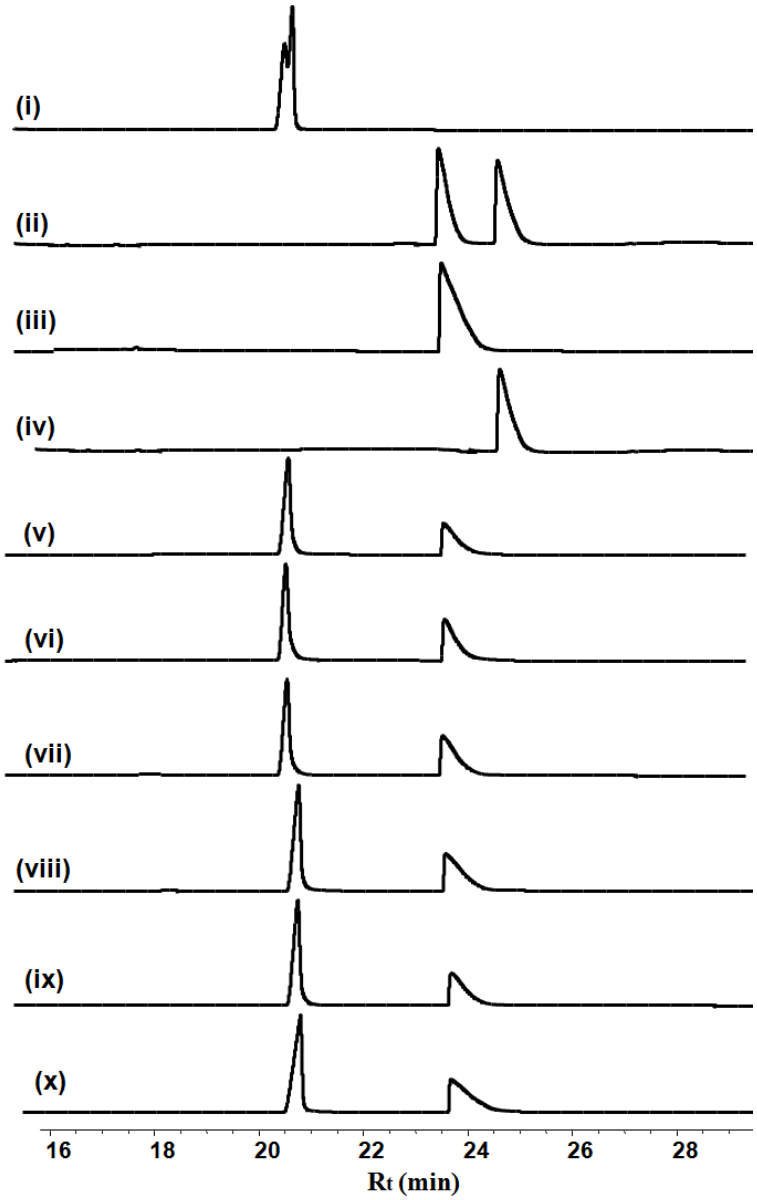


Fig. 2.29 Time Course study for the conversion of (±)-1-Phenyl ethyl acetate by MLC-3 esterase. (i) standard (±)-1-Phenyl ethyl acetate (ii) standard (±)-1-Phenyl ethanol (iii) standard (*R*)-1-Phenyl ethanol (iv) standard (*S*)-1-Phenyl ethanol (v) Conversion of (±)-1-Phenyl ethyl acetate after 6 h incubation (vi) Conversion of (±)-1-Phenyl ethyl acetate after 18 h incubation (vii) Conversion of (±)-1-Phenyl ethyl acetate after 24 h incubation (viii) Conversion of (±)-1-Phenyl ethyl acetate after 30 h incubation (ix) Conversion of (±)-1-Phenyl ethyl acetate after 42 h incubation (x) Conversion of (±)-1-Phenyl ethyl acetate after 48 h incubation .

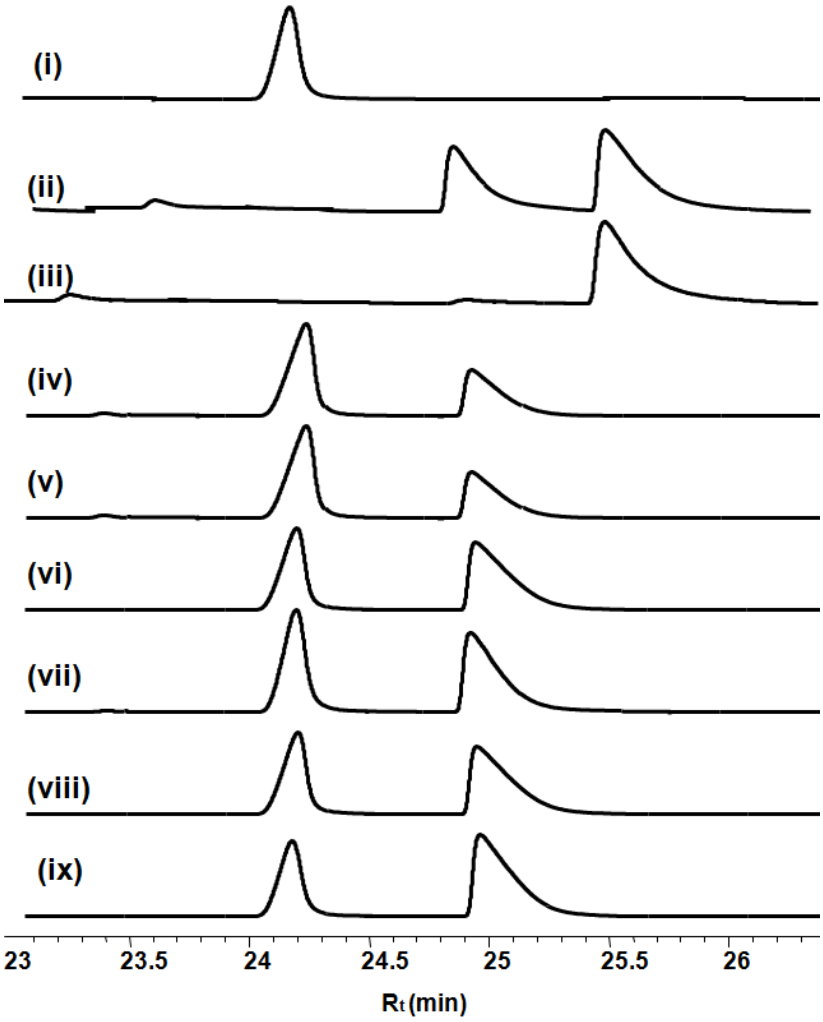


Fig.2.30 Time Course study for the conversion of (±)-3-methyl-1-Phenyl ethyl acetate by MLC-3 esterase.

(i) standard (±)-3-methyl-1-Phenyl ethyl acetate (ii) standard (±)-3-methyl-1-Phenyl ethanol (iii) standard (*S*)-3-methyl-1-Phenyl ethanol (iv) Conversion of (±)-3-methyl-1-Phenyl ethyl acetate after 6 h incubation (v) Conversion of (±)-3-methyl-1-Phenyl ethyl acetate after 18 h incubation (vi) Conversion of (±)-3-methyl-1-Phenyl ethyl acetate after 24 h incubation (vii) Conversion of (±)-3-methyl-1-Phenyl ethyl acetate after 30 h incubation (viii) Conversion of (±)-3-methyl-1-Phenyl ethyl acetate after 42 h incubation (ix) Conversion of (±)-3-methyl-1-Phenyl ethyl acetate after 48 h incubation .

MLC-3 esterase also did not show any reactivity towards compound (**6**) and this can be due to stearic hindrance of methyl group at C2 on phenyl ring interferes the active site pocket of enzyme. Compound (**8**) hydrolysed to (*R*)-(+)-1-phenylpropanol (**8a**) with 99.9 % e.e and 59 % conversion (Fig. 2.31) [Fig. 2.32 (**F**)].

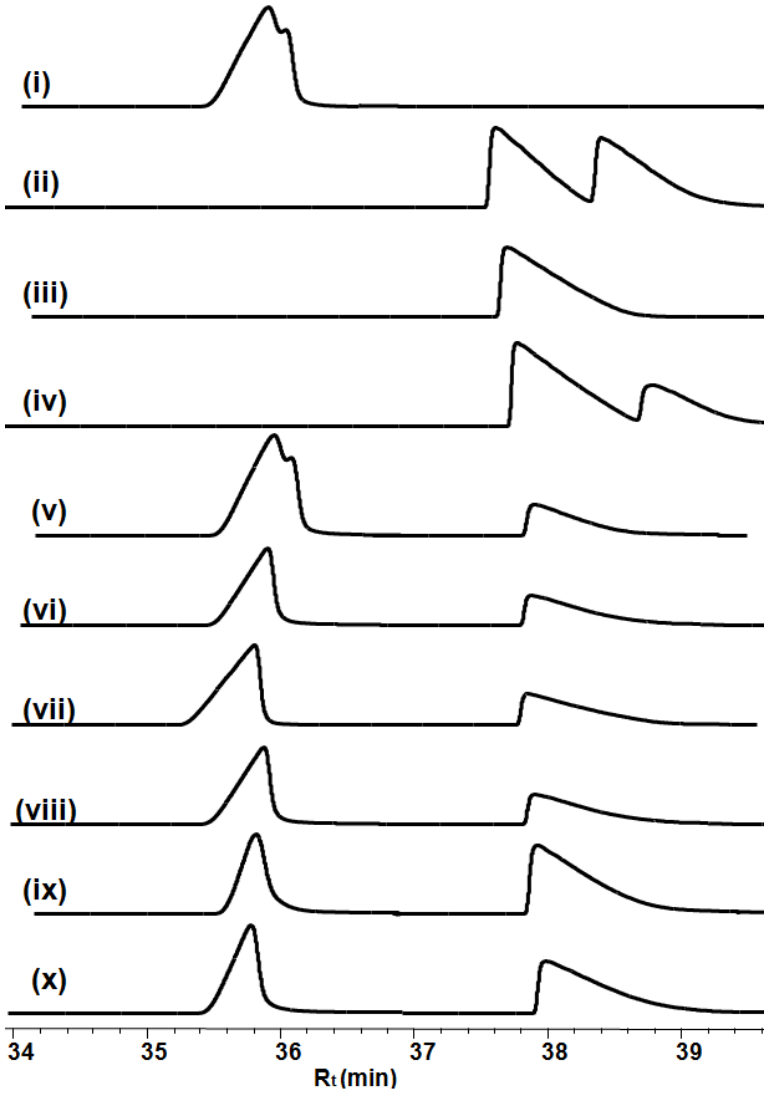
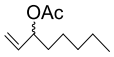
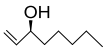


Fig.2.31 Time Course study for the conversion of (±)-1-Phenylpropyl acetate by MLC-3 esterase.

(i) standard (±)-1-Phenylpropyl acetate (ii) standard (±)-1-Phenylpropanol (iii) standard (*R*)-1-Phenylpropanol (iv) Co injection of (±)-1-Phenylpropanol and (*R*)-1-Phenylpropanol (v) Conversion of (±)-1-Phenylpropyl acetate after 6 h incubation (vi) Conversion of (±)-1-Phenylpropyl acetate after 18 h incubation (vii) Conversion of (±)-1-Phenylpropyl acetate after 24 h incubation (viii) Conversion of (±)-1-Phenylpropyl acetate after 30 h incubation (ix) Conversion of (±)-1-Phenylpropyl acetate after 42 h incubation (x) Conversion of (±)-1-Phenylpropyl acetate after 48 h incubation .

In conclusion, MLC-3 esterase was found to be a highly efficient and selective for the (*R*)-acetates and having activity towards wide spectrum of acyclic and aromatic acetates.

Entry	Compounds	Conversion (%)	Product	Time (h)	Configuration and % e.e*
1	 **(1)**	93	 **(1a)**	48	*R*,99.9
2	 **(2)**	56.5	 (**2a**)	48	*R*,99.9
3	 **(3)**	--	**NR**[#]	48	**NR**[#]
4	 **(4)**	66	 **(4a)**	48	*R*,3
5	 **(5)**	47.5	 **(5a)**	48	*R*,99.9
6	 **(6)**	--	**NR**[#]	48	**NR**[#]
7	 **(7)**	61	 **(7a)**	48	*R*,99.9
8	 (**8**)	59	 (**8a**)	48	*R*,99.9

*e.e = enantiomeric excess
NR= No Reaction

Table 2.5 Enantioselective hydrolysis of racemic acetates using MLC-3 esterase [Racemic acetate concentration= 4 mg, MLC-3 esterase enzyme concentration and specific activity =2 mg, 5.2 IU/mg. All incubations were done at 20 ºC and 200 rpm in incubator shaker for 48 h].

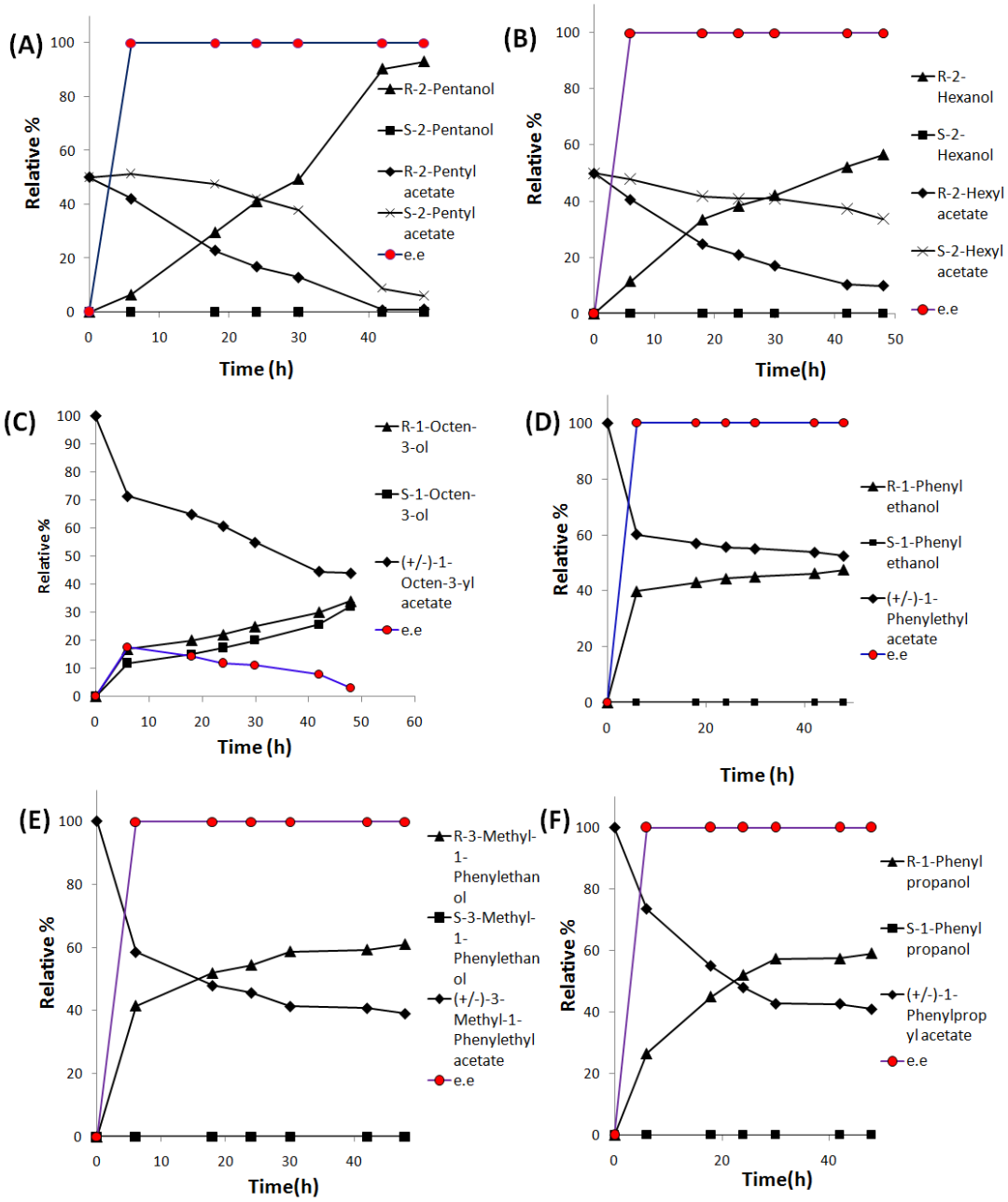


Fig. 2.32 Time-course experiment of kinetic resolution achieved using MLC-3 esterase in graphical representation with **(A)** (±)-2-pentyl acetate **(B)** (±)-2-hexyl acetate, **(C)** (±)-1-octen-3-yl acetate, **(D)** (±)-1-phenylethyl acetate, (**E**) (±)-3-methyl-1-phenylethyl acetate, **(F)** (±)-1-phenylpropyl acetate respectively.

2.3.2.9 Molecular docking analysis

The molecular docking studies were carried out between (*R*) and (*S*)-acetates of the substrates and MLC-3 esterase enzyme. (*R*) and (*S*)-acetates of six compounds [(**1**),(**2**),(**4**),(**5**),(**6**) and (**8**)] were individually docked with the protein model of MLC-3 esterase obtained using Swiss Modeler server. Docking of ligands with the enzyme provides the information about binding pose and atomic interaction between stereo selective pocket of MLC-3 esterase and the ligand. Interaction in active site residues and reactive centers of the substrates are stearically well positioned in the case of favorable interactions. Binding affinity and docking of ligands in the active site displayed that (*R*)-acetates of compounds bind strongly and fits very well in the active site of enzyme while substrate (*S*)-acetates showed stearic hindrance when binding in the active site pocket (Fig. 2.33) (Table 2.6).

Acyclic acetates such as (*R*)-2-pentyl acetate and (*R*)-2-hexyl acetate found to be interacting strongly with less free energy compared to (*S*)-2-pentyl acetate and (*S*)-2-hexyl acetate in the active site pocket of MLC-3 esterase [Fig. 2.33 (1a) and (1b)] [Fig. 2.33 (2a) and (2b)].

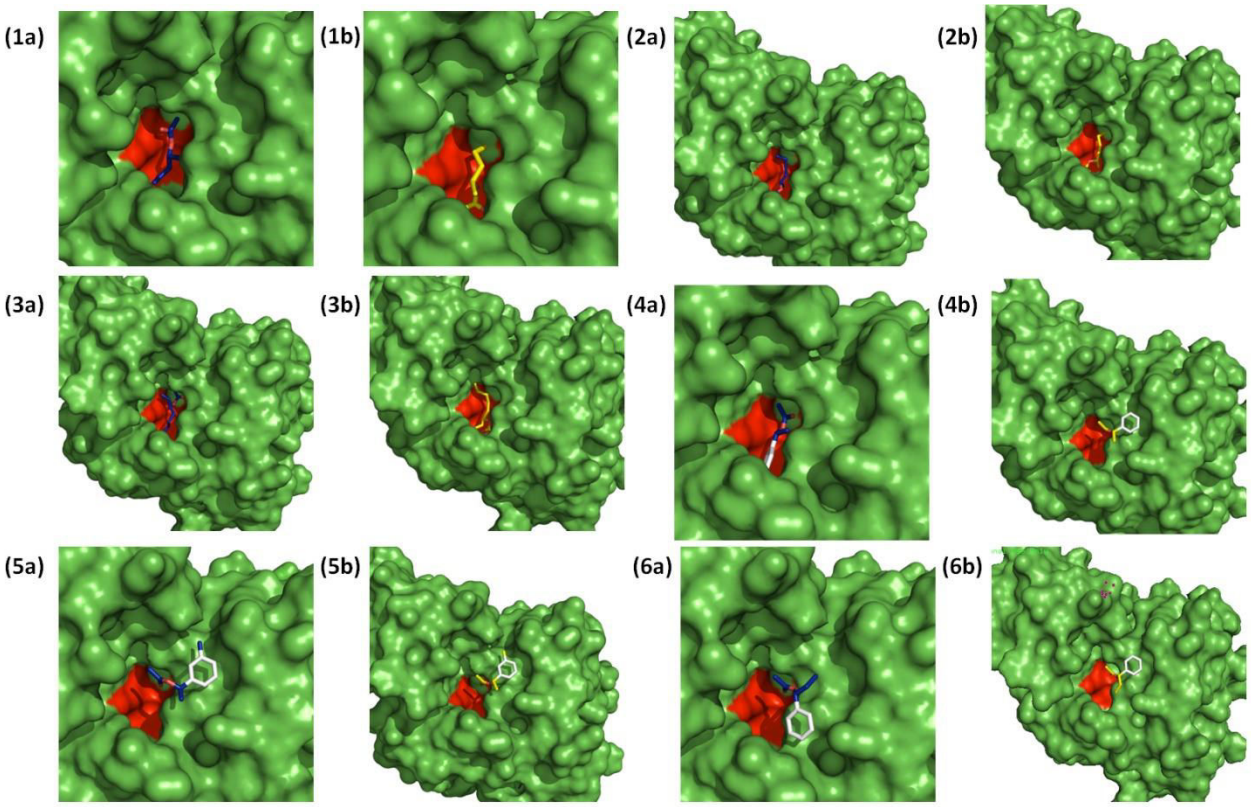


Fig.2.33 Docking analysis of various substrates at the active site pocket of MLC-3 esterase protein model. (**1a**)(*R*)-2-pentyl acetate, (**1b**) (*S*)-2-pentyl acetate, (**2a**) (*R*)-2-hexyl acetate, (**2b**) (*S*)-2-hexyl acetate, (**3a**) (*R*)-1-octen-3-yl acetate, (**3b**) (*S*)-1-octen-3-yl acetate, (**4a**) (*R*)-1-phenylethyl acetate, (**4b**) (*S*)-1-phenylethyl acetate, (**5a**) (*R*)-3-methyl-1-phenylethyl acetate, (**5b**) (*S*)-3-methyl-1-phenylethyl acetate, (**6a**) (*R*)-1-phenylpropyl acetate, (**6b**) (*S*)-1-phenylpropyl acetate. Amino acid residues highlighted in red are active sight residues containing GHSLG motif.

Similarly ligands such as (*R*)-1-phenylethyl acetate [Fig. 2.33 (4a)], (*R*)-3-methyl-1-phenylethyl acetate [Fig.2.33 (5a)] and (*R*)-1-phenylpropyl acetate [Fig.2.33 (6a)] were binding deep inside the stereo selective pocket with minimum free energy as compared to their counterparts such as (*S*)-1-phenylethyl acetate [Fig. 2.33 (4b)], (*S*)-3-methyl-1-phenylethyl acetate [Fig. 2.33 (5b)] and (*S*)-1-phenylpropyl acetate [Fig.2.33 (6b)].

Sr. no.	Substrates	Binding affinity (Kcal/mol)	
		(*R*)-acetate	**(*S*)-acetate**
1	2-Pentyl acetate	-4.7	-4.4
2	2-Hexyl acetate	-4.6	-4.4
3	1-octen-3-yl acetate	-4.6	-4.2
4	1-Phenylethyl acetate	-5.4	-5.1
5	3-Methyl-1-Phenylethyl acetate	-5.5	-5.3
6	1-Phenylpropyl acetate	-5.1	-4.8

Table 2.6 Binding affinity in Kcal/mol of (*R*) and (*S*) acetates with MLC-3 esterase after molecular docking studies.

Binding poses and binding affinity energies suggests that MLC-3 esterase utilizes (*R*)-acetates strictly to convert them into corresponding (*R*)-alcohols. Docking studies were found to be in well corroboration with *in vitro* studies depicting that MLC-3 esterase is (*R*)-selective esterase which preferred (*R*)-acetates only over the (*S*)-acetates.

2.4 Conclusion

In this work, we have successfully isolated, cloned and characterized two novel esterases SLC-6 and MLC-3 from metagenomic libraries having high catalytic efficiency and enantioselectivity towards wide range of economically important acyclic and aromatic acetates. Esterases SLC-6 and MLC-3 were expressed in *E. coli* as host and protein was purified using Ni-NTA agarose chromatography. Purified SLC-6 esterase showed optimum pH and temperature of 7.5 and 30 ºC respectively. SLC-6 esterase is highly selective for *p*-nitro phenol butyrate and its activity was enhanced due to Mg^{+2}, Na^{+1} and K^{+1} ions. SLC-6 esterase is highly stable in polar solvents such as DMSO while shows *R* and *S* selectivity of > 90 % e.e towards substrate (±)-2-pentyl acetate (**1**), (±)-2-hexyl acetate (**2**), (±)-3-hexyl acetate (**3**), (±)-1-phenyl ethyl acetate (**5**), (±)-3-methyl-1-phenyl ethyl acetate (**7**) and (±)-1-phenylpropyl acetate (**8**). MLC-3 esterase is alkaline stable enzyme with optimum pH and temperature of 8.0 and 20 ºC respectively. MLC-3 esterase is highly selective for *p*-nitrophenol butyrate and its activity was enhanced due to Mg^{+2}, $Na^{+1}$and K^{+1} ions. MLC-3 esterase is highly active in ethanol and shows (*R*) selectivity towards (±)-2-pentyl acetate (**1**), (±)-2-hexyl acetate (**2**), (±)-1-phenyl ethyl acetate (**5**), (±)-3-methyl-1-phenyl ethyl acetate (**7**) and (±)-1-phenylpropyl acetate (**8**) with 99.9 % e.e value to form corresponding alcohols. In conclusion, novel esterases SLC-6 and MLC-3 are reported first time and can be successfully used on commercial level for the preparation of enantiopure chiral enantiomers/synthons.

2.5 References

(1) Arpigny, J. L.; Jaeger, K. E. *Biochem. J.* **1999**, *343*, 177-183.

(2) Bornscheuer, U. T. *FEMS Microbiol. Rev.* **2002**, *26*, 73-81.

(3) Casas-Godoy, L.; Duquesne, S.; Bordes, F.; Sandoval, G.; Marty, A. *Methods mol. biol.* **2012**, *861*, 3-30.

(4) Romano, D.; Bonomi, F.; de Mattos, M. C.; Fonseca, T. d. S.; Ferreira de Oliveira, M. d. C.; Molinari, F. *Biotechnol. Adv.* **2015**, *33*, 547-565.

(5) Torrelo, G.; Hanefeld, U.; Hollmann, F. *Catal. Lett.* **2015**, *145*, 309-345.

(6) Jaeger, K. E.; Dijkstra, B. W.; Reetz, M. T. *Ann. Rev. Microbiol.* **1999**, *53*, 315.

(7) Jaeger, K. E.; Ransac, S.; Dijkstra, B. W.; Colson, C.; Vanheuvel, M.; Misset, O. *Fems Microbiology Reviews* **1994**, *15*, 29-63.

(8) Jaeger, K. E.; Reetz, M. T. *Trends Biotechnol.* **1998**, *16*, 396-403.

(9) Adlercreutz, P. *Chem. Soc. Rev.* **2013**, *42*, 6406-6436.

(10) Ansorge-Schumacher, M. B.; Thum, O. *Chem. Soc. Rev.* **2013**, *42*, 6475-6490.

(11) Dlugy, C.; Wolfson, A. *Bioproc. Biosyst. Eng.* **2007**, *30*, 327-330.

(12) Gotor-Fernandez, V.; Brieva, R.; Gotor, V. *J. Mol. Catal. B-Enzym.* **2006**, *40*, 111-120.

(13) Kumar, A.; Dhar, K.; Kanwar, S. S.; Arora, P. K. *Biol. proced. online* **2016**, *18*, 2-2.

(14) Kennedy, J.; O'Leary, N. D.; Kiran, G. S.; Morrissey, J. P.; O'Gara, F.; Selvin, J.; Dobson, A. D. W. *J. Appl. Microbiol.* **2011**, *111*, 787-799.

(15) Lopez-Lopez, O.; Cerdan, M. E.; Siso, M. I. G. *Curr. Prot. Pept. Sci.* **2014**, *15*, 445-455.

(16) Popovic, A.; Tchigvintsev, A.; Tran, H.; Chernikova, T. N.; Golyshina, O. V.; Yakimov, M. M.; Golyshin, P. N.; Yakunin, A. F. *Adv. exp. med. biol.* **2015**, *883*, 1-20.

(17) Tuffin, M.; Anderson, D.; Heath, C.; Cowan, D. A. *Biotechnol. J.* **2009**, *4*, 1671-83.

(18) Berlemont, R.; Pipers, D.; Delsaute, M.; Angiono, F.; Feller, G.; Galleni, M.; Power, P. *Rev. Argent. Microbiol.* **2011**, *43*, 94-103.

(19) Bunterngsook, B.; Kanokratana, P.; Thongaram, T.; Tanapongpipat, S.; Uengwetwanit, T.; Rachdawong, S.; Vichitsoonthonkul, T.; Eurwilaichitr, L.

Biosci. Biotechnol. Biochem. **2010**, *74*, 1848-1854.

(20) Chu, X. M.; He, H. Z.; Guo, C. Q.; Sun, B. L. *Appl. Microbiol. Biotechnol.* **2008**, *80*, 615-625.

(21) De Santi, C.; Ambrosino, L.; Tedesco, P.; de Pascale, D.; Zhai, L.; Zhou, C.; Xue, Y. F.; Ma, Y. H. *Biotechnol. Progr.* **2015**, *31*, 890-899.

(22) Fu, C. Z.; Hu, Y. F.; Xie, F.; Guo, H.; Ashforth, E. J.; Polyak, S. W.; Zhu, B. L.; Zhang, L. X. *Appl. Microbiol. Biotechnol.* **2011**, *90*, 961-970.

(23) Jeon, J. H.; Kim, J. T.; Kim, Y. J.; Kim, H. K.; Lee, H. S.; Kang, S. G.; Kim, S. J.; Lee, J. H. *Appl. Microbiol. Biotechnol.* **2009**, *81*, 865-874.

(24) Jiang, X. W.; Xu, X. W.; Huo, Y. Y.; Wu, Y. H.; Zhu, X. F.; Zhang, X. Q.; Wu, M. *Arch. Microbiol.* **2012**, *194*, 207-214.

(25) Park, H.-J.; Jeon, J. H.; Kang, S. G.; Lee, J.-H.; Lee, S.-A.; Kim, H.-K. *Protein Expres. Purif.* **2007**, *52*, 340-347.

(26) Lee, M. H.; Hong, K. S.; Malhotra, S.; Park, J. H.; Hwang, E. C.; Choi, H. K.; Kim, Y. S.; Tao, W. X.; Lee, S. W. *Appl. Microbiol. Biotechnol.* **2010**, *88*, 1125-1134.

(27) Jeon, J. H.; Lee, H. S.; Kim, J. T.; Kim, S. J.; Choi, S. H.; Kang, S. G.; Lee, J. H. *Appl. Microbiol. Biotechnol.* **2012**, *93*, 623-631.

(28) Oh, K. H.; Nguyen, G. S.; Kim, E. Y.; Kourist, R.; Bornscheuer, U.; Oh, T. K.; Yoon, J. H. *J. Mol. Catal. B-Enzym.* **2012**, *80*, 67-73.

(29) Kim, H. J.; Jeong, Y. S.; Jung, W. K.; Kim, S. K.; Lee, H. W.; Kahng, H.-Y.; Kim, J.; Kim, H. *Mol. Biotechnol.* **2015**, *57*, 781-792.

(30) Petrovskaya, L. E.; Novototskaya-Vlasova, K. A.; Spirina, E. V.; Durdenko, E. V.; Lomakina, G. Y.; Zavialova, M. G.; Nikolaev, E. N.; Rivkina, E. M. *FEMS Microbiol. Ecol.* **2016**, *92*.

(31) Bradford, M. M. *Anal. Biochem.* **1976**, *72*, 248-254.

(32) Yadav, K. N. S.; Adsul, M. G.; Bastawde, K. B.; Jadhav, D. D.; Thulasiram, H. V.; Gokhale, D. V. *Bioresource Technol.* **2011**, *102*, 10663-10670.

(33) Harris, E. M. S.; Aleshin, A. E.; Firsov, L. M.; Honzatko, R. B. *Biochemistry* **1993**, *32*, 1618-1626.

(34) van Gunsteren, W. F. *Biomolecular Simulation: The GROMOS96 Manual and User Guide*; Biomos ; Zurich, **1996**.

(35) Morris, G. M.; Huey, R.; Lindstrom, W.; Sanner, M. F.; Belew, R. K.; Goodsell, D. S.; Olson, A. J. *J. Comput. Chem.* **2009**, *30*, 2785-2791.

(36) Hart, W. E.; Rosin, C.; Belew, R. K.; Morris, G. M. In *Optimization in Computational Chemistry and Molecular Biology: Local and Global Approaches*; Floudas, C. A., Pardalos, P. M., Eds.; Springer US: Boston, MA, **2000**, p 209-229.

(37) Schomburg, I.; Chang, A.; Placzek, S.; Soehngen, C.; Rother, M.; Lang, M.; Munaretto, C.; Ulas, S.; Stelzer, M.; Grote, A.; Scheer, M.; Schomburg, D. *Nucleic Acids Res.* **2013**, *41*, D764-D772.

Chapter 3

Cloning, characterization & utilization of esterases/ lipases isolated from culturable microorganisms.

Chapter 3

Cloning, characterization & utilization of esterases /lipases isolated from culturable microorganisms.

Esterases/ lipases are ubiquitous enzymes found in nature. They are isolated from plants, animals and microorganisms. Microorganisms are the easiest source of these enzymes and so are isolated from various environmental niches such as soil, water, sea sediments, pond water etc. In this chapter, we have isolated lipases and esterases from microorganisms. We have cloned, expressed and characterized (*R*)-enantioselective pEST-1 esterase from *Pseudomonas pseudoalcaligenes* NCIM 2864. pEST-1 esterase is encoded by a gene with 612 bp ORF region and produces an enzyme which have optimum pH and temperature of 8.0 and 20 ºC respectively. Biochemical characterization was carried out using pEST-1 esterase and it was able to hydrolyse racemic acetates to (*R*)-alcohols in aqueous system. pEST-1 esterase could able to carry out selective de-esterification of (±)-2-pentyl acetate, (±)-2-hexyl acetate, (±)-1-phenyl ethyl acetate and (±)-1-phenylpropyl acetate to corresponding (*R*)-alcohol in quantitative manner [> 99 % enantiomeric excess (e.e)].Another microorganism *Yarrowia lipolytica* NCIM 3639 is Tween 80 isolated psychrophillic yeast which produces cell bound and an extracellular lipase (400 kD) after induction with olive oil and Tween 80 respectively. *Yarrowia lipolytica* NCIM 3639 produced cold active lipases could able to carry out kinetic resolution of (±)-lavandulyl acetate. Total RNA was isolated from un-induced (control) and Tween 80 induced *Yarrowia lipolytica* NCIM 3639 cells and was further subjected to transcriptome sequencing. Transcripts generated were then functionally annotated using Pfam analysis and total of nine new and uncharacterised lipases were identified.

3.1. Cloning, expression and biochemical characterization of alkaline stable (*R*)-enantioselective esterase isolated from *Pseudomonas pseudoalcaligenes* NCIM 2864.

3.1.1 Introduction

Esterase belongs to hydrolase family of enzymes (carboxylester hydrolases; EC. 3.1.1.1). Esterases catalyse formation or cleavage of low carbon chain or simple esters unlike lipases which reacts with higher carbon chain compounds. Esterases have same reaction mechanism as that of lipases where serine, aspartate and histidine amino acids are involved in the activity. Esterase shows consensus sequence as GXSXG (Gly-X-Ser-X-Gly) which is found around serine in the active site pocket.[1] Esterases are active over wide range of pH and temperatures, stable in many organic solvents and shows high regio-stereo specificity. Due to these favourable parameters esterases are found to be versatile biocatalysts and are often used commercially for production of optically pure compounds in fine chemicals synthesis.[2, 3]

Esterases are ubiquitous enzymes and they are reported from many plants, animals and microorganisms. Microbial esterases are widely used in industry due to ease in availability of microorganisms, easy to scale up and low cost of production. In literature, there are reports on synthesis of fine chemicals using commercial esterase such as pig liver esterase.[4,5] Esterases have also been reported for kinetic resolution of tertiary alcohols, [6-10] phenylalkyl carboxylic acids, [2] 1, 2-O-Isopropylideneglycerol esters, [11] dl-menthyl acetate, [12] 2-acetoxyphenylacetic acids [13] and for synthesis (*S*)-ketoprofen ethyl esters.[14-16] Esterases from *Bacillus* genus such as *Bacillus subtillis* [6,12] and from *Pseudomonas* genus such as *Pseudomonas* species, [15,17-22] *Pseudomonas fluorescens,* [23-30] *Pseudomonas putida,* [9,31-35] *Pseudomonas mandelii,* [36,37] *Pseudomonas aeruginosa,* [38,39], *Pseudomonas stutzeri,* [40] *Pseudomonas vesicularis,* [41] *Pseudomonas marginata* [42] are also reported. Although lipase [43] and a cutinolytic esterase [44] are reported, very little is known on enantioselective esterase from *Pseudomonas pseudoalcaligenes*. Herein, first time we report an (*R*)-enantioselective esterase from *Pseudomonas pseudoalcaligenes* NCIM 2864. A 612 bp *pEST-1* gene encoding pEST-1 esterase was cloned, over expressed and functionally characterised using recombinant *Escherichia coli* cells. pEST-1 esterase enantioselectively hydrolysed four racemic acetates to its corresponding (*R*)-alcohol with > 99 % e.e value.

3.1.2 Material and method

3.1.2.1 Chemicals and reagents

3.1.2.1.1 Media and kits

Peptone, yeast extract, malt extract, glucose, agar, Luria Bertani broth (L.B broth) used for bacterial culture maintenance were purchased from Himedia Laboratories Limited, Mumbai. All the kits used for DNA isolation and recombinant DNA technology experiments were purchased from Invitrogen, USA.

3.1.2.1.2 Other chemicals

p-nitrophenol (*p*-nitrophenol) esters (C2: *p*-nitrophenol acetate, C4: *p*-nitrophenol butyrate, C5: *p*- nitrophenol valerate, C8: *p*- nitrophenol octanoate, C10: *p*-nitrophenol decanoate, C12: *p*- nitrophenol dodecanoate, C14: *p*- nitrophenol myristate, C16: *p*- nitrophenol palmitate, C18: *p*- nitrophenol stearate) (C2-C18) were purchased from Sigma Aldrich, USA.

Racemic alcohols (±)-2-pentanol, (±)-2-hexanol, (±)-3-hexanol, (±)-1-octen-3-ol, (±)-1-phenylethanol, (±)-2-methyl-1-phenylethanol, (±)-3-methyl-1-phenylethanol and (±)-1-phenylpropanol were purchased from Sigma Aldrich/Fluka, USA. Racemic esters (±)-2-pentyl acetate (**1**), (±)-2-hexyl acetate (**2**) were purchased from Sigma Aldrich/Fluka, USA. Esters such as (±)-3-hexyl acetate (**3**), (±)-1-octen-3-yl acetate (**4**), (±)-1-phenylethyl acetate (**5**), (±)-2-methyl-1-phenylethyl acetate (**6**), (±)-3-methyl-1-phenylethyl acetate (**7**) and (±)-1-phenylpropyl acetate (**8**) were purchased from Sigma Aldrich USA. All the pure enantiomeric (*R*) and (*S*) standards of alcohols were purchased from Sigma Aldrich, USA.

3.1.2.2 Bacterial strain, plasmid and culture collection

A gram negative bacterium *Pseudomonas pseudoalcaligenes* NCIM 2864 was collected from the culture collection, National Collection of Industrial Microorganism (NCIM), NCL, Pune, India. pET 32-a (Novagen) plasmid was used for the cloning and over expression of the recombinant protein. *E. coli* DH5α and Rosetta gami (DE3) (Novagen) cells were used for the transformation and over expression of recombinant protein respectively.

3.1.2.3 DNA isolation from *Pseudomonas pseudoalcaligenes* NCIM 2864

Genomic DNA of *Pseudomonas pseudoalcaligenes* NCIM 2864 was isolated from 12 h grown culture using Pure Link Genomic DNA mini kit (Invitrogen).

Protocol for isolation of genomic DNA:

1) Set a water bath or heat block at 55 °C.

2) Harvest up to 2×10^{9} cells of Gram negative bacteria (~1 mL of overnight *P. pseudoalcaligenes* culture) by centrifugation.

3) Resuspend the cell pellet in 180 µL " Pure Link Genomic Digestion Buffer" . Add 20 µL Proteinase K to lyse the cells. Mix well by brief vortexing.

4) Incubate the tube at 55°C with occasional vortexing until lysis is complete (30 min.).

5) Add 20 µL RNase A to the lysate, mix well by brief vortexing, and incubate at room temperature for 2 min.

6) Add 200 µL PureLink ™ genomic Lysis/Binding Buffer and mix well by vortexing to obtain a homogenous solution.

7) Add 200 µL of 96–100% ethanol to the lysate. Mix well by vortexing for 5 sec. to yield a homogenous solution.

8) Remove a spin column in a collection tube from the package.

Binding of DNA:

9) Add the lysate (~640 µL) prepared with PureLink® Genomic Lysis/Binding Buffer and ethanol to the PureLink Spin Column.

10) Centrifuge the column at 10,000 X g for 1 minute at room temperature.

11) Discard the collection tube and place the spin column into a clean collection tube.

Washing of DNA:

12) Add 500 µL Wash Buffer 1 prepared with ethanol to the column.

13) Centrifuge column at room temperature at 10,000 X g for 1 min.

14) Discard the collection tube and place the spin column into a clean PureLink ™ collection tube supplied with the kit.

15) Add 500 µL Wash Buffer 2 prepared with ethanol to the column.

16) Centrifuge the column at maximum speed for 3 min. at room temperature. Discard collection tube.

Elution of DNA:

17) Place the spin column in a sterile 1.5 mL micro centrifuge tube.

18) Add 25 µL of sterile nuclease free water or PureLink Genomic Elution Buffer to the column.

19) Incubate at room temperature for 1 min. Centrifuge the column at maximum speed for 1 min. at room temperature. The tube contains purified genomic DNA.

20) Store the purified DNA at –20 °C.

3.1.2.4 Cloning of pEST-1 esterase gene

Total of 11 primer sets were generated from nucleotide sequences of 3 genome databases of *Pseudomonas pseudoalcaligenes* (CECT 5344, KF707, AD6) available in NCBI database (Table 3.1). Nucleotide sequences encoding for esterase/lipases were selected for primer designing. Gene of size 612 bp encoding *pEST-1* esterase was amplified from genomic DNA using "AccuPrime *Pfx* DNA polymerase" (Invitrogen) and routine PCR techniques. pEST-1 gene was amplified using following primers: Forward primer 5'- GAT GAT GGA TCC ATG ACC GCA TCC ATC CTC TAT AT -3' and Reverse primer 5'- ATC ATC AAG CTT TCA ATT GAA CGC GGA AAA GTC G -3' (Underlined are restriction sites for Bam HI and Hind III respectively). PCR conditions were as follows: One cycle: 95 ºC for 5 min. (Initial denaturation), 35 cycles: 95 ºC for 30 sec. (denaturation), 65 °C for 30 sec.(primer annealing) and 68 ºC for 1 min. (DNA amplification), One cycle: 10 min. at 68 ºC (final hold) and then at 4 ºC for infinite time.

Primer Set		Primer Names	Primer Sequences
1	Forward	PRAFP1	5'- ATG AAC ATG ACC ATG ACC AA -3'
	Reverse	PRARP1	5'- TCA GCC ATC CCA GTC CGT GT -3'
2	Forward	PRAFP2	5'- ATG TCC CTC GAC CCC CAG AT -3'
	Reverse	PRARP2	5'- TCA GTT CAG CGC GTG GCG CA -3'
3	Forward	PRAFP3	5'- GTG ATC CAC CTG GCC GGT TG -3'
	Reverse	PRARP3	5'- TCA GGC CCC CTC GTG GGC GA -3'
4	Forward	PRAFP4	5'- ATG AAA TCG ATC AGC ACC CT -3'
	Reverse	PRARP4	5'- TTA CTT CAG GTG ATC TTT CA -3'
5	Forward	PRAFP5	5'- ATG CGC CAC GAT GCC TTC TGG -3'
	Reverse	PRARP5	5'- TCA TGA GGG CTC CTT GGC GA -3'
6	Forward	PRAFP6	5'- GTG ATC CAC CTG GCC GGT TG -3'
	Reverse	PRARP6	5'- TCA GGC CCC CTC GTG GGC GA -3'
7	Forward	PRAFP7	5'- ATG GGC GCG GAA CTT CGA GG -3'
	Reverse	PRARP7	5'- CTA GCG CAC AAG GGC GTC TG -3'
8	Forward	PRAFP8	5'- ATG TCC AGT CTG CCG CTG TAT TTC C -3'
	Reverse	PRARP8	5'- CTA GCA CTT CAT CGG CGC ATC CGG A -3'
9	Forward	PRAFP9	5'- ATG AAG AAC AAT AAA ACC CTG CTC GC -3'
	Reverse	PRARP9	5'- TTA GAG CCC GGC GTT CTT CAG GCG AT -3'
10	Forward	PRAFP10	5'- ATG ACC GCA TCC ATC CTC TAT ATA CAC GGC -3'
	Reverse	PRARP10	5'- TCA ATT GAA TGC GGA AAA GTC GGT ATC ACG -3'
11	Forward	PRAFP11	5'- ATG GTA GTG AAC AAT GGC TGA TGA AGA GTG -3'
	Reverse	PRARP11	5'- TCA GTA GCG CCG CGT GCT CAT CAG -3'

Table 3.1 Set of forward and reverse primers used for screening of esterases/lipases in PCR amplification using genomic DNA of *P. pseudoalcaligenes* NCIM 2864.

PCR product obtained was digested with Bam-HI and Hind-III restriction enzymes and ligated into pET 32-a vector (Novagen). Resultant plasmid pEST-1-pET 32-a construct was subsequently transformed into *E. coli* DH5α cells. Plasmid was then extracted and sequenced to check for the undesired mutations.

3.1.2.5 Over expression and purification of recombinant pEST-1 esterase

Plasmid construct pEST-1-pET32-a was further transformed into Rosetta gami (DE3) cells for over expression of pEST-1 esterase. One colony with transformed pEST-1-pET32 a construct was inoculated into 5 mL culture of Luria Bertani (L.B) broth [tryptone (10 g), yeast extract (5 g), sodium chloride (10 g), pH-7.2] with antibiotics ampicillin (100 µg/mL), chloramphenicol (34 µg/mL) and tetracycline (25 µg/mL) and incubated at 37 ºC overnight in incubator shaker at 200 rpm. In secondary inoculum, 0.5 mL of overnight grown culture was then transferred to 50 mL L.B media with appropriate antibiotic selection and concentration. These flasks were then incubated at 37 ºC for 8 h and 10 mL of the well grown culture was then transferred into 1 litre of L.B media. When OD_{600} reached 0.5-0.6, culture was induced with IPTG at the final concentration of 1mM. Induced culture was further incubated at 16 ºC for 12 h and 200 rpm. After induction, cell pellet was kept on ice for 15 min. and cells were pellet down at 4000 X g for 20 min. at 4 ºC using centrifuge (Beckman & Coulter).

Cell pellet thus obtained was lysed with lysis buffer [Tris-HCl buffer (50 mM) pH 8.0, 300 mM NaCl, 0.1 % Triton X-100, 1 mM EDTA, 2 mM BME, 10 % glycerol] and lysozyme with the concentration of 1 mg/mL using probe sonicator. 6 sonic pulses (80 % amplitude) each with 30 sec. on and 30 sec. off cycles for 10 min. were used to completely lyse the cells. Lysed cell extract was subjected to centrifugation at 10,000 X g for 15 min. to clarify the cell debris and get a clear supernatant containing soluble protein fraction. This fraction was then kept for binding with charged Ni-NTA agarose for 1 h at room temperature. Protein bound Ni-NTA agarose was transferred to clean glass column and washed with wash buffers [Tris-HCl buffer (50 mM) pH 8.0, 300 mM NaCl, 10 % glycerol] with different gradients of imidazole concentrations. Two gradients of 20 mM and 60 mM imidazole containing wash buffers were passed over the Ni-NTA agarose matrix to remove the undesired protein contamination. Final elution of protein was carried out with elution buffer [Tris-HCl buffer (50 mM) pH 8.0, 300 mM NaCl, 100 mM imidazole, 10 % glycerol] to get well purified protein. Purity of protein was then checked using SDS-PAGE and quantification was done using Bradfords method and BSA as standard.[45]

3.1.2.6 SDS PAGE Electrophoresis

The purity and molecular weight of protein of pure enzyme were determined by SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis). 5% stacking gel and 12 % resolving gel of pH 6.8 and 8.8 respectively were used for the study. Electrophoresis was performed with 80V fixed voltage. After electrophoresis, gel was stained with Coomassie Brilliant Blue (CBB) and molecular weight of the purified enzyme was compared with standard protein marker.

3.1.2.7 Enzyme activity assay

Esterase activity was assayed using *p*-nitrophenol palmitate (*p*-nitrophenol palmitate) as a substrate for purified enzyme as reported earlier.[46] The assay conditions were as follows: *Solution A*: 37 mg of *p*-nitrophenol palmitate in 10 mL isopropanol. *Solution B*: 0.1 g Gum Arabic and 0.4 g Triton X-100 in 90 mL distilled water. 1 mL of solution A was mixed with 9 mL of solution B to make substrate solution. In the final reaction mixture, 0.9 mL of substrate solution was added into 0.1 mL buffer (potassium phosphate buffer, 10 mM, pH 7.0) and 0.1 mL (100 µg) esterase enzyme. Assay mixture was incubated at 37ºC for 30 min. under shaking condition to quantitate the release of *p*-nitrophenol in the reaction. Blank reaction was kept under similar conditions without enzyme. The release of *p*-nitrophenol from *p*-nitrophenol palmitate was spectrophotometrically determined at 410 nm using Cary 300 UV visible spectrophotometer (Varian). One unit of esterase activity was defined as the amount of enzyme releasing 1 µmol *p*-nitrophenol in 1 min under the assay conditions used. Experiments were conducted in triplicates and average values were calculated.

3.1.2.8 Molar extinction coefficient of *p*-nitrophenol

Molar extinction coefficient of *p*-nitrophenol was calculated at pH 8.0 in potassium phosphate buffer (10 mM). Three sets of each concentrations ranging from 20-200 µM of *p*-nitrophenol (99.9 % pure) were made and optical density was measured at 410 nm using UV visible spectrophotometer. Graph was plotted to calculate molar extinction coefficient. Molar extinction coefficient values for *p*-nitrophenol in potassium phosphate buffer (10 mM) at pH 8.0 were determined to be 15,000 mol^{-1}. cm^{-1}.

3.1.2.9 Effect of pH and temperature on enzyme activity

To investigate the effect of pH on pEST-1 esterase activity, buffers of various pH were prepared (Table 3.2). Enzymatic reaction was done under standard assay condition as mentioned earlier.

pH range	Buffers used	Molarity
4-4.5	Acetate buffer	100 mM
6-8	Potassium phosphate buffer	100 mM
8.5- 10.5	Glycine-sodium hydroxide buffer	100 mM
11-12	Na_2HPO_4- sodium hydroxide buffer	100 mM

Table 3.2 Buffers of various pH ranges used in the enzyme assay.

To study effect of temperature on the enzyme activity, initial reaction velocity (V_0) of the enzyme was calculated at various temperatures (10 ºC, 15 ºC, 20 ºC, 25 ºC, 30 ºC, 35 ºC, 40 ºC, 45 ºC, 50 ºC). In 0.9 mL of substrate solution containing 0.1 mL buffer of specific pH, 0.1 mL of purified enzyme (100 ng) was added and initial reaction velocity (V_0) was calculated for the enzyme at 410 nm using UV-visible spectrophotometer (Cary 300, Varian).

3.1.2.10 Determination of substrate specificity of the enzyme

To determine the substrate specificity of the pEST-1 esterase, *p*-nitrophenol esters of various acyl chain lengths (C2, C4, C5, C8, C10, C12, C14, C16 and C18) were used. The enzymatic reaction was carried out under standard assay condition as mentioned earlier at the optimum pH and temperature of the enzyme. *p*-nitrophenol esters (C2-C18) used were at a final concentration of 100 µM in the reaction mixture.

3.1.2.11 Effect of metal ions on enzyme activity

For determining the effect of metal ions on the activity of purified enzyme, enzyme assays were performed using various divalent and monovalent salt such as $CaCl_2$, $MgCl_2$, $NiCl_2$, NaCl, $CoCl_2$, $CuCl_2$, KCl, $FeSO_4$, $ZnCl_2$, $HgCl_2$ and NH_4Cl.

Enzymatic reaction was carried out under standard assay conditions and the final salt concentration used in the reaction was 5 mM.

3.1.2.12 Effect of various polar and non polar solvents on the stability of the enzyme

To study the stability of the enzyme in different organic solvents, various polar and non polar solvents such as toluene, ethanol, isopropanol, isoamyl alcohol, dimethyl formamide (DMF), acetonitrile, dimethyl sulfoxide (DMSO), n-hexane, carbon tetrachloride (CCl_4), dichloromethane (DCM), tetrahydrofuran (THF) and chloroform were used. The enzyme was pre-incubated with organic solvent (final concentration of 25 % v/v of solvent to enzyme solution) for 1 h at room temperature. This pre-incubated enzyme was further checked for the activity under standard assay conditions.

3.1.2.13 Effect of chelating agents, inhibitors and detergents on enzyme activity

For determining the effect of chelating agent (EDTA), reducing agents [dithiothreitol (DTT), 2-mercapto ethanol (β-ME), sodium dodecyl sulphate (SDS)] and detergents (Triton X-100, Tween 80 and CHAPS), enzyme was assayed with their presence in enzyme reaction mixture. EDTA, DTT, β-ME and CHAPS were used at the final concentration of 10 mM while Triton X-100, Tween-80 and SDS were used at the final concentration of 10 %, 10 % and 1 % respectively in the enzyme assay mixture. All other assay conditions were as per standard conditions.

3.1.2.14 Determination of the kinetic parameters of the pEST-1 esterase enzyme

Michaelis Menten graph was plotted using GraphPad Prism software to calculate kinetic parameters (K_m,V_{max}, K_{cat}, K_{cat} /K_m) of the enzyme. The initial rate of hydrolysis of *p*-nitrophenol butyrate was determined by using various concentration range of *p*-nitrophenol valerate (50 µM to 2200 µM) under standard conditions with purified pEST-1 esterase enzyme (100 nM). Optimum pH of 8.0 and optimum temperature of 20 ºC were used for determining the kinetic parameters of the enzyme.

3.1.2.15 Enantioselective hydrolysis of chiral racemic esters

pEST-1 esterase enzyme was studied for the enantioselective hydrolysis eight [(±)-2-pentyl acetate (**1**), (±)-2-hexyl acetate (**2**), (±)-3-hexyl acetate (**3**), (±)-1-octen-3-yl acetate (**4**), (±)-1-phenylethyl acetate (**5**), (±)-2-methyl-1-phenylethyl acetate (**6**), (±)-3-methyl-1-phenylethyl acetate (**7**) and (±)-1-phenylpropyl acetate (**8**)] commercially important racemic acyclic and aromatic acetates. 1450 µL of pEST-1 esterase enzyme

(1.3 mg, 1.35 IU/mg) was dissolved in 1550 µL of potassium phosphate buffer (pH-8.0, 100 mM) to make final volume of assay to 3 mL. 3 mg of racemic acetate was added to each assay reaction and was incubated at 20 ºC for 200 rpm in an incubator shaker. Aliquots of 200 µL were taken out at the interval of 6, 12, 18, 24, 30, 36, 42, 48 h and each was extracted twice with 400 µL of ethyl acetate (EtOAc) solvent. The upper extracted solvent layer was collected and passed over anhydrous sodium sulphate to inject in GC-FID having injector with split ratio of 1:10 for chiral resolution analysis.

3.1.2.16 Chiral GC Analysis

Chiral gas chromatographic analysis (Agilent GC-FID, 7890 A Agilent, USA) was performed using three chiral stationary phases.

1) Astec CHIRALDEX G-TA (2,6-di-O-pentyl-3-trifluoroacetyl derivative of γ-cyclodextrin, length: 30 m, I.D: 0.25 mm x film thickness: 0.12 µm, supelco, Sigma Aldrich, USA).

2) Astec CHIRALDEX B-DP (2, 3-di-O-propionyl-6-t-butyl silyl derivative of β-cyclodextrin, length: 30 m, I.D: 0.25 mm x film thickness: 0.12 µm, supelco, Sigma Aldrich, USA).

3) Astec CHIRALDEX B-DM (2,3-di-O-methyl-6-t-butyl silyl derivative of β-cyclodextrin, length: 30 m, I.D: 0.25 mm x film thickness: 0.12 µm, supelco, Sigma Aldrich, USA).

A flame ionisation detector (FID) temperature: 200 ºC, nitrogen as carrier gas (flow rate: 1mL/min) and front inlet (temperature: 200 ºC, split ratio: 1:10). All the enantiomers were well separated in GC-FID and enantiomeric excess, conversion values were calculated. Absolute configurations for all compounds were determined by co-injecting racemic mixture of alcohols with pure standards of (*R*) or (*S*) enantiomers into chiral GC.

3.1.2.17 GC/GC-MS conditions

A) While for (±)-2-pentanol and (±)-2-hexanol enantiomeric separation was done on GC column "Astec CHIRALDEX G–TA" capillary column (30 m x 0.25 mm x 0.12 µm, Supelco) with following temperature programme-

Oven temperature programme: Initial temperature maintained at 35 ºC for 1 min, followed by a temperature gradient from 35 ºC to 65 ºC at 1 ºC min^{-1} and then raised to a final temperature of 180 ºC with a 10 ºC min^{-1} rise.

Retention times (R_t): (*S*)-2-pentanol, (*R*)-2-pentanol, (*S*)-2-pentyl acetate & (*R*)-2-pentyl acetate were eluted at retention times of 13.2, 13.6, 17.8 and 18.6 min respectively**.**

Retention times (R_t): (*S*)-2-hexanol, (*R*)-2-hexanol, (*S*)-2-hexyl acetate & (*R*)-2-hexyl acetate eluted at retention times of 20.4, 21.25, 28.17 & 29.99 min respectively.

B) Chiral GC separation of (±)-3-hexanol, (±)-1-octen-3-ol, (±)-1-phenyl ethanol, (±)-3-methyl-1-phenyl ethanol and (±)-1-phenylpropanol was done on "Astec CHIRALDEX B-DP" capillary column (30 m x 0.25 mm x 0.12 µm, Supelco) with the following oven temperature programme

1) **For (±)-3-hexanol and (±)-3-hexyl acetate:**

Oven temperature programme: Initial temperature maintained at 40 ºC for 1 min, followed by a temperature gradient from 40 ºC to 60 ºC at 1 ºC min^{-1} and then raised to final temperature of 200 ºC at the rate of 10 ºC $min^{-1}$and kept on hold for 3 min.

Retention times (R_t): (±)-3-hexyl acetate, (*R*)-(-)-3-hexanol and (*S*)-(+)-3-hexanol were eluted at retention times of 14.9 min, 15.3 min, 17.9 min and 18.7 min respectively.

2) For (±)-1-octen-3-ol and (±)-1-octen-3-yl acetate:

Oven temperature programme: Initial temperature maintained at 40 ºC for 1 min, followed by a temperature gradient from 40 ºC to 80 ºC at 5 ºC min^{-1} and from 80 ºC to 105 ºC at 1 ºC min^{-1}.It was then raised to final temperature of 200 ºC at the rate of 10 ºC $min^{-1}$and kept on hold for 5 min.

Retention times (R_t): (±)-1-octen-3-yl acetate, (*R*)-(-)-1-octen-3-ol and (*S*)-(+)-1-octen-3-ol were eluted at retention times of 16.47 min, 16.6 min and 17.3 min respectively.

3) For (±)-1-phenyl ethanol and (±)-1-phenyl ethyl acetate:

Oven temperature programme: Initial temperature maintained at 40 ºC for 1 min, followed by a temperature gradient from 40 ºC to 90 ºC at 5 ºC min^{-1} and from 90 ºC to

110 ºC at 1 ºC min^{-1}.It was then raised to final temperature of 200 ºC at the rate of 10 ºC $min^{-1}$and kept on hold for 3 min.

Retention times (R_t): (±)-1-phenylethyl acetate, (*R*)-(+)-1-phenyl ethanol and (*S*)-(-)-1-phenyl ethanol were eluted at retention times of 21.2 min, 23.4 min and 24.4 min respectively.

4) For (±)-3-methyl-phenyl ethanol and (±)-3-methyl-phenyl ethyl acetate:

Oven temperature programme: Initial temperature maintained at 50 ºC, followed by a temperature gradient from 50 ºC to 115 ºC at 3 ºC min^{-1} and from 115 ºC to 135 ºC at 1 ºC min^{-1}.It was then raised to final temperature of 200 ºC at the rate of 10 ºC $min^{-1}$and kept on hold for 5 min.

Retention times (R_t): (±)-3-methyl-1-phenylethyl acetate, (*R*)-(+)-3-methyl-1-phenyl ethanol and (*S*)-(-)-3-methyl-1-phenyl ethanol were eluted at retention times of 24.2 min, 24.9 min and 25.33 min respectively.

5) For (±)-1-phenylpropanol and (±)-1-phenylpropyl acetate:

Oven temperature programme: Initial temperature maintained at 40 ºC for 1 min, followed by a temperature gradient from 40 ºC to 70 ºC at 5 ºC min^{-1} and from 70 ºC to 105 ºC at 1 ºC min^{-1}.It was then raised to final temperature of 200 ºC at the rate of 10 ºC $min^{-1}$and kept on hold for 3 min.

Retention times (R_t): (±)-1-phenylpropyl acetate, (*R*)-(+)-1-phenylpropanol and (*S*)-(-)-1-phenylpropanol were at retention times of 35.2 min, 37.8 min and 38.6 min respectively.

C) Chiral GC separation of (±)-2-Methyl-1-phenyl ethanol was done on "Astec CHIRALDEX B-DM" capillary column (30 m x 0.25 mm x 0.12 µm, Supelco) with following oven temperature programme:-

1) For (±)-2-Methyl-1-phenyl ethanol and (±)-2-Methyl-1-phenyl ethyl acetate:

Oven temperature programme: Initial temperature maintained at 50 ºC, followed by a temperature gradient from 50 ºC to 115 ºC at 3 ºC min^{-1} and from 115 ºC to 135 ºC at 1 ºC min^{-1}.It was then raised to final temperature of 200 ºC at the rate of 10 ºC $min^{-1}$and kept on hold for 5 min.

Retention times (R_t): (±)-2-methyl-1-phenylethyl acetate, (*R*)-(+)-2-methyl-1-phenyl ethanol and (*S*)-(-)-2-methyl-1-phenyl ethanol were eluted at retention times of 19.7 min, 25.2 min and 26.53 min respectively.

3.1.2.18 Nucleotide Sequence Accession Number

The nucleotide sequence of pEST-1 esterase gene is available at Gen Bank database as Gen Bank: KX495763.

3.1.2.19 Molecular docking studies

Homology modeling was performed using SwissModel server to predict three-dimensional structure of pEST-1 esterase. X-ray crystal structure of bacterial esterase (PDB ID: 4FLE) were used as templates for homology modeling.[47] Predicted models were energy minimized using GROMOS 43BI force field [48] and then assessed for its quality using ProSA and Ramchandran plot analysis. Docking studies were performed using AutoDock 4.2 version.[49] Ligands and receptor files were optimized for docking studies and converted from .pdb to .pdbqt format. The grid was set around active site residues of pEST-1 esterase with dimensions of 20 x 20 x 20 Å. The docking parameters were configured on a LGA calculation of 10,000 runs. Energy evaluations were set to 1,500,000 and 27,000 generations. Population size was set to 150 and the rate of gene mutation and the rate of gene crossover were set to 0.02 and 0.8 respectively. [50] The obtained conformations were later summarized, collected and extracted by using Autodock Tool. Geometry of resulting complexes was studied using the PyMol molecular viewer utility (The PyMOL Molecular Graphics System, Version 1.5.0.4 Schrödinger, LLC).

3.1.3 Results and Discussion

3.1.3.1 Isolation of genomic DNA from *P. pseudoalcaligenes* NCIM 2864

Genomic DNA of *Pseudomonas pseudoalcaligenes* NCIM 2864 was isolated from overnight grown culture using "Pure Link Genomic DNA mini kit" (Invitrogen). Good quality of genomic DNA was isolated (Fig 3.1) from culture as assessed by $A_{260/230}$ and $A_{260/280}$ ratios.

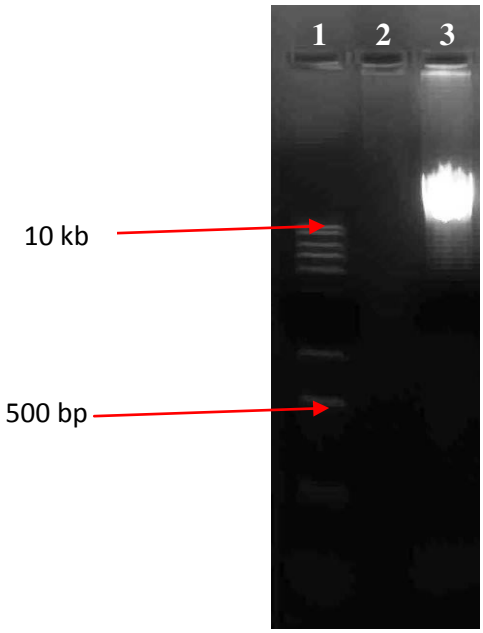


Fig. 3.1 Agarose gel of isolated genomic DNA from *P. pseudoalcaligenes* NCIM 2864. **Lane 1**:-1kb DNA ladder, **Lane 3**:-genomic DNA from *P. pseudoalcaligenes* NCIM 2864.

(Nanodrop readings: - $A_{260/230}$ = 2.11, $A_{260/280}$ = 1.97)

3.1.3.2 Cloning and sequence analysis of the pEST-1 esterase gene

Nucleotide sequence of the pEST-1 esterase gene of *P. pseudoalcaligenes* consists of 612 bp open reading frame (ORF) region.pEST-1 esterase gene was amplified using "Accuprime *pfx* polymerase" (Fig.3.2) and was cloned into pET 32-a vector.

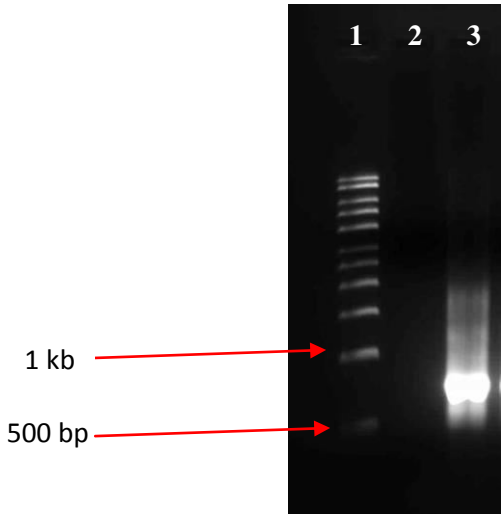


Fig. 3.2 Agarose gel for PCR amplification of pEST-1 esterase. **Lane 1**: 1kb DNA ladder **Lane 3**: PCR amplified *pEST-1* esterase gene (612 bp)

Positive colonies identified from colony PCR (Fig. 3.3) using T7 forward and reverse primers were picked and inoculated for plasmid isolation and the plasmids sent for sequencing to check the frame and for any unwanted mutations.

- **Agarose gel for colony PCR:**

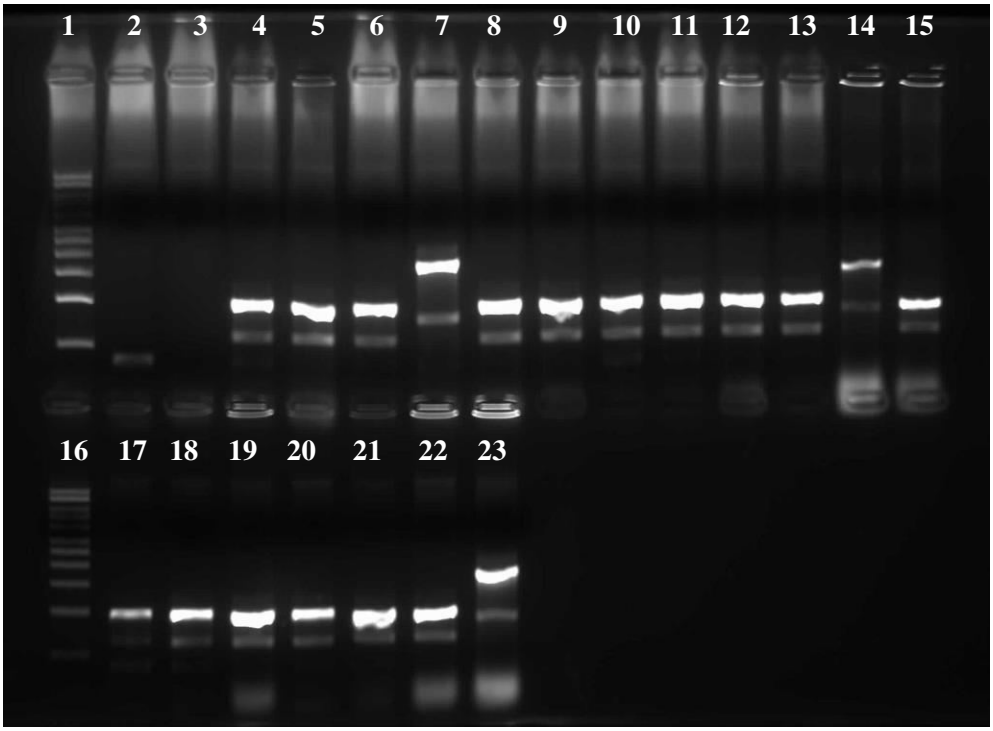


Fig. 3.3 Agarose gel for colony PCR of pEST-1 esterase cloning into pET 32-a vector. **Lane 1, 16**: 1kb DNA ladder, **Lane 2**: Negative control, **Lane 3 to 23**: colony PCR product of colonies 1 to 20.

Nucleotide and amino acid sequence of pEST-1 esterase:

- **pEST-1 esterase (612 bp)**

ATGACCGCATCCATCCTCTATATACACGGCCTCAACAGTTCGCCGGCCTCGCTCAAG
GCCAGCCAGTTGAGCCGTGCCATGGCTCATCTGGGCCTGGAAAACCAGTTGCGCATA
CCGGCCCTGCATCATCATCCGCGTCAGGCCATCACGCAGTTGCAGGCGCTGATCAGC
GAACTGGGCGCGCCGCTACTGGTGGGCAGCTCACTGGGCGGCTACTACGCCACTTAC
CTGGCCGAGCAGCACGGGCTCAAGGCACTGCTGATCAATCCGGCCGTGCAGCCGCAC
CTGCGTTTCGACGGCTACCTGGGCCCACAGAAGAATTACTACAGCGACGAGACCTGG
GACCTCACCGAGGATCACGTCCGCGCCCTGGCTGAACTCGACGTTGCAGCGCCGAAC
GACCCGGCGCGTTACCAGGTGTGGCTGCAAACCGGCGACGAAACCCTCGACTACCGC
GACGCCGAGCGTTACTACCGTGCCTGCGCCCTGCGCATCCAGGCCGGTGGCGACCAC
GGTTTCCAGGGCTTTACCGAACACTTGCCGGCACTCTTCGCATTCGCCGACATTAGCG
CCACACTCTGGCGTGATACCGACTTTTCCGCGTTCAATTGA

- **Amino acid sequence of pEST-1 esterase (204 a.a)**

MTASILYIHGLNSSPASLKASQLSRAMAHLGLENQLRIPALHHHPRQAITQLQALISELGA
PLLVGSSLGGYYATYLAEQHGLKALLINPAVQPHLRFDGYLGPQKNYYSDETWDLTED
HVRALAELDVAAPNDPARYQVWLQTGDETLDYRDAERYYRACALRIQAGGDHGFQGF
TEHLPALFAFADISATLWRDTDFSAFN-

pEST-1 esterase gene encodes a 204 amino acid sequence and shows a conserved pentapeptide GSSLG which belongs to GXSXG motif. This GXSXG motif is found in many serine hydrolases family proteins. pEST-1 esterase shows Abhydrolase_6 domain which belongs to esterase/lipase super family (Fig.3.4).

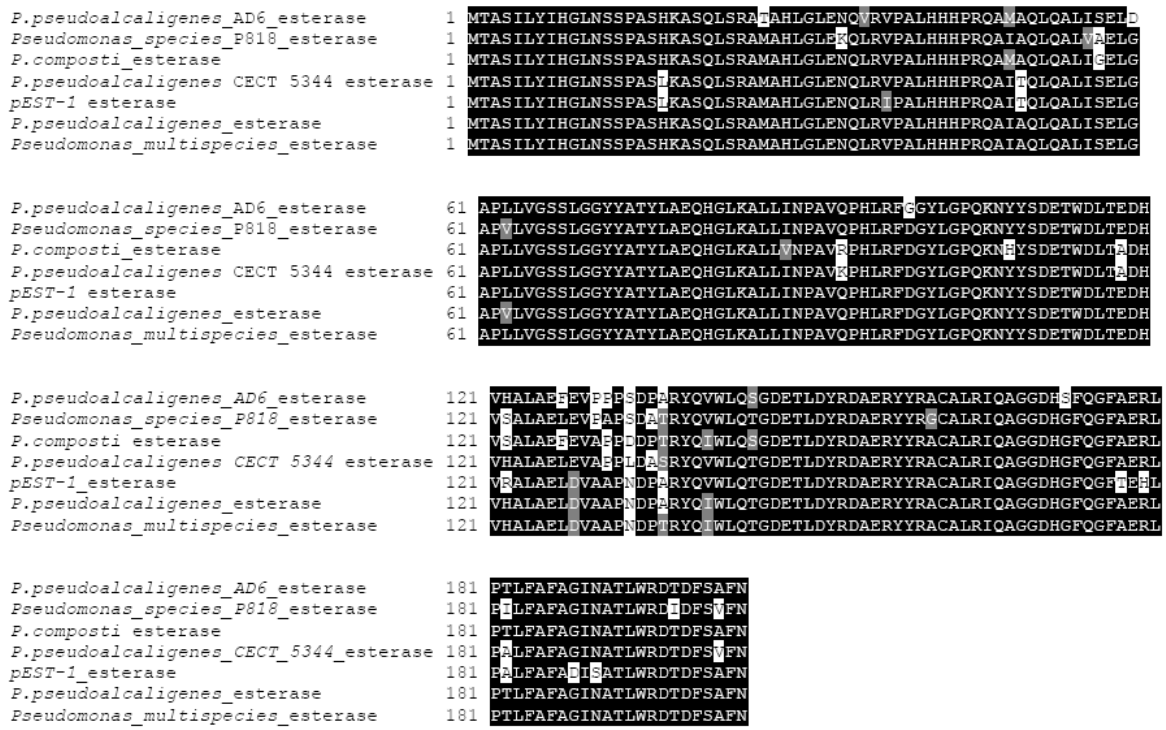


Fig. 3.4 Multiple sequence alignment of pEST-1 esterase with close homologue esterase sequences.The amino acid sequence corresponds to pEST-1 esterase (GI accession no. KX495763), *Pseudomonas multispecies esterase* (WP_059390329.1, 95 % identity), *Pseudomonas pseudoalcaligenes* esterase (WP_017678636.1, 95 % identity), *Pseudomonas pseudoalcaligenes* CECT 5344 esterase (WP_003459142.1, 93 % identity), *Pseudomonas pseudoalcaligenes* AD6 esterase (WP_037003433.1, 90 % identity with pEST-1 esterase), *Pseudomonas species P818 esterase* (WP_024306728.1, 90 % identity), *Pseudomonas composti* (WP_061237415.1, 89 % identity). Residues that are 100% conserved are shadowed in black, and those between 75 % and 100 % are shadowed grey.

3.1.3.3 Phylogenetic analysis

BlastP results of the pEST-1 esterase enzyme shows match with closely related *Pseudomonas* family esterases. Phylogenetic analysis studies revealed that pEST-1 esterase is closely associated with various esterases sequence of multiple families of *Pseudomonas* genus. pEST-1 esterase is closely related with *P. pseudolacaligenes* strains such as AD6, CECT 5344 and with *P. mendocina* esterase on evolutionary scale. pEST-1 esterase is distantly related to esterase enzymes of other *Pseudomonas* species such as *P. fluroscenes*, *P. brassicacearum*, *P. parafulva*, *P. veronii*, *P. thivervalensis* on evolutionary basis as shown in Fig. 3.5.

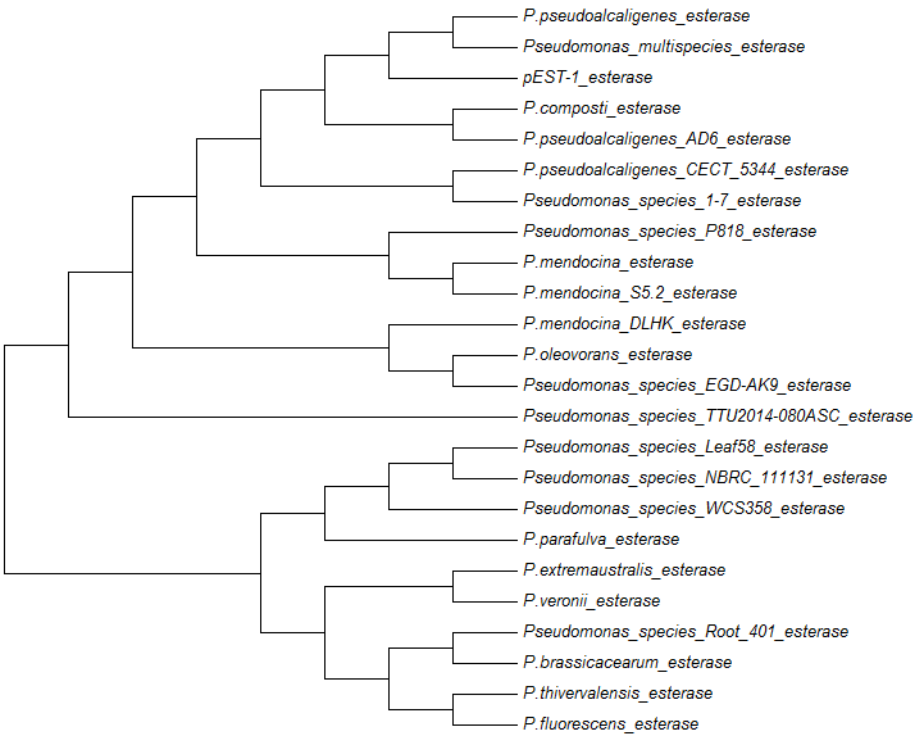


Fig. 3.5 Phylogenetic tree of pEST-1 esterase and other closely related esterase enzymes. The phylogenetic analysis was performed by the neighbour joining method using MEGA 6.0 software. The values at nodes indicate the bootstrap percentage of 1,000 replicates.

3.1.3.4 Expression and purification of the recombinant pEST-1 esterase enzyme

Recombinant pEST-1 esterase enzyme was over expressed in pET 32-a vector using Rosetta gami (DE3) bacterial cells. Over expressed cells were then lysed and protein was purified using Ni-NTA agarose chromatography technique. Purified protein was analyzed for purity on 12 % sodium dodecyl sulphate polyacryl amide gel electrophoresis (SDS-PAGE) gel (Fig. 3.6). Protein corresponded well with their expected mass of ~40 kD due to additional *Trx* solubilisation domain and His-tag present in pET 32-a vector.

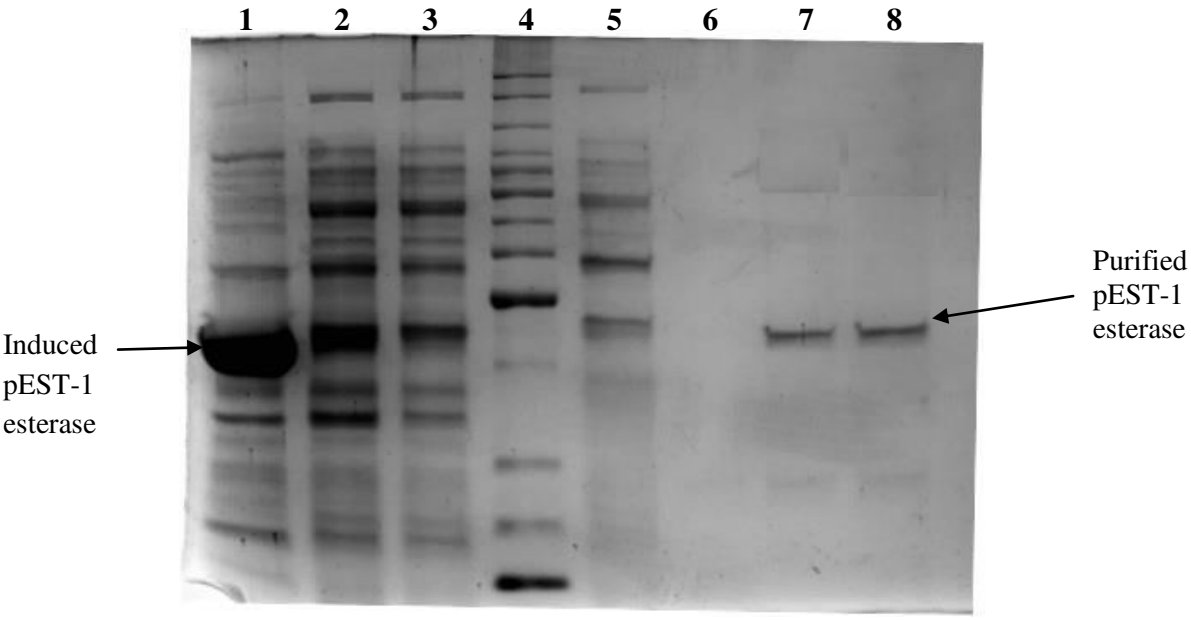


Fig. 3.6 SDS gel for purification of pEST-1 esterase using Ni-NTA agarose chromatography. **Legends:-Lane 1**: Induced pellet of pEST-1 esterase expressed in pET 32-a vector. **Lane 2**: Induced supernatant of pEST-1 esterase expressed in pET 32-a vector. **Lane 3**: Unbound fraction. **Lane 4**: Protein marker. **Lane 5**: 20 mM imidazole wash fraction. **Lane 6**: 60 mM imidazole wash fraction. **Lane 7**: Elution fraction 1 (100 mM).**Lane 8**: Elution fraction 2 (100 mM).

3.1.3.5 Effect of pH and temperature on the activity of pEST-1 esterase

Optimum pH and temperature studies were performed with the pure pEST-1 esterase enzyme. Buffers of pH range 4-11 were used with *p*-nitrophenol valerate as a substrate for the assay reaction wherein pEST-1 esterase showed optimum pH of 8.0. Optimum activity at pH 8.0 suggests this enzyme to be an alkaline stable esterase [Fig. 3.7 (**A**)].

Initial reaction velocities of the enzymatic reactions were calculated at various temperature ranges from 10 ºC to 60 ºC. pEST-1 esterase showed highest activity at temperature of 20 ºC thus can be assumed as microorganism of psychrophilic in origin [Fig. 3.7 (**B**)].

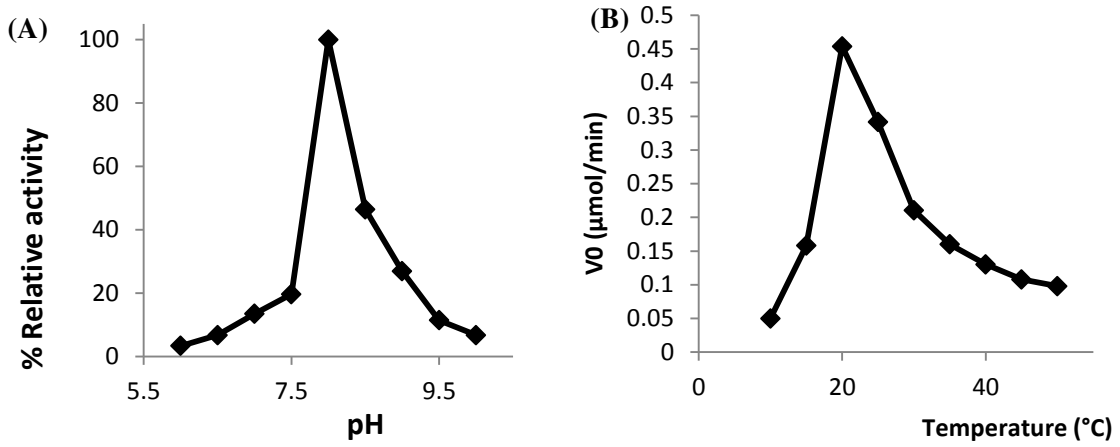


Fig. 3.7 Study of optimum pH and temperature of pEST-1 esterase enzyme (**A**) optimum pH study of pEST-1 esterase (**B**) optimum pH study of pEST-1 esterase. All the experiments were carried out in triplicates and error bars shows the standard deviation.

3.1.3.6 Determination of substrate specificity and effect of metal ions on the activity of pEST-1 esterase

Different carbon length esters of *p*-nitrophenol were tested for checking the substrate specificity of pEST-1 esterase enzyme pEST-1 esterase was found to be highly active towards *p*-nitrophenol valerate (C5) and activity was decreasing towards higher carbon length of esters (C6-C18). Higher activity towards short chain esters of *p*-nitrophenol confirms enzyme to be an esterase (Fig. 3.8).

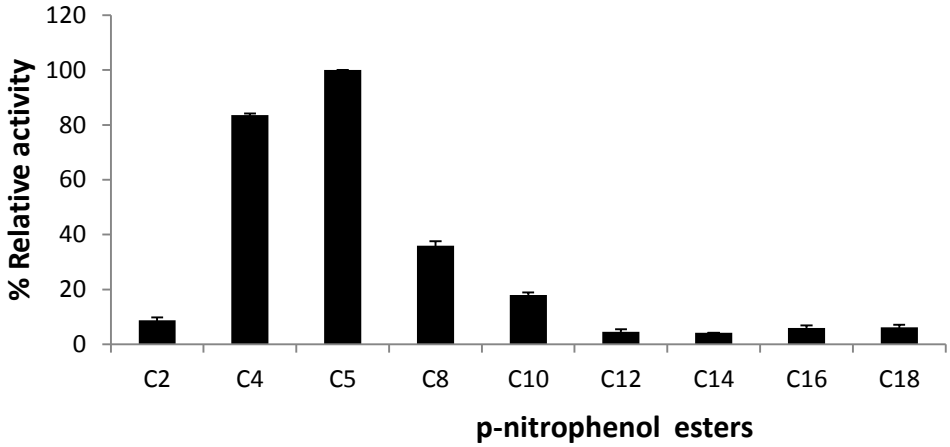


Fig. 3.8 Determination of substrate specificity of pEST-1 esterase using *p*-nitrophenol esters. (C2: *p*- nitrophenol acetate, C4:*p*- nitrophenol butyrate, C5:*p*- nitrophenol valerate, C8:*p*-nitrophenol octanoate, C10:*p*- nitrophenol decanoate, C12: *p*- nitrophenol dodecanoate, C14: *p*-nitrophenol myristate, C16: *p*- nitrophenol palmitate, C18: *p*- nitrophenol stearate). All the experiments were carried out in triplicates and error bars shows the standard deviation.

Presence of different metal ions in the enzyme assay mixture either enhances or decreases enzyme activity. $FeSO_4$, $CuCl_2$, $NiCl_2$ and $ZnCl_2$ decreased pEST-1 esterase activity to 31.4, 41.08, 56.9 and 50.04 % respectively while $HgCl_2$,$CoCl_2$,$CaCl_2$ and NH_4Cl decreased enzyme activity to 65.5 ,77.8,86.8 and 72.2% respectively. Monovalent and divalent salts such as $MgCl_2$, NaCl and KCl increased the enzyme activity to 104,104.1 and 103.8 % respectively.

Though esterase doesn't need cofactor for their activity, increase in the activity of enzymes by addition of ions such as Mg^{+2}, K^{+1} and Na^{+1} may be due to binding of these ions to allosteric sites of the enzyme and positively modulating the enzyme conformation to enhance the activity. (Fig. 3.9)

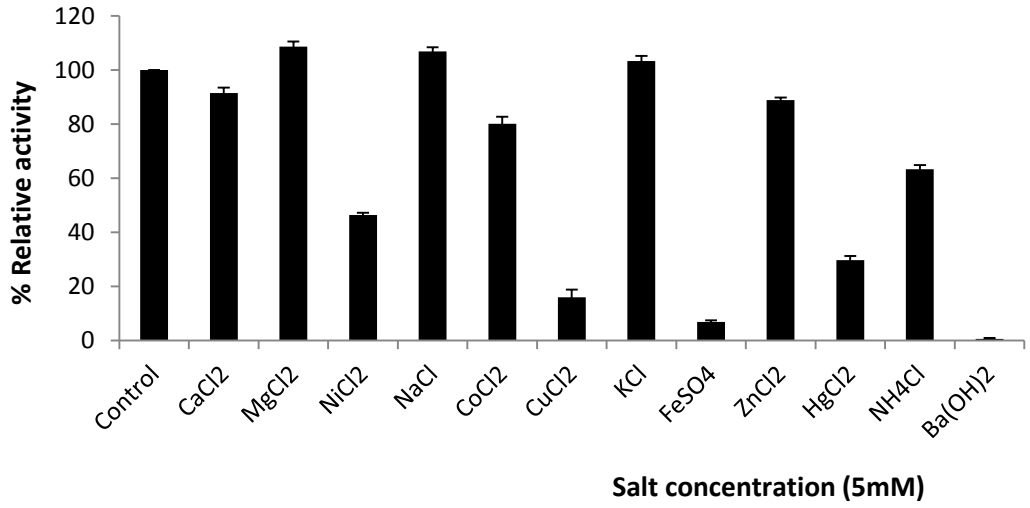


Fig. 3.9 Determination of effects of various metal salt ions on the pEST-1 esterase enzyme. All the experiments were carried out in triplicates and error bars shows the standard deviation.

3.1.3.7 Effect of organic solvents, chelating agent and inhibitors on the activity of pEST-1 esterase

Stability of pEST-1 esterase was analysed in the presence of various polar and non-polar solvents (25 % v/v) (Fig.3.10). Solvents such as isoamyl alcohol, chloroform, toluene, carbon tetrachloride and n-hexane reduced enzyme activity drastically to 18.5 %, 36.5 %, 46.5 %, 41.4 % and 52 % respectively compared to control (enzyme without solvents). Furthermore, solvents such as dimethylforamide (DMF), acetonitrile, ethanol, isopropanol, dimethylsulfoxide (DMSO) and tetrahydrofuran (THF) reduced enzyme activity to 73.4 %, 83.4 %, 90.2 %, 97.8 %, 98 % and 98.7 % respectively. Solvent

dicholoromethane (DCM) was shown to enhance the enzyme activity to 111.3 %. Above results suggests pEST-1 esterase to be quite stable in both polar and non-polar solvents.

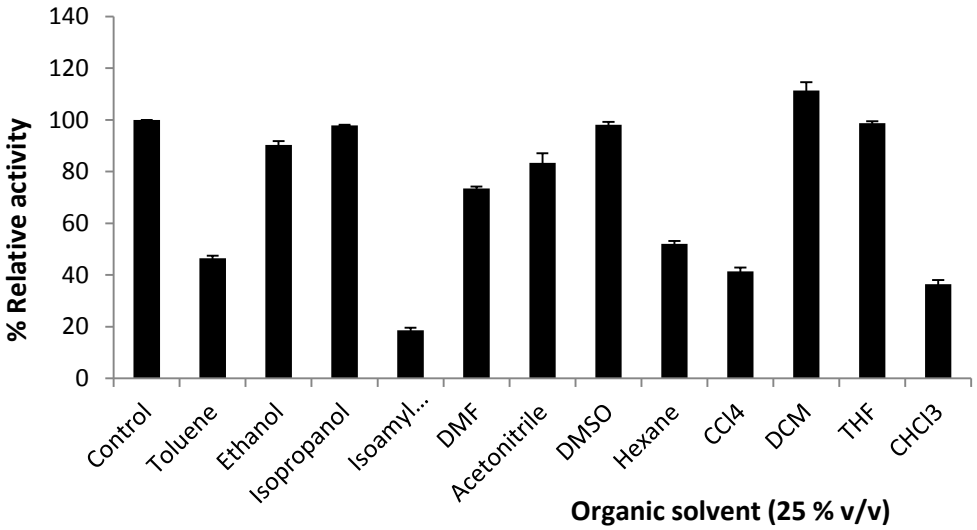


Fig.3.10 Determination of stability of pEST-1 esterase in different polar and non-polar organic solvents. All the experiments were carried out in triplicates and error bars shows the standard deviation.

pEST-1 esterase activity was checked in the presence of chelators, enzyme inhibitors and detergents (Fig.3.11). Metal ion chelator EDTA reduced enzyme activity drastically to 18.4 % suggesting that the enzyme do require presence of some metal ions for their optimal activity. Enzyme inhibitors such as dithiothreitol (DTT), 2-β-mercaptoethanol (β-ME) and sodium dodecyl sulphate (SDS) reduced enzyme activity drastically to 0.5 %,0.15 % and 21.6 % respectively suggesting a possible loss of conformation or inhibition of enzyme by the inhibitors. Non ionic detergents such as Triton X-100, Tween 80 reduced enzyme activity to 14.4 % and 17.8 % respectively whereas zwitterionic detergent, CHAPS increased enzyme activity to 121 %.

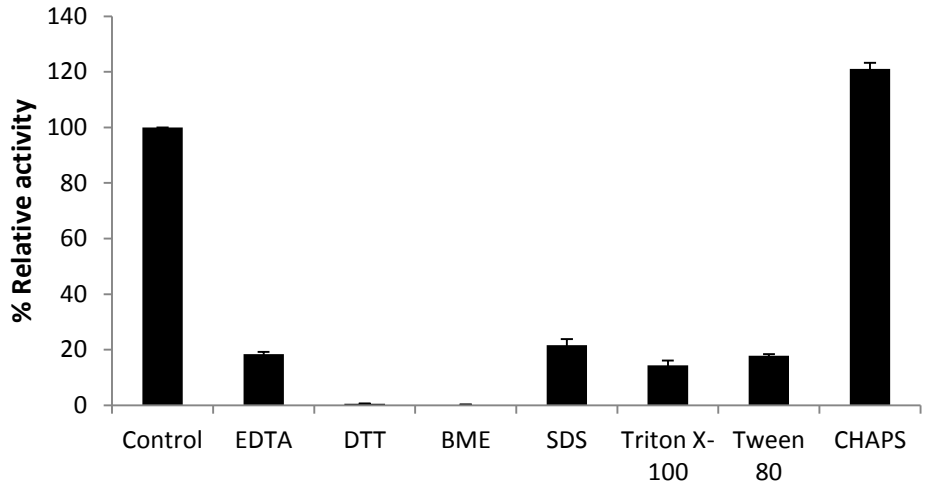


Fig. 3.11 Effect of chelator, enzyme inhibitors and detergents on pEST-1 esterase. All the experiments were carried out in triplicates and error bars shows the standard deviation.

3.1.3.8 Enzyme kinetics study with of pEST-1 esterase

Kinetic parameters of the pEST-1 esterase were calculated plotting Michealis-Menten graph using substrate *p*-nitrophenol valerate at its optimum pH and temperature of 8.0 and 20 ºC respectively (Fig.3.12).

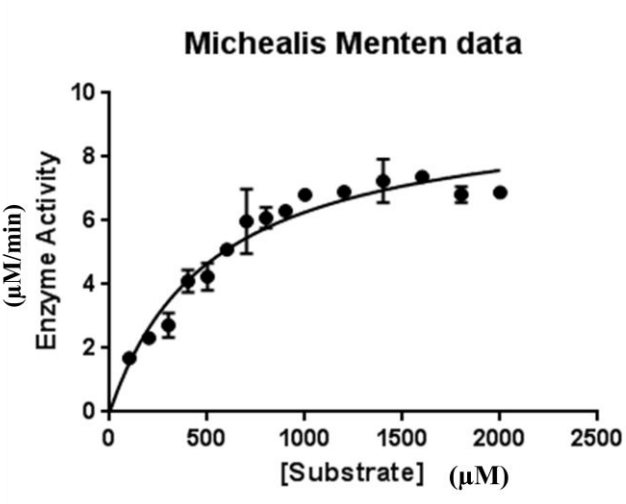


Fig. 3.12 Michaelis Menten graph for the pEST-1 esterase enzyme.

pEST-1 esterase enzyme (100 nM) kinetics was studied using various concentration ranges (50-2200 µM) of substrate *p*-nitrophenol valerate. Kinetic parameters such as V_{max}, K_m, K_{cat} and K_{cat}/K_m calculated for the pEST-1 esterase were 9.5 $\mu M.min^{-1}$, 529 µM, 1.5 s^{-1} and 2987 $M^{-1}.s^{-1}$ respectively (Table 3.3).

The kinetic parameters calculated for pEST-1 esterase was comparable with many carboxyl esterases present in BRENDA enzyme database.[51]

Enzyme	Substrate	V_{max} ($\mu M.min^{-1}$)	K_m (μM)	K_{cat} (S^{-1})	K_{cat}/K_m ($M^{-1}.S^{-1}$)
pEST-1 esterase	C5	9.5	529	1.5	2987

Table 3.3 Kinetic parameters of pEST-1 esterase calculated using Michaelis-Menten graph.

3.1.3.9 Enantioselective hydrolysis of racemic acyclic/aromatic acetates by pEST-1 esterase

Time course study for kinetic resolution via enantioselective hydrolysis of eight racemic acetates [(±)-2-pentyl acetate (**1**), (±)-2-hexyl acetate (**2**), (±)-3-hexyl acetate (**3**), (±)-1-octen-3-yl acetate (**4**), (±)-1-phenylethyl acetate (**5**), (±)-2-methyl-1-phenylethyl acetate (**6**), (±)-3-methyl-1-phenylethyl acetate (**7**) and (±)-1-phenylpropyl acetate (**8**)] were carried out using purified pEST-1 esterase (Fig. 3.17). Hydrolysis reactions of acetates were carried out at 20 ºC and 200 rpm in incubator shaker (Table 3.4).

Acyclic acetates such as (**1**) was hydrolysed to (*R*)-(-)-2-pentanol (**1a**) with 99.9 % e.e and 21 % conversion in 48 h by pEST-1 esterase (Fig. 3.13) [Fig. 3.17 (**A**)].

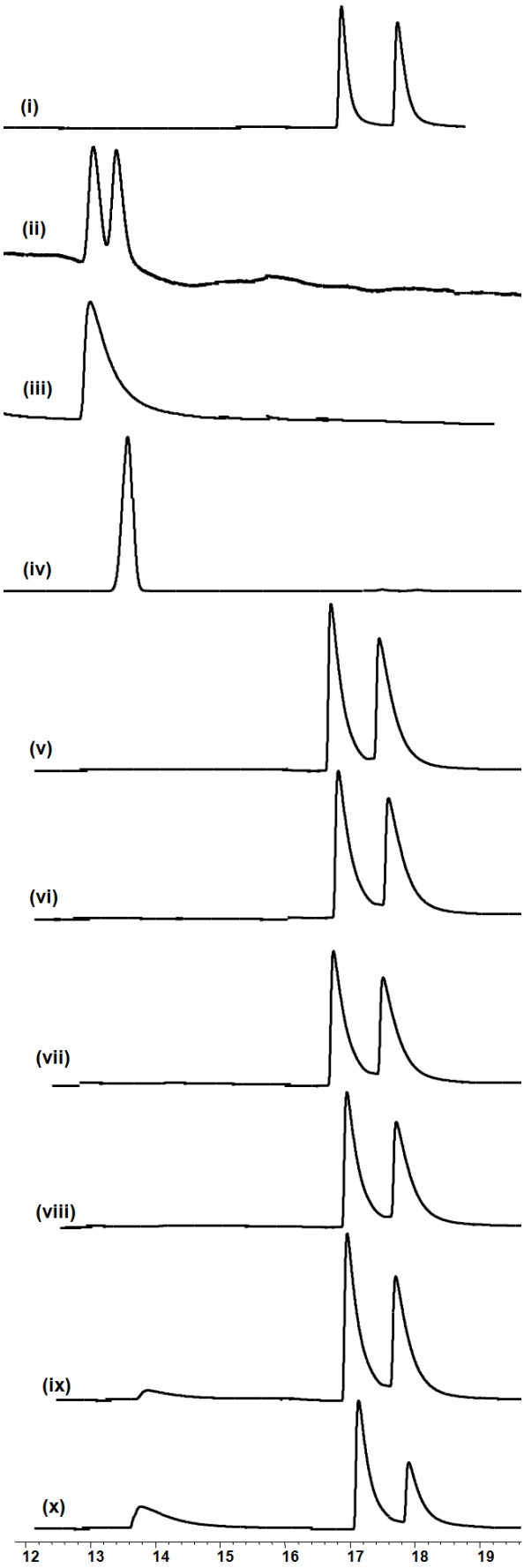

(i)
(ii)
(iii)
(iv)
(v)
(vi)
(vii)
(viii)
(ix)
(x)
12
13
14
15
16
17
18
19

Fig. 3.13 Time Course study for the conversion of (±)-2-Pentyl acetate by pEST-1 esterase. (i) standard (±)-2-pentyl acetate (ii) standard (±)-2-pentanol (iii) standard (*S*)-(+)-2-pentanol (iv) Standard (*R*)-(-)-2-pentanol (v) Conversion of (±)-2-pentyl acetate after 6 h incubation (vi) Conversion of (±)-2-pentyl acetate after 18 h incubation (vii) Conversion of (±)-2-pentyl acetate after 24 h incubation (viii) Conversion of (±)-2-pentyl acetate after 30 h incubation (ix) Conversion of (±)-2-pentyl acetate after 42 h incubation (x) Conversion of (±)-2-pentyl acetate after 48 h incubation .

Acyclic acetates (**3**) and (**4**) were not at all hydrolysed by pEST-1 esterase suggesting no substrate recognition/specificity by the pEST-1 esterase enzyme active site.

Aromatic acetates (**5**) and (**7**) were converted to respective (*R*)-1-phenyl ethanol (**5a**) (Fig. 3.14) [Fig. 3.17 (**B**)] and (*R*)-1-phenyl ethanol (**7a**) (Fig. 3.15) [Fig. 3.17 (**C**)] with 99.9 %, 84 % e.e and 8.5 %, 11 conversion respectively at the end of 48 h.

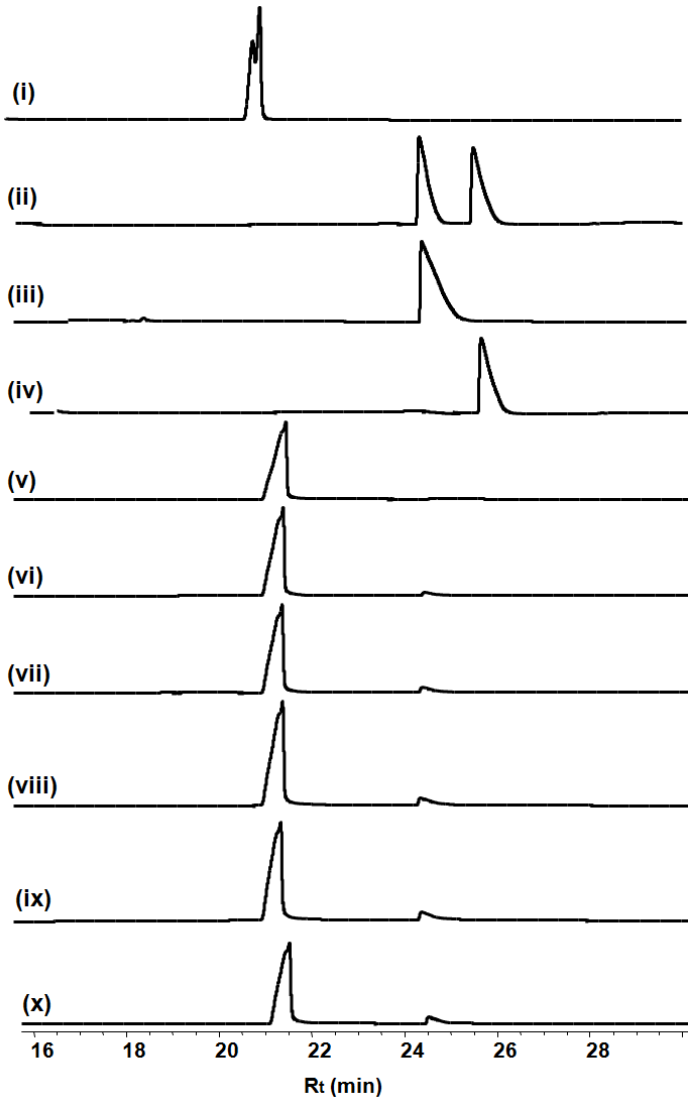
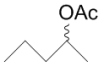
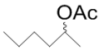
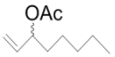


Fig. 3.14 Time Course study for the conversion of (±)-1-Phenyl ethyl acetate by pEST-1 esterase.

(i) standard (±)-1-Phenyl ethyl acetate (ii) standard (±)-1-Phenyl ethanol (iii) standard (*R*)-1-Phenyl ethanol (iv) standard (*S*)-1-Phenyl ethanol (v) Conversion of (±)-1-Phenyl ethyl acetate after 6 h incubation (vi) Conversion of (±)-1-Phenyl ethyl acetate after 18 h incubation (vii) Conversion of (±)-1-Phenyl ethyl acetate after 24 h incubation (viii) Conversion of (±)-1-Phenyl ethyl acetate after 30 h incubation (ix) Conversion of (±)-1-Phenyl ethyl acetate after 42 h incubation (x) Conversion of (±)-1-Phenyl ethyl acetate after 48 h incubation .

Substrate (**8**) was enantioselectively hydrolysed to (*R*)-1-phenylpropanol (**8a**) with 99.9 % e.e with 5 % conversion after 48 h incubation with pEST-1 esterase (Fig. 3.16) [Fig. 3.17 (**D**)]. Compound (**6**) was not hydrolysed by the enzyme suggesting the interference of methyl group present at second position of phenyl ring with the active site pocket.

Entry	Substrate	% Conversion	Product	Configuration and % e.e
1	 (**1**)	21	 (**1a**)	*R*, 99.9
2	 (**2**)	**NR**[#]	 (**2a**)	--
3	 (**3**)	**NR**[#]	**--**	**--**
4	 (**4**)	**NR**[#]	**--**	**--**
5	 (**5**)	8.5	 (**5a**)	*R*, 99.9
6	 (**6**)	**NR**[#]	**--**	**--**
7	 (**7**)	11	 (**7a**)	*R*, 84
8	 (**8**)	5	 (**8a**)	*R*, 99.9

NR-No reaction, Time of incubation for each entry = 48 h, Substrate concentration for each entry = 3 mg

Table 3.4 Hydrolytic kinetic resolution of racemic acyclic and aromatic acetates by pEST-1 esterase.

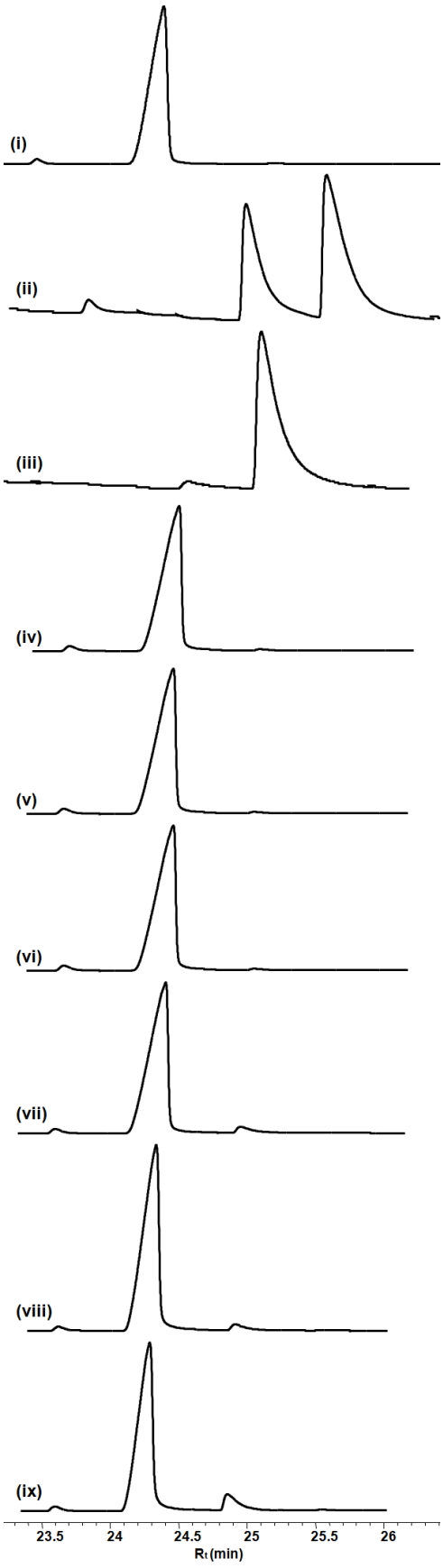

(i)
(ii)
(iii)
(iv)
(v)
(vi)
(vii)
(viii)
(ix)
23.5
24
24.5
25
25.5
26
Rt (min)

Fig. 3.15 Time Course study for the conversion of (±)-3-methyl-1-Phenyl ethyl acetate by pEST-1 esterase.

(i) standard (±)-3-methyl-1-Phenyl ethyl acetate (ii) standard (±)-3-methyl-1-Phenyl ethanol (iii) standard (*S*)-3-methyl-1-Phenyl ethanol (iv) Conversion of (±)-3-methyl-1-Phenyl ethyl acetate after 6 h incubation (v) Conversion of (±)-3-methyl-1-Phenyl ethyl acetate after 18 h incubation (vi) Conversion of (±)-3-methyl-1-Phenyl ethyl acetate after 24 h incubation (vii) Conversion of (±)-3-methyl-1-Phenyl ethyl acetate after 30 h incubation (viii) Conversion of (±)-3-methyl-1-Phenyl ethyl acetate after 42 h incubation (ix) Conversion of (±)-3-methyl-1-Phenyl ethyl acetate after 48 h incubation .

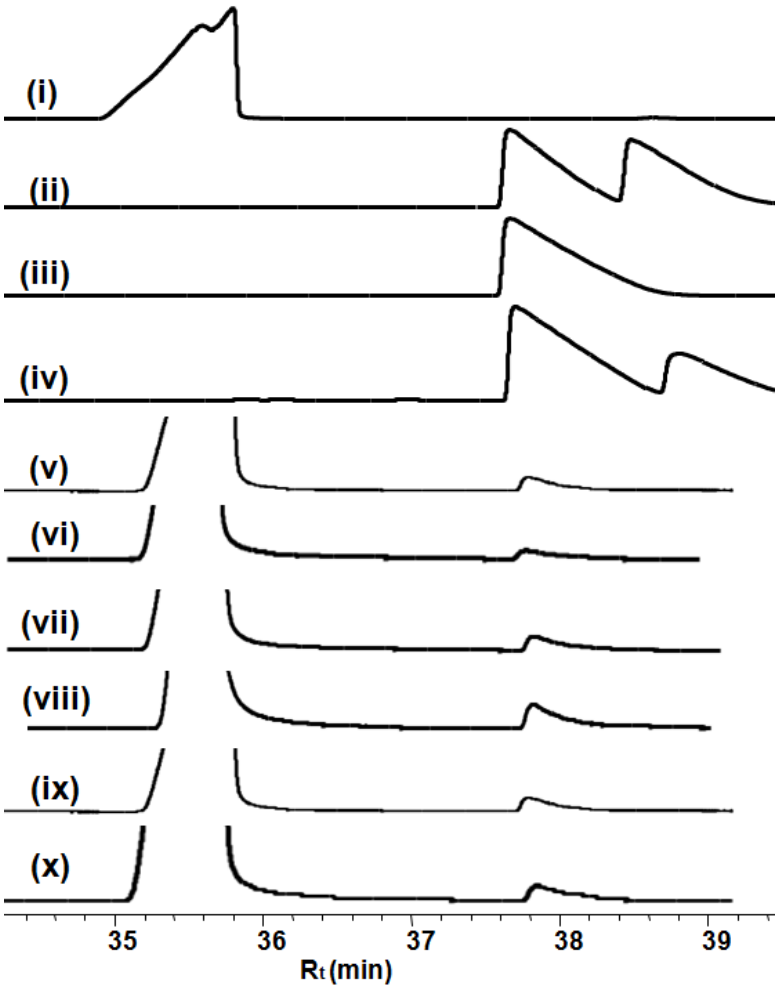


Fig.3.16 Time Course study for the conversion of (±)-1-Phenylpropyl acetate by pEST-1 esterase.

(i) standard (±)-1-phenylpropyl acetate (ii) standard (±)-1-phenylpropanol (iii) standard (*R*)-1-phenylpropanol (iv) Co-injection of (±)-1-phenylpropanol and (*R*)-1-phenylpropanol (v) conversion of (±)-1-phenylpropyl acetate after 6 h incubation (vi) Conversion of (±)-1-phenylpropyl acetate after 18 h incubation (vii) Conversion of (±)-1-phenylpropyl acetate after 24 h incubation (viii) Conversion of (±)-1-phenylpropyl acetate after 30 h incubation (ix) Conversion of (±)-1-phenylpropyl acetate after 42 h incubation (x) Conversion of (±)-1-phenylpropyl acetate after 48 h incubation.

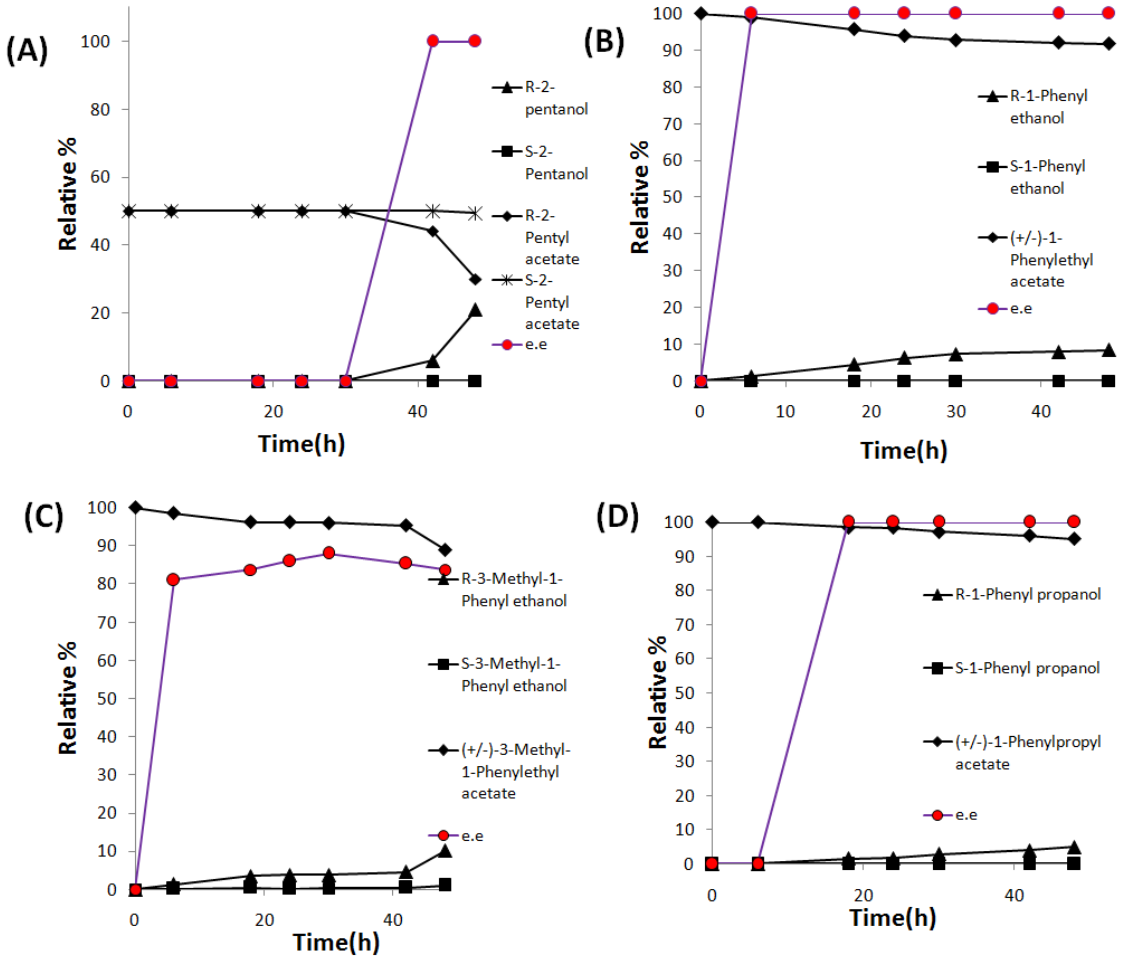


Fig. 3.17 Time-course experiment of kinetic resolution achieved using pEST-1 esterase in graphical representation with **(A)** (±)-2-pentyl acetate **(B)** (±)-1-phenylethyl acetate, (**C**) (±)-3-methyl-1-phenylethyl acetate, **(D)** (±)-1-phenylpropyl acetate respectively.

Above results suggests that pEST-1 is able to hydrolyse racemic acetates to corresponding (*R*)-alcohol with e. e value > 99 %. pEST-1 esterase seems to be active against small carbon chain esters over the long chain esters. Thus pEST-1 esterase can be used commercially used for the preparation of enantiopure (*R*)-alcohols.

3.1.3.10 Molecular docking analysis

The molecular docking studies were carried out between (*R*) and (*S*)-acetates of the substrates and pEST-1 esterase enzyme. (*R*) and (*S*)-acetates of four compounds [(**1**),(**5**),(**7**) and (**8**)] were individually docked with the protein model of pEST-1 esterase. Docked complexes provide the information about binding pose and atomic interaction between active site of pEST-1 esterase and substrates. Interaction in active site residues

and reactive centers of the substrates are stearically well positioned in the case of favorable interactions.

Binding energies and docking of ligands in the active site displayed that (*R*)-acetates of compounds bind fit inside the active site, while substrate (*S*)-acetates showed steric hindrance in the binding pocket (Fig. 3.18) [Table 3.5]. (*R*)-2-pentyl acetate found to be interacting relatively stronger with the active site residue of pEST-1 esterase as compared to (*S*)-2-pentyl acetate [Fig 3.18 (1a) and (1b)].

Similarly, aromatic acetates such as (*R*)-1-phenylethyl acetate [Fig 3.18 (2a)], (*R*)-3-methyl-1-phenylethyl acetate [Fig 3.18 (3a)] and (*R*)-1-phenylpropyl acetate [Fig 3.18 (4a)] were binding strongly deep inside the binding pocket with minimum free energy as compared to substrates like (*S*)-acetates such as (*S*)-1-phenylethyl acetate [Fig 3.18 (2b)], (*S*)-3-methyl-1-phenylethyl acetate [Fig 3.18 (3b)] and (*S*)-1-phenylpropyl acetate [Fig 3.18 (4b)].

Binding poses and energy suggest that pEST-1 esterase utilizes (*R*)-acetates to convert them in (*R*)-alcohols. Docking studies were found to be in well corroboration with *in vitro* studies depicting that pEST-1 esterase mostly preferred (*R*)-acetates are over the (*S*)-acetates.

Sr. no.	**Substrates**	**Binding affinity (Kcal/mol)**	
		(*R*)-acetate	**(*S*)-acetate**
1	2-Pentyl acetate	-3.5	-3.4
2	1-Phenylethyl acetate	-4.7	-4.5
3	3-Methyl-1-Phenylethyl acetate	-5.1	-5.0
4	1-Phenylpropyl acetate	-4.8	-4.7

Table 3.5 Binding affinity of (*R*) and (*S*) acetates with pEST-1 esterase after molecular docking studies.

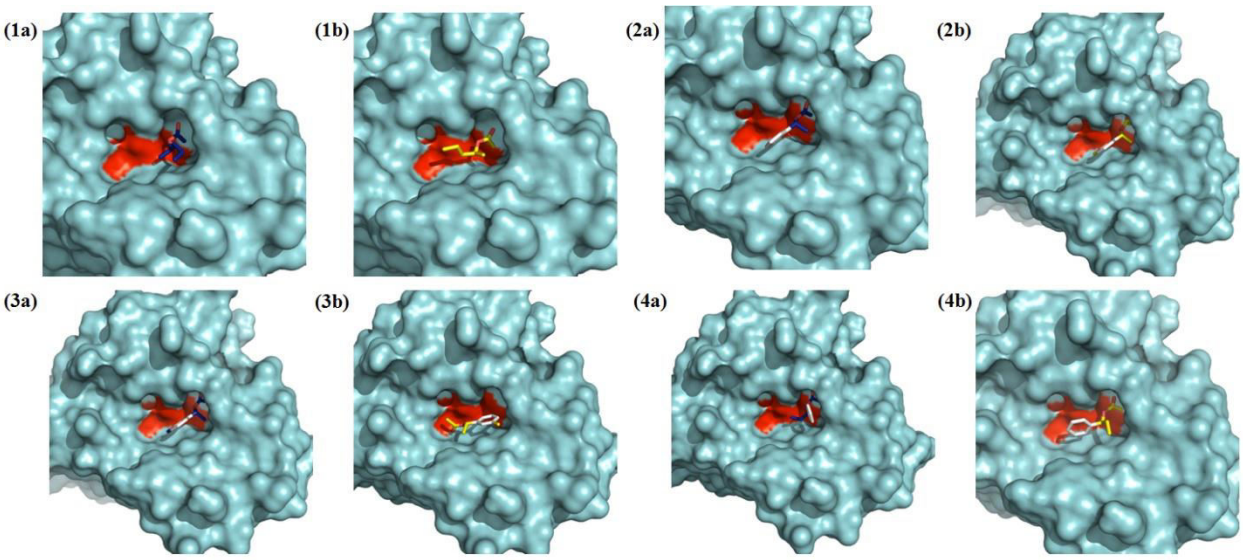


Fig.3.18 Docking analysis of various substrates at the active site pocket of pEST-1 esterase protein model. (**1a**)(*R*)-2-pentyl acetate, (**1b**) (*S*)-2-pentyl acetate, (**2a**) (*R*)-1-phenylethyl acetate, (**2b**) (*S*)-1-phenylethyl acetate, (**3a**) (*R*)-3-methyl-1-phenylethyl acetate, (**3b**) (*S*)-3-methyl-1-phenylethyl acetate, (**4a**) (*R*)-1-phenylpropyl acetate, (**4b**) (*S*)-1-phenylpropyl acetate. Amino acid residues highlighted in red are active sight residues containing GSSLG motif.

3.2 RNA isolation, transcriptome sequencing & identification of new uncharacterized lipases from *Yarrowia lipolytica* NCIM 3639.

3.2.1 Introduction

Yarrowia lipolytica is hemiascomycetous, non-conventional, dimorphic and non-pathogenic aerobic yeast. It is usually found in hydrophobic environments such as hydrophobic substrates rich in alkanes and fats. It can be isolated from cheese, yogurts, detergents, meat and known to have various industrial applications.[52,53] *Yarrowia lipolytica* is widely used for production of citric, isocitric acids, γ-decalactone and enzymes such as acid or alkaline proteases, lipases. *Yarrowia lipolytica* grows optimally at 30 º-34 ºC but psychrophillic strain is also reported.[46] Lipase is one of the key enzymes produced by *Y. lipolytica* , which has many industrial applications.

There are many lipases such as LIP2, [54] LIP 8, [55,56] LIP 11, [57] LIP 9, [58] LIP 14, [56] and LIP 18 [56] are reported from *Yarrowia lipolytica* till date. It remains a potential source of finding a novel biocatalyst in future also. *Yarrowia lipolytica* lipases are used for the bioremediation and production of biosurfactant, [52,53,59,60] production of γ-decalactone, [61] citric acid [62] and intracellular lipids.[63] They are also used for waste water treatment coming from oil industry, [64, 65] preparation of chiral intermediates for pharmaceutical industry [66-68] and food processing industry. [52, 53, 59]

Yarrowia lipolyitca NCIM 3639 is psychrophillic yeast isolated from Tween 80 detergent and produces two cold active lipases. It produces cell bound lipase when induced with olive oil and a 400 kDa extracellular lipase when induced with Tween 80 detergent and are shown to be involved in chiral resolution of (±)-lavandulyl acetate.[46] Thus cold active lipases from this yeast remain to be a potential source of identifying a novel biocatalyst having commercial importance.

In this work, we have tried out finding novel cold active lipases through transcriptome sequencing. We have isolated total RNA from Tween 80 induced and non-induced *Yarrowia lipolyitca* NCIM 3639 sample and sent it for transcriptome sequencing. These transcripts were then functionally annotated using Pfam domains and novel lipases were identified from the database.

3.2.2 Materials and Methods

3.2.2.1 Media and kits

Peptone, yeast extract, malt extract, glucose, agar were purchased from Hi-media Laboratories Limited Mumbai, India. Kit used for RNA isolation is "Spectrum ^{TM} Plant Total RNA Kit" which was purchased from Sigma Aldrich, USA. All the kits used for recombinant DNA technology experiments were purchased from Invitrogen, USA.

3.2.2.2 Other chemicals

Tween–80, Triton X-100 were obtained from Merck India Ltd. 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulphonate (CHAPS), Sodium dodecyl sulphate (SDS), Dithiothreitol (DTT), β-mercaptoethanol (β-ME), EDTA were obtained from Sigma Aldrich (USA). All other chemicals were of analytical grade.

p-nitrophenol esters (C2: *p*-nitrophenol acetate, C4: *p*- nitrophenol butyrate, C5: *p*- nitrophenol valerate, C8: *p*- nitrophenol octanoate, C10: *p*- nitrophenol decanoate, C12: *p*- nitrophenol dodecanoate, C14: *p*- nitrophenol myristate, C16: *p*- nitrophenol palmitate, C18: *p*- nitrophenol stearate) (C2-C18) were purchased from Sigma Aldrich, USA.

3.2.2.3 Microorganism

A yeast strain used in the study *Yarrowia lipolytica* NCIM 3639 was collected from the culture collection, National Collection of Industrial Microorganism (NCIM), NCL, Pune, India.

3.2.2.4 Microorganisms and growth media

Yeast strain of *Yarrowia lipolytica* NCIM 3639 was maintained on MGYP agar slopes containing malt extract (0.3 %), glucose (1.0 %), yeast extract (0.3 %), peptone (0.5 %) and agar (2.0 %). Synthetic oil-based (SOB) medium was used as production medium which contained $NaNO_3$ (0.05 %), $MgSO_4.7H_2O$ (0.05 %), KCl (0.05 %), KH_2PO_4 (0.2 %), yeast extract (0.1 %), bacto-peptone (0.5 %) and Tween-80 (1.0 %). The initial pH of the medium was adjusted to 5.5 with 0.1 N sodium hydroxide (NaOH) or HCl prior to sterilization.

3.2.2.5 Inoculum preparation and lipase production

A loopful culture of *Yarrowia lipolytica* was inoculated in 10 ml MGYP liquid medium and grown on a rotary shaker with shaking at 150 rpm at 20 ºC for 48 h. 5 mL of fully grown culture was then transferred to 250 mL flask containing 50 ml of MGYP medium and incubated for 48 h at 20 ºC. This inoculum (5 %) was used for lipase production wherein it was added in 250 ml conical flasks containing 70 ml of SOB medium. These flasks were incubated on a rotary shaker at 150 rpm at 20 ºC for 48 h. The cells were harvested by centrifugation at 8000 X g for 20 min at 4 °C. Cell pellet was then used for isolation of total RNA.

3.2.2.6 Isolation of total RNA from *Yarrowia lipolytica* NCIM 3639

Isolation of total RNA from *Yarrowia lipolytica* NCIM 3639 was carried out using "Spectrum ™ Plant Total RNA Kit". Procedure for total RNA isolation is as follows:

- **Grinding cell pellet:**

1) Harvested cells of *Y. lipolytica* were submerged in liquid nitrogen as soon as possible to prevent RNA degradation.

2) Cells were grinded to a fine powder in liquid nitrogen using bead beater machine. Cycles of 6 minutes each were given twice for each sample to get fine powder of cells.

3) Weigh cells approximately 50 mg after liquid nitrogen has evaporated from in a 2 mL micro centrifuge tube, pre-chilled on dry ice or in liquid nitrogen. Keep the weighed sample on dry ice or at –70 °C before lysis solution is added.

4) Assemble column and collection tube insert a filtration column into a 2 mL collection tube and close the lid.

- **Prepare Lysis Solution/2-ME Mixture:** Prepare lysis solution in a conical tube by adding 10 µl of 2-Mercaptoethanol to every 1 ml of lysis Solution and mix briefly.

4) Add 500 µL lysis solutions to 50 mg sample and vortex immediately and vigorously for at least 30 sec.

5) Incubate the sample at 56 °C for 3–5 min. and pellet cellular debris by centrifuging the sample at maximum speed for 3 min.

6) Pipette the lysate supernatant into a filtration column (blue retainer ring) seated in a 2 mL collection tube by positioning the pipette tip at the bottom of the tube but away from the pellet.

- **Binding the RNA**

7) Pipette 500 µl of binding solution into the clarified lysate and mix immediately and thoroughly by pipetting at least 5 times or vortex briefly.

8) Pipette 700 µl of the mixture into a binding column seated in a 2 mL collection tube. Close the cap and centrifuge at maximum speed for 1 min. to bind RNA and decant the flow-through liquid and return the column to the collection tube.

9) Return the column to the collection tube and pipette the remaining mixture to the column and repeat the centrifugation and decanting steps.

10) First column wash: Pipette 500 µl of **wash solution 1** into the column. Close the cap and centrifuge at maximum speed for 1 min. and decant the flow-through liquid and return the column to the collection tube.

11) Second column wash: Pipette 500 µl of the diluted **wash solution 2** into the column. Close the cap and centrifuge at maximum speed for 30 sec. and discard the flow-through liquid and return the column to the collection tube.

12) Third column wash: Pipette another 500 µl of the diluted wash solution 2 into the column, close the cap and centrifuge at maximum speed for 30 sec. and discard the flow-through liquid and return the column to the collection tube.

13) Dry column centrifuge the column at maximum speed for 1 min. to dry.

14) Elution: Pipette 50 µl of elution solution directly onto the binding matrix inside the column. Close the cap and let the tube sit for 1 min. Centrifuge at maximum speed for 1 min. to elute. Purified RNA is now in the flow through eluate and ready for immediate use or storage at –20 °C (short term) or –70 °C (long term).

3.2.2.7 Quantification of total RNA

Total RNA was quantified using a spectrophotometer (NanoDrop, Thermo Scientific) by measuring optical density (O.D) of isolated total RNA in 10 mM TE buffer. RNA purity was checked by comparing the ratios of the absorbance at wavelengths 230/260 and 260/280. The integrity of total RNA was confirmed by visualising rRNA (28S and 18S rRNA) bands on 1.5 % agarose gels using GelRedTM (Biotium).

3.2.2.8 Transcriptome sequencing

Library preparation for RNA of *Y. lipolytica* cells was performed using Illumina TruSeq RNA library protocol outlined in "TruSeq RNA Sample Preparation Guide" (Part # 15008136; Rev. A; Nov 2010) in Genotypic Technology's Genomics facility. 1 µg of Total RNA was subjected to Poly-A purification of mRNA. Purified mRNA was fragmented for 2 minutes at elevated temperature (94 ℃) in the presence of divalent cations and reverse transcribed with Superscript III Reverse transcriptase by priming with random hexamers. Second strand cDNA was synthesized in the presence of DNA polymerase-I and RnaseH. The cDNA was cleaned up using HighPrep PCR (MAGBIO, Cat# AC-60050). Illumina Adapters were ligated to the cDNA molecules after end repair and addition of A base. SPRI cleanup was performed after ligation. The library was amplified using 8 cycles of PCR for enrichment of adapter ligated fragments. The prepared library was quantified using Nanodrop and validated for quality by running an aliquot on High Sensitivity Bio analyzer Chip (Agilent) [Fig.3.20 (**A**) and (**B**)].

3.2.2.9 *De novo* transcriptome assembly

Total of 82.9 million raw reads were generated [Control sample (41.6 million) and Tween 80 induced sample (41.3 million)] with read length of 151 bp and were quality checked using FastQC1 software to obtain high quality read data. Raw reads were filtered by discarding the reads containing adaptor sequence and poor quality raw reads to obtain 75.8 million processed reads [Control sample (38.2 million) and Tween 80 induced sample (37.6 million)]. *De novo* assembly of the clean raw reads was done and unigenes/transcripts were generated with optimised hash length of 25 using "Trinity" software.

3.2.2.10 Transcriptome annotation

To assign molecular function of unigenes in biological system functional annotations were performed. Open Reading Frames (ORFs) of all unigenes were predicted in all six frames by "Virtual Ribosome" online program. The longest ORFs were selected for each unigenes and submitted to Pfam database to identify protein domain, architecture and their families. Unigenes assigned with Pfam IDs were also searched against NCBI database using ncbi-BLAST-2.2.29. Unigenes assigned to Pfam ID's were then searched against already known protein families (Pfam) belonging to esterase/lipase family of enzymes.

3.2.3 Results and Discussion

3.2.3.1 Isolation of total RNA from *Y. lipolytica* NCIM 3639 strain

High quality of total RNA was isolated from *Y. lipolytica* samples [control and Tween 80 induced] using "**Spectrum ™ Plant Total RNA Kit**" (Sigma Aldrich). Quantity and purity was checked using Nanodrop and integrity of RNA was checked on agarose gel (Fig.3.19).

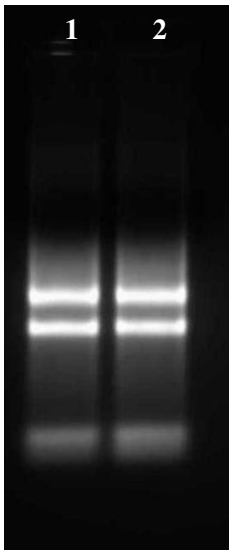


Fig. 3.19 Agarose gel electrophoresis of total RNA isolated from *Y. lipolytica* NCIM 3639.**Lane 1:** Total RNA isolated from *Y. lipolytica* control sample. **Lane 2:** Total RNA isolated from *Y. lipolytica* Tween 80 induced samples.

Ratios of $A_{260/230}$ and $A_{260/280}$ were found to be 2.33 and 2.51 for *Y. lipolytica* (control) sample while 2.40 and 2.33 for *Y. lipolytica* (induced) sample respectively. High quality RNA thus obtained with no DNA contamination was used for transcriptome sequencing.

3.2.3.2 Transcriptome sequencing

3.2.3.2.1 cDNA library preparation and sequencing

cDNA library was constructed from the mRNA purified from total RNA isolated from the control and Tween 80 induced sample from *Yarrowia lipolytica* NCIM 3639 cells. It was then subjected to sequencing using Illumina GAII Analyzer. cDNA library was constructed and amplified by PCR to enrich the adaptor ligated fragments (Fig. 3.20). The cDNA library formed was then sequenced and total of 82.9 million raw reads were generated [Control sample (41.6 million) and Tween 80 induced sample (41.3

million)] with read length of 151 bp were generated. Primary QC check for raw reads was performed using "FastQC1" software. Total of 75.8 million processed reads [Control sample (38.2 million) and Tween 80 induced sample (37.6 million)] were generated after adapter trimming and low quality trimming to get better quality processed reads.

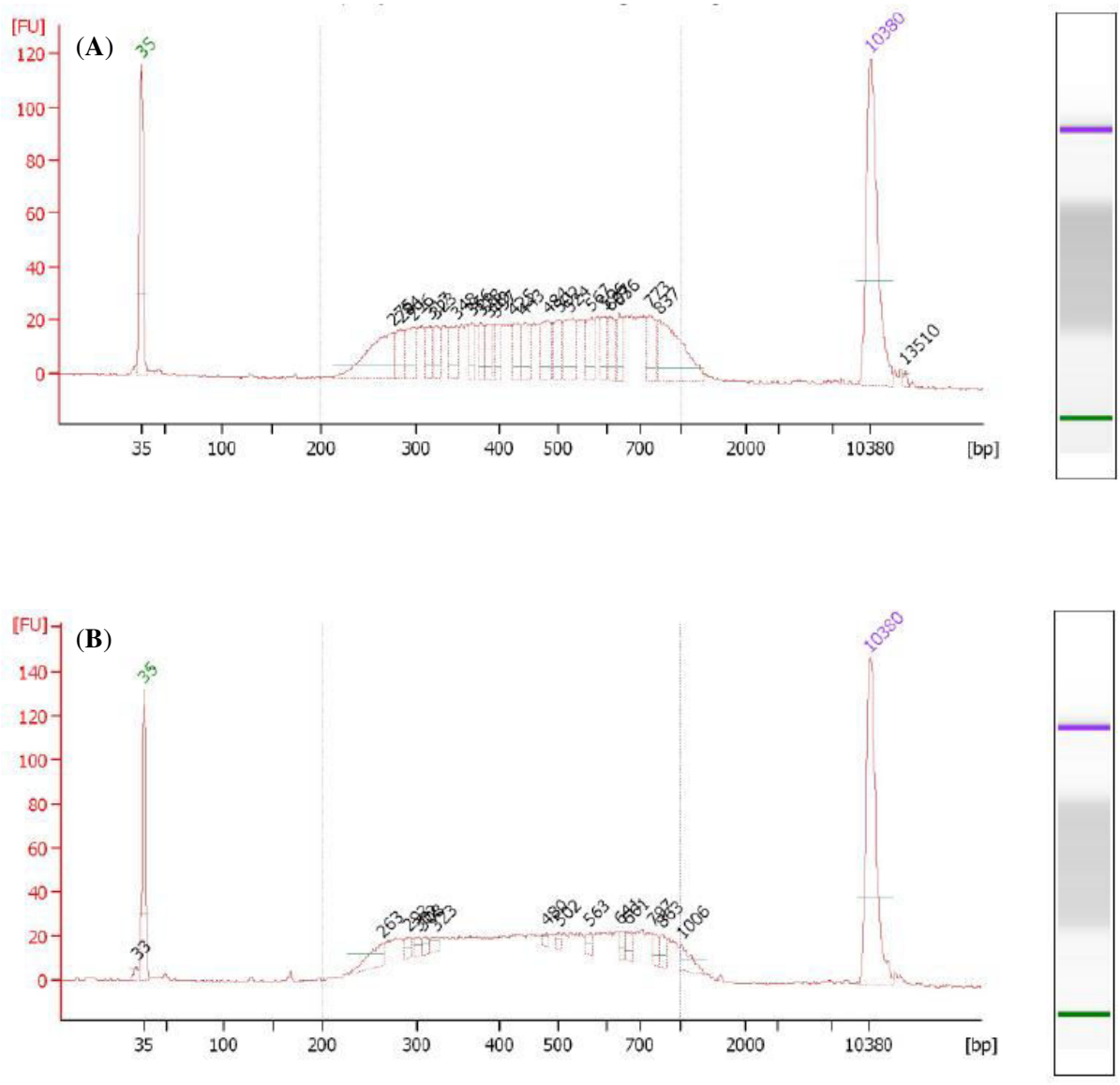
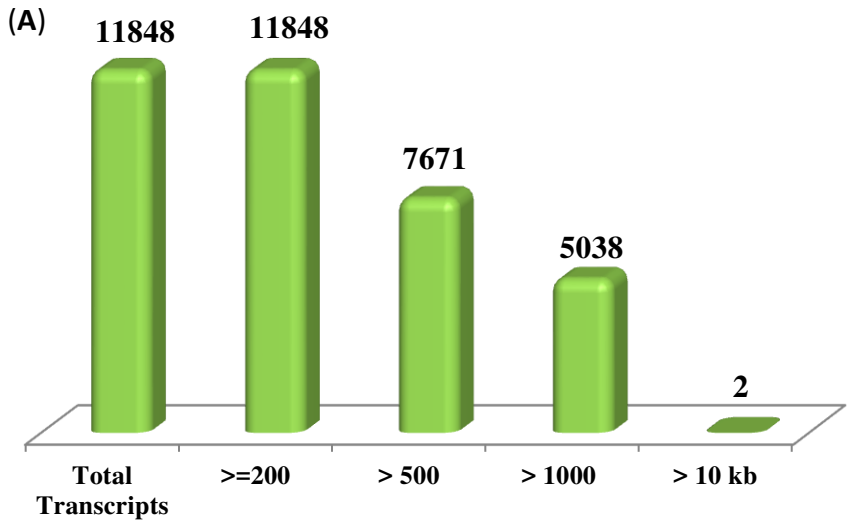


Fig. 3.20 Bio analyzer profile of amplified adaptor ligated fragments (**A**) Bio analyzer profile for *Y. lipolytica* (control) sample (**B**) Bio analyzer profile for *Y. lipolytica* (induced) sample

3.2.3.2.2 *De novo* transcriptome assembly

Total of 75.8 million high quality reads [Control sample (38.2 million) and Tween 80 induced sample (37.6 million)] generated with read length of 151 bp were subjected to *De novo* assembly using "Trinity" software. Total of 34959 [*Y. lipolytica* control sample (11848) and *Y. lipolytica* induced sample (23111)] contigs of size ≥ 200 bp were generated with optimised hash length of 25 (Table 3.6). Average transcript length for *Y. lipolytica* control sample and *Y. lipolytica* induced sample were 1115.5 and 707.1 with N50 values of 1706 and 1396 respectively. In *Y. lipolytica* control sample 7671 transcripts (64.74 %), 5038(42.52 %) and 2 (0.000168 %) transcripts were of size greater than or equal to 500 bp, 1 kb and 10 kb respectively [Fig. 3.21 (**A**)]. Further in *Y. lipolytica* induced sample 7858 transcripts (34 %), 5008 (21.66 %) and 3 (0.000129 %) transcripts were of size greater than or equal to 500 bp,1 kb and 10 kb respectively [Fig. 3.21 (**B**)]. This good quality of assembled transcripts was then subjected to functional annotation.



***Y. lipolytica* sample (Control)**

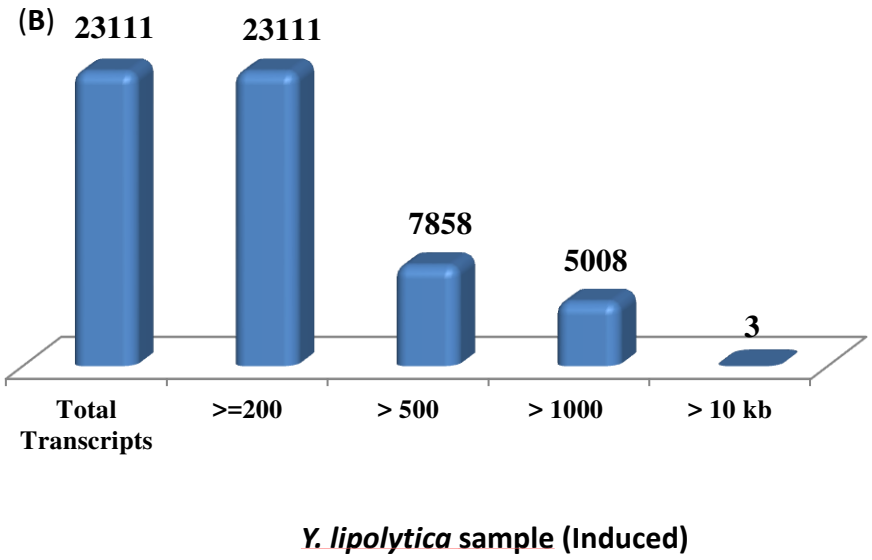


Fig. 3.21 Transcript length distribution graph (**A**) Transcript length distribution for *Y. lipolytica* control sample (**B**) Transcript length distribution for *Y. lipolytica* Tween-80 induced sample.

"Trinity" Assembly statistics		
Sample name	*Y. lipolytica* (control) sample	*Y. lipolytica* (induced) sample
Tools used	Trinity	
Hash length	25	
Transcripts generated	11848	23111
Maximum Transcript length	11569	11574
Minimum Transcript length	201	201
Average Transcript length	1115.5	707.1
Median Transcript length	1708	263
Total Transcript Length	13216447	16342785
Total number of Non-ATGC characters	0	0
Transcripts >=200 bp	11848	23111
Transcripts > 500 bp	7671	7858
Transcripts > 1 kb	5038	5008
Transcripts > 10 kb	2	3
N50 value	1706	1396

Table 3.6 Transcriptome assembly statistics.

3.2.3.2.3 Functional annotation of unigenes

Functional annotation of unigenes allows structural and functional characterization of unigenes involved in different physiological processes in a particular biological system. We have identified and assigned unigenes to their functions using Pfam analysis and NCBI search.

3.2.3.2.4 Pfam analysis

Pfam is a database composed of data about various protein families containing information about protein domain and protein families represented as multiple sequence alignments using hidden Markov models.[69] For the functional annotation of unigenes, we first made an Open Reading Frame (ORF) prediction analysis using online software tool "Virtual Ribosome-V1.1". 25085 unigenes [combined of *Y. lipolytica* (control) sample and *Y. lipolytica* (induced) sample] of size ≥ 500 bp were submitted to Virtual Ribosome to predict ORF of maximum length for each unigene in all six frames. Total of 25049 unigenes (99.85 %) were identified as having ORF starting at an ATG codon. Out of which 25085 unigenes, 36 (0.0014 %) unigenes were without ORF while 25049 unigenes were with ORF of ≥ 10 amino acid lengths. In 25085 unigenes, 203 unigenes (0.008 %) were with ORF ≤ 10 amino acid lengths (Fig. 3.22).

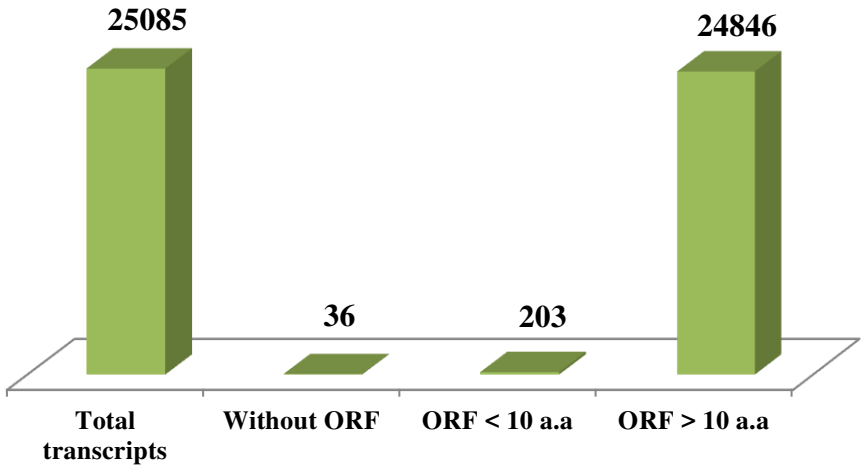


Fig. 3.22 ORF prediction using Virtual Ribosome-V-1.1

Total of 24846 unigenes were subjected to Pfam analysis among which 16561 (66.65 %) unigenes were assigned Pfam IDs while 8285 (33.34 %) unigenes were not

assigned any Pfam IDs (Fig. 3.23). From the Pfam family database, total of 21 families were selected which were belonging to esterase/lipase as shown in Table 3.5. 8285 unigenes having assigned Pfam IDs were then searched for the known Pfam IDs belonging to esterase/lipase class of enzymes as in Table 3.7. Out of 21 families searched, our *Yarrowia lipolytica* transcriptome database showed match with the 5 protein families.

Pfam IDs of the matching familes were **PF01764** [(Lipase_3, Lipase (class 3)], **PF03893** [Lipase3_N, Lipase 3 N-terminal region], **PF00561** [Abhydrolase_1, Esterase/ Hydrolase family], **PF07859** [Abhydrolase_3, alpha/beta hydrolase fold] and **PF00135** [CO esterase, Carboxylesterase family] (Table 3.7).

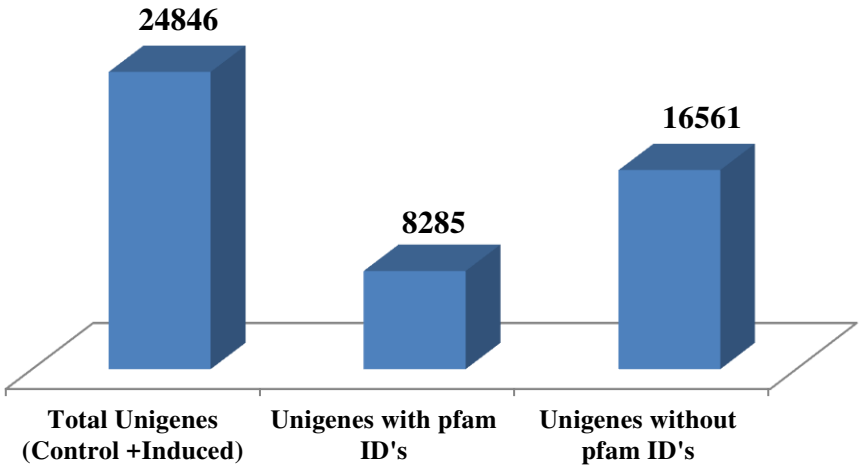


Fig. 3.23 Pfam analysis of transcript having ORF ≥10 amino acid. 24846 unigenes assigned with Pfam ID were subjected to search against NCBI Nr-database and Pfam IDs.

Sr. No	Pfam ID No.	Protein Family name	Description
1	PF00151	Lipase	Lipase
2	PF01674	Lipase_2	Lipase (class 2)
3	**PF01764**	**Lipase_3**	**Lipase (class 3)**
4	**PF03893**	**Lipase3_N**	**Lipase 3 N-terminal region**
5	PF03583	LIP	Secretory lipase
6	PF00657	Lipase_GDSL	GDSL-like Lipase/Acylhydrolase
7	PF13472	Lipase_GDSL_2	GDSL-like Lipase/Acylhydrolase family
8	PF14606	Lipase_GDSL_3	GDSL-like Lipase/Acylhydrolase family
9	PF16255	Lipase_GDSL_like	GDSL-like Lipase/Acylhydrolase
10	PF04083	Abhydrolase_lipase	Partial alpha/beta-hydrolase lipase region
11	**PF00561**	**Abhydrolase_1**	**Esterase/ Hydrolase family**
12	PF02230	Abhydrolase_2	Phospholipase/Carboxylesterase
13	**PF07859**	**Abhydrolase_3**	**alpha/beta hydrolase fold**
14	PF12695	Abhydrolase_5	Alpha/beta hydrolase family
15	PF06259	Abhydrolase_8	Alpha/beta hydrolase
16	PF10081	Abhydrolase_9	Alpha/beta-hydrolase family
17	PF15420	Abhydrolase_9_N	Alpha/beta-hydrolase family N-terminus
18	**PF00135**	**CO esterase**	**Carboxylesterase family**
19	PF14607	GxDLY	N-terminus of Esterase_SGNH_hydro-type
20	PF00756	Esterase	Putative esterase
21	PF04083	N-terminal part of an alpha/beta hydrolase domain	Partial alpha/beta-hydrolase lipase region

Table 3.7 List of protein families and their Pfam IDs belonging to esterase/lipase class of enzymes**.** (Protein families marked are in **bold** and matching with our *Yarrowia lipolytica* transcriptome database).

From our transcriptome database, total of 33 unigenes were assigned to five protein familes belonging to esterase/lipase families as discussed earlier. Among 33 unigenes, thirteen (13) unigenes belong to Pfam ID **PF01763**, four (4) belong to Pfam ID

PF00135, one (1) belongs to Pfam ID **PF03893**, 4 belongs to Pfam ID **PF07859** and 11 belongs to Pfam ID **PF00561** (Fig. 3.24).

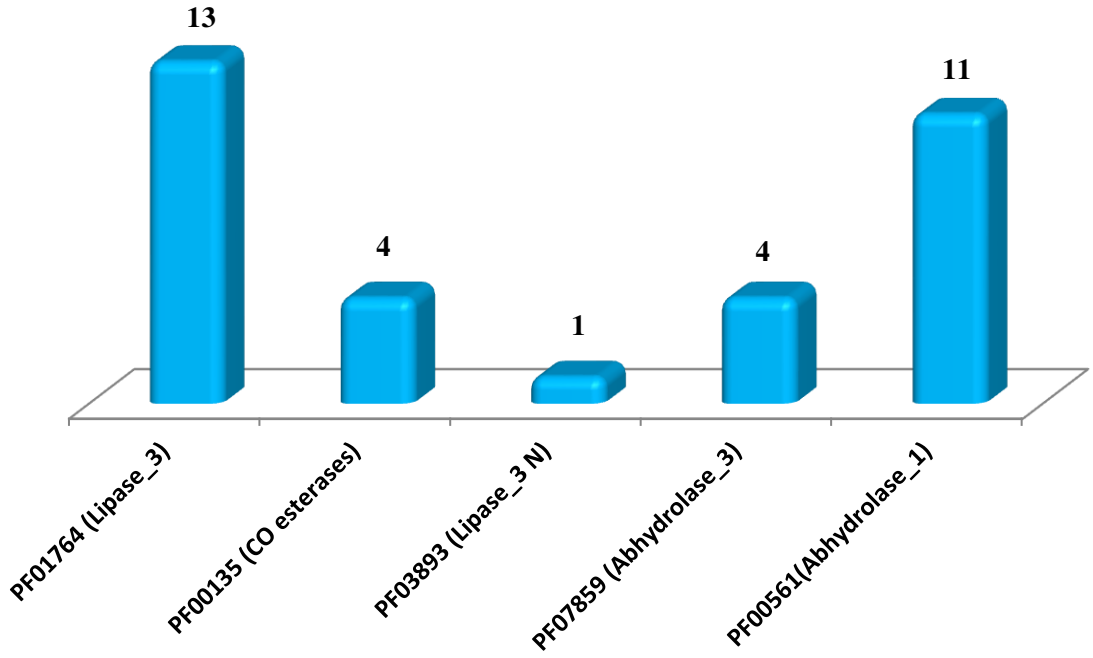


Fig. 3.24 Distribution of 33 unigenes among five protein families belonging to esterase/lipase class of enzymes.

Out of 33 unigenes matched in our transcriptome database nine (9) were found out to be new, uncharacterised lipases not functionally characterised so far. All these 9 unigenes were searched in NCBI database and showed match with the existing database as shown in the Table 3.6. Among 9 new uncharacterised unigenes, 5 unigenes [Master_Control_11942, Master_Control_9565, Master_Control_2245, Master_Control_3587, Master_Control_1834] belongs to protein family **PF00561** [Abhydrolase_1, Esterase/ Hydrolase family]. Two (2) unigenes [Master_Control_2476, Master_Control_2691] belongs to protein family **PF01764** [(Lipase_3, Lipase (class 3)] while other 2 unigenes [Master_Control_11278, Master_Control_2016] belongs to protein family **PF07859** [Abhydrolase_3, alpha/beta hydrolase fold]. These nine unigenes were analysed using NCBI protein database and it showed match with existing *Y. lipolytica* genes as shown in Table 3.8.The transcript Master_control_11492 showed match with gene YALI0B18854p of *Yarrowia lipolytica CLIB122* strain with 100 % query coverage and 99 % identity.

Sr.No	Transcript name	NCBI Protein blast match (Gene name /microorganism)	Query coverage	E value	Identity (%)	Pfam ID	Protein family	Comments
1	Master_Control_11942	YALI0B18854p [*Yarrowia lipolytica*]	100	0	99	PF00561	**Abhydrolase_1**	New Uncharacterized Lipase
2	Master_Control_9565	YALI0E00836p [*Yarrowia lipolytica*]	100	0	100	PF00561	**Abhydrolase_1**	New Uncharacterized Lipase
3	Master_Control_2245	YALI0C07326p [*Yarrowia lipolytica*]	100	0	100	PF00561	**Abhydrolase_1**	New Uncharacterised Lipase
4	Master_Control_3587	YALI0D08404p [*Yarrowia lipolytica*]	100	0	100	PF00561	**Abhydrolase_1**	New Uncharacterized Lipase
5	Master_Control_1834	YALI0E19899p [*Yarrowia lipolytica*]	100	0	100	PF00561	**Abhydrolase_1**	New Uncharacterized Lipase
6	Master_Control_2476	YALI0F06358p [*Yarrowia lipolytica*]	100	0	99	PF01764	**Lipase_3**	New Uncharacterised Lipase
7	Master_Control_2691	LIPY8p	100	0	99	PF01764	**Lipase_3**	New Uncharacterised Lipase
8	Master_Control_11278	YALI0E03806p [*Yarrowia lipolytica*]	100	1.00E-179	100	PF07859	**Abhydrolase_3**	New Uncharacterized Lipase
9	Master_Control_2016	YALI0C06732p [*Yarrowia lipolytica*]	96	0	100	PF07859	**Abhydrolase_3**	New Uncharacterized Lipase

Table 3.8 List of new and uncharacterised lipase identified from *Y. lipolytica* NCIM 3639 transcriptome database.

Master_Control_9565, Master_Control_2245, Master_Control_3587 and Master_Control_1834 showed match with genes YALI0E00836p. YALI0C07326p, YALI0D08404p and YALI0E19899p respectively with 100 % query coverage and identity with *Yarrowia lipolytica CLIB122* strain.

Master_Control_2476 and Master_Control_2691 showed match with genes YALI0F06358p and LIPY8p with 100 % query coverage and 99 % identity. Master_Control_11278 and Master_Control_2016 showed match with YALI0E03806p and YALI0C06732p with 100 % identity and 100 %, 96 % query coverage respectively. Therefore all mentioned lipases are all reported in NCBI database and in literature [70] but still remains to be functionally characterised.

- **Amino acid sequences of nine new uncharacterized lipases:-**

1) Transcript name: Master_control_11942

Amino acid sequence:

MNPTIERSARLADYVWSFLGAHKVVVGLFLAAYFIFKSRDHVKITKPRKPLELHLKNGKTESLDAL
IQQVPGLKNGDTLWYNPLKILNHVQTIMASQNDLHGTDLVYYARRMVDFDDGVQIAADYVVPAP
KTPEEKAAWKKGLEYKPEENTPKFPVRTRYKQPQEMELDHSDDSKPMLILLHGLTGGSYESYVRA
VVAKITSQYNASADGTLVDFECVVMNTRGCARTTIKTPELFNGCWTEDVRRFVKDMRKRYPNRR
FYMVGFSLGASILANYLGQEAEDIDIEAACVVANPWDLCASYYALRSSWSGRHLYNPQMAKNLL
RMLRNHREVMKRNPIYDESLTKHVKSIVDFDNLFTAPMFGFDTATDYYRHGSSCNRIMNIRVPTLI
LHALDDPVAPGFQLPYHEIKRNPYTVMAATNHGGHIGWFHWGKDKRWFPSKIAGFFSQFEKEVD
HTKDNNTKVERLERRWENDRLRAVRYD

2) Transcript name: Master_control_9565

Amino acid sequence:

MKFRRTKVVKENPFSGLVSDQEIAHVPEYQLESGVTIYNVPIAYKTWGVLNEAGNNAMVICHALT
GSADVSDWWGPLIGPGRAFDPTRFFIVCLNSLGSPYGSASPCTADNTPGADKNTYYGPEFPLVTVR
DDVNIHRLVLDDLGIKQIACCIGGSMGGMLTLEYAFFGKDYVRTFVALATSARHSAWCISWGEAQ
RQCIYSDPKYDDGYYSFEDPPSSGLGAARMAALLTYRSRDSFENKFGRDTPDPTRHKTINGPQRRG
PQTASEEHWLIHNDGHMRGKKMADEEGSSTPTANGVSQPSSVSTNGSILDDTSSVSSSSSFVPLAV
KKKPPTHFSAQNYLRYQSDKFTKRFDANCYIAITRKLDTHDVSRERAESVEAALQTLEQNALIIGIK
SDGLFTFAEQELIAANVKNSHLVTIDSPEGHDAFLLDFALINKEIVEFLAANVPDIINTNGVSWEEA
KVGELGKSSLFGEAEVEITQW

3) Transcript name: Master_control_2245

Amino acid sequence:

MPTEQVNDTPITPERRELKVPNKANPLSVAESFKQYFFADGIEKAEFKVLSMLPFFPETDGKRKAK
VCETEIKKNTYINDFHITNTEQPAYDSSSSRTLVMLHGWGTGMGLWFENLDVISSLPGWNVHVLD
NPGMGRSTRETFNISQHKDDPDGRKMVAETEEWYLSRLEAWREKKGLERFVLLGHSLGGYIASIY
AMKYPNRVERLILVSPVGVEHINGVSLDGSISDGSDGAGAVNGAERPSSAGSSSTAPSNASSKQPL
GLHAGSMAPVESPDTPQSPGATSNDSNLGTQPTGEDSVVSQEEPHPGREFEVGEFDDIPDDIFSSLK
PVVSENQIDVHLGADLEKRREQNKLLAYMWQSHYSFIGAVRIAGPMGPKLVARWSYIRFQQLPTE
QRDIMHIYAYRIFAGKASGERGLTRLMAPFCIARLPIIDRITKIKCPSFWIYGENDWMNTSAGHEAA
KRLNKLGRPAYYKIVAEAGHHIYLDNIDAFNKSVVNYLKDADEDGRI

4) Transcript name: Master_control_3587

Amino acid sequence:

MLLQPLKTAFGTSARSLGMSRLGPVSCRSLTPSIATKRTLVGHASSYETTETVELAWDVQEVPNPT
RTPLVFMHGLFGHKANHHTVSKKLAADLNCNVYGLDLRNHGQSPHNPRHDYIALASDVERWINE
VMGGKEVILIGHSMGAKTAMAVALRHPELVKYLIPVDNSPVDTSLSSDFPKYIRGMLEIQHKKVK
NSKEAQEIMRKYEPDQMIRYFLLNNLRKTDDKDEPMKFRVPVEILGKSLGYLGDWPFFAEDGRFT
KPTLFIRGTKSKYVADEFLPAIGQFFPNFEIKDIECGHWVISQKPDEFMRDVREFVEKHDDKAAEEA
KLDADA

5) Transcript name: Master_control_1834

Amino acid sequence:

MAHLTKEYYEPFHHDVILGGKRWHYLDIPPEGKDNGRVLVLVHGFPDFWYGWRHQIPVFRKRGH
RIILPTLMGFPGSEVPEPPAMEEFEENEDGINIYTELGQEDDCRELHFYGFKFFADCMAELLKKLNI
KSATFLGHDWGAHYVPKVWAYHPEIVDAISSACWYYQVPEPEWVPLTDFSDKWPTTKYQLQFG
GDAVNNIGPGMIPFFLRRSYTVGANFDGEPDPEAPMHMTEEEFAVYEEHFSKEKRSLAGPFTYYRS
RKLNWEQDKENFLDKGATKKDLTVNVPYLYIGSTNDIALIPEMSMHLDEYVEKGKLTREHVPTSH
WALFEAPDQINKIYVDWLDKLDKTSKL

6) Transcript name: Master_control_2476

Amino acid sequence:

MTDTFKKYPDYSLKVTGHSMGGAASFIHGINMKTRGYDPYVVASGQPLVGNQALADYNDRLFFG
DKPDFLRQDSGRRYWRLTHKGDLVPQIPFWSPFQQPGGEIYIDYVLSDPPLDSLKVCDGQDNPNC
NYSSNMVNSAITGTLLWAHFQYFVVFTLCGVNYWSTHIHG

7) Transcript name: Master_control_2691

Amino acid sequence:

MVSLSARIKDFFSVLLLGAATITPSTQTAGVSQGFYDFARDFAHLSNIAYCVNAPITPLNPDFTCGN
SCKHFPDIELVKTFGGNFFKTSITGYLAVDHVKKEKYVVFRGTFSLADAITDMQFPLSPFLVDVPAL
NTFSANDTTAEAQTHCEGCKIHDGFSKAFTETWGNIGEDLQKHLDANPDYQLYVTGHSLGAAVA
LLGATSIKLKGYDPILINYGQPRVGNKPFAEFINKLWFGEGNGLEITPERKLYRMTHWNDIFVGLPN
WEGYTHSNGEVYINNRFINPPLKDVISCAGGENSKCYRSSFSLLSQINLLQNHLAYIDYIGYCALNI
GRRELADQEHYTGPYYYGHRSEEDFKKLGLELSTPQVEN

8) Transcript name: Master_control_11278

Amino acid sequence:

MTTITKLTYGPDPRQYSNVWIPKGSKAPSHWVIFMHGGAWVDYKQTEKDGDELMEAVVDGNV
WGASIEYRLAPEVSGKTFAEDVYNAIKSIFDKYPAAPWTMIGHSAGAFHSLKLDTDVKEKGGFAS
PTNIILSEGIYDLRTLVDDHPTYSYFTNPAWGEDKAKWDEESPLLHHQACPKINYTIVHSDIDELVP
FDGQPQLLAKKWKQQGVPFDFEVIRGLKHNDVFISSEFAKIANEIIQKSS

9) Transcript name: Master_control_2016

Amino acid sequence:

MPAIFPDNGSMVSDDMSSYSRDVLSLMVPRTRRPPAVTLPYIPSPNPFHTLDLYLPESGLCPTTKWL
IFIHGGYWRDASQSKDVGASILTRLPPHWAGASIDYRLSPEVEHPDHLHDVTAAVQFLRNAYGIKT
AVILAHGAGACMAFQYVAARLSLGETWVKHIVGSGGVYDLLDVSQSSPCYKSYILDAYGADAEN
WDDSSPARLEWDALYGPDVADLKFTLVHSKHDTLVPLRQVLQFEDTLTKAGFTATLRLVDIHDH
DAVLETSDLINVVLAICEEMDHKEEQQYLRLLE

3.3 Conclusion

In the present work, we have successfully isolated, cloned and over-expressed a novel 612 bp *pEST-1* esterase gene from *Pseudomonas pseudoalcaligenes* NCIM 2864. pEST-1 esterase was over expressed in *E. coli* and was purified using Ni-NTA agarose chromatography. Purified pEST-1 enzyme is an alkaline stable esterase having optimum pH and temperature of 8.0 and 20 ºC respectively. pEST-1 esterase is highly specific for *p*-nitrophenol valerate substrate among all *p*-nitrophenol esters screened. pEST-1 esterase activity was enhanced by addition of Na^{+1}, K^{+1} and Mg^{+2} metal ions and was found to be stable in polar and non-polar organic solvents such as ethanol, isopropanol, DMSO and dichloromethane. pEST-1 esterase was inhibited by EDTA, DTT, SDS and β-ME. pEST-1 esterase enantioselectively hydrolysed (±)-2-pentyl acetate, (±)-2-hexyl acetate, (±)-1-phenylethyl acetate and (±)-1-phenylpropyl acetate to corresponding (*R*)-alcohols with > 99 % e.e. In conclusion, due to parameters such as high stability in various polar and non polar solvents, optimal activity at lower temperatures and ability to hydrolyse wide range of substrates (racemic acetates) to (*R*)-alcohol makes pEST-1 esterase a potential biocatalyst for preparation of chiral precursors in asymmetric organic synthesis.

Yarrowia lipolytica is highly useful yeast which is widely used for its potential industrial applications. Lipases from *Yarrowia lipolytica* are used in industry because of its application in lipid degradation, bakery industry and synthesis of fine chemicals. In the present work, we were successful in identifying nine new uncharacterised lipases from transcriptome analysis of a psychrophillic yeast *Yarrowia lipolytica* NCIM 3639. Cloning and over expression of one of the transcript (Master_control_2691) were done in pPICZ-α vector (*Pichia pastoris*) but were unable to get good expression levels of protein. In summary, these cold active lipases could work as future potential biocatalysts having many industrial applications.

3.4 References

(1) Bornscheuer, U. T. *FEMS Microbiol. Rev.* **2002**, *26*, 73-81.

(2) Fernandez-Alvaro, E.; Kourist, R.; Winter, J.; Boettcher, D.; Liebeton, K.; Naumer, C.; Eck, J.; Leggewie, C.; Jaeger, K.-E.; Streit, W.; Bornscheuer, U. T. *Microb. Biotechol.* **2009**, *3*, 59-64.

(3) Romano, D.; Bonomi, F.; de Mattos, M. C.; Fonseca, T. d. S.; Ferreira de Oliveira, M. d. C.; Molinari, F. *Biotechnol. Adv.* **2015**, *33*, 547-565.

(4) Lange, S.; Musidlowska, A.; Schmidt-Dannert, C.; Schmitt, J.; Bornscheuer, U. T. *Chembiochem* **2001**, *2*, 576-582.

(5) Musidlowska, A.; Lange, S.; Bornscheuer, U. T. *Angew. Chem-Int Edit.* **2001**, *40*, 2851-2853.

(6) Heinze, B.; Kourist, R.; Fransson, L.; Hult, K.; Bornscheuer, U. T. *Protein. Eng. Des. Sel.* **2007**, *20*, 125-131.

(7) Herter, S.; Giang-Son, N.; Thompson, M. L.; Steffen-Munsberg, F.; Schauer, F.; Bornscheuer, U. T.; Kourist, R. *Appl. Microbiol. Biot.* **2011**, *90*, 929-939.

(8) Kourist, R.; Bornscheuer, U. T. *Appl. Microbiol. Biot.* **2011**, *91*, 505-517.

(9) Rehdorf, J.; Behrens, G. A.; Giang-Son, N.; Kourist, R.; Bornscheuer, U. T. *Appl. Microbiol. Biot.* **2012**, *93*, 1119-1126.

(10) Yan, Q.-j.; Yang, S.-q.; Duan, X.-j.; Xu, H.-b.; Liu, Y.; Jiang, Z.-q. *J. Mol. Catal. B-Enzym.* **2014**, *109*, 76-84.

(11) Godinho, L. F.; Reis, C. R.; Tepper, P. G.; Poelarends, G. J.; Quax, W. J. *Appl. Environ. Microb.* **2011**, *77*, 6094-6099.

(12) Gong, Y.; Xu, G.-C.; Zheng, G.-W.; Li, C.-X.; Xu, J.-H. *J. Mol. Catal. B-Enzym.* **2014**, *109*, 1-8.

(13) Ju, X.; Pan, J.; Yu, H.-L.; Li, C.-X.; Xu, J.-H. *Biochem. Eng. J.* **2011**, *57*, 63-68.

(14) Choi, G. S.; Kim, J. Y.; Kim, J. H.; Ryu, Y. W.; Kim, G. J. *Protein Expres. Purif.* **2003**, *29*, 85-93.

(15) Kim, G. J.; Choi, G. S.; Kim, J. Y.; Lee, J. B.; Jo, D. H.; Ryu, Y. W. *J. Mol. Catal. B-Enzym.* **2002**, *17*, 29-38.

(16) Wei, T.; Feng, S.; Mao, D.; Yu, X.; Du, C.; Wang, X. *J. Mol. Catal. B-Enzym.* **2012**, *85-86*, 23-30.

(17) Dong, J.; Zhao, W.; Gasmalla, M. A. A.; Sun, J.; Hua, X.; Zhang, W.; Han, L.; Fan, Y.; Feng, Y.; Shen, Q.; Yang, R. *J. Mol. Catal. B-Enzym.* **2015**, *121*, 53-63.

(18) Kim, G. J.; Lee, E. G.; Gokul, B.; Hahm, M. S.; Prerna, D.; Choi, G. S.; Ryu, Y. W.; Ro, H. S.; Chung, B. H. *J. Mol. Catal. B-Enzym.* **2003**, *22*, 29-35.

(19) Lee, E. G.; Won, H. S.; Ro, H. S.; Ryu, Y. W.; Chung, B. H. *J. Mol. Catal. B-Enzym.* **2003**, *26*, 149-156.

(20) Shimada, Y.; Nagao, T.; Sugihara, A.; Iizumi, T.; Yui, T.; Nakamura, K.; Fukase, T.; Tominaga, Y. *Biochim. Biophys. Acta* **1993**, *1174*, 79-82.

(21) Wicka, M.; Wanarska, M.; Krajewska, E.; Pawlak-Szukalska, A.; Kur, J.; Cieslinski, H. *Acta Biochim. Pol.* **2016**, *63*, 117-125.

(22) Xu, F.; Chen, S.; Xu, G.; Wu, J.; Yang, L. *Biotechnol. Biop. Eng.* **2015**, *20*, 473-487.

(23) Cheeseman, J. D.; Tocilj, A.; Park, S.; Schrag, J. D.; Kazlauskas, R. J. *Acta Crystallogr. D.* **2004**, *60*, 1237-1243.

(24) Choi, K. D.; Jeohn, G. H.; Rhee, J. S.; Yoo, O. J. *Agr. Biol. Chem. Tokyo* **1990**, *54*, 2039-2045.

(25) Khalameyzer, V.; Fischer, I.; Bornscheuer, U. T.; Altenbuchner, J. *Appl. Environ. Microb.* **1999**, *65*, 477-482.

(26) Kim, S.; Tri Duc, N.; Kim, K. K.; Kim, T. D. *Acta Crystallogr. F.* **2012**, *68*, 1374-1377.

(27) Krebsfanger, N.; Schierholz, K.; Bornscheuer, U. T. *J. Biotechnol.* **1998**, *60*, 105-111.

(28) Krebsfanger, N.; Zocher, F.; Altenbuchner, J.; Bornscheuer, U. T. *Enzyme Microb. Tech.* **1998**, *22*, 641-646.

(29) Torres, L. L.; Schliessmann, A.; Schmidt, M.; Silva-Martin, N.; Hermoso, J. A.; Berenguer, J.; Bornscheuer, U. T.; Hidalgo, A. *Org. Biomol. Chem.* **2012**, *10*, 3388-3392.

(30) Zocher, F.; Krebsfanger, N.; Yoo, O. J.; Bornscheuer, U. T. *J. Mol. Catal. B-Enzym.* **1998**, *5*, 199-202.

(31) Dou, S.; Kong, X.-D.; Ma, B.-D.; Chen, Q.; Zhang, J.; Zhou, J.; Xu, J.-H. *Biochem. Bioph. Res. Co.* **2014**, *446*, 1145-1150.

(32) Hasona, A.; York, S. W.; Yomano, L. P.; Ingram, L. O.; Shanmugam, K. T. *Appl. Environ. Microb.* **2002**, *68*, 2651-2659.

(33) Ma, B.-D.; Yu, H.-L.; Pan, J.; Liu, J.-Y.; Ju, X.; Xu, J.-H. *Bioresource Technol.* **2013**, *133*, 354-360.

(34) Ozaki, E.; Sakimae, A. *J. Ferment. Bioeng.* **1997**, *83*, 535-539.

(35) Shaw, S.-Y.; Chen, Y.-J.; Ou, J.-J.; Ho, L. *Enzyme Microb. Tech.* **2006**, *39*, 1089-1095.

(36) Hong, S.; Lee, C.; Jang, S.-H. *Biotechnol. Lett.* **2012**, *34*, 1051-1055.

(37) Lee, C.; Kim, J.; Hong, S.; Goo, B.; Lee, S.; Jang, S.-H. *Appl. Biochem. Biotech.* **2012**, *169*, 29-40.

(38) Kovacic, F.; Bleffert, F.; Caliskan, M.; Wilhelm, S.; Granzin, J.; Batra-Safferling, R.; Jaeger, K.-E. *FEBS Open. Bio.* **2016**, *6*, 484-493.

(39) Lee, J. H.; Rhee, S. K.; Kim, C. H. *Enzyme Microb. Tech.* **2004**, *35*, 563-567.

(40) Nicolay, T.; Devleeschouwer, K.; Vanderleyden, J.; Spaepen, S. *Appl. Environ. Microb.* **2012**, *78*, 2533-2542.

(41) Sakai, K.; Fukuba, M.; Hasui, Y.; Moriyoshi, K.; Ohmoto, T.; Fujita, T.; Ohe, T. *Biosci. Biotech. Bioch.* **1998**, *62*, 2000-2007.

(42) Wagner, U. G.; Solkner, B.; Petersen, E. I.; Schlacher, A.; Schwab, H.; Kratky, C. *Acta Crystallogr. D.* **1997**, *53*, 596-598.

(43) Lin, S. F. *J. Ferment. Bioeng.* **1996**, *82*, 448-451.

(44) Inglis, G. D.; Yanke, L. J.; Selinger, L. B. *Can. J. Microbiol.* **2011**, *57*, 902-913.

(45) Bradford, M. M. *Anal. Biochem.* **1976**, *72*, 248-254.

(46) Yadav, K. N. S.; Adsul, M. G.; Bastawde, K. B.; Jadhav, D. D.; Thulasiram, H. V.; Gokhale, D. V. *Bioresource Technol.* **2011**, *102*, 10663-10670.

(47) Harris, E. M. S.; Aleshin, A. E.; Firsov, L. M.; Honzatko, R. B. *Biochemistry* **1993**, *32*, 1618-1626.

(48) van Gunsteren, W. F. *Biomolecular Simulation: The GROMOS96 Manual and User Guide*; Biomos ; Zurich, **1996**.

(49) Morris, G. M.; Huey, R.; Lindstrom, W.; Sanner, M. F.; Belew, R. K.; Goodsell, D. S.; Olson, A. J. *J. Comput. Chem.* **2009**, *30*, 2785-2791.

(50) Hart, W. E.; Rosin, C.; Belew, R. K.; Morris, G. M. In *Optimization in Computational Chemistry and Molecular Biology: Local and Global Approaches*; Floudas, C. A., Pardalos, P. M., Eds.; Springer US: Boston, MA, **2000**, p 209-229.

(51) Schomburg, I.; Chang, A.; Placzek, S.; Soehngen, C.; Rother, M.; Lang, M.; Munaretto, C.; Ulas, S.; Stelzer, M.; Grote, A.; Scheer, M.; Schomburg, D. *Nucleic Acids Res.* **2013**, *41*, D764-D772.

(52) Brigida, A. I. S.; Amaral, P. F. F.; Coelho, M. A. Z.; Goncalves, L. R. B. *J. Mol. Catal. B-Enzym.* **2014**, *101*, 148-158.

(53) Goncalves, F. A. G.; Colen, G.; Takahashi, J. A. *Scientific World J.* **2014**, *2014*, 476207-476207.

(54) Aloulou, A.; Benarouche, A.; Puccinelli, D.; Spinelli, S.; Cavalier, J. F.; Cambillau, C.; Carriere, F. *Eur. J. Lip. Sci. Technol.* **2013**, *115*, 429-441.

(55) Kamoun, J.; Schue, M.; Messaoud, W.; Baignol, J.; Point, V.; Mateos-Diaz, E.; Mansuelle, P.; Gargouri, Y.; Parsiegla, G.; Cavalier, J. F.; Carriere, F.; Aloulou, A. *BBA Mol. Cell Biol. L.* **2015**, *1851*, 129-140.

(56) Kumari, A.; Gupta, R. *Biotechnol. Lett.* **2012**, *34*, 1733-1739.

(57) Kumari, A.; Baronian, K.; Kunze, G.; Gupta, R. *Protein Expres. Purif.* **2015**, *110*, 138-144.

(58) Syal, P.; Gupta, R. *Appl. Biochem. Biotechnol.* **2015**, *176*, 110-124.

(59) Bankar, A. V.; Kumar, A. R.; Zinjarde, S. S. *Appl. Microbiol. Biotechnol.* **2009**, *84*, 847-865.

(60) Fickers, P.; Marty, A.; Nicaud, J. M. *Biotechnol. Adv.* **2011**, *29*, 632-644.

(61) Aguedo, M.; Gomes, N.; Escamilla Garcia, E.; Wache, Y.; Mota, M.; Teixeira, J. A.; Belo, I. *Biotechnol. Lett.* **2005**, *27*, 1617-1621.

(62) Kamzolova, S. V.; Shishkanova, N. V.; Morgunov, I. G.; Finogenova, T. V. *FEMS Yeast Res.* **2003**, *3*, 217-222.

(63) Papanikolaou, S.; Aggelis, G. *Eur. J. Lip. Sci. Technol.* **2010**, *112*, 639-654.

(64) Pereira, M. R.; Mercaldi, G. F.; Maester, T. C.; Balan, A.; de Macedo Lemos, E. G. *Plos One* **2015**, *10*.

(65) Saygun, A.; Sahin-Yesilcubuk, N.; Aran, N. *J. Am. Oil Chem. Soc.* **2014**, *91*, 1521-1530.

(66) Cui, C. X.; Xie, R.; Tao, Y. F.; Zeng, Q. Q.; Chen, B. Q. *Biocatal. Biotransfor.* **2015**, *33*, 38-43.

(67) Liu, Y.; Guo, C.; Liu, C. Z. *Catal. Lett.* **2014**, *144*, 1552-1556.

(68) Zhou, X. Y.; Gao, Q.; Feng, W.; Pan, K. H. *Chem. Eng. Technol.* **2013**, *36*, 1249-1254.

(69) Finn, R. D.; Coggill, P.; Eberhardt, R. Y.; Eddy, S. R.; Mistry, J.; Mitchell, A. L.; Potter, S. C.; Punta, M.; Qureshi, M.; Sangrador-Vegas, A.; Salazar, G. A.; Tate, J.; Bateman, A. *Nucleic Acids Res.*, *44*, D279-D285.

(70) Dujon, B.; Sherman, D.; Fischer, G.; Durrens, P.; Casaregola, S.; Lafontaine, I.; de Montigny, J.; Marck, C.; Neuveglise, C.; Talla, E.; Goffard, N.; Frangeul, L.; Aigle, M.; Anthouard, V.; Babour, A.; Barbe, V.; Barnay, S.; Blanchin, S.; Beckerich, J. M.; Beyne, E.; Bleykasten, C.; Boisrame, A.; Boyer, J.; Cattolico, L.; Confanioleri, F.; de Daruvar, A.; Despons, L.; Fabre, E.; Fairhead, C.; Ferry-Dumazet, H.; Groppi, A.; Hantraye, F.; Hennequin, C.; Jauniaux, N.; Joyet, P.; Kachouri, R.; Kerrest, A.; Koszul, R.; Lemaire, M.; Lesur, I.; Ma, L.; Muller, H.; Nicaud, J. M.; Nikolski, M.; Oztas, S.; Ozier-Kalogeropoulos, O.; Pellenz, S.; Potier, S.; Richard, G. F.; Straub, M. L.; Suleau, A.; Swennen, D.; Tekaia, F.; Wesolowski-Louvel, M.; Westhof, E.; Wirth, B.; Zeniou-Meyer, M.; Zivanovic, I.; Bolotin-Fukuhara, M.; Thierry, A.; Bouchier, C.; Caudron, B.; Scarpelli, C.; Gaillardin, C.; Weissenbach, J.; Wincker, P.; Souciet, J. L. *Nature,* **2004**, *430*, 35-44.

Chapter 4

Biocatalysis: Enzyme and whole cell mediated

kinetic resolution of

α/β - amyrin and commercially important racemic esters

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Biocatalysis is a green approach to obtain commercially valued chiral synthons with high enantioselectivity, low cost and in large scale. This is achieved through use of either whole cell or free/ immobilised enzymes. Separation of structural isomers from its mixture is a difficult task with very few reports on enzyme mediated resolution of racemic mixture. In this study, α/β – amyrins were shown, for first time, to be resolved from its mixture by use of a commercial lipase *Candida rugosa* lipase (CRL) out of the 16 commercial lipases screened. CRL preferred β-amyrin over α-amyrin when amyrin mixture (α-amyrin: β-amyrin: 78:22) was incubated in n-hexane solvent at 37 ºC in presence of vinyl acetate as acyl donor. A novel whole cell biocatalyst *Fusarium proliferatum* NCIM 1105 was first time shown to be used for the efficient kinetic resolution of racemic acetates to enantiopure (*R*)-alcohols. Eight acyclic and aromatic acetates were enantioselectively hydrolyzed to give corresponding (*R*)-alcohols with > 90 % enantiomeric excess (e.e) and 100 % conversion using resting cell method. Kinetic resolution was found to be one pot two step reaction where racemic acetates were first hydrolyzed to racemic alcohols wherein (*S*)-alcohol was selectively oxidized to corresponding ketone leaving enantiopure (*R*)-alcohol in the fermentation broth.

4.1 Kinetic resolution of structural isomers α and β amyrin using *Candida rugosa* lipase (CRL)

4.1.1 Introduction

Triterpenoids are known for their diverse biochemical and pharmaceutical properties.[1-6] Specifically, α/β-amyrin and their derivatives, structurally characterized as pentacyclic triterpenoids,[7] formed through cyclization of 2,3-oxidosqualene catalyzed by triterpene cyclase, are shown to possess a broad spectrum of biological and pharmacological activities, including anti-inflammatory,[8,9] anti-nociceptive[10], insecticidal,[11,12] antidepressant,[13] anti-arthritic,[14] gastroprotective,[15] anti-hyperglycemic,[16] anti-microbial,[17,18] and cytotoxic activities.[19] α/β-amyrin are structural isomers in relation and possess basic skeletons of two different subgroups of pentacyclic triterpenoids, that is, ursane and oleanane, respectively. The only structural variation in them lies in the E-ring methyl group occupying the position either at C-19 (α-amyrin) or C-20 (β-amyrin) (Fig. 4.1).

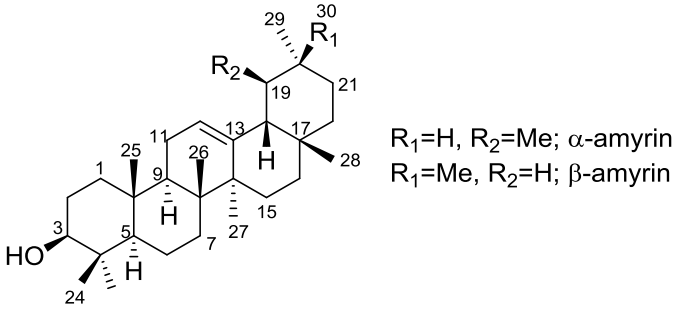
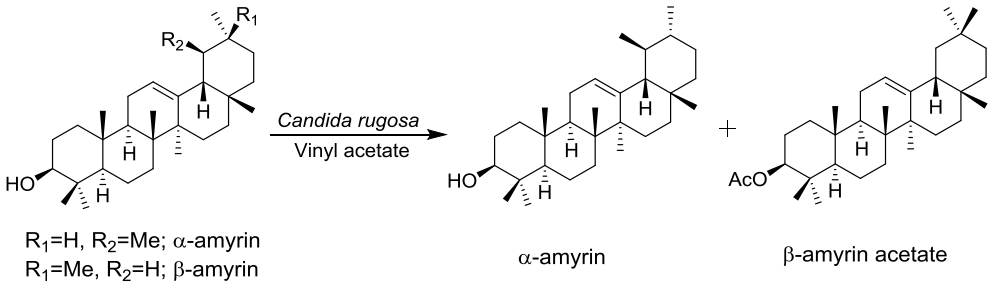


Fig. 4.1 The structures of α and β-amyrins.

In the last decade, α/β-amyrin and their semi-synthesized derivatives have been investigated extensively for their bioactivities and underlying molecular mechanisms.[13,14,18-24] Unfortunately, many of these studies have been carried out with a mixture of α and β-amyrin instead of its pure components.[10, 13,15,18,19,21,23,24] In fact, the plant resources usually contain a complex mixture of structurally similar pentacyclic triterpenoids with wide variation in their relative abundance. The mixture of pentacyclic triterpenoids shows hardly any chromatographic separation due to their high structural and functional resemblance. As a result chromatographic techniques are truly ineffective and tedious for separating individual components. Very few reports are available till date

on purification of α and β-amyrin from their mixture. Literature study has revealed only a HPLC based semi-preparative purification procedure with a long run time.[25]

On the other hand, lipase mediated trans-esterification is a smart choice for the kinetic resolution of isomeric alcohols. Practically, lipases are preferred biocatalyst for their high stability, broad substrate specificity, independency of coenzymes, and equal efficacy in both the aqueous/organic medium.[26,27] Although, lipases have been widely applied for the kinetic resolution of racemic alcohols or desymmetrization of meso-diols and separation of diastereomers or geometrical isomers in a few instances,[28] their application for the separation of structural/constitutional isomers is rare. In this work, we have demonstrated the *Candida rugosa* type VII lipase (CAS No. 9001-62-1 and abbreviated as CRL) mediated trans-esterification of α and β amyrin leading to their kinetic separation. In fact, it is the first report on the lipase-catalyzed separation of two structurally isomeric triterpene alcohols (Scheme 4.1).



Scheme 4.1 Kinetic separation of α and β-amyrin mixture by *Candida rugosa* (*CRL*).

Sixteen commercial lipases from various sources were screened for the trans-esterification of the mixture of α and β amyrin using vinyl acetate as the acyl donor in three different solvent systems (n-hexane, di-isopropyl ether and toluene). Screening experiments clearly indicated that CRL was able to carry out acetylation of β-amyrin in an efficient manner compared to its isomer α-amyrin.

4.1.2 Materials and methods

4.1.2.1 Plant material

Plumeria obtusa (*P. obtusa*) plant material used in the study for extracting latex was collected from the premises of NCL, Pune, India.

4.1.2.2 Chemicals and reagents

All the acyl donors used such as vinyl acetate, vinyl stearate, vinyl benzoate, vinyl laurate, vinyl propionate, vinyl cinnamate, vinyl decanoate, vinyl butyrate and all AR grade solvents were purchased from Sigma aldrich, USA

4.1.2.2 Enzymes

Lipases such as CAL-B (Novozyme 435), CAL-A, Amano lipase M (from *Mucor javanicus*), Amano lipase A (from *Aspergillus niger*), Amano lipase PS (immobilized on diatomite), Amano lipase G (from *Penicillium camemberti*), Amano lipase AK (from *Pseudomonas fluorescens*), lipase from *Thermomyces lanuginosa*, lipase from *Penicillium camemberti*, lipase from wheat germ, lipase from *Candida rugosa*, lipase from Porcine pancreas type-II, lipase from *Candida rugosa* immobilized on immobead 150, lipase from *Rhizopus niveus*, lipase from *Rhizopus arrhizus*, lipase from *Candida rugosa* type-VII were all purchased from Sigma Aldrich, USA.

4.1.2.3 Extraction of α and β amyrin mixture

Latex (175 g) collected from *P. obtusa* was taken in water (400 mL) and extracted with ethyl acetate (600 mL X 6). Ethyl acetate layers were pooled together and concentrated to get 33 g white solid, which upon fractionation by column chromatography using gradient mixture of ethyl acetate–hexane furnished two fractions. Less polar fraction (8.2 g) contained the mixture of α, β-amyrin acetate and lupeol acetate whereas more polar fraction (12.3 g) was consisted of deacetylated analogues of the same pentacyclic triterpenoids. From the alcohol fraction, mixture of α and β-amyrin (4.8 g, α: β = 78:22) was obtained through argentation column chromatography.

4.1.2.4 Screening of different acyl donors for the reaction

Acyl donors: Vinyl donors used for screening were vinyl acetate, vinyl stearate, vinyl benzoate, vinyl laurate, vinyl propionate, vinyl cinnamate, vinyl decanoate and vinyl butyrate.

4.1.2.5 Screening conditions

Amyrin mixture (1.0 mg), lipase (5.0 mg), and vinyl acetate (10% of total reaction volume of 2.0 mL) in various solvents were incubated at 200 rpm for 24 h at temperature directed by manufacturer's instructions. In time course studies and screening experiments, the aliquot of reaction mixture (100 µL) was drawn, filtered, and diluted to 0.5 mL (total volume) using n-Hexane. 1.0 µL of this diluted reaction mixture was subjected to GC/GC–MS analyses.

4.1.2.6 GC and GC-MS conditions

GC-Instrument: Agilent 7890 attached with hydrogen flame ionization detector, **Column:** HP-5 (30 m X 0.32 mm X 0.25 µm, J and W Scientific), Carrier gas: Nitrogen with flow rate 1.0 mL/min,

Oven temperature program: Initially the column was maintained at 150 ºC for 2 min followed by a temperature gradient from 150 ºC to 320 ºC at 10° C/min and finally the temperature was maintained at 320 ºC for 11 min, injector: 300 ºC, detector: 280 ºC, split ratio: 1:10. Retention time (Rt in min): 20.4 (β-amyrin), 20.8 (α-amyrin), 21.2 (β-amyrin acetate) and 21.6 (α-amyrin acetate).

GC–MS instrument: Agilent 7890A GC coupled with 5975C mass detector, column HP-5-MS (30 m X 0.25 mm X 0.25 µm, J and W Scientific) and other conditions are maintained similar as GC.

4.1.2.7 Optimized separation protocol

CRL (200.0 mg) was added to the assay mixture containing α and β amyrin mixture (16.0 mg, 78:22, respectively) and 0.5 mL vinyl acetate in n-hexane. The total volume of assay mixture was 5.0 mL. This reaction mixture was incubated at 37 ºC on an incubator shaker with 200 rpm for six days. After this incubation period, the reaction mixture was subjected to flash column chromatography on silica gel (60–120 mesh) to

obtain α-amyrin (11.0 mg, 97.5%). The acetate fraction contained both α and β-amyrin acetates. To obtain pure β-isomer, the assay was carried out under the same conditions except the vinyl acetate was 0.05 mL and incubation period was 24 h. At the end of this incubation period, β-amyrin acetate (1.0 mg, 99.9%) was obtained in pure form after subjecting to column chromatography under similar conditions. For large scale purification, the volume of the assay mixture was increased proportionately.

4.1.3 Results and discussion

Candida rugosa lipase *(CRL)* carried out acetylation of β-amyrin in a very efficient manner, whereas α-amyrin was found to be a very poor substrate for *CRL* mediated trans-esterification reaction. These observations were further confirmed by the kinetic studies of CRL mediated acetylation of pure α- and β-amyrin (Fig. 4.3).

After the incubation period of 48 h, CRL converted 38 % of β-amyrin (βA-OH) to β-amyrin acetate (βA-OAc) in presence of vinyl acetate as the acyl donor [Fig. 4.2 (A)]. However, less than 1 % of α-amyrin acetate (αA-OAc) formation was observed when α-amyrin (αA-OH) was used as the substrate with similar assay conditions [Fig. 4.2 (B)]. The variable catalytic activity of *CRL* with respect to α- and β-amyrin was efficiently exploited for the kinetic separation of individual isomers from amyrin mixture obtained from *Plumeria obtusa* latex. The mixture of α and β-amyrin (78:22) [Fig. 4.3(B)] was obtained by subjecting the ethyl acetate extract [Fig. 4.3 (A)] of the *P. obtusa* latex to successive column chromatography.

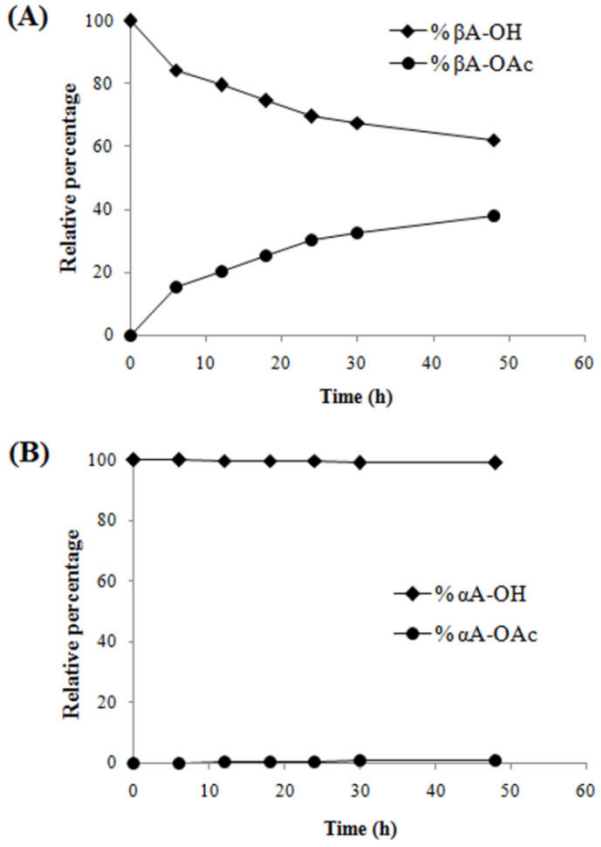


Fig. 4.2 Kinetic studies of *CRL* catalyzed trans-esterification of pure (**A**) β-amyrin and (**B**) α-amyrin. Pure α or β-Amyrin (1.0 mg), *CRL* (12.5 mg) and vinyl acetate (10% of total reaction volume of 2.5 mL) in n-hexane were incubated at 37 ºC and 200 rpm.

CRL mediated trans-esterification conditions such as preference of solvent, choice and effective concentration of acyl donor and concentration of *CRL* were optimized to get the best separation. Among various solvents screened, acyclic ethers (diisopropyl ether, diethyl ether, tert-butyl methyl ether) and alkanes (n-hexane, n-pentane) were found to be better solvent systems for *CRL* mediated trans-esterification. In fact, n-hexane was found to be the most suitable solvent for *CRL* mediated trans-esterification, as indicated by GC analyses.

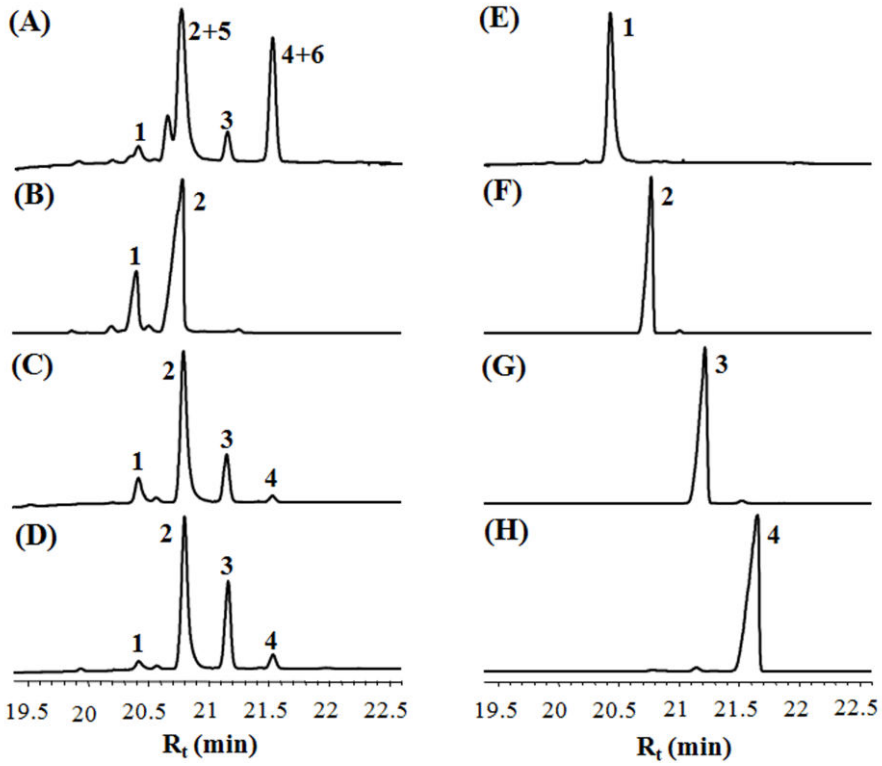


Fig. 4.3 GC-FID chromatograms of (**A**) *P. obtusa* extract, (**B**) α and β-amyrin mixture obtained from *P. obtusa* extract, (**C**) *CRL* mediated trans-esterification reaction mixture after 1 day, (**D**) after 5 days, (**E**) Pure β-amyrin, (**F**) Pure α-amyrin, (**G**) Pure β-amyrin acetate, (**H**) Pure α-amyrin acetate. Labeling of compounds, **1**: β-amyrin, **2**: α-amyrin, **3**: β-amyrin acetate, **4**: α-amyrin acetate, **5**: Lupeol, **6**: Lupeol acetate.

Among eight different acyl donors used for *CRL* mediated trans-esterification of β-amyrin, only vinyl acetate was found to be utilized as the acyl donor by lipase system. To determine the optimized concentration of acyl donor, trans-esterification reaction was carried out with variable proportions of vinyl acetate in n-hexane. The percentage of

acetylated product (% conversion) was in the same range at various concentrations (1.0, 2.0, 4.0, 10.0, 20.0 and 60.0 %) total reaction volume respectively) of vinyl acetate during 24 h of incubation period; though at 10.0 % concentration of vinyl acetate slightly higher formation of acetylated products was observed [Fig. 4.4 (A)]. Interestingly, lower vinyl acetate concentrations led to the higher abundance of βA-OAc in the acetate mixture and this condition can be utilized for the purification of β-isomer.

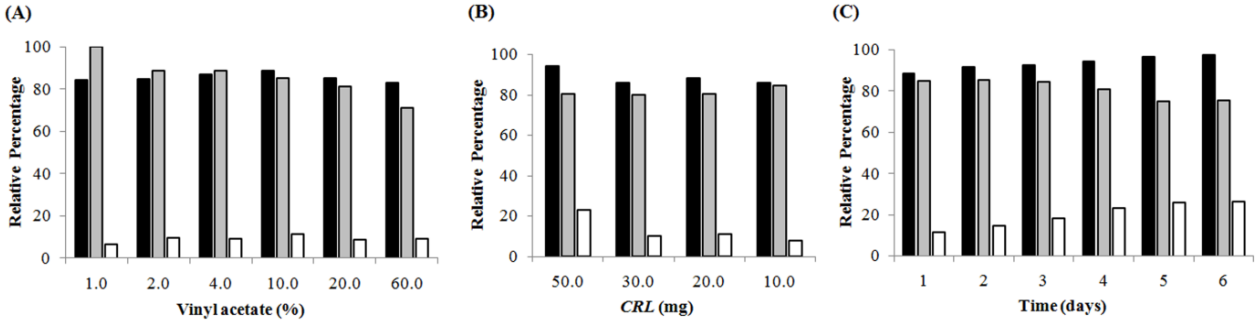


Fig. 4.4 (**A**) Optimization study of effective vinyl acetate concentration. Amyrin mixture (4.0 mg), *CRL* (50.0 mg) and vinyl acetate (1.0, 2.0, 4.0, 10.0, 20.0 and 60.0 % of total reaction volume of 5.0 mL) in n-hexane were incubated for 24 h. (**B**) Optimization study of effective *CRL* concentration. Amyrin mixture (4.0 mg), *CRL* (50.0, 30.0, 20.0 and 10.0 mg) and vinyl acetate (10 % of total reaction volume of 5.0 mL) in n-hexane were incubated for 96 h. (**C**) Time course experiment of *CRL* catalyzed separation of amyrin mixture from *Plumeria obtusa*. Amyrin mixture (4.0 mg), *CRL* (50.0 mg) and vinyl acetate (10 % of total reaction volume of 5.0 mL) in n-hexane were incubated together and monitored at an interval of 1 d. All the reaction mixtures were incubated at 37 ºC and 200 rpm throughout. (■ % of α-amyrin in alcohol mixture, ▩ % of β-amyrin acetate in acetate mixture, □ % conversion).

The effective concentration of *CRL* was optimized by performing the reaction with 10.0, 20.0, 30.0 and 50.0 mg of *CRL* respectively with 4.0 mg of amyrin mixture as the substrate and 10 % of vinyl acetate as the acyl donor. Progress (% conversion) of the reaction and enrichment of the individual isomers (α-amyrin in alcohol and β-amyrin acetate in acetate mixture) was monitored at an interval of 24 h through GC and GC-MS for all four sets of reaction containing different concentrations of *CRL*. Although after 24 h all the sets showed similar progress and enrichment, at the end of 96 h highest percentage of conversion (23.0 %) was achieved with 50 mg CRL per 4.0 mg amyrin mixture. Also, the highest abundance of α-amyrin (94.4 %) in alcohol mixture after 96 h was attained with same *CRL* concentration of 50 mg/ 4.0 mg amyrin mixture [Fig. 4.4 (B)]. Therefore, 4.0 mg amyrin mixture and 50 mg *CRL* in total reaction volume of 5.0

mL (containing 10 % vinyl acetate in n-hexane) was found to be most suitable *CRL* catalyzed trans-esterification condition.

Time course experiment was performed to monitor time dependent progress and enrichment of *CRL* mediated trans-esterification product [Fig. 4.3(C), 4.3 (D) and 4.4(C)]. Reaction containing 4 mg amyrin and 50 mg *CRL* in total reaction volume of 5.0 mL (with 10 % vinyl acetate in n-hexane) was monitored at an interval of 24 h for 6 days. Results indicated after 6 days of incubation period, percentage conversion reached highest (26.3 %) with 97.5 % purity of α-amyrin in alcohol mixture. Prolonging the incubation period did not yield noticeable changes in the levels of α-amyrin [Fig. 4.4(C)]. Hence, incubation period of 6 days can be considered as most effective to achieve highest abundance of α-amyrin in alcohol mixture.

In conclusion, *Candida rugosa* lipase (*CRL*) catalyzed separation protocol was developed for the separation of structurally isomeric triterpene mixture of α- and β-amyrin. *CRL* selectively acetylated β-amyrin, thus elevating the percentage of α-amyrin in alcohol mixture and β-amyrin acetate in acetate mixture. n-Hexane and vinyl acetate were found to be better organic solvent and acyl donor respectively for the *CRL* mediated trans-esterification. Highest percentage of β-amyrin acetate in acetate mixture was obtained at lower concentration of vinyl acetate in the early stage of incubation period (24 h). On the other hand, highly pure α-amyrin was obtained by incubating *CRL* with α- and β-amyrin mixture for prolonged incubation period (6 d). This separation procedure might be useful for the large-scale separation of α- and β-amyrin for their detailed biological and commercial applications.

4.2 Fungal mediated kinetic resolution of racemic acetates to (R)-alcohols using *Fusarium proliferatum* NCIM 1105.

4.2.1 Introduction

Enantiomerically pure low molecular weight alcohols and their corresponding acetates are widely useful chemicals in industry as well as academia. They are important ingredients in the perfumery industry due to their volatility and unique odour.[29-31] Also, strategic utilization of these molecules as the chiral precursors for the asymmetric syntheses of complex organic molecules is well-known.[32, 33] For example, acyclic alcohols such as (*R*)-(-)-lavandulol (**1a**) exists naturally as a major component of the lavender essential oil and is used in perfumery and cosmetic industry. (*R*)-(-)-lavandulyl propionate has been investigated as the sex pheromone in mealybug, whereas its (*S*)-enantiomer was found to be inactive (Fig. 1).[34] Both the enantiomers of 2-hexyl acetate (**2**) and 2-heptyl acetate (**3**) along with their corresponding alcohols are used as useful constituents in flavour and fragrance industry. On the other hand, (*R*)-(-)-2-hexanol (**2a**) and (*S*)-(+)-2-hexanol (**2b**) were used in the preparation of key intermediates in the total synthesis of anti-viral glycolipid cycloviracin B_1.[35] *(R)*-(-)-2-heptanol (**3a**) was utilised in resolving the racemic mixture of a key intermediate in the synthesis of 2,3,4,5-tetrahydro-1*H*-1-benzdiazepine derivatives, known to be a strong vasopressin V_2 receptor agonist.[36]

Resolution of the racemic mixture of these alcohols/acetates is highly challenging and available chromatographic techniques are ineffective to achieve the desired resolution, especially in preparative scale separation. Enzyme mediated kinetic resolution of such alcohols using lipases/alcohol dehydrogenases have been reported in the literature.[31, 37-42] For example, resolution of (±)-lavandulol has been achieved previously with various lipases such as *Candida antarctica* lipase B (CAL B), [37] *Hog pancreas* lipase,[43] *Porcine pancreas* lipase,[42] and *Yarrowia lipolytica* lipase. [44] However, enzyme catalyzed resolution process is associated with several disadvantages such as higher cost, low substrate concentration, instability and cofactor dependency of the enzymes in several occasions. On the other hand, whole cell biocatalysis offers an inexpensive choice in which enzymes are stable within cellular environment and the microbial cells itself act as the source of cofactors for the biocatalyst mediated conversion.[45, 46] In the present study, we have

developed a one-pot two-step de-esterification/oxidation kinetic resolution process for nine acyclic and aromatic acetates to furnish enantiopure (*R*)-alcohols using whole-cells of *Fusarium proliferatum* (National Collection of Industrial Organisms/NCIM, catalogue no. 1105) (Fig. 1). Substrate concentration and incubation period were optimized and the efficiency of kinetic resolution process [enantiomeric excess (e.e)] was evaluated for each of the substrates. Furthermore, preparative scale resolution was successfully achieved on (±)-1-phenylethyl acetate (**6**) to isolate (*R*)-(+)-1-phenylethanol (**6a**).

4.2.2 Materials and method

4.2.2.1 Chemicals and media

Media ingredients, salts and acids were purchased from Merck (India) and Himedia laboratories, Mumbai.

4.2.2.2 Microorganisms used

Microorganisms used in the study were either isolated from soil or obtained from culture collection such as National Collection of Industrial Microorganisms (NCIM), NCL, Pune, and MTCC-IMTECH, Chandigarh. All the fungal cultures were maintained and propagated on potato dextrose agar (PDA) slants and preserved at 4 ºC. Before propagation, purity of each culture was monitored using microscopic techniques.

4.2.2.2.1 Maintenance and propagation of the organisms

The main stock of cultures was maintained on Czapek-Dox agar or Potato Dextrose agar (PDA) slants.

Potato Dextrose Agar [PDA]:

Ingredient	Quantity
Potato	200 g
Dextrose	20 g
Agar	20 g
Distilled water	1000 mL

Table 4.1: Composition of Potato Dextrose Agar

The potatoes were cut into small cubes, boiled until it became soft, filtered through cheesecloth, the filtrate was then made up to 1000 ml volume. To this dextrose and agar were added, pH was adjusted to 7, prior to adding agar. The mixture was boiled to melt agar. Aliquots (8 mL) of the hot homogenous solution were distributed into test tube (20 mL size) which were then plugged with cotton and autoclaved at 15 psi for 20 min. The tubes were kept in slant position while hot and allowed to solidify. To prepare solid base agar flasks, 100 mL of the homogenous solution were dispensed into 500 mL Erlenmeyer flask, plugged and autoclaved. The flasks were then kept on a flat surface while hot and allowed to solidify.

4.2.2.2.2 Czapek-Dox-Agar Slants

2.0 g of agar was weighed into 100 ml of modified Czapek-Dox medium and the slants prepared as described above. The solidified slants were kept at 30 ºC for a day to check for any possible contamination. The slants (/flasks) thus prepared were inoculated under aseptic conditions from the stock culture and incubated at 30 ºC for 5 days to ensure good sporulation. The slants were then stored at 4 ºC till further use.

4.2.2.2.3 Growth of the Microorganisms

Sterile water (10 mL) was added to a sporulated slant and the spores were loosened by scraping the surface gently with an inoculation loop. 1.5 ml of this spore suspension was added to each 250 mL Erlenmeyer flask containing 50 ml of sterile modified Czapek-Dox medium. Different constituents of the modified Czapek Dox media (Table 4.2) were supplemented in 1 litre de-ionized water and pH of the media was adjusted to 5.8 using 1 M K_2HPO_4. The flasks thus inoculated were kept on a rotary shaker (200 rpm) at 30 ºC for required period of incubation.

Ingredient	Quantity per liter
Dextrose	30.0 g
Sodium nitrate	3.0 g
Di-potassium hydrogen phosphate	1.0 g
Potassium chloride	0.5 g
Magnesium sulphate	0.5 g
Yeast extract	0.5 g
Corn steep liquor	7.0 mL
Ferrous sulphate	10.0 mg

Table 4.2 Composition of modified Czapek Dox media.

4.2.2.2 Other chemicals

Racemic alcohols (±)-lavandulol, (±)-2-hexanol, (±)-2-heptanol, (±)-3-hexanol, (±)-1-octen-3-ol, (±)-1-phenylethanol, (±)-2-methyl-1-phenylethanol, (±)-3-methyl-1-phenylethanol and (±)-1-phenylpropanol were purchased from Sigma aldrich/Fluka, USA.

Racemic esters (±)-lavandulyl acetate (**1**), (±)-2-hexyl acetate (**2**) and (±)-2-heptyl acetate (**3**), (±)-3-hexyl acetate (**4**), (±)-1-octen-3-yl acetate (**5**), (±)-1-phenylethyl acetate (**6**), (±)-2-methyl-1-phenylethyl acetate (**7**), (±)-3-methyl-1-phenylethyl acetate (**8**) and (±)-1-phenylpropyl acetate (**9**) were purchased from Sigma aldrich, USA. All the pure enantiomeric standards were purchased from Sigma aldrich, USA.

4.2.2.3 GC/GC-MS columns

HP-5, HP-Chiral columns were purchased from Agilent (J and W scientific), USA while Astec CHIRALDEX G-TA, B-DP, B-DM columns were purchased from Supelco, Sigma Aldrich, USA.

4.2.2.4 Fermentation Conditions

4.2.2.4.1 Inoculation of fermentation media with fungal culture

Sterile water (10 mL) was added to a well sporulated slant and the spores were loosened by scraping the surface gently. Sterile PDA slants were streaked with the loop containing spores, incubated at 30 ºC till well sporulation was observed and stored at 4 ºC for further use. 1.5 mL of above spore suspension was added to 250 mL Erlenmeyer flasks containing 50 mL of sterile modified Czapek Dox medium. All the inoculation was carried out in laminar air flow under sterile conditions. The flasks were then incubated at 30 ºC on a rotary shaker (200 rpm) for 48 h.

4.2.2.4.2 Screening experiment

To a well-grown culture (50 mL modified CZ media, pH of which was adjusted to 5.8 using sterile 1 M K_2HPO_4), 0.6 g/L of (±)-lavandulyl acetate in 0.2 mL acetone was added and incubated at 30 °C and 200 rpm for 3 days. After this incubation period, mycelia and broth were extracted separately with dichloromethane (three times) and analyzed by TLC and chiral GC-FID/GC-MS. All the screening experiments were analyzed by comparing with corresponding substrate control and organism control.

4.2.2.4.3 Biotransformation using whole cell method

Fermentations were carried out in modified Czapek Dox medium (C.Z). The pH of the medium was adjusted to 5.8 with 1 M K_2HPO_4. Flasks (250 mL) containing 50 mL of sterile medium were inoculated with 1.5 mL (3 % v/v) of a spore suspension from a 2-3-day-old culture grown on potato dextrose agar (PDA) slants and were incubated at 30 ºC on a rotary shaker (200 rpm) for 48 h. After growth period, the pH of the medium was adjusted to 7.0 by the addition of sterile 1 M K_2HPO_4, substrate in acetone (0.2 mL) was added to each flask, and the incubation was continued for an additional period, as required. Control experiments were also run with the substrate but without microorganism and with microorganism but without substrate. In time course experiments, incubations were carried out for 1-5 days and the metabolites formed at the end of each incubation period were monitored by chiral GC and GC-MS analyses.

4.2.2.4.4 Substrate concentration study

In substrate concentration studies, different concentration of substrate (±)-lavandulyl acetate ranging from 0.4 to 1 g/L were added to flasks containing 48 h grown cultures of *Fusarium proliferatum* NCIM 1105 in 50 mL media. Flasks were then incubated at 30 ºC on rotary shaker (200 rpm) for 5 days. At the end of the incubation period, mycelia were separated from fermentation media by filtration through muslin cloth. Both fermentation media and mycelia were separately extracted twice with equal volume of dicholomethane (50 mL X 3) and crude extracts were analyzed by TLC and chiral GC/GC-MS. The concentration of the substrate at which enantiopure (*R*)-(-)-lavandulol with highest conversion rate observed was further used for time course study and large-scale fermentation.

4.2.2.4.5 Time-course experiment

In time course experiment, the optimum concentration of substrate standardized from substrate concentration study, was aseptically added to 48 h grown culture and incubation was continued. At every specific interval of incubation period, aliquots were drawn for extraction and analysis of the metabolites, until 5 days. The product formed were extracted in dichloromethane and monitored by TLC and chiral GC/GC-MS analyses.

4.2.2.4.6 Resting cell experiment

Resting cell experiments were carried out using 2 g of fully grown mycelia which washed with phosphate buffer (0.1M, pH 7.2). This mycelia was then incubated with substrate (racemic acetates) having concentration of 0.1 g/Litre in 50 mL phosphate buffer (0.1 M, pH 7.2 with 0.2 % Glucose) in 250 mL conical flask. These flasks were then incubated on a rotary shaker at 200 rpm and 30 ºC for various time intervals of 1 h to 36 h depending on the substrate in use. At the end of incubation period, the contents from the flask were filtered and extracted as described earlier. Control experiment was also done without substrate and without micro-organism. The organic phase was concentrated and analyzed by GC-FID (Chiral GC) and GC-MS.

4.2.2.4.7 Preparative scale fermentation

1 litre CZ (Czapex Dox) media was prepared in 2.8 litre Nalgene flask and freshly grown *Fusarium proliferatum* NCIM 1105 culture (3 % v/v) was added into it. These flasks were then incubated at 200 rpm and 30ºC for 48 h. Substrate [(±)-1-Phenylethyl acetate] at the concentration of 0.4 g/L in 2 mL acetone was directly added into it. After incubating these flasks for 3 days, mycelia were filtered using muslin cloth. Separated mycelia and filtered media was extracted with Dichloromethane (DCM) thrice (1 L X 3 times) and filtrate was then passed though anhydrous sodium sulphate. Filtrate thus extracted was concentrated to get crude extract which was further purified over silica gel column (230-400 mesh) to get enantiopure (*R*)-(+)-1-Phenyl ethanol. Product was then characterised by NMR, Optical rotation and Chiral GC/GCMS techniques.

4.2.2.5 Metabolite extraction procedure

At the end of the incubation period, the contents from all of the flasks were pooled, the pH was adjusted to 5.5-6.0, and the mixture was filtered. The filtrate (broth) and mycelia were separately extracted with CH_2Cl_2 (DCM) twice. The two CH_2Cl_2 (DCM) extracts were combined, concentrated, and separated into acidic and neutral fractions by treatment with 5 % sodium bicarbonate solution. The bicarbonate phase was acidified and re-extracted with CH_2Cl_2. The acidic fraction contained compounds derived

from the organism and hence was not processed further. The neutral fraction was subjected to column chromatography and to isolate the metabolites in the pure form.

4.2.2.6 GC/GC-MS conditions

A) GC-MS was performed on a HP 5975C mass selective detector interfaced with a HP 7890A GC instrument. GC and GC-MS analyses for (±)-lavandulol was done on HP-CHIRAL-20B (20 % β-cyclodextrin) capillary column (30 m X 0.32 mm X 0.25 µm, J and W Scientific) with the following oven temperature program-

1) For (±)-lavandulol and (±)-lavandulyl acetate

Oven temperature programme: Initially the column temperature was maintained at 80 °C for 1 min, followed by a temperature gradient from 80 ºC to 140 ºC at 2 ºC min^{-1} and then raised to a final temperature of 230 ºC with a 10 ºC min^{-1} rise for 10 min.

Retention times (R_t): (*S*)-lavandulol and (*R*)-lavandulol and (±)-lavandulyl acetate were eluted at retention times of 21.9 min, 22.23 min and 24.6 min, respectively.

B) While for (±)-2-hexanol and (±)-2-heptanol chiral GC separation was done on GC column "Astec CHIRALDEX G–TA" capillary column (30 m X 0.25 mm X 0.12 µm, Supelco) with following:

Oven temperature programme: - Initial temperature maintained at 35 ºC for 1 min, followed by a temperature gradient from 35 ºC to 65 ºC at 1 ºC min^{-1} and then raised to a final temperature of 180 ºC with a 10 ºC min^{-1} rise with no holding time.

Retention times (R_t): (*S*)-2-hexanol, (*R*)-2-hexanol, (*S*)-2-hexyl acetate and (*R*)-2-hexyl acetate eluted at retention times of 20.4, 21.25, 28.17 and 29.99 min respectively.

Retention times (R_t): (*S*)-2-heptanol, (*R*)-2-heptanol, (*S*)-2-heptyl acetate and (*R*)-2-heptyl acetate were eluted retention times of of 29.77, 30.22, 34.58 and 35.14 min respectively.

C) Chiral GC separation of (±)-3-hexanol, (±)-1-octen-3-ol, (±)-1-phenyl ethanol, (±)-3-methyl-1-phenyl ethanol and (±)-1-phenylpropanol was done on "Astec CHIRALDEX B-DP" capillary column (30 m X 0.25 mm X 0.12 µm, Supelco) with following oven temperature programme-

Oven temperature programme: Initial temperature maintained at 50 ºC for 1 min, followed by a temperature gradient from 50 ºC to 70 ºC at 0.5 ºC min^{-1}, a temperature gradient from 70 ºC to 110 ºC at 1 ºC min^{-1} and then raised to final temperature of 200 ºC at the rate of 10 ºC $min^{-1}$and kept on hold for 3 min.

1) For (±)-3-hexanol and (±)-3-hexyl acetate

Retention times (R_t): (±)-3-hexyl acetate, (*R*)-(-)-3-hexanol and (*S*)-(+)-3-hexanol were eluted at retention times of (11.6, 11.8) min, 15.2 min and 15.7 min respectively.

2) For (±)-1-octen-3-ol and (±)-1-octen-3-yl acetate

Retention times (R_t): (±)-1-octen-3-yl acetate, (*R*)-(-)-1-octen-3-ol and (*S*)-(+)-1-octen-3-ol were eluted at retention times of (35.5, 36.2) min, 42.8 min and 44.0 min respectively.

3) For (±)-1-phenyl ethanol and (±)-1-phenyl ethyl acetate

Retention times (R_t): (±)-1-phenylethyl acetate, (*R*)-(+)-1-phenyl ethanol and (*S*)-(-)-1-phenyl ethanol were eluted at retention times of (55.1, 55.7) min, 66.6 min and 69.3 min respectively.

4) For (±)-3-methyl-phenyl ethanol and (±)-3-methyl-phenyl ethyl acetate

Retention times (R_t): (±)-3-methyl-1-phenylethyl acetate, (*R*)-(+)-3-methyl-1-phenyl ethanol and (*S*)-(-)-3-methyl-1-phenyl ethanol were eluted at retention times of 69.8 min, 84.7 min and 85.7 min respectively.

6) For (±)-1-phenylpropanol and (±)-1-phenylpropyl acetate

Retention times (R_t): (±)-1-phenylpropyl acetate, (*R*)-(+)-1-phenylpropanol and (*S*)-(-)-1-phenylpropanol were at retention times of (64.1, 64.5) min, 70.2 min and 71.5 min respectively.

D) Chiral GC separation of (±)-2-Methyl-1-phenyl ethanol was done on "Astec CHIRALDEX B-DM" capillary column (30 m X 0.25 mm X 0.12 μm, Supelco) with following oven temperature programme:-

Oven temperature programme: Initial temperature maintained at 50 ºC for 1 min, followed by a temperature gradient from 50 ºC to 70 ºC at 0.5 ºC min^{-1}, a temperature

gradient from 70 ºC to 110 ºC at 1 ºC min^{-1} and then raised to final temperature of 200 ºC at the rate of 10 ºC $min^{-1}$and kept on hold for 3 min

1) For (±)-2-Methyl-1-phenyl ethanol and (±)-2-Methyl-1-phenyl ethyl acetate

Retention times (R_t): (±)-2-methyl-1-phenylethyl acetate, (*R*)-(+)-2-methyl-1-phenyl ethanol and (*S*)-(-)-2-methyl-1-phenyl ethanol were eluted at retention times of 67.8 min, 74.3 min and 71.5 min respectively.

4.2.3 Results and discussion

(±)-Lavandulyl acetate (**1**), an acyclic racemic ester was used as the model substrate for the whole-cell mediated resolution of esters. Among various fungal system screened (*Aspergillus*, *Fusarium*, *Neurospora*, *Cunninghamella* etc), *Fusarium proliferatum* (National Collection of Industrial Microorganisms, Catalogue No. 1105) efficiently converted (±)-lavandulyl acetate to (*R*)-(-)-lavandulol (**1a**) with very high enantiomeric excess (e.e) (Table 4.3). Bio catalytic resolution was validated with two independent control experiments; substrate control (substrate without organism) and organism control (organism without substrate). Both the control experiments did not show any evidence for the formation of (*R*)-(-)-lavandulol (**1a**) as analyzed by GC-FID and GC-MS. In addition, the resolution achieved through the resting cell of *F. proliferatum* confirmed the enzyme to be constitutive to the whole-cell used. Substrate concentration 0.6 gL^{-1} was found to be optimum to achieve the highest e.e and the higher concentration led to the diminished e.e and slower conversion rate. After 3 days of incubation, (±)-lavandulyl acetate (**1**) was converted to (*R*)-(-)-lavandulol (**1a**) (95.3%, 99.8 % e.e).

Sr no.	Micro-organism	NCIM Catalogue no.	% GC Area			e.e value (%)
			(1b)	(1a)	(1)	
1	*Fusarium prolifertaum*	1105	0.1	99.9	--	99.8
2	*Aspergillus niger*	582	23.1	18.2	58.7	36
3	*Aspergillus niger*	589	26.6	12.5	60.9	36
4	*Neurospora crassa*	910	30.6	28.8	40.6	31
5	*Aspergillus gigantius*	568	38.5	61.5	--	23
6	*Cunnighamella irregularis*	1278	28.5	18.6	52.9	21
7	*Aspergillus niger*	612	45.8	31.5	22.7	18.4
8	*Aspergillus foetidus*	510	36.9	26.1	37.0	17.0
9	*Aspergillus niger*	572	24.7	19.0	56.3	12.9

Incubation period= 72 h, Concentration of (**1**) = 0.6 gL^{-1}

Table 4.3 Screening of various fungal systems for the biotransformation of (±)-lavandulyl acetate.

Time-course experiment was carried out to investigate the time-dependent progress and mechanistic pathway of the kinetic resolution [Fig. 4.8(A)]. In early stage of the incubation period (12 h), (±)-lavandulyl acetate was (**1**) converted to (*R*) (**1a**) and (*S*)-lavandulol (**1b**) (*R*: *S* 45.9:49.4) with 95.3% conversion. With prolonged incubation up to 3 days, the relative abundance of (*R*)-lavandulol (**1a**) increases to 99.8 % and the percentage of (*S*)-lavandulol (**1b**) steeply diminished to 0.18 % in the fermentation broth. Continuing the incubation up to 5 days didn't alter the relative abundance of the individual enantiomers in the reaction mixture. To further improve the rate of kinetic resolution the biotransformation was carried out with the resting cell of *F. proliferatum* with 0.1 gL^{-1} concentration of (±)-lavandulyl acetate (**1**) [Fig. 4.8 (B)]. Time-course experiment with the resting cell revealed that after 6 h of incubation period, the abundance of (*R*)-lavandulol (**1**) starts increasing in the fermentation broth which reaches up to 94.4 % (96.8 % e.e) after 24 h with quantitative consumption of the acetate [Fig.4.5].

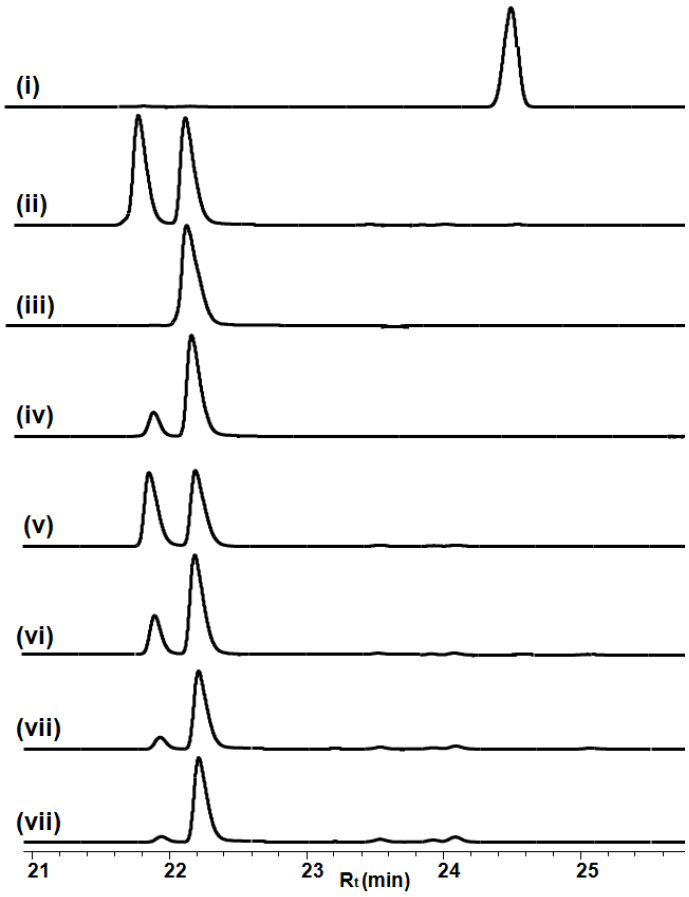


Fig. 4.5 Time Course study for the conversion of (±)-lavandulyl acetate by *F. proliferatum*.

(i) Standard (±)-Lavandulyl acetate (ii) Standard (±)-Lavandulol (iii) Standard (*R*)-Lavandulol (iv) Co injection of (±)-Lavandulol and (*R*)-Lavandulol (v) Conversion of (±)-Lavandulyl acetate after 6 h incubation with *F. proliferatum* (vii) Conversion of (±)-Lavandulyl acetate after 12 h incubation with *F. proliferatum* (viii) Conversion of (±)-Lavandulyl acetate after 18 h incubation with *F. proliferatum,* (viii) Conversion of (±)-Lavandulyl acetate after 24 h incubation with *F. proliferatum*

To investigate the substrate scope of the whole-cell biocatalyst four racemic acyclic esters [(±)-2-hexyl acetate (**2**) , (±)-2-heptyl acetate (**3**), (±)-3-hexyl acetate (**4**), (±)-1-octen-3-yl acetate (**5**)] and four aromatic esters [(±)-1-phenylethyl acetate (**6**), (±)-2-methyl-1-phenylethyl acetate (**7**), (±)-3-methyl-1-phenylethyl acetate (**8**) and (±)-1-phenylpropyl acetate (**9**)] were chosen as the substrates (Table 4.4). The kinetic resolution of these substrates was assessed by incubating with resting cells of *F. proliferatum* and time-course study [Fig. 4.8(C-F)]. Resting cell experiments with the acyclic esters [(**2**), (**3**), (**4**) and (**5**)] were carried out at substrate concentration of 0.1 gL^{-1}. Esters (**2**) and (**3**) yielded enantiomerically pure (*R*)-alcohols (**2a**) and (**3a**) (100 % and 99.9 % e.e) [Fig.4.6 and Fig.4.7] respectively after 6 h of incubation [Fig. 4.8(C) and 4.5(D)].

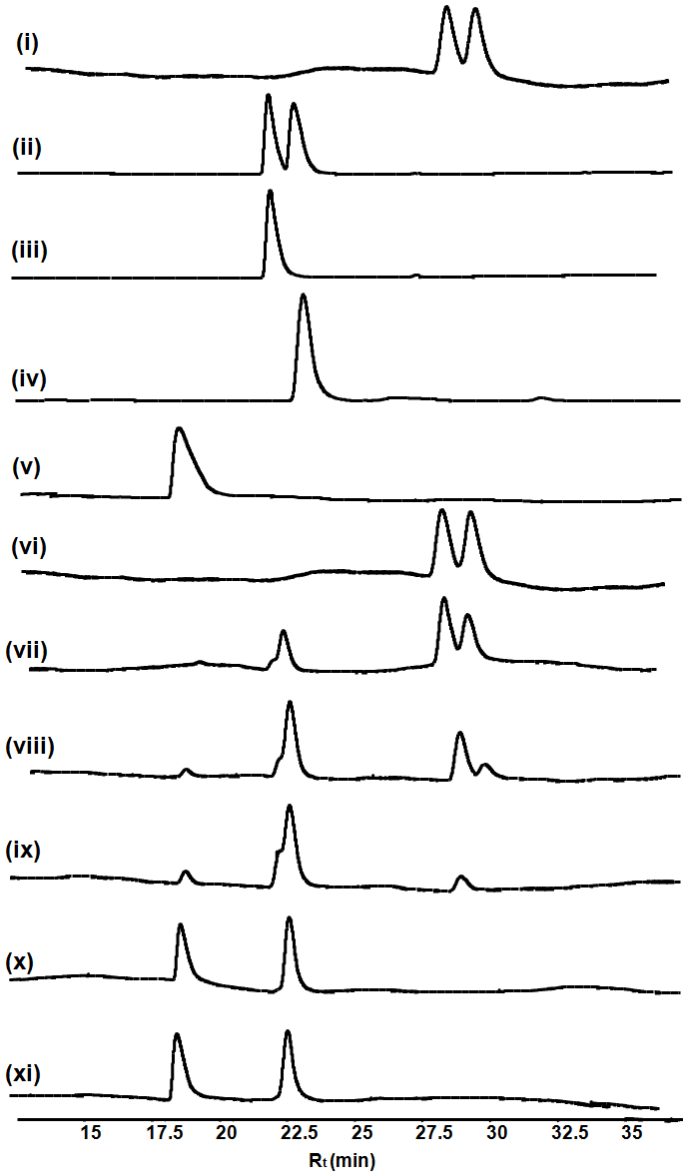


Fig.4.6 Time Course study for the conversion of (±)-2-Hexyl acetate by *F. proliferatum*

(i) Standard (±)-2-Hexyl acetate (ii) Standard (±)-2-Hexanol (iii) Standard (*S*)-(+)-2-Hexanol (iv) Standard (*R*)-(-)-2-Hexanol (v) Standard 2-Hexanone (vi) Conversion of (±)-2-Hexyl acetate after 1 h incubation with *F. proliferatum* (vii) Conversion of (±)-2-Hexyl acetate after 2 h incubation with *F. proliferatum* (viii) Conversion of (±)-2-Hexyl acetate after 3 h incubation with *F. proliferatum,* (ix) Conversion of (±)-2-Hexyl acetate after 4 h incubation with *F. proliferatum* (x) Conversion of (±)-2-Hexyl acetate after 5 h incubation with *F. proliferatum* (xi) Conversion of (±)-2-Hexyl acetate after 6 h incubation with *F. proliferatum.*

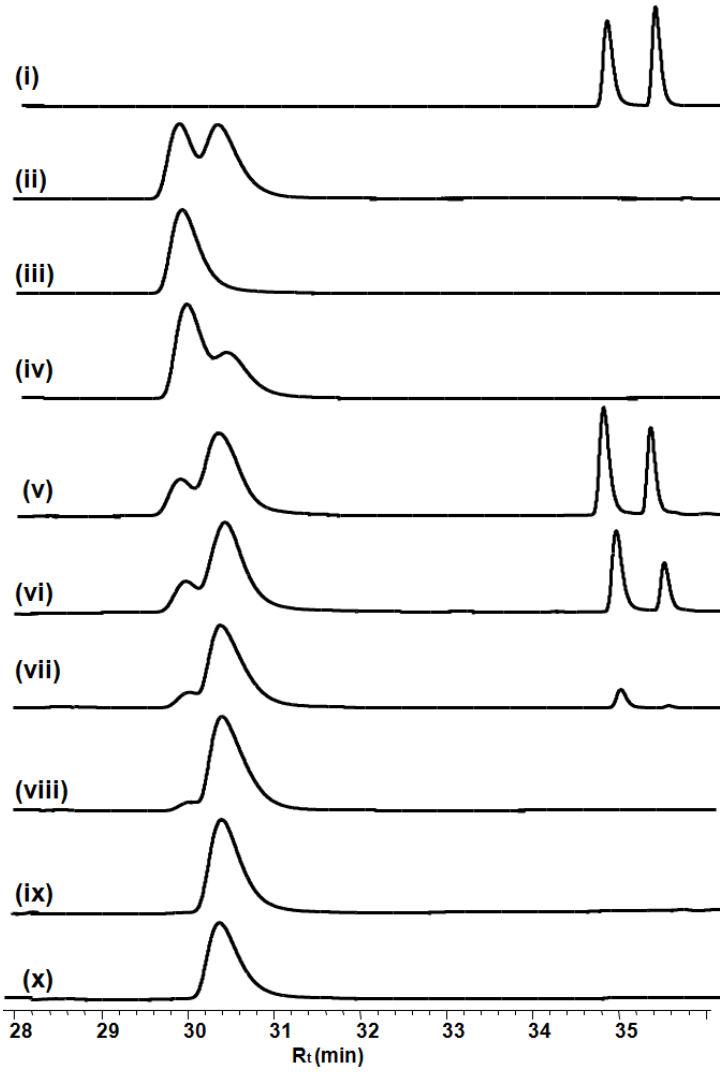


Fig. 4.7 Time Course study for the conversion of (±)-2-Heptyl acetate by *F. proliferatum*

(i) Standard (±)-2-Heptyl acetate (ii) Standard (±)-2-Heptanol (iii) Standard (*S*)-(+)-2-Heptanol (iv) Co injection of (±)-2-Heptanol and (*S*)-(+)-2-Heptanol (v) Conversion of (±)-2-Heptyl acetate after 1 h incubation with *F. proliferatum* (vi) Conversion of (±)-2-Heptyl acetate after 2 h incubation with *F. proliferatum* (vii) Conversion of (±)-2-Heptyl acetate after 3 h incubation with *F .proliferatum* (vii) Conversion of (±)-2-Heptyl acetate after 4 h incubation with *F. proliferatum* (ix) Conversion of (±)-2-Heptyl acetate after 5 h incubation with *F. proliferatum* (x) Conversion of (±)-2-Heptyl acetate after 6 h incubation with *F. proliferatum.*

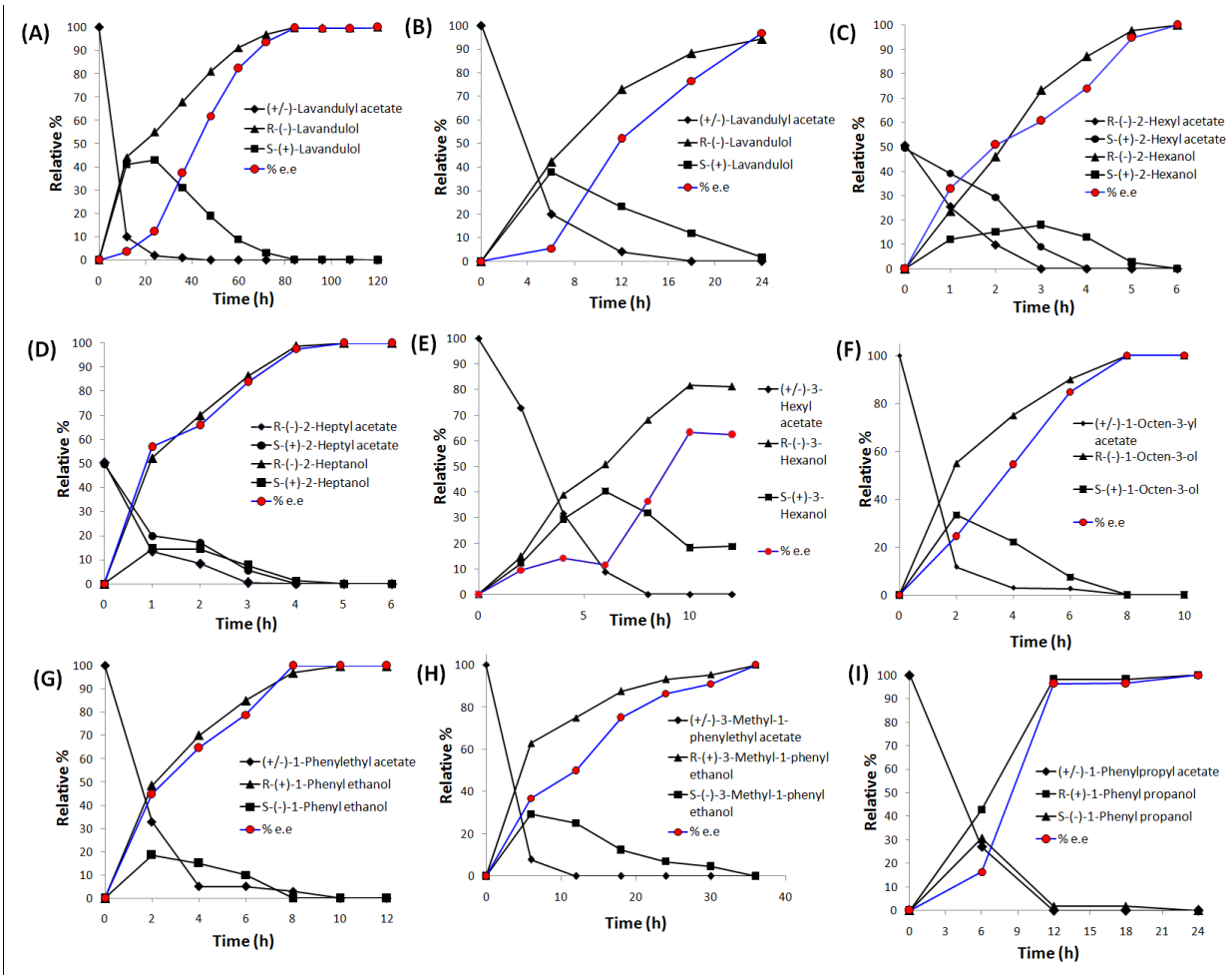


Fig. 4.8 Time-course experiment of kinetic resolution achieved using *F. proliferatum* in graphical representation: (A) whole-cell incubated with (±)-lavandulyl acetate with 0.6 gL^{-1} concentration; **(B-K)** resting-cell incubated with (±)-lavandulyl acetate **(B)**, (±)-2-hexyl acetate **(C)**, (±)-2-heptyl acetate **(D)**, (±)-3-hexyl acetate **(E)**, (±)-1-octen-3-yl acetate **(F)**, (±)-1-phenylethyl acetate **(G)**, (±)-3-methyl-1-phenylethyl acetate **(H),** (±)-1-phenylpropyl acetate **(I)** respectively with 0.1 gL^{-1} substrate concentration.

While esters (**4**) and (**5**) yielded corresponding (*R*)-alcohols (**4a**) and (**5a**) [Fig.4.9 and Fig.4.10] (100 % and 62.5% e.e, 99.9% e.e) respectively after incubation of 12 h and 8 h respectively [Fig.4.8 (E), 4.8 (F)].

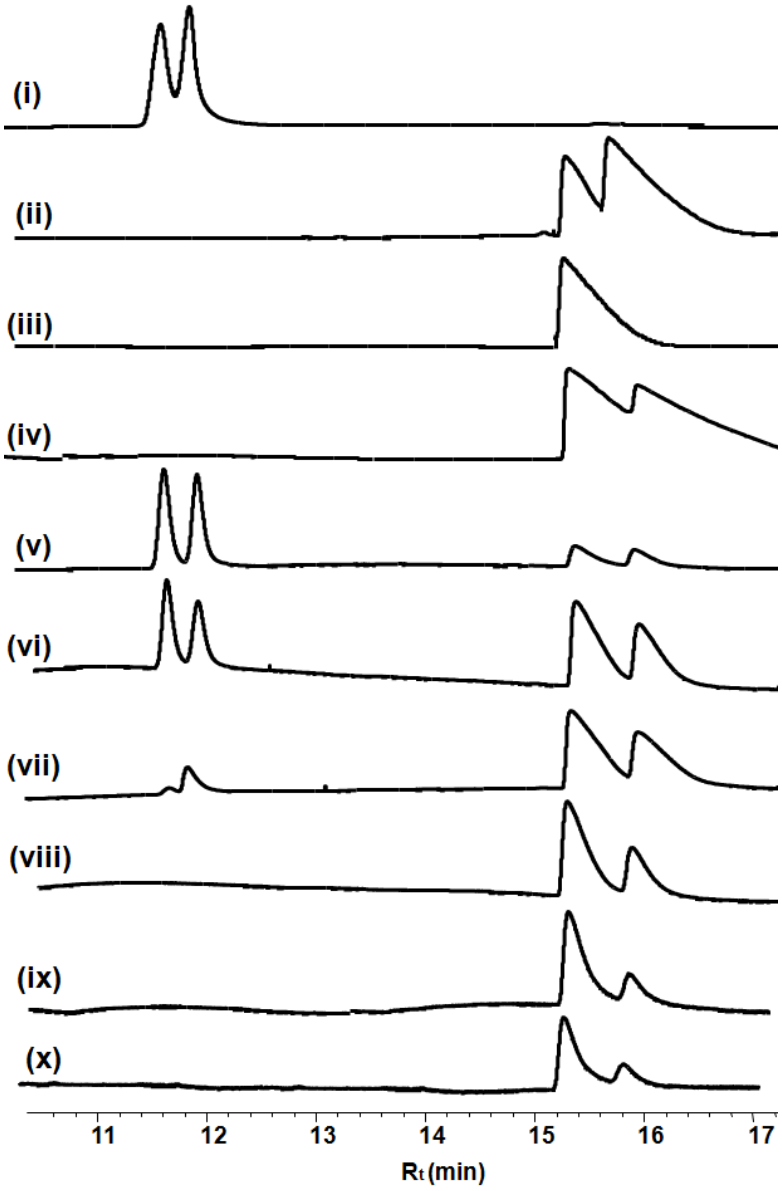


Fig.4.9 Time Course study for the conversion of (±)-3-Hexyl acetate by *F. proliferatum* (i) standard (±)-3-Hexyl acetate (ii) standard (±)-3-Hexanol (iii) standard (*R*)-3-Hexanol (iv) Co injection of (±)-3-Hexanol and (*R*)-3-Hexanol (v) Conversion of (±)-3-Hexyl acetate after 2 h incubation with *F. proliferatum* (vi) Conversion of (±)-3-Hexyl acetate after 4 h incubation with *F. proliferatum* (vii) Conversion of (±)-3-Hexyl acetate after 6 h incubation with *F. proliferatum* (viii) Conversion of (±)-3-Hexyl acetate after 8 h incubation with *F. proliferatum* (ix) Conversion of (±)-3-Hexyl acetate after 10 h incubation with *F. proliferatum* (x) Conversion of (±)-3-Hexyl acetate after 12 h incubation with *F. proliferatum.*

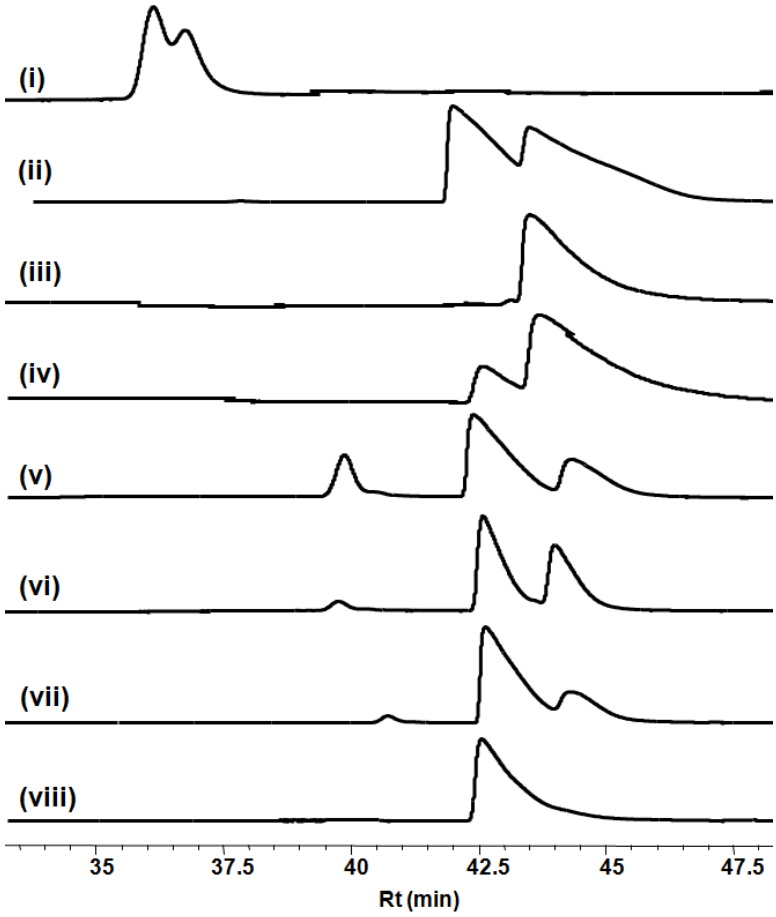


Fig. 4.10 Time Course study for the conversion of (±)-1-octen-3-yl acetate by *F. proliferatum* (i) standard (±)-1-Octen3-yl acetate (ii) standard (±)-1-Octen3-ol (iii) standard (*S*)-1-Octen3-ol (iv) Co-injection of (±)-1-Octen3-ol and (*S*)-1-Octen3-ol (v) Conversion of (±)-1-Octen3-yl acetate after 2 h incubation with *F. proliferatum* (vi) Conversion of (±)-1-Octen3-yl acetate after 4 h incubation with *F. proliferatum* (vii) Conversion of (±)-1-Octen3-yl acetate after 6 h incubation with *F. proliferatum* (viii) Conversion of (±)-1-Octen3-yl acetate after 8 h incubation with *F. proliferatum*

Similar experiments were performed with the racemic aromatic esters [(**6**), (**7**), (**8**) and (**9**)] at the concentration 0.1 gL^{-1}. Time course study indicated that (±)-1-phenylethyl acetate (**6**) and (±)-3-Methyl-1-phenylethyl acetate (**8**) acetate were converted to (*R*)-(+)-1-phenyl ethanol (**6a**) and (*R*)-(+)-3-methyl-1-phenyl ethanol (**8a**) quantitatively (100 %, 99.9 % e.e) after 12 and 36 h [Fig.4.11 and Fig.4.13] of incubation respectively [Fig. 4.8 (G), Fig.4.8 (H)]. While there was no enantioselectivity observed with ester (**7**) using whole cells of *F. proliferatum* (Fig.4.12) may be due to stearic hindrance of 2-C methyl group present on aromatic ring interfering the stereo-selective pocket of enzyme.

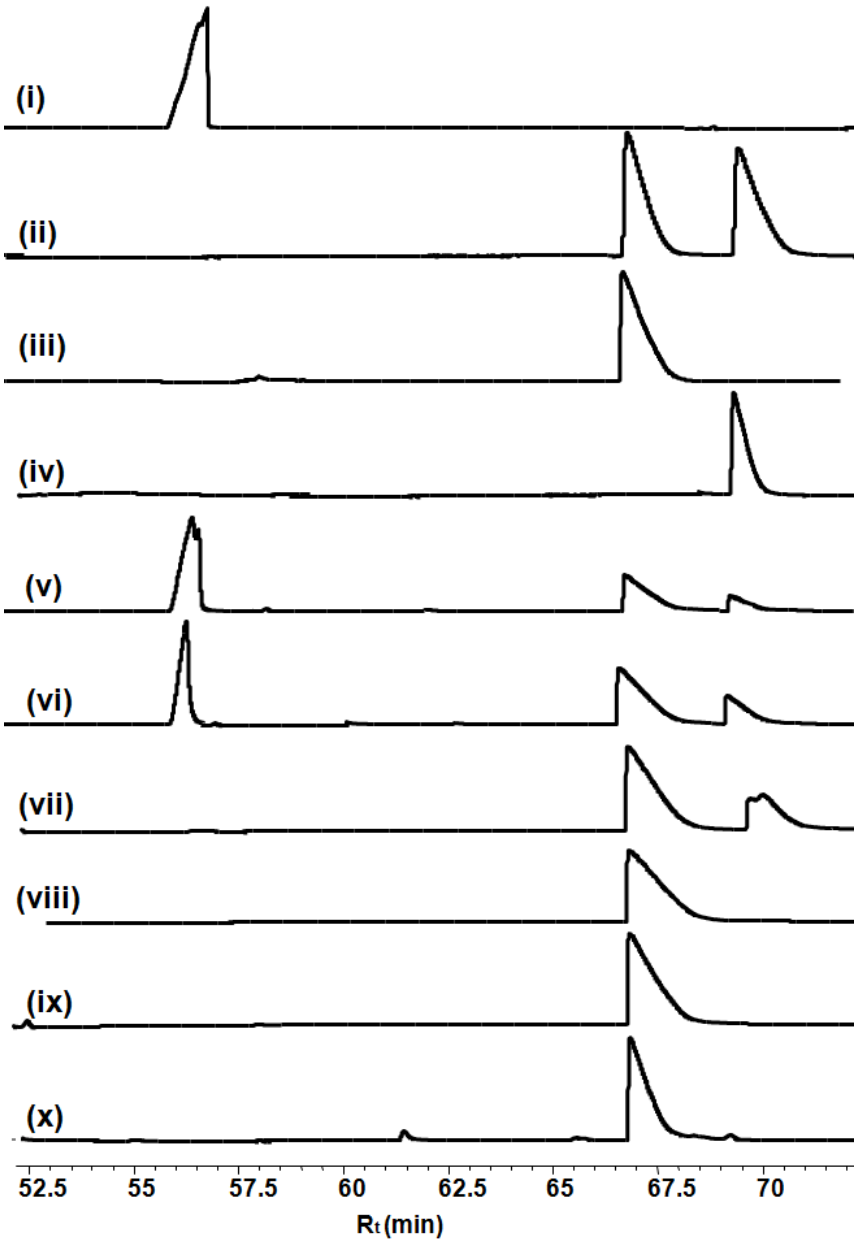


Fig.4.11 Time Course study for the conversion of (±)-1-Phenyl ethyl acetate by *F. proliferatum* (i) standard (±)-1-Phenyl ethyl acetate (ii) standard (±)-1-Phenyl ethanol (iii) standard (*R*)-1-Phenyl ethanol (iv) standard (*S*)-1-Phenyl ethanol (v) Conversion of (±)-1-Phenyl ethyl acetate after 2 h incubation with *F. proliferatum* (vi) Conversion of (±)-1-Phenyl ethyl acetate after 4 h incubation with *F. proliferatum* (vii) Conversion of (±)-1-Phenyl ethyl acetate after 6 h incubation with *F. proliferatum* (viii) Conversion of (±)-1-Phenyl ethyl acetate after 8 h incubation with *F. proliferatum* (ix) Conversion of (±)-1-Phenyl ethyl acetate after 10 h incubation with *F. proliferatum* (x) Conversion of (±)-1-Phenyl ethyl acetate after 12 h incubation with *F. proliferatum*

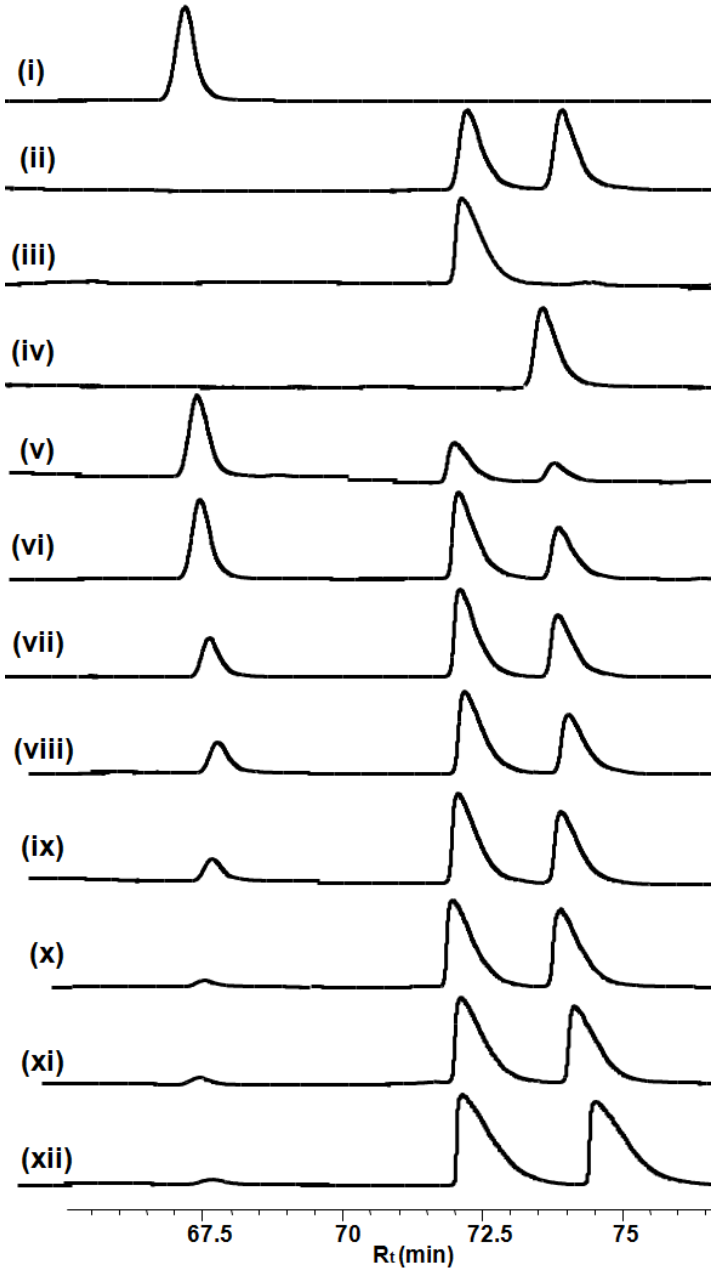


Fig. 4.12 Time Course study for the conversion of (±)-2-methyl-1-Phenyl ethyl acetate by *F. proliferatum* (i) standard (±)-2-methyl-1-phenyl ethyl acetate (ii) standard (±)-2-methyl-1-phenyl ethanol (iii) standard (*R*)-2-methyl-1-Phenyl ethanol (iv) standard (*S*)-2-methyl-1-Phenyl ethanol (v) Conversion of (±)-2-methyl-1-Phenyl ethyl acetate after 6 h incubation with *F. proliferatum* (vi) Conversion of (±)-2-methyl-1-Phenyl ethyl acetate after 12 h incubation with *F. proliferatum* (vii) Conversion of (±)-2-methyl-1-Phenyl ethyl acetate after 18 h incubation with *F. proliferatum* (viii) Conversion of (±)-2-methyl-1-Phenyl ethyl acetate after 24 h incubation with *F. proliferatum* (ix) Conversion of (±)-2-methyl-1-Phenyl ethyl acetate after 30 h incubation with *F. proliferatum* (x) Conversion of (±)-2-methyl-1-Phenyl ethyl acetate after 36 h incubation with *F. proliferatum* (xi) Conversion of (±)-2-methyl-1-Phenyl ethyl acetate after 42 h incubation with *F. proliferatum* (xii) Conversion of (±)-2-methyl-1-Phenyl ethyl acetate after 48 h incubation with *F. proliferatum*

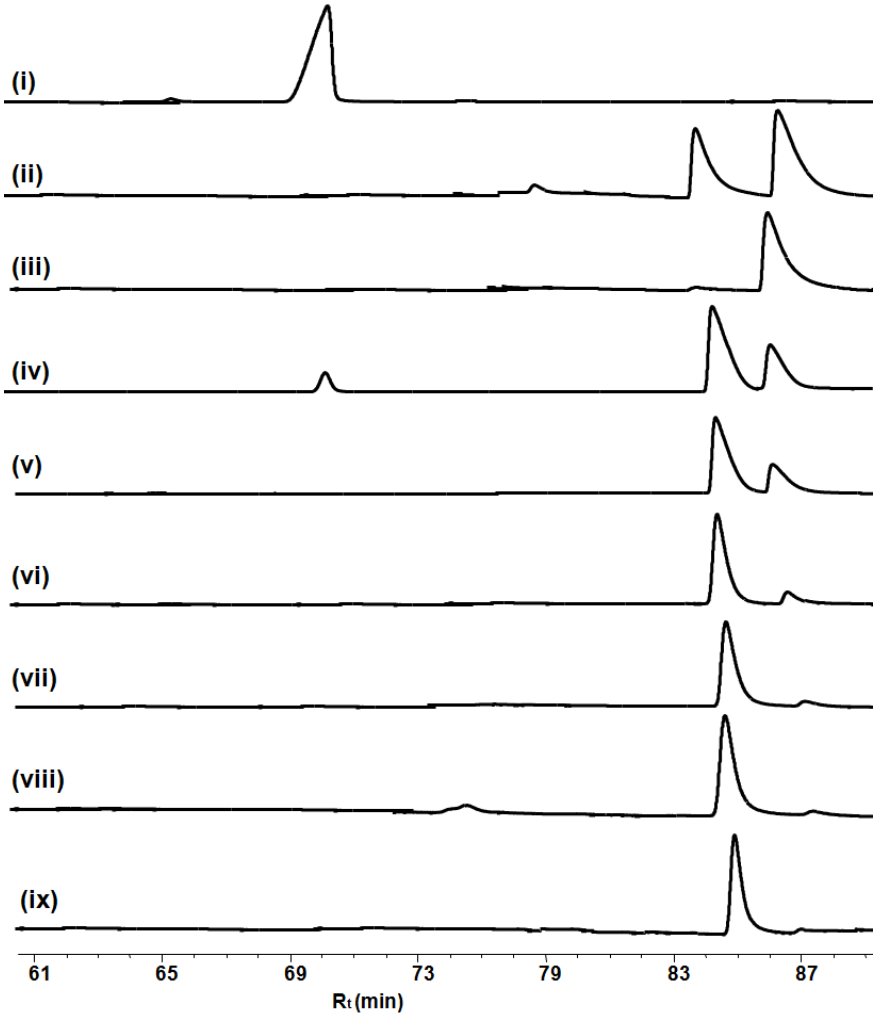


Fig. 4.13 Time Course study for the conversion of (±)-3-methyl-1-Phenyl ethyl acetate by *F. proliferatum* (i) standard (±)-3-methyl-1-Phenyl ethyl acetate (ii) standard (±)-3-methyl-1-Phenyl ethanol (iii) standard (*S*)-3-methyl-1-Phenyl ethanol (iv) Conversion of (±)-3-methyl-1-Phenyl ethyl acetate after 6 h incubation with *F. proliferatum* (v) Conversion of (±)-3-methyl-1-Phenyl ethyl acetate after 12 h incubation with *F. proliferatum* (vi) Conversion of (±)-3-methyl-1-Phenyl ethyl acetate after 18 h incubation with *F. proliferatum* (vii) Conversion of (±)-3-methyl-1-Phenyl ethyl acetate after 24 h incubation with *F. proliferatum* (viii) Conversion of (±)-3-methyl-1-Phenyl ethyl acetate after 30 h incubation with *F. proliferatum* (ix) Conversion of (±)-3-methyl-1-Phenyl ethyl acetate after 36 h incubation with *F. proliferatum*

(±)-1-phenylpropyl acetate (**9**) was converted to (*R*)-(+)-1-phenylpropanol (**9a**) (100%, 98.2% e.e) after 24 h of incubation (Fig.4.14) with the resting cell of *F. proliferatum* [Fig.4.8 (I)].

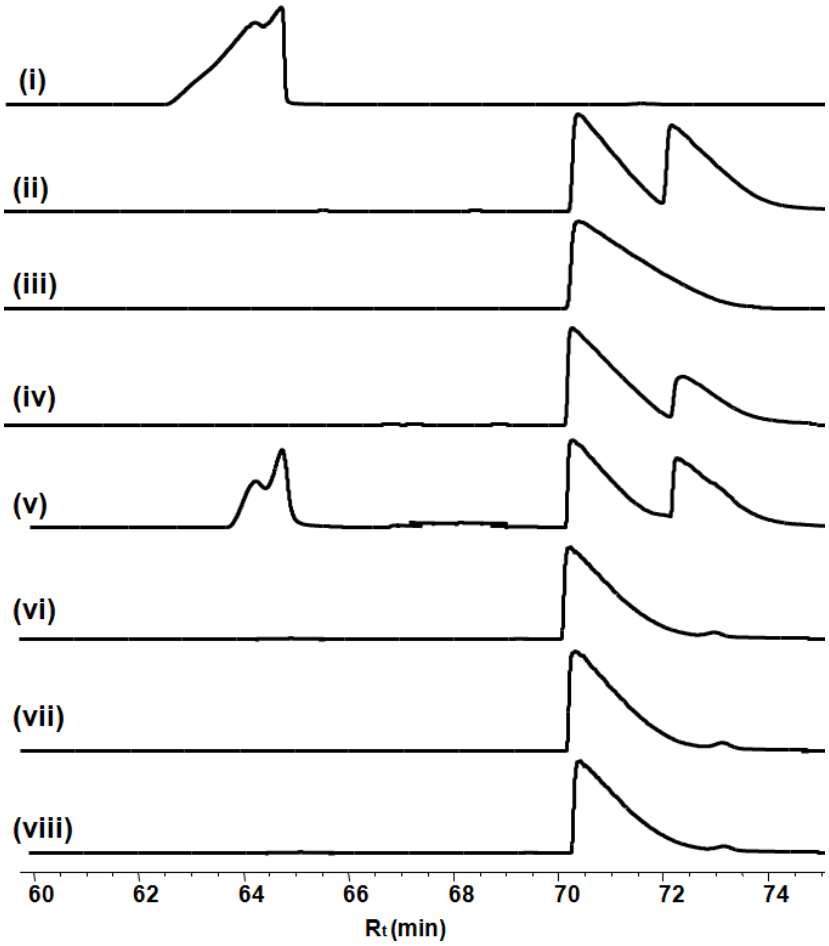


Fig.4.14 Time Course study for the conversion of (±)-1-Phenylpropyl acetate by *F. proliferatum* (i) standard (±)-1-Phenylpropyl acetate (ii) standard (±)-1-Phenylpropanol (iii) standard (*R*)-1-Phenylpropanol (iv) Co injection of (±)-1-Phenylpropanol and (*R*)-1-Phenylpropanol (v) Conversion of (±)-1-Phenylpropyl acetate after 6 h incubation with *F. proliferatum* (vi) Conversion of (±)-1-Phenylpropyl acetate after 12 h incubation with *F. proliferatum* (vii) Conversion of (±)-1-Phenylpropyl acetate after 18 h incubation with *F. proliferatum* (viii) Conversion of (±)-1-Phenylpropyl acetate after 24 h incubation with *F. proliferatum*

Time-course study has indicated during initial period of incubation racemic acetate is converted into individual enantiomeric alcohols at the same rate and after a certain period of incubation the percentage of (*R*)-alcohol starts increasing in the reaction mixture (Fig. 4.8).

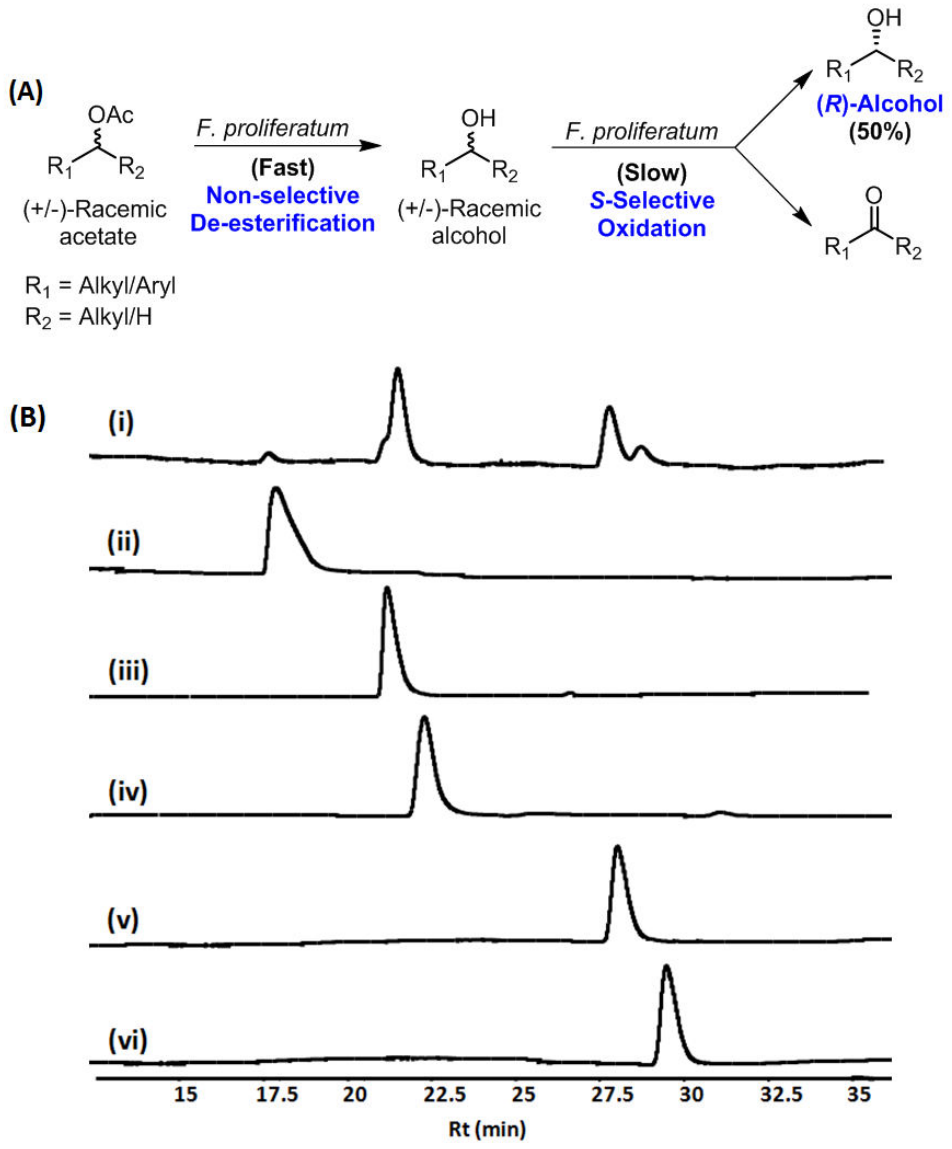
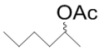
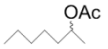
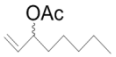


Fig. 4.15 (A) Schematic representation of de-esterification/oxidation one-pot two-step kinetic resolution process of acyclic/aromatic acetates by *F. proliferatum* **(B)** GC-FID chromatograms of **(i)** extracted reaction mixture of (±)-2-hexyl acetate with resting cell of *F. proliferatum* after 2 h of incubation period, **(ii)** standard 2-hexanone **(iii)** standard (*S*)-(+)-2-hexanol, **(iv)** standard (*R*)-(-)-2-hexanol **(v)** standard (*S*)-(+)-2-hexyl acetate and **(vi)** standard (*R*)-(-)-2-hexyl acetate.

Entry	Substrate†	Time (h)	Product	Configuration and % e.e
1	 (**1**)	24	 (**1a**)	*R*, 96.8
2	 (**2**)	6	 (**2a**)	*R*, 99.9
3	 (**3**)	6	 (**3a**)	*R*, 99.9
4	 (**4**)	12	 (**4a**)	*R*, 62.5
5	 (**5**)	8	 (**5a**)	*R*, 99.9
6	 (**6**)	12	 (**6a**)	*R*, 97.5
7	 (**7**)	48	 (**7a**)	0
8	 (**8**)	36	 (**8a**)	*R*, 99.9
9	 (**9**)	24	 (**9a**)	*R*, 98.2

† Substrate concentration: 0.1 gL^{-1} and substrate conversion: 100 % for each entry

Table 4.4 Kinetic resolution of various racemic acyclic/aromatic acetates achieved through resting cell of *F. proliferatum* (Substrate concentration: 0.1 gL^{-1} and substrate conversion: 100% for each entry)

Careful analysis of the GC and GC-MS chromatograms of the extracted fermentation broth revealed the presence of corresponding ketones in case of all nine substrates [(**2**), (**3**), (**4**), (**5**), (**6**), (**7**), (**8**) and (**9**)] during later stage of incubation [Fig. 4.15 (B)]. This result indicated a selective oxidation of (*S*)-alcohol to the corresponding ketone with faster rate in comparison to (*R*)-alcohol, leading to kinetic resolution with high e.e [Fig. 4.15 (A)]. To further substantiate this pathway of kinetic resolution, racemic mixture of alcohols was incubated with *F. proliferatum* which resulted in decrease in the relative abundance of (*S*)-alcohol in the reaction mixture along with the formation of corresponding prochiral ketone (Fig.4.16).

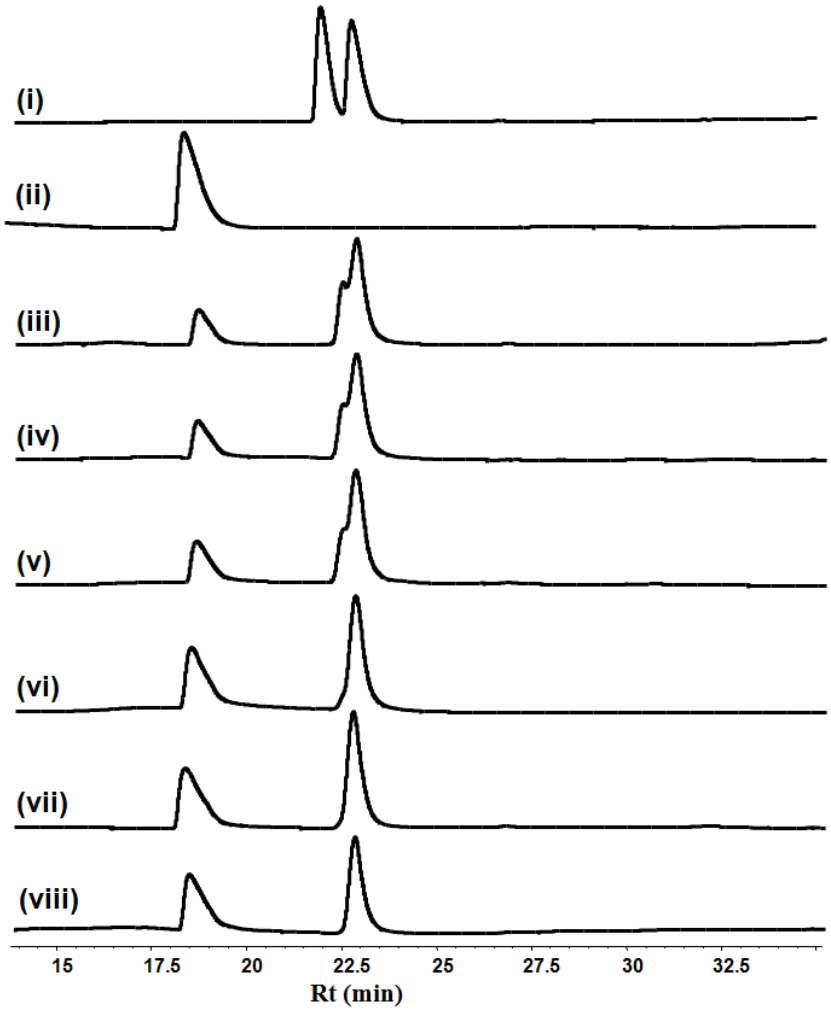


Fig. 4.16 Time Course study for the conversion of (±)-2-Hexanol by *F. proliferatum*
(i) Standard (±)-2-Hexanol (ii) Standard 2-Hexanone, (iii) Conversion of (±)-2-Hexanol after 1 h incubation with *F. proliferatum* (iv) Conversion of (±)-2-Hexanol after 2 h incubation with *F. proliferatum* (v) Conversion of (±)-2-Hexanol after 3 h incubation with *F. proliferatum* (vi) Conversion of (±)-2-Hexanol after 4 h incubation with *F. proliferatum* (vii) Conversion of (±)-2-Hexanol after 5 h incubation with *F. proliferatum* (viii) Conversion of (±)-2-Hexanol after 6 h incubation with *F. proliferatum.*

Similar case studies were performed using individual (*R*) and (*S*)-alcohol with *F. proliferatum* which showed selective oxidation of (*S*)-alcohol to corresponding ketone while (*R*)-alcohol remained as it was in fermentation broth (Fig.4.17 and Fig.4.18).

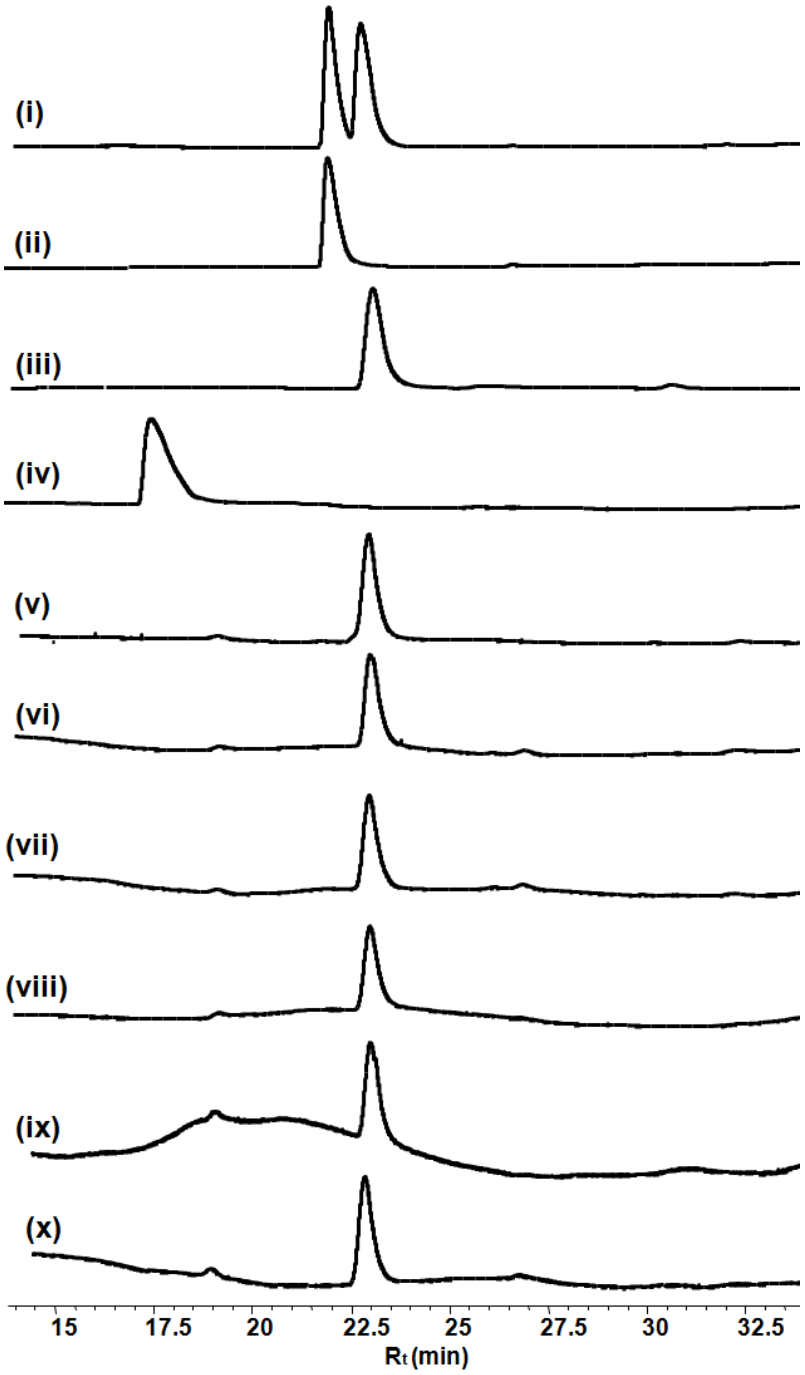


Fig. 4.17 Time Course study for the conversion of (*R*)-(-)-2-Hexanol by *F. proliferatum*
(i) Standard (*R*)-(-)-2-Hexanol (ii) standard (*S*)-(+)-2-hexanol, (iii) standard (*R*)-(-)-2-hexanol (iv) standard 2-Hexanone (v) Conversion of (*R*)-(-)-2-Hexanol after 1 h incubation with *F. proliferatum* (vi) Conversion of (*R*)-(-)-2-Hexanol after 2 h incubation with *F. proliferatum* (vii) Conversion of (*R*)-(-)-2-Hexanol after 3 h incubation with *F. proliferatum* (viii) Conversion of (*R*)-(-)-2-Hexanol after 4 h incubation with *F. proliferatum* (ix) Conversion of (*R*)-(-)-2-Hexanol after 5 h incubation with *F. proliferatum* (x) Conversion of (*R*)-(-)-2-Hexanol after 6 h incubation with *F. proliferatum.*

GC and GC-MS analysis of the assay extract obtained by incubating corresponding prochiral carbonyl compounds did not show presence of corresponding reduced alcohol metabolites. GC and GC-MS analyses of the assay extract obtained by incubating (±)-lavandulyl acetate (**1**) with *F. proliferatum* did show presence of corresponding aldehyde metabolite.

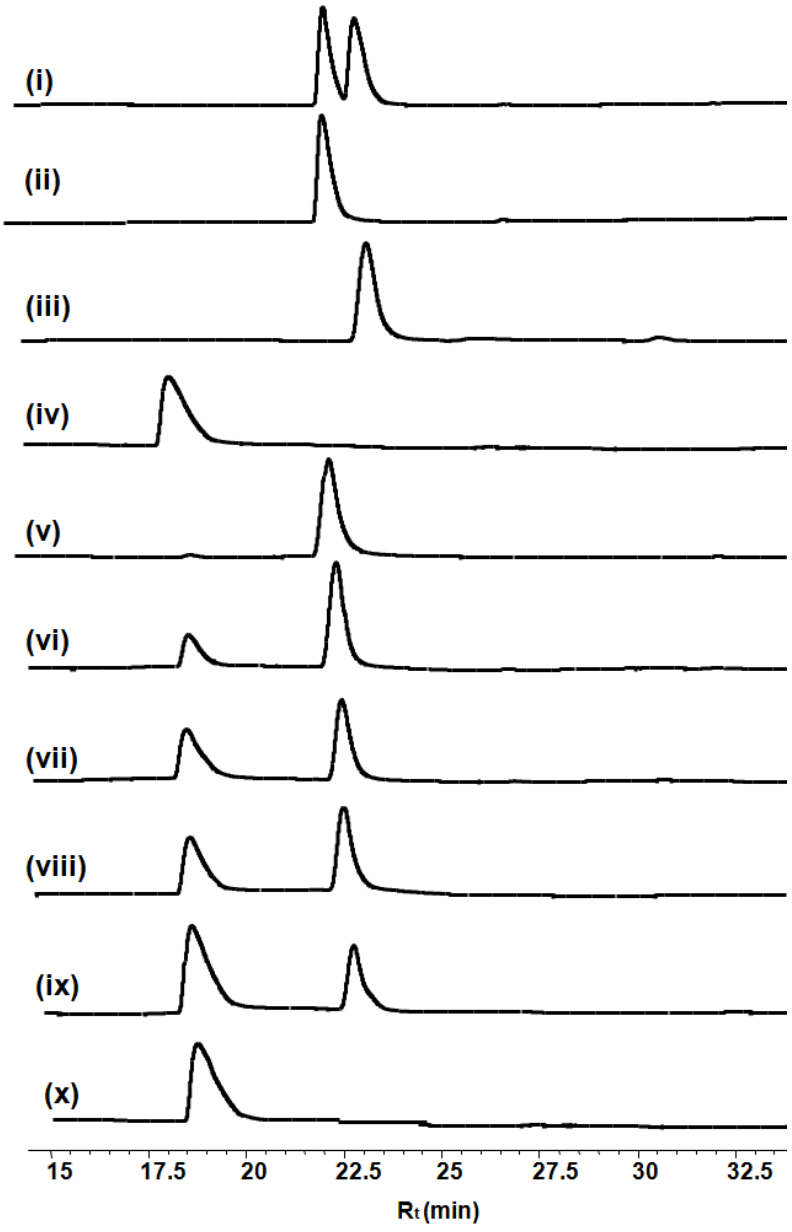


Fig. 4.18 Time Course study for the conversion of (*S*)-(-)-2-Hexanol by *F. proliferatum*
(i) Standard (±)-2-Hexanol (ii) standard (*S*)-(+)-2-hexanol (iii) standard (*R*)-(-)-2-hexanol (iv) standard 2-Hexanone (v) Conversion of (*S*)-(+)-2-Hexanol after 1 h incubation with *F. Proliferatum* (vi) Conversion of (*S*)-(+)-2-Hexanol after 2 h incubation with *F. Proliferatum* (vii) Conversion of (*S*)-(+)-2-Hexanol after 3 h incubation with *F. Proliferatum* (viii) Conversion of (*S*)-(+)-2-Hexanol after 4 h incubation with *F. Proliferatum* (ix) Conversion of

(*S*)-(+)-2-Hexanol after 5 h incubation with *F. proliferatum* (x) Conversion of (*S*)-(+)-2-Hexanol after 6 h incubation with *F. proliferatum*

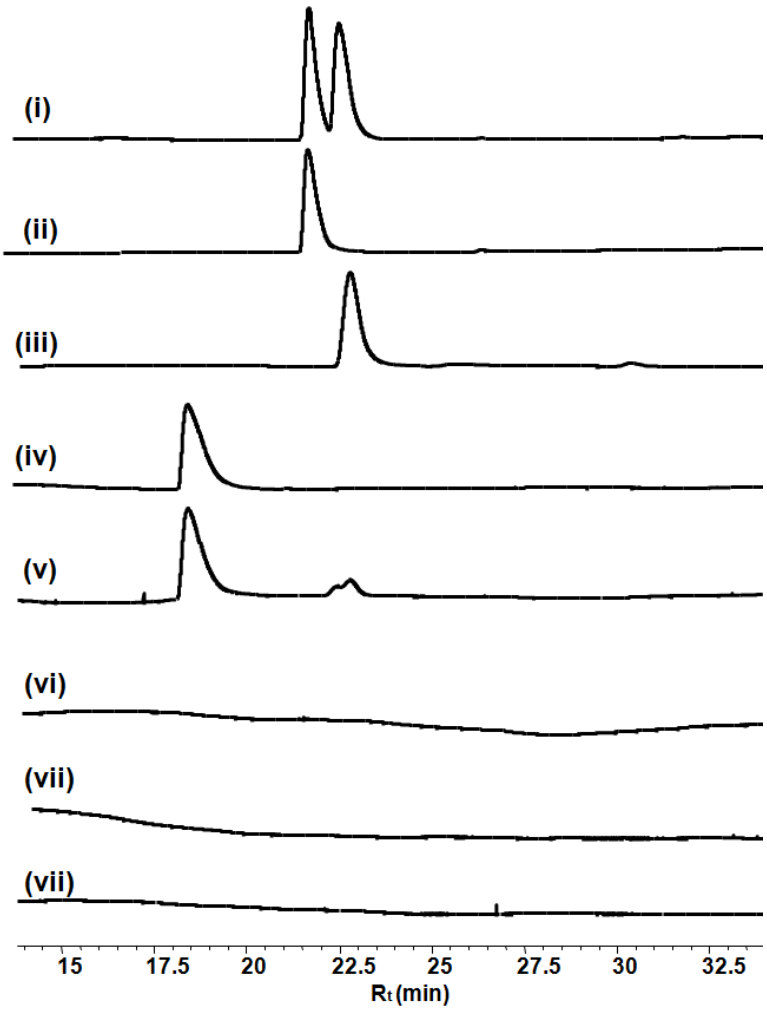


Fig. 4.19 Time Course study for the conversion of 2-Hexanone by *F. proliferatum*
(i) Standard (±)-2-Hexanol (ii) standard (*S*)-(+)-2-hexanol (iii) standard (*R*)-(-)-2-hexanol (iv) Standard 2-Hexanone (v) Conversion of 2-Hexanone after 6 h incubation with *F. Proliferatum*, (vi) Conversion of 2-Hexanone after 12 h incubation with *F. Proliferatum* (vii) Conversion of 2-Hexanone after 18 h incubation with *F. Proliferatum* (viii) Conversion of 2-Hexanone after 24 h incubation with *F. proliferatum*

Scale-up studies were carried out in shake flasks (100 mL media) containing (±)-1-phenylethyl acetate (**6**) as substrate. Substrate concentration studies carried out using varied substrate concentration (0.2, 0.4, 0.8, 1.2, 2.0 and 2.4 gL^{-1}) indicated that the organism could transform (**6**) in to (*R*)-(+)-1-phenylethanol (**6a**) at the optimum substrate concentration of 0.4 gL^{-1}. Increase in the substrate concentration decreased the level of metabolite formation as well as enantiomeric excess (e.e). Time course experiments

indicate that *F. proliferatum* could transform 100 % of (**6**) into (**6a**) at the end of 3 days of incubation period. The fermentation volume was scaled up to 1.0 L with substrate concentration 0.4 gL^{-1}.

Purification of the alcohol fraction from 1.0 L fermentation medium containing 0.4 g racemic acetate (**6**) resulted in isolation of 0.14 g (*R*)-(+)-1-phenylethanol (**6a**) (yield 47 %) with 99.9 % e.e. Purified (*R*)-(+)-1-phenylethanol (**6a**) was further characterised by various analytical techniques such as NMR (^{1}H, ^{13}C and DEPT-135) (Appendix-4), GC-FID/MS (chiral) (Fig. 4.20) and optical rotation (Appendix-4). Thus, the fungal system, *F. proliferatum* can be used for the large-scale production of the corresponding (*R*)-alcohols through kinetic resolution of acyclic and aromatic acetates with fine tuning of the fermentation conditions.

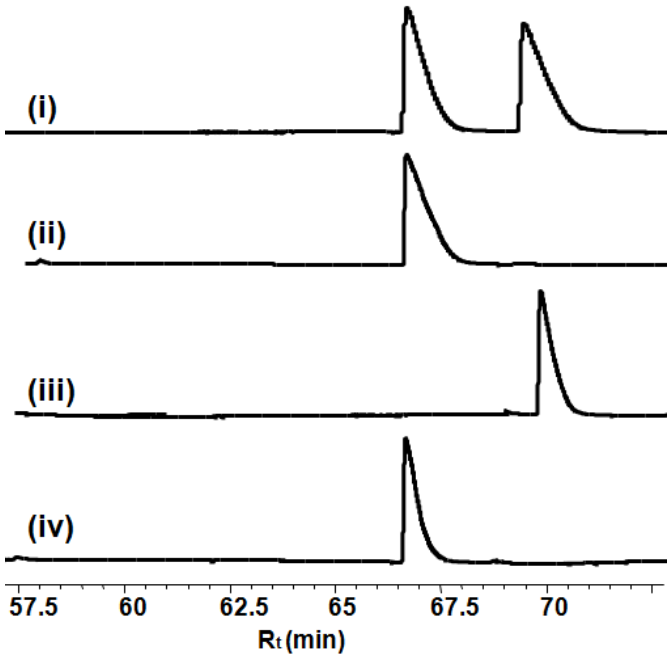


Fig. 4.20 Preparative scale production and purification of (±)-1-Phenyl ethyl acetate using *F. proliferatum* (i) Racemic (±)-1-Phenyl ethanol, (ii) Standard (*R*)-(+)-1-Phenyl ethanol, (iii) Standard (*S*)-(-)-1-Phenyl ethanol, (iv) Large scale purified (*R*)-(+)-1-Phenyl ethanol.

4.3 Conclusion

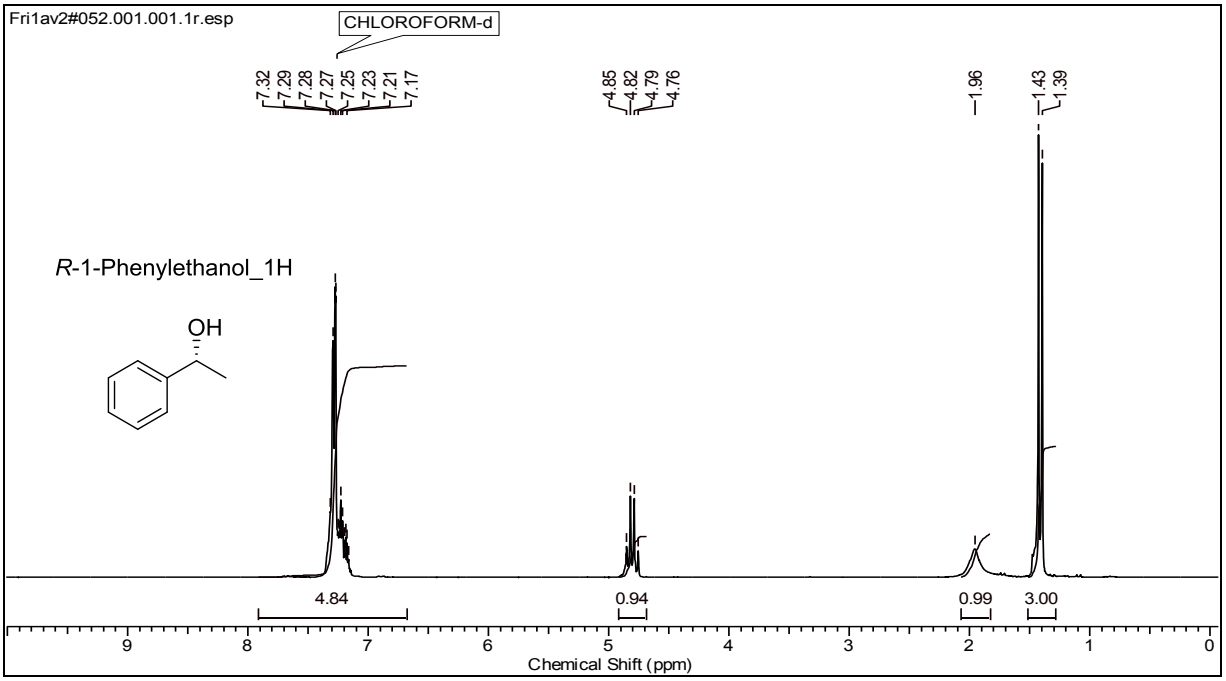
In conclusion, *Candida rugosa* lipase (CRL) catalyzed separation protocol was developed for the separation of structurally isomeric triterpene mixture of α and β-amyrin. CRL was able to carry out acetylation of β-amyrin into β-amyrin acetate in a selective and efficient manner at lower concentration of vinyl acetate in the early stage of incubation period (24 h). On the other hand, α-amyrin was obtained by incubating CRL with α and β-amyrin mixture for prolonged incubation period (6 days). n-hexane and vinyl acetate were found to be better organic solvent and acyl donor, respectively, for the CRL mediated trans-esterification. This separation procedure might be useful for the large-scale separation of α and β-amyrin for their detailed biological and commercial applications.

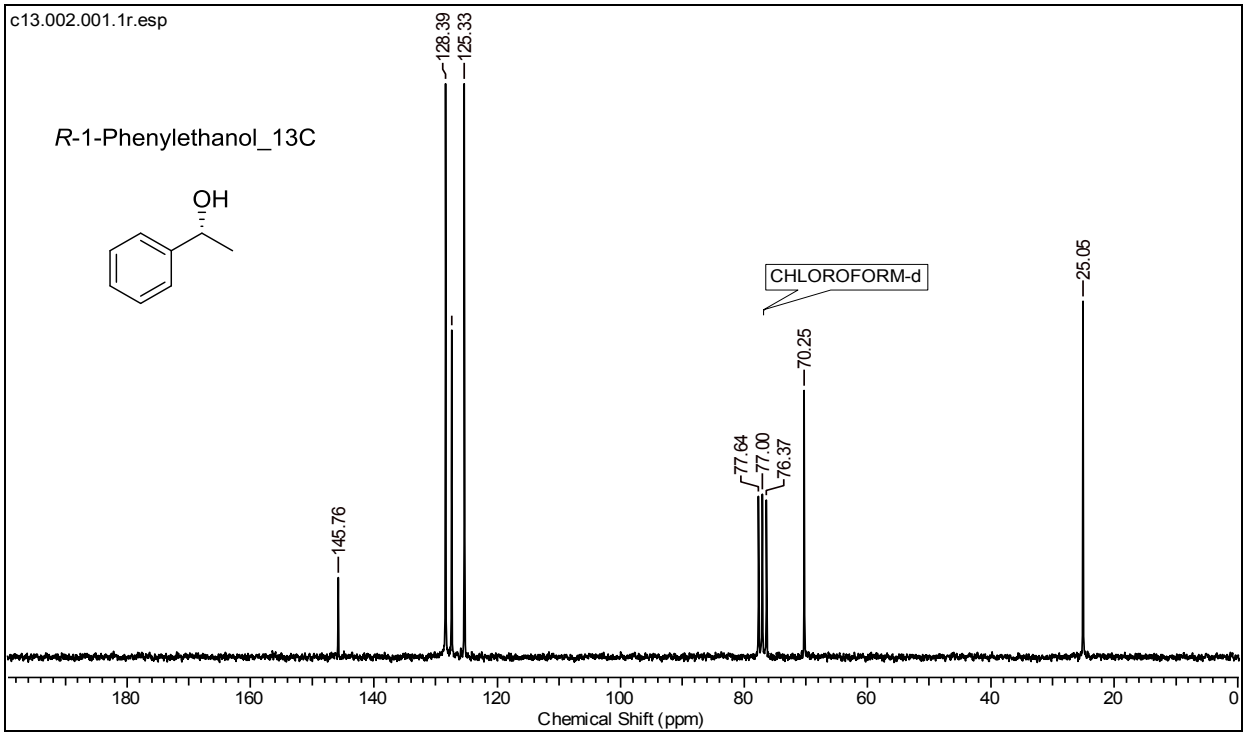
An efficient one-pot two step de-esterification /oxidation biocatalytic technique was developed for the kinetic resolution of acyclic and aromatic acetates by using the whole-cells of *Fusarium proliferatum*. The fungal system could able to carry out the kinetic resolution of four racemic acyclic esters [(±)-2-hexyl acetate (**2**) , (±)-2-heptyl acetate (**3**) and (±)-1-octen-3-yl acetate (**5**)] and four aromatic esters [(±)-1-phenylethyl acetate (**6**), (±)-3-methyl-1-phenylethyl acetate (**8**) and (±)-1-phenylpropyl acetate (**9**)] into corresponding (*R*)-alcohols in an efficient manner with high enantiomeric excess. Enantioselective hydrolysis of (±)-1-phenylethyl acetate (**6**) to (*R*)-(+)-1-phenylethanol (**6a**) was successfully scaled up to preparative scale, which indicated great potential of the developed process to be applied in large-scale preparation of enantiopure (*R*)-alcohols.

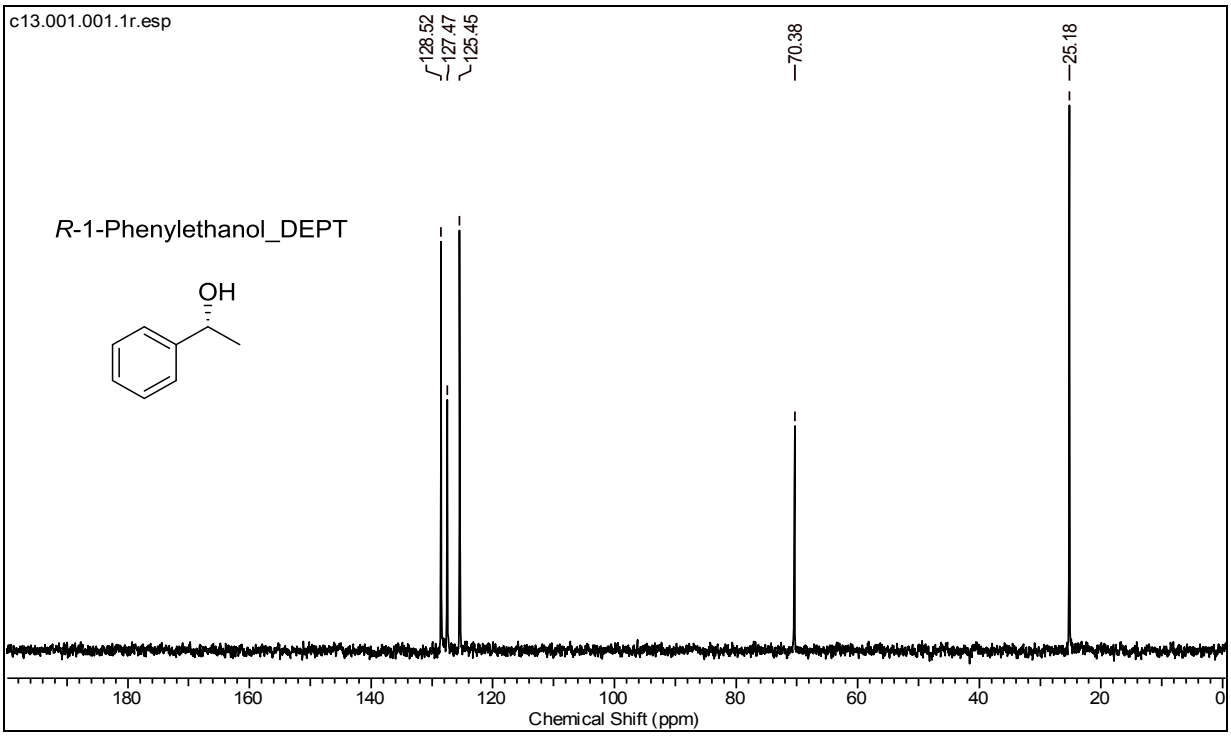
Appendix 4:

1) Analytical data of *R*-(+)-1-Phenyl ethanol:-

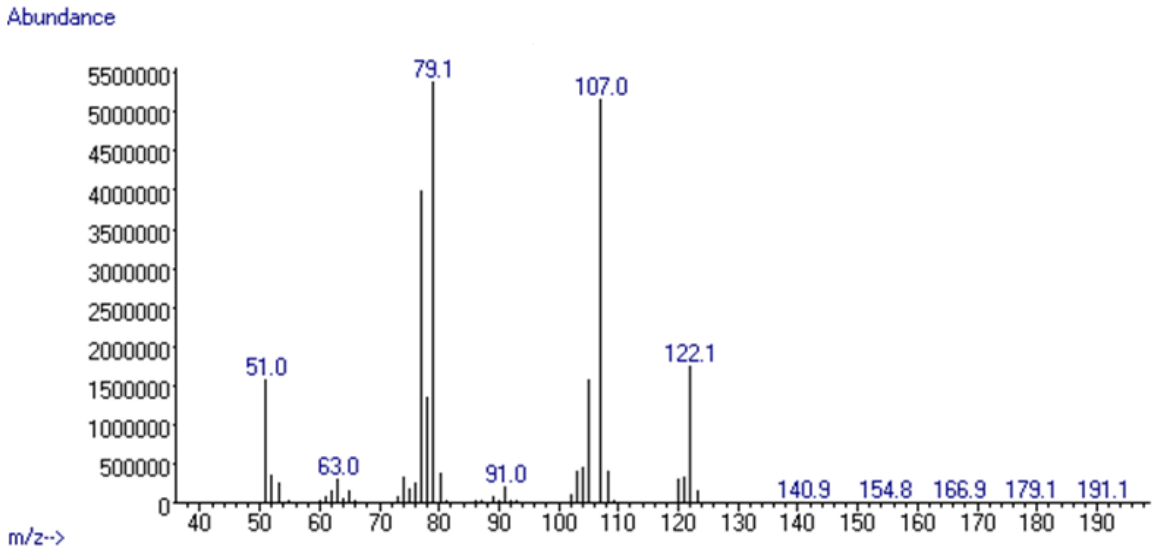
1) 1H, 13C, DEPT NMR







2) Mass fragmentation of *R*-(+)-1-Phenyl ethanol



3) Optical rotation

1) (*R*)-(+)-1-Phenyl ethanol

Specific optical rotation: $[\alpha]^{25}D$ =+36.2.

4.4 References

(1) Dzubak, P.; Hajduch, M.; Vydra, D.; Hustova, A.; Kvasnica, M.; Biedermann, D.; Markova, L.; Urban, M.; Sarek, J. *Nat. Prod. Rep.* **2006**, *23*, 394-411.

(2) Ikeda, Y.; Murakami, A.; Ohigashi, H. *Mol. Nutr. Food Res.* **2008**, *52*, 26-42.

(3) Kuo, R. Y.; Qian, K. D.; Morris-Natschke, S. L.; Lee, K. H. *Nat. Prod. Rep.* **2009**, *26*, 1321-1344.

(4) Petronelli, A.; Pannitteri, G.; Testa, U. *Anti-Cancer Drugs* **2009**, *20*, 880-892.

(5) Roy, A.; Saraf, S. *Biol. Pharm. Bull.* **2006**, *29*, 191-201.

(6) Salvador, J. A. R.; Moreira, V. M.; Goncalves, B. M. F.; Leal, A. S.; Jing, Y. K. *Nat. Prod. Rep.* **2012**, *29*, 1463-1479.

(7) Sheng, H. M.; Sun, H. B. *Nat. Prod. Rep.* **2011**, *28*, 543-593.

(8) Melo, C. M.; Morais, T. C.; Tome, A. R.; Brito, G. A. C.; Chaves, M. H.; Rao, V. S.; Santos, F. A. *Inflamm. Res.* **2011**, *60*, 673-681.

(9) Otuki, M. F.; Vieira-Lima, F.; Malhairos, K.; Yunes, R. A.; Calixto, J. B. *Eur. J. Pharmacol.* **2005**, *507*, 253-259.

(10) Soldi, C.; Pizzolatti, M. G.; Luiz, A. P.; Marcon, R.; Meotti, F. C.; Mioto, L. A.; Santos, A. R. S. *Bioorgan. Med. Chem.* **2008**, *16*, 3377-3386.

(11) Chenniappan, K.; Kadarkari, M. *Parasitol. Res.* **2012**, *110*, 2117-2124.

(12) Kannan, S.; Vijayakumar, B.; Sureshkumar, C.; Mohankumar, R.; Narasimhan, S. *Asian J. Chem.* **2013**, *25*, 1167-1168.

(13) Aragao, G. F.; Carneiro, L. M. V.; Junior, A. P. F.; Vieira, L. C.; Bandeira, P. N.; Lemos, T. L. G.; Viana, G. S. D. *Pharmacol. Biochem. Be.* **2006**, *85*, 827-834.

(14) Kweifiookai, G.; Demunk, F.; Rumble, B. A.; Macrides, T. A.; Cropley, M. *Res. Commun. Mol. Path.* **1994**, *85*, 45-55.

(15) Oliveira, F. A.; Vieira-Junior, G. M.; Chaves, M. H.; Almeida, F. R. C.; Santos, K. A.;Martins, F. S.; Silva, R. M.; Santos, F. A.; Rao, V. S. N. *Planta Med.* **2004**, *70*, 780-782.

(16) Narender, T.; Khaliq, T.; Singh, A. B.; Joshi, M. D.; Mishra, P.; Chaturvedi, J. P.; Srivastava, A. K.; Maurya, R.; Agarwal, S. C. *Eur. J. Med. Chem.* **2009**, *44*, 1215-1222.

(17) Diaz-Ruiz, G.; Hernandez-Vazquez, L.; Luna, H.; Wacher-Rodarte, M. D.; Navarro-Ocana, A. *Molecules* **2012**, *17*, 12603-12611.

(18) Johann, S.; Soldi, C.; Lyon, J. P.; Pizzolatti, M. G.; Resende, M. A. *Lett. Appl.*

Microbiol. **2007**, *45*, 148-153.

(19) Barros, F. W. A.; Bandeira, P. N.; Lima, D. J. B.; Meira, A. S.; de Farias, S. S.; Albuquerque, M.; dos Santos, H. S.; Lemos, T. L. G.; de Morais, M. O.; Costa-Lotufo, L.V.; Pessoa, C. D. *Bioorgan. Med. Chem.* **2011**, *19*, 1268-1276.

(20) Chicca, A.; Marazzi, J.; Gertsch, J. *Brit. J. Pharmacol.* **2012**, *167*, 1596-1608.

(21) Lima-Junior, R. C. P.; Sousa, D. I. M.; Brito, G. A. C.; Cunha, G. M.; Chaves, M. H.;Rao, V. S. N.; Santos, F. A. *Inflamm. Res.* **2007**, *56*, 487-494.

(22) Marcon, R.; Luiz, A. P.; Werner, M. F. D.; Freitas, C. S.; Baggio, C. H.; do Nascimento,F. P.; Soldi, C.; Pizzolatti, M. G.; Santos, A. R. S. *Brain Res.* **2009**, *1295*, 76-88.

(23) Melo, C. M.; Carvalho, K.; Neves, L.; Morais, T. C.; Rao, V. S.; Santos, F. A.; Brito, G.A. D.; Chaves, M. H. *World J. Gastroentero.* **2010**, *16*, 4272-4280.

(24) Vitor, C. E.; Figueiredo, C. P.; Hara, D. B.; Bento, A. F.; Mazzuco, T. L.; Calixto, J.B. *Brit. J. Pharmacol.* **2009**, *157*, 1034-1044.

(25) Dias, M. O.; Hamerski, L.; Pinto, A. C. *Quim. Nova* **2011**, *34*, 704-U161.

(26) Ghanem, A.; Aboul-Enein, H. Y. *Chirality* **2005**, *17*, 1-15.

(27) Theil, F. *Chem. Rev.* **1995**, *95*, 2203-2227.

(28) Tanikawa, S.; Suzuki, K.; Ono, M.; Akita, H. *J. Mol. Catal. B-Enzym* **2004**, *31*, 103-110.

(29) Breuer, M.; Ditrich, K.; Habicher, T.; Hauer, B.; Kesseler, M.; Sturmer, R.; Zelinski,T.*Angew. Chem. Int. Edit.* **2004**, *43*, 788-824.

(30) Schmid, A.; Dordick, J. S.; Hauer, B.; Kiener, A.; Wubbolts, M.; Witholt, B. *Nature,* **2001**, *409*, 258-268.

(31) Tewari, Y. B.; Vanderah, D. J.; Rozzell, J. D. *J. Mol. Catal. B-Enzym.* **2003**, *21*, 123-131.

(32) Patel, J. M. *J. Mol. Catal. B-Enzym.* **2009**, *61*, 123-128.

(33) Patel, R. N. *Curr. Opin. Biotechnol.* **2001**, *12*, 587-604.

(34) de Alfonso, I.; Hernandez, E.; Velazquez, Y.; Navarro, I.; Primo, J. *J. Agric. Food Chem.* **2012**, *60*, 11959-11964.

(35) Furstner, A.; Albert, M.; Mlynarski, J.; Matheu, M.; DeClercq, E. *J. Am. Chem. Soc* **2003**, *125*, 13132-13142.

(36) Kondo, K.; Kan, K.; Tanada, Y.; Bando, M.; Shinohara, T.; Kurimura, M.; Ogawa, H.; Nakamura, S.; Hirano, T.; Yamamura, Y.; Kido, M.; Mori, T.; Tominaga, M. *J.*

Med Chem. **2002**, *45*, 3805-3808.

(37) Cross, H.; Marriott, R.; Grogan, G. *Biotechnol. Lett.* **2004**, *26*, 457-460.

(38) de los Rios, A. P.; van Rantwijk, F.; Sheldon, R. A. *Green Chem.* **2012**, *14*, 1584-1588.

(39) Ferreira, H. V.; Rocha, L. C.; Severino, R. P.; Porto, A. L. M. *Molecules* **2012**, *17*, 8955-8967.

(40) Frings, K.; Koch, M.; Hartmeier, W. *Enzyme Microb. Tech.* **1999**, *25*, 303-309.

(41) Wang, Y. H.; Wang, R.; Li, Q. S.; Zhang, Z. M.; Feng, Y. *J. Mol. Catal. B-Enzym.* **2009**,*56*, 142-145.

(42) Zada, A.; Harel, M. *Tetrahedron-Asymmetr.* **2004**, *15*, 2339-2343.

(43) Zada, A.; Dunkelblum, E. *Tetrahedron-Asymmetr.* **2006**, *17*, 230-233.

(44) Yadav, K. N. S.; Adsul, M. G.; Bastawde, K. B.; Jadhav, D. D.; Thulasiram, H. V.; Gokhale, D. V. *Bioresource Technol.* **2011**, *102*, 10663-10670.

(45) Haldar, S.; Kolet, S. P.; Thulasiram, H. V. *Green Chem.* **2013**, *15*, 1311-1317.

(46) Haldar, S.; Mulani, F. A.; Aarthy, T.; Thulasiram, H. V. *J. Org. Chem.* **2015**, *80*, 6490-6495.

List of Publications and Patents

Publications

1) **Dipesh D. Jadhav**, Harshal S. Patil, Patil S. Chaya and H. V. Thulasiram*.Fungal mediated kinetic resolution of racemic acetates to (*R)*-Alcohols by *Fusarium proliferatum.* ***Tetrahedron Letters,*** volume 57, Issue 41, 12 October 2016, Pages 4563-4567.

2) Swati P. Kolet, **Dipesh D. Jadhav**, Balaraman Priyadarshini, Bhagyashree N. Swarge, Hirekodathakallu V. Thulasiram*. Fungi mediated production and practical purification of (*R*)-3-quinuclidinol, ***Tetrahedron Letters***, Volume 55, Issue 43, 22 October 2014, Pages 5911–5914.

3) Saikat Haldar, Balaji Kale, **Dipesh D. Jadhav**, H.V. Thulasiram*.Lipase mediated seperation of triterpene isomers, α and β-amyrins, ***Tetrahedron Letters,*** Volume 55, Issue 19, 7 May 2014, Pages 3122–3125.

4) K.N. Sathish Yadav , M.G. Adsul, K.B. Bastawde , **Dipesh D. Jadhav** , H.V. Thulasiram, D.V. Gokhale*.Differential induction, purification and characterization of cold active lipase from *Yarrowia lipolytica NCIM 3639****, Bioresource Technology***, Volume 102, Issue 22, November 2011, Pages 10663-10670.

5) **Dipesh D. Jadhav**, Patil S. Chaya, Rakesh S. Joshi and H.V. Thulasiram*. Cloning, expression and biochemical characterization of an alkaline stable (*R*)-*e*nantioselective esterase isolated from *Pseudomonas pseudoalcaligenes* NCIM 2864. (***Manuscript under preparation***).

6) **Dipesh D. Jadhav**, Ajit Singh, Chaya Patil, Rakesh Sharma*, H.V. Thulasiram*. Cloning, expression and utilisation of novel enantioselective esterases from metagenomic libraries**.** ***(Manuscript under preparation)***

7) **Dipesh D. Jadhav**, Avinash Pandreka, Chaya Patil, H.V.Thulasiram*. Transcriptome analysis and isolation of novel lipases from *Yarrowia lipolytica* NCIM 3639**.** ***(Manuscript under preparation)***

Patent

1) **Dipesh D. Jadhav**, Niloferjahan K. Siddiqui, Swati P.Kolet, H.V.Thulasiram*. A process of chiral resolution of cyclic and acyclic acetates to enantiomerically pure (*R*)-alcohols. **Publication number: -** WO2015063796 A4.

List of symposium and conferences

1) Gave **Oral Presentation** of poster entitled -"Chiral resolution of important flavour and Fragrance compounds using Lipases." in the Symposium "**Biodesign India 1.0**" held by University of Kerala, Trivandrum between October 7-9th 2010.
2) **Presented poster** entitled "Chiral resolution of important flavor and Fragrance compounds using Lipases." On National science day held in CSIR-NCL on 26-28th February 2011.
3) **Presented poster** in International symposium on "**Indo-Mexico workshop on Biotechnology: Beyond borders**" organized by CSIR-NCL, Pune and CINVESTAV Mexico between 7-9th October, 2013.
4) **Presented poster** entitled "One pot tandem biocatalytic resolution of esters to enantiopure *R*-alcohol catalysed by versatile fungus *Fusarium proliferatum* NCIM 1105"in "**International symposium on Bioorganic Chemistry" (ISBOC 2015)** held in IISER, Pune between 11-15th January 2015.
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Curriculum Vitae

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PROFILE SYNOPSIS

- **PhD** (**Biotechnology**) with **over 6.5 years** of research experience in the field of Molecular Biology, heterologous protein expression and purification, analytical techniques (HPLC, GC-FID, GC-MS, GC-HRMS and LC-MS) and synthetic organic chemistry.
- Strong theoretical knowledge, technical expertise with independent handling and monitoring of the instruments- **GC-FID**, **GC-MS**, **GC-HRMS**, **HPLC and LC-MS**.
- Expertise in the field of Biotransformation/Biocatalysis, synthetic organic chemistry.
- Strong analytical and problem solving skills with excellent time management, people management, interpersonal and self learning skills.
- Possesses excellent written and verbal communication skills with interest in playing instruments, sports and reading about current affairs.

EDUCATION

PhD (Biotechnology) January 2010-Present

CSIR-National Chemical Laboratory Pune, India

Dissertation – Isolation, characterization and utilization of novel lipases.

M. Sc – Biophysics (67.9 %) June 2007-March 2009

Department of Biophysics University of Mumbai, Mumbai

Thesis - Understanding development and isolation of the microbial flora from the flower compost and to study their interaction with nanoparticles.

B. Sc – Biotechnology (65.5 %) June 2004-March 2007

Ramniranjan Jhunjhunwala College University of Mumbai,

AWARDS AND ACHIEVEMENTS

- **Teaching fellowship award-** CSIR-NET (LS) June 2009
- **Doctoral fellowship award-** ICMR-JRF fellowship July 2009
- Stood **Third (3rd)** in Mumbai University M. Sc (Biophysics) exam 2009
- Awarded Mumbai University fellowship for academic excellence during post graduation**.** 2007

RELATED RESEARCH EXPERIENCE

- **Research Assistant –** CSIR-IGIB, New Delhi, Jan. 2015 – Feb.2015

  Trained on Isolation of metagenomic DNA and preparing fosmid/ pUC based libraries for identifying novel biocatalyst under the guidance of Dr. Rakesh Sharma.

- **Research Assistant**-Bhabha Atomic Research Centre, Mumbai Jan 2008

  Trained on preparation of liposome from synthetic fatty acid and their characterisation using fluroscenes spectrometry technique under guidance of Dr.B.N.Pandey. These liposome's were then used for targeted DNA delivery in bacteria and animals.

SKILLS

➢ Wet lab/Experimental skills:

- Genomic and Metagenomic DNA isolation.
- Total RNA isolation, Transcriptome annotation and analysis.
- Molecular cloning and vector design, PCR.
- **Biocatalysis**: Chiral/Kinetic resolution of prochiral drugs or their intermediates using whole cells and enzymes like Lipases, Alcohol oxidoreductases.

- **Whole cell and enzyme mediated biotransformation**

  Use of Biotransformation technique to increase bioavailability through processes like hydroxylation.
- **Upstream processing:** Whole cell fermentation, heterologous protein expression in *S. cerevisiae, E. coli.*
- **Downstream processing:** Protein purification (Ultra filtration, Ion exchange chromatography, Ni-NTA agarose chromatography, Affinity chromatography, Gel filtration), Metabolite extraction.
- Large scale fermenter level production and optimisation.
- **Synthetic organic chemistry:**

  Synthesis of prochiral molecules like Flavour fragrance compounds (Acyclic alcohols & acetates, acyclic/cyclic/sesquiterpenoids and their derivatives) and API's (1-Phenylethanol and their derivatives).
- Silica gel chromatography/Compound purification.

➢ Analytical skills:-

- **HPLC** (Analytical and semi preparative): (Waters).
- **GC-FID/Chiral GC**: (Agilent 7890 A).
- **GC-MS**: (Agilent 7890 A/5975C MSD).
- **GC-HRMS**: (Agilent 7200 Accurate Mass Q-TOF).
- **FPLC**: (*GE* AKTA AVANT 25).
- **1 D NMR** (C13, H1, DEPT), Mass fragmentation analysis and structure prediction.

➢ Management skills

- Has trained 3 Masters Project trainees and 4 project assistants during the tenure of Ph.D.
- Managed, handled and maintained **HPLC**, **GC-FID**, **GC-MS** (Single quad.) and **GC-HRMS** (Q-TOF) of the lab single handedly.
- Managed finance committee for consecutive 2 years (2012 & 2013) for the cultural event "***R.S.M"*** held in National Chemical Laboratory, Pune, India.

➢ **IT skills:-**

- MS-Office.
- Use of search engines and academic databases such as SciFinder, Web of Science, Google scholar for advanced research functions.
- Use of bioinformatics tools (NCBI blast, Expasy, ClustalW etc.) and softwares such as VMD, spdbv.

PUBLICATIONS AND PATENTS

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