

**NOVEL CUTINASES FROM *THERMOBIFIDA FUSCA*:
PRODUCTION, CHARACTERIZATION AND APPLICATIONS**

A THESIS

submitted by

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of

DOCTOR OF PHILOSOPHY



**DEPARTMENT OF BIOTECHNOLOGY
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Dedicated to my wife and Parents



INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI

DEPARTMENT OF BIOTECHNOLOGY

STATEMENT

I do hereby declare that the matter embodied in this thesis is the result of investigations carried out by me in the Department of Biotechnology, Indian Institute of Technology Guwahati, Guwahati, India under the supervision of Dr. Veeranki Venkata Dasu.

In keeping with the general practice of reporting scientific observations, due acknowledgements have been made wherever the work described is based on the findings of other investigators.

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CERTIFICATE

It is certified that the work described in this thesis entitled “**Novel cutinases from *Thermobifida fusca*: production, characterization and applications**” by Mr. Krishnamoorthy Hegde for the award of degree of Doctor of Philosophy is an authentic record of the results obtained from the research work carried out under my supervision in the Department of Biotechnology, Indian Institute of Technology Guwahati, India and this work has not been submitted elsewhere for a degree.

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ABSTRACT

Cutinase is a hydrolytic enzyme that can act on multifarious substrates and share some vital catalytic properties of lipase and esterase. Because of its unique catalytic nature, it is being considered as one of the industrially important enzymes. Some of the useful applications of cutinase include hydrolysis of fats and oils, degradation of synthetic polymers like polyethylene terephthalate, biodegradation and detoxification of fatty acid based toxins, esterification and transesterification reactions. It has a potential to be an efficient biocatalyst in the food industry for synthesis of flavors, petrochemical industry for synthesis of biodiesel and preparation of house-hold detergents. This enzyme was first reported in fungus, *F. solani* and mainly produced by phytopathogenic fungi, but there are several bacteria, which are known to produce cutinase.

Thermobifida fusca is a rod shaped, thermophilic organism found in decaying organic matter and is a major degrader of plant cell wall. The optimum growth temperature of *Thermobifida fusca* is 55°C. The genus *Thermobifida* is comprised of two members, the other being *Thermobifida alba*. Both members of the genus are acid fast, Gram-positive aerobic organisms. It appears to degrade all major plant cell wall polymers except lignin and pectin and can grow on most simple sugars and carboxylic acids. The organism produces several industrially relevant extracellular enzymes including cutinase, glycoside hydrolases, cellulases, xylanases etc. The extracellular enzymes of *T. fusca* have been studied extensively because of their thermostability, activity in a broad pH range, and their resistivity to various effector molecules such as, organic solvents and surfactants.

In this study, two cutinase encoding genes, *cut1* (NCBI Acc. No. JN129499.1) and *cut2* (NCBI Acc. No. JN129500.1) from *T. fusca* NRRL B-8184 were cloned into pET22b(+) and expressed in *E. coli* BL21 (DE3) separately. Both the cutinases were purified to homogeneity using C-terminal (His)₆ tag and Ni-NTA column. The sequencing of the cloned genes revealed 960 and 906 bp length for *cut1* and *cut2*, respectively with 93% similarity in amino acids among them. The homogeneity of the purified enzyme was investigated by SDS-PAGE. The purified Cut1 and Cut2 showed molecular weight of ~30 and ~29 kDa, respectively. Cutin hydrolytic activity of the purified enzyme was confirmed by using cutin analog substrate, p-NMSH, which showed 0.218 ± 0.06 and 0.395 ± 0.17 U mg⁻¹ for Cut1 and Cut2, respectively. Purified Cut1 and Cut2 showed specific activity of 542.5 ± 2.7 and 643.4 ± 3.4 U mg⁻¹, respectively against pNPB. Further, cutinolytic activity of purified Cut1 and Cut2 was confirmed with substrate, tomato cutin.

Various physiochemical parameters (growth temperature, IPTG conc., and cell density for induction) were optimized using Luria Bertani (LB) medium to achieve optimal production of recombinant Cut1 and Cut2. The optimum levels of growth temperature, IPTG and cell density (A_{600 nm}) were found to be 37°C, 0.1 mM and 0.75, respectively. At these optimum conditions, the recombinant cutinase yield in LB medium was found to be 101 ± 0.29 and 112 ± 0.20 U mL⁻¹, respectively for Cut1 and Cut2 after 6 h post-induction. In the process, the effect of (His)₆ tag on growth and production of recombinant cutinase was also carried out, which showed no significant effect of (His)₆ tag on the growth and production.

Further, the development of medium to enhance the production of recombinant Cut1 and Cut2 was carried out under optimum physiochemical conditions by screening of five different commercially available media viz., LB, NB, M9, M9-ZB and TB. Among tested media, TB

was found to be the best suited medium for growth and higher production of cutinase. Furthermore, it was observed that addition of 2 mM MgSO_4 and 1 mM NaCl in TB medium (called MTB) increased the growth by 1.5-fold and cutinase production was around 1.1-fold higher than TB and ~ 2.5 -fold higher than LB medium. In addition, different substrates, i.e. glucose and glycerol at different concentrations were also studied to evaluate the effect of substrate on growth and production in the MTB medium, which showed superior growth and production among the screened medium. Glucose was not found to be a suitable substrate for higher production and showed considerable reduction in growth and production. Nevertheless, glycerol at 4 mL L^{-1} concentration showed highest cutinase yield for both Cut1 and Cut2. Assessment of effect of individual medium components of MTB on the production of recombinant cutinase was studied using Taguchi's design technique. According to Taguchi's three-level design matrix, 27 runs were performed with different combinations of medium components at shake flask level and activity (U mL^{-1}) was considered as response to statistically analyze the effect of medium components on cutinase production using the statistical software package MINITAB. The results showed that tryptone and MgSO_4 had significant effect on the production of both Cut1 and Cut2. These findings on the significance of the individual parameters and their contribution on cutinase production was further validated by ANOVA of the results which showed the R^2 of 99.23 and 99.02% of medium components attributed to the production of Cut1 and Cut2, respectively. Based on the effects of each of the seven variables investigated in the study on recombinant cutinases production, tryptone, 2 g L^{-1} ; yeast extract, 24 g L^{-1} ; glycerol, 4 mL L^{-1} ; KH_2PO_4 , 1.15 g L^{-1} ; K_2HPO_4 , 12.54 g L^{-1} ; MgSO_4 , 0.61 g L^{-1} ; and NaCl, 0.058 g L^{-1} with medium pH 7.3 were determined as optimal conditions for the maximum production of recombinant cutinase. At above

combination of medium components, yield of 318 ± 0.73 and 316 ± 0.90 U mL⁻¹ for Cut1 and Cut2, respectively was achieved, which was 1.2-fold higher as compared with un-optimized condition (260 ± 1.1 and 263 ± 0.77 U mL⁻¹, respectively for Cut1 and Cut2). This experimental result was very close to the statistically predicted value of 319 and 315 U mL⁻¹, respectively for Cut1 and Cut2. After optimization was done using Taguchi orthogonal design, the overall production was found to be 11 fold higher than production in wild strain of *T. fusca*. Furthermore, the cellular localization of expressed cutinase in *E. coli* was also investigated in the optimized MTB medium. The result showed that 57 and 61% of Cut1 and Cut2, respectively localized in the periplasm and 28% of the recombinant protein was localized in the cytoplasm. The extracellular secretion for Cut1 and Cut2 was found to be only 15 and 11%, respectively.

The molecular mass determined by MALDI-TOF analysis for Cut1 and Cut2 was found to be 30.1906 kDa and 29.6812 kDa, respectively. 2-D electrophoresis showed that isoelectric point (pI) of Cut1 and Cut2 was 7.7 and 7.5, respectively. Optimum pH of Cut1 and Cut2 for the hydrolysis of p-nitrophenyl butyrate was in the range of 7.0-8.5 with maximum activity at pH 8, and its optimum temperature was found to be 55°C. Studies on thermal stability showed that both the cutinases are highly stable at 37°C, with retention of more than 60% relative activity even after 150 h and more than 40% activity were retained after 40 h at 55°C.

The kinetic parameter for Cut1 and Cut2 was investigated using pNPB, which showed highest activity among the tested substrates. K_m (in micromolar per liter) and k_{cat} (in seconds) for Cut1 was found to be 131 ± 9 and 178 ± 8 and for Cut2 was found to be 89 ± 17 and 253 ± 11 , respectively. The results showed that Cut2 have the highest affinity towards pNPB. Cut1

exhibited lesser catalytic efficiency with a k_{cat}/K_m of $1.35 \text{ s}^{-1} \mu\text{M}^{-1}$; whereas, Cut2 showed almost double catalytic efficiency, with k_{cat}/K_m of $2.84 \text{ s}^{-1} \mu\text{M}^{-1}$.

The deactivation rate constants (k_d) and the half-life of cutinase were estimated at different combinations of pH and temperature. The minimum values of k_d observed for Cut1 and Cut2 were 0.045 and 0.039 min^{-1} , respectively. The combinations of pH and temperature at which the above mentioned minimum deactivation rate constant was observed to be 8 and 45°C for both Cut1 and Cut2, respectively. The deactivation process was found to be faster at pH below 7 and above 8 for both the cutinases. It was found that maximum half-life time of Cut2 was 17.59 h, showing that this enzyme is more stable than Cut1 (15.3 h) at optimum conditions of pH and temperature (pH 8 and 45°C , respectively). Thermodynamic parameters, viz., change in enthalpy (ΔH^*), entropy (ΔS^*), free energy (ΔG^*) and activation energy of thermal deactivation of cutinase were also calculated in the temperature range from 45°C to 80°C at different pH (6.0-9.0). Enthalpy of Cut1 and Cut2 increased as pH increased till pH 8. However, there was a decrease in enthalpy of Cut1 and Cut2 at pH 9. It was also observed that the ΔH^* value of Cut2 was ~ 1.5 fold higher than Cut1 in the range of pH studied (pH 6-9), which indicates that Cut2 is thermodynamically more stable than Cut1. Both the cutinases showed negative entropy values, which was increased with increase in the pH.

Study on hydrolytic ability with different acyl chain length showed that both the cutinases had increasing tendency till C4 acyl chain length. However, the catalytic efficiency decreased with the increase in acyl chain from C4. Nevertheless, Cut1 showed better catalytic ability towards longer chains compared with Cut2. So, both Cut1 and Cut2 exhibited very low levels of activity against the long-chain substrate, p-nitrophenyl palmitate (C16) as compared with the activity against pNPB at 50°C . Investigation on the effect of various effectors on the

enzyme stability showed that both Cut1 and Cut2 in general, showed excellent organic solvent stability, with no drastic decreases in residual activity in most of the studied organic solvents at 40% (v/v). Various surfactants showed no significant negative effect on stability of both the cutinases. Both the cutinases showed slight enhancement in the activity in the presence of mono cations, Na^+ and K^+ whereas, inhibitor EDTA showed no inhibitory effect on activity and urea had less inhibitory effect.

The fluorescence and CD spectra of purified Cut1 and Cut2 at different pH (pH 6-9) at 25°C showed no considerable difference in the secondary structure. However, both the cutinases showed emission maximum around 331 nm and no shift in emission spectra was observed at any of the pH studied indicating that the blue shift in the emission spectra would be due to inaccessible tryptophan residues. Quenching study with iodide further confirmed that all the tryptophan residues are buried in hydrophobic pockets as there was no noticeable quenching even at 1.5 M KI. CD spectroscopy revealed that both the cutinases are α -helix protein with characteristic minima at 208 nm and 220 nm. Unfolding study with GdnHCl and urea was carried out for both the cutinases. A loss of organized secondary structure in the presence of GdnHCl was observed for both Cut1 and Cut2. However, the pattern of unfolding was different for Cut1 and Cut2 with faster unfolding in case of Cut1 compare to Cut2 at lower GdnHCl concentration. Nevertheless, urea was found to be ineffective in denaturing both the cutinases.

Homology modeling of the Cut1 and Cut2 revealed that both the enzymes are a monomeric in nature, folded into α/β domain. It consists of central stranded β sheet flanked by α helices. This model also revealed a potential Ser-His-Asp catalytic triad in which the nucleophilic Ser is located in a sharp turn called the “nucleophile elbow,” which is commonly observed in the

α/β -hydrolases. The “oxyanion hole” is predicted to be formed by the backbone amides of Met and Tyr (Met189 and Tyr118 for Cut1 and Met171 and Tyr100 for Cut2). There is a disulfide bond present in both Cut1 (Cys299-Cys317) and Cut2 (Cys281-Cys299) in the C-terminal region.

Immobilization studies of Cut1 and Cut2 on different supports to enhance the stability and activity was investigated. It was observed that both the cutinases showed enhancement in thermal stability and activity when adsorbed on Zeolite and Celite-545. Covalent coupling to chitosan beads also showed considerable increase in thermostability, reusability and storage stability. Nevertheless, covalent coupling of Cut1 and Cut2 to magnetic nanoparticles (MNPs) showed drastic decrease in thermostability. There was also considerable decrease in activity upon reuse and storage, indicating MNP not to be a suitable support for immobilization of cutinase from *T. fusca*.

Short chain alkyl esters are well appreciated for fruity flavors they provide. These are mainly applied to the fruit-flavored products like jam, jelly, beverages, wine and dairy. To evaluate the industrial applicability and performance of cutinase during esterification and transesterification reactions, experiments were performed to assess the synthesis of terpenoid, butyl and isopropyl esters using both free and immobilized cutinases. Recombinant cutinase of *T. fusca* NRRL B-8184 was found to be active in catalyzing the synthesis of alkyl esters in organic solvent (n-hexane). The optimal temperature for the enzyme catalyzed synthesis was found to be 50°C for terpenoid, butyl and isopropyl esters. The maximum conversion (%) during synthesis of isopropyl ester was obtained with Cut2, which showed 47% conversion as a free cutinase and the immobilization on Zeolite and Celite-545 increased the conversion yield by 10 and 16%, respectively. However, Cut1 showed lesser conversion

yield in comparison to Cut2 for isopropyl ester synthesis. The maximum conversion (%) during synthesis of terpenoid (geraniol) ester was obtained for acetic acid (80%), butyric acid (71%) and valeric acid (61%) with Cut1 as biocatalyst, reflecting the increasing specificity of the enzyme for short-chain length fatty acids. There was a nominal increase of 3-5% conversion yield with Cut1 upon immobilization on Zeolite, whereas the Cut1 immobilized on Celite-545 showed 6-10% increase in conversion, which is better than the performance of Cut1 immobilized on Zeolite. In comparison to Cut1, the conversion yield with Cut2 was somewhat low for terpenoid ester synthesis. Cut1 and Cut2 showed 89 and 66% conversion respectively, for butyl butyrate synthesis when used as a free enzyme without any immobilization. However, immobilization of Cut1 (which showed better conversion rate in comparison to Cut2) on Zeolite and Celite-545 showed only 4% increase in the conversion yield. In general it was found that Celite-545 and zeolite are the best suited support for immobilization, which showed considerable increase in catalytic efficiency for both the cutinases. Nevertheless, chitosan immobilization was found to be inefficient in increasing the catalytic efficiency for ester synthesis.

Experiments were also performed to evaluate the performance of cutinase as a catalyst for transesterification to yield methyl, ethyl esters and lactate esters. However, though *T. fusca* cutinase was found to be active in catalyzing the transesterification of tributyrin and lactic acid, the ester yield was less than 35% for tributyrin and 20% for lactic acid, which makes it unsuitable catalyst for long chain fatty acid based catalysis.

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ABBREVIATIONS AND NOTATIONS

Abbreviations

Amp	Ampicillin
ANOVA	Analysis of variance
AOT	Sodium di-2-ethyl hexyl sulfocinate
APES	3-aminopropyltriethoxysilane
ARS	Agricultural research service
ATPS	Aqueous two-phase systems
BBP	Butyl benzyl phthalate
BTF3	Boron trifluoride
CBB	Coomassie brilliant blue
CBM	Carbohydrate-binding modules
CCD	Central composite design
CD	Circular dichorism
cDNA	Complementary dioxyribonucleic acid
CHCA	α -Cyano-4-hydroxycinnamic acid
CL	ϵ -caprolactone
CMC	Critical micelle concentration
CTAB	Cetyl trimethylammonium bromide
DCW	Dry cell weight (g L^{-1})
DEAE	Diethylaminoethyl
DEHP	Di-(2-ethylhexyl)-phthalate

Abbreviations

DEPA	N,N-diethyl-2-phenylacetamide
DF	Degree of freedom
DMF	Dimethylformamide
DMP	Dimethyl phthalate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DO	Dissolved oxygen (%)
DPrP	Dipropyl phthalate
DSC	Differential scanning calorimetry
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EPR	Electron paramagnetic resonance
FAEE	Fatty acid ethyl ester
FAME	Fatty acid methyl ester
FESEM	Field emission scanning electron microscopy
FFA	Free fatty acids
FID	Flame ionization detector
FTIR	Fourier transform infrared
GA	Glutaraldehyde
GC	Gas chromatography
GdnHCl	Guanidine hydrochloride

Abbreviations

HPLC	High-performance liquid chromatography
Ig	Immunoglobulin
IMAC	Immobilized metal affinity chromatography
IPTG	Isopropyl β -D-1-thiogalactopyranoside
LB	Luria broth
LC-MS	Liquid chromatography–mass spectrometry
MALDI-TOF MS	Matrix assisted laser desorption ionization time-of-flight mass spectrometry
MNPs	Magnetic nanoparticles
MRE	Mean residue ellipticity
mRNA	Messenger ribose nucleic acid
MS	Mean square
MTB	Modified terrific broth
NATA	N-Acetyl-L-tryptophanamide
NaY	Sodium form of zeolite
NB	Nutrient broth
NCBI	National center for biotechnology information
NEB	New England Biolabs
Ni-NTA	Nickel-nitrilotriacetic acid
NMR	Nuclear magnetic resonance
NRRL	Northern regional research laboratories
OD	Optical density
OmpA	Outer membrane protein A

Abbreviations

PA	Polyamide
PA 6,6 fiber	Polyamide 6,6 fiber
PAGE	Polyacrylamide gel electrophoresis
PAN	Polyacrylonitrile
PAT	Polyalkylene terephthalates
PB	Plackett-Burman
PBS	Poly-(butylene succinate)
PBSA	Poly-(butylene succinate-co adipate)
PCR	Polymerase chain reaction
PDL	ω -pentadecalactone
PEG	Polyethyleneglycol
PET	polyethylene terephthalate
<i>phoA</i>	Signal peptide for alkaline phosphatase
pKa	Acid dissociation constant
PMSF	Phenyl methyl sulphonyl fluoride
pNMSH	p-Nitrophenyl (16 methyl sulphone ester) hexadecanoate
pNPA	p-nitrophenyl acetate
pNPB	p-Nitrophenyl butyrate
pNPH	p-nitrophenyl hexanoate
pNPP	p-Nitrophenyl palmitate
pNPV	p-nitrophenyl valerate
PTT	Poly(trimethylene terephthalate)
PVDF	Polyvinylidene fluoride

Abbreviations

RMSD	Root-mean-square deviation
RMSE	Root mean square error
RNA	Ribonucleic acid
ROP	Ring-opening polymerizations
rRNA	Ribosomal ribonucleic acid
RSM	Response surface methodology
S/N	Signal-to-noise
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SOC	Super optimal broth
sp.	Species
SS	Sum of square
SSD	Sum of squares of the differences
SSF	Solid state fermentation
TB	Terrific broth
TCA	Tri-chloro acetic acid
TDOC	Sodium taurodeoxycholate
TEA	Triethylamine
TFA	Trifluoroacetic acid
TFB	Transformation buffer
THF	Tetrahydrofuran
TLC	Thin layer chromatography

Abbreviations

TM	Trade mark
Tris	Tris hydroxymethyl amino methane buffer
UV	Ultraviolet
w.r.t	With respect to
XPS	X-ray photoelectron spectroscopy
(His) ₆	A polyhistidine-tag with six histidine
2-D	2-Dimensional
3-D	3-Dimensional
3PET	Bis(benzoyloxyethyl) terephthalate

Notations

°C	Degree centigrade
%	Percentage
bp	Base pair
dB	Decibel
E_A	Activation energy
g	Gram
g	Gravitational acceleration
h	Hour
kb	Kilobase
k_{cat}	Turnover numbers (s^{-1})
k_{cat}/K_m	Specificity constant ($M^{-1}s^{-1}$)

Notations

k_d	Deactivation constant (h^{-1})
kDa	Kilo dalton
K_m	Michaelis constant (mM)
L	Litre
M	Molar (mol L^{-1})
min	Minute
mL min^{-1}	Milliliter per minute
mmolL	Milimolar
M_r	Molecular weight of enzyme (kDa)
nm	Nanometer
nmoL	Nanomolar
pI	Isoelectric point
R^2	Regression coefficient
rpm	Revolution per minute
s	Second
U	Unit of enzyme activity
$\text{U L}^{-1} \text{h}^{-1}$	Unit of enzyme activity per liter per hour
U mg^{-1}	Unit of enzyme activity per milligram
U mL^{-1}	Unit of enzyme activity per milliliter
v	Reaction velocity
v/v	Volume/volume
$V_{\max}$	Maximal reaction velocity ($\text{U } \mu\text{g}^{-1}$)

Notations

w/v	Weight/volume
w/w	Weight/weight
Y	Predicated response
ΔG^*	Change in free energy
ΔH^*	Change in enthalpy
ΔS^*	Change in entropy

Greek letter

μl	Microlitre
μ_m	Maximum specific growth rate (h^{-1})
μM	Micromolar ($\mu\text{mol L}^{-1}$)
μmol	Micromoles
$\text{\AA}$	Angstrom
C α	Central carbon atom in an amino acid
h	Planck's constant
Δ	Delta
κ	Boltzmann constant
σ_s	Specific saturation magnetization

CHAPTER 1

INTRODUCTION

1.1 Generalities

Cutinase (3.1.1.74) is an enzyme whose name derives from their ability to degrade cutin polymers of higher plant that is composed of hydroxy and epoxy fatty acid polyesters (Carvalho *et al.*, 1999; Purdy and Kolattukudy, 1975). Cutinase is a serine esterase that belongs to α/β hydrolase family (Longhi *et al.*, 1999). Crystallographic study of cutinases from *Fusarium solani* (Martinez *et al.*, 1992), *Aspergillus oryzae* (Liu *et al.*, 2009), *Glomerella cingulate* (Nyon *et al.*, 2009) and *Thermobifida alba* (Kitadokoro *et al.*, 2012) revealed some common feature about its structure that they have serine in its catalytic site and unlike most of the lipases where the catalytic site is buried under an amphipathic 'lid like' structure. The catalytic site of cutinases are solvent accessible and not buried under amphipathic loop. The plant cuticle that acts as a protecting layer in plant, which prevents desiccation as well as pathogen attack (Fett *et al.*, 1992a) is composed of fatty acids usually of n-C16 and n-C18. The very first step of infection in plants requires the enzymatic hydrolysis of cutin. Besides cutin, cutinases are also able to hydrolyze several synthetic esters and show activity on short- and long-chain fatty acid triacylglycerols. This feature of cutinase was not observed in most of the lipases other than *Candida antarctica* lipase B (Longhi *et al.*, 1996). Activity of the lipases is greatly enhanced in the presence of a lipid–water interface, whereas cutinases do not require any interfacial activation (Verger and Haas, 1976). On the other side, esterases though do not require any interfacial activation and can act in aqueous media; they can efficiently catalyze only short chain fatty acids (Longhi *et al.*, 1999). Interestingly, cutinases are active in both aqueous solution as well as water-lipid interface and can act on both soluble and emulsified triglycerides (Verger and Haas, 1976). This feature of sharing enzymatic capabilities of true esterase and true lipase makes cutinase unique.

In vitro, cutinases display hydrolytic activity towards a broad variety of esters, from soluble synthetic esters to insoluble long-chain triglycerides (Carvalho *et al.*, 1998; Dutta *et al.*, 2009). Therefore, cutinases have been evaluated as a potential catalyst in degradation of

polymers such as PET (Acero *et al.*, 2011; Baker *et al.*, 2012; Heumann *et al.*, 2006; Nimchua *et al.*, 2008; Oeser *et al.*, 2010; Ronkvist *et al.*, 2009; Vertommen *et al.*, 2005; Zang *et al.*, 2013), cotton scouring (Degani *et al.*, 2002), dairy industry for hydrolysis of milk fat, household detergents, oleochemical industry, synthesis of structured triglycerides polymers and surfactants (Dekoster *et al.*, 1995), synthesis of short chain fatty acids (Dutta and Dasu, 2011), synthesis of ingredients for personal-care products, pharmaceuticals and agrochemicals containing one or more chiral centers (Carvalho *et al.*, 1998a; Melo *et al.*, 2003). Some of these processes are already implemented in industry (Carvalho *et al.*, 1998a), while others are still under evaluation at research level. Another interesting field of application of cutinase is in esterification and transesterification reactions. At low water activities, transesterification of fats and oils or stereo selective esterification of alcohols can be achieved using cutinase (De Barros *et al.*, 2009a; De Barros *et al.*, 2009b; De Barros *et al.*, 2010a; De Barros *et al.*, 2010b; Sarazin *et al.*, 1995). The esters produced from short-chain fatty acids have applications as flavoring agents in food industry. Although lipases can be used for the hydrolysis and synthesis of esters, active in organic solvents and have wide substrate specificity, they still have a number of shortcomings. The most important hurdle of lipases is their relatively large size and instability under industrial process conditions. Cutinase has also found to be a potent catalyst for transesterification. Transesterification is the reaction of fats or oils with an alcohol to form esters and glycerol. Alcohol combines with the triglycerides to form glycerol and esters. A catalyst is usually used to improve the reaction rate and yield. Methyl and ethyl esters synthesized by transesterification can be used to enrich diesel fuels. For instance, the transesterification (alcoholysis) reaction of butyl acetate with hexanol in organic media (isooctane) has been evaluated using cutinase in several systems (Cunnah *et al.*, 1996; Carvalho *et al.*, 1997a; Serralha *et al.*, 1998). Reports are also available on methanolysis of triolein catalyzed by cutinases (Badenes *et al.*, 2010a; Badenes *et al.*, 2010b; Badenes *et al.*, 2011a).

1.2 Cutinase producing microorganisms

In general, phyto-pathogenic microbial species are known to produce cutinase (Ferreira *et al.*, 2004; Fett *et al.*, 1992a; Fett *et al.*, 1999; Fett *et al.*, 2000; Macedo and Pio, 2005). Microorganisms capable of producing substantial quantities of enzymes have importance in the industrial sector (Haki and Rakshit, 2003). Source dependent variation in

physical/biochemical/molecular properties, such as optimum pH, temperature, metal ion requirement, substrate specificity, inhibition pattern and molecular weight of cutinase produced by microbial species has been well documented till date (Fett *et al.*, 1992b; Lin and Kolattukudy, 1978; Pio and Macedo, 2007; Rispoli and Shah, 2007). Furthermore, many researchers are continuously involved in efforts to isolate and identify the microbial biosystems producing enzymes suitable for industrial as well as commercial applications directly and indirectly (Kumar and Takagi, 1999).

Though both bacterial and fungal sources are reported to produce cutinases, phytopathogenic fungi are the first microorganism to be reported to produce cutinase (Purdy and Kolattukudy, 1975). Extensive studies have been done on the production, purification and characterization of fungal cutinases (Koller and Parker, 1989; Purdy and Kolattukudy, 1975; Rispoli and Shah, 2007; Soliday and Kolattukudy, 1976; Trail and Koller, 1990; Trail and Koller, 1993; Wang *et al.*, 2000). In contrast, there are only few studies done so far on bacterial cutinases (Chen *et al.*, 2008, Chen *et al.*, 2009; Dutta *et al.*, 2013; Fett *et al.*, 1992a; Fett *et al.*, 1999; Sebastian and Kolattukudy, 1988). Bacterial cutinases are different from fungal cutinase with respect to their thermal and pH optima and stability. It has been observed by Sebastian *et al.*, (1987) that bacterial cutinase is stable even at 70°C, whereas fungal cutinases loses its activity at 45°C.

Thermobifida fusca (formerly known as *Thermomonospora fusca*) is an aerobic, moderately thermophilic, filamentous soil bacterium that is a major degrader of plant cell walls in heated organic materials, such as compost heaps, rotting hay, manure piles, or mushroom growth medium (Bachmann *et al.*, 1999). Its extracellular glycoside hydrolases (cellulases and xylanases) have been studied extensively because of their thermostability, broad pH range (4 to 10), and high activity. It appears to degrade all major plant cell wall polymers except lignin and pectin and can grow on most simple sugars and carboxylic acids. It belongs to the phylum of *Actinobacteria* and was first isolated from decaying wood (Bellamy and Wilson, 1977). Apart from cutinase, *T. fusca* has been the source organism for isolating and studying multiple secreted cellulases and other carbohydrate-degrading enzymes (Ghangas and Wilson, 1988; Hu and Wilson, 1988).

Recently, cutinase from *Thermobifida fusca* have been shown its superiority over other cutinases in many aspects like thermostability, high activity in broad pH range, surfactant and organic solvent tolerance, which could have great biotechnological promise in many industrial

applications (Chen *et al.*, 2008; Chen *et al.*, 2010). Reportedly there are two genes having cutinase activity in *T. fusca* with 93% homology among them (Chen *et al.*, 2008; Chen *et al.*, 2010). Interestingly, despite the high level homology, they showed different biochemical properties and substrate specificity (Chen *et al.*, 2010). Hence, detailed study needed in the case of *T. fusca* cutinases on biochemical and biophysical properties as well as ability to catalyze various substrates.

1.3 Production of recombinant protein

In recent years, the recombinant proteins have been utilized as tools for cellular and molecular biology with more and more applications in several fields become a commercial reality. Efficient recombinant strategies for the production of recombinant proteins are gaining increasing importance due to the benefits like higher production efficiencies and lower costs of the final product, which are essential for obtaining a commercially viable process.

E. coli is one of the earliest and most widely used hosts for the production of heterologous proteins (Demain and Vaishnav, 2009; Terpe, 2006). Some of the advantages with *E. coli* system include rapid growth and expression, ease of culture, high product yields (Swartz, 1996) and cost effective downstream processing. Several commercial proteins are being massively produced in *E. coli* system. This system is excellent for functional expression of non-glycosylated proteins. Moreover, *E. coli* genetics are far better understood than those of any other microorganism. Recent progress in the fundamental understanding of transcription, translation, and protein folding in *E. coli*, together with the availability of improved genetic tools, is making this bacterium more valuable than ever for the expression of complex eukaryotic proteins (Demain and Vaishnav, 2009).

1.4 Statistical approach for optimization of process parameters for enhanced production of cutinase

Optimization of production of any metabolite plays a vital role to study the technical and economic feasibility of the process in the commercial level. It involves the study of a number of physicochemical parameters, such as medium composition, pH, and temperature. In any bioprocess, the improvement in productivity of any metabolite could be achieved through manipulation of nutritional and physiochemical parameters. Statistical methodologies involving optimization of interaction of several factors at a time are generally preferred over traditional single factor at time optimization methods due to their several advantages (Dasu

and Panda, 2000; Kumar *et al.*, 2009) such as statistically designed experiments minimize the error in determining the effect of parameters in an economical manner (Dasu and Panda, 2002; Sharma and Satyanarayana, 2006). Recently, the use of statistical approaches involving Taguchi and response surface methodology has been widely used for media optimization and for the understanding of interactions among various parameters using a minimum number of experiments (Kalil *et al.*, 2000; Taguchi, 1990). The Taguchi's method has been used widely in industrial process design, principally in developmental trials (Taguchi, 1990). The basic principle of this method serves as screening filters, which examine the effects of many process variables and identify those factors, which have major effects as process using a single trial with a few experiments.

1.5 Purification and characterization of cutinase

Downstream processing of biomolecules obtained from fermentation broths is a fundamental step in process biotechnology since it often represents the major manufacturing cost. The economic viability of a biochemical process depends not only on improvements achieved in the production but also on innovations and optimization of downstream processes (Haki and Rakshit, 2003; Wheelwright, 1987). Though protein purification requires specific strategy for purification depending on the nature of protein, various methods are employed to enrich or purify a protein of interest from other proteins and components in a crude cell lysate or other sample. Affinity chromatography has emerged as one of the most powerful methods among all purification techniques, whereby the protein of interest is purified by virtue of its specific binding properties to an immobilized ligand in a single step. Immobilized metal affinity chromatography (IMAC) using (His)₆ tag is one of the popular affinity purification methods used for purification of recombinant proteins. His-tag based purification has a clear advantage over other methods in their relatively strong binding to immobilized metal ion affinity chromatography (IMAC) resin and their small size, which commonly eliminates the need for proteolytic removal.

After purification, characterization of the purified enzyme is very much necessary before it is exploited commercially. The enzymatic hydrolysis depends on several physicochemical factors. The proportion of substrate-to-enzyme, temperature of incubation, and pH influence the rate of reaction to a large extent (Naidu and Panda, 2003). For any industrially important enzyme, the stability in presence of organic solvent, surfactants, metal ions etc. needs to be

evaluated. Biophysical characterization plays a vital role in an overall analytical strategy of enzymes, augmenting biological and biochemical methods used for process and product development of macromolecules. These techniques are critical for understanding the structure and behavior of proteins under different stress conditions, and they are essential tools for understanding enzyme-substrate binding behavior and conducting characterization studies for product development.

1.6 Immobilization strategy to enhance the activity and stability of cutinase

At present enzyme technology is considered as a powerful alternative to conventional process technology in various biotechnological and analytical fields. The benefits of biocatalyst over chemical catalysts are many, these include its capability to achieve diverse chemical reactions under mild environmental conditions with high efficiency and specificity, remarkably efficient product conversion, low undesirable by-products and increased product yield. Such features of biocatalyst have made them a key target of exploitation by many emerging biotechnology and biopharmaceutical industries (D'Souza, 1999; Mateo, 2007; Zhang *et al.*, 2012). Regardless of all these advantages, the use of enzymes in industrial application has been slowed down by various factors (Garcia-Galan *et al.*, 2011), for instance, recovery of soluble enzyme from the reaction mixture and their recycle in subsequent reactions. To make use of enzyme economically feasible, recovery and reuse is vital (Garcia-Galan *et al.*, 2011). A comprehensive research in the field of enzyme technology has provided various approaches to tackle such problems, among which enzyme immobilization technology is one, which offers promising economic exploitation of even expensive enzymes in various industries like bioprocessing, biopharmaceuticals, waste water treatment, development of biosensors, etc. Several strategies are employed for immobilization and various factors influence the performance of immobilized enzymes based on the properties of enzyme. Some of the popular immobilization method includes adsorption or carrier-binding method, cross-linking or covalent binding method, entrapment method and microencapsulation method (Datta *et al.*, 2012; Garcia-Galan *et al.*, 2011; Mateo *et al.*, 2007). However, the characteristics of the support, reactive groups and immobilization conditions need to be carefully selected to be able to achieve the maximum immobilization. Some of the successful immobilization strategies employed to enhance the activity of cutinases from various sources (*F. solani pisi*, *F. roseum culmorum*, *C. gloeosporoides*, *F. roseum sambucinum* and *Pseudomonas spp.*)

involves adsorption on zeolite Y and polyamide Accurel PA6 (Serralha *et al.*, 2004), immobilized onto silica derivatives like, Biosil-NH₂ and Biosil –dextran-NH₂ (Goncalves *et al.*, 1999, Goncalves *et al.*, 1996a) and microencapsulated in reversed micelles of surfactants (Melo *et al.*, 1995b).

1.7 Esterification and transesterification reactions catalyzed by cutinase

Short chain fatty acids esters are commonly used in the manufacturing of flavors and fragrances because of their fruity odor. These esters are in high demand and are widely used in the food, beverage, cosmetic, and pharmaceutical industries. Fatty acid esters of alcohols are also of increasing economic interest in many industries involving a wide range of applications; viz., bio-carburants, biosurfactants, biolubricants, solvents, hydraulic and drilling fluids, dispersing agents, cosmetics (Dossat *et al.*, 2002) and biodiesel (Xie and Ma, 2010). Although flavor esters are currently produced by chemical synthesis, there is an increasing inclination towards natural flavors, rather than chemically synthesized flavors. However, the same flavor and fragrance esters prepared by enzymatic synthesis may be labeled as “natural” (Gillies *et al.*, 1987; Larios *et al.*, 2004). Lipases and esterases are commonly used for the enzymatic production of a wide range of ester products in non-conventional media. Nevertheless, most of the lipases have higher affinity towards longer-chain length substrates, and low molecular weight substrate may have some inhibitory effect on enzymes (Abbas and Comeau, 2003; Hari-krishna and Karanth, 2002). In case of esterases, except few, most esterases loss their reactivity when the acyl chain length of substrate is more than two (Macarie and Baratti, 2000; Torres *et al.*, 2009). Recently, cutinases (Cunnah *et al.*, 1996; De Barros *et al.*, 2009a; De Barros *et al.*, 2010a; Dutta and Dasu, 2011; Pinto-Sausa *et al.*, 1994; Sarazin *et al.*, 1992; Sarazin *et al.*, 1995; Sebastiao *et al.*, 1993; Serralha *et al.*, 2001) were employed for the synthesis of some alkyl esters. Cutinases are believed to be a group of hydrolytic enzyme intermediate between lipase and esterase, which are capable of hydrolyzing various soluble esters as well as emulsified triacylglycerol. As the esterification reaction is reversible, the enzymes can also be employed to catalyze the formation of alcohols and fatty acids from esters. However, in aqueous solutions, the hydrolysis reaction is favored over synthesis reaction as the equilibrium is strongly shifted towards the starting reagents and esters cannot be synthesized. To overcome this difficulty, reaction media containing very small amounts of water or comprised of organic solvents can be used to bring about a

chemical equilibrium shift towards ester. Recently, few studies on the production of methyl/ethyl ester of oil mixtures have also been carried out using cutinase from *F. solani pisi* (Badenes *et al.*, 2010a; Badenes *et al.*, 2011).

1.8 Cutinase catalytic site

To date, five cutinase crystal structures had been resolved, this includes *F. solani*, *A. oryzae*, *G. cingulate*, *Cryptococcus* and recently, *T. alba* (Kitadokoro *et al.*, 2012; Kodama *et al.*, 2009; Liu *et al.*, 2009; Longhi *et al.*, 1997; Martinez *et al.*, 1992; Nyon *et al.*, 2009).

The overall structural studies of cutinase reveals that all the cutinases belong to the class of serine esterases and to the super family of the α/β hydrolases in which the nucleophilic serine is located at the center of an extremely sharp turn between a central β -strand surrounded by α -helices. The consensus sequence G-X-S-X-G with active serine in the catalytic triad is seen in all the cutinases. They have solvent exposed catalytic triad “Ser-Asp-His” and an oxyanion binding site that stabilizes the transition state *via* hydrogen bonds with two main chain amide groups (Nicolas *et al.*, 1996). One to two disulfide bridges are seen in cutinase that helps to the stabilization of the global molecular folding of cutinase. The binding of cutinase to interfaces seems not to require a main-chain rearrangement, as in the case of lipases, but only the reorientation of few lipophilic side chains.

1.9 Cutinase mechanism of action

Cutinases show catalytic machinery similar to those present in serine proteases. The catalytic triad is a coordinated structure consisting of three essential amino acids: histidine, serine and aspartic acid, which play an essential role in the cleaving ability of the cutinase.

The general catalytic mechanism is shown in the Fig. -. In brief, the reaction occurs as follows. The substrate binds to the surface of the cutinase such that the scissile bond is inserted into the active site of the enzyme, with the carbonyl carbon of this bond positioned near the nucleophilic serine. The serine has an -OH group that is able to act as a nucleophile, attacking the carbonyl carbon of the ester bond of the substrate. A pair of electrons on the histidine nitrogen has the ability to accept the hydrogen from the serine -OH group, thus coordinating the attack of the ester bond. The carboxyl group on the aspartic acid in turn hydrogen bonds with the histidine, making the nitrogen atom mentioned above much more electronegative. The reaction stabilization is also brought by additional amino acids, Met and

Tyr (Bacterial cutinase) or Gly and Ser (Fungal cutinase) in the vicinity of the active site, by formation of oxyanion hole.

1.10 Enzyme reaction kinetics

Enzyme kinetics is the study of the chemical reactions that are catalysed by enzymes. In enzyme kinetics, the reaction rate is measured and the effects of varying the conditions of the reaction is investigated. Studying an enzyme's kinetics in this way can reveal the catalytic mechanism of particular enzyme, its role in catalysis, how its activity is controlled, and how an inhibitor might inhibit the enzyme.

Unlike uncatalysed chemical reactions, enzyme-catalysed reactions display saturation kinetics. For a given enzyme concentration and for relatively low substrate concentrations, the reaction rate increases linearly with substrate concentration; the enzyme molecules are largely free to catalyse the reaction, and increasing substrate concentration means an increasing rate of enzyme catalysis. However, at relatively high substrate concentrations, the reaction rate asymptotically approaches the theoretical maximum due to the fact that all the active sites of enzyme are almost occupied and the reaction rate is determined by the intrinsic turnover rate of the enzyme. The substrate concentration midway between these two limiting cases is denoted by K_M .

Michaelis–Menten kinetics is one of best-known models of enzyme kinetics. The model takes the form of an equation describing the rate of enzymatic reactions, by relating reaction rate (v) to the concentration of a substrate (s). Its formula is given by,

$$V = (V_{\max} \times [s]) / (K_M + [s])$$

Where, $V_{\max}$ represents the maximum rate achieved by the system, at maximum (saturating) substrate concentrations, s is the substrate, The Michaelis constant K_m is the substrate concentration at which the reaction rate is half of $V_{\max}$. Biochemical reactions involving a single substrate are often assumed to follow Michaelis-Menten kinetics.

However, multi-substrate reactions follow complex rate equations that describe how the substrates bind and in what sequence. Depending on the substrates involved and the binding of substrates, it can be classified as, The Sequential Mechanism (Ordered Sequential Mechanism and Random Sequential Mechanism) and the Non-sequential or “Ping-Pong” Mechanism.

Inhibitors are compounds that decrease the rate of an enzyme-catalyzed reaction. The study of enzyme inhibition has enhanced our knowledge of specificity and the nature of functional

groups at the active site. The activity of certain enzymes is regulated by a feedback mechanism such that an end product inhibits the enzyme's function in an initial stage of a sequence of reactions. The action of an inhibitor on an enzyme can be described as either reversible or irreversible. In reversible inhibition, an equilibrium exists between the enzyme and the inhibitor. There are three important types of reversible inhibition: competitive inhibition, noncompetitive inhibition, and uncompetitive inhibition. In irreversible inhibitions, inhibition progressively increases with time. Complete inhibition results if the concentration of the irreversible inhibitor exceeds that of the enzyme.

1.8 Objectives and scope

Cutinase (EC 3.1.1.74) is a carboxylic esterase belong to the superfamily of serine α/β hydrolases that degrade cutin. Apart from cutin, its capabilities to act on many other substrates like, synthetic short and long chain fatty acids, synthetic esters, polymers, cotton scouring and degradation of toxic substances made it one of the potent commercial enzymes in biotechnological applications like dairy, oleochemical industries, degradation of plastics, synthesis of structured polymers, agro chemicals, synthesis of short chain aroma compound, detergent industries etc. Though both fungal and bacterial species have been reported to produce cutinase, very less reports are available on bacterial cutinases. Moreover, bacterial cutinase shows several advantages over fungal cutinase such as, higher thermostability, better resistance to rigorous reaction conditions and wide range of applicability.

Thermobifida fusca is an aerobic, moderately thermophilic, filamentous soil bacterium that is a major degrader of plant cell walls in heated organic materials. *T. fusca* has been the source organism for isolating and studying multiple secreted cellulases, carbohydrate degrading, and other enzymes. The extracellular enzymes of *T. fusca* have been studied extensively because of their thermostability, activity in a broad pH range, and their resistivity to various effector molecules such as, organic solvents and surfactants. Nevertheless, cutinases from *T. fusca* has not been explored exhaustively and no information is available on several aspects of cutinase from *T. fusca* such as, biochemical, biophysical and structural properties of this enzyme and their application potentials.

The present research focuses on understanding the importance of two homologous, thermostable cutinases, Cut1 and Cut2 from *Thermobifida fusca*. In the present study we have shown that optimization of the medium components can substantially increase the production

of recombinant *T. fusca* cutinase (11 folds compare to wild type *T. fusca*). The key findings of the present study also showed that *T. fusca* cutinases are active over a broad range of pH (6.8-9.0) and temperature (40-80°C) with superior thermostability to cutinases from any other sources reported. *T. fusca* cutinases are highly resistant to several organic solvents (*viz.*, Acetone, Acetonitrile, Ethanol, and Tetrahydrofuran, n-Hexane) and surfactants (*viz.*, Sodium deoxycholate, Sodium taurodeoxycholate) which could have a potential application in esterification and transesterification and detergent formulations. Further, biochemical, biophysical and structural characterization of the homologous cutinases of *T. fusca* showed that despite their highly homologous structure, they have different substrate specificity towards short chain fatty acid synthetic esters (C₂-C₆), different organic solvent and surfactant tolerance level. Protein unfolding studies of the *T. fusca* cutinase revealed that both the cutinases are stable at pH ranging from 6-9 at 25°C with no considerable change in the secondary structure and found to be α -helix in nature and they are highly resistant to urea induced unfolding. Unfolding study also revealed that Cut2 is more resistant to unfolding by GdnHCl compare to Cut1 due to the different surface electrostatic properties of these enzymes. In the present study we have also attempted to enhance the stability and activity of the *T. fusca* cutinase by various immobilization techniques. Chitosan, zeolite and Celite-545 was found to be an appropriate immobilization support to enhance the activity and stability of the cutinase in aqueous medium. However, only Zeolite and Celite-545 was found to be a suitable support for the cutinase catalyzed reaction in organic media. The present study also demonstrates that cutinase can be used as a catalyst for various esterification and transesterification reactions to synthesize esters of geraniol, isopropanol, butyric acid etc. Based on an extensive literature survey, industrial importance of cutinase and the need to obtain new cutinase with better stability, activity and versatile applicability, the present investigation was focused on the production of cutinases from *T. fusca* NRRL B-8184. In order to evaluate the possible establishment of its potential applications in industries, the following objectives have envisaged.

- Molecular cloning, expression and purification of cutinases from *T. fusca*.
- Production optimization of recombinant cutinases in *E. coli* system.
- Biochemical, biophysical and structural characterization of recombinant cutinases from *T. fusca*.

- Study on increasing the stability and activity of cutinases by immobilization.
- Applications of cutinase.
 - Esterification reactions catalyzed by cutinase.
 - Transesterification reactions catalyzed by cutinase.

1.9 Organization of the thesis

The presentation of the work has been divided into five chapters. The current **Chapter 1** presents a general introduction, objective and scope of the present work. While the literature that supports the present work is presented in **Chapter 2**. **Chapter 3** includes the details of the materials and methods adopted in the present study. Details of the cloning, expression, purification of cutinase, optimization of medium components in shake flask, characterization and applications of cutinase from *T. fusca* NRRL B-8184 are presented in this chapter. It also provides technical information about the analytical methods adopted in the present work. **Chapter 4** contains the results and discussion, where the results of recombinant cutinase construction, purification, optimization of medium components for higher cutinase production, biochemical, biophysical and structural characterization, and applications of cutinases from *T. fusca* NRRL B-8184 are presented and thoroughly discussed. The chapter also emphasizes on some esterification and transesterification reactions catalyzed by cutinases from *T. fusca* NRRL B-8184. **Chapter 5** draws summary and appropriate conclusions based on the previous results and discussion. This chapter also provides some useful recommendations to carry out further work in this field.

CHAPTER 2

REVIEW OF LITERATURE

2.1 Cutinase: an overview

Cutinase (EC 3.1.1.74) is one of the members of serine hydrolase superfamily and belongs to α/β hydrolase fold family (Jelsch *et al.*, 1998; Longhi *et al.*, 1997b; Martinez *et al.*, 1992) that degrade cutin, the cuticular polymer of higher plants, which is a polyester composed of hydroxy and epoxy fatty acids (Purdy and Kolattukudy, 1975). Cutin is one of the components of the cuticle composed of an insoluble polymer built mainly from esterified C16 and C18 hydroxy and epoxy fatty acids embedded in a layer of waxy material of cuticle (Fig. 2.1) (Fauth *et al.*, 1998; Purdy and Kolattukudy, 1975). Ester bonds predominate in cutin, although presence of peroxide bridges and ether linkages has also been reported. The structure of the cutin and the waxes largely determine the physical and chemical properties of the plant cuticle, which plays an important role in the interaction between the plant and its environment (Watson and Kolattukudy, 1972). The cuticle present in various parts of plant acts as a protective layer to prevent desiccation and microbial and insect attack (Fett *et al.*, 1992a). The enzymatic hydrolysis of cutin is one of the first steps of most of the infections. Cutinase aid the fungal pathogens to penetrate into their hosts by disrupting the cuticle layer (Kolattukudy, 1985; Kolattukudy *et al.*, 1995; Kolattukudy 1992; Köller *et al.*, 1991; Köller *et al.*, 1995; Koller and Kolattukudy, 1982; Köller *et al.*, 1982; Koller and Yao, 1996). Cutin monomers generated by the action of fungal cutinase were shown to act as signals for fungal gene

activation (Kolattukudy *et al.*, 1995) and also to contribute to induction of fungal appressoria (Francis *et al.*, 1996; Gilbert *et al.*, 1996).

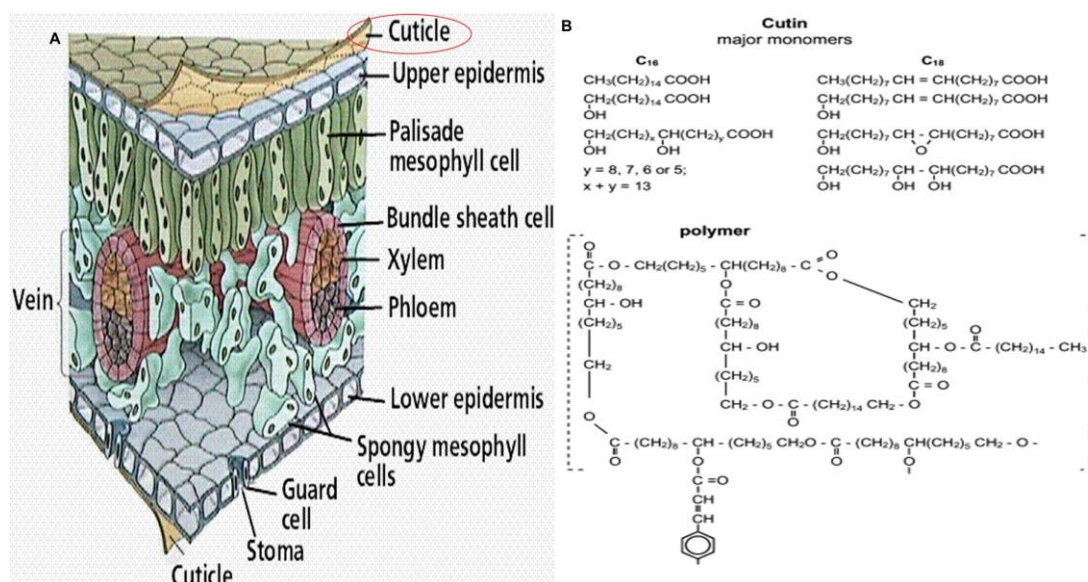


Fig. 2.1 Typical plant leaf cuticle (A) and structure of cutin (B) (adopted from Purves *et al.*, 1995 and Kolattukudy, 1981)

Some of the studies also showed the release of other cutinolytic esterases from germinating spores of several plant pathogens (Deising *et al.*, 1992; Fan and Köller, 1998; Francis *et al.*, 1996; Köller *et al.*, 1982; Nicholson and Kunoh, 1995; Pascholati *et al.*, 1993; Yao and Köller, 1995), and their involvement in surface penetration (Köller *et al.*, 1995; Köller and Yao, 1996) or in the attachment of spores to host surfaces (Nicholson and Kunoh, 1995). In most of the cases, phyto-pathogenic fungi species are known to produce cutinase (Fett *et al.*, 1992; Fett *et al.*, 1999, Fett *et al.*, 2000; Macedo and Pio, 2005; Ferreira *et al.*, 2004) nevertheless, in the past decade several bacterial sources also been reported (Chen *et al.*, 2008; Chen *et al.*, 2010; Fett *et al.*, 1992a, 1992b; Sebastian and Kolattukudy, 1988). There are also reports that some of the plant pollen produces cutinase like enzymes (Maiti *et al.*, 1979).

2.2 *Thermobifida fusca*: an overview

Thermobifida fusca (formerly known as *Thermomonospora fusca*) is an aerobic, moderately thermophilic, filamentous soil bacterium that is a major degrader of plant cell walls in heated organic materials, such as compost heaps, rotting hay, manure piles, or mushroom growth medium (Bachmann *et al.*, 1999). Its extracellular glycoside hydrolases (cellulases and xylanases) have been studied extensively because of their thermostability, broad pH range (4 to 10), and high activity. It appears to degrade all major plant cell wall polymers except lignin and pectin and can grow on most simple sugars and carboxylic acids. It belongs to the phylum of *Actinobacteria* and was first isolated from decaying wood (Bellamy and Wilson, 1977). Apart from cutinase, *T. fusca* has been the source organism for isolating and studying multiple secreted cellulases and other carbohydrate-degrading enzymes (Ghangas and Wilson, 1988; Hu and Wilson, 1989). A recent genome sequence analysis by Lykidis *et al.*, (2006) showed that *T. fusca* has a single circular chromosome of 3,642,249 bp predicted to encode 3,117 proteins and 65 RNA species with a coding density of 85%. Genome analysis revealed the existence of 29 putative glycoside hydrolases in addition to the previously identified cellulases and xylanases. The analysis of proteome revealed an increased content of charged (Asp, Glu, Lys and Arg) versus polar (Asn, Gln and Thr) residues: This is common feature of organisms with optimum growth temperature higher than 55°C, thus making it one of the important sources of organisms for various industrial enzymes (Suhre and Claverie, 2003).

Several strains belonging to various *Thermobifida* species have been tested for their ability to hydrolyse the insoluble plant polyester cutin (Fett *et al.*, 1999). Recently, two cutin hydrolyzing enzymes were reported from *T. fusca* (Chen *et al.*, 2008) with superior thermostability, active in broad range of pH, organic solvent, and surfactant tolerance and

active on various synthetic substrates. There are also reports on production of *T. fusca* cutinase in wild type and recombinant organisms by shake flask, batch and fed-batch cultivation strategy (Chen *et al.*, 2011; He *et al.*, 2008; 2009; Su *et al.*, 2012; 2013) and evaluation of cutinase as a biocatalyst in several applications such as cotton scouring and polymer degradation (Acero *et al.*, 2011; Baker *et al.*, 2012; Eberl *et al.*, 2009; Heumann *et al.*, 2006; Zhang *et al.*, 2010). Table 2.1 summarizes some of the industrially important enzymes identified in *T. fusca* strain YX (Lykidis *et al.*, 2006).

Table 2.1 Summary of hydrolytic enzymes identified in *T. fusca* strain YX (modified from Lykidis *et al.*, 2006)

Locus Signal tag	Family	Enzyme description	Binding module
Tfu0082	CE_1	Acetyl xylan esterase	
Tfu2473	CE_9	6P-NAG deacetylase/6P-NAGal deacetylase	
Tfu2990	CE_3	Acetyl xylan esterase	Cellulose, CBM2 LBP
Tfu2788	CE_4	Acetyl xylan esterase/chitin deacetylase	Cellulose, CBM2
Tfu0584	GH_13	Amylase	
Tfu1345	GH_15	Amylase/dextranase	
Tfu1613	GH_31	Amylase/isomaltase	
Tfu0582	GH_13	Amylase/pullulanase	
Tfu0833	GH_13	Amylase/pullulanase	
Tfu0985	GH_13	Amylase/pullulanase	Starch, CBM20
Tfu2205	GH_77	Amylomaltase or 4-glucanotransferase	
Tfu2712	GH_5	Cellulase	Cellulose, CBM3
Tfu2176	GH_9	Cellulase (E4)	Cellulose, CBM2, CBM3
Tfu0580	GH_18	Chitinase	
Tfu1213	GH_11	Endo 1,4- β -D-xylanase	Cellulose, CBM2
Tfu0901	GH_5	Endocellulase (E5)	Cellulose, CBM2
Tfu0868	GH_18	Exochitinase	
Tfu1614	GH_95	Fucosidase	
Tfu0915	GH_2	Galactosidase	
Tfu1615	GH_42	Galactosidase	

Locus Signal tag	Family	Enzyme description	Binding module
Tfu0084	CE_14	GlcNAc-Pl deacetylase	
Tfu2130	GH_81	Glucanase	
Tfu1629	GH_1	Glucosidase/galactosidase	
Tfu2768	GH_4	Glucuronidase/galactosidase/ glucosidase	
Tfu2594	GH_23	Lysozyme	
Tfu2168	PL_1	Pectate-lyase (EC 4.2.2.2)	
Tfu2009		Rhamnogalacturonan lyase	Cellulose, CBM2
Tfu3044	GH_65	Trehalase/maltose phosphorylase	
Tfu0882		Triacylglycerol lipase/cutinase	
Tfu0883		Triacylglycerol lipase/cutinase	
Tfu2923	GH_10	Xylanase	Cellulose, CBM2
Tfu1616	GH_43	Xylanase/galactan galactosidase	
Tfu1612	GH_74	Xyloglucanase, endo- β -1,4- glucanase	Cellulose, CBM2
Tfu1607	GH_3	Xylosidase/acetylhexosaminidase	β -1,3-Glucan, CBM6
Tfu1627	GH_9	β -1,4-Endoglucanase (E1)	Cellulose/amorphous/ xylan, CBM2, CBM4
Tfu1074	GH_6	β -1,4-Endoglucanase (E2)	Cellulose, CBM2
Tfu0620	GH_6	β -1,4-Exocellulase (E3)	Cellulose, CBM2
Tfu1959	GH_48	β -1,4-Exocellulase (E6-celF)	Cellulose, CBM2
Tfu0937	GH_1	β -Glucosidase/cellobiase	
Tfu0900	GH_5	β -Mannanase (EC 3.2.1.78)	Cellulose, CBM2

2.3 Cutinases, lipases and esterases: an overview

Hydrolases are the class of enzyme that shows very wide substrate specificity. They can hydrolyse esters, amides, glycosides, peptides, ketonic substrates and halides. This versatile nature of the hydrolases made them one of the eminent industrial enzymes today. Esterases, lipases and cutinases are the group of biotechnologically important enzymes which share many structural properties as well as substrates in common. For instance, the enzymes which show esterase activity are also able to hydrolyse non-ester bonds, which raise an interesting argument on the terminology and classification of these enzymes (Junge and Kirsch, 1973).

Previously, this group of enzymes was classified according to their known substrate specificity. For example, esterases are group of enzyme that hydrolyses ester linkages in the presence of a water molecule. Generally, an esterase is specific for either the alcohol or the acid moiety of the substrate, but not for both (Fojan, 2000). A classification scheme proposed by Whitaker (1972), for esterases was based on their specificity for the acid moiety of the substrate, such as the carboxylic esterases which catalyses the hydrolysis of carboxylic acid esters. There are several categories present in esterases group viz., carboxyl esterases, aryl esterases, acetyl esterases, cholin esterases and cholesterol esterases, which catalyses the hydrolysis of carboxylic acid ester, aryl acid ester, acetyl esters and cholesterol esters, respectively. On the other hand, lipases also belong to this group of hydrolytic enzymes. These enzymes were classified on the basis either of their substrate specificity or place of enzymatic action (Carriere *et al.*, 1998). In general, it was observed that esterases preferentially hydrolyse ester bonds of shorter chain fatty acids, whereas lipases display their activity towards long chain fatty acid esters. It appears that one of the key factors contributing towards the substrate specificity is the physical state of the substrate. Long-chain fatty acids are poorly soluble (emulsions) or insoluble thus, the lipase has capacity to identify an insoluble or densely aggregated substrate in their emulsions. Since, lipases are active towards aggregated substrates; lipase activity is directly correlated with the total substrate area and not with the substrate concentration (Verger, 1998). Esterase activity is found to be highest towards more water soluble substrates. Numerous esterases and lipases have been studied over the last decades. X-ray crystallographic, as well as NMR studies have provided information at the atomic level about the 3D-structure of these proteins. The growing knowledge of protein 3D-structures has prompted an attempt to classify proteins according to

their fold. Hydrolases are found in the α/β fold group, also called the α/β hydrolase fold (Scharg and Cygler, 1997). All lipases as well as the majority of the esterases share this fold. The triacylglycerol lipases contain all fungal, bacterial and pancreatic lipases. On the other hand, the esterases are split into groups such as the cutinase group, acetylcholine esterase group, all having an α/β hydrolase fold. The lid-like structure that many lipases display has triggered the formulation of mechanistic models involving lid-motion (Derewenda *et al.*, 1992). The putative spatial movement of the lid, associated with catalysis will expose hydrophobic patches, which in the closed lid conformation are solvent inaccessible. This phenomenon of increase in activity at the lipid-water interface due to spatial movement of lid is known as an interfacial activation. Nevertheless, esterases (and a few lipases) do not display a lid structure, thus they are active on soluble substrates in aqueous media. Besides the above mentioned esterases, and lipases, the third class of enzymes is cutinase which shows no significant structural rearrangements upon binding to a substrate analogue, representing an important feature of cutinase, which is probably shared only by *Candida antarctica* lipase B (Longhi *et al.*, 1996). In contrast to the lipases, activity of which is greatly enhanced in presence of a lipid–water interface, cutinases do not display or display little interfacial activation, being active on both soluble and emulsified triglycerides (Verger and Haas, 1976). Thus, sometimes cutinases are referred as connecting link between lipase and esterase.

2.4 Cutinase sources and production

Several fungal, bacterial and some plant sources have been reported to produce cutinase (Fett *et al.*, 1992a; Fett *et al.*, 1992b; Fett *et al.*, 1999; Fett *et al.*, 2000; Kim *et al.*, 2003; Lin and

Kolattukudy, 1980; Maiti *et al.*, 1979; Purdy and Kolattukudy, 1975; Pio and Macedo, 2007; Sebastian and Kolattukudy, 1988; Sebastian *et al.*, 1987). However, the main problems with the plant sources are that the cultivation, maintenance and isolation of enzyme in large scale are much more complicated than microbes. Table 2.2 summarizes the various organisms producing cutinases and Fig. 2.2 shows the phylogenetic relationship of some of the cutinase producing organisms generated based on the 18S ribosomal RNA.

Table 2.2 Cutinase Producing micro-organisms

Source	Genus	Species	Reference
Bacteria	<i>Corynebacterium</i> sp.,		Sebastian <i>et al.</i> , 1987
	<i>Pseudomonas</i>	<i>aeruginosa</i>	Fett <i>et al.</i> , 1992b
		<i>cepacia</i>	Sebastian <i>et al.</i> , 1987, Dutta <i>et al.</i> , 2013
		<i>mendocina</i>	Kim <i>et al.</i> , 2003
		<i>pseudoalcaligenes</i>	Fett <i>et al.</i> , 1992b
		<i>putida</i>	Sebastian and Kolattukudy, 1988
		<i>Syringae</i> pvar <i>tomato</i>	Bashan <i>et al.</i> , 1985
	<i>Streptomyces</i>	<i>badius</i>	Fett <i>et al.</i> , 1992a
		<i>Scabies</i>	Lin and Kolattukudy, 1980
	<i>Thermoactinomyces</i>	<i>vulgaris</i>	Fett <i>et al.</i> , 2000
	<i>Thermobifida</i>	<i>alba</i>	Ribitsch <i>et al.</i> , 2011
		<i>cellulolysitica</i>	Acero <i>et al.</i> , 2011
		<i>fusca</i>	Chen <i>et al.</i> , 2008; Fett <i>et al.</i> , 1999
	<i>Thermomonospora</i>	<i>acidiscabies</i>	Fett <i>et al.</i> , 1992a
Fungi	<i>Alternaria</i>	<i>brassicicola</i>	Trail and Koller, 1993
	<i>Ascochyta</i>	<i>rabiei</i>	Tenhaken <i>et al.</i> , 1997
	<i>Aspergillus</i>	<i>nidulans</i>	Denise Castro-Ochoa <i>et. Al.</i> , 2011
		<i>oryzae</i>	Liu <i>et al.</i> , 2009
	<i>Botryotinia</i>	<i>fuckeliana</i>	Gindro <i>et al.</i> , 1999; Skamnioti <i>et al.</i> , 2008
	<i>Botrytis</i>	<i>cinerea</i>	van der Vlugt-Bergmans <i>et al.</i> , 1997
	<i>Colletotrichum</i>	<i>gloeosporioides</i>	Chen <i>et al.</i> , 2007
		<i>kahawae</i>	Chen <i>et al.</i> , 2007
		<i>lindemuthianum</i>	Rispoli and Shah, 2008

Source	Genus	Species	Reference
	<i>Coprinopsis</i>	<i>cinerea</i>	Kontkanen <i>et al.</i> , 2009
	<i>Emericella</i>	<i>nidulans</i>	Skamnioti <i>et al.</i> , 2008
	<i>Fusarium</i>	<i>graminearum</i>	Ettinger <i>et al.</i> , 1987
		<i>oxysporium</i>	Pio and Macedo, 2007
		<i>roseum culmorum</i>	Soliday and Kolattukudy, 1976
		<i>Solani pisi</i>	Purdy and Kolattukudy, 1975
	<i>Gibberella</i>	<i>zeae</i>	Skamnioti <i>et al.</i> , 2008
	<i>Glomerella</i>	<i>cingulata</i>	Nyon <i>et al.</i> , 2009
	<i>Humicola</i>	<i>insolens</i>	Ronkvist <i>et al.</i> , 2009
	<i>Magnaporthe</i>	<i>grisea</i>	Skamnioti <i>et al.</i> , 2008
	<i>Monilinia</i>	<i>fruticola</i>	Wang <i>et al.</i> , 2002
	<i>Penicillium</i>	<i>citrinum</i>	Liebminger <i>et al.</i> , 2007
	<i>Rhizoctonia</i>	<i>solani</i>	Parker and Köller, 1998
	<i>Trichoderma</i>	<i>harzianum T34</i>	Rubio <i>et al.</i> , 2008
	<i>Venturia</i>	<i>inaequalis</i>	Trail and Koller, 1990
Pollen	<i>Arabidopsis</i>	<i>thaliana</i>	Takahashi <i>et al.</i> , 2010
	<i>Tropaeolum</i>	<i>majus</i>	Maiti <i>et al.</i> , 1979
Yeast	<i>cryptococcus spp.</i>		Masaki <i>et al.</i> , 2005
	<i>Neurospora</i>	<i>crassa</i>	Skamnioti <i>et al.</i> , 2008

2.4.1 Cutinase production in wild type organisms

There are many cutinase producing fungi and bacteria isolated and screened till date (Table 2.2). However, only few of them are studied further in context of production, characterization and applications. As per production in wild strain is concerned, microbial cutinases are generally produced by submerged culture (Fett *et al.*, 1992; Fett *et al.*, 1999; Fett *et al.*, 2000; Ferreira *et al.*, 2004; Macedo and Pio, 2005) albeit, solid state fermentation has also been reported (Macedo and Fraga, 2007). The production of wild type cutinase is influenced by several factors which include the type and concentration of carbon and nitrogen sources, culture pH and temperature, and dissolved oxygen concentration (Du *et al.*, 2007). It was also reported that cutin can be used as a carbon source and/or an inducer for achieving high level

of cutinase yield (Fett *et al.*, 1992; Fett *et al.*, 1999; Fett *et al.*, 2000; Lin and Kolattukudy, 1978).

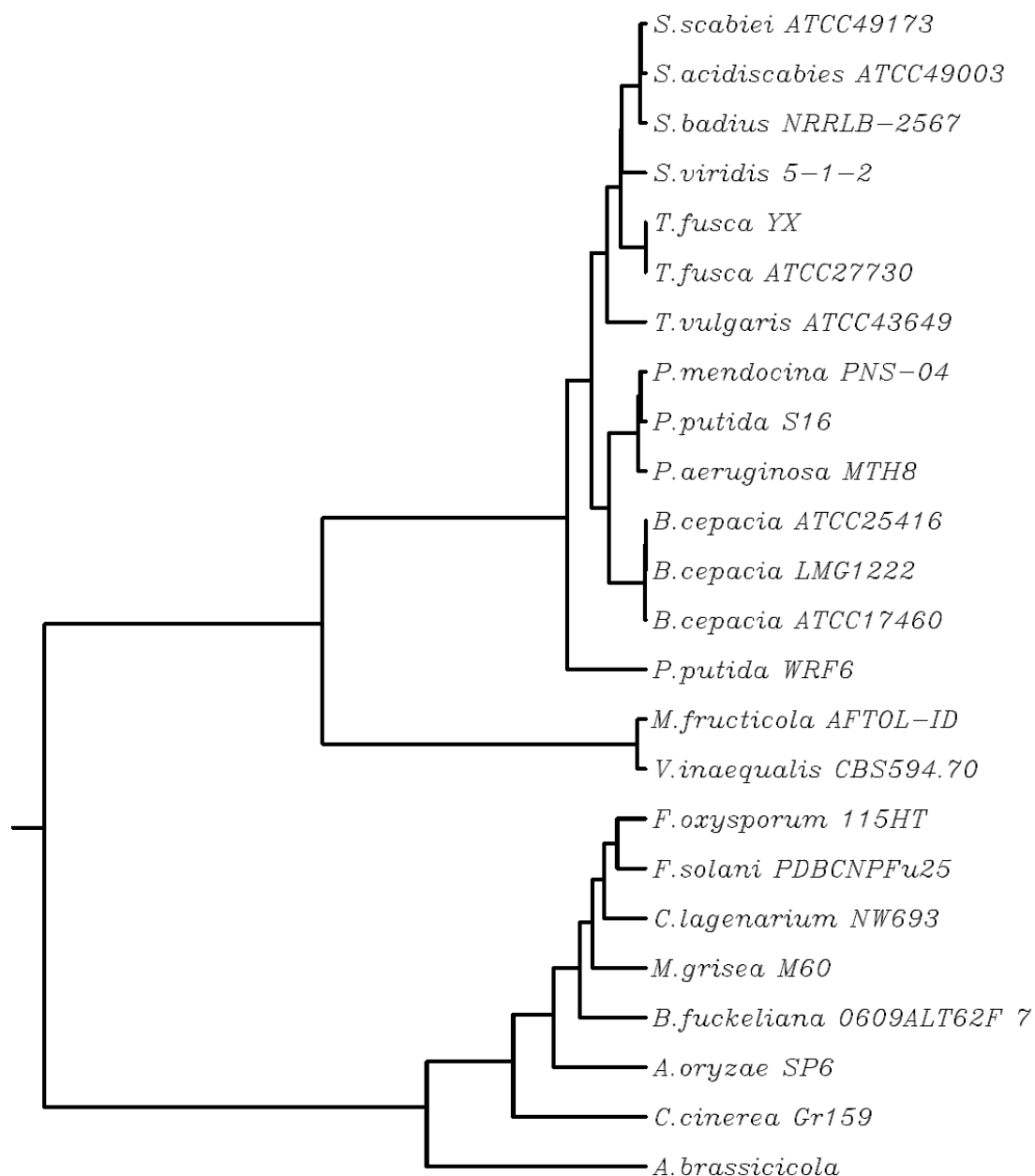


Fig. 2.2 Phylogenetic relationship of the cutinase producing organisms. The phylogenetic tree was constructed using UPGMA method on online construction tool from ExPASy online server (http://www.expasy.org/phylogeny_evolution).

Lin and Kolattukudy (1978) reported that cutin hydrolysate induces the secretion of an extracellular cutinase by *F. solani* f. sp. *pisi*. They observed that the rate of cutinase

production depends on the amount of cutin hydrolysate added to the medium containing glucose and saturation was achieved at $80 \mu\text{g mL}^{-1}$ of cutin. Glucose was found to be a repressor for the production of cutinase. The production of cutinase was induced by cutin hydrolysate and exogenous labeled phenylalanine was found to be incorporated into cutinase. The study also shown that the induction of cutinase by cutin hydrolysate was not inhibited by actinomycin D and stimulated (=100%) by cordycepin. Lin and Kolattukudy (1978) reported that extracellular cutinase production induced by cutin hydrolysate was a cycloheximide-sensitive process. Experiments with derivatives and analogues of ω -hydroxy C16 acid indicated that the free hydroxyl group at the ω -position was the most important factor for induction of cutinase activity. *n*-Aliphatic primary alcohols with 14 or more carbon atoms have displayed induction of cutinase, among which *n*-C16 was found to be the most effective inducer (Lin and Kolattukudy, 1978). These results are in the favor of the fact that cutin monomers act as a chemical signal, which induces extracellular cutinase production. Studies have been conducted on the production of cutinase with other substrates (viz., olive oil, soy oil, sunflower oil and palm oil) besides cutin (Pio and Macedo, 2007). A high level of enzymatic activity (from 11 to $22.68 \mu\text{Mol min}^{-1} \text{mL}^{-1}$) was observed by Pio and Macedo (2007) after 48 h of fermentation from *F. oxysporium* using flaxseed oil as a carbon source. This flaxseed oil is a low cost carbon source as compared to cutin. Rispoli and Shah (2007) observed that the K_2HPO_4 had a positive influence on the production of cutinase by *C. lindemuthianum* and MgSO_4 had a minimal effect on the production of cutinase. Due to change in the chemical activity of a particular chemical species like substrate concentration in the neighborhood of a microbial cell may cause any given physiological variable of the cell like specific growth rate to increase, to decrease, to fluctuate, or to show no apparent change.

When the concentration of substrate is continuously varied from low to high values in an aqueous media of microbes, several patterns of dependence of physiological variables may be observed. At very low concentrations, the effect was negligible on gross measures of metabolic activity, such as specific growth rate, respiration rate, rate of protein synthesis, etc. If the nutrient is essential for growth or the production of enzyme of interest, the initial value of the physiological parameter at very low concentrations may be zero or even negative if it represents a rate function. By increasing concentration of that nutrient, the physiological parameter (growth or enzyme production) will increase due to stimulation of the metabolism of the micro-organisms. Finally, a concentration of the nutrient is reached at which further increase in concentrations do not increase the physiological parameter. This occurs at this stage due to either there is limited supply of some other environmental factor or cells themselves have reached their own limit for the present culture conditions. Further increasing the concentration of the chosen nutrient or substrate will eventually cause decrease (the nutrient or substrate inhibition) in physiological parameter. This type of microbial behavior has vital role in the metabolic activities of microorganisms in industrial fermentation, biological waste treatment processes, infected plants or animals, and in other parts of the biosphere. In this case, reliable and tractable mathematical models relating the physiological state of microorganism to the state of its surroundings facilitates the analysis of such processes. So, there are several models reported to analyze the relation between specific growth rate and substrate concentrations. Among these, some models have focused either on the stimulatory range of concentrations or on inhibitory concentrations or analyze both stimulatory and inhibitory domains of substrate action.

2.4.2 Cutinase production in recombinant organisms

In order to obtain an efficient and low cost production, several methods have been assessed for the production of cutinases in recombinant systems from various sources, such as *E. coli*, *S. cerevisiae* and *P. pastoris*.

High level production of recombinant cutinase from *F. solani f. pisi* was reported in *E. coli* WK6 (Lauwereys *et al.*, 1991) and *P. pastoris* (Kwon *et al.*, 2009). Cultivation of recombinant *S. cerevisiae* SU50 for the production of cutinase in batch, fed batch and continuous culture was studied by Ferreira *et al.*, (2004). They found that the fed batch cultivation gives higher productivity and effective purification compared to other strategies when fed with glucose and galactose. A *F. solani f. pisi* cDNA library containing the cutinase gene was also expressed behind the signal peptide, alkaline phosphatase (*phoA*) in order to direct the cutinase to the periplasm region in *E. coli* WK6 host (Lauwereys *et al.*, 1991). Backlund *et al.*, (2008) used *E. coli* K12 strain 0:17 to express a cutinase from *F. solani pisi* cloned into a high copy number plasmid pK4ZZ having OmpA signal peptide. They investigated how the specific feed rate of glucose during fed batch cultivation can be used to influence the cell physiology which leads to the retention of selected periplasmic products. The study showed that an increased feed rate led to a higher production rate but also to an increased specific leakage, which reduced the periplasmic retention. Sagt *et al.*, (1998) achieved the yield of 25 mg per g (DCW) by fed batch fermentation for the *F. solani* cutinase expressed in *S. cerevisiae* under control of the *GAL7* promoter. An efficient production system for *F. solani f. pisi* cutinase in *S. cerevisiae* was developed, and point mutations were introduced into the cutinase gene to optimize lipase activity (Sagt and Verrips, 1995). The original signal peptide was replaced by a yeast β -D-fructofuranosidase peptide, and the gene

was cloned in a MIRY plasmid containing the Pgal7 promoter (Longhi *et al.*, 1996; Sagt and Verrips, 1995). The transformant was grown in a fed-batch culture and extracellular cutinase was accumulated in the fermentation medium. Other studies on cloning of the cutinase gene from *F. solani f. pisi* have also been reported in a Charon 35 vector (Soliday *et al.*, 1989). Further, studies involving *Aspergillus* strains and *S. cerevisiae* harboring other plasmids have also been reported. A cDNA from *F. solani f. sp. Pisi* cutinase was cloned and expressed in *S. cerevisiae* SU50 and *A. awamori* using expression vector pUR7320 and pUR7382 (van Gemeren *et al.*, 1995). The efficiencies of secretion of the wild-type (CY000) and hydrophobic mutant (CY028) cutinase (created by site directed mutagenesis) by the *S. cerevisiae* strain SU50 were compared. The CY000 strain secreted up to 25 mg cutinase g⁻¹ (dry weight), whereas CY028 secreted only 2 mg g⁻¹. Pulse-chase experiments were showed that the introduction of two exposed hydrophobic patches in cutinase resulted in higher immunoaffinity with immunoglobulin (Ig) heavy-chain binding protein (BiP), which could cause the retention of cutinase in the endoplasmic reticulum of the CY028 mutant (Sagt *et al.*, 1998). *A. japonicus* strains have also been used as host cells, with these containing a recombinant DNA sequence encoding cutinase operably linked to a fungal promoter (Berka *et al.*, 1995). A cutinase cDNA synthetic derivative was expressed in *A. awamori* AW4-20 using an *Awamori* endoxylanase II (ex1A) gene promoter and terminator. Fusions of the cutinase gene to the endoxylanase II promoter with four different pre-pro- or pre-sequences were constructed from synthetic oligonucleotides. The multicopy strains showed a 6- to 12-fold-increase in production of extracellular cutinase relative to the single-copy strains (van Gemeren *et al.*, 1995; van Gemeren *et al.*, 1996). Recently, Chen *et al.*, (2008) expressed genes encoding the mature forms of Tfu 0882 and Tfu 0883 within vector pET20b(+)

expression, containing a C-terminal (His)₆ tag and signal peptide PelB, which allows the heterologously expressed proteins to be secreted. The pNPB hydrolyzing activity in the culture supernatant of transformed *E. coli* cells harboring plasmid pET20b-Tfu 0883 was 180 units mL⁻¹, which was 9.5-fold higher than that of cutin-induced *T. fusca* cells and *E. coli* cells harboring pET20b-Tfu 0882 plasmid was 69 units mL⁻¹, which was 3.6-fold higher than that of cutin-induced *T. fusca* cells. In another work (Chen *et al.*, 2011), glycine and sodium taurodeoxycholate (TDOC) were used to increase the secretion of recombinant *T. fusca* cutinase expressed in *E. coli* BL21 (DE3). The study showed that supplementation of 100 mM glycine and 1 mM TDOC in the TB medium remarkably decreased the periplasmic storage of recombinant cutinase and the activity in the culture medium reached to 146 and 149 U mL⁻¹ after 54 h of culturing, respectively. The influence of number of signal peptide cleavage site on extracellular production of recombinant cutinase was also studied for recombinant cutinase from *T. fusca* (Chen *et al.*, 2011). In their study, recombinant *E. coli* that expressed precursor recombinant cutinase in pET vector with one and two signal peptide cleavage sites were constructed and expressed. The investigation showed that the increase of signal peptide cleavage site can improve the extracellular production of recombinant cutinase in *E. coli*. Su *et al.*, (2012) also have worked towards the extracellular secretion of recombinant cutinase Tfu 0883 from *T. fusca* in *E. coli* BL 21 (DE3). In the study, *T. fusca* cutinase was fused with the specific signal peptide of α -hemolysin secretion system and expressed in *E. coli* BL21 (DE3). In addition, HlyB and HlyD, strain-specific translocation components of α -hemolysin secretion system were co-expressed to facilitate the enzyme expression. The result showed that the cultivation of this engineered cells had an extracellular secretion of 334 U mL⁻¹,

which was 2.5 times higher than that of type II secretion system routinely used in *E. coli* BL21 (DE3) expression system.

2.5 Purification of cutinases

The purification of cutinases from wild type microbial sources have involved separation of the culture from the fermentation broth, selective concentration by precipitation using ammonium sulfate or organic solvents such as chilled acetone. The crude enzyme is then subjected to chromatography (usually affinity, ion exchange and/or gel filtration). A number of reports are available on the purification of cutinases from a range of microorganisms (Almeida *et al.*, 1998; Chen *et al.*, 2007; Du *et al.*, 2007; Sebastian and Kolattukudy, 1988) using various purification techniques (Fig. 2.3). The recent advances in recombinant DNA technology and the use of affinity tags have proven a very efficient and economically feasible technique for purifying an enzyme.

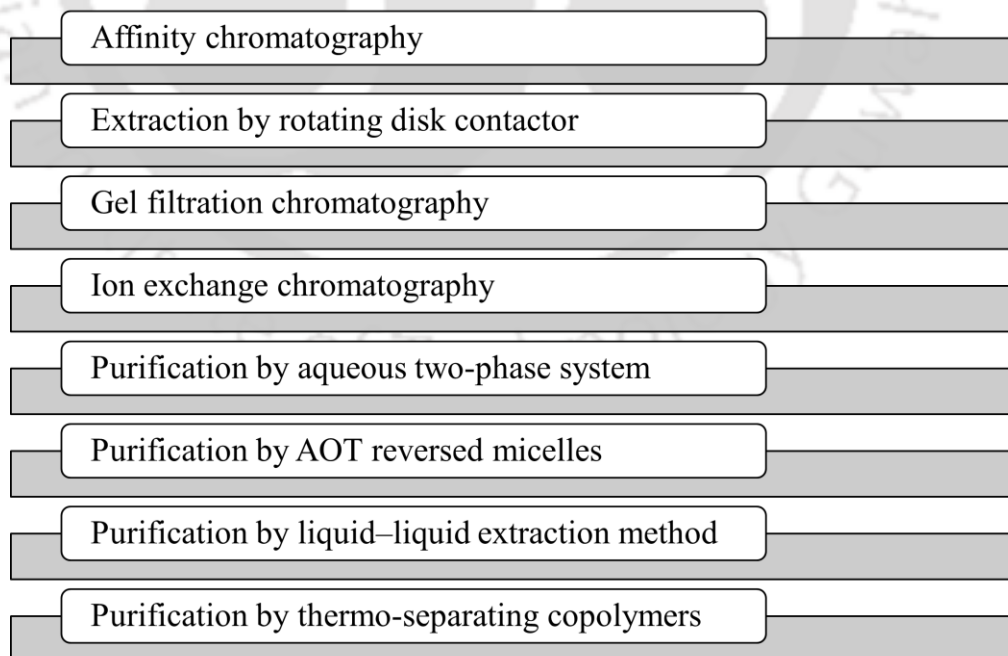


Fig. 2.3 Strategies employed for purification of cutinases

The compounds such as 3-n-octylthio-1,1,1-trifluoro-2-propanone (OTFP) (Abdel-Aal *et al.*, 1985), 8-mercapto-1,1,1-trifluoro-2-octanone (MTFO) (Abdel-Aal *et al.*, 1986) and 3-(4-mercaptobutylthio)-1,1,1-trifluoro-2-propanone (MBTFP) (Shiotsuki *et al.*, 1994) have been used as affinity ligands to purify juvenile hormone esterases from several insect species. Due to the small difference in the I_{50} of OTFP and MBTFP (Shiotsuki *et al.*, 1994) for *Monolinia fructicola* cutinase, OTFP was chosen to elute the affinity column (Wang *et al.*, 2000). Another compound of interest in view of affinity chromatography for cutinase purification is Trifluoromethyl ketones (TFK) (Wang *et al.*, 2000). This is due to their similarity with the transition state of ester hydrolysis by forming a hemiketal link with the serine at the enzyme's active site.

Cutinase can also be purified by aqueous two-phase system, depending on its partition coefficient in the two phases. Almeida *et al.*, (1998) have reported that the efficient and inexpensive cutinase purification could be achieved in PEG–starch derivatives ATPSs (aqueous two-phase systems). They showed that the concentration and type of salts have significant effect on the cutinase partition behaviors. In the absence of salts, the purification of cutinase was found to be inefficient in PEG-HPS (polyethylene glycol-hydroxypropyl starch) systems. Partition behavior seems to be influenced minimally by changing PEG average molecular mass (from 1000 to 8000). Liquid–liquid extraction of proteins using reversed micelles is a very simple procedure to isolate and purify lipases. In the first step, proteins are microencapsulated into the water pool of the surfactant aggregates. Then, the solubilized proteins are back extracted into a new aqueous phase. The extraction and back extraction of pure cutinase was established by Carneiro-da-Cunha *et al.*, (1996b) using the anionic surfactant, AOT, in isooctane for *F. solani* cutinase. The amount of cutinase that can

be solubilized in the reverse micelles is strongly dependent on the pH, the maximum obtained for pH 7.2 (97% cutinase solubilization), which is probably related to the isoelectric point (pI). However, pH 9 was the value selected for use in further extraction, due to the higher activities displayed by the enzyme at this pH value. Cutinase has been recovered from the reverse micellar phase into a 50 mM Tris-HCl-buffered solution at pH 9 with 100 mM KCl at 20°C. The extraction of cutinase directly from the fermentation medium and from suspended cells by the use of AOT reversed micelles also suggests that the extractant plays an important role in the recovery of the intracellular cutinase, probably due to cell disruption caused by the surfactant. The application of the AOT reversed micellar system to the extraction of cutinase allowed activity yields in the range of 5% to 50% with purification factors varying from 1.2 to 10.2 from fermentation medium to protein solution after removal of cell debris. Extraction columns with rotary motion, like the perforated rotating disk contactor, allow continuous extraction and are alternatives to the conventional liquid-liquid extraction equipment. The main advantage of this system is the higher mass transfer rate that leads to higher separation efficiencies. This system was applied successfully to the extraction of a pure cutinase solution from disrupted *E. coli* cells. The optimal conditions for the direct extraction of the enzyme from media containing cell debris led to an extraction yield of 54.4% (Carneiro-da-Cunha *et al.*, 1994a; Carneiro-da-Cunha *et al.*, 1996a). A single-step process for the recovery and partial purification of cutinase from *E. coli* cell debris, using an ATPS of 25% w/w PEG-1000 and 10% w/w sodium phosphate (pH 4.8), was developed to replace osmotic shock and acid precipitation (Sebastiao *et al.*, 1997). The yield and purification degree obtained were 91% and 4.1, respectively that corresponds to an increase of 14% in yield. Moreover, the ATPS separation process was completed in a few min (20 min), in comparison with the time-

consuming, expensive multistep process. A model to predict the cutinase partition coefficients in this biphasic system was developed by Sebastiao *et al.*, (1997). This model accounts for both charge-independent and electrostatic effects. The partition coefficients were satisfactorily predicted in PEG-1000 and phosphate ATPS in the pH range of 6.0 to 9.0. More recently, the recovery of cutinase has been performed with alternative thermo-separating copolymers, namely ethylene oxide (EO) and propylene oxide (PO). These polymer solutions demix into two macroscopic phases when heated above a certain temperature. One of the phases is enriched in the polymer and the other is depleted. The EOPO copolymers can form two-phase systems with other polymers, like Reppal or dextran, and salts, suitable for extraction of enzymes. One of the main advantages of using these polymers in ATPS is the possibility of heating the EOPO-rich phase above the cloud-point temperature, enabling both polymer recycling and its removal from the target product solution (Cunnah *et al.*, 1998). Merz *et al.*, (2009) purified cutinase from *Coprinopsis cinerea* by foam fractionation method. They recovered esterase type enzymes from culture supernatants of *C. cinerea*. Beside the preservation of the enzymatic activity, no solvents or other substances, which have to be separated in additional work-up steps, had to be added during the foam fractionation process. Further advantages of foam fractionation comprise its mechanical and operational simplicity and therefore, low investment and operating costs. Optimization of various process parameters, such as pH, gas flow rate velocity, temperature and the foaming period, offers the potential to adapt the foaming process to various enzyme sources and matrices. Taken together, foam fractionation is an ecologic and economic method with the potential to open new alternatives for efficient and quick enzyme purification strategies. On the 70 mL scale, Merz *et al.*, (2009) observed that the recovery of activity of 79% with an enrichment factor of

10.5 at pH 7 and 20 mL air min⁻¹ within 15 min. Another widely used technique for purification of cutinase is ion-exchange chromatography. Chen *et al.*, (2007) used a two-stage chromatography process for purifying cutinases from *Colletotrichum kahawae* and *Colletotrichum gloeosporioides*. They purified cutinases using a DEAE matrix (weak anionic exchanger) at pH 8.5 as the first chromatographic step devoid of proteins with no NPBase (p-Nitrophenyl butyrate) activity and also separated from the other 40 kDa proteins. An additional chromatographic step using an SP matrix (strong cationic exchanger) bound additional proteins with no activity to the matrix and allowed purification of cutinase to electrophoretic homogeneity. Cutinases purified in this method should have a pI within the range 7.5–8.5 so that they neither bound to DEAE at pH 8.5 nor to SP at pH 7.6. Sebastian and Kolattukudy (1988) purified cutinase from the culture filtrate by acetone precipitation followed by chromatography on DEAE-cellulose, QAE-Sephadex, Sepharose 6B, and Sephadex G-100. The enzyme was found to be a monomer by SDS-PAGE and gel filtration of the native enzyme obtained through a Sephadex G-100 column. Sebastian and Kolattukudy (1988) could obtain 200 fold purified protein with 3% yield. Chen *et al.*, (2007) purified cutinases from *C. kahawae* and *C. gloeosporioides* in six steps and achieved purification fold of 15.5 and 16.5, respectively. Gindro and Pezet (1999) purified cutinase from *Botrytis cinera* in four steps with 2.9% yield and 66.5 purification fold.

2.6 Cutinase structure

To date, five cutinase crystal structures had been resolved, this includes *F. solani*, *A. oryzae*, *G. cingulate*, *Cryptococcus* and recently, *T. alba* (Kitadokoro *et al.*, 2012; Kodama *et al.*, 2009; Liu *et al.*, 2009; Longhi *et al.*, 1997; Martinez *et al.*, 1992; Nyon *et al.*, 2009).

Fusarium solani f. pisi cutinase is a 196 amino acids enzyme with a molecular weight of 22 kDa. The three dimensional structure of *F. solani f. pisi* cutinase was solved at 1.6 Å resolution (Martinez *et al.*, 1992) and the resolution was extended at 1.0 Å (Jelsch *et al.*, 1998; Longhi *et al.*, 1997). *F. solani* cutinase is a compact single domain molecule of 45×30×30 Å³ in size. Its structure includes a slightly twisted five-parallel-stranded β-sheet covered by four helices on either side of the sheet (Fig. 2.4 A). Two flexible loops including residues 80–87 and 180–187 with hydrophobic amino acids constitute the binding site of cutinase with catalytic triad Ser120, Asp175, and His188, accessible to the solvent. The stretch Gly-Tyr-Ser-Gln-Gly containing the active site Ser120 has even stronger homology with the consensus sequence Gly-(Tyr or His)-Ser-X-Gly commonly present in lipases. Two disulfide bridges exist in cutinase: one between Cys31 and Cys109 that helps to the stabilization of the global molecular folding and the other between Cys171 and Cys178. Reduction of the disulfide bridges results in complete inactivation of the enzyme. The above mentioned loops 80-87 and 180-188, bearing hydrophobic amino acids (Leu81, Gly82, Ala85, Leu86, Pro87, Leu182, Ileu183 and Val184), may constitute the interfacial binding site (Martinez *et al.*, 1992). Despite the existence of two side chain bridges of amino acids Leu81 and Val184, and Leu182 and Asn84, the catalytic serine of cutinase is not buried under surface loops, but is accessible to solvent and substrate.

Cutinases differ from classical lipases, as they do not exhibit interfacial activation. The absence of a flap, masking the active-site serine, as in other lipases, probably explains why cutinase is not activated by the presence of interfaces. The binding of cutinase to interfaces seems not to require a main-chain rearrangement, as in the case of lipases, but only the reorientation of few lipophilic side chains, for example Leu81 and Leu182, that play the role

of a 'mini' flap. Another important feature on cutinase structure that differ from lipase is that the oxyanion hole is pre-formed in cutinase instead of being induced by ligand binding and seems to be stabilized by cutinase Ser42 side chain (Nicolas *et al.*, 1996).

The crystal structure of *A. oryzae* resolved at 1.75 Å (Liu *et al.*, 2009) revealed that it is a monomeric protein with an α/β fold hallmarked by a central β -sheet of 5 parallel strands surrounded by 10 α -helices. As a member of the α/β hydrolases, this enzyme possesses an active site composed of the catalytic triad residues Ser126, Asp181, and His194 (Fig. 2.4 B). The catalytic site is surrounded by the two hydrophobic surfaces composed of residues 87-93 within helix 3, and residues 186-194 that represents the loop between helices 9 and 10 as well as the first 3 residues of the latter. *A. oryzae* cutinase bears an oxyanion hole composed of Ser48 and Gln127 backbone amides that are critical in polarizing the ester bond of the substrate and stabilizing the transition state of the formed substrate oxyanion (Fig. 2.4 B). The *A. oryzae* structure contains a unique disulfide bond between Cys63 and Cys76 that ties helix 2 to strand 2 of the central β -sheet. This disulfide bond has not been previously reported for any cutinase or hydrolase structures (Liu *et al.*, 2009). The other two disulfide bonds, Cys37-Cys115 connecting the loop between helix 1 and strand 1 with the loop between helix 4 and strand 3 and Cys177-Cys184 linking the loop following strand 5 to helix 9, are well-conserved in cutinase structures, including that from *F. solani*. Sequence analysis suggests that this disulfide bond is unique for the cutinases from the *Aspergillus* family (sharing sequence identity of 50-77% with *A. oryzae*) and a few other filamentous fungi, such as *Neosartorya fischeri* (53% sequence identity) and *E. nidulans* (52% sequence identity). The cutinases from *F. Solani* and *G. cingulata* represent another group of filamentous fungi that do not have this special disulfide bond, although they share about 50% sequence identity with

the *A. oryzae* enzyme (Liu *et al.*, 2009). Upon overlay, the *A. oryzae* and *F. solani* structures have a similar fold with an overall rms deviation of 1.02 Å, main chain deviation of 0.87 Å, and Cα deviation of 0.84 Å. However, comparison of the two sequences reveal that the *A. oryzae* cutinase is shorter than that of *F. solani* as exhibited by smaller loops in the N and C-terminal regions, the N-terminal region (residues Thr26 to Asp30), the loops in between helix 1 and strand 1 (residues Gly35 to Pro49), as well as helix 2 and strand 2 (residues Ser71 to Asp73), helix 10 (residues Ser199 to Asp203), and C-terminal residues beyond helix 10 based on the *A. oryzae* structure deviate significantly from that of *F. solani* structure (Liu *et al.*, 2009).

G. cingulata cutinase structure was solved at 1.9 Å by Nyon *et al.*, (2009). Analysis of these structures reveals that it shares 50% fold and sequence identity with *F. solani* cutinase (Fig. 2.4 C). The catalytic triad (Ser136, Asp191, and His204) adopts an unusual configuration with the putative essential His204 swung out of the active site into a position where it is unable to participate in catalysis, with the imidazole ring 11 Å away from its expected position. Such a model implies that the 196–205 loop must undergo a significant conformational rearrangement in order to form the active state of the enzyme.

Recently crystal structure of a cutinase (Est119) from *T. alba* AHK119 was determined at a resolution of 1.76 Å by Kitadokoro *et al.*, (2012). Overall structure of Est119 displays a typical α/β-hydrolase fold consisting of a central twisted β-sheet of nine β-strands that are flanked by nine α-helices. Est119 adopts a three-layer α/β sandwich fold, the central β-sheet being flanked by the α-helices α1, α2, α7, α8 and α9 on one side and α3, α4, α5 and α6 on the other. Catalytic site consists of Ser169, His247 and Asp215, which is located on the loops between the β-sheets and helices (Fig. 2.4 D). The oxyanion hole is formed by the main chain

amides of Met170 and Tyr99. There is a disulfide bond present in the monomer (Cys280-Cys298) in the C-terminal region. The overall secondary structure of Est119 is very similar to that of *S. exfoliatus* lipase (SEL) (Kitadokoro *et al.*, 2012) with highest Z-score of 46.2, RMSD of only 0.7 Å for 256 C α atoms and 63% sequence identity.

The overall structural studies reveals that all the cutinases belong to the class of serine esterases and to the super family of the α/β hydrolases in which the nucleophilic serine is located at the center of an extremely sharp turn between a β -strand and an α -helix. They have one or more disulfide bridges among which one is essential for the catalytic activity. They show catalytic machinery similar to those present in serine proteases. They are characterized by the catalytic triad Ser, His, Asp residues, and by an oxyanion binding site that stabilizes the transition state *via* hydrogen bonds with two main chain amide groups (Nicolas *et al.*, 1996).

2.7 Biochemical properties of cutinase

In order to optimize the enzyme performance under process conditions, to explore the possibilities of catalytic efficiency on various substrates, to make an enzyme economically more feasible and efficient and to enhance the shelf-life of the enzyme, the characteristics of a purified enzyme must be studied very well. Characterization of an enzyme generally includes studying the molecular properties, substrate specificity, physical and chemical parameters influencing the catalysis (pH, temperature *etc.*), tolerance to various effectors such as detergents, metal ions, inhibitors, organic solvents *etc.*

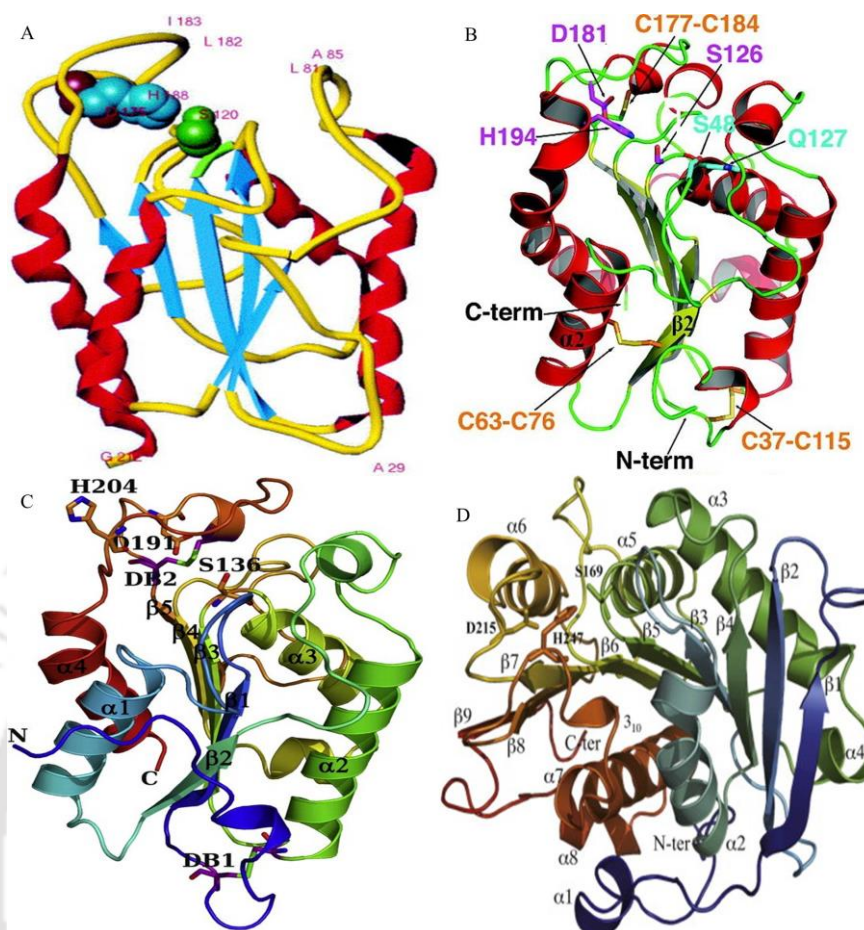


Fig. 2.4 Structure of *F. solani* (A), *A. oryzae* (B), *G. cingulata* (C) and *T. alba* cutinase with the helices, strands, and catalytic residues [Adopted from Longhi and Cambillau, 1999 (A) Liu *et al.*, 2009 (B), Nyon *et al.*, 2009 (C) and Kitadokoro *et al.*, 2012 (D)]

2.7.1 Molecular properties

In general molecular weight of most of the fungal cutinase are reported to be around 20-26 kDa (Koller and Parker, 1989). Four different fungal cutinases were identified by Chen *et al.*, (2007) out of which two were of 21 kDa and other two were of 40 kDa. The amino acid compositions of fungal cutinases from five different strains were compared and discussed in detail by Kollar and Parker (1989). The amino acid composition of bacterial cutinase has been studied in detail by Sebastian *et al.*, (1987) and was reported to be distinctly different from that of fungal or plant cutinases. However, for any cutinase, this holds true that unlike most

lipases; the catalytic serine is not buried under an amphipathic loop but is accessible to solvent and substrate. This explains why cutinase does not require interfacial activation (Carvalho *et al.*, 1998a; Longhi *et al.*, 1999). The cutinase isolated from *P. putida* and *T. fusca* was found to be 30 kDa and 29 kDa molecular weight, respectively. The molecular weight of cutinases isolated from *F. solani pisi* and *C. kahawae* were found to be 21 kDa and 22.1 kDa, respectively (Chen *et al.*, 2007). Sebastian and Kolattukudy (1988) reported that generally the molecular weight of bacterial cutinase lies between the molecular weight of fungal cutinase (22 kDa) and pollen cutinase (40 kDa). Although fungal cutinase has been extensively characterized, the molecular identity of bacterial cutinase has remained as a mystery. Chen *et al.*, (2008) discussed in their study, giving the evidence indicating Tfu 0882 and Tfu 0883 from the actinomycete *T. fusca* function as cutinases: (i) a 29-kDa protein was secreted into the *T. fusca* culture upon cutin induction and exhibited cutinase activity (ii) N-terminal amino acid sequencing of the 29-kDa protein matched two proteins, Tfu 0882 and Tfu 0883, which are 93% identical in sequence; (iii) both Tfu 0882 and Tfu 0883 are able to hydrolyse cutin, resulting in monomeric products typical of fungal cutinase. *P. putida* cutinase was found to be 30 kDa (Sebastian and Kolattukudy, 1988), whereas *P. cepacia* cutinase was reported to be of 26.25 kDa (Dutta *et al.*, 2013). Table 2.3 summarizes the size of cutinase enzyme of various fungal and bacterial sources.

Table 2.3 Cutinase and their molecular weight

Source	Genus	Species	Protein size (kDa)	Reference
Bacteria	<i>Pseudomonas</i>	<i>cepacia</i>	26.25	Dutta <i>et al.</i> , 2013
		<i>putida</i>	30	Sebastian and Kolattukudy, 1988
	<i>Thermobifida</i>	<i>fusca</i>	29.22 /28.99*	Chen <i>et al.</i> , 2010

Source	Genus	Species	Protein size (kDa)	Reference
		<i>alba</i>	30	Thumarat <i>et al.</i> , 2011
		<i>cellulolysitica</i>	28.1	Ribitsch <i>et al.</i> , 2012
Fungi	<i>Alternaria</i>	<i>brassicicola</i>	24	Koschorreck <i>et al.</i> , 2010
	<i>Ascochyta</i>	<i>rabiei</i>	22	Tenhaken <i>et al.</i> , 1997
	<i>Aspergillus</i>	<i>oryzae</i>	20.6	Liu <i>et al.</i> , 2009
	<i>Botryotinia</i>	<i>fuckeliana</i>	38.7	Skamnioti <i>et al.</i> , 2008
	<i>Coprinopsis</i>	<i>cinerea</i>	18.8	Kontkanen <i>et al.</i> , 2009
	<i>Emericella</i>	<i>nidulans</i>	30.1	Skamnioti <i>et al.</i> , 2008
	<i>Fusarium</i>	<i>oxysporium</i>	21.7	Rocha <i>et al.</i> , 2008
		<i>roseum culmorum</i>	24.3	Soliday <i>et al.</i> , 1976
		<i>Solani pisi</i>	21.8/20.4 /22**	Purdy and Kolattukudy, 1975; Brissos <i>et al.</i> , 2008
	<i>Gibberella</i>	<i>zeae</i>	36.4	Skamnioti <i>et al.</i> , 2008
	<i>Glomerella</i>	<i>cingulata</i>	21	Nyon <i>et al.</i> , 2009
	<i>Humicola</i>	<i>insolens</i>	20	Hunsen <i>et al.</i> , 2007
	<i>Magnaporthe</i>	<i>grisea</i>	41.2	Skamnioti <i>et al.</i> , 2008
	<i>Monilinia</i>	<i>fructicola</i>	22.2	Wang <i>et al.</i> , 2002
	<i>Trichoderma</i>	<i>harzianum T34</i>	29	Rubio <i>et al.</i> , 2008
	<i>Venturia</i>	<i>inaequalis</i>	21.7	Koeller <i>et al.</i> , 1989
Pollen	<i>Arabidopsis</i>	<i>thaliana</i>	39.2	Takahashi <i>et al.</i> , 2010
Yeast	<i>Neurospora</i>	<i>crassa</i>	39.2	Skamnioti <i>et al.</i> , 2008

* Two cutinase genes, namely, Tfu 0882 (29.22 kDa) and Tfu 0883 (28.99 kDa) reported

** Two cutinase genes namely, Cut1 (21.8) and Cut II (20.4) was reported by Purdy and Kolattukudy, 1975 and Brissos *et al.*, 2008 reported a mutant variety to be of 22 kDa

2.7.2 Substrate specificity

In general, almost all type of cutinases showed a trend of increasing K_m , and decreasing V_{max} as the chain length of acyl group increased (Sebastian and Kolattukudy, 1988). The bacterial cutinase catalyses hydrolysis of p-nitrophenyl esters of C4 to C16 fatty acids. However, these changes were not as large as those observed with some fungal cutinases. As the chain length of the acyl moiety increased from C2 to C12, the V_{max} of many fungal cutinases decreased

from 200 to 1000-fold (Sebastian and Kolattukudy, 1988). Liu *et al.*, (2009) studied the kinetics of *A. oryzae* and *F. solani* cutinases on a set of model *p*-nitrophenyl ester substrates: *p*-nitrophenyl acetate (pNPA), *p*-nitrophenyl butyrate (pNPB), *p*-nitrophenyl valerate (pNPV), and *p*-nitrophenyl hexanoate (pNPH). The influence of chain length on cutinase activity studied via Michaelis-Menten kinetics showed that the *A. oryzae* cutinase demonstrated a preference for pNPV resulting in a K_m of 0.04 μM , followed by pNPB and pNPH with K_m 's of 0.21 and 0.29 μM , respectively. The short pNPA chain yielded a >120-fold higher K_m of 4.96 μM . The highest catalytic efficiency was observed for pNPB and pNPV with a k_{cat}/K_m of 3.49 and 3.32 $\mu\text{M}^{-1} \text{min}^{-1}$, respectively. This was followed by pNPH and pNPA with a k_{cat}/K_m of 1.34 and 0.07 $\mu\text{M}^{-1} \text{min}^{-1}$, respectively. In contrast, *F. solani* cutinase showed an overall preference for short chain substrate pNPA with a k_{cat}/K_m of 2.53 $\mu\text{M}^{-1} \text{min}^{-1}$ followed by pNPV, pNPB, and pNPH, respectively. In another study, substrate specificity of (Baker *et al.*, 2012) the cutinase from *A. brassicicola*, *A. fumigatus*, *H. insolens* showed that *A. fumigatus* cutinase exhibited a 6.5-fold lower K_m (8.9 μM) for pNPB in comparison to *F. solani* cutinase (1.36 μM) whereas, *H. insolens* and *A. brassicicola* cutinase demonstrated better pNPB recognition with K_m values of 2.46 and 1.96 μM , respectively. Dutta *et al.*, (2013) and Sebastian and Kolattukudy (1988) in a separate kinetic study with the substrate pNPB, reported a K_m of 0.23 and 0.27 mM for *P. cepacia* and *P. putida* cutinase, respectively. Chen *et al.*, (2010) studied the kinetic constants of *T. fusca* cutinases, Tfu 0882, Tfu 0883 and observed that they exhibit better affinity (lowest K_m value) for the lower acyl chain length substrates in comparison to *F. solani* cutinase. In addition, the catalytic efficiency (K_{cat}/K_m) of Tfu 0883 was twice as much as that of Tfu 0882 regardless of an identical structure of their active sites.

Unlike lipases, cutinases hydrolyse tributyrin below its Critical Micelle Concentration (CMC) effectively. Cutinase activity in this case is accounted for the accessibility of its active site and by the deep embedding of tributyrin in the hydrophobic catalytic crevice. This embedding reduces the unfavorable interactions between the acyl chains of tributyrin and the surrounding bulk water. At concentrations exceeding the CMC, the binding of tributyrin at the lipid water interface and its burying in the active site cleft would result in an almost complete removal of the tributyrin from the lipidic phase. Flipsen *et al.*, (1996) investigated the hydrolysis of lipid by cutinase from *F. solani pisi* with the oil-drop tensiometer technique. It had been observed that the decrease of oil-water interface tension ($\gamma_{o/w}$) during lipid hydrolysis was related to cutinase adsorption to an oil-drop and hydrolysis of a natural long chain triglyceride. Hydrolysis of triglycerides and analogues by cutinase have been widely studied in several reaction systems, namely in AOT reversed micelles (Melo *et al.*, 1995b) and in a biphasic system. In the two-phase system, the conversion of triolein was followed by the use of a monolayer technique (Flipsen *et al.*, 1996). An optimum pH in the range of 9-10 was found in both systems. The apparent kinetic constants for triolein (Melo *et al.*, 1995b) were determined: $K_{cat}=1630 \text{ min}^{-1}$, $K_m=395 \text{ mM}$ and $K_{cat}/K_m=4.1 \text{ min}^{-1} \text{ mM}^{-1}$. The kinetic analysis of the hydrolysis of other triglycerides, tricaprylin, trilaurin and trimyristin was also performed. Cutinase presented higher activity and specificity for the shorter chain triglyceride under study and with tricaprylin, $K_m=132 \text{ mM}$ and $K_{cat}/K_m=8.2 \text{ min}^{-1} \text{ mM}^{-1}$ was observed. These results were in accordance with others obtained on the selectivity and specificity of cutinase (Mannesse *et al.*, 1995a).

2.7.3 Effect of inhibitors and metal ions

Active serine directed reagents such as di-isopropylfluorophosphate and (O,O)-diethyl-(3,5,6-trichloro)-2-pyridylphosphorothioate and phenylmethanesulfonylfluoride (PMSF) were found to be a potential inhibitors of both bacterial and fungal cutinases. Histidine modifying reagents like Diethylpyrocarbonate (DEPC) was also reported to inhibit the cutinases. Phenylboronic acid, a transition state analog of serine hydrolases, inhibits cutinase activity. The inhibition of cutinase by phenylboronic acid was competitive in nature (Sebastian and Kolattukudy, 1988). Thiol-directed reagents, p-hydroxymercuribenzoate and N-ethylmaleimide completely inhibited pollen cutinase whereas the bacterial and fungal cutinase was hardly inhibited by them even at higher concentration (Sebastian and Kolattukudy, 1988, Maiti *et al.*, 1979). Sulfhydryl group disrupting agents such as Hg^{2+} and p-chloromercuribenzoic were found to be inhibitory for the bacterial and fungal cutinases (Chen *et al.*, 2010; Dutta *et al.*, 2013, Speranza and Macedo, 2013).

Speranza and Macedo (2013) studied the effect of various metal ions on the crude (unpurified) cutinase from *F. oxysporum*. The study showed that the enzyme was activated in the presence of divalent metal ions, Ca, Fe, Mn and a monovalent cation Na at 1 mM concentration. However, Co^{2+} Mn^{2+} , 4-Cloromercuribenzoic and Sodium lauryl sulfate at higher concentration (10 mM) and Hg^{2+} at 1 mM concentration completely inhibited the enzyme. Dutta *et al.*, (2013) in their study on purified cutinase from *P. cepacia* found that divalent metal ions, Mn, Ni, or Ca at 1 mM concentration did not exhibit any significant impact on the enzyme activity, whereas, Zn, Fe, Hg or Cu showed an inhibitory effect. It was also observed in the study that some of the divalent metal ions (Mg^{2+} , Ca^{2+} and Co^{2+}) and monovalent metal ions (K^{+} and Na^{+}) were found to enhance the enzyme activity at 1 mM

concentration. EDTA at concentration up to 10 mM did not affect the activity of the cutinase. Chen *et al.*, (2010) studied the effect of different metal ions on recombinant cutinase activity from *T. fusca* and *F. solani pisi*. Like in the case of *P. cepacia* cutinase, EDTA did not affect activities of the *T. fusca* cutinases, however Zn^{2+} , Fe^{2+} , or Pb^{2+} showed a medium inhibitory effect and Cr^{2+} and Hg^{2+} were found to be potentially inhibitory. The study also showed that presence of 1mM of divalent metal ions such as Mn, Co, Ni, Mg, Ba, Cu, or Ca did not exhibit a significant effect on the enzyme activity.

2.7.4 Organic solvent, surfactant tolerance

Application of industrial enzymes often involves relatively harsh conditions such as the presence of surfactants and organic solvents.

The stability of the enzyme in organic solvents makes them more attractive for synthetic (esterification and transesterification) reactions. Generally, enzyme catalyzed esterification reaction is used between fatty acid and alcohol to synthesize alkyl esters, but this reaction is reversible. Thus, at higher water concentration, the equilibrium for the enzyme-catalyzed reaction shifts to the reverse direction forming again the initial reactants. To use the enzyme for the synthesis of esters, it must have stability at minimum water concentration and with organic solvents (de Barros *et al.*, 2009a, 2009b; Dutta and Dasu, 2011). Cutinases have been reported to show esterification and transesterification activity and have potential for use in the food industry for synthesis of various short chain fatty acid esters and in production of biodiesel (Cunnah *et al.*, 1996; Serralha *et al.*, 1998), therefore it is important to evaluate their stability in organic solvents. Speranza and Macedo (2013) reported that *F. oxysporum* cutinase in the unpurified form shows a significant increase in activity in presence of octanol, 2-nonanol, hexane, octane, isooctane and decane. A possible explanation for this increase in

activity may be attributable to changes in conformation of the enzyme during incubation caused by the interaction of solvent molecules with the enzyme. However, the result also showed that use of acetone, methanol, ethanol, propanol and butanol in reaction system leads to inactivation of the *F. oxysporum* cutinase. In another study, Speranza *et al.*, (2011) evaluated the stability of cutinases from *F. oxysporum* produced in different solid-state fermentation. The enzyme produced in medium with soybean rind and rice bran was relatively stable in the presence of hexane showing 71% and 66%, respectively of their activities compared with control. The enzyme produced in medium with *Jatropha curcas* seed cake was fairly stable in the presence of organic solvents, its activity remained high in all solvents evaluated, highlighting the hexane, butanol and propanol. The results indicate that the cutinase from *F. oxysporum* produced by submerged or solid-state fermentation is quite stable to the presence of organic solvents. Chen *et al.*, (2010) also studied the solvent tolerance of the cutinases. *T. fusca* cutinases, Tfu 0882 and Tfu 0883 exhibited excellent tolerance to methanol, ethanol, acetone, n-hexane, and dimethyl sulfoxide, but were less stable in isopropanol and butanol. In contrast, *F. solani* cutinase, FspC was very unstable in these solvents except in n-hexane in which nearly 70% activity remained. They concluded that, compared with fungal cutinase, the bacterial cutinases from *T. fusca* are far more suitable for applications in organic solvents. Dutta *et al.*, (2013) reported that the cutinase from *P. cepacia* has shown better stability in the solvents, viz. butanol, isooctane, chloroform, n-hexane and isoamyl alcohol, than any previously reported cutinase (Chen *et al.*, 2010). Thus the enzyme purified from *P. cepacia* has potential to be used in several industries.

The activity of the *P. cepacia* cutinase was tested in the presence of the nonionic surfactants Triton X-100 and Tween 80 and the anionic surfactants SDS and sodium deoxycholate (Dutta

et al., 2013). Triton X-100 and Tween 80 did not affect the enzyme activity at a concentration of 1 mM or 10 mM, whereas sodium deoxycholate slightly enhanced the cutinase activity at both 1 and 10 mM. In the presence of 1 mM or 10 mM of SDS, the activity of cutinase was decreased. A similar observation was also reported by Chen *et al.*, (2010) for cutinase from *Thermobifida fusca*. Chen *et al.*, (2010) found that at concentrations of 1 mM and 10 mM, Triton X-100 and Tween 20 inhibited cutinases from *T. fusca* but did not significantly reduce the *F. solani* cutinase activity. They also observed that SDS inhibited cutinases from *T. fusca* and *F. solani* but TDOC, on the other hand, stimulated *F. solani* cutinase activity with a 23.11% increase at 1 mM and a 73.65% increase at 10 mM. It did not have a significant effect on the *T. fusca* cutinase activity. Chen *et al.*, (2010) also performed inhibition kinetics for SDS to evaluate its inhibitory efficiency on the three cutinases. They determined inhibition constants by a double-reciprocal plot ($1/v$ vs. $1/[pNPB]$) of the initial reaction rate at varying concentrations. SDS appeared to be a competitive inhibitor for all three enzymes with a K_{iSDS} of 1385.6 μM for Tfu 0882, 944.4 μM for Tfu 0883 and 657.5 μM for FspC. Compared with the other two enzymes, Tfu 0882 appeared to be more resistant to SDS and may be more favorable for applications in detergent formulations.

2.7.5 Effect of pH and temperature on cutinase function

Optimum pH for cutinases from *F. solani f. pisi* (Soliday and Kolattukudy, 1976) and *F. roseum culmorum* is 10 (Soliday and Kolattukudy, 1976). Whereas, *V. inaequalis* cutinase showed the optimum pH between 5 to 6 (Koller *et al.*, 1989). The bacterial and fungal cutinase characterized using tritiated apple cutin as the substrate to release of soluble cutin components from the insoluble labeled cutin shows very low rate of cutin hydrolysis below pH 7, but the rate increased rapidly as the pH was raised from 7.5 to 10.0 showing a wide

range of pH optimum between 8.5 to 10.5 (Sebastian and Kolattukudy, 1988; Soliday and Kolattukudy, 1976). With increasing amounts of cutin, the rate of hydrolysis increased linearly up to 4 mg mL⁻¹ of cutin for bacterial cutinase (Sebastian and Kolattukudy, 1988) and 6 mg mL⁻¹ for pollen cutinase (Maiti *et al.*, 1979) and further increase in the substrate content of the reaction mixture did not change the rate. The slight decrease in the hydrolysis rate might be due to the absorption of the enzyme on the insoluble cutin polymer. Rectilinear rates of cutin hydrolysis were observed up to 20 min for bacterial cutinase (Sebastian and Kolattukudy, 1988) and 5 h incubation time for pollen cutinase (Maiti *et al.*, 1979). Several studies to evaluate the optimum pH of cutinases have been done with p-nitrophenyl esters of fatty acids and in most of the cases it was found that the cutinases shows activity in a broad range of pH with an optimal pH in the neutral to mild alkaline, however, there are few exceptions such as in case of *V. inaequalis* and *F. oxysporum* which showed optimum activity at acidic pH and *F. solani f. pisi* and *F. roseum culmorum* which has an optimum activity at highly alkaline pH.

Most of the cutinase assays have been performed at 25-37°C. However, there are few cutinases which showed higher temperature optima and thermostability. For instance, Chen *et al.*, (2009) evaluated the thermostability of Tfu 0882, Tfu 0883 and *F. solani pisi* cutinase at 40 and 60°C and found that the activity of Tfu 0882 and Tfu 0883 increased initially during incubation at both temperatures, whereas *F. solani pisi* cutinase exhibited a similar initial increase at 40°C, but a simple exponential decay at 60°C. *T. fusca* cutinases exhibited superior thermostability, with residual activities of over 80% after 160 h at 40°C or over 50% after 40 h at 60°C. In contrast, *F. solani pisi* cutinase was significantly less stable, with 50% of activity retained after 85 h at 40°C or after 5 min at 60°C. Dutta *et al.*, (2013) reported that

a combined effect of pH and temperature are crucial for the *P. cepacia* cutinase. In their study they found that maximum cutinase activity was obtained at pH 8.0 and 40°C, and decreased significantly (50%) when the pH was decreased to 6.5. The activity was also decreased sharply above and below the optimum temperature range (35-45°C) with almost 65% loss of its original activity at 60°C. *F. oxysporum* cutinase was reported to retain its activity almost completely in the temperature range of 30 and 50°C when incubated for 1 h (Speranza and Macedo, 2013). However, at 60°C the enzyme retains 20% of its activity, decreasing slowly with increasing temperature and at 100°C the enzyme retains only 9.3% of its activity. The highest activity for *A. brassicicola* cutinase was observed at 40°C (Koschorreck *et al.*, 2010) and increasing the temperature to above 50°C resulted in complete inactivation of the enzyme. The thermal stability of *A. brassicicola* cutinase investigated by incubating the enzyme at 40°C and pH 8.0 for 5 days showed a remarkable retention of 84% of its initial activity, indicating high stability of the enzyme at 40°C. Hunsen *et al.*, (2008) studied the ring-opening polymerizations (ROP) of ϵ -caprolactone (CL) and ω -pentadecalactone (PDL) using *H. insolens* cutinase (HIC) immobilized on Lewatit (ion exchange resin) as a catalyst at different temperatures in the range 50–90°C. The optimal temperature for HIC-catalyzed bulk ROP of CL was found to be 70°C. A large reduction in HIC activity for CL polymerization was found when the reaction temperature was increased from 70 to 80°C. Further increase of reaction temperature to 90°C resulted in complete loss of HIC activity for this reaction.

2.8 Biophysical properties of cutinase

A protein's function depends on its ability to acquire its native structure. The measurement of protein stability and how a protein fold into a well ordered native structure is essential for

elucidating protein function *in vivo*, engineering the improved stability of biocatalytic enzymes, and formulating proteins for various industrial applications and therapeutic delivery. According to the crystal structure of the *F. solani* cutinase, the opposite side of the active center has a single L-tryptophan (Trp) residue Trp69 involved in a hydrogen bond with an alanine (Ala) residue, located in the β -sheet, close to one of the two disulfide bridges (Cys31 and Cys109) (Longhi *et al.*, 1997) which strongly quenches the fluorescence of Trp by both static and dynamic quenching mechanisms. A study involving fluorescence spectroscopy, fluorescence decay curves and fluorescence anisotropy decays of the single Trp of cutinase at different pH values (4 to 8) and temperatures, below and above the protein-melting temperature showed that the Trp fluorescence spectrum was red shifted and sharply increases in intensity (four-fold) on protein melting with higher intensity at pH 8, indicating that cutinase is more stable at higher pH. The fluorescence decays below the melting temperature could be fitted only with a sum of four exponentials with a very fast decay component (0.05 ns) attributed to the Trp fluorescence quenching by a disulfide bridge in its vicinity. Above the melting temperature, the shortest lifetime component disappears, possibly because of the break of the Ala-Trp hydrogen bond that keeps the Trp in the vicinity of the disulfide bridge. The four-fold increase in fluorescence intensity observed after protein melting is not completely explained by the increase in the average lifetime, which means that a static quenching component exists, probably from the Trp-Cys complexes. By irradiation with UV light at room temperature, the disulfide bridge breaks, whereas the short lifetime component of the Trp decay remains, suggesting that irradiation preserves the Ala-Trp hydrogen bond. Thus, the increase in Trp fluorescence quantum yield is mainly due to the suppression of the static quenching from the Trp-cystine complexes. The Trp fluorescence anisotropy decays can

be fitted with a sum of two exponentials above and below protein denaturation. The short component results from the local motion of the Trp. The longer one is due to protein rotation below protein melting and to the backbone protein dynamics above it (Martinho *et al.*, 2003). A tryptophan mediated photo reduction of disulfide bond has been studied by Prompers *et al.*, 1999. The study indicated that prolonged irradiation of the *F. solani* cutinase in the tryptophan absorption band causes an increase of the tryptophan fluorescence quantum yield by an order of magnitude. Structural analysis of the protein with combination of NMR spectroscopy and chemical detection of free thiol groups with a sulfhydryl reagent showed that the unusual fluorescence behavior of Trp69 in cutinase is caused by the breaking of the disulfide bond between Cys31 and Cys109 upon irradiation, while the amide-aromatic hydrogen bond between Ala32 and Trp69 remains intact. Similar, elaborated investigations on UV-induced change in the three-dimensional structure, photochemical reaction kinetics and lifetimes of transient chemical species created upon UV excitation of aromatic residues in the cutinase from *F. solani* has also been reported by Petersen *et al.*, (2002, 2009). A comparative analysis of the unfolding and inactivation of cutinases from *F. solani pisi* and *H. insolens* (HiC) was studied in the presence of guanidine hydrochloride (GdnHCl) and bis-(2-ethylhexyl) sodium sulfosuccinate (AOT) by Ternstrom *et al.*, (2005) using fluorescence and circular dichroism (CD) techniques. The study indicated that the unfolding is a co-operative two state process under these denaturants and in presence of AOT reverse micelles, the cutinases unfold with mono-exponential rates, indicating a two-state process. The free energy of denaturation calculated by linear extrapolation of equilibrium data in water yielded very similar values for both the cutinases with averages of $-11.6 \text{ kcal mol}^{-1}$ and $-2.6 \text{ kcal mol}^{-1}$ for GdnHCl and AOT, respectively, indicating the AOT denatured state to be less destabilised

than the GdnHCl denatured state, relative to the native state in water. Far-UV CD and fluorescence spectroscopy data revealed that AOT denatured state retains some secondary structure, while GdnHCl denatured state is essentially unstructured for all the cutinases and the cutinases are more compact in AOT denatured state compared to GdnHCl denatured state. It was also observed in the activity measurements that both AOT and GdnHCl denatured cutinases are practically inactive in the context of catalysis.

A comparative analysis of the biophysical parameter of five cutinases from *A. brassicicola* (AbC), *A. fumigatus* (AfC), *A. oryzae* (AoC), *H. insolens* (HiC), and *F. solani* (FsC) was studied recently by Baker *et al.*, (2012). Near UV CD analysis at different pH revealed that AoC, AbC, AfC, and HiC shows a similar characteristic peaks at pH 8. However, at pH 5, FsC exhibited an increase in the negative ellipticity followed by a dramatic decrease at pH 3. In contrast, all other cutinases demonstrated a loss of signal with decrease in pH as compared to pH 8, with HiC maintaining a greater fraction of its overall structure and AbC exhibiting the largest loss in structure at low pH conditions. Thermal unfolding study revealed that all the cutinases unfold cooperatively exhibiting a single transition during unfolding, suggesting a two-state mechanism of unfolding. This was also confirmed by DSC by the observation of a single endothermic transition peak. Thermal denaturation study also showed that FsC showed a loss in thermostability with T_m 's of 56.3°C, 52°C, and 26.3°C, under pH 8, 5, and 3, respectively, whereas, AoC was more thermostable with a T_m of 58°C at pH 8.0. Upon decreasing the pH to 5.0, an increase in T_m of 3°C was observed in contrast to the decrease in T_m of FsC. Further decrease in pH to 3.0 resulted in a large decrease (23°C) in T_m of AoC. This pattern of increase and then decrease in thermostability at pH 5.0 and 3.0 was also observed for AfC and HiC, while AbC followed the overall trend described for FsC. In

another study (Melo *et al.*, 2001), stabilization effect of trehalose, and mannosylglycerate on temperature and guanidine hydrochloride induced unfolding of the *F. solani* cutinase at acidic pH (pH 4.5) was investigated. The results indicated that the protein thermal unfolding approached a two-state process, whereas the presence of trehalose at 0.5 M increased the temperature at which 50% of cutinase is unfolded by 3°C. However, the unfolding induced by guanidine hydrochloride was not a two state process with the presence of a stable intermediate molten globule structure in presence and absence of trehalose, and mannosylglycerate. The free energy calculation showed that trehalose and mannosylglycerate stabilize the folded cutinase relative to the intermediate by 1.4-1.6 and 1.6 kcal mol⁻¹ and the intermediate relative to the unfolded state by 1.0 and 1.5 kcal mol⁻¹, respectively.

2.9 Strategies for improvement of cutinase activity and stability

Study of industrially important enzymes in the context of stability, improved activity and economically feasible usage like many of today's most interesting scientific problems, demands a multidisciplinary perspective. Enzyme engineering, in particular, has much to gain from an integration of chemistry, molecular biology and biotechnology. By exploiting these tools, we hope to better understand how enzymes work and evolve. It is also expected to provide researchers of the future with useful catalysts for diverse applications. The following two subsections discuss on the various strategies employed to improve the usability of cutinase in various fields.

2.9.1 Genetic engineering strategy

Egmond and Vlieg (2000) studied the properties of wild-type *F. solani* cutinase in relation with the effects brought about by several site-directed mutagenesis of the enzyme (Table 2.4).

The study showed that extreme stereo-selectivity can be exploited by mutagenesis and the chain length preference can be adjusted to some extent by introduction of hydrophobic and aromatic residues in the substrate binding region. Koschorreck *et al.*, (2010) reported that the substrate specificity of the cutinase (CUTAB1) from *A. brassicicola* can be increased substantially for the longer chain length fatty acid substrates. They used a point mutation study, where, a mutation A84F in the small helical flap of CUTAB1 significantly increased the activity of the enzyme towards longer chain substrate, p-NPP by 11 folds compare to wild type CUTAB1. Brissos *et al.*, (2008) addressed the inactivation of *F. solani* cutinase in the presence of anionic surfactant, dioctyl sulfosuccinate sodium salt (AOT) by creating a complete saturation library. In this study, the cutinase gene from *F. solani pisi* was mutated to incorporate 19 possible amino acid exchanges at each of the 214 amino acid positions. The resulting library was screened for active variants with improved stability in the presence of the AOT. Twenty-four sites in cutinase were discovered where amino acid replacements resulted in a 2–11-fold stability increase as compared to the wild-type enzyme. It was also believed that the mutants showing substitutions in the large hydrophobic crevice (S54D, S57D, S61D, K65P, R196A), that is thought to be the region more involved in the unfolding by anionic surfactants, are very important to obtain an enzyme less sensitive to AOT. Cutinases from *F. solani*, *T. fusca* and have been successfully tailored to favorably alter its specificity towards degradation of various synthetic polymers. Cutinase from *F. solani pisi* was genetically modified near the active site, by site-directed mutagenesis, to enhance its activity towards polyethylene terephthalate (PET) and polyamide 6,6 (PA 6,6) fibers (Araujo *et al.*, 2007). The mutations L81A, N84A, L182A, V184A and L189A were done to enlarge the active site in order to better fit a larger polymer chain. It was found that the mutation

L81A and L182A showed an activity increase of four- and five-fold, respectively, for PET fibers and L182A showed the one- and two-fold higher ability to biodegrade aliphatic polyamide substrates when compared with the wild type cutinase. Silva *et al.*, (2011) genetically modified the bacterial cutinase, Tfu 0883 from *T. fusca* by site-directed mutagenesis to enhance its activity on polyethylene terephthalate (PET). The mutation I218A was designed to create space and the double mutation Q132A/T101A was designed both to create space and to increase hydrophobicity. The activity of the double mutant on the soluble substrate p-nitrophenyl butyrate increased two-fold compared to wild-type cutinase, while on PET both single and double mutants exhibited considerably higher hydrolysis efficiency. The new mutations tailored the catalytic site for PET, increasing the affinity of cutinase to this hydrophobic substrate and the ability to hydrolyse it. It was also observed in the study that the replacement of specific amino acids at the active site was an effective approach for the improvement of the Tfu 0883 cutinase capacity to hydrolyze polyester surfaces. Zhang *et al.*, (2010) reported that fusion of a cutinase from *T. fusca* and a carbohydrate-binding modules (CBMs) from *T. fusca* cellulase Cel6A (CBMCel6A) or *Cellulomonas fimi* cellulase CenA (CBMCenA) to the carboxyl terminus of *T. fusca* cutinase displayed a superior activity half-life of 53 h at their optimal temperature of 50°C compare to wild type *T. fusca* cutinase. Moreover, the binding activity on cotton fiber was enhanced by 2% for cutinase-CBMCel6A and by 28% for cutinase-CBMCenA in absence of pectinase, whereas in the presence of pectinase, the binding activity was enhanced by 40% for the former and 45% for the latter. Notably, a dramatic increase of up to 3-fold was observed in the amount of released fatty acids from cotton fiber by both cutinase-CBM fusion proteins when acting in concert with pectinase. In another study (Zhang *et al.*, 2013), cutinase–CBMCenA fusion protein was

genetically modified in the carbohydrate binding module (CBM) binding sites, by site-directed mutagenesis, to enhance its activity toward polyethylene terephthalate (PET) fiber. The hydrolysis of PET was investigated by replacing each of W14L/Y, W50L/Y and W68L/Y in the CBM domain. Enzyme characterization showed that the mutants displayed similar thermostability and pH stabilities in response to the native enzyme and W68L and W68Y, among all the mutants, exhibited significant improvement in binding and catalytic efficiency (1.4-1.5 fold) toward PET fiber when compared to that of the native enzyme. Recently, Ribitsch *et al.*, (2013) studied the effect of fusion of a cellobiohydrolase I binding modules from *Hypocrea jecorina* (CBM) and polyhydroxyalkanoate depolymerase from *Alcaligenes faecalis* (PBM) with cutinase from *T. cellulosylitica* (Thc Cut1) on sorption and hydrolysis of synthetic polymer PET. The result showed that the fusion enzymes were active both on the insoluble PET model substrate bis(benzoyloxyethyl) terephthalate (3PET) and on PET and Thc Cut1-CMB showed 3.8 fold enhancement in hydrolytic activity compare to wild type Thc Cut1. Matama *et al.*, (2013) engineered a wild type *F. solani* cutinase by fusion with the carbohydrate binding module N1 from *Cellulomonas fimi* to increase the catalytic efficiency for the cellulose substrates such as Avicel or cotton fabrics. The study showed that engineered cutinase was able to esterify the hydroxyl groups of cellulose with distinct efficiencies depending on the acid substrate/solvent system used and the cutinase treatment resulted in relative increases of 31 and 9% when octanoic acid and vegetable oil were used as substrates, respectively. The *G. cingulata* cutinase has been genetically modified by site directed mutagenesis to enhance its thermostability and substrate specificity (Iuan *et al.*, 2013). The mutation studies were performed with the aim of altering the surface electrostatics as well as removing a potentially deamidation-prone asparagine residue. In one of the mutation study,

the N177D cutinase variant was affirmed to be more resilient to temperature increase with a 2.7-fold increase in half-life at 50°C as compared with wild-type enzyme. Another double variant, L172K/N177D exhibited higher enzymatic performance towards phenyl ester substrates of longer carbon chain length and enhanced thermal stability.

Table 2.4 List of single mutation sites in *F. solani* cutinase, for which variants were produced and investigated. Activities were obtained from incubations with olive oil emulsions (olive oil and gum arabic, both 3% by weight) in a pH stat apparatus at pH 9.0, with 20 mM NaCl and 10 mM CaCl₂ at 30°C. [Adopted from Egmond and Vlieg (2000)]

Residue	Position	Changed into	Activity w.r.t wild-type (%)
Ala	29	Ser	64
Ala	79	Asn, Gly	n.d., 50
Ala	85	Phe, Trp	136, 109
Ala	164	Arg	41
Ala	185	Leu	96
Ala	195	Ser	38
Ala	199	Cys	0
Arg	17	Asn, Glu	31, 34
Arg	78	Asn,Leu	34, 49
Arg	88	Ala	39
Arg	96	Asn	57
Arg	156	Asn, Glu, Leu,Lys	n.d.,79, 71, 115
Arg	196	Glu,Leu, Lys	45, 44, 38
Arg	208	Ala	64
Asn	84	Ala, Asp,Leu	5, 0, 5
Asn	161	Asp	63
Asn	172	Lys	45
Asp	33	Ser	74
Asp	83	Ser	62
Asp	111	Asn	39
Asp	134	Ser	37
Glu	201	Lys	54
Gly	26	Ala	32
Gly	192	Gln	44
Ile	24	Ser	4
Ile	183	Phe	25

Residue	Position	Changed into	Activity w.r.t wild-type (%)
Ile	204	Lys	66
Insertion	49a	Ile	52
Leu	99	Lys	78
Leu	114	Tyr	20
Leu	182	Trp	19
Leu	189	Phe	109
Lys	151	Arg	29
Lys	168	Leu	83
Met	98	Cys	35
Ser	42	Ala	0
Ser	54	Glu, Lys, Ile, Trp	34, 96, n.d., 89
Ser	92	Arg	50
Ser	120	Ala	0
Thr	18	Glu, Ile, Val	n.d., n.d., 90
Thr	19	Val	35
Thr	45	Ala, Lys	98,74
Thr	50	Val	25
Thr	80	Asp	32
Thr	144	Cys	54
Thr	167	Leu	54
Thr	173	Lys	119
Thr	179	Tyr	131
Trp	69	Tyr	12
Tyr	38	Phe	62

n.d., not detected

2.9.2 Immobilization strategy

Various supports and immobilization techniques have been evaluated for increasing the stability and activity of cutinase catalyzed reactions in several applications like, esterification and transesterification.

To enhance the activity, *F. solani* f. *pisi* cutinase was immobilized on sodium form of zeolite Y (NaY) and polyamide Accurel PA6 by Serralha *et al.*, (2004) and used to catalyse the alcoholysis reaction of butyl acetate with hexanol in isooctane. They observed that the increase in temperature enhances the specific activity of cutinase for both NaY and Accurel

PA6 preparations. The pH and hexanol concentration did not affect the enzyme activity for NaY preparation but at higher pH enzyme activity decreased for Accurel PA6. At higher temperature enzyme activity increased with the increasing concentration of hexanol. Cutinase stability and activity was enhanced measurably when it was immobilized onto zeolites (Goncalves *et al.*, 1996b, Goncalves *et al.*, 1995). A BSTR (batch stirred tank reactor) was developed by Gonclaves *et al.*, (1998) for utilization of the immobilized cutinase on the hydrolysis of triglycerides in non-conventional media. High conversions were observed using 4% water and 96% organic phase in the medium (Gonclaves *et al.*, 1998). Cutinase immobilized onto silica derivatives, Biosil-NH₂ and Biosil-dextran-NH₂ retains almost 97% and 58% of initial activity, respectively after 50 days at 4°C (Goncalves *et al.*, 1999, Goncalves *et al.*, 1996a). Tricaprylin hydrolysis by cutinase adsorbed onto NaY zeolite (Gonçalves *et al.*, 1996b), covalent coupled on porous silica (Gonçalves *et al.*, 1995) and entrapped in calcium alginate (Gonçalves *et al.*, 1998) has also been studied. The optimum pH was around 8 for the three cutinase preparations and the highest specific activity was obtained for the adsorbed cutinase. Entrapped cutinase presented a Michaelis-Menten type of kinetics for substrate concentrations below 200 mM and substrate inhibition was observed at 400 mM. The apparent kinetic constants were found to be $k_{cat}=6.51 \text{ min}^{-1}$ and $K_m=35.6 \text{ mM}$ (Gonçalves *et al.*, 1995) and the apparent specific constant (k_{cat}/K_m) was $0.183 \text{ min}^{-1}\text{M}^{-1}$. For covalent binding immobilization, diffusional limitations were present at low substrate concentrations (up to 35 mM) and substrate inhibition was observed for concentrations higher than 150 mM. With the adsorbed cutinase a k_{cat} value of 1.74 min^{-1} and an apparent K_m of 186 mM were obtained (Gonçalves *et al.*, 1995). Encapsulation of cutinase in gel matrices, e.g. calcium alginate, seems to be an inadequate method for cutinase immobilization; because of

the very high hydrophilic character of the alginate polymer, both partition and diffusion effects hinder the catalytic action of cutinase, as it occurs for other lipases acting on lipophilic substrates. The specific activity of the encapsulated cutinase was found to be 16-fold lower than a covalent coupled cutinase preparation and 40-fold lower than cutinase adsorbed onto zeolites (Gonçalves *et al.*, 1996a; Gonçalves *et al.*, 1995). Alternatively, cutinase has been microencapsulated in reversed micelles of surfactants in organic media and successfully used for hydrolysis (Melo *et al.*, 1995b), esterification (Cunnah *et al.*, 1996; Pinto-Sousa *et al.*, 1994; Sebastião *et al.*, 1993; Sebastião *et al.*, 1992) and transesterification (Carvalho *et al.*, 1997a; Carvalho *et al.*, 1998b; Cunnah *et al.*, 1996; Papadimitriou *et al.*, 1996). Reversed micelles were found to be suitable system to promote biocatalysis in organic media. The solubilization of the enzyme in the water pool of reversed micelles favors the retention of the catalytic activity, the enzyme being protected against the negative effect of the solvent on its structure. Furthermore, encapsulation in reversed micelles provides a very high interfacial area, making possible the solubilization of hydrophilic and hydrophobic substrates and often enhancing enzyme activity. Separation of products and recovery of enzyme is also possible and constitutes another research field regarding the use of micelles. One of the most important parameters for optimal enzyme activity in reversed micelles is the molar ratio of water to surfactant, often called w_o . A distinct maximum in enzymatic activity is usually found when it was measured as a function of w_o . The size of the reversed micelles is related to this parameter. At low w_o values, the micelles may be too small to accommodate the enzyme molecules; at high values, the large amount of water present may interfere with the enzymatic reaction. Therefore, cutinase microencapsulated in reversed micelles shows an optimal activity at w_o 6 for the esterification of oleic acid with hexanol in AOT/isooctane (Sebastião

et al., 1992; Sebastião *et al.*, 1993) and at w_o 7-8 for the esterification of hexanoic acid with hexanol in CTAB (Cunnah *et al.*, 1996). Moreover, for the transesterification of butyl acetate with hexanol the optimal w_o range is 5-8 (Carvalho *et al.*, 1997a). Concerning the hydrolysis of triolein the activity still increased up to w_o 30 (Melo *et al.*, 1995b). Recently, cutinase (HiC) from the ascomycete *H. insolens* was immobilized onto microporous resin Diaion HP20 to assess the synthesis of functionalised acrylic esters by transesterification. Immobilized enzyme showed a 95.4% conversion of 6-mercapto-1-hexanol to 6-mercaptohexyl acrylic ester after 6 h at 40°C in the presence of 0.025% (w/w) water without formation of by-products in a solvent free system (Kazenwadel *et al.*, 2012).

2.10 Cutinase function and applications

The elucidation of cutinase structure by crystallographic methods and protein engineering, has given the opportunity to clarify its mechanism of action and to improve the understanding of cutinase structure-function relationships (Jelsch *et al.*, 1998; Longhi *et al.*, 1996; Martinez *et al.*, 1993), and to widen the range of potential applications (Okkels 1997a; Okkels 1997b; Genencor, 1989; Unilever, 1994a; Unilever, 1994b). The ability of cutinases to hydrolyze a variety of short and long chain esters, various polymeric substrates and its stability and activity in presence of oxidizing agents, detergents and protease makes it industrially very important enzyme, especially for textile industry, detergent industry, pharmaceutical industry, food industry, agriculture and many more (Fig. 2.5). Cutinases have been suggested for use with chemical agents such as fungicides and pesticides to obtain better penetration and adhesion of the chemical agents to softened plant surfaces (Iwasaki and Hioki, 1991; Poulouse and Kolattukudy, 1995). Cutinases also have been suggested for use with surfactants in

detergents (Kolattukudy and Poullose, 1988). Recently, Point *et al.*, (2013) showed that *F. solani* cutinase can be used for enantioselective synthesis of non-racemic monocyclic enolphosphonate analogues of Cyclophostin, which indicates that cutinase also has promising potentials in pharmaceuticals, especially in designing novel drugs. It has been found that cutinases possess surprising properties which make it superior to lipases for use in enzyme-catalysed reactions. Cutinases are able to hydrolyse both water soluble esters as well as emulsified water insoluble triacylglycerols (Lauwereys *et al.*, 1991). The catalytic properties of cutinases seem to be intermediate between esterases and lipases (Martinez *et al.*, 1992). The thermostability of cutinases in aqueous solution is quite high compare to most of the lipases. One of the most neglected aspects of enzyme technology is enzyme stability in organic solvents. However, cutinases were stable under conventional hydrolysis conditions, allowing repeated use of the enzyme and thus making the economics of such a process attractive (De Geus *et al.*, 1990). Several industrial applications of cutinases are mentioned below. Some important reactions catalysed by cutinase are given in the Table 2.5.

2.10.1 Cutinase in polymer degradation

Cutinases display hydrolytic activity toward a broad variety of polymers such as poly-(butylene succinate) (PBS), poly-(butylene succinate-co adipate) (PBSA) (Maeda *et al.*, 2005), polyamide 6,6 (PA 6,6), vinyl acetate (co-monomer of acrylic fiber) fibers, polyamide 6,6 (PA 6,6) fibers, and polyethylene terephthalate (PET) fibers (Arujo *et al.*, 2007; Silva *et al.*, 2005c). Some of the advances in the research of polymer degradation using engineered cutinases have already been discussed in the section 2.9. This subsection further discusses selected literatures reported on the hydrolysis of various polymers by cutinases from different sources.

Maeda *et al.*, (2005) used biodegradable plastics, PBS and PBSA as fermentation substrates for the filamentous fungus *A. oryzae* and showed that this fungus could grow under culture

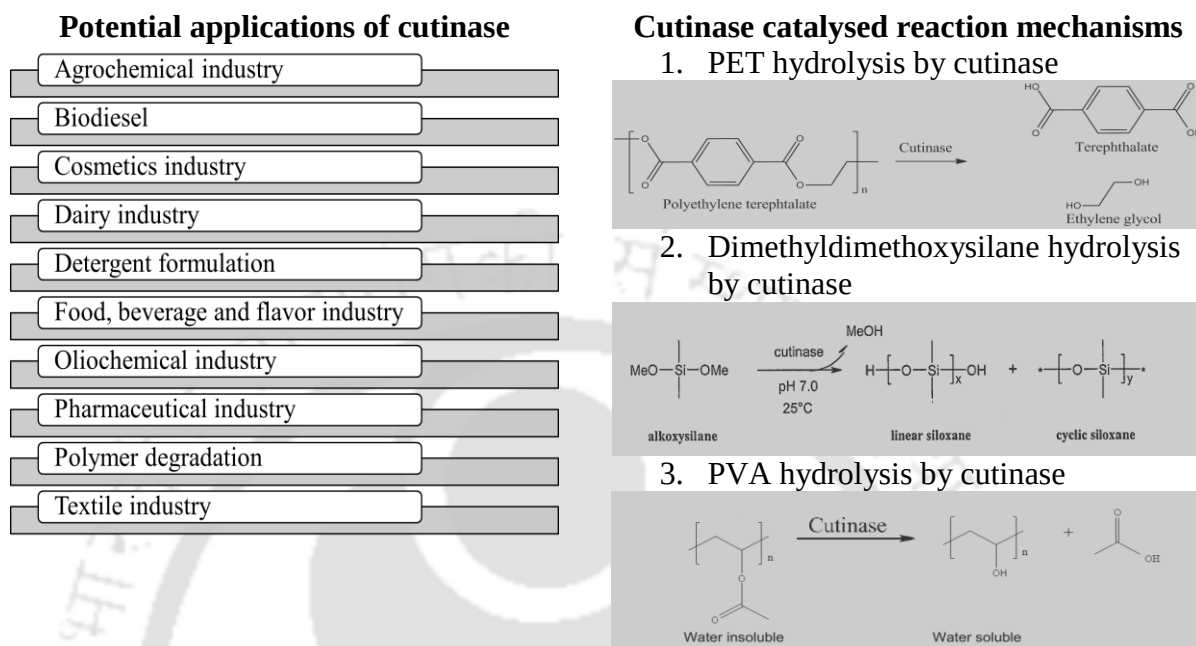


Fig. 2.5 Potential applications of cutinase and some cutinase catalyzed hydrolytic reactions

conditions that contained emulsified PBS and emulsified PBSA as the sole carbon source and digest PBS and PBSA. Purification and biochemical characterization of the PBS/PBSA degrading enzyme showed that it was a cutinase. Sulaiman *et al.*, (2012) cloned a gene encoding a cutinase homolog, LC-cutinase, from a fosmid library of a leaf-branch compost metagenome by functional screening using tributyrin agar plates. LC-cutinase which showed 57.4% identity to *T. fusca* cutinase had an ability to degrade poly(ϵ -caprolactone) and PET. The specific PET degrading activity of LC-cutinase was determined to be very high, 12 mg h⁻¹ mg⁻¹ of enzyme at pH 8.0 and 50°C. Cutinase from *F. solani* and *A. oryzae* have also been reported to hydrolyse the polymer, poly(ϵ -caprolactone) (Liu *et al.*, 2009). Eberl *et al.*, (2009) reported that the treatment of poly(trimethylene terephthalate) (PTT) fabrics with

cutinase improved the dyeability with a significant increase in *K/S* value. Hydrolysis of high molecular weight biodegradable plastics [polybutylene succinate, poly(ϵ -caprolactone), and poly(3-hydroxybutyrate)] and polylactic acids by cutinase from *Cryptococcus* sp. strain S-2 also have been studied (Masaki *et al.*, 2005). Researchers have shown that the vinyl acetate moieties in polyacrylonitrile (PAN) can be hydrolysed by cutinases and lipases, making this approach applicable to most commercially available PANs (Guebitz *et al.*, 2008; Matama *et al.*, 2006). Studies have been done on evaluation of polyamide (PA) hydrolysis using cutinase from *F. solani* (Silva *et al.*, 2007). This cutinase was also genetically modified to achieve higher activity on synthetic polymers (Araujo *et al.*, 2007). Polyalkyleneterephthalates (PAT) is another group of polymers which have been studied as a hydrolytic substrate for cutinases along with lipases and esterases (Guebitz and Paulo, 2008). A comparative study on lipase and cutinase catalyzed surface esterification of hemicellulose; arabinoxylan (AX) films showed that cutinase from *F. solani* can efficiently catalyse surface acetylation when shorter alkyl chain substrate (vinyl acetate) was used, whereas the lipases (*Mucor javanicus*, *Rhizopus oryzae* and *Candida rugosa*) have preference towards the longer alkyl chain substrate (vinyl stearate), as shown by surface steatation (Stepan *et al.*, 2013). Several reports are available on degradation of PET and PET derivatives by cutinases from *F. solani*, *F. oxysporum*, *T. fusca*, *T. alba* and *Pencillium citrinum* (Ribitsch *et al.*, 2011; 2013; Thumarat *et al.*, 2011). Vertommen *et al.*, (2005) studied the hydrolysis of different solid model substrates of PET by *F. solani* cutinase in aqueous heterogeneous system. The extent of hydrolysis was detected by measuring the amount of (soluble) degradation products in solution using reversed-phase HPLC. The result showed that the crystallinity of the PET greatly affects the capability of the enzyme to hydrolyze the ester bonds, displaying relatively high activity towards an

amorphous polyester film and little activity on a highly crystalline substrate. It was also observed in the study that the enzyme is sufficiently stable and hydrolysis rate on the amorphous substrate maintained at sufficient high level over a long period of time of at least five days. Eberl *et al.*, (2009) reported that cutinases from *T. fusca* and *F. solani* can efficiently hydrolyse the bis(benzoyloxyethyl) terephthalate (3PET) and PET in the form of fabrics and films. The hydrolysis was monitored by MALDI-TOF-MS, LC-MS, cationic dyeing and XPS analysis. The study also shown that the presence of the plasticizer *N,N*-diethyl-2-phenylacetamide (DEPA), increased hydrolysis rates of semi-crystalline PET films and fabrics.

2.10.2 Cutinase in textile related industry

The treatment of wool fabrics with protease is an environmentally friendly technique and has been intensively explored as an alternative of the commercial chlorine-Hercosett process to provide shrink resist property to wool fabrics (Schumacher *et al.*, 2001). The cuticle membrane in the wool surface is mainly composed of naturally occurring lipids connecting cysteine residues via thioester or ester bonds and covalently cross linked isopeptide via amide bonds, which makes the wool surface highly hydrophobic. Chemical or physical treatments, such as alkali, oxidation, chlorination, or plasma treatments can dislodge some fatty acids bonds in the wool surface, break some disulfide crosslinks and provide polar functional groups. However, some chemical pretreatments have the disadvantages of causing excessive fiber damages and uneven treatments of wool surface. Enzymes, such as lipases and the chemically modified proteases, have the great potential application in wool processing without causing significant damage to wool fibers. The enzymatic process based on the chemically modified proteases has been investigated by several groups (Shen *et al.*, 2007;

Silva *et al.*, 2005a; Silva *et al.*, 2006) achieving a satisfactory anti-felting effect without any significant weight loss. Lipases are expected to remove the hydrophobic complexes and long chain fatty acids in the wool surface, which might promote the succeeding proteolytic reactions. However, several studies (Hutchinson *et al.*, 2007; Monlleó *et al.*, 1994,) revealed that lipase treatment of wool fibers do not changes the surface of wool significantly and negligible difference in the wettability was observed. Thus, the efficacy of lipases in wool processing is still argumentative. Cutinases display hydrolytic activity towards a broad variety of aliphatic esters (Pocalyko *et al.*, 1998; Silva *et al.*, 2005b; Silva *et al.*, 2007). Recently, Agrawal *et al.*, (2008) demonstrated that cutinase treatment enhanced the degradation of cotton waxes and increased the hydrolytic rate of pectinase during cotton scouring. Since, the outmost bound lipids in the wool surface are a complex mixture of aliphatic lipids, cutinase might have the potential application in the pretreatment of wool fibers. Wang *et al.*, (2009) showed that the wettability of wool fabric was improved after cutinase (*T. fusca* WSH04) treatment compared with that of the sample pretreated with hydrogen peroxide. Another advantage of cutinase treatment is that the partial dislodgement of the lipid-rich outer layer and the increase of the wettability caused by the cutinase pretreatment make the succeeding proteolysis with protease easily occur from the underlying protein layer on wool surface. Several works have been done in order to improve the degradation of cotton seed coat. Yan *et al.*, (2009) has reported that cutinase was capable of degrading the cotton seed coat. The removal of aliphatic components by cutinase enables other enzymes to penetrate into the inner of cotton seed coat. Cutinase can potentially improve the degradation of cotton seed coat during cotton fabric bio-scouring process. Cotton contains various non-cellulose components such as waxes (2.5%), pectins (1.2%), cuticles (3%), and so on (Lee *et al.*, 2009). These non-

cellulose components prevent the uniform dyeing of the fabric and can decrease its absorbency. Pectinase, lipase, and cutinase are examples of enzymes used for hydrolyzing the non-cellulose components of cotton. Cutinase can hydrolyse the cuticle layer of cotton. Accordingly, hydrolyzing the cuticle layer, a large portion of which comprises the non-cellulose components of cotton, can improve the fabric dyeing properties and the moisture regain. Furthermore, auxiliaries such as nonionic surfactants and salts can facilitate cutinase hydrolysis of the cuticle layer of cotton. A nonionic surfactant such as Triton X-100 helps to reduce the enzyme penetration time (Lee *et al.*, 2009) and some salts such as calcium chloride serve as activators to help dissolve the proteins into solution, thereby increasing the enzymatic activity. Optimization of the dyeing properties and moisture regain of cotton was possible by applying this cutinase treatment in the presence of a Triton X-100 and calcium chloride. Lee *et al.*, (2009) reported that the optimum condition for scouring of cotton to be a pH and temperature of 9.0 and 50°C, respectively at cutinase concentration of 100%, and a treatment time of 60 min. The weight loss of cutinase-treated cotton fabrics was 2.5% because the cuticle layer of the cotton fiber was effectively hydrolyzed. Cutinase treatment did not affect to decrease of the moisture regain or the *K/S* value.

2.10.3 Cutinase in ester synthesis

Cutinase has been used for synthesis of short chain fatty acid esters that has applications as flavoring agents in food industry (Sebastião *et al.*, 1992; Sebastião *et al.*, 1993). Although, lipases can be used for the hydrolysis and synthesis of esters, are active in organic solvents and have wide substrate specificity, still they have a number of shortcomings. The most important shortcoming of lipases is their relatively large size and instability under industrial process conditions. Despite the known ability of cutinases to catalyse the hydrolysis of esters,

use of cutinases have been suggested only for very limited purposes until now, viz., successful utilization as catalyst for synthesis of esters in laboratory scale. Cutinases can be used for the hydrolysis of a variety of ester substrates including monoesters, such as p-nitrophenyl butyrate, and triglycerides, such as triolein and tributyrin.

Cutinases can also be used for the direct synthesis of esters starting from alcohols and organic acids (e.g., fatty acids). This derives from the reversibility of the cutinase reaction. The synthesis of fatty acid esters by cutinase has been analyzed in reversed micelles of both anionic, AOT, (Sebastião *et al.*, 1992; Sebastião *et al.*, 1993) and cationic, CTAB, (Cunnah *et al.*, 1996) surfactants. The oleic acid esterification with aliphatic alcohols by microencapsulated cutinase in AOT reversed micelles showed that cutinase has a preference for C5 to C6 alcohols, reflecting both the intrinsic selectivity of the enzyme and the different accessibility of the alcohol substrates to the cutinase active site (Sebastião *et al.*, 1992; Sebastião *et al.*, 1993). The effect of the fatty acid chain length on the esterification of hexanol was also evaluated and the maximum activity was obtained with butyric acid, confirming the cutinase selectivity for short chain substrates. The same reactions were also performed with cutinase encapsulated in CTAB reversed micelles (Cunnah *et al.*, 1996) and similar conclusions can be drawn; the cutinase activity was maximal for the esterification of hexanol with butyric acid. However, the cutinase activity in AOT reversed micelles was higher than in CTAB reversed micelles. The esterification of hexanol with butyric acid was also investigated by other authors (Carvalho *et al.*, 1998a) using cutinase adsorbed on a macroporous polypropylene support in both water-immiscible (hexane and diisopropyl ether) and water-miscible (acetonitrile) solvents. The esterification of hexanoic acid with hexanol has also been performed in some studies (Cunnah *et al.*, 1996; Sereti *et al.*, 1997). However,

the initial reaction rate in supercritical CO₂ was considerably lower (around 1.1 nmol min⁻¹ mg⁻¹ protein) than that in CTAB reversed micelles, (14 nmol min⁻¹ mg⁻¹ protein) (Cunnah *et al.*, 1996; Sereti *et al.*, 1997). In addition, the equilibrium in the supercritical reaction medium was reached after 5 days. The esterification of caprylic acid with butanol was also performed with lyophilized cutinase (Sarazin *et al.*, 1992; Sarazin *et al.*, 1995) using a NMR tube as a probe of the spectrometer. Around 80% of esterification could be achieved after 7 h. The ester synthesis of butyric acid and 2-butanol was carried out by cutinase microencapsulated in nonionic surfactant, phosphatidylcholine (Pinto-Sousa *et al.*, 1994). The enzymatic activity for the synthesis of butyl butyrate increased with increasing substrates concentrations according to a Michaelis-Menten kinetics. However, the inhibition of cutinase was observed at higher than 500 mM and 200 mM for 2-butanol and butyric acid, respectively. Dutta and Dasu (2011) also studied the synthesis of short chain alkyl esters catalysed by *P. cepacia* cutinase. In their study, ability of *P. cepacia* cutinase to synthesize butyl esters of various chain lengths and the specificity for acid chain length and alcohol was evaluated. The maximum conversion (%) during synthesis of ester was obtained for butyric acid (C4) and valeric acid (C5) with butanol reflecting the specificity of the enzyme for short-chain length fatty acids. In case of alcohol specificity, butanol was found to be most preferred substrate by the enzyme and conversion (%) decreased with increasing carbon chain length of alcohol used in the esterification reaction. The esterification of lauric acid and pentanol with cutinase microencapsulated in AOT reversed micelles was performed as a model system to study the structural and catalytic properties of the enzyme by using EPR spectroscopy of the labeled active site (Papadimitriou *et al.*, 1996). The effect of water content on cutinase activity was assessed, the maximum being at w₀=9. Up to w₀ 9, there was an increase of both activity and

active site mobility. As the water content of the system became higher, the mobility of the bound spin label stabilized, whereas the enzymatic activity dropped considerably. Kinetic studies allowed the determination of the apparent kinetic parameter, K_m 208 mM and 60 mM for pentanol and lauric acid, respectively. The synthesis of oleoyl glycerides, monoolein, diolein and triolein, catalysed by lyophilized cutinase was demonstrated (Melo *et al.*, 1995a) using the monomolecular film technique previously used to study the kinetics of lipase hydrolysis. The water sub phase was replaced by glycerol and a film of oleic acid was initially spread on the glycerol surface. More than 50% of the oleic acid film was acylated after 7 minutes of reaction. De Barros *et al.*, (2009a; 2009b; 2010a; 2010b) studied the ability of cutinase to catalyse the esterification of short chain alkyl esters in isooctane, miniemulsion system. De Barros *et al.*, (2009a) observed higher ester yields and initial reaction rates in the esterification of butyric (C4) and valeric acid (C5) as compared with the shorter or longer chain length acid. It was also reported by De Barros *et al.*, (2009b) that cutinase has substrate specificity towards short chain fatty acids (C4-C5) for synthesis of ethyl esters by esterification in iso-octane, whereas the specificity shifted to C10-C18 when cutinase was used in miniemulsion system. In another study, De Barros *et al.*, (2010b) observed that a higher stability of cutinase can be achieved when esterification reactions were carried out in fed batch mode using consecutive feeding pulse of substrate (alcohol).

2.10.4 Cutinase in transesterification reactions

Cutinase has been found to be one of the potent catalysts for transesterification reactions. Transesterification is the reaction of fats or oils with an alcohol to form esters and glycerol. The transesterification (alcoholysis) reaction of butyl acetate with hexanol in organic media (isooctane) has been evaluated using cutinase in several systems (Carvalho *et al.*, 1998a;

Carvalho *et al.*, 1997a; Cunnah *et al.*, 1996; Serralha *et al.*, 1998). The effect of the reaction conditions, viz., water content, temperature, buffer molarity, pH, surfactant bis (2-ethyl-1-hexyl) sodium sulfosuccinate (AOT), hexanol and butyl acetate concentrations on the transesterification activity of cutinase microencapsulated in AOT reversed micelles were evaluated using the factorial design methodology (Carvalho *et al.*, 1997a). The alcoholysis of methyl propionate with propanol using cutinase at several hydration levels were studied in several gas solid systems (Lamare and Legoy, 1995; Lamare *et al.*, 1997; Parvaresh *et al.*, 1992). Recently, transesterification of triolein catalysed by cutinase microencapsulated in AOT reverse micelle was studied in small scale (Badenes *et al.*, 2010a; Badenes *et al.*, 2010b; Badenes *et al.*, 2011a) and reactor (Badenes *et al.*, 2011b). Badenes *et al.*, (2010a) have obtained higher conversion of alkyl ester for transesterification of triolein with butanol than ethanol and methanol, due to the lower toxicity of butanol. The three step addition of ethanol was improved the conversion during transesterification of triolein (Badenes *et al.*, 2010b). The optimum alcohol concentration, enzyme concentration and w_o obtained by Badenes *et al.*, (2010a) were about 300 mM, 1 mg mL⁻¹ and 2.7, respectively. In membrane reactor, Badenes *et al.*, (2011b) could able to achieve high productivity of 500 g product day⁻¹ g⁻¹ of enzyme.

2.10.5 Cutinase in detergent and laundry industry

Cutinases have been evaluated as a lipolytic enzyme in laundry and dishwashing detergent formulations (Egmond and van Bommel, 1997; Okkels, 1997a; Uniliver, 1994a). They have oxidative stability in H₂O₂. They have good stability in a temperature range of about 20-50°C which is ideal from a cleaning point of view. Further, many lipases are not stable at pH 8–11 where most cleaning compositions are used. Cutinases are also stable in the presence of other enzymes; e.g., proteases and hence are ideal for mixtures of enzymes. The addition of

cutinase to conventional cleaning compositions does not create any special use limitation (Kolattukudy and Poulouse, 1994). For all these reasons, it has been found that cutinases are more beneficial than commercial lipase, lipolaseTM, for removing of triacylglycerols and to hydrolyse the fats without calcium (Egmond and van Bommel, 1997).

Table 2.5 Cutinase catalyzed hydrolytic and synthetic reactions

Type of reaction		Substrates	Enzymatic Preparation	Reference
Hydrolysis	Triglyceride	Triolein	Reverse micelle AOT/isooctane	Melo <i>et al.</i> , 1995b
			Aqueous/triolein biphasic medium	Flipsen <i>et al.</i> , 1996
			Free enzyme	Chen <i>et al.</i> , 2008
		Tricaprylin	Immobilization onto zeolites	Gonçalves <i>et al.</i> , 1996a
			Entrapment in calcium alginate	Gonçalves <i>et al.</i> , 1995
			Covalent binding on porous silica	Gonçalves <i>et al.</i> , 1995
	Esters	p-nitrophenyl valerate	Micelles of SDS, Triton X-100	Pocalyko and Tallman, 1998
		p-nitrophenyl palmitate	Immobilization on dextran and derevetized silica supports	Gonçalves <i>et al.</i> , 1998
			Free enzyme	Chen <i>et al.</i> , 2008, Sebastian and Kolattukudy 1988
		Methyl-,ethyl-, propyl propionate	Gas/solid system	Lamare <i>et al.</i> , 1997

Type of reaction		Substrates	Enzymatic Preparation	Reference
Synthesis	Esterification	Oleic acid + hexanol	Reversed micelles of AOT/isooctane	Sebastiao <i>et al.</i> ,1992, 1993
		Caprylic acid + butanol	Substrates in organic media	Sarazin etal.,1992, Sarazin <i>et al.</i> ,1995
		Butyric acid + 2-butanol	Phosphatidylcholine/is ooctane reversed micelles	Pinto-Sousa <i>et al.</i> , 1994
		Oleic acid + glycerol	Organic solvents	Melo <i>et al.</i> , 1995a
		Hexanoic acid + hexanol	CTAB reversed micelles	Cunnah <i>et al.</i> , 1996
			Immobilization onto Accurel EP 100 in SC CO ₂	Sereti <i>et al.</i> , 1997
		Lauric acid + pentanol	Reversed micelles of AOT/isooctane	Papadimitriou <i>et al.</i> , 1996
		Short chain fatty acid (C4-C18) + ethanol	Lyophilized enzyme in organic media	De Barros <i>et al</i> 2009a
		Caproic acid + ethanol	Lyophilized enzyme in organic media	De Barros <i>et al.</i> , 2010a
				De Barros <i>et al.</i> , 2010b
		Methyl propionate + propanol	Gas/solid system	Lamare and Legoy, 1995 ,Lamare <i>et al.</i> , 1997

Type of reaction		Substrates	Enzymatic Preparation	Reference
Transestrification		Butyl acetate + hexanol	Reversed micelles of AOT/isooctane	Carvalho <i>et al.</i> , 1997a; Carvalho <i>et al.</i> , 1998b
			Reversed micelles of CTAB/isooctane	Cunnah <i>et al.</i> , 1996
			Immobilization onto zeolites	Serralha <i>et al.</i> , 1998
		Methanol + Triolien	Reversed micelles of AOT/isooctane	Badenes <i>et al.</i> , 2010a
			Reversed micelles of AOT/isooctane	Badenes <i>et al.</i> , 2010b
			Reversed micelles of AOT/isooctane	Badenes <i>et al.</i> , 2011a

2.10.6 Cutinase in biodegradation/detoxification

Cutinase can also be used in many industrial waste water treatment processes where fat content of the waste water limits the degree of treatment e.g. leather industry. Cutinase from a genetically modified *S. cerevisiae* was reported in the tested reaction conditions, to display higher activities than the commercial Defat 50 lipase in the degreasing of a solid waste from the leather industry with a specific activity approximately three times higher than Defat 50 (Teles FRR, 2001).

Another very important feature of cutinase is that it can be used to degrade many toxic substances. For instance, cutinase has been found to degrade malathion (Kim *et al.*, 2005a). Malathion is an organophosphate insecticide and acaricide that have been used for the control of insects on field crops, fruits, vegetable, livestock and also extensively used to prevent

mosquitoes, flies, household insect, animal parasites and head body lice as substitute for DTT (Barlas, 1996; Chambers, 1992; Rettich, 1980). It persistently remains in oily products for a long period (Racke, 1992) and affects central nervous system of invertebrates, immune system of higher vertebrate wildlife, adrenal glands, liver and blood of fish (El-Dib, 1996; Galloway, 2003; Senanayake, 1987). *F. oxysporum* cutinase can degrade malathion much faster than yeast esterase with high stability (Kim *et al.*, 2005a). *F. oxysporum* cutinase also been reported to degrade endocrine disrupting chemicals like Butyl benzyl phthalate (BBP) (Kim *et al.*, 2002), di-(2-ethylhexyl)-phthalate (DEHP) (Kim *et al.*, 2003), dihexyl phthalate (DHP) (Kim *et al.*, 2007), dipropyl phthalate (DPrP) and dimethyl phthalate (DMP) (Kim *et al.*, 2005b). Phthalate esters are predominantly used in the manufacture of polyvinyl chloride to make it flexible, a lesser degree in paints lacquers and cosmetics (Chang *et al.*, 2004; Sung *et al.*, 2003). Recently, the most commonly occurring phthalates such as DPrP and DMP were classified as priority pollutants by the United States Environmental Protection Agency (Sung *et al.*, 2003).

2.10.7 Cutinase in dairy industry

Enzymes are widely used in dairy industry as a catalyst for the production of dairy flavors to enhance food flavor via lipolysis of the triacylglycerides present in milk fat to produce free fatty acids (FFA). Fatty acids provide characteristic flavors to many dairy foods, especially cheese and dairy flavor concentrates. Short-chain fatty acids such as butyric and caproic acids are volatile and provide aroma flavor to the dairy food products. Depending on the length of fatty acid chain the flavor nature changes. As the long chain fatty acids (C10–C18) have soapy or bitter flavors, they are generally not suitable for food production. Since most of the lipases have higher specificity towards long chain fatty acids alternative source of enzymes

are always in demand. Though there are lesser reports available on hydrolysis of dairy products by cutinase, they have been shown to hydrolyse fatty acids from butter (Horii *et al.*, 2010). The secretory form of *A. oryzae* cutinase, which was produced in recombinant *A. oryzae* and *S. cerevisiae* as a fusion protein with α -agglutinin was investigated for the hydrolysis of fatty acids from salt-free commercially available butter. The study showed that cutinase secreted in recombinant *A. oryzae* was able to release 16.8 mol% of butyric acid from butter whereas, cutinase displayed on the cell surface of the yeast *S. cerevisiae* as a fusion protein with α -agglutinin released butyric acid at a 2.7-fold rate (45.4 mol %) higher than that of the secreted form. It was also shown in the study that the yeasts carrying two copies of cutinase genes into their chromosomes released free fatty acids rapidly and showed 2-fold higher lipase activity compared with yeasts carrying one copy of the cutinase gene (Horii *et al.*, 2010).

2.10.8 Cutinase in fruit industry

Cutinase can be used in fruit industry for degrading a part of insoluble material of the membrane of fruits so as to enhance the water permeability across the membrane. This technique is useful for making dehydrated fruits and also for delivering some synthetic substances like sweeteners, flavor enhancers, preservatives, stabilizers into the fruits (Poulouse and Boston, 1994).

CHAPTER 3

MATERIALS AND METHODS

3.1 Chemicals and reagents

All restriction enzymes, polymerases, ligase and dNTPs used in cloning work were purchased from NEB (USA). All chemicals used in the expression study and enzyme purification experiments were procured from Sigma-Aldrich (India). Chemicals and reagents used in the medium development study were of analytical grade and obtained from HiMedia (India) or Merck (India). Chemicals and markers used in SDS PAGE were obtained from Bangalore Genei (India). Most of the chemicals and reagents used in the biochemical and biophysical characterization studies (p-nitrophenyl butyrate, p-nitrophenyl palmitate, p-nitrophenyl caproate, p-nitrophenyl valerate, p-nitrophenyl laurate, Guanidine hydrochloride, urea, DTT, EDTA, SDS, Triton X-100 and PMSF), esterification and transesterification reactions were procured from Sigma-Aldrich (India). All other chemicals used in protein analysis were of analytical grade and obtained from Merck (India).

3.2 Microorganisms and vector

Thermobifida fusca NRRL B-8184 strain used in this study as a source of cutinase was procured from Agricultural Research Service (ARS-Culture collection), USDA, Peoria, USA. *E. coli* DH5 α was procured from Microbial Type Culture Collection, Chandigarh, India and *E. coli* BL21 (DE3) and expression vector pET22b(+) were procured from Novagen Inc., USA. *Thermobifida fusca* NRRL B-8184 strain was grown on nutrient agar medium at 50°C

and maintained as glycerol stock (20% v/v) at $-20 \pm 1^\circ\text{C}$. *E. coli* strains were grown on Luria Bertani medium at 37°C and maintained as glycerol stock (20% v/v) at $-20 \pm 1^\circ\text{C}$. *E. coli* strain harboring pET22b(+) vector or pET22b(+) with cutinase gene construct was grown on Luria Bertani medium supplemented with $100 \mu\text{g mL}^{-1}$ ampicillin at 37°C and maintained as glycerol stock (10% v/v) at $-20 \pm 1^\circ\text{C}$.

3.3 Cultivation medium and culture conditions

3.3.1 Cutinase production from wild type *T. fusca*

The production of cutinase from wild strain, *T. fusca* NRRL B-8184 was performed in the modified basal semisynthetic medium containing (g L^{-1}): glucose, 5.0; beef extract, 3.0; peptone, 15.0; urea, 6.0; KH_2PO_4 , 2.0; KCl, 0.5; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 5.0 and initial pH was maintained at 7. The inoculum was prepared by adding a loop full of freshly prepared pure culture on a slant into 50 mL of autoclaved above mentioned medium containing glucose as the sole source of carbon in a 250 mL Erlenmeyer flask. The culture flask was incubated at 50°C and 200 rpm in an orbital shaking incubator for 10-12 h (Optical density (OD) at $A_{600 \text{ nm}} = 0.6$ to 0.8). A 2% of inoculum from the above seed culture was added to 50 mL of the medium containing (g L^{-1}): tomato cutin, 5.0; beef extract, 3.0; peptone, 15.0; urea, 6.0; KH_2PO_4 , 2.0; KCl, 0.5; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 5.0 (pH 7) in 250 mL Erlenmeyer flasks and incubated in an orbital shaking incubator at 50°C and 200 rpm. Samples were withdrawn at regular intervals to determine cell growth and cutinase activity.

3.3.2 Cutinase production from recombinant *E. coli* BL21 (DE3)

The standard culture method used for the production of recombinant cutinase from *E. coli* BL21 (DE3) was as follows. A single colony of recombinant *E. coli* BL21 (DE3) was

inoculated in to 5 mL of LB medium supplemented with 100 $\mu\text{g mL}^{-1}$ ampicillin and incubated at 37°C and 250 rpm in a shaking incubator for 12 h. 50 mL of LB medium supplemented with 100 $\mu\text{g mL}^{-1}$ ampicillin was inoculated with the above seed culture (around 1%), adjusted for the initial cell density A600 nm of 0.05 and incubated at 37°C and 250 rpm in a shaking incubator. IPTG was added to the culture medium (A600 nm ~0.75) and allowed to grow further. Samples were withdrawn at regular intervals to determine cell growth and cutinase activity.

3.4 Analytical methods

3.4.1 Assay for cutinase

3.4.1.1 The pNP-ester assay

Cutinase activity against p-nitrophenyl esters (pNP-ester) was determined by measuring the amount of p-nitrophenol released by hydrolysis of pNP-ester. The production of p-nitrophenol was monitored at 405 nm. The standard assay was measured in 1 mL volume with 1 mM pNP-ester as a substrate in 50 mM Potassium phosphate buffer (pH 8) containing 4% THF, 10 mM Sodium deoxycholate at 50°C for 5 min. One unit of enzyme activity is defined as release of 1 μmol of p-nitrophenol (pNP) per minute. Specific activity is defined as the activity of an enzyme per milligram of total protein (expressed in $\mu\text{mol min}^{-1} \text{mg}^{-1}$). The method for preparation of p-nitrophenol standard curve was described in appendix A.1.

3.4.1.2 The pNMSH assay

Activity against cutinase specific substrate, pNMSH (p-nitrophenyl (16-methyl sulfone ester) hexadecanoate) (Degani *et al.*, 2006) was determined by measuring the amount of p-nitrophenol released by hydrolysis of pNMSH. The production of p-nitrophenol was

monitored at 405 nm. The standard assay was measured in 1 mL volume with 1 mM pNMSH as a substrate in 50 mM Potassium phosphate buffer (pH 8) containing 4% THF, 10 mM Sodium deoxycholate at 50°C for 1 h. One unit of enzyme activity is defined as release of 1 μ mol of p-nitrophenol (p-NP) per minute. Specific activity is defined as the activity of an enzyme per milligram of total protein (expressed in μ mol min⁻¹ mg⁻¹). The method for preparation of pNMSH and p-nitrophenol standard curve was described in appendix A.4 and appendix A.1, respectively.

3.4.1.3 Cutin hydrolysis

Hydrolysis of plant cutin was analyzed using tomato cutin as follows. 1 mg tomato cutin was added to 5 mL KPO₄ buffer (50 mM, pH 8) containing 1 mg mL⁻¹ enzyme. The hydrolysis was initiated by incubating the sample at 50°C with 200 rpm shaking for 24 h. The fatty acid hydrolyzed was extracted twice with 10 mL n-hexane, derivatized in to methyl ester, filtered through 0.2 micron filter and analyzed by gas chromatography. The method for preparation of tomato cutin and methyl ester derivatization was described in appendix A.5 and appendix A.6, respectively.

3.4.2 Protein determination

The total protein content of the samples was determined according to the Bradford's method. In a standard microtiter plate protein assay, the assay mixture consisted of 1-50 μ L of protein sample and 150 μ L of Bradford reagent. The contents in the sample were mixed and incubated in dark at room temperature for 10 min. The blue color developed by the protein-dye complex was measured at 595 nm in a microtiter plate reader (Tecan,

Switzerland). The protein concentration in the reaction was determined based on a standard curve obtained with bovine serum albumin (Sigma) as standard (appendix A.2).

3.4.3 Dry cell weight (DCW)

For preparation of standard curve of DCW, 1 mL of culture grown at 37°C with 200 rpm shaking was withdrawn at different cell densities (measured at A600 nm), cells were centrifuged at 10000g for 10 min at $4 \pm 1^\circ\text{C}$ and the supernatant was discarded. The pellet was suspended in 5 mL of 50 mM KPO₄ buffer (pH 8) and centrifuged at 10000g for 10 min at $4 \pm 1^\circ\text{C}$. Cells were washed twice with the same buffer and supernatant free cells were transferred to a pre-weighed microcentrifuge tubes, dried to a constant weight at 80°C in an oven. Standard curve between DCW vs. OD at A600 nm was prepared to determine the DCW of unknown samples (appendix A.3).

3.4.4 Fluorescence spectroscopy

Steady state fluorescence was recorded on a FluoroMax spectrofluorimeter (HORIBA Scientific, USA.). Intrinsic tryptophan fluorescence spectra were recorded by exciting the samples at 295 nm with excitation and emission slit widths set at 1:3 ratios. The emission spectra were recorded in the range of 310-450 nm. Baseline corrections were carried out with corresponding buffer without protein in all cases. Each spectrum represented the average of three accumulations. 1 μM enzyme was used for analysis.

3.4.5 Circular dichroism (CD) spectroscopy

CD spectra were recorded using a JASCO J-720 or J-815 CD spectrophotometer (JASCO, Japan). Each spectrum represented the average of three accumulations recorded between wavelengths of 190 and 250 nm, with a 0.1 nm resolution, bandwidth of 1 nm, response time

of 8 sec, and a scan speed 20 nm min^{-1} using a 1 cm or 0.1 cm path length cuvette. All spectra were corrected for background by the subtraction of an appropriate buffer blank. All measurements were made at 25°C . $5 \mu\text{M}$ enzyme was used for secondary structure analysis. Tertiary structure analysis was performed in the near-UV region (250-350 nm) using $10 \mu\text{M}$ enzyme in 50 mM KPO_4 buffer (pH 8). The machine CD units in millidegree (mdeg) obtained was converted to mean residue ellipticity (MRE, θ) for plotting the graph (appendix A.7).

3.4.6 GC analysis of esters

Synthesis of fatty acid ester was analyzed by injecting the diluted aliquots of the reaction mixture in a gas chromatograph (Varian 450-GC). The column temperature was kept at 140°C for 5 min, raised to 220°C at $2^{\circ}\text{C min}^{-1}$ and was maintained at this temperature for 20 min. The temperatures of the injector and detector were set at 250°C . The column used was SLB IL-100 (Supalco) and detector was FID. The carrier gas used was N_2 and a make-up gas was H_2 and air. The molar conversion percentage calculations and the internal GC standards used for the calculations are listed in appendix A.8.

3.5 Cloning and expression of cutinase encoding genes from *T. fusca* NRRL B-8184

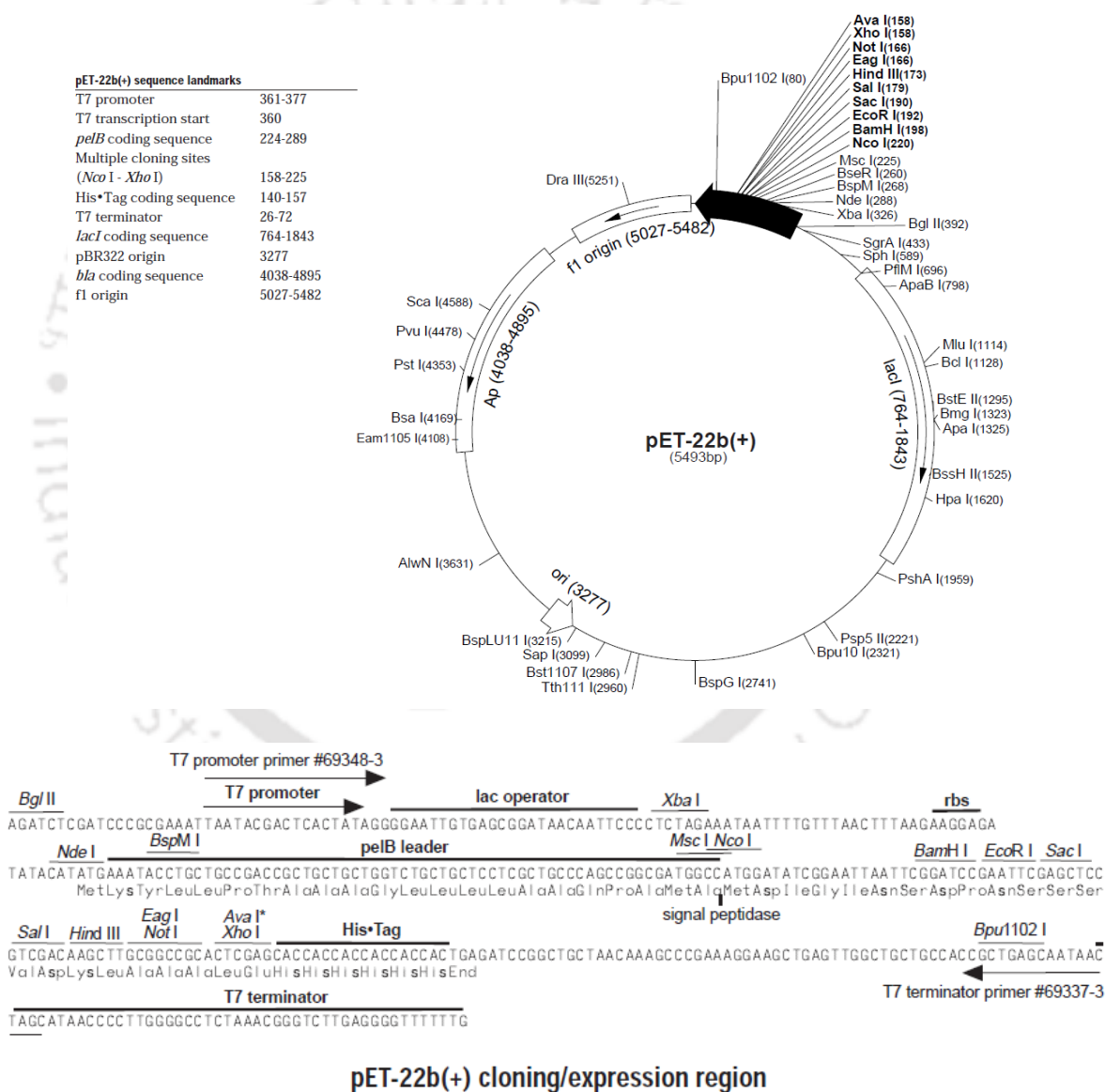
3.5.1 Choice of host and vector

E. coli BL21 (DE3) (Table 3.1) was used as a host for expression of *T. fusca* cutinase genes. Target genes were cloned into pET22b(+) vector (Fig 3.1) in frame with pelB signal peptide for periplasmic localization of recombinant protein and expressed under IPTG inducible T7 promoter.

Table 3.1 Details of the host strain *E. coli* BL21 (DE3) used for expression of cutinase

Strain	Deriv.	Genotype	Description	Ab resistance
BL21 DE3	B834	F ⁻ OmpT hsdS _B (r ⁻ _B m ⁻ _B) gal dcm (DE3)	General purpose expression host [#]	None

[#]Expression means that the strain is a λDE3 lysogen, i.e., it carries the gene for T7 RNA polymerase under lacUV5 control. It is therefore suited for expression from T7 promoters

**Fig. 3.1** Map of pET22b(+) and cloning/region

3.5.2 Primer design

DNA sequences to be amplified were taken from the NCBI data base from the organism *Thermobifida fusca* YX (Accession No. NC_007333) strain and the primer were designed using the software FastPCR. The details of the primers designed for *cut1* and *cut2* are illustrated in Fig. 3.2 and 3.3, respectively.

To study the effect of C-terminal (His)₆ tag on expression of *T. fusca* cutinases in *E. coli* BL21 (DE3) and the cutinase activity, both *cut1* and *cut2* were cloned into pET22b(+) vector with and without C-terminal (His)₆ tag. The details of all the primers used in this study are summarized in Table 3.2.

3.5.3 Isolation of genomic DNA

Genomic DNA was isolated from *T. fusca* NRRL B-8184 using Sigma genomic DNA preparation kit as per the manufacturer's instructions. The purified genomic DNA was analyzed on agarose gel (0.6%) and quantified using spectrophotometer by measuring absorbance at 260 nm (1 OD= 50 µg mL⁻¹).

3.5.4 Isolation of plasmid DNA

Plasmid DNA was isolated from *E. coli* harboring pET22b(+) or pET22b(+) with cutinase using Sigma plasmid DNA isolation kit following the manufacturer's instructions. The purified plasmid DNA was analyzed on agarose gel (0.8%) and quantified using spectrophotometer by measuring absorbance at 260 nm (1 OD= 50 µg mL⁻¹).



Fig 3.2 Design of primers for *cut1*: The *cut1* sequence (shaded grey) to which primers was designed with restriction sites. Forward and reverse primer annealing region to gene (shaded yellow in gene). Forward primer with two restriction sites (*Eco*RI-pink, *Bam*HI-blue) followed by 'a' nucleotide before start codon (marked red) to keep the gene in frame with *pelB* signal peptide. One set of reverse primer was designed with stop codon and the other without stop codon with same restriction sites (*Xho*I-violet, *Hind*III-orange)



Fig 3.3 Design of primers for *cut2*: The *cut2* sequence (shaded grey) to which primers was designed with restriction sites. Forward and reverse primer annealing region to gene (shaded yellow in gene). Forward primer with two restriction sites (*Eco*RI-pink, *Bam*HI-blue) followed by 'a' nucleotide before start codon (marked red) to keep the gene in frame with *pelB* signal peptide. One set of reverse primer was designed with stop codon and the other without stop codon with same restriction sites (*Xho*I-violet, *Hind*III-orange)

Table 3.2 Summary of primers designed for amplification of *cut1* and *cut2* of *T. fusca* to clone into pET22b(+) vector

Gene	primer	Sequence	Restriction sites
<i>cut1</i>	Forward primer	5'-ggaattcggatccaatgccccgcgatgcggcg-3' (34 mer)	<i>EcoRI</i> and <i>BamHI</i>
	Reverse Primer ^a	5'-gaagcttctcgagctagaaggggcaggtggagcg-3' (34 mer)	<i>HindIII</i> and <i>XhoI</i>
	Reverse Primer ^b	5'-gaagcttctcgaggaaggggcaggtggagcgga-3' (34 mer)	<i>HindIII</i> and <i>XhoI</i>
<i>cut2</i>	Forward primer	5'-ggaattcggatccaatggctgtgatgacccccg-3' (34 mer)	<i>EcoRI</i> and <i>BamHI</i>
	Reverse Primer ^a	5'-gaagcttctcgagctagaacgggcaggtggagc-3' (33 mer)	<i>HindIII</i> and <i>XhoI</i>
	Reverse Primer ^b	5'-gaagcttctcgaggaacgggcaggtggagcggt-3' (33 mer)	<i>HindIII</i> and <i>XhoI</i>

^a primer for amplification of genes without C-terminal (His)₆ tag

^b primer for amplification of genes with C-terminal (His)₆ tag

3.5.5 PCR amplification

All diagnostic PCR reactions (including PCR for checking of integrant, PCRs for quick check of plasmids and confirmation of clones) were set up with NEB taq (Cat No. M0273L) and preparative PCRs for amplification of insert to clone into the pET22b(+) vector were set up with high fidelity taq from NEB (Cat No. M0530L). The details of the reaction are summarized in Table 3.3. Cycling conditions followed for all diagnostic PCR and for all preparative PCR are shown in Table 3.4 and Table 3.5, respectively.

Table 3.3 PCR set up used in cloning of cutinase genes

Reagents	Amount
DNA	1-10 ng or as required
10X buffer	2 µL
10mM dNTPs	0.20 µL
Taq Polymerase	0.20 µL
Forward primer (10pM/µL)	0.20 µL
Reverse primer (10pM/µL)	0.20 µL
water	To make up to volume
Total	20 µL

Table 3.4 Conditions for diagnostic PCR of cutinase genes

Step no	PCR condition
1	94°C - 5 min
2	94°C – 1min
3	72°C - 1 min
4	Steps 2-3: 35 cycles
5	72°C-15 min
6	4°C

Table 3.5 Conditions for preparative PCR for cloning of cutinase genes

Step no	PCR condition
1	98°C - 3 min
2	98°C – 10 sec
3	72°C - 45 sec
4	Steps 2-3: 20 cycles
5	72°C-10 min
6	4°C

3.5.6 Restriction digestions

The pET22b(+) expression vector and PCR amplicon of *cut1* or *cut2* (2 µg each) was double digested for 4 h at 37°C with *Bam*HI and *Xho*I in 50 µL reaction volume for directional cloning in to pET22b(+) expression vector as per the manufacturer's instruction (NEB<http://www.neb.com/nebecomm/DoubleDigestCalculator.asp>) and purified by PCR column extraction using HiMedia PCR clean-up kit (HiMedia, India).

3.5.7 Ligation reaction

Ligation reactions were carried out using instant ligation kit from NEB (Cat No. M0202L) at 25°C for 18 h. The ligation reaction was set up at the molar ratio 1:3 (vector: insert) in final volume of 20 µL using 50 ng double digested pET22b(+) vector and corresponding molar quantity of insert as per the manufacturer's instruction. 2-3 µL of ligation mix was used for transformation into *E. coli* DH5α competent cells.

3.5.8 Preparation of competent cells of *E. coli* DH5 α and *E. coli* BL21 (DE3)

E. coli DH5 α and BL21 (DE3) competent cells were prepared by rubidium chloride (RbCl) method using 25 mL culture as follows. The *E. coli* [DH5 α or BL21 (DE3)] culture was grown in Luria-bertani (LB) medium for 12 h and inoculated again in fresh LB medium (1% inoculum) and incubated at 37°C with shaking at 220 rpm. After 2 h ($A_{600\text{ nm}} \sim 0.4-0.5$), the culture was centrifuged at 5000g for 5 min at 4°C and the supernatant was discarded. The cell pellet was suspended in sterile ice cold TFB I solution (30 mM potassium acetate, 100 mM RbCl, 10 mM CaCl₂, 50 mM MnCl₂ and 15% (v/v) glycerol, pH 5.8) and incubated on ice for 10 min. The cell suspension was centrifuged at 4100g for 5 min and the supernatant was discarded. The cells were suspended in sterile ice cold TFB II solution (10 mM MOPS, 75 mM CaCl₂, 10 mM RbCl, and 15% (v/v) glycerol, pH 6.5) and incubated on ice for 1 h. The cell suspension was then dispensed as an aliquot of 50 μL in to sterile tubes. The tubes were flash-freeze with liquid N₂ and stored at -80°C till the further use.

3.5.9 Transformation in to *E. coli* DH5 α and *E. coli* BL21 (DE3)

Both *E. coli* DH5 α and *E. coli* BL21 (DE3) were transformed as follows. 2-3 μL of ligation mix (In case of DH5 α) or 2 ng vector construct with cutinase [In case of BL21 (DE3)] was added to 50 μL competent cells prepared by RbCl method, and mixed gently by tapping and then incubated on ice for 1 hour without shaking. After 1 hour, the vial was incubated for 45 seconds at 37°C water bath followed by a snap cool on ice for 2-3 min. 400 μL SOC (tryptone, 2.0 g L⁻¹; yeast extract, 5.0 g L⁻¹; NaCl, 10 mM; KCl, 2.5 mM; MgCl₂, 10 mM; MgSO₄, 10 mM; glucose, 20 mM) was added to the cells and mixed by gentle tapping of the tube, the whole volume was plated on LB Agar plate supplemented with 100 $\mu\text{g mL}^{-1}$ ampicillin and incubated for 12-14 h at 37°C.

3.5.10 Screening of *E. coli* recombinant clones

All the colonies growing on the ampicillin plates after transformation into DH5 α were streaked on LB agar plate containing ampicillin (100 $\mu\text{g mL}^{-1}$). The cultures were grown at 37°C overnight. A loop full of culture was subjected to tooth-pick mini plasmid preparation (Sambrook and Russell, 2001) followed by confirmation by colony PCR with gene specific primers. Clones showing appropriate size were confirmed by restriction digestion analysis and sequencing.

3.5.11 Expression of cutinase encoding genes in *E. coli* BL21 (DE3)

Cutinase construct in *E. coli* BL21 (DE3) were inoculated in 50 mL LB medium in 250 mL Erlenmeyer flask with ampicillin (100 $\mu\text{g mL}^{-1}$) and incubated at 37°C and 200 rpm. Culture was induced with 0.6 mM IPTG (at A600 nm $\sim$ 0.75), 5 mL aliquot was taken in sterile condition at regular time interval and centrifuged at 8000g for 10 min at 4°C. The cell pellet was suspended in 500 μL of 50 mM Potassium phosphate buffer (pH 7), sonicated for 3 min and centrifuged for 10 min at 10000g. The supernatant was used as crude enzyme source for analysis by pNPB assay and SDS-PAGE.

3.6 Optimization of physical and chemical parameters for the production of

***T. fusca* recombinant cutinases in *E. coli* BL21 (DE3)**

As mentioned in section 3.3.2, the standard culture methods were used for the production of recombinant cutinases. Experiments were performed to determine the optimal level of IPTG for higher level of recombinant protein expression by the addition of various concentration of IPTG (0.1 mM, 0.4 mM, 0.6 mM, 0.8 mM, 1 mM) at cell density A600 nm $\sim$ 0.75 at 37°C. Temperature optimization was done by inducing the cells with optimal IPTG of 0.1 mM at

cell density A600 nm ~ 0.75. The culture was further grown at different temperature viz., 20°C, 25°C, 30°C and 37°C. Induction opportunity was studied at optimal IPTG and temperature (0.1 mM and 37°C, respectively) by adding IPTG to the culture at different A600 nm viz., 0.20, 0.5, 0.75, 1, 1.5, 2.5 and 3. Samples (2 mL each) were taken at regular time interval to measure the growth and cutinase activity by pNPB assay.

3.7 Study on effect of C-terminal (His)₆ tag on production and activity

To study the effect of C-terminal (His)₆ tag on expression and activity of recombinant Cut1 and Cut2 in *E. coli*, the *E. coli* BL21 (DE3) cells harboring pET-cut1 or pET-cut2, with or without C-terminal (His)₆ tag were grown at 37°C in 50 mL LB medium containing 100 µg mL⁻¹ ampicillin. The each culture was induced with 0.1 mM IPTG at A600 nm ~0.75 and incubated at 37°C with 250 rpm shaking for 24 h. During the cultivation, 5 mL of samples were taken at regular time interval to measure the growth and cutinase activity by pNPB assay.

3.8 Screening, selection and optimization of medium for enhanced production of *T. fusca* recombinant cutinases in *E. coli* BL21 (DE3)

3.8.1 Screening and selection of medium for enhanced production of recombinant cutinase

Experiments were performed to screen the best suitable medium for maximum production of recombinant cutinases using different media (LB, NB, M9, M9-ZB, TB and MTB). Further, the TB medium was modified in its composition (called MTB) by addition of

MgSO₄ and NaCl for enhancing the growth and possible cutinase production. The composition of the medium used are listed in Table 3.6.

Table 3.6 Composition of various medium used for screening

S. no	Medium	Composition (per liter)	Medium pH
1	Luria-Bertani (LB)	10 g Tryptone, 5 g Yeast extract, 10 g NaCl	7
2	Nutrient broth (NB)	5 g Peptone, 5 g Sodium chloride, 1.5 g Beef extract, 1.5 g Yeast extract	7.4
3	M9	4 ml glycerol, 0.246 g MgSO ₄ , 0.5 g NaCl, 1g NH ₄ Cl, 3 g KH ₂ PO ₄ , 6 g Na ₂ HPO ₄ •7H ₂ O	7
4	M9-ZB	4 ml glycerol, 10 g N-Z amine, 0.5 g NaCl, 1g NH ₄ Cl, 3g KH ₂ PO ₄ , 6 g Na ₂ HPO ₄ •7H ₂ O	7
5	Terrific broth (TB)	12 g Tryptone, 24 g Yeast extract, 4 ml Glycerol, 2.31 g KH ₂ PO ₄ , 12.54 g K ₂ HPO ₄	7.3
6	Modified TB (MTB)	24 g Yeast extract, 4 ml Glycerol, 2.31 g KH ₂ PO ₄ , 12.54 g K ₂ HPO ₄ , 0.49 g MgSO ₄ , 0.05 g NaCl	7.3

The preparation of seed culture and production medium was done as mentioned in the section 3.3.2. The culture medium was incubated at 37°C and 250 rpm in a shaking incubator, and the induction of recombinant cutinase was done by the addition of 0.1 mM of IPTG.

3.8.2 Effect of carbon sources on the production of recombinant cutinases

In order to select the most suitable substrate(s) for the maximum production of recombinant cutinases, experiments were conducted with various concentrations of two commonly used carbon sources (glucose and glycerol). The basal medium composition was of MTB (Table 3.6) with varying concentrations of glucose or glycerol as a primary carbon source.

3.8.3 Screening and optimization of significantly influencing medium components by Taguchi's experimental design

To maximize the production of recombinant cutinases, Taguchi's method (Taguchi, 1990) was applied for optimization of expression condition of recombinant Cut1 and Cut2 in MTB medium. According to Taguchi's orthogonal array, 27 experiments were performed to evaluate the effect of seven variables of the MTB medium (Table 3.6), which was assumed to have considerable influence on the recombinant protein expression in *E. coli*. According to experimental plan given in the Table 3.7, experiments were performed with each variable represented at three levels. In each experimental run, the response was taken as the cutinase production and corresponding signal-to-noise (S/N) ratio was calculated using Eq. 3.1 with an overall objective of estimating the effects of various parameters on cutinase production, where a large S/N ratio is preferred. All experiments were conducted in duplicates and averages of the results were taken as the response.

$$\frac{S}{N} = -10 \log \left[\frac{\left(\frac{1}{Y^2} \right)}{n} \right] \quad 3.1$$

Where, Y is the response [enzyme activity (U mL⁻¹)], and n is the number of experimental runs. Statistical analysis of the results in the form of analysis of variance (ANOVA) was performed using statistical software package MINITAB® (Version 15.1.00, Minitab Inc., USA).

Table 3.7 A Taguchi's design matrix in real values and corresponding levels (in parenthesis) for screening of production of recombinant Cut1 and Cut2

Run order	Variables and their values						
	A	B	C	D	E	F	G
1	6 (1)	12 (1)	2 (1)	1.15 (1)	6.27 (1)	0.61 (1)	0.05(1)
2	6 (1)	12 (1)	2 (1)	1.15 (1)	12.54 (2)	1.23 (2)	0.29 (2)
3	6 (1)	12 (1)	2 (1)	1.15 (1)	20.00 (3)	1.84 (3)	0.58 (3)
4	6 (1)	24 (2)	4 (2)	2.31 (2)	6.27 (1)	0.61 (1)	0.05 (1)
5	6 (1)	24 (2)	4 (2)	2.31 (2)	12.54 (2)	1.23 (2)	0.29 (2)
6	6 (1)	24 (2)	4 (2)	2.31 (2)	20.00 (3)	1.84 (3)	0.58 (3)
7	6 (1)	36 (3)	8 (3)	3.50 (3)	6.27 (1)	0.61 (1)	0.05 (1)
8	6 (1)	36 (3)	8 (3)	3.50 (3)	12.54 (2)	1.23 (2)	0.29 (2)
9	6 (1)	36 (3)	8 (3)	3.50 (3)	20.00 (3)	1.84 (3)	0.58 (3)
10	12 (2)	12 (1)	4 (2)	3.50 (3)	6.27 (1)	1.23 (2)	0.58 (3)
11	12 (2)	12 (1)	4 (2)	3.50 (3)	12.54 (2)	1.84 (3)	0.05 (1)
12	12 (2)	12 (1)	4 (2)	3.50 (3)	20.00 (3)	0.61 (1)	0.29 (2)
13	12 (2)	24 (2)	8 (3)	1.15 (1)	6.27 (1)	1.23 (2)	0.58 (3)
14	12 (2)	24 (2)	8 (3)	1.15 (1)	12.54 (2)	1.84 (3)	0.05 (1)
15	12 (2)	24 (2)	8 (3)	1.15 (1)	20.00 (3)	0.61 (1)	0.29 (2)
16	12 (2)	36 (3)	2 (1)	2.31 (2)	6.27 (1)	1.23 (2)	0.58 (3)
17	12 (2)	36 (3)	2 (1)	2.31 (2)	12.54 (2)	1.84 (3)	0.05 (1)
18	12 (2)	36 (3)	2 (1)	2.31 (2)	20.00 (3)	0.61 (1)	0.29 (2)
19	20 (3)	12 (1)	8 (3)	2.31 (2)	6.27 (1)	1.84 (3)	0.29 (2)
20	20 (3)	12 (1)	8 (3)	2.31 (2)	12.54 (2)	0.61 (1)	0.58 (3)
21	20 (3)	12 (1)	8 (3)	2.31 (2)	20.00 (3)	1.23 (2)	0.05 (1)
22	20 (3)	24 (2)	2 (1)	3.50 (3)	6.27 (1)	1.84 (3)	0.29 (2)
23	20 (3)	24 (2)	2 (1)	3.50 (3)	12.54 (2)	0.61 (1)	0.58 (3)
24	20 (3)	24 (2)	2 (1)	3.50 (3)	20.00 (3)	1.23 (2)	0.05 (1)
25	20 (3)	36 (3)	4 (2)	1.15 (1)	6.27 (1)	1.84 (3)	0.29 (2)
26	20 (3)	36 (3)	4 (2)	1.15 (1)	12.54 (2)	0.61 (1)	0.58 (3)
27	20 (3)	36 (3)	4 (2)	1.15 (1)	20.00 (3)	1.23 (2)	0.05 (1)

Where, A is tryptone (g L^{-1}), B is Yeast extract (g L^{-1}), C is Glycerol (mL L^{-1}), D is KH_2PO_4 (g L^{-1}), E is K_2HPO_4 (g L^{-1}), F is MgSO_4 (g L^{-1}) and G is NaCl (g L^{-1}).

3.8.4 Validation of the model at predicted optimum levels of chemical parameters

In order to validate the model, experiments were performed at optimal levels of the most significantly influencing variables at shake flask level. The culture method employed for

this study is as described in section 3.3.2. All experiments in shake flask were conducted in duplicates and averages of the results were taken as response.

3.9 Cellular localization of *T. fusca* recombinant cutinases in *E. coli* BL21 (DE3)

Cellular localization of expressed recombinant Cut1 and Cut2 was analyzed using fresh culture pellet taken from IPTG induced culture. 2 mL culture pellet was washed in 200 μ L sterile distilled water, suspended in 100 μ L Buffer A (Tris-HCl, 200 mM; pH 7.5; Sucrose, 20%; EDTA, 1mM; 30 U μ L⁻¹ lysozyme), incubated on ice and 100 μ L of chilled distilled water was added to it. The suspension was centrifuged and the supernatant containing soluble periplasmic fraction was collected for further analysis. The pellet part was suspended in buffer B (Tris-HCl, 10 mM, pH 7.5; KCl, 50 mM; EDTA, 1 mM; Deoxycholate, 0.1%) and incubated on ice for 5 min, centrifuged and the supernatant containing soluble spheroplastic fraction was collected for analysis. To check the presence of inclusion body (IB), 10 mg cell pellet was suspended in 100 μ L lysis buffer (Tris-HCl, 100 mM, pH 8.5; NaCl, 150 mM; EDTA, 10 mM and 0.5 mg mL⁻¹ lysozyme), incubated on ice for 1 h and sonicated for 3 min under cold condition. The suspension was centrifuged and supernatant containing soluble protein and pellet containing inclusion bodies (if any), were collected separately for analysis. Induction medium supernatant was used as source of enzyme to check the extracellular secretion. All the fractions were analyzed by pNPB activity assay and SDS-PAGE.

3.10 Purification of *T. fusca* recombinant cutinases by affinity chromatography

3.10.1 Enzyme production for purification

E. coli BL21 (DE3) cells harboring pET22-*cut1* or pET22-*cut2* were induced with 0.1 mM IPTG in TB medium (Table 3.6) supplemented with 100 $\mu\text{g mL}^{-1}$ ampicillin and grown for 9 h at 37°C with 200 rpm shaking. Experiments were performed as described in the section 3.3.2. After 9 h of induction cells were harvested and centrifuged at 10000*g* for 10 min at 4 $\pm$ 1°C. The pellet was washed once with 50 mM KPO₄ buffer (pH 8) and centrifuged at 8000*g* for 10 min at 4 $\pm$ 1°C and the pellet was used for further processing and purification.

3.10.2 Affinity purification of recombinant cutinases

The cell pellet was suspended in buffer A (NaH₂PO₄, 50 mM; NaCl, 0.5 M; imidazole, 10 mM; lysozyme, 1 mg mL⁻¹; pH 8.0), sonicated for 5 min and centrifuged at 10000*g* at 4 $\pm$ 1°C. The supernatant was loaded into 10 mL Nickel affinity column (Sigma) pre-equilibrated with Buffer A (without lysozyme). The lysate was circulated in the affinity column for 2 h at 1 mL min⁻¹ through peristaltic pump at 4 $\pm$ 1°C for thorough binding of (His)₆ tagged protein to Ni affinity matrix. The column was washed with 10 column volumes of buffer A (without lysozyme) and eluted with 2 column volume of Buffer B (NaH₂PO₄, 50 mM; NaCl, 0.5 M; imidazole, 250 mM). The fractions containing cutinase activity were pooled and dialyzed against 4 liter potassium phosphate buffer (20 mM, pH-8) at 4°C overnight. The purified enzyme was concentrated by ultra-freezing (Christ, ALPHA 1-4), suspended in potassium phosphate buffer (50 mM, pH 8) and used for analysis of purity (SDS PAGE) and activity

(various assay as mentioned in section 3.4.1). The enzyme was dispensed in to small aliquots flash freeze with liquid N₂ and stored at -20°C for long term-storage.

3.11 Biochemical characterization of purified *T. fusca* recombinant cutinases

3.11.1 Homogeneity analysis by SDS PAGE

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) of the purified enzyme was carried out to check the homogeneity of the enzyme preparation and to determine its molecular weight. SDS-polyacrylamide gel electrophoresis was performed in Mini PROTEAN[®] Tetra Cell system (BIO-RAD, USA) using 1.5 mm thick gels, following the method of Laemmli (1970). 10% (w/v) of acrylamide for resolving gel and 5% (w/v) of acrylamide for stacking gel were used. The protein samples were prepared in 0.5 M Tris-HCl buffer (pH 6.8) containing 2.3% (w/v) sodium dodecyl sulfate, 10% (w/v) glycerol, 5% (w/v) β-mercaptoethanol and 0.05% (w/v) bromophenol blue. The sample buffer contained SDS (the anionic detergent) for movement of samples under charged conditions. Loading samples were prepared by mixing 1 part of 5x sample buffer to 4 parts of the enzyme sample. This mixture was incubated at 95°C for 5 min to denature the enzyme for performing the SDS-PAGE.

3.11.2 Molecular weight determination by MALDI-TOF-MS

The intact molecular mass was determined by matrix assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrophotometry. The matrix was prepared in deionized water containing CHCA (10 mg mL⁻¹), 50% acetonitrile and 0.1% TFA. Cutinase was mixed

with matrix (1:1) and 2 μ L of the sample was spotted on plate, dried at room temperature and analyzed.

3.11.3 Isoelectric point (pI) determination

Isoelectric point of purified Cut1 and Cut2 was determined by 2-D electrophoresis on 10% SDS PAGE (17 cm) with pH gradient of 3-10.

3.11.4 Effect of pH on activity and stability of purified enzyme

The optimum pH for cutinase activity was determined under the assay conditions over a pH range of 5-9 using pNPB as substrate for enzyme assay. For pH stability studies, the enzyme preparations were incubated in a pH range of 5-9 for various time intervals at 37°C in the absence of substrate and residual activity was determined using pNPB assay. The buffers, potassium phosphate (50 mM, pH 5-8), Tris HCl (50 mM, pH 8-9) were used for this study.

3.11.5 Effect of temperature on activity and stability of purified enzyme

The temperature dependence of the enzyme was studied by pNPB assay at temperature ranging from 20–80°C. The pNPB assay was set-up at 25°C as mentioned in section 3.4.1.1. The reaction mixture was incubated at various temperatures and residual activity was determined. Thermostability was determined by pre-incubating the purified enzyme for various time intervals at temperatures, 37 and 55°C and analyzing the residual activity by pNPB assay.

3.11.6 Kinetic analysis of cutinase

Kinetic studies were performed with pNPB (0.05–2 mM) as substrate using the continuous spectrophotometric assay. Initial reaction velocities were calculated from the linear region

(60 s) of the reaction progress curve and measured in triplicate by varying the concentration of the substrate. Apparent kinetic constants, K_m were calculated from a double-reciprocal plot [$1/v$ vs. $1/(pNPB)$] of the initial rate data. Results are the average of triplicate assays. k_{cat} was calculated on the basis of molecular weight of the enzymes determined by MALDI-TOF analysis.

3.11.7 Studies on the thermal deactivation kinetics of cutinase

In order to study the thermal stability of cutinase, the enzyme was incubated at four different temperatures between 45°C and 80°C. The pH of the purified enzyme was adjusted to four different levels, viz., 6.0, 7.0, 8.0 and 9.0. The enzyme samples were deactivated at various combinations of pH and temperature as mentioned in the Table 3.8 and aliquots of samples were collected at different intervals of time and were assayed for the residual enzyme activity as described in section 3.4.1.1.

Table 3.8 Experimental condition adopted to study the deactivation of purified cutinase at different pH and temperature

pH	Temperature (°C)
6	45
	55
	70
	80
7	45
	55
	70
	80
8	45
	55
	70
	80

pH	Temperature (°C)
9	45
	55
	70
	80

3.11.8 Estimation of deactivation rate constant

The following first order expression was used to account for the zero activity at a particular temperature and at specified incubation time.

$$\frac{dE}{dt} = -k_d E \quad 3.2$$

so that,

$$\ln \left[\frac{E_t}{E_0} \right] = -k_d t \quad 3.3$$

Where, k_d is enzyme deactivation rate constant (h^{-1}); t is incubation time (h); E_t is the enzyme activity (U mL^{-1}) at time t and E_0 is initial enzyme activity (U mL^{-1}) at time $t=0$. The values of k_d were calculated from the plot of $\ln(E_t/E_0)$ vs. t at a particular temperature.

The half-life of an enzyme was defined as the time required by the enzyme to lose half of its initial activity and can be expressed by the following equation.

$$t_{\frac{1}{2}} = \frac{\ln 2}{k_d} \quad 3.4$$

3.11.9 Estimation of thermodynamic parameters for cutinase deactivation

In order to obtain the change in enthalpies (ΔH^*) and change in entropies (ΔS^*) during enzyme deactivation process, it is necessary to make use of the theory of absolute reaction rates (Eyring, 1935; Kapat and Panda, 1997). The central point of this theory is that the rate of any reaction at a given temperature depends only on the concentration of an energy-rich

activated complex, which is in equilibrium with the inactivated reactants. The deactivation constant is expressed by the following equation.

$$k_d = \frac{\kappa T}{h} \cdot e^{\frac{\Delta S^*}{R}} \cdot e^{\frac{-\Delta H}{RT}} \quad 3.5$$

Or

$$\ln\left(\frac{k_d}{T}\right) = \ln\left(\frac{\kappa}{h}\right) + \left(\frac{\Delta S^*}{R}\right) - \left(\frac{-\Delta H^*}{R}\right)\left(\frac{1}{T}\right) \quad 3.6$$

where, k_d is enzyme deactivation rate constant (h^{-1}); κ is Boltzmann constant ($1.38 \times 10^{-23} \text{ J} \cdot \text{K}^{-1}$); h is Plank's constant ($6.626 \times 10^{-34} \text{ J} \cdot \text{s}$); ΔH^* is change in enthalpy ($\text{J} \cdot \text{mol}^{-1}$); ΔS^* is change in entropy ($\text{J} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$); R is gas constant ($8.314 \text{ J} \cdot \text{M}^{-1} \cdot \text{K}^{-1}$) and T is temperature (K). The values of ΔH^* and ΔS^* were calculated from the slope and intercept of the plot of $\ln(k_d/T)$ versus $1/T$, respectively. Values of change in free energy (ΔG^*) were further estimated by the following relationship.

$$\Delta G^* = \Delta H^* - T\Delta S^* \quad 3.7$$

Where, ΔG^* is change in free energy (J mol^{-1}).

The activation energy (E_A) was calculated from the Arrhenius equation as:

$$k_d = k_0 \exp\left(-\frac{E_A}{RT}\right) \quad 3.8$$

or

$$\ln k_d = \ln k_0 - \left(\frac{E_A}{R}\right)\left(\frac{1}{T}\right) \quad 3.9$$

Where, E_A activation energy (J mol^{-1}) and k_0 is frequency factor (h^{-1}).

The values of E_A and k_0 were estimated from the slope and intercept of the plot of $\ln(k_d)$ versus $1/T$, respectively.

3.11.10 Substrate specificity

The substrate specificity of the purified cutinase was analyzed using the following substrates: (1 mM) of pNP-fatty acyl esters: acetate, C2; butyrate, C4; valerate, C5; octanoate, C8; decanoate, C10; laurate, C12; myristate, C14; and palmitate, C16 as substrates. The activity was expressed as relative activity in comparison to activity with p-nitrophenyl butyrate.

3.11.11 Effect of various solvents on cutinase stability

Stability against organic solvents was studied by incubating the enzyme (100 $\mu\text{g mL}^{-1}$) in 50 mM potassium phosphate buffer (pH 8.0) containing 40% organic solvent (v/v) at 25°C for 1 h and analyzing the residual activity with pNPB (1mM) as a substrate. Based on the literature, the average amount of solvents commonly used in the reaction medium (up to 40%) was selected to study the stability of the enzyme. The activity was expressed as relative activity as compared to solvent free controls. The stability of cutinases over time in few industrially important solvents in perspective of applications like esterification and transesterification was investigated by incubation the enzyme (100 $\mu\text{g mL}^{-1}$) in various organic solvents viz., acetonitrile, butanol, ethanol, methanol, THF and n-hexane at 25°C for various time intervals and analyzing the residual activity by pNPB assay. The activity was expressed as relative activity as compared to solvent free controls incubated in KPO₄ (50 mM, pH 8) buffer at 25°C.

3.11.12 Effect of various surfactants on cutinase activity

Effect of surfactants was studied by incubating the enzyme (100 $\mu\text{g mL}^{-1}$) in 50 mM potassium phosphate buffer (pH 8.0) containing 1 mM or 10 mM surfactant at 37°C for 1 h and analyzing the residual activity with pNPB (1mM) as a substrate. The activity was expressed as relative activity as compared to surfactant free controls.

3.11.13 Effect of various metal ions and inhibitors on cutinase activity

Effect of metal ions (1 mM) and inhibitors on the enzymatic activity was investigated by incubating the enzyme (100 $\mu\text{g mL}^{-1}$) for 10 min at 37°C in 50 mM potassium phosphate buffer (pH 8.0) containing corresponding concentration of additives and residual activity was measured using pNPB as a substrate. The relative activity was expressed as the percentage ratio of the activity of the enzyme incubated with metal ions to that of the untreated enzyme.

3.12 Biophysical characterization of purified *T. fusca* recombinant cutinases

3.12.1 Effect of pH on secondary structure of cutinases

The enzyme solution was prepared in 50 mM KPO₄ buffers at different pH (6 to 9) was incubated at 25°C for 10 min. The change in environment of tryptophan residue present in the protein was analyzed using fluorescence spectroscopy as mentioned in section 3.4.4. The change in secondary structure was analyzed using CD spectroscopy as mentioned in section 3.4.5.

3.12.2 Effect of denaturant (guanidine hydrochloride and urea) on secondary structure of cutinases

Effect of guanidine hydrochloride (GdnHCl) on unfolding of enzymes was studied as follows. The GdnHCl solution in the range of 0-8 M was prepared in 50 mM KPO₄ buffer (pH 8) and enzyme was added to it. The solution was incubated at 25°C for 14-15 h and the spectroscopic measurements were made as mentioned in section 3.4.4 (for fluorescence spectroscopy) and section 3.4.5 (for CD spectroscopy). Appropriate blank with corresponding concentration of GdnHCl without enzyme was used for baseline correction.

To study the effect of urea on unfolding of the enzymes, the urea solution was prepared in the range of 0-8 M in 50 mM KPO₄ buffer (pH 8) and enzyme was added to it. The solution was incubated at 25°C for 14-15 h and the tryptophan fluorescence was measured as mentioned in section 3.4.4. Appropriate blank with corresponding concentration of urea without enzyme was used for baseline correction.

3.12.3 Dynamic quenching of tryptophan fluorescence

Quenching effect of iodide ion on the tryptophan fluorescence of native and urea denatured enzyme (prepared as mentioned in section 3.11.2) was investigated using potassium iodide (KI) in the range of 0-2 M concentration. Appropriate blank with corresponding concentration of urea and KI without enzyme was used for baseline correction. The spectroscopic measurements were made as mentioned in section 3.4.4.

The classical relation employed to describe the collisional quenching process is given by the Stern-Volmer equation,

$$\frac{F_0}{F} = 1 + K_{SV} [Q] \quad 3.10$$

Where, F_0 and F are fluorescence intensities at appropriate emission wavelengths in the absence and the presence of quencher molecules, respectively. The collisional quenching constant K_{SV} gives the slope of the intensity ratio as a function of the quencher concentration $[Q]$.

3.13 Structural analysis of *T. fusca* cutinases by homology modeling

Structural analysis was done based on the homology model constructed using MODELER ModWeb server Ver. SVN.r1368M (Sali lab). Crystal structure of esterase from *Thermobifida alba* (PDB ID: 3VIS) was selected as a template for construction of model. The initial models were refined by energy minimization using DeepView/Swiss-Pdv viewer software package (Guex and Peitsch, 1997). Accuracy of the predicted model was examined by Ramchandran Plot obtained from the PROCHECK (Laskowski *et al.*, 1993) and structural features were validated using different online server programs (PDBsum, VADAR, ModFold server, QMEAN server, SAVER and PDB2PQR Server). For 3D modeling PyMOL Version 1.5 software package was used. Appendix A.9 gives details on the official url of the various web servers used in the structural analysis.

3.14 Studies on immobilization of *T. fusca* recombinant cutinases on various supports

In the present study, different immobilization support was screened to study the effect of immobilization support on *T. fusca* cutinase, and use of immobilized cutinase in subsequent applications like esterification and transesterification reaction. The parameters such as, temperature, pH, incubation time and enzyme concentration was selected to study their effect on immobilization process according to the available methods in the literature for other enzymes or the other cutinases.

3.14.1 Covalent immobilization on chitosan support

3.14.1.1 Preparation of chitosan beads

3.0% (w/v) chitosan beads were prepared by dissolving powdered chitosan in 1.5% (v/v) glacial acetic acid at 50-60°C. The mixture was then added drop wise into 150 mL 1 N KOH solution containing 25% (v/v) ethanol under stirring condition using a syringe needle (21 G). To get uniform sized beads, the syringe was fixed at an appropriate height. The solution was allowed to stand for 1-2 h for hardening of beads. The beads of diameter nearly 0.4-0.5 mm

and uniform shape obtained were immediately washed with sterile double distilled water and stored in 20% ethanol at 4°C until activation with glutaraldehyde.

3.14.1.2 Bead activation and immobilization of cutinase

Chitosan beads were activated at different concentration of glutaraldehyde in the range of 1.5% to 4% (v/v) at room temperature for 3 h. The activated beads were washed extensively with 50 mM potassium phosphate buffer (pH 8) to remove all traces of un-reacted glutaraldehyde. The activated beads were incubated with 0.5 mg mL⁻¹ purified cutinase for 24 h at 4°C for enzyme coupling. To determine optimal pH for cutinase coupling, the chitosan beads activated with 3% (v/v) glutaraldehyde were incubated for 24 h at 4°C with 0.5 mg mL⁻¹ cutinase in 50 mM potassium phosphate buffer in the range of pH 6-9. The beads were washed with 50 mM potassium phosphate buffer to remove un-bound cutinase. The activity of the chitosan coupled and unbound cutinase was measured by pNPB assay as mentioned in section 3.4.1.1. The protein content in the unbound fraction was estimated by Bradford method as mentioned in the section 3.4.2.

The percentage immobilization (percentage activity retention) was calculated as follows:

$$\text{Immobilization (\%)} = \frac{\text{Total specific activity of immobilized cutinase}}{\text{Total specific activity of soluble cutinase}} \times 100 \quad 3.11$$

The total specific activity of immobilized cutinase was determined by subtracting total specific activity of unbound cutinase from total specific activity of soluble enzyme.

3.14.2 Covalent immobilization on magnetic nanoparticles (MNPs)

3.14.2.1 Preparation of MNPs

Magnetic nanoparticle Fe₃O₄ was prepared by co-precipitation of Fe²⁺ (FeCl₂·4H₂O) and Fe³⁺ (FeCl₃·6H₂O) at molar ratio 1:2 in ammonia solution at 25°C. Fig. 3.4 shows the summary of

steps involved in immobilization of cutinase on MNPs. The precipitation was achieved by drop wise addition of NH_4OH to the ferrous and ferric solution (0.3 M final conc.) under vigorous stirring. During the precipitation process, the pH of the solution was maintained at about 10. The precipitate was heated for 30 min at 80°C , cooled to room temperature and washed with distilled water and ethanol several times till the pH drops to 7. The washed MNPs were dissolved in ethanol and used for 3-aminopropyltriethoxysilane (APES) coating. The silica coating on MNPs was achieved by dispersing 1 g of MNPs in ethanol containing 3% (v/v) APES. The suspension was sonicated for 5 min to disperse the MNPs thoroughly followed by rolling the sample bottle overnight at 25°C . The APES-bound magnetic nanoparticles were recovered from the reaction mixture by placing the bottle on a permanent magnet. The supernatant was removed and the precipitates were washed several times with ethanol.

3.14.2.2 Activation and immobilization of cutinase

Cutinase was immobilized on APES-bound magnetic nanoparticles through glutaraldehyde linkage (Fig. 3.4). A 20 mL of 10% (v/v) glutaraldehyde (GA) was added to the precipitates of APES-coated magnetic nanoparticles, and the sample was kept on rolling at 25°C for 4 h. After 4 h, the particles were settled with a magnetization and then washed several times with water to remove all trace of GA. The activated MNPs were incubated with 1 mg mL^{-1} purified cutinase for 24 h at 4°C for enzyme coupling. To determine optimal pH for cutinase coupling, the MNPs activated with 10% (v/v) glutaraldehyde were incubated for 24 h at 4°C with 1 mg mL^{-1} cutinase in 50 mM potassium phosphate buffer in the range of pH 6-9. The MNPs were washed with 50 mM potassium phosphate buffer to remove un-bound cutinase. The activity of the MNP coupled and unbound cutinase was measured by pNPB assay as mentioned in

section 3.4.1.1. The protein content in the unbound fraction was estimated by Bradford method as mentioned in section 3.4.2. The percentage immobilization (percentage activity retention) was calculated as per the eq. 3.11

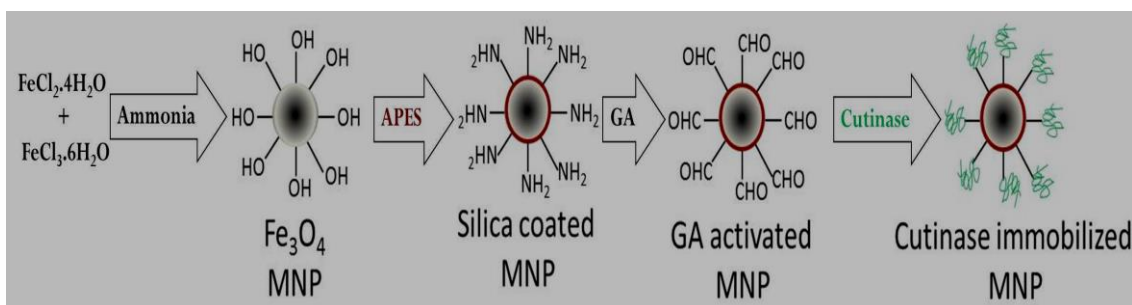


Fig. 3.4 Schematic representation of steps in cutinase immobilization on MNPs

3.14.3 Adsorption on zeolite (NaY) and Celite-545

Immobilization on Zeolite and Celite-545 by adsorption was carried out as follows. 100 mg zeolite or Celite-545 was washed with ethanol thrice followed by a wash with KPO_4 buffer (50 mM, pH 8). 1 mg mL^{-1} enzyme was added to the support and kept on rolling to adsorb at 4°C for 24 hours. Total amount of protein adsorbed was estimated by Bradford assay as mentioned in the section 3.4.2 and the activity was measured by pNPB assay as mentioned in the section 3.4.1.1. The percentage immobilization (percentage activity retention) was calculated as per the eq. 3.11.

3.14.4 Biophysical characterization of cutinase immobilized supports

3.14.4.1 Fourier Transform Infrared (FTIR) Spectra of cutinase immobilized supports

Changes in the biophysical properties of immobilization support of chitosan and MNPs, before and after immobilization of cutinase were analyzed using FTIR (UNICAM Mattson 1000 FTIR spectrophotometer) spectra. For FTIR spectra, samples were crushed with

potassium bromide (KBr) to form a very fine powder. This powder was then compressed into thin pellet for analysis.

3.14.4.2 Field Emission Scanning Electron Microscopy (FESEM) of chitosan beads

The surface morphology of the chitosan beads, glutaraldehyde activated beads, and cutinase immobilized beads were analyzed by FESEM (Carl Zeiss, SIGMA). Samples were coated with a thin layer of gold, prior to examination using FESEM.

3.14.4.3 Vibration sample magnetometer (VSM) analysis of MNPs

The magnetic properties of the MNPs before and after immobilization of cutinase were analyzed by VSM (Lakeshore-7410).

3.14.5 Biochemical characterization of immobilized enzymes

Various biochemical properties of immobilized cutinase viz., coupling pH (In case of chitosan and MNP immobilization), pH and temperature optima and stability, freeze-drying and its effect, storage stability, reusability of immobilized cutinase were investigated. All the experiments were performed in duplicate, separately, using 1 mg sample (in case of MNPs, Zeolite and Celite-545 immobilized samples) or five beads per sample (in case of chitosan immobilized samples) as mentioned in the following sections. In all the cases, cutinase without immobilization and the immobilization support without enzyme were used as positive and negative controls, respectively.

3.14.5.1 The pH and temperature optima

The activity of cutinase as a function of varying pH was measured to determine the pH optima under immobilized condition. Activity of cutinase was measured by pNPB assay in a range of pH varying from 5 to 9 as mentioned in section 3.4.1.1.

The effect of temperature on activity of immobilized cutinase was investigated by incubating samples for 10 min followed by pNPB assay at different temperature ranging from 37 to 80°C.

3.14.5.2 The pH and thermal stability

The ability of immobilized cutinase to retain its activity at different pH was studied by incubating immobilized cutinase in buffer of different pH, ranging from pH 5 to 9. Cutinase immobilized samples were incubated in corresponding buffer for 1 h at 37°C. The buffer was removed and residual activity of the sample was measured by pNPB assay.

Thermal stability of the immobilized cutinase was investigated at 55°C. A series of tubes having immobilized cutinase in 50 mM potassium phosphate buffer (pH 8) was incubated at 55°C. Tubes were removed at regular time intervals to check the residual activity by pNPB assay.

3.14.5.3 Freeze-drying and its effect

The effect of freeze drying on stability and activity of immobilized cutinase was investigated as follows. Cutinase immobilized support was subjected to freeze drying (Christ, ALPHA 1-4) at -50° C under vacuum for 1 h. The freeze dried samples were reconstituted by soaking in 50 mM potassium phosphate buffer (pH 8) for 15 min. These reconstituted samples were then used to measure activity by pNPB assay. The used immobilized cutinase was washed 3 times with 50 mM potassium phosphate (pH 8) and subjected to repeated freeze drying and activity assay to assess the reusability. The relative activity was expressed as the percentage ratio of the activity of the enzyme without freeze dry.

3.14.5.4 Storage stability and enzyme leaching study

Storage stability of the immobilized cutinase at 4°C was measured as a function of pNPB activity at different time intervals as follows. The immobilized cutinase was stored in 50 mM potassium phosphate buffer (pH 8) at 4°C and taken at regular time interval to measure the activity by pNPB assay. In order to study the leaching of coupled enzyme (if any) in a course of time, the storage buffer was used as a source of enzyme to check the activity by pNPB assay.

3.14.5.5 Reusability of immobilized cutinase

The reusability of the immobilized enzyme was assessed as follows. After each activity assay, the immobilized cutinase was removed and washed with 50 mM potassium phosphate buffer (pH 8), to remove any residual substrate within the chitosan beads and stored in the same buffer at 4°C until further use. They were then reintroduced into fresh assay buffer, and the enzyme activity was determined. The relative activity was expressed as the percentage ratio of the activity of the enzyme to first re-usage cycle.

3.15 Studies on applications of *T. fusca* cutinases in various esterification and transesterification reactions

In the standard protocol, ester synthesis was carried out in screw-capped micro reaction vial as bioreactors. Unless otherwise specified, 3 mg mL⁻¹ of enzyme in lyophilized powder form was added to 2 mL of n-hexane containing 0.25 M fatty acid and 0.25 M alcohol. All reagents are previously dried over 4 Å molecular sieves. The tubes were incubated at 50°C with 180 rpm shaking. At regular intervals, 100 µL of the mixture were transferred to 1.5 mL micro centrifuge tube and centrifuged at 10,000g for 1 min to remove the suspended enzyme

particles. The progress of reaction was monitored by gas chromatography as described in sections 3.4.6. Various biophysical and biochemical parameters were optimized to investigate the catalytic efficiency, alcohol:acid molar ratio, amount of initial water content and catalytic temperature.

Table 3.9 shows various esterification and transesterification reactions carried out using cutinase (free as well as immobilized) as a biocatalyst. A comparative study on catalytic efficiency of free as well as cutinase immobilized on various supports was carried out using equal amount of enzymes in terms of unit mg^{-1} of protein (in case of free enzyme) or support (in case of immobilized enzyme).

Table 3.9 Various esterification and transesterification reactions performed for recombinant *T. fusca* cutinases

Reaction	Alcohol	Acid
Isopropyl esters		
Isopropyl oleate	Isopropanol	Oleic acid
Geraniol ester		
Geranyl acetate	Geraniol	Acetic acid
Geranyl butyrate		Butyric acid
Geranyl valerate		Valeric acid
Geranyl decanoate		Decanoic acid
Butyl esters		
Butyl butyrate	Butanol	Butyric acid
Butyl valerate		Valeric acid
Butyl octanoate		Octanoic acid
Butyl decanoate		Decanoic acid
FAME/FAEE [#]		
FAME	Methanol	Tributyryn
FAEE	Ethanol	Tributyryn
Lactic acid esters		
Ethyl lactate	Ethanol	Lactic acid

[#] FAME, Fatty Acid Methyl Ester
FAEE, Fatty Acid Ethyl Ester

CHAPTER 4

RESULTS AND DISCUSSION

Cutinase is a hydrolytic enzyme that degrades cutin, short-chain fatty acid esters as well as emulsified triacylglycerol. This enzyme has immense potential in different industries as discussed in chapter 2. In the following studies, the cloning of cutinase genes from *T. fusca* NRRL B-8184, production of recombinant cutinases in *E. coli*, purification, characterization and applications of *T. fusca* NRRL B-8184 cutinases have been carried out.

4.1 Cutinase production from wild type *T. fusca*

In order to validate the *T. fusca* NRRL B-8184 strain obtained from NRRL, the production of cutinase from the wild type *T. fusca* NRRL B-8184 was performed as described in the section 3.3.1. Cutinase activity was estimated by pNPB assay and cutinase specific substrate, pNMSH according to the method described in section 3.4.1.1 and 3.4.1.2, respectively. The enzyme activity towards pNPB and pNMSH was found to be 58 ± 1.2 and $4.7 \times 10^{-2} \pm 4.9 \times 10^{-3}$ U mL⁻¹, respectively. He *et al.*, (2009) reported a similar production yield (52.4 U mL⁻¹) earlier for wild type *T. fusca* WSH03-11 with butyrate as the carbon source.

4.2 Cloning and expression of cutinase encoding genes from *T. fusca* NRRL B-8184

4.2.1 Cloning of cutinase encoding genes from *T. fusca* NRRL B-8184

Amplification of *cut1* and *cut2* was carried out with gene specific primers to express as fusion protein with C-terminal (His)₆-tag (Section 3.5.2). To study the effect of (His)₆ tag on expression and activity of recombinant proteins, amplification of each gene was performed with stop codon for expressing the recombinant proteins without (His)₆ tag (Section 3.5.2). After amplification of the genes by PCR, products of expected sizes of *Cut1* (~960 bp), *cut2* (~906 bp) of *T. fusca* NRRL B-8184 was observed in agarose gel (1.0%) electrophoresis as shown in Fig. 4.1.

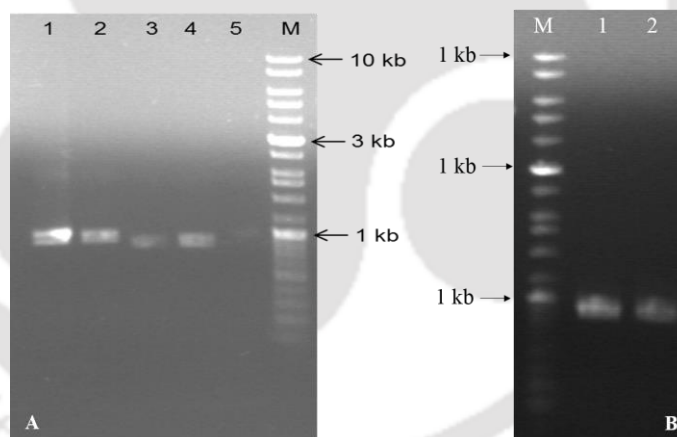


Fig. 4.1 PCR amplification of genes encoding cutinase with and without stop codon. (A) Lane 1, PCR amplicon of *cut1* without stop codon; Lane 2, PCR amplicon of *cut1* with stop codon; Lane 3, PCR amplicon of *cut2* without stop codon; Lane 4, PCR amplicon of *cut2* with stop codon; Lane 5, Negative PCR control; M, 1 kb molecular marker. (B) Lane 1 and 2, PCR amplicon of *cut2* without stop codon and with stop codon, respectively; M, 1 kb molecular marker

The amplified PCR products and pET22b(+) were double digested with *Bam*HI and *Xho*I restriction enzyme and purified with commercially available purification kit. Purified DNA fragments were cloned into the expression vector, pET22b(+) in frame with pelB leader

sequence with and without C-terminal (His)₆ tag and transformed in to *E. coli* DH5 α . Conformation of positive clones was carried out by tooth-pick mini plasmid preparation to screen the putative positive clones followed by PCR (Fig. 4.2 A and B), restriction digestion with *Bam*HI and *Xho*I (Fig.4.2 C and D) and nucleotide sequencing. The vector map of the constructed pET22b(+)_*cut1* or pET22b(+)_*cut2* is represented in Fig. 4.3.

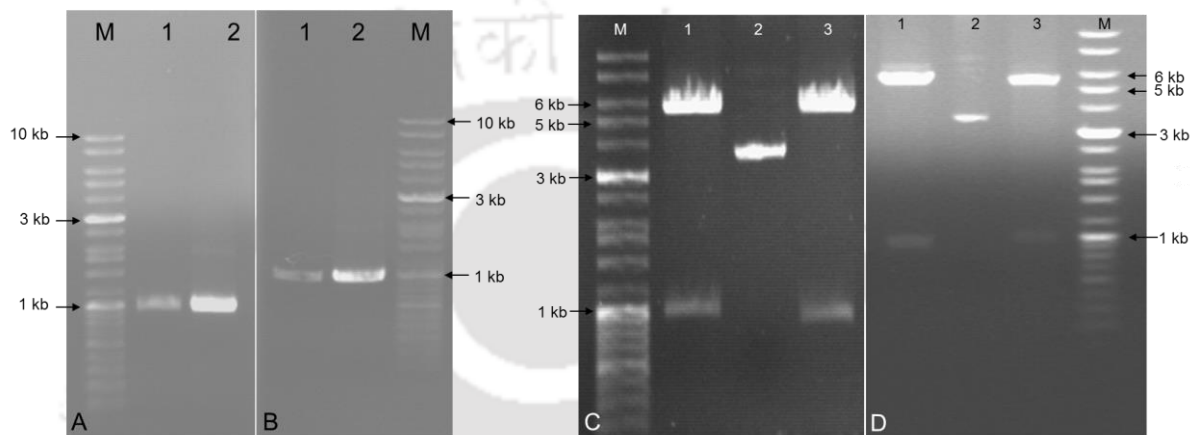


Fig. 4.2 Confirmation of cutinase genes cloned into pET22b(+) expression vector by PCR and restriction digestion. (A) PCR amplification of cloned *cut1* with (Lane1) and without (Lane2) stop codon. (B) PCR amplification of cloned *cut2* with (Lane1) and without (Lane2) stop codon. (C) *Bam*HI/*Xho*I double restriction digestion of cloned *cut1* (Lane1) and *cut2* (Lane3) with stop codon. (D) *Bam*HI/*Xho*I double restriction digestion of cloned *cut2* (Lane1) and *cut1* (Lane3) without stop codon. Lane 2 in C and D represents double digested pET22b(+) vector without insert. M. 1 kb molecular marker

4.2.2 Nucleotide sequencing and analysis of cutinase construct in pET22b(+) vector

PCR and restriction digestion analysis confirmed putative pET22b(+)_*cut1* and *cut2* clone was further confirmed by gene sequencing using T7 promoter and T7 terminator specific primers. BLASTn of the sequence of the genes revealed 960 bp and 906 bp length for *cut1* and *cut2*, respectively (Fig. 4.4) with 93% homology among them. BLASTx result of the *cut1* showed 100% homology to the available triacylglycerol lipase; *tfu 0882* of *Thermobifida fusca* YX (Acc. no. YP_288943.1), whereas, the *cut2* showed 100% homology to the

available triacylglycerol lipase; *tfu 0883* of *Thermobifida fusca* YX (Acc. No. YP_288944.1). The nucleotide sequence of *cut1* and *cut2* has been deposited in NCBI with Acc. No. JN129499.1 and JN129500.1, respectively. Both the genes were found to be in frame with *pelB* signal peptide and T7 promoter without any mutation.

4.2.3 Expression of cutinase encoding genes in *E. coli* BL21 (DE3)

The pET expression system is one of the most powerful systems developed for the cloning and expression of recombinant proteins in *E. coli* (pET expression manual). Target genes are cloned in pET plasmids under control of strong bacteriophage T7 transcription and translation signals. The expression is induced by providing a source of T7 RNA polymerase in the host cell. T7 RNA polymerase is so selective and active that, when fully induced, almost all of the cell's resources are converted to target gene expression; the desired product can comprise more than 50% of the total cell protein within a few h after induction. Although this system is extremely powerful, it is also possible to attenuate the expression level simply by lowering the concentration of inducer. Decreasing the expression level may enhance the soluble yield of some target proteins. Another important benefit of this system is its ability to maintain target genes transcriptionally silent in the uninduced state. All these features provide great flexibility and the ability to optimize the expression of a wide variety of target genes (pET expression manual).

The pET22b(+) expression vector construct with nucleotide sequence confirmed *cut1* or *cut2* was transformed in to an IPTG inducible expression host, *E. coli* BL21 (DE3) and experiments were carried out by inducing the culture with IPTG according to the methods described in Section 3.5.11 for the production of recombinant cutinase. Fig. 4.5 represents the SDS-PAGE showing the expression of recombinant cutinases (~30 kDa) in LB medium.

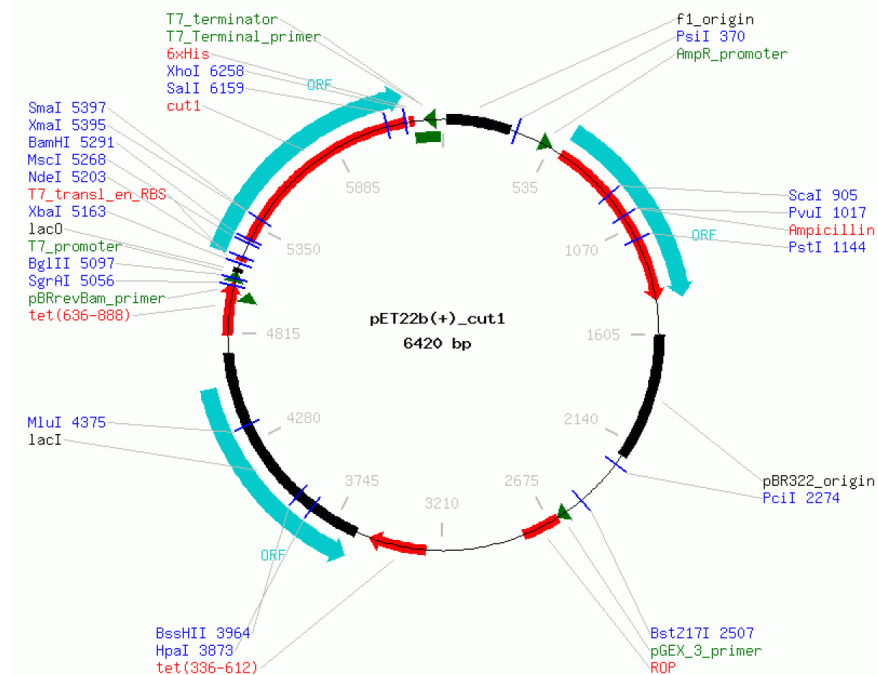
Under unoptimized physiochemical parameters, the recombinant cutinase production in the LB medium was found to be 87 ± 1.7 and 93 ± 2.1 U mL⁻¹ for Cut1 and Cut2, respectively.

The following section discusses the optimization of the physiochemical parameters for the production of recombinant cutinases in *E. coli* BL21 (DE3).

4.3 Optimization of physical and chemical parameters for the production of *T. fusca* recombinant cutinases in *E. coli* BL21 (DE3)

Physical and chemical parameter such as IPTG, growth temperature plays a vital role in production of recombinant proteins. To study the effect of IPTG concentration, temperature and cell density on the expression of recombinant cutinases, experiments were performed according to the methods described in the section 3.6 in the LB medium. The optimal concentration of IPTG was found to be 0.1 mM for higher expression of both the recombinant cutinases (Fig. 4.6 A), though there was no remarkable difference in the production yield in the range of IPTG (0.1 to 1 mM) studied. A series of growth temperature, in the range of 20 to 37°C had been tried, among which the highest expression (97.3 ± 2.2 and 107.8 ± 2.1 U mL⁻¹ in terms of pNPB activity for Cut1 and Cut2, respectively) was observed at 37°C (Fig. 4.6 B). It was observed that the cell density at which the induction of recombinant protein by IPTG plays a vital role for achieving higher level of expression. Addition of IPTG at cell density A_{600 nm} < 0.5 or > 1 gave very poor cutinase expression (Fig. 4.6 C). The expression of both Cut1 and Cut2 was found to be 28 ± 1 and 12 ± 0.91 U mL⁻¹ at cell density of A_{600 nm} 0.2 and 3, respectively. While, the cell density A_{600 nm} 0.75 was found to be the optimum with 98 ± 1.2 and 110 ± 0.62 U mL⁻¹ production, respectively for Cut1 and Cut2.

A



B

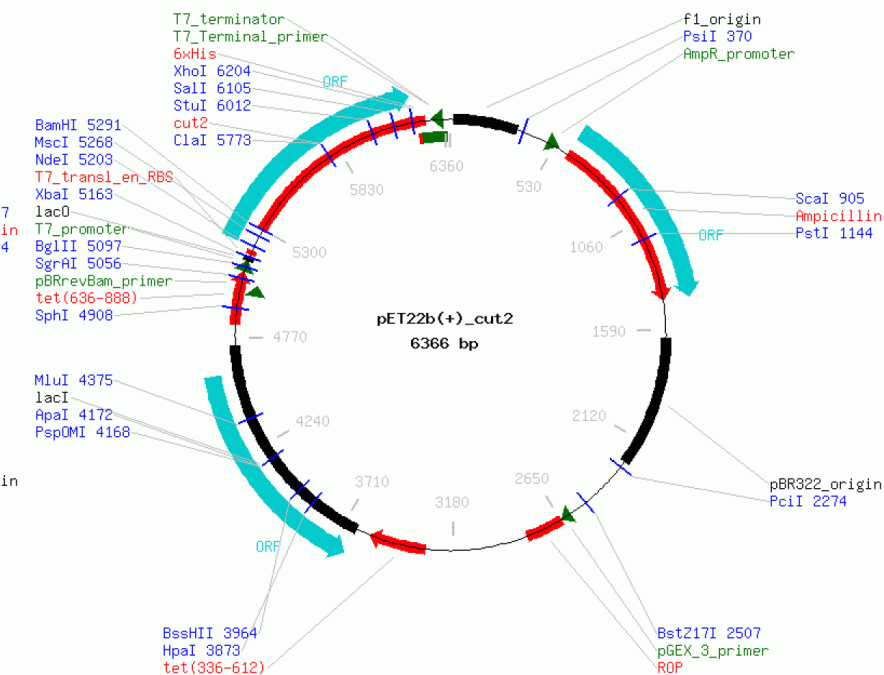


Fig. 4.3 Map of the pET22b(+) construct of *cut1* (A) and *cut2* (B) showing the inserted gene between *Bam*HI and *Xho*I, IPTG inducible T7 promoter , T7 terminator, C-terminal (His)₆ tag and other features of the vector

(A) Nucleotide and amino acid sequence of *Cut1*

```

atgccccgcatgcccgcggcccgccctgcacagaaccgaaggagcgtgcaatggct
M P P H A A R P G P A Q N R R G R A M A
gtgattacccccgcgggagcgtcttccctgctctcccgggcactgcgcttcaccgcc
V I T P R R E R S S L L S R A L R F T A
gcggctgccacagcgttctgtaccgcggtcagcctggcgcccccgctcatgccgcaac
A A A T A L V T A V S L A A P A H A A N
ccctacgagcgcggccccaaccgaccgacccctgctcgaagcccgagcggcccttc
P Y E R G P N P T D A L L E A R S G P F
tccgtgagtgaagaacggcctcccgcttcgggtgctgacgggttcggcgggcgaccatc
S V S E E R A S R F G A D G F G G G T I
tactccccggggagaacaacacctacgggtgctgacgttcccccggtacacgggc
Y Y P R E N N T Y G A V A I S P G Y T G
accagggcctctgtgcctgggtggcgagcgcacgcctccacggcttcgtcgtcatc
T Q A S V A W L G E R I A S H G F V V I
accatcgacaccaacaccacccctcgaccagccggacagccgggccccgcagctcaacgcc
T I D T N T L D Q P D S R A R L A S Q
gcgctggactacatgatcaacgacgcctcgtccgcggtgcgacggcgatcgacagcagc
A L D Y M I N D A S S A V R S R I D S S
cgactggcggtcatgggccaactccatggcgggcgggcgacccctgctgtggcctcccag
R L A V M G H S M G G G G T L R L A S Q
cgtcccgacctgaaggccgccatcccgctcaccctggcgaccccaacaagaactggagc
R P D L K A A I P L T P W H L N K N W S
agtgtgcgggttcccaccctcatcatcgtgctgacctggacacccatcgtccggctcctc
S V R V P T L I I G A D L D T I A P V L
acccacgcccggcccttctacaacagcctcccgacctcgatcagcaaggcctacctggag
T H A R P F Y N S L P T S I S K A Y L E
ctggacggcgcaacccacttcgccccgaacatccccaacaagatcatcggaagtacagc
L D G A T H F A P N I P N K I I G K Y S
gtcgcctggctcaagcgggttcgtcgacaacgacacccgctacacccagttcctctgcccc
V A W L K R F V D N D T R Y T Q F L C P
ggaccgcgcgacggactcttcggcgaggtcgaagagtaccgctccacctgccccttctag
G P R D G L F G E V E E Y R S T C P F *

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(B) Nucleotide and amino acid sequence of *Cut2*

```

atggctgtgatgacccccgcgggagcgtcttccctgctctcccgagctctgcaagtg
M A V M T P R R E R S S L L S R A L Q V
acggctgcgggtgccacagcgttctgtgaccgcggtcagcctggcgcccccgctcatgcc
T A A A A T A L V T A V S L A A P A H A
gccaaacctacgagcgcggccccaaccgaccgacgcctgctcgaagccagcagcggc
A N P Y E R G P N P T D A L L E A S S G
cccttctccgtcagcgcaggagaacgtctcccggttgagcgcagcggcttcggcgggc
P F S V S E E N V S R L S A S G F G G G
accatctactaccgcgggagaacaacacctacgggtgcggtggcgatctcccccggtac
T I Y Y P R E N N T Y G A V A I S P G Y
accggcactgaggttccatcgctgggtggcgagcgcacgcctccacggcttcgtc
T G T E A S I A W L G E R I A S H G F V
gtcatcaccatcgacaccatcaccacccctcgaccagccggacagccggcgagcagctc
V I T I D T I T T L D Q P D S R A E Q L
aacgcgcgctgaaccacatgatcaaccgggctcctccacgggtgcgagccggatcgat
N A A L N H M I N R A S S T V R S R I D
agcagccgactggcggtcatgggccaactccatggcgggcgggcgacccctgctctggcc
S S R L A V M G H S M G G G G T L R L A
tcccagcgtcccgacctgaaggccgccatcccgctcaccctggtgcacctcaacaagaac
S Q R P D L K A A I P L T P W H L N K N
tggagcagcgtcacctgcccgcgacgtgatcatcgggccgaccccgacacgatcgcggccg
W S S V T V P T L I I G A D L D T I A P
gtcgcacgcacgcgaaacgggttctacaacagcctgcccagctccatcagcaaggcctac
V A T H A K P F Y N S L P S S I S K A Y
ctggagctggacggcgcaacccacttcgccccgaacatccccaacaagatcatcggaag
L E L D G A T H F A P N I P N K I I G K
tacagcgtcgcctggctcaagcgggttcgtcgacaacgacacccgctacacccagttcctc
Y S V A W L K R F V D N D T R Y T Q F L
tgccccggacccgcgcgacggactcttcggcgaggtcgaagagtaccgctccacctgcccc
C P G P R D G L F G E V E E Y R S T C P
ttctag
F *

```

Fig. 4.4 Nucleotide and amino acid sequence of *cut1* (A) and *cut2* (B) cloned in to pET22b(+) vector

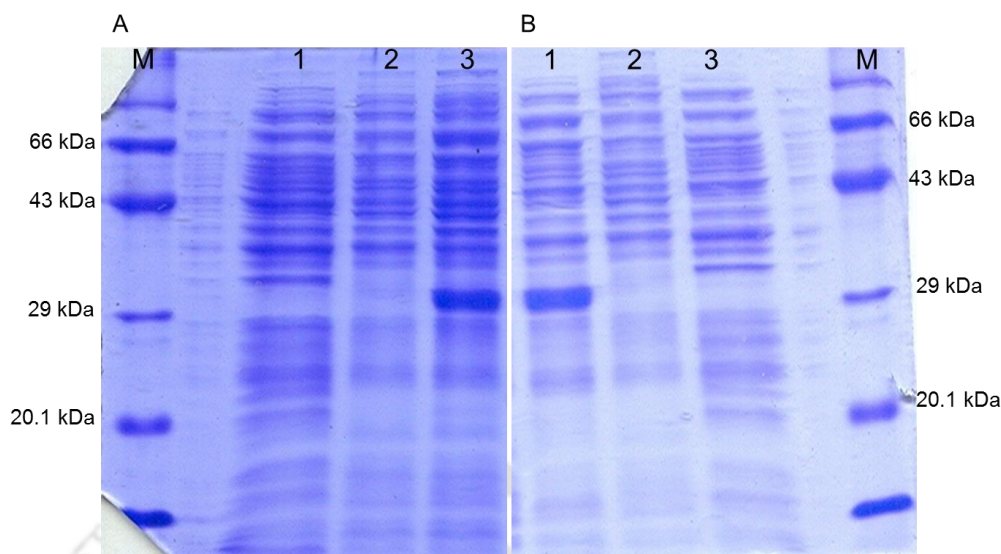


Fig. 4.5 A 12% SDS-PAGE analysis of recombinant cutinase expression in LB medium for Cut1 (A) and Cut2 (B) after 6 h induction. (A) Lane 1, *E. coli* BL21 (DE3) uninduced; lane 2, Cell lysate of *E. coli* BL21 (DE3) with pET22b(+)_cut1 uninduced; lane 3, Cell lysate of *E. coli* BL21 (DE3) with pET22b(+)_cut1 induced with IPTG; (B) Lane 1, Cell lysate of *E. coli* BL21 (DE3) with pET22b(+)_cut2 induced with IPTG; lane 2, Cell lysate of *E. coli* BL21 (DE3) with pET22b(+)_cut2 uninduced; lane 3, *E. coli* BL21 (DE3) uninduced

Under the optimal levels of IPTG concentration, temperature and cell density (0.1 mM, 37°C and A600 nm 0.75), the maximum expression of Cut1 and Cut2 was observed to be 101 ± 0.29 and 112 ± 0.20 U mL⁻¹, respectively, after 6 h post induction.

The following section discusses the effect of (His)₆ tag on the growth and production of recombinant cutinase in *E. coli* BL21 (DE3).

4.4 Study on effect of C-terminal (His)₆ tag on the production and activity

Effect of C-terminal (His)₆ tag on growth, and activity is depicted in Fig 4.7. Though there are several reports on positive and negative effect of (His)₆ tag on the expression of recombinant proteins, solubility and activity of recombinant cutinase from *F. solani* (Kwon *et al.*, 2009) and other enzymes (Arnau *et al.*, 2006), in this case, the expression of enzyme with or without

(His)₆ tag showed no considerable difference and there was negligible difference in growth pattern (Fig. 4.7 A). Effect of (His)₆ tag on enzyme activity was also studied by pNPB assay. Surprisingly, there was no difference in either activity or specific activity of the enzymes (Fig. 4.7 B). Hence, we selected the cutinase with C-terminal (His)₆ tag for all further studies, as it is more reliable in downstream processing as compare to non-tagged enzyme.

Further, screening, selection and optimization of the medium were investigated for enhanced production of recombinant cutinases in *E. coli* BL21 (DE3). The results are discussed in the following section.

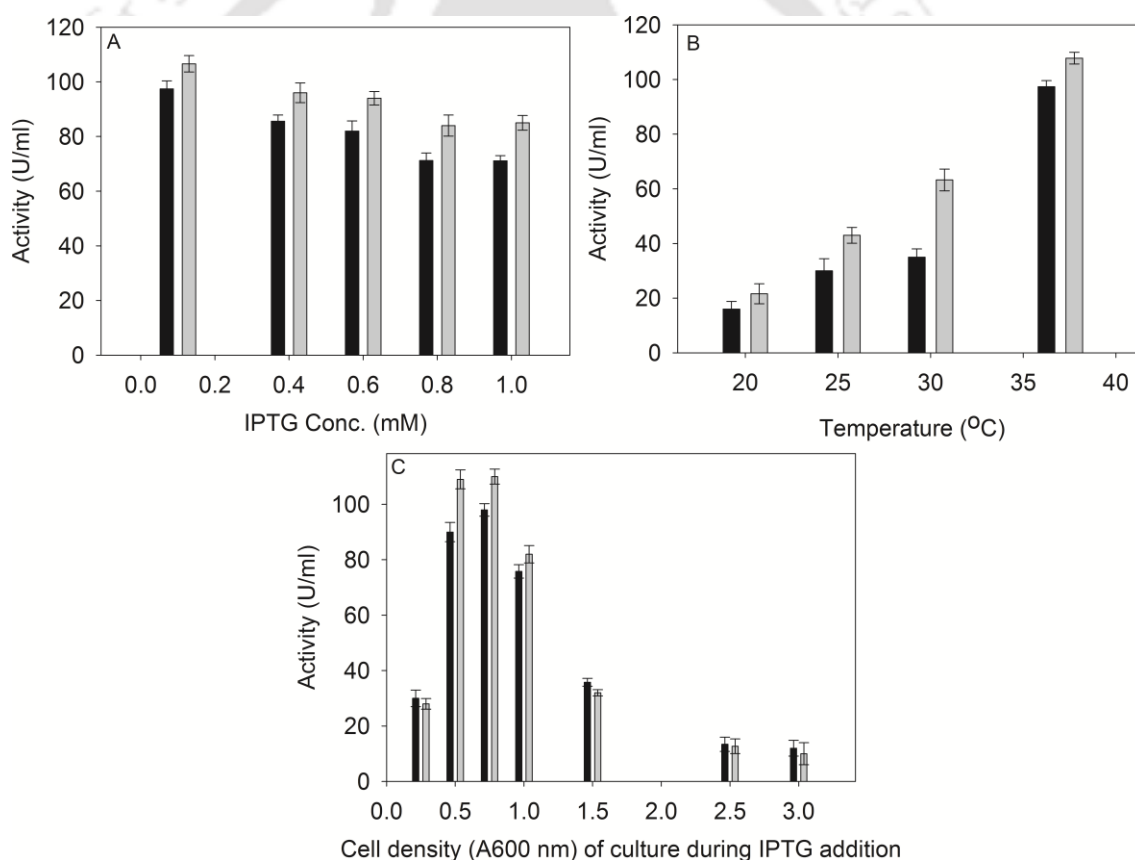


Fig. 4.6 Physiochemical parameter optimization for expression. (A) Effect of IPTG on expression of Cut1 (black bars) and Cut2 (grey bars), (B) Effect of temperature on expression of Cut1 (black bars) and Cut2 (grey bars). (C) Effect of cell density at A600 nm of culture during addition of IPTG on expression of Cut1 (black bars) and Cut2 (grey bars). Error bar represents the average of two experiments with $\pm$ SD

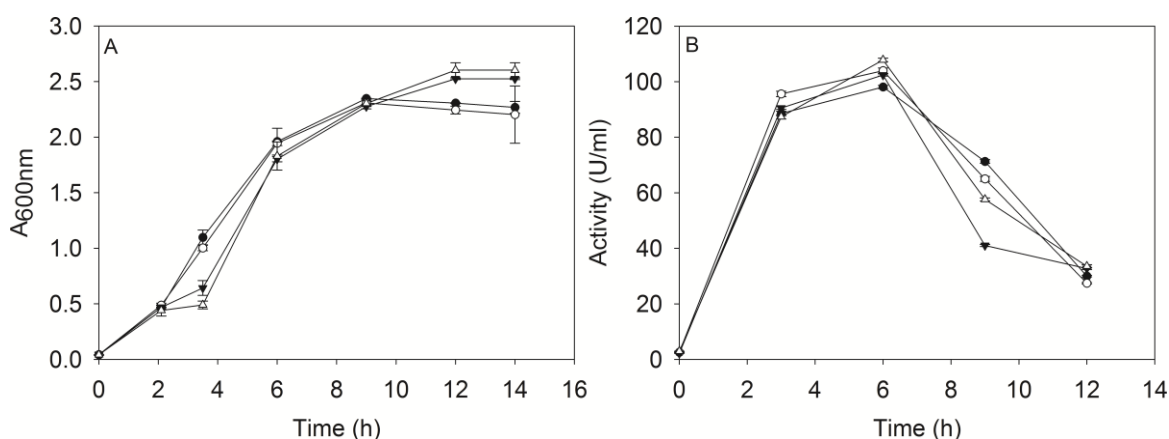


Fig. 4.7 Comparison of effect of C-terminal (His)₆ tag on growth (A) and the cutinase activity (B) between the *E. coli* BL21 (DE3) expressing C-terminal (His)₆ tagged Cut1 (filled circles), Cut2 (filled inverted triangles) and non-tagged Cut1 (empty circles) and Cut2 (empty triangles). Error bar represents the average of two experiments with $\pm$ SD

4.5 Screening, selection and optimization of medium for enhanced production of *T. fusca* recombinant cutinases in *E. coli* BL21 (DE3)

4.5.1 Screening and selection of medium for enhanced production of recombinant cutinases

The goal of optimizing production of recombinant proteins is to produce the highest amount of functional product per unit volume per unit time. For *E. coli*, or any other fermentation system, the level of accumulation of a recombinant protein is dependent on the final cell density and the specific activity of the protein, which in turn depends on medium formulation. According to Lee (1996) and Kleman and Strohl (1994), typical strategies taken for optimizing the production of a recombinant protein are, choice of culture medium, mode of cultivation, strain improvement, and expression system control. To evaluate the cost effective media formulation for achieving high cell density and production, five different available media were screened and the best suited medium was further modified for the enhancement of production.

Summary of growth in each of the medium using shake flask cultures is depicted in Fig. 4.9 A and B. TB medium showed longer exponential phase and higher cell density as compare to any other screened medium. A systematic study on effect of MgSO_4 on cell density of *E. coli* has been published by Studier (2005). The study indicates that addition of MgSO_4 in growth medium at concentration of 1 mM to 2 mM almost doubled the cell density of *E. coli* BL21 (DE3). There are various other reports on increase in cell density by the addition of MgSO_4 . Abdulkarim *et al.*, (2009) reported that addition of 0.5% NaCl considerably enhance the *E. coli* growth at 37°C. It was also observed in the present study that the addition of 2 mM MgSO_4 and 1 mM NaCl to TB medium (called MTB) showed highest cell density (4.7 ± 0.56 and $4.3 \pm 0.74 \text{ g L}^{-1}$ DCW for Cut1 and Cut2, respectively) among all the screened medium, which accounts for the increase in growth by 1.5 fold as compare to TB medium (Fig. 4.9 A and B). Based on the obtained result, if we assume that the levels of accumulation of recombinant proteins is constant and independent of media composition, then the increase in biomass yields alone will serve to increase the production of a given recombinant protein. To analyze the above said prediction and effect of media composition on the production of recombinant Cut1 or Cut2, the relative level of expression was studied in each of the six media. All the media were inoculated to get an initial cell density of $A_{600 \text{ nm}} \sim 0.05$ and induced at $A_{600 \text{ nm}} 0.75$ to eliminate the effects caused by differences in expression due to cell growth stage. However, the final cell densities were different due to the different growth-rates attained in each of the medium. Recombinant protein accumulation was assessed by enzyme assay using pNPB and SDS-PAGE.

Summary of cutinase produced in different medium is depicted in Fig. 4.9 C and D. Highest level of expression was observed after 6 h post-induction in case of LB, NB, M9-ZB, and M9

medium and started to decline thereafter. But in case of TB and MTB medium, there was increase in expression from 3 h with highest activity at 9 h of post induction and slow decrease in production rate with time as compared with other screened medium (Fig. 4.9 C and D). MTB medium showed highest level of cutinase production with around 1.1-fold higher than TB and ~2.5-fold higher than LB medium. One of the explanations for such differential expression profile would be the limiting nutrients in case of LB, NB, M9-ZB, and M9 media. The other possible reason is the sharp change in medium pH. It was observed that pH of the non-buffered medium was increased from pH 7 (initial) to 8.9 and 8.5 just after 6 h induction in case of LB and NB, respectively. However, the pH was decreased to 6 and 6.7 in case of M9 and M9-ZB, respectively, though it was buffered medium (Table 4.1). It is a well-known fact that most of the *E. coli* is best grown in the pH range of 7-8 and sharp change in pH above or below this optimal level might be deleterious to growth and expression. Growth overwhelming the buffering capacity of KH_2PO_4 and Na_2HPO_4 would be one of the reasons for pH change in M9-ZB and M9 medium. Interestingly, TB and MTB showed least deviation in pH (0.1 to 0.2) even after 20 h (Table 4.1). Keen observation of expression and medium pH change, showed that controlled pH is one of the important criteria in achieving higher expression. The decrease in the final pH in medium other than LB and NB (Which does not contain glucose or glycerol in the medium composition) is due to the presence of carbon source glycerol. When sugars such as glycerol and glucose are used as carbon source in *E. coli* cells, they produce acetic acid which lowers the medium pH. Thus, the MTB medium with 2 mM MgSO_4 and 1 mM NaCl was used for the production of recombinant cutinase from *E. coli* BL21 (DE3). Under unoptimized levels of medium components, the production was found to be 260 ± 1.1 and $263 \pm 0.77 \text{ U mL}^{-1}$, respectively for Cut1 and Cut2 in MTB

medium. These data show that medium composition along with media pH has a significant effect on the accumulation of recombinant proteins.

Table 4.1 pH and activity profile of recombinant cutinases in different medium

Medium	Initial pH	Cut 1			Cut 2		
		pH after 6 h	pH after 20 h	Highest activity (U mL ⁻¹) ^a	pH after 6 h	pH after 20 h	Highest activity (U mL ⁻¹) ^a
LB	7	8.9 ± 0.1	8.9 ± 0.1	101 ± 0.29	8.8 ± 0.3	8.9 ± 0.1	112 ± 0.20
NB	7.4	8.5 ± 0.1	8.5 ± 0.1	148 ± 0.12	8.5 ± 0.1	8.5 ± 0.1	152 ± 0.90
M9	7	6.0 ± 0.2	6 ± 0.1	203 ± 1.0	6.1 ± 0.2	6.2 ± 0.2	188 ± 1.78
M9-ZB	7	6.7 ± 0.1	6.7 ± 0.1	157 ± 1.2	6.8 ± 0.1	6.8 ± 0.1	165 ± 1.32
TB	7.3	7.4 ± 0.1	7.4 ± 0.1	230 ± 0.98	7.4 ± 0.1	7.6 ± 0.1	236 ± 1.43
MTB ^b	7.3	7.4 ± 0.1	7.4 ± 0.2	260 ± 1.1	7.3 ± 0.1	7.4 ± 0.2	263 ± 0.77

^a highest activity was observed after 6 h post induction in LB and NB and 8 h post induction in rest of the medium, ^bTB with 2 mM MgSO₄ and 1 mM NaCl. Results are the average of two experiments with ± SD

4.5.2 Effect of carbon sources on the production of recombinant cutinase

The effect of different concentrations of glucose and glycerol as a carbon source on the growth and production of recombinant cutinase from *E. coli* BL21 (DE3) is presented in Fig. 4.8. Experiments were performed according to the method described in the section 3.8.2 with *E. coli* BL21 (DE3) harboring Cut2 as a model organism, as there was not much difference in the growth and production profile of Cut1 and Cut2 in the earlier studies (Section 4.5.1). It was observed that there was a constant increase in growth as well as production in the presence of glucose as a substrate in the range of 1 to 4 g L⁻¹. However, the growth as well as the production of cutinase was much lower in the medium containing glucose as a source of carbon in comparison to glycerol (Fig. 4.8). Maximum cell density was observed at around 12 h of cultivation in the medium supplemented with 0.4% glycerol and the glycerol concentration below or above 0.4% showed relatively poor cell growth. Maximum enzyme production was observed after 9 h of post induction in the medium containing 0.4% glycerol.

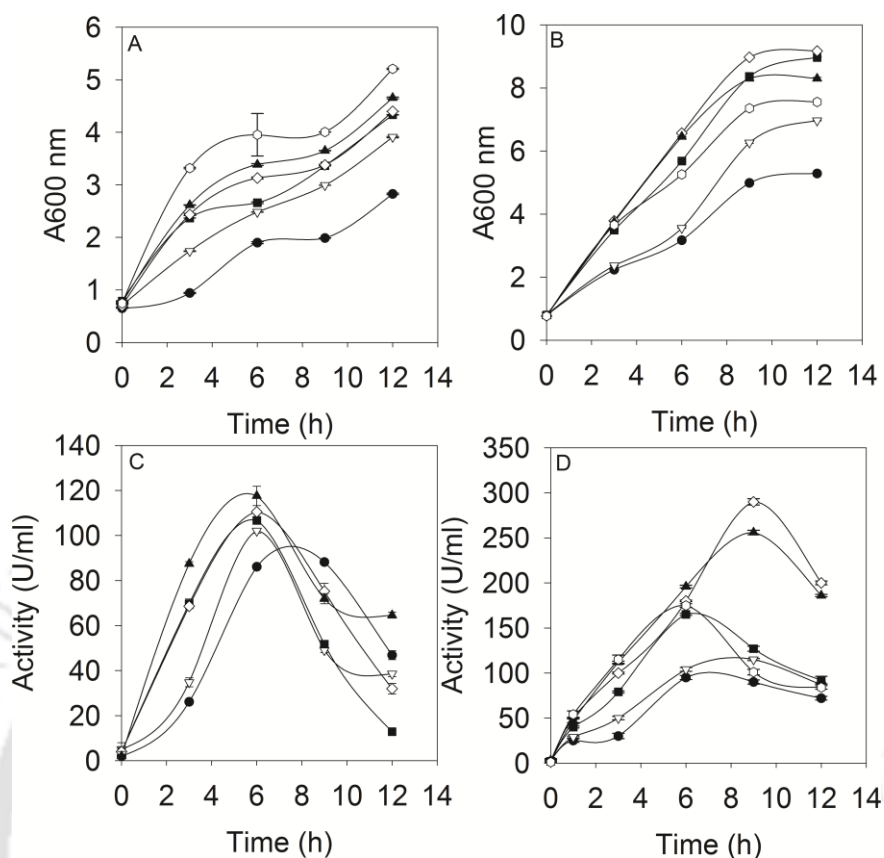


Fig. 4.8 Effect of glucose and glycerol on growth and production of recombinant Cut2 in *E. coli* BL21 (DE3). (A) Growth in presence of 0 (filled circle), 0.5 (inverted triangle), 1 (square), 2 (diamond), 3 (triangle) and 4 (hexagon) g L⁻¹ glucose. (B) Growth in presence of 0 (filled circle), 1 (inverted triangle), 2 (square), 4 (diamond), 6 (triangle) and 8 (hexagon) mL L⁻¹ glycerol. (C) Cutinase production in presence of 0 (filled circle), 0.5 (inverted triangle), 1 (square), 3 (diamond), 4 (triangle) g L⁻¹ glucose. (D) Cutinase production in presence of 0 (filled circle), 1 (inverted triangle), 2 (square), 4 (diamond), 6 (triangle) and 8 (hexagon) mL L⁻¹ glycerol. Error bar represents the average of two experiments with $\pm$ SD

The production yield of the recombinant cutinase in the medium containing glucose showed highest activity at 6 h post induction. However, the production was significantly low in comparison to medium with glycerol. A similar catabolic repressive effect on the recombinant enzyme expression with IPTG induced *E. coli* was reported earlier with glucose and lactose (Mondin *et al.*, 2002; Ramey, 2002). It has also been reported that the acetate formation is faster in the culture having glucose as substrate in comparison to glycerol, which would interfere with the growth of the bacteria. In summary, growth and production of recombinant

cutinase in the medium containing glycerol was found to be much higher than with glucose. Hence, further studies have been carried out using glycerol as a substrate.

4.5.3 Screening of significantly influencing medium components by Taguchi's experimental design

Based on the results obtained in media screening for higher expression (section 4.5.1 and 4.5.2), MTB medium was selected for further assessment of effect of individual medium components on the production using Taguchi's method (Taguchi, 1990). To optimize the levels of MTB components (Table 3.6), experiments were performed according to the Taguchi's three-level design matrix. The design matrix and the corresponding results are shown in Table 4.2. The variation in medium components and cutinase production (in units per milliliter) profiles inferred that the production of cutinase is associated with the medium components. Wide variation of enzyme activity from 179.43 ± 3.86 and 177.50 ± 0.90 (run No. 9) to 302.72 ± 0.36 and 303.40 ± 0.74 U mL⁻¹ (run No. 26) for Cut1 and Cut2, respectively, reflects the importance of the optimization of medium constituents. Furthermore, to understand which of these variables affected cutinase production in a significant manner, their ranking was accessed based on the calculated delta S/N ratio. In general, delta value for a factor, calculated by measuring the difference between the highest and lowest characteristic average S/N ratio of the factor indicates its relative significance over others on a given response: higher value of delta for a factor denoting a larger significant effect than others. And, while S/N ratio indicates effect of factors on a response, delta S/N ratio can be used as a criterion for ranking factors of their effects on the response (Saudagar and Singhal, 2007). In this study, based on the delta S/N ratio obtained for each factor, the seven parameters were ranked accordingly, and the results are presented in Table 4.3. According to these ranking,

tryptone had the maximum effect while MgSO_4 had the second highest effect on both Cut1 and Cut2 production. Tryptone is rich in amino acids and peptides. Its amino acid and peptide compositions enhance the growth. Magnesium (Mg^{2+}) is required for several cellular enzymatic reactions as well as cell growth. Adding Mg^{2+} in the medium increase the cell density with increased aeration. K_2HPO_4 is used as a source of Potassium as well as buffering agent. Better the buffering capacity, lesser fluctuation of the pH, thus, a better growth. Nevertheless, NaCl and glycerol had the least effect on Cut1 and Cut2 recombinant cutinase production, respectively. These findings on the significance of the individual parameters and their contribution on cutinase production was further validated by ANOVA of the results. Main effects of the factors on the cutinase production showed low P value (<0.05 ; α -level) of all the terms in ANOVA reflecting the high significance of the term. Hence, it indicated that the combined effects of all the independent variables significantly contributed to maximize the production of recombinant cutinase. The goodness of the model was checked by coefficient of determination, R^2 , which implies that the sample variation of 99.23 and 99.02%, respectively for Cut1 and Cut2 production is attributed to the medium components. The percent contribution of variance for each term in the ANOVA (Table 4.3) showed that tryptone and MgSO_4 contributed highest and second highest, respectively for the total cutinase production in case of both Cut1 and Cut2. These findings of the effects of parameters on cutinase production by ANOVA are in good agreement with those observed earlier from the factors ranking based on their delta S/N ratio.

4.5.3.1 Selection of optimum levels of parameters for enhancing the cutinase production

Based on the effects of each of the seven variables investigated in the study on recombinant cutinases production, particular levels of the variables caused a significant increase in the

mean response compared with other levels of the variables. viz., level 1 of each of the variables of KH_2PO_4 , MgSO_4 , and NaCl , level 2 of yeast extract, K_2HPO_4 , and glycerol, and level 3 of tryptone were found to exhibit a significant positive effect on the response (Table 4.3). Thus, tryptone, 2 g L^{-1} ; yeast extract, 24 g L^{-1} ; glycerol, 4 mL L^{-1} ; KH_2PO_4 , 1.15 g L^{-1} ; K_2HPO_4 , 12.54 g L^{-1} ; MgSO_4 , 0.61 g L^{-1} ; and NaCl , 0.058 g L^{-1} with medium pH 7.3 were chosen as optimal conditions for the maximum production of recombinant cutinase according to the method described by Saudagar and Singhal (2007).

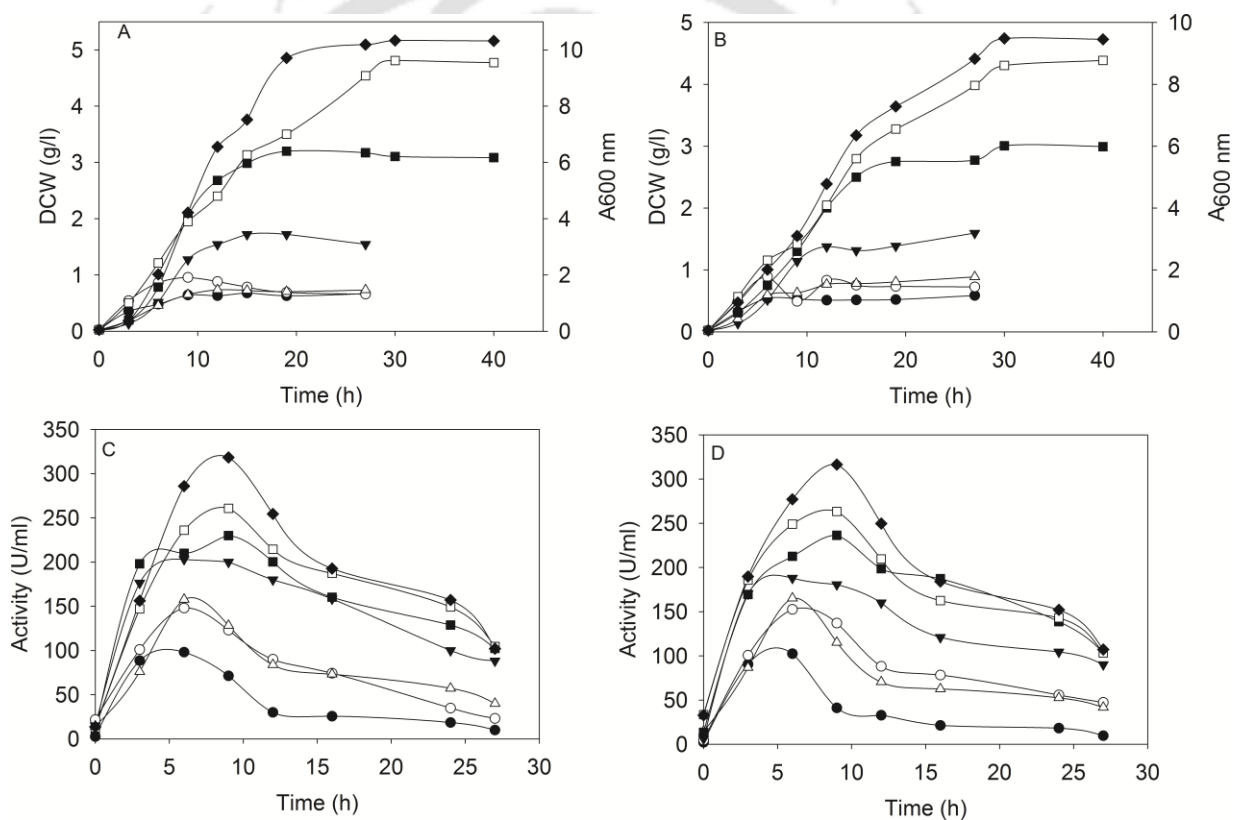


Fig. 4.9 Effect of medium on growth and production of cutinase. Growth pattern for Cut1 (A) and Cut2 (B) and Production profile for Cut1 (C) and Cut2 (D) in terms of activity in LB (filled circles), NB (empty circles), M9 (filled inverted triangles), M9 ZB (empty triangles), TB (filled squares), MTB (empty squares), and Taguchi optimized MTB (filled diamonds) screened medium. The values are average of duplicates with SD $\pm$ 0.2-1.2

Table 4.2 A Taguchi's design matrix in real values and corresponding levels (in parenthesis) with experimental and predicted values of cutinase production for Cut1 and Cut2

Run order	Variables and their values							Cut1 activity (U mL ⁻¹)		Cut2 activity (U mL ⁻¹)	
	A	B	C	D	E	F	G	Observed ^a	Predicted	Observed ^a	Predicted
1	6 (1)	12 (1)	2 (1)	1.15 (1)	6.27 (1)	0.61 (1)	0.05(1)	238.75 ± 0.85	234.28	237.12 ± 0.43	236.53
2	6 (1)	12 (1)	2 (1)	1.15 (1)	12.54 (2)	1.23 (2)	0.29 (2)	231.02 ± 0.18	232.26	226.70 ± 0.32	227.93
3	6 (1)	12 (1)	2 (1)	1.15 (1)	20.00 (3)	1.84 (3)	0.58 (3)	185.51 ± 0.75	188.74	199.04 ± 0.13	198.41
4	6 (1)	24 (2)	4 (2)	2.31 (2)	6.27 (1)	0.61 (1)	0.05 (1)	240.18 ± 0.40	243.81	238.46 ± 0.76	238.98
5	6 (1)	24 (2)	4 (2)	2.31 (2)	12.54 (2)	1.23 (2)	0.29 (2)	246.29 ± 0.30	241.8	233.70 ± 0.07	230.39
6	6 (1)	24 (2)	4 (2)	2.31 (2)	20.00 (3)	1.84 (3)	0.58 (3)	197.42 ± 0.47	198.28	198.08 ± 0.78	200.87
7	6 (1)	36 (3)	8 (3)	3.50 (3)	6.27 (1)	0.61 (1)	0.05 (1)	220.03 ± 0.18	220.87	213.40 ± 0.85	213.47
8	6 (1)	36 (3)	8 (3)	3.50 (3)	12.54 (2)	1.23 (2)	0.29 (2)	215.59 ± 0.33	218.85	202.78 ± 0.82	204.87
9	6 (1)	36 (3)	8 (3)	3.50 (3)	20.00 (3)	1.84 (3)	0.58 (3)	179.43 ± 3.86	175.33	177.50 ± 0.90	175.35
10	12 (2)	12 (1)	4 (2)	3.50 (3)	6.27 (1)	1.23 (2)	0.58 (3)	225.55 ± 0.48	228.33	218.51 ± 0.49	220.25
11	12 (2)	12 (1)	4 (2)	3.50 (3)	12.54 (2)	1.84 (3)	0.05 (1)	210.60 ± 0.09	211.07	213.17 ± 0.88	213.12
12	12 (2)	12 (1)	4 (2)	3.50 (3)	20.00 (3)	0.61 (1)	0.29 (2)	242.15 ± 0.90	238.89	243.66 ± 0.12	241.97
13	12 (2)	24 (2)	8 (3)	1.15 (1)	6.27 (1)	1.23 (2)	0.58 (3)	260.97 ± 0.54	261.71	261.17 ± 0.77	261.2
14	12 (2)	24 (2)	8 (3)	1.15 (1)	12.54 (2)	1.84 (3)	0.05 (1)	245.52 ± 0.25	244.44	253.63 ± 0.81	254.07
15	12 (2)	24 (2)	8 (3)	1.15 (1)	20.00 (3)	0.61 (1)	0.29 (2)	271.93 ± 0.90	272.27	283.37 ± 0.86	282.92
16	12 (2)	36 (3)	2 (1)	2.31 (2)	6.27 (1)	1.23 (2)	0.58 (3)	235.63 ± 0.68	232.11	236.57 ± 0.95	234.8
17	12 (2)	36 (3)	2 (1)	2.31 (2)	12.54 (2)	1.84 (3)	0.05 (1)	214.24 ± 0.58	214.84	228.05 ± 0.50	227.67
18	12 (2)	36 (3)	2 (1)	2.31 (2)	20.00 (3)	0.61 (1)	0.29 (2)	239.75 ± 0.03	242.67	254.37 ± 0.00	256.52
19	20 (3)	12 (1)	8 (3)	2.31 (2)	6.27 (1)	1.84 (3)	0.29 (2)	225.96 ± 0.29	222.62	224.60 ± 0.99	220.81
20	20 (3)	12 (1)	8 (3)	2.31 (2)	12.54 (2)	0.61 (1)	0.58 (3)	283.55 ± 0.20	283.29	278.64 ± 0.20	277.83
21	20 (3)	12 (1)	8 (3)	2.31 (2)	20.00 (3)	1.23 (2)	0.05 (1)	267.08 ± 0.52	270.67	259.02 ± 0.08	263.61
22	20 (3)	24 (2)	2 (1)	3.50 (3)	6.27 (1)	1.84 (3)	0.29 (2)	221.72 ± 1.29	221.53	215.75 ± 0.74	211.78
23	20 (3)	24 (2)	2 (1)	3.50 (3)	12.54 (2)	0.61 (1)	0.58 (3)	283.01 ± 0.10	282.19	268.60 ± 0.60	268.8

Run order	Variables and their values							Cut1 activity (U mL ⁻¹)		Cut2 activity (U mL ⁻¹)	
	A	B	C	D	E	F	G	Observed ^a	Predicted	Observed ^a	Predicted
24	20 (3)	24 (2)	2 (1)	3.50 (3)	20.00 (3)	1.23 (2)	0.05 (1)	268.56 ± 0.66	269.58	250.80 ± 0.28	254.58
25	20 (3)	36 (3)	4 (2)	1.15 (1)	6.27 (1)	1.84 (3)	0.29 (2)	239.61 ± 0.31	243.14	239.23 ± 0.55	246.99
26	20 (3)	36 (3)	4 (2)	1.15 (1)	12.54 (2)	0.61 (1)	0.58 (3)	302.72 ± 0.36	303.81	303.40 ± 0.74	304.01
27	20 (3)	36 (3)	4 (2)	1.15 (1)	20.00 (3)	1.23 (2)	0.05 (1)	295.81 ± 0.23	291.19	298.15 ± 0.31	289.79

^aResults are the average of two experiments with ± SD

Table 4.3 Response table for S/N ratio (dB), relative ranking and percentage contribution of variables for Cut1 and Cut2

Level	Cut1						
	A	B	C	D	E	F	G
1	46.68	47.34	47.38	47.96	47.38	48.19	47.72
2	47.52	47.86	47.69	47.52	47.82	47.91	47.48
3	48.43	47.43	47.57	47.15	47.43	46.54	47.43
Delta ^a	1.74	0.51	0.31	0.81	0.44	1.65	0.28
Rank	1	4	6	3	5	2	7
% contribution	40.8	3.6	1.4	9.2	3.4	39.4	1.0
Level	Cut2						
	A	B	C	D	E	F	G
1	46.57	47.32	47.4	48.08	47.28	48.18	47.69
2	47.7	47.73	47.63	47.53	47.73	47.66	47.42
3	48.24	47.46	47.49	46.9	47.5	46.67	47.4
Delta ^a	1.67	0.41	0.23	1.19	0.45	1.52	0.28
Rank	1	5	7	3	4	2	6
% contribution	39.0	2.3	1.0	19.9	3.5	31.9	1.1

^a Difference between maximum and minimum S/N ratio values

Where, A is tryptone (g L⁻¹), B is Yeast extract (g L⁻¹), C is Glycerol (mL L⁻¹), D is KH₂PO₄ (g L⁻¹), E is K₂HPO₄ (g L⁻¹), F is MgSO₄ (g L⁻¹) and G is NaCl (g L⁻¹)

4.5.3.2 Validation of the model at predicted optimum levels of chemical parameters

To validate the proposed experimental methodology, recombinant cutinase production was performed at the optimum level of each individual factor (section 4.5.3.1). The experimental data showed an enhanced cutinase yield of 318 ± 0.73 and 316 ± 0.90 U mL⁻¹ for Cut1 and Cut2, respectively (Fig. 4.9), which was 1.2 fold higher as compared with un-optimized conditions (260 ± 1.1 and 263 ± 0.77 U mL⁻¹, respectively for Cut1 and Cut2). This experimental result was very close to the statistically predicted value of 319 and 315 U mL⁻¹, respectively for Cut1 and Cut2. The verification revealed a high degree of accuracy of the model of more than 99.0%, which is an evidence for the model validation under the investigated conditions. Fig. 4.10 shows the SDS-PAGE of cutinase produced in different medium. It is clear from the figure that, there is remarkable increase in the production after optimization.

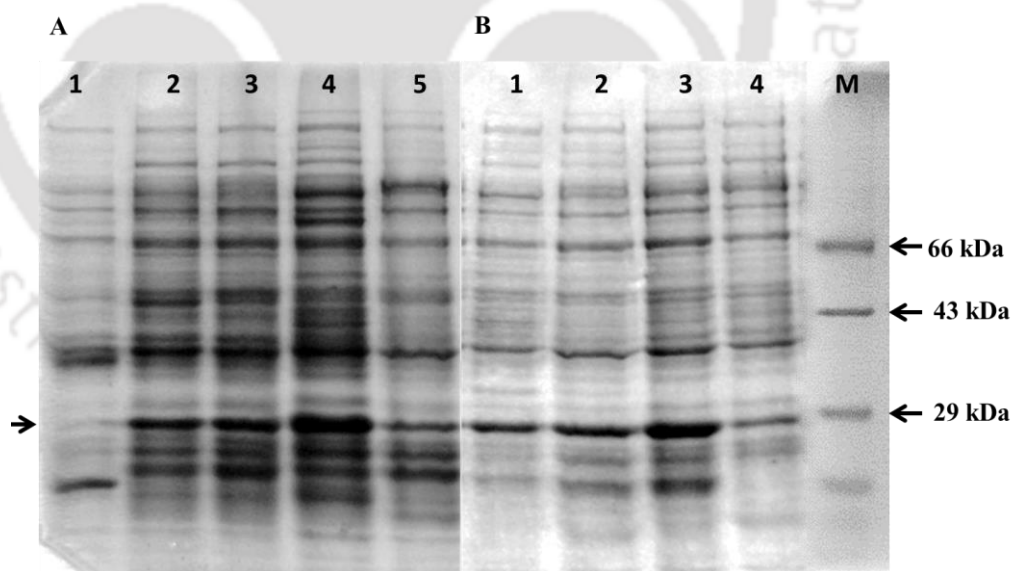


Fig. 4.10 A 12% SDS-PAGE analysis of expression profile in various medium for Cut1 (A) and Cut2 (B) after a 9 h induction. Cells were diluted to get A600 nm ~1, lysed, and 20 μ L supernatant was loaded. Black arrow shows the position of Cut1 or Cut2. (A) Lane 1, *E. coli* BL21 (DE3) uninduced; lanes 2–5, Cut1 expression in M9, TB, Taguchi optimized MTB, and LB medium. (B) Lanes 1–4, Cut2 expression in M9, TB, Taguchi optimized MTB, and LB medium. M, molecular weight marker

4.6 Cellular localization of *T. fusca* recombinant cutinases in *E. coli* BL21 (DE3)

The main purpose of recombinant protein expression is often to obtain a high degree of accumulation of soluble product in the bacterial cell and most of the time as extracellular secretion or in to periplasmic space due to lesser proteolytic activity in these regions as compare to cytoplasm. In addition, recombinant protein purification is simpler due to fewer contaminating proteins in the periplasm or extracellular medium. This strategy is not always accepted by the metabolic system of the host and in some situations a cellular stress response is encountered.

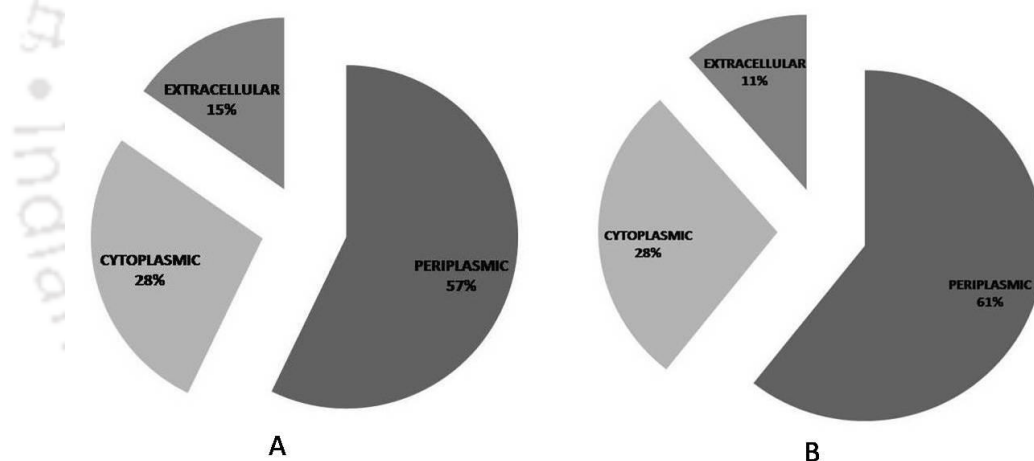


Fig. 4.11 Cellular localization of recombinant Cut1 (A) and Cut2 (B) analysed by pNPB activity assay

Depending on the medium components and other factors like culture temperature, IPTG concentration etc. target recombinant protein accumulate as biologically inactive, often misfolded, insoluble aggregates called inclusion bodies (Villaverde and Carrio, 2003). To account the soluble recombinant protein production and to check the formation of inclusion bodies, the cellular localization of the protein was studied. It was found that for Cut1 and

Cut2, respectively 57 and 61% located in periplasm, and 15 and 11% was seen in extracellular medium. Only 28% for both Cut1 and Cut2 was observed in cytoplasm (Fig. 4.11) and SDS-PAGE showed no inclusion bodies.

4.7 Purification of *T. fusca* recombinant cutinases by affinity chromatography

Recombinant cutinases with C-terminal (His)₆ tag was purified by IMAC using Ni-resin under native conditions. The homogeneity of the purified enzyme was investigated by SDS-PAGE on a 15% (w/v) polyacrylamide gel (Fig. 4.12). The purified Cut1 and Cut2 showed molecular weight of ~30 and ~29 kDa, respectively. Cutin hydrolytic activity of the purified enzyme was confirmed by using cutin analog substrate, pNMSH, which showed 0.218 ± 0.06 and 0.395 ± 0.17 U mg⁻¹ for Cut1 and Cut2, respectively. Purified Cut1 and Cut2 showed specific activity of 542.5 ± 2.7 and 643.4 ± 3.4 U mg⁻¹, respectively against pNPB.

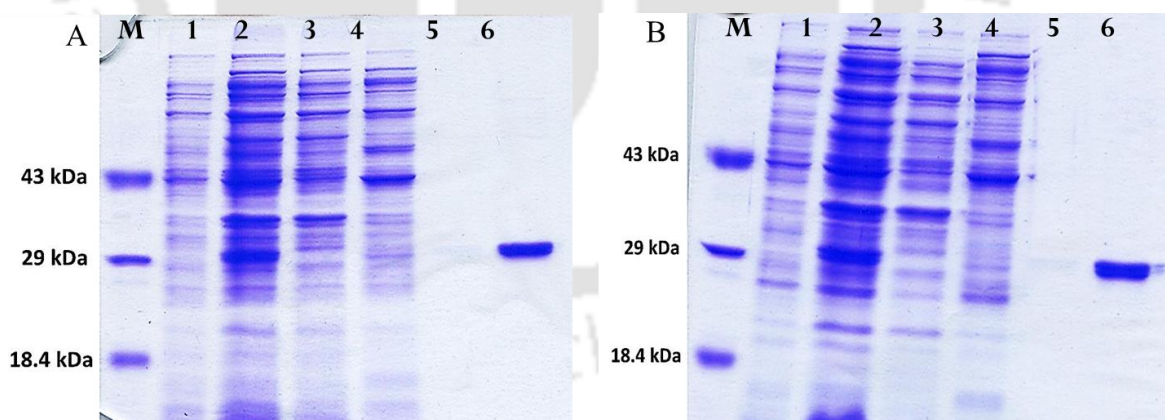


Fig. 4.12 A 15% SDS-PAGE analysis of the Cut1 (A) and Cut2 (B) at various steps of (His)₆ tag purification. M: Molecular weight marker, Lane 1. Cell lysate of pET22b(+)_{cut1} (A) or pET22b(+)_{cut2} un-induced Lane 2. Cell lysate of induced Cut1 (A) or Cut2 (B), Lane 3. Cut1 (A) or Cut2 (B) flow through, Lane 4. Cut1 (A) or Cut2 (B) wash through, Lane 5. Purified Cut1 (A) or Cut2 (B)

4.8 Biochemical characterization of purified *T. fusca* recombinant cutinases

Every enzyme needs to be characterized prior to evaluation for any industrial applications because the optimum condition for maximum enzyme activity and effect of different environmental factors on the activity should be known for using the enzyme without losing the activity. Therefore following studies have carried out to characterize the cutinase enzyme.

4.8.1 Homogeneity, molecular weight and isoelectric point of the purified cutinases

The homogeneity of the *T. fusca* recombinant cutinases purified by IMAC was analysed on 15% SDS-PAGE. Both the purified cutinases (Cut1 and Cut2) were found to be homogeneous, as evident from SDS-PAGE (Fig. 4.12). The molecular mass of cutinase was found to be ~30 kDa and ~29 kDa, respectively for Cut1 and Cut2.

The molecular mass of the protein was also confirmed by MALDI-TOF-MS analysis, according to which Cut1 was found to be 30.1906 kDa and size of Cut2 was determined as 29.6812 kDa (Fig. 4.13). The molecular mass of the recombinant cutinases from *T. fusca* NRRL B-8184 was very close to previously reported cutinase from other bacterial sources. Chen *et al.*, (2008) found that the molecular mass of the *T. fusca* cutinases, Tfu 0882 and Tfu 0883 to be 29.22 and 28.99 kDa, respectively. The molecular mass of cutinases isolated from *P. putida* (Chen *et al.*, 2008) was found to be 30 kDa. Gindro and Pezet (1999) reported that the molecular mass of the purified cutinase from *B. cinera* was 26.5 kDa as calculated from SDS-PAGE molecular markers. The molecular mass of cutinases isolated from *F. solani pisi* and *C. kahawae* were found to be 21 and 22.1 kDa, respectively (Chen *et al.*, 2007). Sebastian and Kolattukudy (1988) reported that generally the molecular mass of bacterial cutinase lies between the molecular mass of fungal cutinase (22 kDa) and pollen cutinase (40 kDa). The

results obtained for purification of cutinase in the present investigation were in agreement with results reported by Sebastian and Kolattukudy (1988).

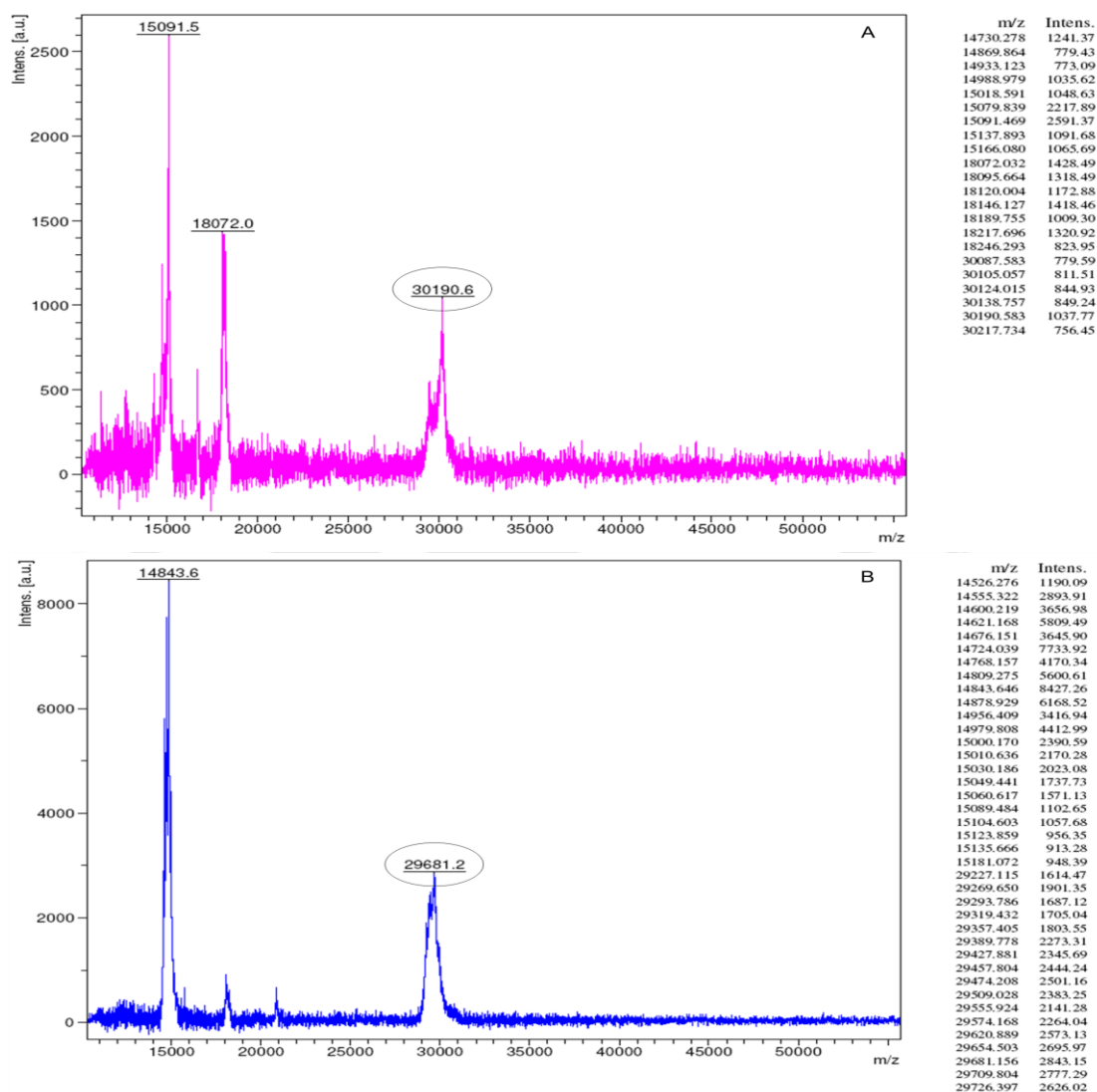


Fig. 4.13 Molecular weight of the purified Cut1 (A) and Cut2 (B) as determined by MALDI-TOF-MS

2-D electrophoresis and isoelectric focusing showed the isoelectric point (pI) of Cut1 and Cut2 to be 7.7 and 7.5, respectively (Fig. 4.14). Reported pI of some other cutinases includes *M. fructicola*; pI 8.4 (Wang *et al.*, 2002), *F. solani*; pI 8 (Pedersen *et al.*, 2006) and *F. oxysporum*; pI 8.1 (Rocha *et al.*, 2008).

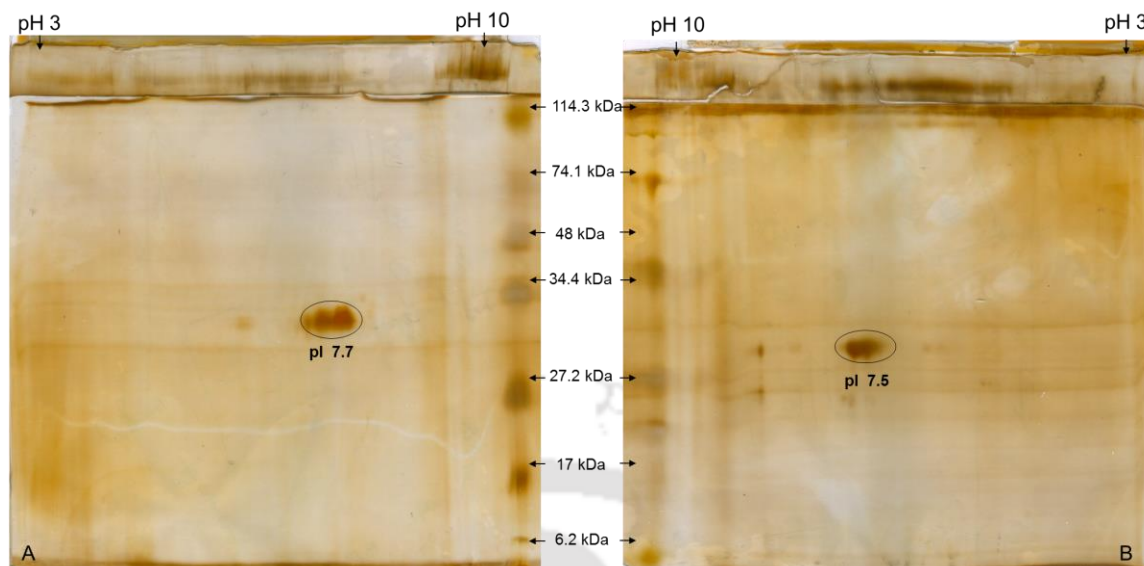


Fig. 4.14 2-D electrophoresis gel showing the pI of the purified Cut1 (A) and Cut2 (B)

4.8.2 Effect of pH and temperature on activity and stability of cutinases

Both the purified recombinant cutinases of *T. fusca* NRRL B-8184 were active over a broad range of pH (6.8–9.0) with an optimum pH of 8 (Fig. 4.15). Both cutinases showed more than 75% activity in the pH range of 6.8 to 9; however the enzyme showed higher stability at alkaline pH range (pH 8.0–9.0) with retention of more than 75% of its original activity at pH 9. Similar results were reported earlier for pH optima for cutinase from *T. fusca* (Chen *et al.*, 2008) and *P. cepacia* (Dutta *et al.*, 2013).

Optimum temperature of both Cut1 and Cut2 was found to be 55°C, while both the enzymes showed more than 60% activity in the range of 40 to 80°C (Fig. 4.16). These results are in accordance with earlier reports on the temperature optimums of cutinases from *T. fusca* (Chen *et al.*, 2008). The thermostability of Cut1 and Cut2 was investigated at 37 and 55°C (Fig. 4.17). Interestingly, the activity of both Cut1 and Cut2 was increased initially up to 10 h of incubation at both the temperatures, with residual activities of over 75% even after 125 h at 37°C (Fig. 4.17 B). Nevertheless, only over 38% activity was retained at 55°C (Fig. 4.17 A).

Similar findings of thermostability were reported earlier by Chen *et al.*, (2008) for cutinases from *T. fusca*.

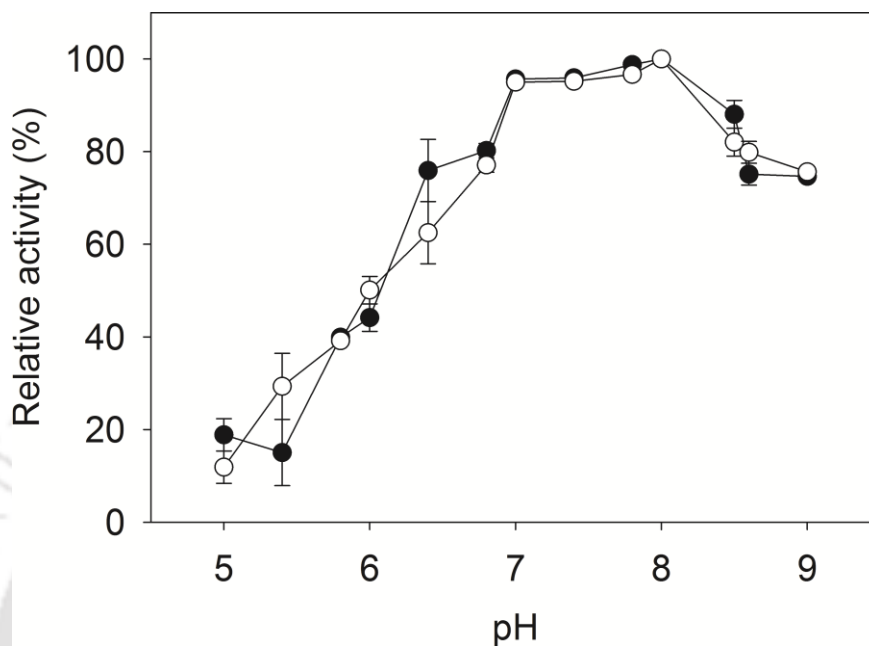


Fig. 4.15 Effect of pH on activity of the Cut1 (filled circle), Cut2 (open circle). Error bar represents the average of two experiments with $\pm$ SD

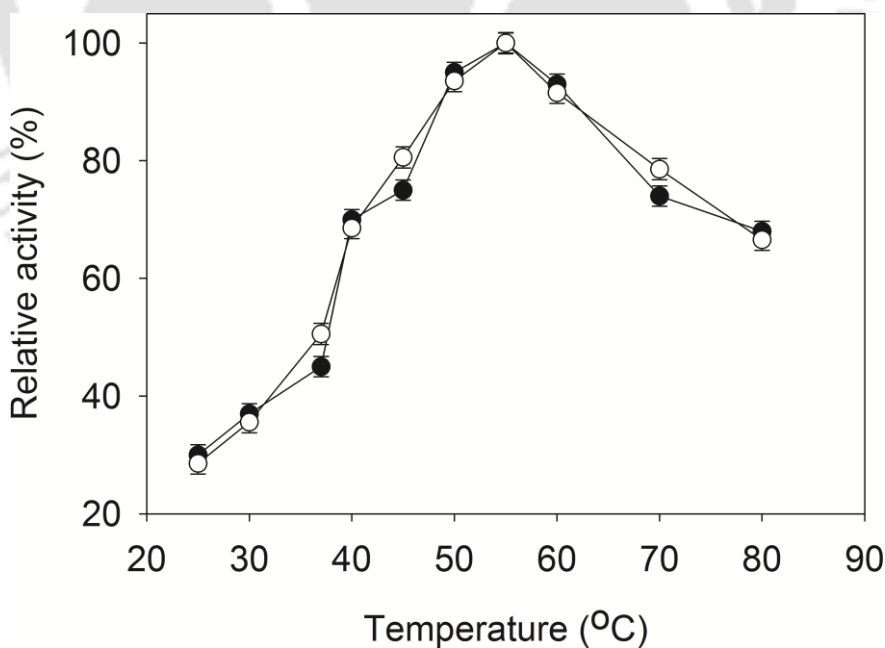


Fig. 4.16 Effect of temperature on activity of the Cut1 (filled circle), Cut2 (open circle). Error bar represents the average of two experiments with $\pm$ SD

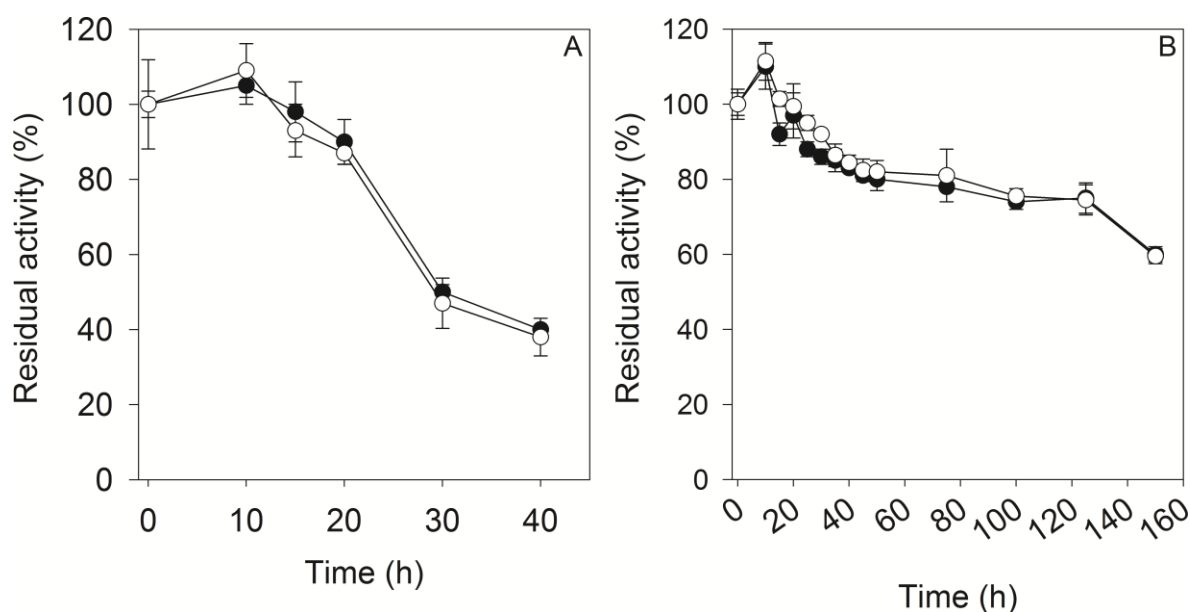


Fig. 4.17 Thermostability of Cut1 (filled circles) and Cut2 (empty circles) at 55°C (A) and 37°C (B). Error bar represents the average of two experiments with $\pm$ SD

4.8.3 Kinetic analysis of cutinase

The kinetic parameter for Cut1 and Cut2 was investigated using pNPB, which showed highest activity among all the substrates. The values of K_m and V_{max} of cutinase were determined by steady state kinetic analysis as described in the section 3.11.6. K_m , V_{max} and k_{cat} values calculated for Cut1 and Cut2 was found to be respectively, 131 and 89 $\mu\text{M L}^{-1}$ (K_m), 3.2 and 3.8 $\mu\text{M s}^{-1}$ (V_{max}), 178 and 253 s^{-1} (k_{cat}). The results showed that both the cutinases showed Michaelis-Menten kinetics with Cut2 showing the higher affinity for pNPB. Cut1 exhibited lesser catalytic efficiency with a k_{cat}/K_m of $1.35 \text{ s}^{-1} \mu\text{M}^{-1}$; whereas, Cut2 showed almost double catalytic efficiency, with k_{cat}/K_m of $2.84 \text{ s}^{-1} \mu\text{M}^{-1}$. Similar kinetic behavior was reported earlier by Chen *et al.*, (2010) for *T. fusca* cutinase Tfu 0882 and Tfu 0883 at different conditions. The K_m (mM L^{-1}) for bacterial cutinases from *P. cepacia* was observed to be 0.23 (Dutta *et al.*, 2013). Previously, Sebastian and Kolattukudy (1988) reported that the K_m of 0.27 mM for *P. putida* cutinase. A similar K_m of 0.272 was reported for cutinase from *F. solani pisi* (Chen

et al., 2010). Compare to other cutinases, both Cut1 and Cut2 from *T. fusca* showed lower K_m for pNPB.

4.8.4 Studies on the thermal deactivation kinetics of cutinases

The deactivation rate is proportional to the active enzyme concentration, and k_d (deactivation rate constant is the proportional constant). Cut1 and Cut2 were deactivated under various combinations of pH and temperature as described in the section 3.11.7. The extent of deactivation was measured by the deactivation rate, the deactivation process was modeled as first-order kinetics and the deactivation rate constant was evaluated (Section 3.11.8). The effect of pH and temperature on half-life time ($t_{1/2}$) and corresponding k_d values are shown in Table 4.4. The minimum value of k_d observed for Cut1 and Cut2 was 0.045 and 0.039 min⁻¹, respectively. The combinations of pH and temperature at which the above mentioned minimum deactivation rate constant have been observed are 8 and 45°C for both Cut1 and Cut2, respectively. The deactivation process was found to be faster at pH below 7 and above 8 for both the cutinases. This is possibly due to disulfide exchange, which usually occurs at near neutral and alkaline conditions (Munch and Tritsch, 1990). However, the rate of deactivation was faster for Cut1 in comparison to Cut2 (Table 4.4). It was also observed that with increase in the temperature above 70°C, the deactivation occurs faster, irrespective of pH for both the cutinases. Furthermore, the observation of interrelationship between conformational stability and enzyme activity suggested that in naturally occurring enzymes one cannot expect to find stability at temperatures far above than that of growth of an organism (Daniel, 1996). The results obtained in the present study also indicate that optimum pH and temperature lie near that of the growth condition. The maximum half-life time of Cut2 was 17.59 h, showing that this enzyme is more stable than Cut1 (15.3 h) at optimum conditions of pH and temperature

(pH 8 and 45°C, respectively) (Table 4.4). The variation in stability noticed for these two enzymes may probably be due to their differences in tertiary structure.

Table 4.4 Effect of temperature at different pH on deactivation constant (k_d) and half life time ($t_{1/2}$) of the purified enzyme

pH	Temperature (°C)	Cut1		Cut2	
		K_d (h ⁻¹)	$t_{1/2}$ (h ⁻¹)	K_d (h ⁻¹)	$t_{1/2}$ (h ⁻¹)
6	45	0.06	11.514	0.048	14.411
	55	0.07	9.973	0.066	10.471
	70	0.098	7.091	0.152	4.566
	80	0.169	4.094	0.25	2.771
7	45	0.048	14.396	0.041	16.989
	55	0.054	12.884	0.05	13.808
	70	0.078	8.836	0.104	6.691
	80	0.139	4.978	0.243	2.848
8	45	0.045	15.369	0.039	17.593
	55	0.052	13.215	0.045	15.541
	70	0.075	9.205	0.113	6.15
	80	0.154	4.505	0.232	2.983
9	45	0.055	12.534	0.058	11.869
	55	0.064	10.78	0.069	10.017
	70	0.113	6.156	0.159	4.357
	80	0.172	4.021	0.285	2.43

4.8.5 Estimation of thermodynamic parameters for cutinase deactivation

The thermodynamic parameters were estimated according to the method described in the section 3.11.9. The change in enthalpy (ΔH^*) and entropy (ΔS^*) for recombinant cutinase from *T. fusca* NRRL B-8184 were calculated by transition state theory (eq. 3.6) and the results are shown in

Table 4.5. Solvent and structural effects are the two major factors, which influence the numerical values of ΔH^* and ΔS^* .

Enthalpy change and deactivation energy of Cut1 and Cut2 were calculated within a temperature range of 45 to 80°C. It has been reported that enthalpy change of enzymes should be in the range of 20 to 150 kJ mol⁻¹ (Amico *et al.*, 2003). As depicted in Table 4.5, ΔH^* of Cut1 and Cut2 deactivation was within this range in the buffers studied at all the temperatures, which indicates that these enzymes maintained their rate of reaction even during incubation at different pH and temperatures. Furthermore, enthalpy of Cut1 and Cut2 increased as pH increased (Table 4.5) till pH 8. However, there was a decrease in enthalpy of Cut1 and Cut2 at pH 9. This result shows that both the cutinases were more stable at pH 7 and 8. However, it was interesting to observe that ΔH^* value of Cut2 was ~1.5 fold higher than Cut1 in the range of pH studied; inferring Cut2 to be thermodynamically more stable than Cut1. Enthalpy derives from the energy of the non-covalent interactions within the polypeptide chain, the hydrophobic interactions, H-bonds and ionic bond. Thus, the substantial difference in the enthalpy for Cut1 and Cut2 indicates that there is a considerable tertiary structural difference between Cut1 and Cut2, although they are 93% homologous to each other at amino acid level.

The temperature dependency of first-order deactivation rate constant was studied by Arrhenius equation (Eq. 3.9). The activation energy (E_A) estimated are shown in Tables 4.5. It was observed that the deactivation energy is maximum at optimum pH for both Cut1 and Cut2, however, Cut2 showed ~1.7 fold higher deactivation energy at pH 6 and 7 and ~1.4 fold higher deactivation energy at pH 8 and 9 in comparison to Cut1. The higher deactivation energy suggests that Cut2 require more energy to get deactivated compare to Cut1.

The measurement of entropy change during unfolding of protein molecule is very much helpful in enhancing the thermostability of proteins of known 3-dimensional structure. This can be achieved by selective amino acid substitutions, which decreases the configurational entropy change of unfolding thereby increases the stability of the protein molecule (Dclerk, 2003). In the present investigation, it was observed that the entropy values are negative for both the enzymes in all the cases (Table 4.5), which is unique in biocatalytic systems. The possible reason for negative entropy could be due to the formation of charged particles around the enzyme molecule and the ordering of solvent molecules or compaction of the enzyme molecules (Gohel and Naseby, 2007; Gummadi, 2003). Both Cut1 and Cut2 showed increase in entropy with increase in pH (Table 4.5). The probable reason is that enzyme gets unfolded during deactivation with the increase in pH or it may be due to the ordering of solvent molecules. Though the difference in the entropy change is marginal in the range of pH studied, apparently, Cut1 had lower entropy in comparison to Cut2. Furthermore, the negative ΔS^* also signifies that the transition states of the cutinases were found to be ordered. Similar results have been observed for the amylase of *Bacillus lichiniiformis* (Foster *et al.*, 1980), the chitinase of *T. harzianum* (Kapat and Panda, 1997) and the chitinase from *Pantoea dispersa* (Gohel and Naseby, 2007). This result also indicates that cutinases were altered in the direction of partially unfolded transition state but the flexibility implies decreased conformational entropy of the folded state, which is favorable to thermodynamic stability. Thus, it may be concluded on the basis of entropy that the mechanism of deactivation for Cut1 and Cut2 is more or less similar although the stability of Cut1 and Cut2 might be different due to the tertiary structure.

For both the cutinases, ΔG^* increased with increasing temperature (Table 4.5). However, there was a marginal difference and both the cutinases showed almost similar free energy change with increasing temperature. Moreover, there was no or very small difference in the ΔG^* value in the range of pH studied entails the fact that pH had negligible effect on denaturing the enzymes or in other words; both the cutinases are active in broad pH.

Table 4.5 Thermodynamic parameters estimated for thermal deactivation of Cut1 and Cut2

pH	ΔH^* (KJ mol ⁻¹)	ΔS^* (KJ mol ⁻¹ K ⁻¹)	ΔG^* (KJ mol)	E_A (KJ/mol)	R ² of plot of ln(kd/T) v/s (1/T)	R ² of plot of ln(kd) v/s (1/T)
Cut1						
6	25.941	-0.188	0.086-0.092 [#]	26.406	0.963	0.914
7	26.956	-0.187	0.086-0.093 [#]	27.357	0.948	0.903
8	31.434	-0.173	0.087-0.093 [#]	34.228	0.957	0.963
9	28.12	-0.182	0.086-0.092 [#]	30.906	0.958	0.965
Cut2						
6	42.298	-0.138	0.086-0.091 [#]	45.083	0.985	0.986
7	44.157	-0.135	0.087-0.092 [#]	46.943	0.925	0.933
8	45.877	-0.13	0.087-0.092 [#]	48.661	0.932	0.939
9	40.805	-0.142	0.086-0.091 [#]	43.59	0.953	0.958

[#] The temperature range is 45-80°C

4.8.6 Substrate specificity

The specificity of the Cut1 and Cut2 towards various chain length esters was investigated using p-nitrophenyl-fatty acyl esters. As depicted in Table 4.6, both the enzymes showed higher specificity towards short-chain fatty acid esters. The hydrolytic activity was found to be highest in case of esters of C2-C4 fatty acid. However, the catalytic efficiency decreased with the further increase in acyl chain from C4. Nevertheless, Cut1 showed better catalytic ability towards longer chains compared with Cut2. In summary, both Cut1 and Cut2 exhibited

very low levels of activity against the long-chain substrate, p-nitrophenyl palmitate (C16) as compared with the activity against pNPB at 50°C. Other studies have also indicated that cutinases showed specificity for short and medium carbon chain of fatty acids (Chen *et al.*, 2010; Dutta *et al.*, 2013; Pio and Macedo, 2007).

Table 4.6 Specificity of the cutinases towards the acyl chain length of different esters

Acyl chain length	Residual activity (%)	
	Cut1	Cut2
Acetate (C2)	85.7 ± 1	75.0 ± 1
Butyrate (C4)	100 ± 2	100 ± 2
Valerate (C5)	72.7 ± 7	52.3 ± 1
Caprylate (C8)	61.7 ± 1	48.6 ± 3
Decanoate (C10)	35.9 ± 4	24.5 ± 2
Lautate (C12)	31.2 ± 5	21.8 ± 1
Myristate (C14)	26.3 ± 5	16.2 ± 2
Palmitate (C16)	20.4 ± 5	15.1 ± 4

Cutin hydrolyzing ability of *T. fusca* cutinase, Cut1 and Cut2 was evaluated at optimal temperature and pH using tomato cutin as substrate according to the method mentioned in the section 3.4.1.3 and analysed by GC according to the method described in the section 3.4.6. As shown in Table 4.7, the C16 and C18 family fatty acid monomers released after enzymatic reaction were slightly different for Cut1 and Cut2. Cutin treatment with Cut1 released higher amount of 16-Hydroxyhexadecanoic acid and Octadecanoic acid in comparison to Cut2, whereas, Cut2 treatment showed slightly higher hydrolytic product of Hexadecanoic acid. Similar results were reported by Chen *et al.*, (2008) for *T. fusca* cutinases. In summary, the

substrate specificity experiments showed that both the cutinase can efficiently hydrolyse cutin and despite the high homology between them, they show different affinity for various natural and synthetic substrates studied.

Table 4.7 Specificity of the cutinases towards cutin hydrolysis

Hydrolysis product	GC Area (%)	GC Area (%)
	Cut1	Cut2
9-Hexadecenoic acid	0.11 ± 0.07	0.07 ± 0.02
16-Hydroxyhexadecanoic acid	4.2 ± 0.76	3.6 ± 0.45
Hexadecanoic acid	8.4 ± 1.3	9.6 ± 2.1
Octadecanoic acid	7.9 ± 0.94	6.8 ± 0.32

4.8.7 Effect of various solvents on cutinase stability

The use of organic solvents in biocatalytic reactions has some advantages such as increased solubility of organic substrates insoluble in water and the ability to shift the thermodynamic equilibrium of many processes for the synthesis path like in the esterification and transesterification reactions (production of biodiesel). Generally, enzyme catalyzed esterification reactions between fatty acid and alcohol to synthesize alkyl esters are reversible. So, at higher water concentration, the equilibrium for enzyme catalyzed reaction shifts to reverse direction and forming again initial reactants. Thus, the enzyme must have stability at minimum water concentration and organic solvents (De Barros *et al.*, 2009a) for synthesis of esters. The effect of various organic solvents on the enzyme stability is shown in Table 4.8. Both Cut1 and Cut2 in general, showed extreme stability, with no drastic decreases in residual activity in most of the studied organic solvents at 40% (v/v) (Table 4.8). In fact, dimethyl sulphoxide and dimethyl formamide showed slight enhancement in enzyme activity for both Cut1 and Cut2. Acetone, acetonitrile, ethanol, and tetrahydrofuran (THF) had no adverse effect on both the Cut1 and Cut2 even after incubation for 1 h at 25°C. Negligible loss of

residual activity was observed with butanol, methanol, n-hexane, 1-heptanol, and triethyl amine. However, 1-hexanol, benzene, dichloro methane, hexyl acetate considerably decreased the residual activity. It is also worth noting that some of the organic solvent showed higher inhibitory effect on Cut1 (hexyl acetate, isopropanol, triethyl amine) in comparison to Cut2, whereas only 1-Hexanol had higher inhibitory effect on Cut2 in comparison to Cut1.

Table 4.8 Solvent stability of the cutinases

Solvents (40% v/v)	Residual activity (%)	
	Cut1	Cut2
None	100 ± 1	100 ± 3
1-Heptanol	63 ± 1	62 ± 1
1-Hexanol	32 ± 1	14 ± 1
Acetone	100 ± 1	103 ± 3
Acetonitrile	102 ± 2	100 ± 2
Benzene	21 ± 3	23 ± 1
Butanol	90 ± 1	83 ± 1
Dichloro methane	26 ± 3	39 ± 4
DMSO	109 ± 3	114 ± 3
DMF	110 ± 10	106 ± 4
Ethanol	95 ± 1	100 ± 1
Hexyl acetate	17 ± 1	37 ± 2
Isopropanol	25 ± 2	67 ± 3
Isoamyl Alcohol	93 ± 2	94 ± 2
Methanol	98 ± 1	92 ± 3
n-Hexane	93 ± 1	90 ± 2
THF	100 ± 6	101 ± 6
Triethyl amine	76 ± 7	103 ± 7

Studies on stability of both the cutinases over time was also carried out in few industrially important solvents that are commonly used in applications like esterification, and transesterification as a substrate or an organic media. Effect of acetonitrile, butanol, ethanol, methanol, THF and n-hexane were investigated for effect on stability and activity of cutinases. As depicted in Fig. 4.18, both Cut1 and Cut2 retained more than 68% activity over 25 h in acetonitrile, ethanol, methanol, THF, and n-hexane. Nevertheless, Cut2 showed very less stability in butanol with loss of almost 88% activity over 25 h, whereas Cut1 lost only 60 % activity.

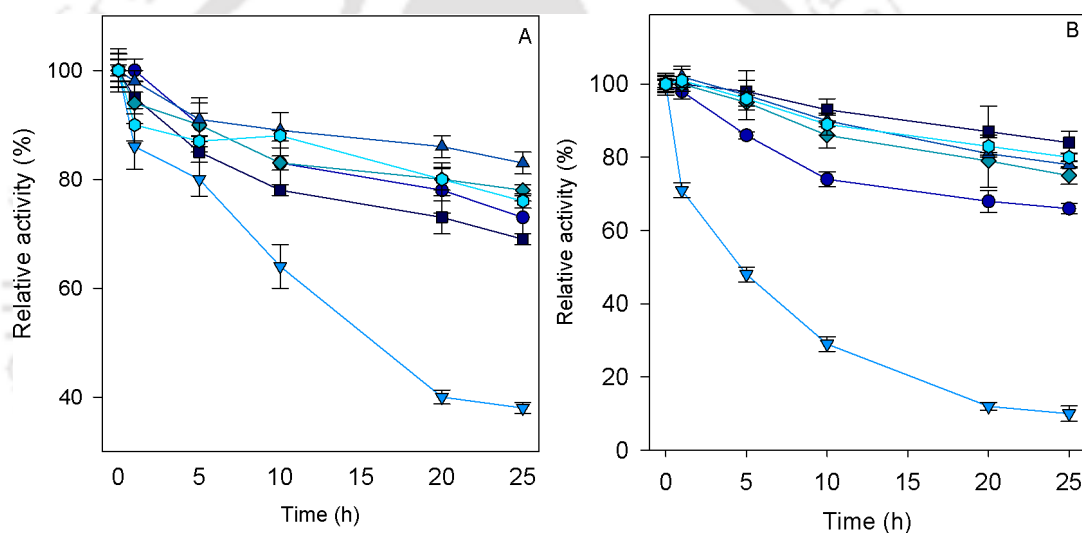


Fig. 4.18 Effect of solvent on stability of Cut1 (A) and Cut2 (B), Acetonitrile (circle), Butanol (inverted triangle), Ethanol (square), Methanol (diamond), n-Hexane (triangle), THF (hexagon). Cutinases were incubated at 25°C in each of the organic solvents, and aliquots were withdrawn at regular time intervals to study the residual activity by pNPB assay

4.8.8 Effect of various surfactants on cutinase activity

Cutinase is being investigated as a potent cleansing agent in detergent compositions (Adams *et al.*, 2012; Kolattukudy and Poulouse, 1994). One of the basic criteria for enzyme to be used in detergent or laundry composition is that it should be tolerant to various types of detergents since the enzyme often needs to go through relatively harsh conditions. In order to study the

effect of various detergents on stability and activity, the performance of the cutinase was investigated in the presence of the nonionic surfactants, Triton X-100, Tween 80, Tween 20, and the anionic surfactants deoxycholate, sodium deoxycholate, sodium taurodeoxycholate, and SDS as described in the section 3.11.12 (Table 4.9).

Table 4.9 Effect of different surfactants on cutinase activity

Surfactant	Residual activity (%)	
	Cut1	Cut2
None	100 ± 1	100 ± 1
Deoxycholate (10 mM)	76 ± 5	75 ± 3
SDS (1 mM)	91 ± 3	70 ± 4
Sodium deoxycholate (10 mM)	100 ± 3	105 ± 3
Sodium taurodeoxycholate (10 mM)	110 ± 4	124 ± 2
Triton X-100 (1 mM)	45 ± 2	65 ± 2
Tween-20 (1 mM)	65 ± 5	72 ± 3
Tween-80 (1 mM)	40 ± 1	76 ± 1

In presence of Triton X-100 or Tween 80 at 1 mM concentration, Cut1 showed more than 50% decrease in its residual activity, whereas Cut2 was comparatively stable with loss of ~35% residual activity. Tween 20 showed 35 and 28% inhibition for Cut1 and Cut2, respectively. In case of anionic detergents, SDS showed more inhibitory effect on Cut2 as compared with Cut1, whereas deoxycholate had similar inhibitory effect on both the cutinases. Sodium deoxycholate had no inhibitory effect on any of the two cutinases, surprisingly, sodium taurodeoxycholate showed enhancement in activity by 10 and 24% for Cut1 and Cut2, respectively. The possible explanation would be the bulky side chain of the anionic surfactant which may bind to the hydrophobic sites of proteins preventing their aggregation and rendering them more stable (Chen *et al.*, 2010). Similar kind of observation was also reported by Chen *et al.*, (2010) and Dutta *et al.*, (2013) for cutinase from *T. fusca*

and *P. cepacia*, respectively. These characteristics of the enzyme make them suitable for use in laundry industry, where it has to undergo many harsh conditions. Also, the enzyme is more suitable for any kind of detergent formulations.

4.8.9 Effect of various metal ions and inhibitors on cutinase activity

To determine whether the cutinase requires any metal cofactor for activity, it was incubated with the metal chelator EDTA or metal ions and then assayed for activity against pNPB as described in the section 3.11.13. Both Cut1 and Cut2 showed slight enhancement of 15-20% in its activity in the presence of NaCl, whereas, KCl and NiCl₂ enhanced the activity of Cut2 by 10% but had no adverse effect on the activity of Cut1. These metal ions are known to enhance the activity in *T. fusca* and other cutinases from *P. cepacia*, *F. oxysporum* and *F. solani* cutinase. Though cutinase is not a metalloprotein and does not require metal ions for its activity, the presence of these metal ions probably change the electrostatic properties of the enzyme, which favor the catalysis of the substrate in a better way. No significant effect on enzyme activity was observed when they were incubated with BaCl₂, CoCl₂, CuSO₄, MgCl₂, MgSO₄, and MnCl₂. Nevertheless, FeSO₄, PbCl₂, and ZnSO₄ have shown considerable negative effect with decrease in more than 50% activity, and AgNO₃ and RbCl drastically inhibited the activity up to 95% for both the cutinases. CrCl₂ and HgCl₂ showed complete inhibitory effect on both the cutinases (Table 4.10).

The effects of various inhibitors on cutinase are shown in Table 4.11. Phenylmethylsulfonyl fluoride, a typical modification reagent for Ser, and diethylpyrocarbonate, a modification reagent for His, drastically inhibited the reaction by more than 50% at 1 mM, whereas at 5 mM, there was no detectable activity for both Cut1 and Cut2. Among the other inhibitors investigated, urea showed slight inhibition of 17% for Cut1; however, 30% inhibition was observed with Cut2. Ethylenediaminetetraacetic acid (EDTA) had no effect on the activity of both the cutinases.

Table 4.10 Effect of different metal ions on cutinase activity

Metal ions (1 mM)	Residual activity (%)	
	Cut1	Cut2
None	100 ± 2	100 ± 1
AgNO ₃	7 ± 1	10 ± 2
BaCl ₂	86 ± 3	92 ± 1
CoCl ₂	98 ± 3	101 ± 2
CrCl ₂	> 0.5	> 0.5
CuSO ₄	68 ± 2	74 ± 1
FeSO ₄	45 ± 2	45 ± 4
HgCl ₂	> 0.5	> 0.5
KCl	105 ± 3	112 ± 4
MgCl ₂	85 ± 3	92 ± 7
MgSO ₄	98 ± 1	96 ± 1
MnCl ₂	103 ± 4	105 ± 4
NaCl	115 ± 1	120 ± 1
NiCl ₂	105 ± 3	111 ± 3
PbCl ₂	46 ± 2	47 ± 5
RbCl	5 ± 1	7 ± 1
ZnSO ₄	35 ± 5	41 ± 3

Table 4.11 Effect of different inhibitors on cutinase activity

Inhibitors (1 mM)	Residual activity (%)	
	Cut1	Cut2
None	100 ± 3	100 ± 3
DEPC	44 ± 1	46 ± 1
EDTA	101 ± 2	103 ± 2
PMSF	52 ± 2	41 ± 3
Urea	83 ± 4	70 ± 4

In summary, the cutinase hydrolytic activity was not dependent on the metal ions as it did not show much variation in the presence of the metal ions or metal chelator (EDTA). Inhibitory effect of PMSF and DEPC suggests that the active site consists of Ser and His amino acids, which upon inhibition abolish the activity.

4.9 Biophysical characterization of cutinases

A protein's function depends on its ability to acquire its native structure. The measurement of protein stability and how a protein fold into a well ordered native structure is essential for elucidating protein function *in vivo*, engineering the improved stability of biocatalytic enzymes, and formulating proteins for therapeutic delivery (Mogk, 2002).

4.9.1. Effect of pH on secondary structure of cutinases

With respect to tryptophan fluorescence measurements, Cut1 and Cut2 are an excellent example for comparative studies, because the number and positions of tryptophan residues are highly conserved in both structures (Fig. 4.29). Fig. 4.19 A and B shows the intrinsic fluorescence spectra of Cut1 and Cut2, respectively at different pH upon excitation at 295 nm. Native Cut1 and Cut2 gave emission maximum ($\lambda_{\max}$) of 331 and 332 nm, respectively. Fig. 4.19 further shows that $\lambda_{\max}$ remains constant over pH range from 7 to 8 while the intensity gradually drops when pH decreased from 7 to 6 or increased from 8 to 9. The reduction in fluorescence intensity at lower pH might be caused by quenching of fluorescence by hydrogen ions and neutralization of COO^- on aspartic and glutamic acid residues in the vicinity of fluorophore (Cowgill, 1975; Malavasic *et al.*, 1996). The observed decrease in fluorescence at higher pH may be explained in terms of deprotonation of tyrosine phenolic groups and other positively charged groups of amino acid side chains (Malavasic *et al.*, 1996). A blue-shifted

$\lambda_{\max}$ of cutinases in the range of pH studied indicates that all the four tryptophan residues of the Cut1 or Cut2 are completely in apolar environment or partially accessible (Cowgill, 1975).

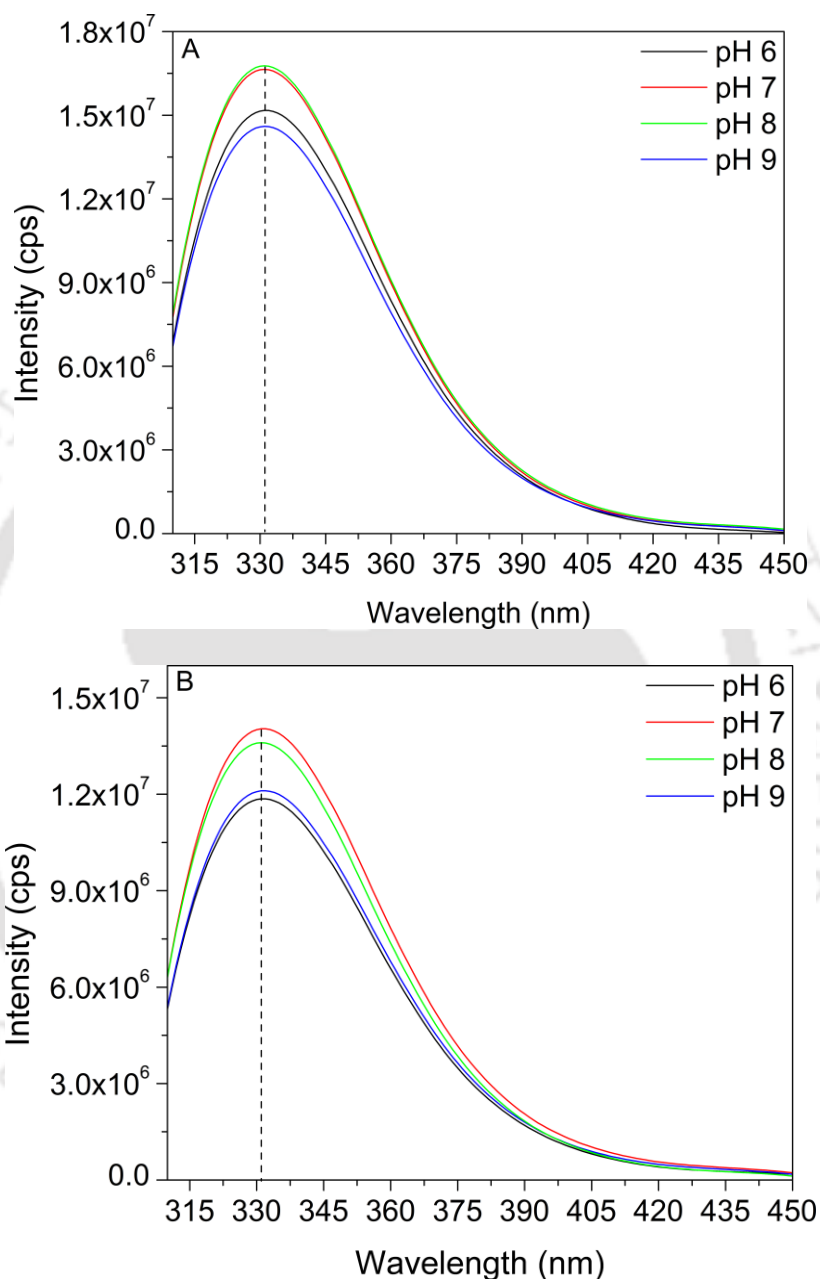


Fig. 4.19 Tryptophan fluorescence spectra of Cut1 (A) and Cut2 (B) at different pH at 25°C

In order to analyze whether one or more Trp residues are partially accessible or completely buried in hydrophobic pockets, a quenching studies with iodide was carried out. As depicted in Fig. 4.20 there was a very small decrease in fluorescence intensity upon addition of

quencher up to 1 M KI. However, further increase in the quencher (iodide) concentration to 1.5 M had no effect on fluorescence intensity which indicates that the Trp residues are probably buried in the hydrophobic region and partially accessible to the solvent exposed environment.

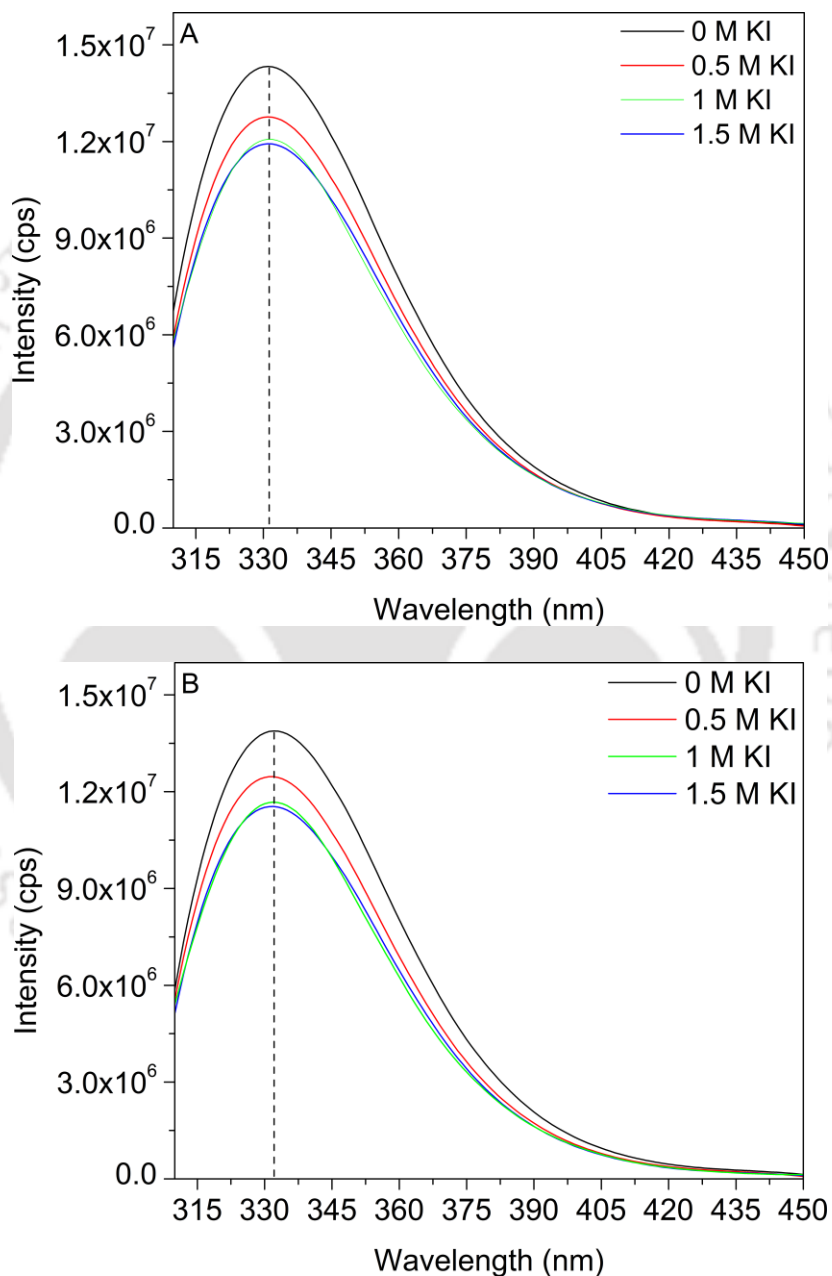


Fig. 4.20 Tryptophan fluorescence spectra of Cut1 (A) and Cut2 (B) in presence of different concentration of quencher (iodide). Fluorescence measurements were obtained in 50 mM KPO₄ buffer (pH 8) at 25°C

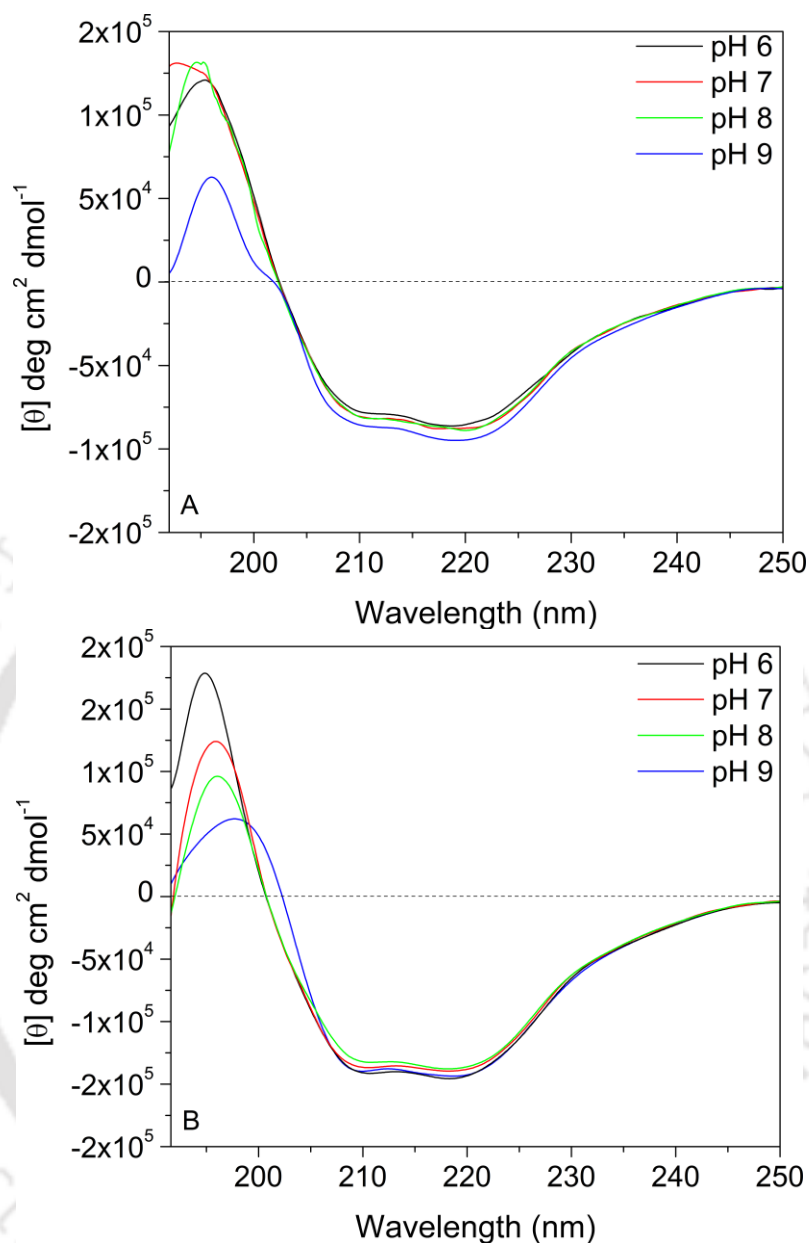


Fig. 4.21 Far UV CD spectra of Cut1 (A) and Cut2 (B) at different pH at 25°C

The far-UV CD measurement of both the cutinases revealed two well-resolved negative peaks at 220 nm and at 208 nm. Strong negative ellipticities at 208 and 220 nm suggest that *T. fusca* cutinases in the native state is composed of α -helix and β -sheet-rich regions which belong to the α/β class of proteins. CD measurement at varying pH (6-9) showed no noticeable change in the secondary structure at any of the pH at 25°C (Fig. 4.21 A and B). This is in accordance

with the studies on activity (Section 4.8.2), where it was observed that both the cutinases are stable and active in terms of activity in the range of pH 6-9.

Near-UV CD spectra provide a measure of the level of interactions of side chain aromatic rings with other groups such as side chain amide carboxylate groups and main chain peptide bonds and are applicable in assessing the asymmetric environment of the aromatic residues in the tertiary structure of proteins (Kelly *et al.*, 2000). In the aromatic region, the CD spectrum of native Cut1 and Cut2 exhibits a long positive peak at 251 nm and there was a variation in the spectra in the region 256-290 nm which shows slight increase in the ellipticity for Cut2 (Fig.4.22).

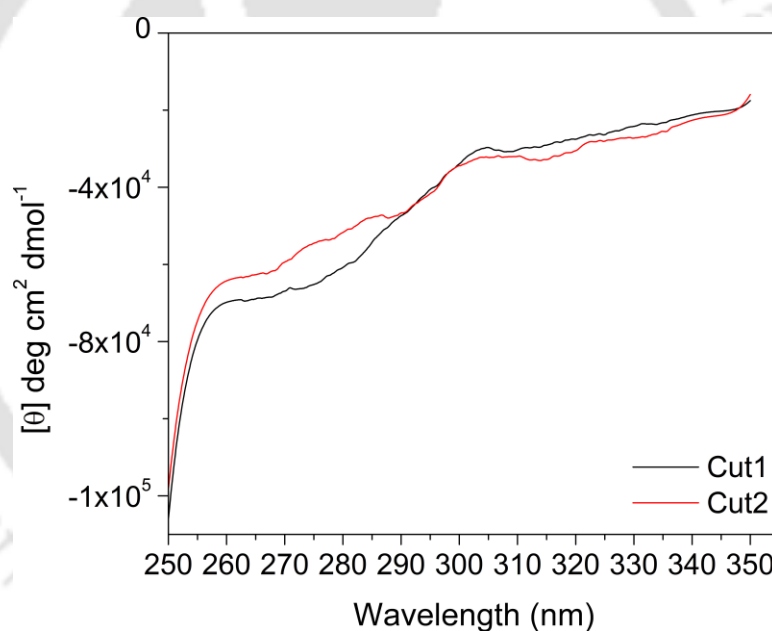


Fig. 4.22 Near UV CD spectra of Cut1 and Cut2. CD measurements were obtained in 50 mM KPO4 buffer (pH 8) at 25°C

As the near UV absorption of the disulfide bond occurs near 260 nm the positive long peak near 251 nm is probably due to the disulfide bond (Kelly *et al.*, 2000). However, the intensity depends on a number of factors including the dihedral angle of the disulfide bond, the C-S-S bond angle and the effects of neighboring groups and the left handed or right handed sense of

the disulfide bond (Kelly *et al.*, 2000). As discussed in the homology modeling study (Section 4.10), the disulfide bond in Cut1 is left handed and the Cut2 is right handed (Fig. 4.29), thus the difference in the intensity at 250-260 nm would be due to this reason. The difference in the near UV spectra in the region 256-290 nm further shows that the Phe and Tyr residues order on tertiary structure are not similar in Cut1 and Cut2.

4.9.2 Effect of guanidine hydrochloride (GdnHCl) on secondary structure of cutinases

To investigate the effect of GdnHCl on the secondary and tertiary structure unfolding of Cut1 and Cut2, fluorescence and CD spectral analysis with different concentration of GdnHCl was carried out as described in the section 3.12.2.

As depicted in the Fig 4.23, initially, both the cutinase showed similar behavior up to 2 M GdnHCl, with decrease in fluorescence $\lambda_{\max}$ intensity at 1 M GdnHCl and then increase in the $\lambda_{\max}$ intensity at 2 M GdnHCl (Fig.4.23 A and B). This may be due to the differential exposure of solvent accessible amino acids in the presence of different concentration of GdnHCl. However, it was interesting to observe that at GdnHCl concentration above 2 M both the cutinases showed different unfolding behavior. In case of Cut1 there was a noticeable 64.3% decrease in the $\lambda_{\max}$ intensity and a red shift of 13 nm at 3 M GdnHCl whereas Cut2 showed only 2 nm red shift with 18.3% decrease in $\lambda_{\max}$ intensity. The major decrease in $\lambda_{\max}$ intensity (68%) and a red shift of 15 nm occurred at 4 M GdnHCl for Cut2 indicating that Cut2 is more resistant to GdnHCl induced unfolding.

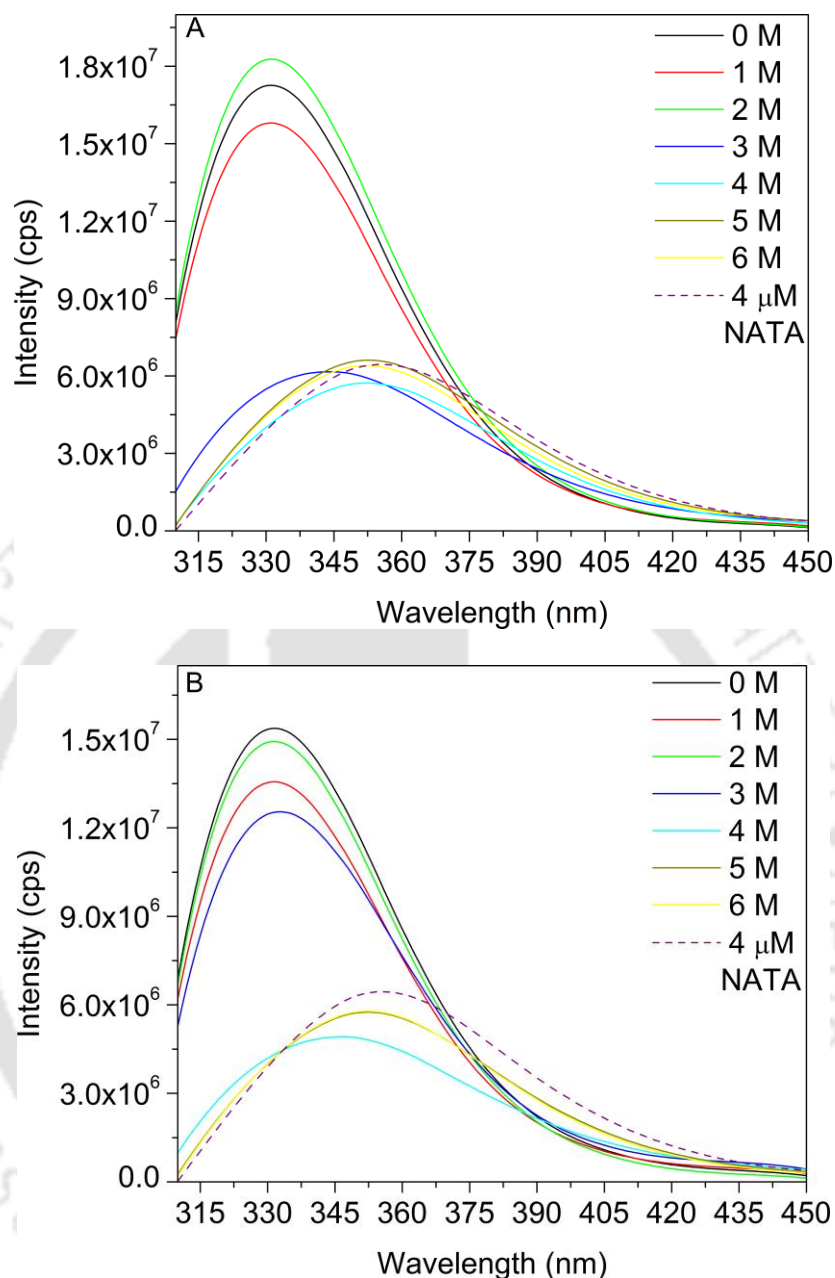


Fig. 4.23 Tryptophan fluorescence spectra of Cut1 (A) and Cut2 (B) in presence of different concentration GdnHCl (M). Fluorescence measurements were obtained in 50 mM KPO₄ buffer (pH 8) at 25°C

However, unlike Cut2, there was a drop in λ_{max} intensity at 4 M GdnHCl concentration for Cut1 and a red shift of 8 nm in comparison to λ_{max} intensity at 3 M GdnHCl. It was presumed that the reason for decrease in intensity of λ_{max} intensity for Cut1 at 4 M GdnHCl (not seen in case of Cut2) and further increase at 5 and 6 M GdnHCl concentration may be due to

differential binding of GdnHCl (thus, the difference in unfolding) followed by two enzymes due to the variation in the surface electrostatic properties of the two enzymes (Section 4.10 and Fig. 4.30). Increasing the GdnHCl concentration further, showed a red shift for both the cutinases and no additional decrease in λ_{max} intensity was seen above 5 M concentration, indicating that both the cutinases are completely unfolded at 5 M GdnHCl concentration. Comparison of λ_{max} fluorescence intensity with 4 μM N-acetyl-L-tryptophanamide (NATA) shows the decrease in fluorescence intensity for Cut2 at 4 and 5 M GdnHCl (not observed in case of Cut1 at same concentration of GdnHCl) which may be due to aggregation of hydrophobic regions which quite often occurs for non-native states of protein (Vieille and Zeikus, 2001).

In principle, changes in Trp fluorescence can be caused both by structural changes and changes in the polarity of the environment induced by the presence of denaturants. Thus, to distinguish simple changes in the polarity of external environment from conformational changes, the change in the near UV CD spectrum with increasing GdnHCl concentration was investigated. As shown in Fig. 4.24 A and B, addition of GdnHCl leads to a pronounced loss of ordered secondary structure in the far-UV CD spectrum in both the cutinases, as more GdnHCl is titrated in, there is a general increase in ellipticity but the greatest change occurs between 1 and 3 M in case of Cut1 and 3 to 4 M in case of Cut2. At 3 M GdnHCl, the protein is essentially in the form of β -sheets and above 4 M GdnHCl, both the cutinases turns into a featureless random coil structure. This is in accordance with Trp fluorescence spectra, where the major transition in fluorescence occurs at same concentration of GdnHCl (Fig. 4.23). It is also evident from Fig. 4.25 A and B, that the equilibrium unfolding in GdnHCl is a two-state process, where only the native and denatured states are significantly populated.

Ternstrom *et al.*, (2005) in their study with cutinases from *F. solani* and *H. insolens* found the similar two-stage unfolding with GdnHCl.

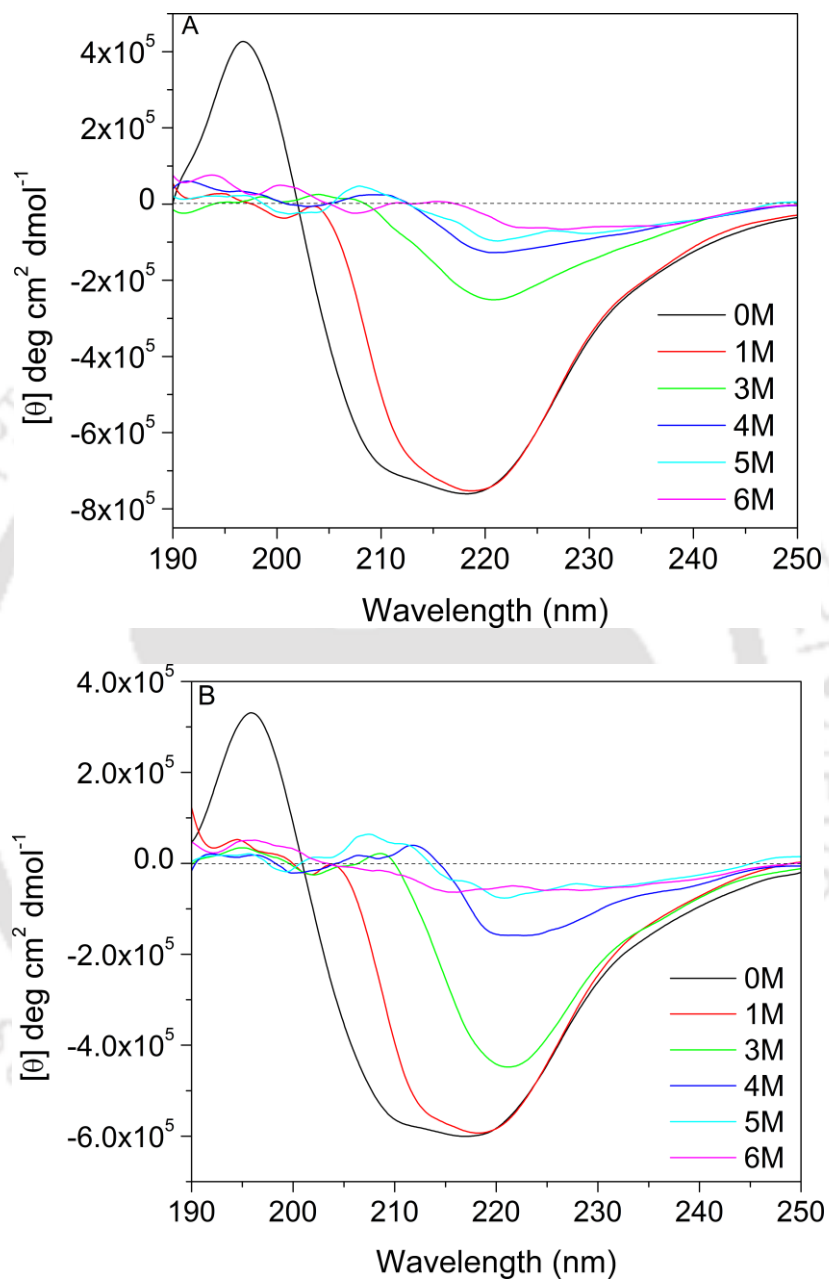


Fig. 4.24 Far UV CD spectra of Cut1 (A) and Cut2 (B) in presence of different concentration GdnHCl (M). CD measurements were obtained in 50 mM KPO₄ buffer (pH 8) at 25°C

Thus, to summarize the structural stability in presence of GdnHCl, the comparison of Cut1 and Cut2 yields a difference in the resistivity to unfolding, with Cut2 showing considerably higher tolerance level for GdnHCl unfolding compare to Cut1 at lower concentration of GdnHCl. On the basis of numerous studies on thermostability of proteins, it is well known that many different mechanisms are utilized to achieve thermal adaptation (Jaenicke *et al.*, 1996; Perl *et al.*, 2000; Vieille and Zeikus, 2001).

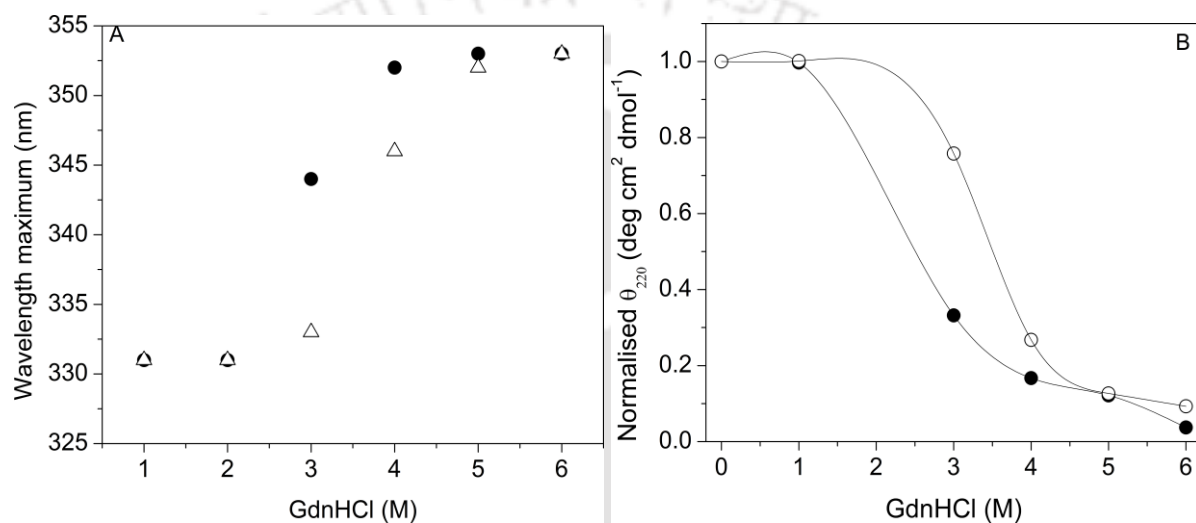


Fig. 4.25 (A) Effect of GdnHCl on the tryptophan fluorescence maxima of Cut1 (filled circle) and Cut2 (triangle). (B) Concentration dependence of equilibrium unfolding of Cut1 (filled circle) and Cut2 (open circle) in presence of GdnHCl. The 220 nm CD signals at different concentrations of GdnHCl were normalized with respect to the control signal (in the absence of GdnHCl) and plotted against GdnHCl concentrations

Considering the enormous variety of potential mechanisms, it is assumed that even quite often a combination of several different mechanisms plays a decisive role. Thus, it can be inferred that the prime reasons for the higher stability would be due to the more compactness of the Cut2 structure, which is resisting the unfolding process, difference in solvent accessible hydrophobic or electrostatic properties and the difference in the N-terminal region of the two cutinases (Fig. 4.27), which is believed to play a key role in the unfolding of cutinases (Ternstrom *et al.*, 2005). All these factors might be contributing synergistically to the

variation in the structural integrity and stability of the two cutinases. Moreover, GdnHCl is a monovalent salt having both ionic and chaotropic effects. Guanidine is present in a fully protonated Gdn^+ form at pH below 11 (pK_a 11) thus, the presence of Gdn^+ and Cl^- influence the stability properties of proteins. At low concentrations, Gdn^+ and Cl^- ions are assumed to mask the positively and negatively charged amino acid side chains, thereby reducing or even totally eliminating any stabilizing or destabilizing electrostatic interactions. The effect of GdnHCl and NaCl has been reported on RNase T1 (Mayr and Schmid, 1993) where compaction of native enzyme is interpreted in terms of stabilization by cation (Gdn^+ and Na^+) binding to the negatively charged moieties of protein which has also been demonstrated for glucose oxidase (Ahmad *et al.*, 2001). In the present case also, we presume that the initial slight increase in stability of Cut2 observed in the lower concentration of GdnHCl denaturation may be explained in terms of the masking of electrostatic repulsions at low GdnHCl concentrations. As it is evident from the Fig. 4.30 that surface electrostatic charge of both the cutinases are very different, despite their high structural resemblance, one of the reason for the differential behavior in unfolding of Cut1 and Cut2 below 4 M GdnHCl would be due to the dissimilarity in binding of the Gdn^+ ions to the proteins to push the equilibrium toward the unfolded state, where the equilibrium is reached faster for Cut1 in comparison to Cut2. As both the protein contain ion binding sites of varying affinity and specificity, degree of stabilization of proteins by denaturants could vary in a fairly common process. However, at higher concentration of GdnHCl, despite the difference in electrostatic surface properties in the cutinases, GdnHCl act as a classic denaturant.

4.9.3 Denaturation with urea

Like in the case of GdnHCl denaturation the tryptophan emission spectrum of cutinases as probes was used to study the urea induced unfolding at 25°C. As depicted in Fig. 4.26, there was a very small increase in $\lambda_{\max}$ intensity at 1 M urea for both the cutinases, whereas the accompanying increase in $\lambda_{\max}$ intensity was very large for both the cutinases at 2 M urea (by about 48.8% for Cut1 and 65.3% for Cut2 relative to the native protein). However, further increasing the urea concentration gave a different Trp fluorescence pattern for both the cutinases. Cut1 showed gradual drop in the $\lambda_{\max}$ with an increase in the urea concentration up to 8 M urea with a red shift of 2 nm at 8 M urea concentration. Whereas Cut2 showed drop in $\lambda_{\max}$ only up to 4 M urea, and further increasing the urea concentration up to 8 M had no significant effect on the $\lambda_{\max}$ intensity, though there was a red shift of ~ 1 nm at 8 M urea. Thus, it was hypothesized that both the cutinases are highly resistant to urea induced unfolding and probably at 8 M urea there is a slight unfolding, which was evident by red shift in fluorescence intensity by 1-2 nm due to exposure of Trp residue to solvent accessible environment. To confirm this hypothesis, quenching studies with iodide was carried out as described in the section 3.12.3. In the quenching experiment, 1 M KI was added to each of the urea denatured samples before taking the fluorescence measurement, incubated at 25°C for 5 min and fluorescence measurements were taken. As seen in Fig. 4.26, there was no noticeable change in the pattern of fluorescence in both the enzymes till 6 M urea, except the reduction in $\lambda_{\max}$, which was also seen in native enzymes (Fig. 4.20). However, at 8 M urea there was a blue shift in $\lambda_{\max}$ for both the cutinases and a further drop in $\lambda_{\max}$.

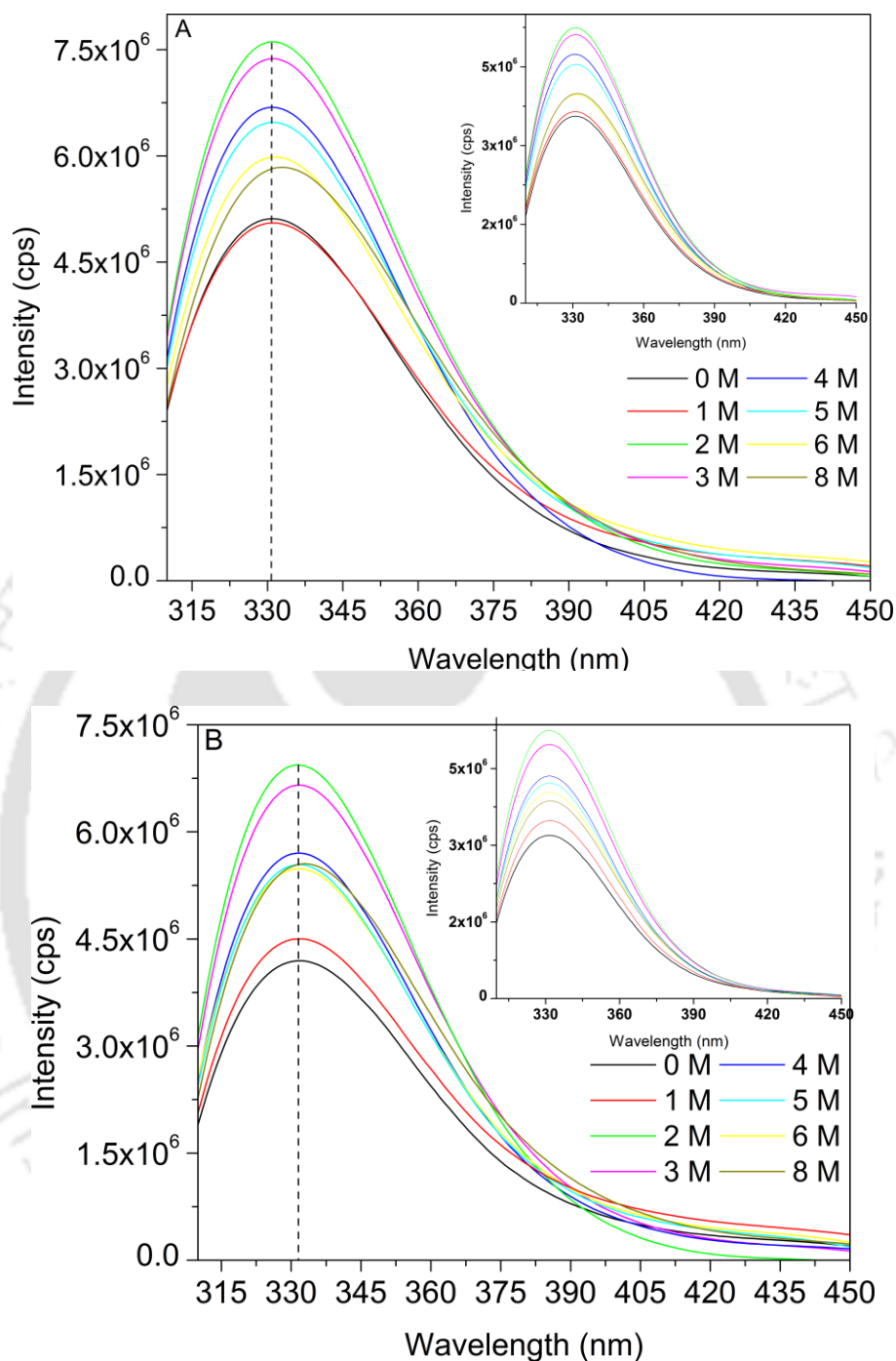


Fig. 4.26 Tryptophan fluorescence spectra of Cut1 (A) and Cut2 (B) in presence of different concentrations urea (M). Inset Fig. shows the fluorescence in presence of corresponding concentration of urea and quencher (iodide). Fluorescence measurements were obtained in 50 mM KPO₄ buffer (pH 8) at 25°C

Thus, according to the changes observed in urea denatured Trp spectra with and without quencher one may speculate that slight unfolding occurs only at 8 M urea for both the cutinases and in lower concentration of urea there is only change in the microenvironment of the enzymes without any unfolding.

Thus, to summarize the structural stability of Cut1 and Cut2 in presence of urea, there was no unfolding for both the cutinases up to the concentration of 8 M, at which there was a small shift (~ 2 nm) in the fluorescence λ_{max} , which indicates the high resistivity towards unfolding in presence of urea. In contrast to GdnHCl, the urea molecule is uncharged thus; it is not expected to have any significant effect on the intermolecular and intramolecular electrostatic interactions in all the proteins (Monera *et al.*, 1994). There are reports which supports the fact that urea is insufficient in unfolding of other proteins (Dubey and Jagannadham, 2003).

4.10 Structural characterization of *T. fusca* cutinases

The homology model of Cut1 and Cut2 was obtained through Modeller online server; ModWeb (Eswar *et al.*, 2003; Sali and Blundell, 1993) using 1.76 Å resolution structure of cutinase (Est119) from *Thermobida alba* AHK119 (PDB ID: 3VIS) with 85 and 82% sequence identity for Cut1 and Cut2, respectively. The residues from 57 to 319 and 39 to 301 positions of Cut1 and Cut2 respectively, were aligned with template sequence (Fig. 4.27). The pairwise sequence alignment between the model and template showed that most of the secondary structures were evolutionarily conserved with minimum gaps or insertions. As depicted in Fig. 4.28, the core secondary structural elements of cutinase were organized into a mixed α/β structure consisting of 9 β -strands and 10 (Fig. 4.28 A) or 11 (Fig. 4.28 B) α -helices in case of Cut1 and Cut2, respectively connected by several loops.

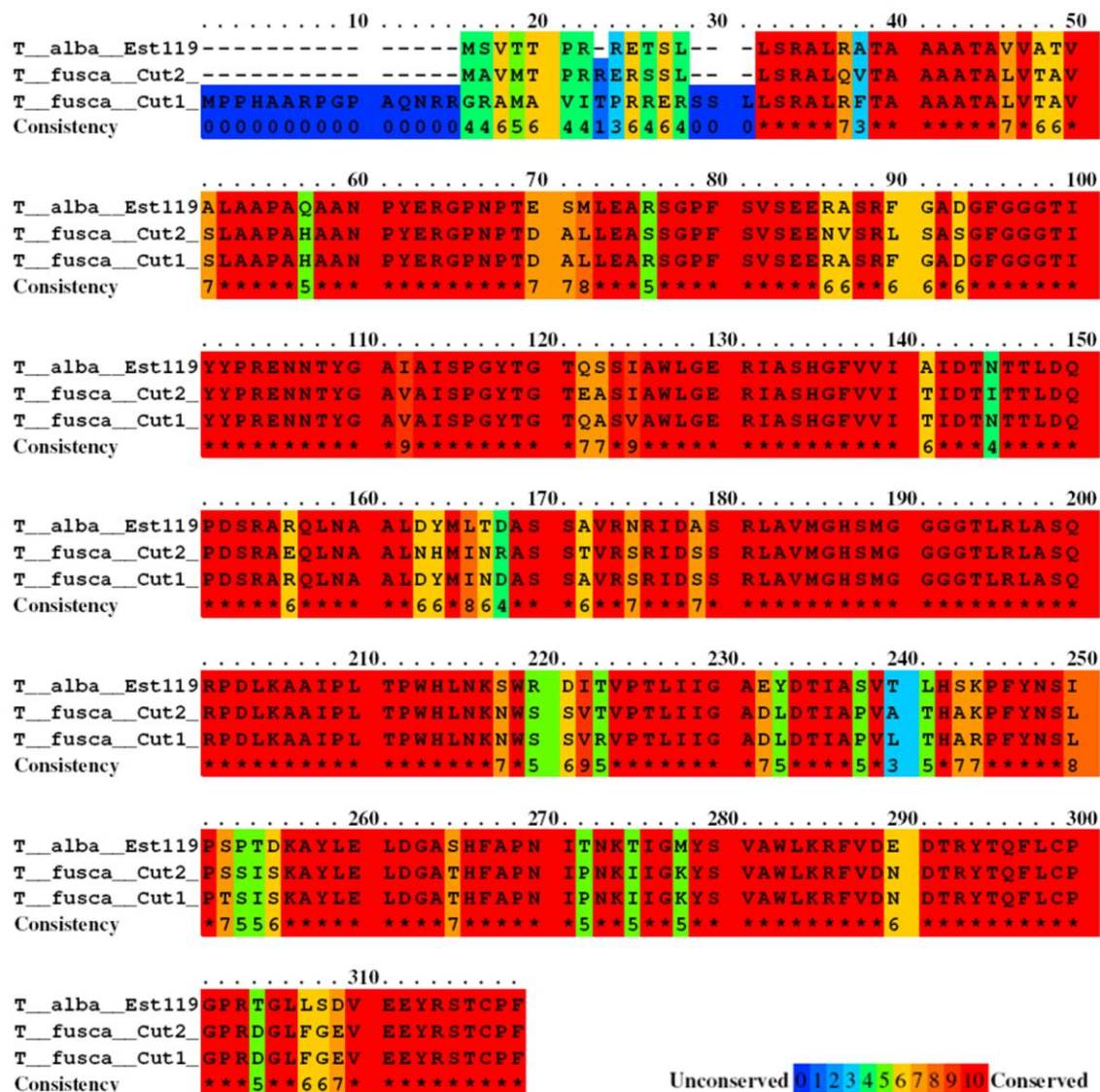


Fig. 4.27 Structural alignment of Cut1 and Cut2 with Est 119

This model also revealed a potential Ser-His-Asp catalytic triad (Ser188-His266-Asp234 in case of Cut1 and Ser170-His248-Asp216 in case of Cut2) in which the nucleophilic Ser (Ser188 in case of Cut1 and Ser170 in case of Cut2) is located in a sharp turn called the “nucleophile elbow,” which is commonly observed in the α/β -hydrolases (Fig.4.29 A and B). The oxyanion hole is predicted to be formed by the backbone amides of Met and Tyr (Met189 and Tyr118 for Cut1 and Met171 and Tyr100 for Cut2) (Kitadokoro *et al.*, 2012).

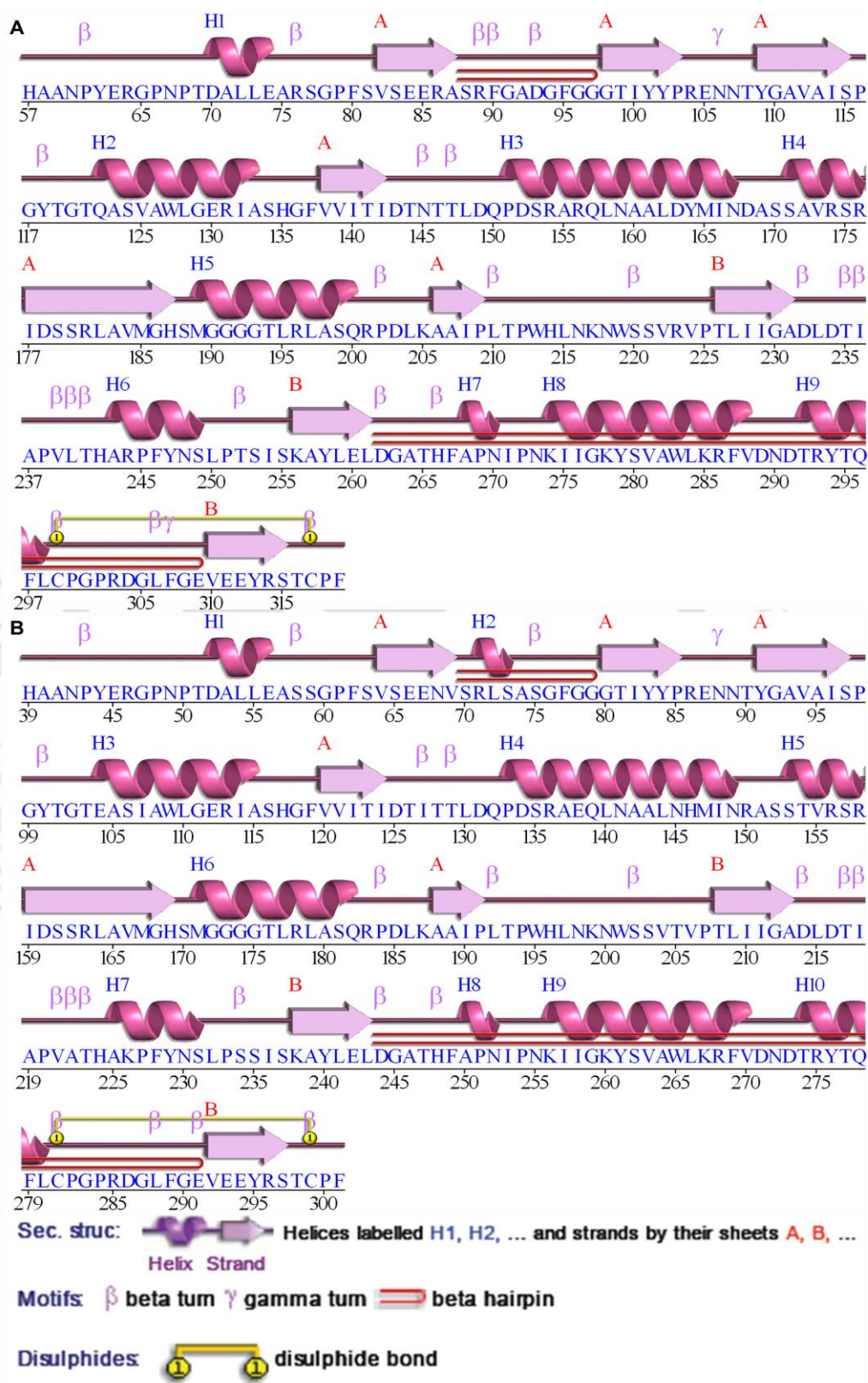


Fig. 4.28 Secondary structure of the Cut1 (A) and Cut2 (B) predicted by homology modeling

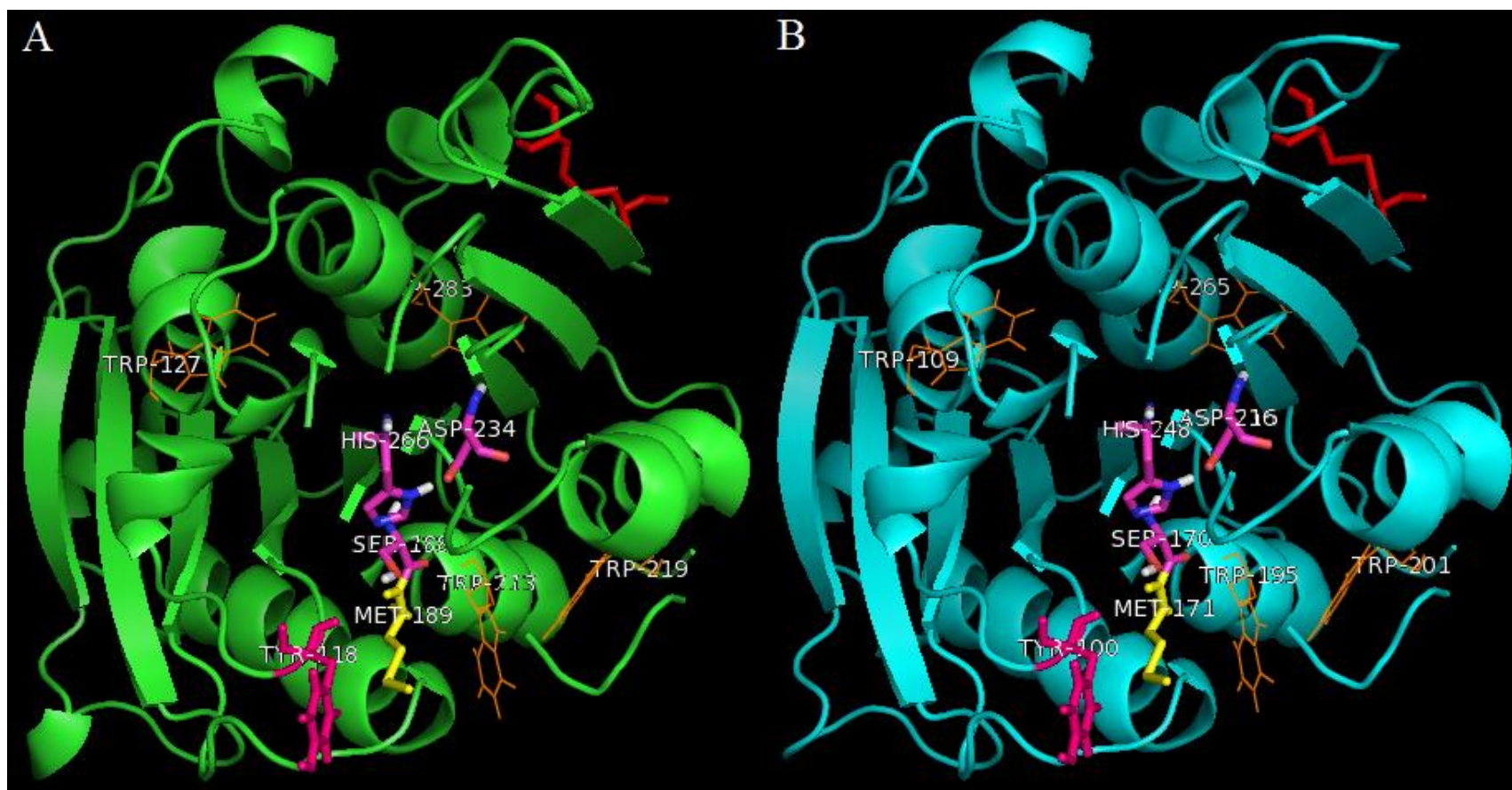


Fig. 4.29 Ribbon diagram of the Cut1 (A) and Cut2 (B) structure predicted by homology modeling. The catalytic triad, which is composed of Ser188-His266-Asp234 in case of Cut1 and Ser170-His248-Asp216 in case of Cut2, are shown as sticks. The oxyanion hole is predicted to be formed by the backbone amides of Met189 and Tyr118 for Cut1 and Met171 and Tyr100 for Cut2 are shown as yellow and pink sticks. Four Trp residues, Trp127, Trp213, Trp219, Trp283 of Cut1 and Trp105, Trp195, Trp201, Trp265 of Cut2 are shown as orange sticks. Disulfide bonds of Cut1 (Cys299-Cys317) and Cut2 (Cys281-Cys299) are shown as red sticks

Notably, similar to fungal cutinase (Liu *et al.*, 2009; Longhi *et al.*, 1997; Martinez *et al.*, 1992), the enzyme does not contain a lid insertion that is commonly observed in lipases (Longhi *et al.*, 1997; Martinez *et al.*, 1992), resulting in a nucleophilic serine that is exposed to the solvent. There is a disulfide bond present in both Cut1 (Cys299-Cys317) and Cut2 (Cys281-Cys299) in the C-terminal region. As depicted in Fig 4.29, all the 4 Trp residues in Cut1 and Cut2 are either buried in the hydrophobic pocket or partially exposed to the solvent accessible environment. Further, exploration of the electrostatic surface of the cutinases showed that the surface electrostatic properties of the two cutinases were fairly dissimilar. Both the cutinases showed a patch of highly electronegative regions, however, majority of the surface region of Cut1 was electropositive and Cut2 was found to be slightly electronegative (Fig. 4.30).

4.10.1 Structural assessment of the 3-D model

The superimposition of Cut1 or Cut2 onto the template structure showed a RMSD of 0.267 and 0.257 Å for C α pairs of Cut1 and Cut2, respectively, signifying the overall tertiary structure of the model bears close resemblance to its template. Validation of the cutinase model was carried out using different structure validation tools as mentioned in section 3.13. Summary of the various validations to evaluate the goodness of the model are listed in Table 4.12. The calculated Ramachandran plot using PROCHECK compares well with that of the template (Est119) exhibiting 90.6% residues in core, 8.7% in additional allowed, 0.5% in generously allowed, and 0.2% within disallowed regions (Kitadokoro *et al.*, 2012). Altogether, the main chain and side chain parameters as evaluated by PROCHECK and Ramachandran plot (Fig. 4.31) and the high scoring values obtained using various other structure validation tools (Table 4.12) confirm the overall accuracy of the model. In

conclusion, it was evident from the structural data that the Cut1 and Cut2 are structurally very similar, with minor changes in the secondary structure. However, the hydrophobicity and the surface electrostatic properties of the two enzymes are considerably different.

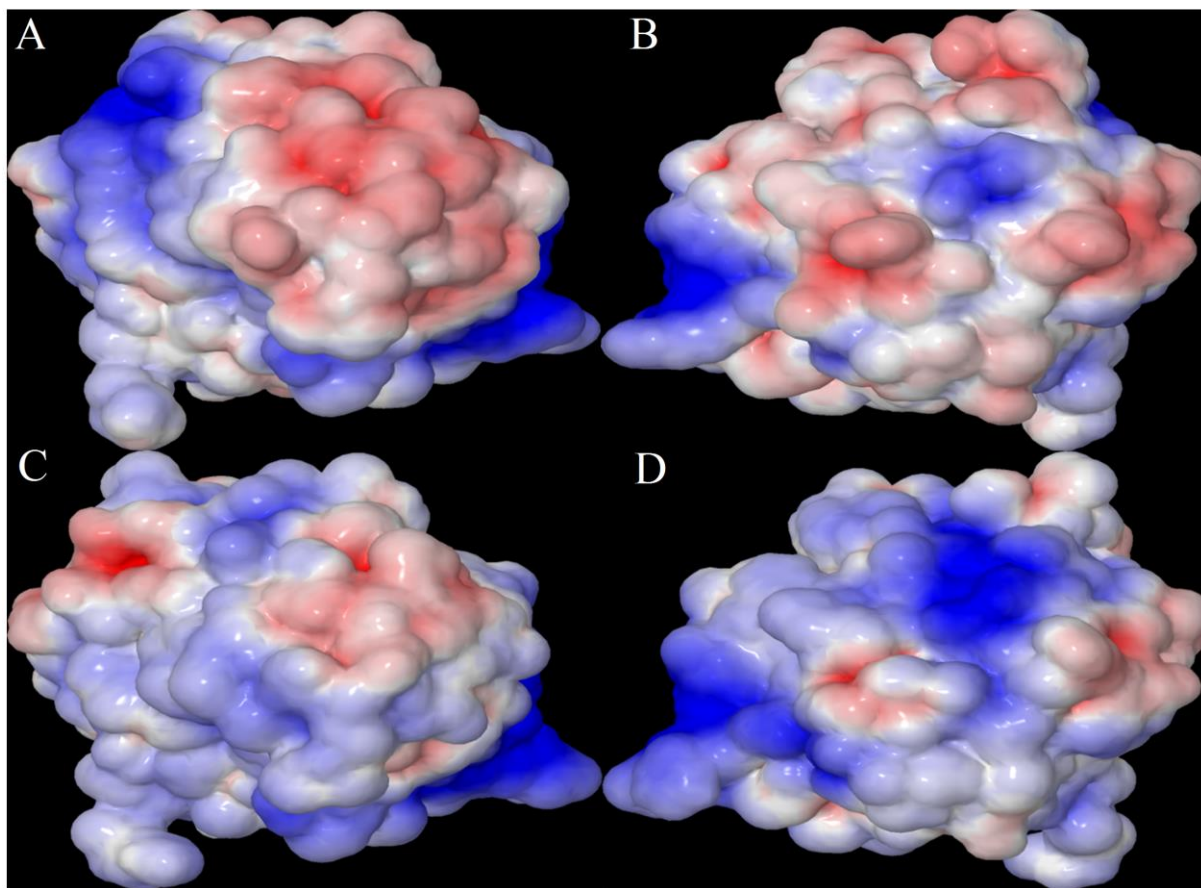


Fig. 4.30 The electrostatic surface representation of Cut1 (A; front view, B; rear view) and Cut2 (C; front view, D; rear view) model. The red and blue color represents the electropositive and electronegative surface regions, respectively

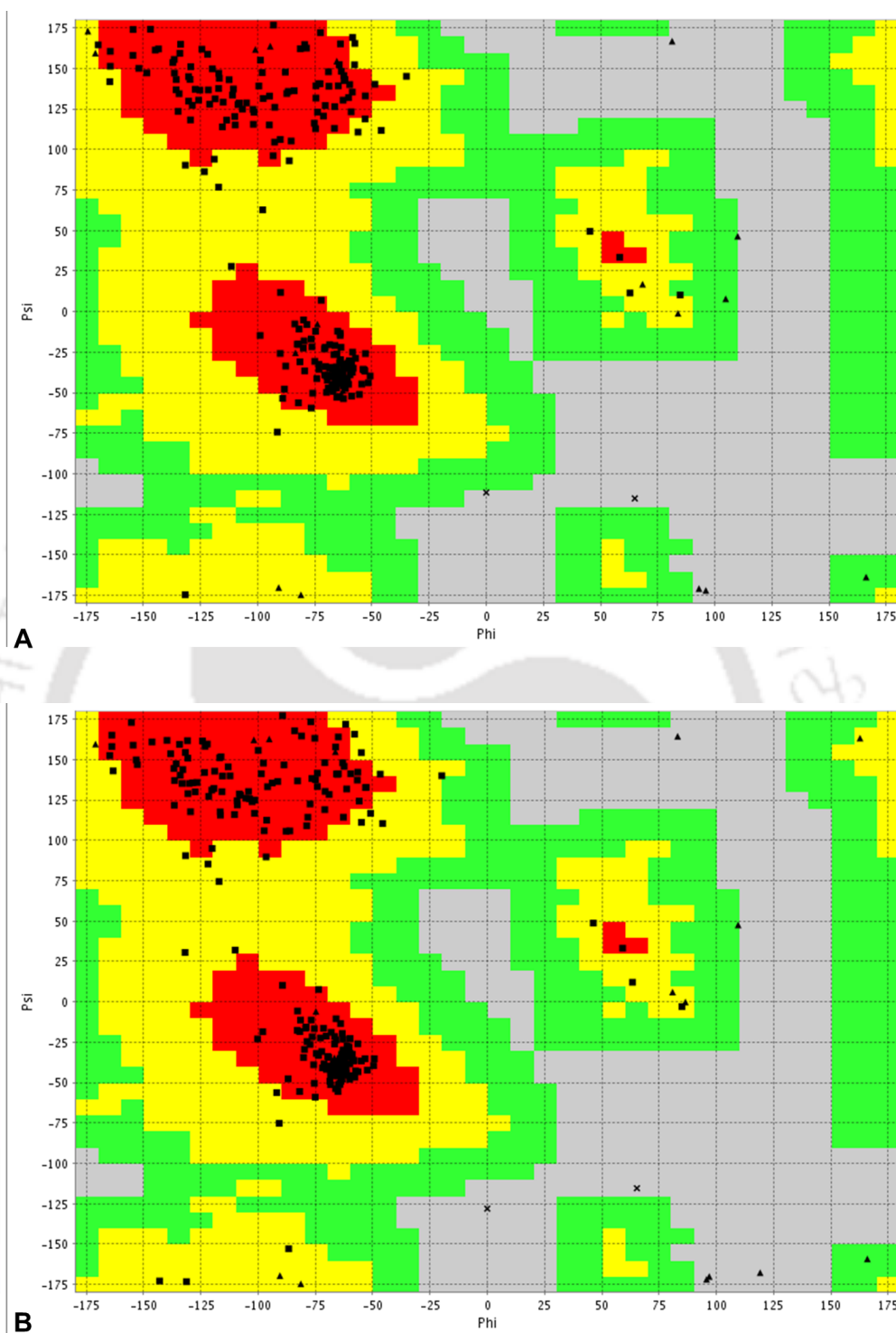


Fig. 4.31 Ramachandran plot for the Cut1 (A) and Cut2 (B) model generated using PROCHECK server. Color and symbol represents Core region (red), Allowed (yellow), generous (green), disallowed (grey), glycine residues (triangle). The percentage of amino acids in each region is listed in Table 4.12

Table 4.12 Quality scores of modeled Cut1 and Cut2 protein using different quality control programs

Parameter	Results		Significance	Program
	Cut1	Cut2		
Ramachandran plot	91.3% core, 8.2% allowed, 0.0% generous, 0.5% disallowed	90.5% core, 8.6% allowed, 0.5% generous, 0.5% disallowed	--	PROCHECK
G-factor	0.01	0.01	Values below -0.5 is unusual	PROCHECK
VERIFY3D	98.11	89.02	Percentage of residues with average 3D–1D score >0.2.	SAVES
ERRAT	92.941	92.52	Percentage of the protein for which the calculated error value falls below the 95% rejection limit	SAVES
Q score	0.63	0.64	QMEAN score of the whole model reflecting the predicted model reliability ranging from 0 to 1.	QMEAN
Z-score	-1.53	-1.42	--	QMEAN
Mean fractional volume	10444	10348.3	--	VADAR

4.11 Studies on immobilization of *T. fusca* recombinant cutinases on various supports

Immobilization technology offers promising economic exploitation of even expensive enzymes in several catalytic reactions in applications like, esterification and transesterification. Various supports and immobilization techniques were evaluated for increasing the stability and activity of recombinant *T. fusca* cutinases.

4.11.1 Covalent immobilization on chitosan support

The Chitosan beads of $\sim 0.42 \pm 0.035$ mm size weighing $\sim 5.68 \pm 0.66$ mg per wet bead were prepared (Fig. 4.32) and then activated by glutaraldehyde according to the method described in section 3.14.1.2. In addition to activation of chitosan beads, glutaraldehyde also cross-links the chitosan, thereby providing resistance against lower pH. The two terminal aldehyde groups of glutaraldehyde react with amino groups of D-glucosamine units of different chains, resulting in cross-linking of those chains through glutaraldehyde. The irreversible Schiff's base linking of aldehyde with amino group provides operational stability to beads.

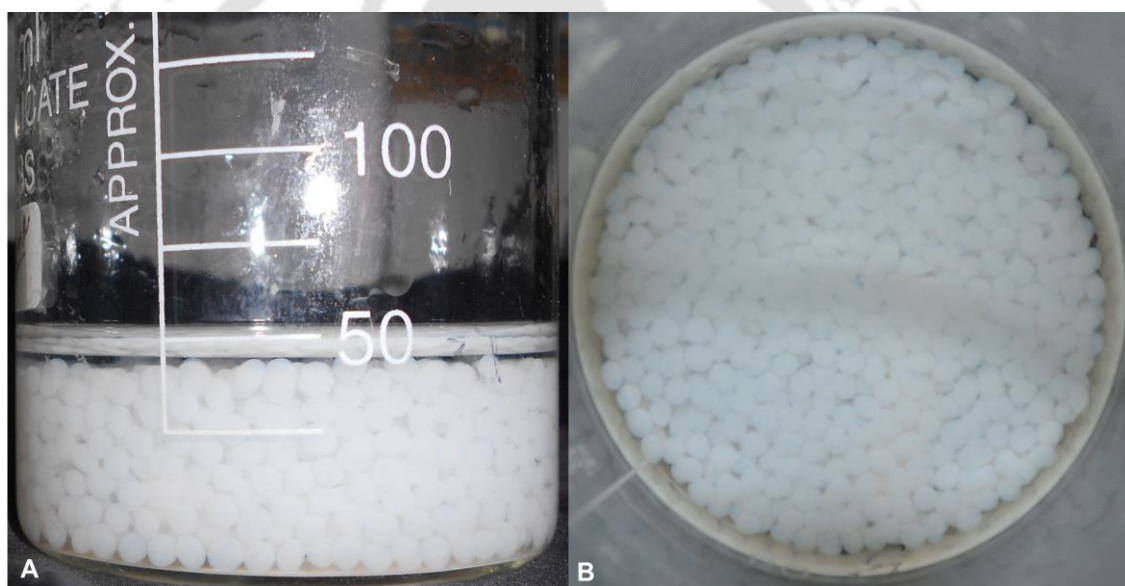


Fig. 4.32 Wet chitosan beads before immobilization of enzymes (A) and after immobilization of cutinase (B)

4.11.1.1 Activation and immobilization of cutinase

Effect of glutaraldehyde concentration on activation of chitosan beads and immobilization of cutinase was investigated in the range of 1.5 to 4% at pH 8. Most efficient glutaraldehyde concentration was found to be 3% for both Cut1 and Cut2 with 67 and 65% immobilization, respectively (Table 4.13). Lower concentration of glutaraldehyde showed lesser

immobilization efficiency, which could be due to the fact that not enough aldehyde groups were generated on surface of chitosan beads to couple enough cutinase. Nevertheless, at higher glutaraldehyde concentration there was no significant decrease in coupling efficiency but the chitosan beads become brittle. This could be due to the fact that, glutaraldehyde not only activates the chitosan beads but it also cross-links the chitosan molecules. Higher concentration of glutaraldehyde increases inter molecular crosslinking which leads to increase in stiffness of the chitosan beads thus, making them brittle.

The pH plays a vital role in coupling of enzyme to glutaraldehyde activated chitosan through the amino group. It is a well-known fact that the ionization state of various functional groups present on the enzyme predominantly depends on the pH of the surrounding environment. The effect of pH on cutinase coupling with chitosan beads was investigated in the range of pH 6 to 9. As shown in Table 4.13, the best suited pH range for ideal cutinase immobilization was found to be pH 8 to 9, with more than 50% immobilization, precisely, the optimum pH was found to be pH 8.5, with 74 and 71% immobilization for Cut1 and Cut2, respectively. At lower pH, the immobilization was substantially decreased, which could be due to improper ionization state of surface amino groups of cutinase.

4.11.1.2 Properties of cutinase immobilized chitosan beads

The structural changes in chitosan after glutaraldehyde activation were analyzed by IR spectroscopy and FESEM. As depicted in Fig. 4.33, the peak at 1495 cm^{-1} represents the C-N bond resulting from a cross-linking reaction. A reaction occurs between aldehyde groups of glutaraldehyde molecules and the amino group of chitosan through Schiff base linkage. Linkage of both terminal aldehyde groups with separate chitosan molecules results in cross-linking, while the linkage of only one aldehyde group results in the activation of surface for

immobilization (Singh *et al.*, 2011). Other characteristic absorption peaks of chitosan were observed at 3000-3500 cm^{-1} (OH, NH_2), 1656 (CONH amide band II) and 1085 cm^{-1} (may be attributed to polysaccharide structure).

Table 4.13 Immobilization condition optimization for Cut1 and Cut2 on glutaraldehyde activated chitosan beads

	pH ^a	Immobilization (%)		Specific activity per bead	
		Cut1	Cut2	Cut1	Cut2
Variation of coupling pH	6	25 ± 0.2	21 ± 0.7	3 ± 0.3	3 ± 0.6
	6.5	30 ± 0.6	32 ± 0.3	4 ± 0.3	4 ± 0.1
	7	38 ± 0.2	40 ± 0.5	6 ± 0.1	6 ± 0.6
	7.5	48 ± 0.8	51 ± 0.8	6 ± 0.2	6 ± 0.1
	8	69 ± 0.7	67 ± 0.4	7 ± 0.2	7 ± 0.1
	8.5	74 ± 0.5	71 ± 0.4	7 ± 0.4	7 ± 0.9
	9	71 ± 0.1	69 ± 0.2	6 ± 0.1	6 ± 0.7
	GA% ^b				
Variation of glutaraldehyde (GA) %	1.5	46 ± 0.4	50 ± 0.9	5 ± 0.2	5 ± 0.7
	2	61 ± 0.3	59 ± 0.0	6 ± 0.4	7 ± 0.9
	3	67 ± 0.2	65 ± 0.5	8 ± 0.9	8 ± 0.6
	4	64 ± 0.6	62 ± 0.1	4 ± 1.0	5 ± 0.6

± correspond to the standard deviation of two determinations

^a Coupling pH optimization was done using 3% Glutaraldehyde (GA) activated chitosan beads

^b Glutaraldehyde (GA) % optimization was done at coupling pH 8

FESEM analysis of the chitosan beads at different stages of immobilization showed increase in the roughness of the beads upon glutaraldehyde treatment and subsequent cutinase immobilization (Fig 4.34). The increase in coarseness may be due to activation of the surface with glutaraldehyde and attachment of the enzyme on the activated surface.

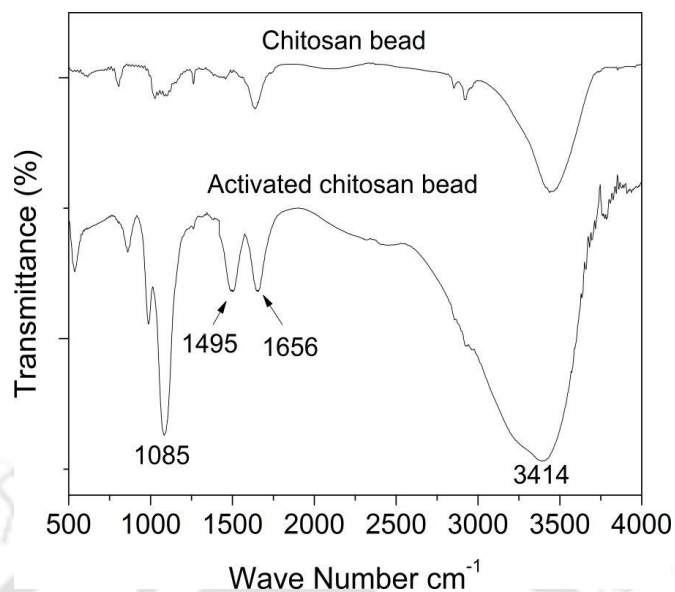


Fig 4.33 Comparison of FTIR spectra of normal chitosan beads and glutaraldehyde activated chitosan beads for confirmation of glutaraldehyde activation

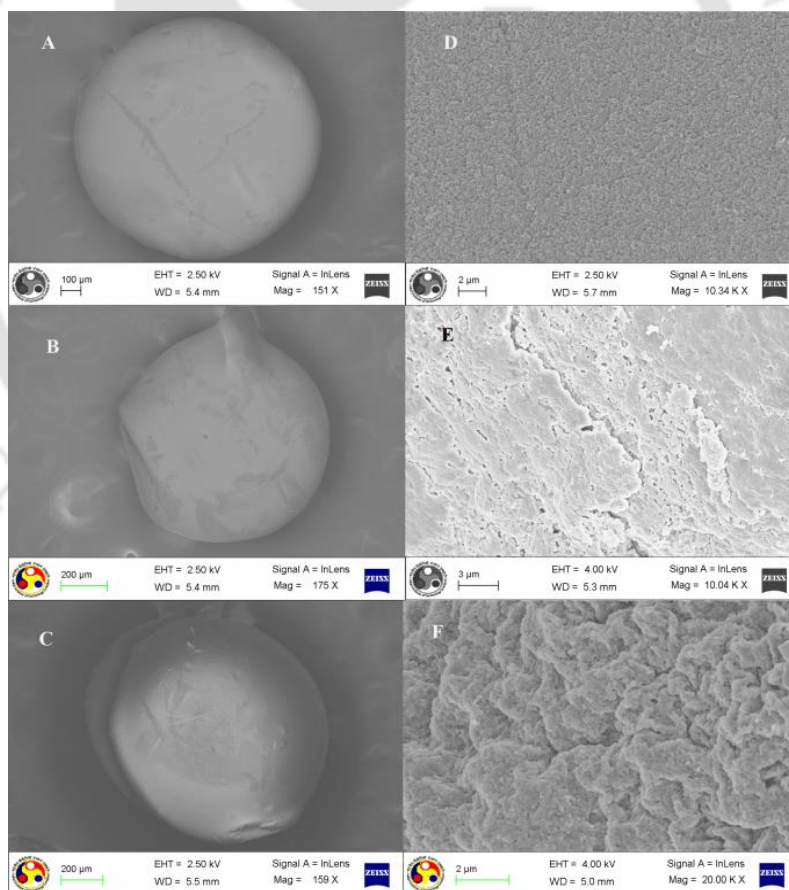


Fig. 4.34 FESEM images of chitosan bead and detailed surface view of normal chitosan bead (A and D), glutaraldehyde-activated chitosan bead (B and E), and immobilized chitosan bead (C and F)

4.11.1.3 Biochemical characterization of cutinases immobilized on chitosan

The evaluation of behavior of cutinase under immobilized conditions is essential as the efficiency of catalysis might be greatly affected by varying pH and temperature. Thus, a prior knowledge of the immobilized enzyme performance at various pH and temperature would be a beneficial step in carrying out the experimental part of various applications. The biochemical characterization of the immobilized cutinases was carried out according to the method described in the section 3.14.5.

4.11.1.3.1 The pH and temperature optima

It was observed that free cutinase has broader pH optima as compare to immobilized cutinase (Fig. 4.35 A). Though free as well as immobilized cutinase showed highest activity at pH 8, immobilized Cut1 as well as Cut2 showed considerably lesser activity at acidic pH (below pH 7) but were more active in alkaline pH (above pH 9) in comparison to free cutinase. One possible reason for the reduction in activity for immobilized cutinase at acidic pH could be due to the interaction of residual charges of the activated chitosan residues with coupled enzyme resulting in the unfavorable alteration of active site. Similar results were observed for some other enzymes in previous studies (Esawy *et al.*, 2006; Krajewska *et al.*, 2005; Gonzalez-Saiz *et al.*, 2011). Thus, the immobilized cutinase is efficient at pH 8 or higher pH as compare to free cutinase, which showed comparatively broader pH curve.

The temperature optima of the immobilized Cut1 and Cut2 were found to be 55°C (Fig. 4.35 B). The operational stability of the immobilized cutinase was considerably higher in the range of temperature studied. Increase in the operational stability at higher temperature is a reflection of conformational rigidity which makes immobilized enzyme resistant to denaturation.

4.11.1.3.2 The pH and thermal stability

Both the immobilized Cut1 and Cut2 were found to be highly stable at pH 7.5 to 9, with more than 80% activity retention. However, there was noticeable activity loss at pH 9 to 11 (Fig. 4.36 A). Thermal stability of the immobilized cutinase was carried out at 55°C, which was found to be the optimal operational temperature. Moreover most of the industrial processes are employed in the range of 40-60°C for carrying out any catalytic reactions. As depicted in Fig. 4.36 B, the deactivation process was faster after 12 h for free cutinase in comparison to immobilized cutinase. There was almost 60% activity retention even after 50 h for immobilized cutinase, whereas the free cutinase activity was dropped to 40% at 40 h which indicates that the chitosan immobilized cutinases are thermally more stable in comparison to free cutinase.

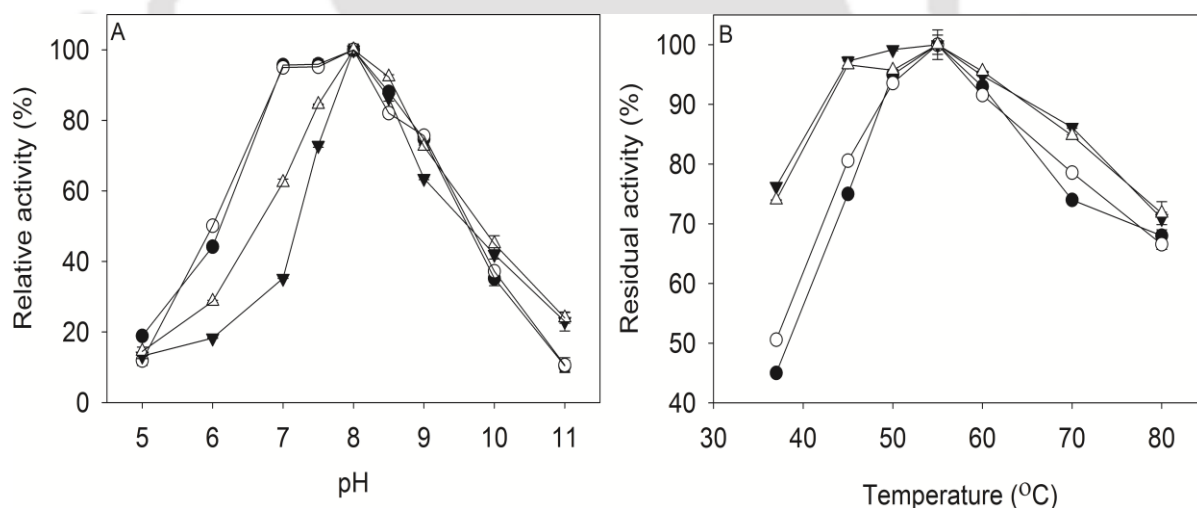


Fig. 4.35 (A) Effects of pH on activity of Cut1 (filled down-pointing triangle) and Cut2 (open up-pointing triangle) immobilized on chitosan beads, in comparison to free Cut1 (filled circle) and Cut2 (open circle). (B) Effects of temperature on activity of Cut1 (filled down-pointing triangle) and Cut2 (open up-pointing triangle) immobilized on chitosan beads, in comparison to free Cut1 (filled circle) and Cut2 (open circle). Error bars correspond to the standard deviation of two replicates

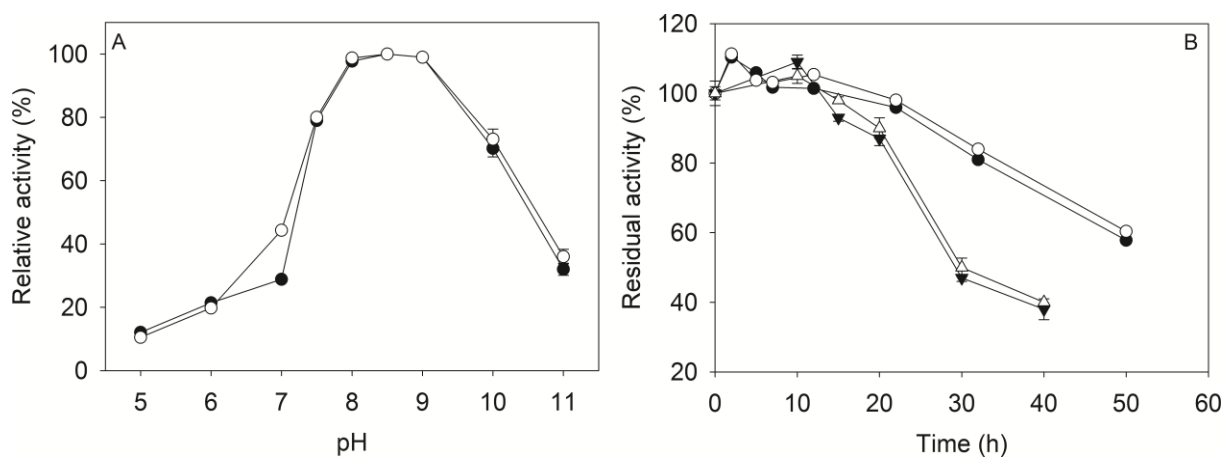


Fig. 4.36 (A) Stability of Cut1 (filled circle) and Cut2 (open circle) immobilized on chitosan beads in different pH. (B) Thermostability of Cut1 (filled circle) and Cut2 (open circle) immobilized on chitosan beads, at 55°C in comparison to free Cut1 (filled down-pointing triangle) and Cut2 (open up-pointing triangle). Error bars correspond to the standard deviation of two replicates

4.11.1.3.3 Freeze-drying and its effect

Most of the catalytic applications like esterification and transesterification using cutinase are carried out in different organic media such as isooctane, hexane, and other organic solvents where the high water contents are not the desirable criteria (Carvalho *et al.*, 1999). Thus, the method of choice in the case of free enzyme is lyophilization whereas in case of immobilized enzymes, they are freeze dried or desiccated to remove water contents.

The ability of the chitosan immobilized cutinase to retain its activity after freeze drying was investigated. As shown in Fig. 4.37 A, both the immobilized Cut1 and Cut2 showed good stability after freeze drying, with no loss of activity even after 3 freeze cycles. However, the chitosan beads become brittle and powdery after 3 freeze drying cycles. After first freeze drying cycle, there was slight increase in the relative activity of both Cut1 and Cut2. The possible reason would be that during coupling by glutaraldehyde, the protein molecules (enzyme) not only couples to chitosan but some protein molecules couple among themselves and some protein molecules might be coupled in wrong orientation which might not favor the

active site for proper hydrolysis. As the number of freeze drying increases, the chitosan beads become powdery; this might be releasing some of the enzyme molecules to free solution, which include wrongly oriented and self-coupled, thus favoring the proper hydrolysis to enhance the activity after 1st freeze dry cycle. In summary, cutinase immobilized with chitosan beads showed good resistance with freeze drying which could be a promising application in esterification and transesterification reactions.

4.11.1.3.4 Storage stability, reusability and enzyme leaching study for immobilized cutinase

Reusability and storage stability are the most important criteria in enzyme immobilization for economic and cost effective application of enzyme in various industrial processes.

Both the immobilized Cut1 and Cut2 showed good reusability and storage stability at 4°C (Fig. 4.37 B). More than 90% of the activity was retained even after reusing for 9 times. Both the immobilized cutinase showed more than 50% activity even after 13 days storage at 4°C. However, after 11 uses, there was a gradual decrease in activity which could be explained by inactivation by denaturation and stripping of the enzyme upon repeated use. The possible enzyme leaching during storage was studied by pNPB assay using immobilized enzyme storage buffer as a source of enzyme. There was no noticeable activity found even after 15 days in immobilized enzyme storage buffer, which confirms that the loss in activity after repeated reuse (after 11 cycles) is due to either enzyme deactivation or leakage of enzyme from support upon use.

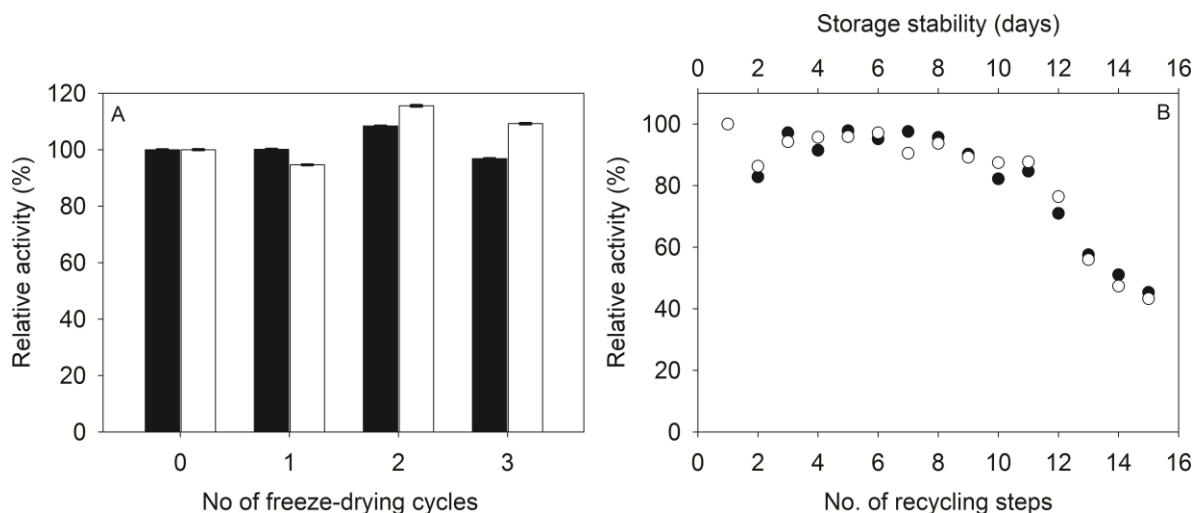


Fig. 4.37 (A) Effect of freeze drying on activity of chitosan immobilized Cut1 (black bar) and Cut2 (white bars). (B) Reusability and storage stability of chitosan immobilized Cut1 (filled circle) and Cut2 (open circle). Error bars correspond to the standard deviation of two replicates

4.11.2 Covalent immobilization on magnetic nanoparticles (MNPs)

In the past decade, MNPs have been considered as one of the important immobilization support for various catalytic applications. Simple magnetic nanoparticles are non-porous nanomaterials, which could have a disadvantage of being damaged due to erosion caused by unwanted interactions with reacting agents (Lu *et al.*, 2007; Tran *et al.*, 2012). One way of tackling such problems is to develop a layer of resistant materials such as silica, chitosan, PVA (Chastellain *et al.*, 2004; Hua *et al.*, 2009; Tran *et al.*, 2012) around the MNPs to create a hybrid MNPs which can protect them from being eroded due to environmental reactions.

4.11.2.1 Preparation of MNPs, activation with glutaraldehyde and immobilization of cutinase

The magnetic nanoparticles were prepared and coated with silica layer according to the method described in section 3.14.2.1. The silica coated MNPs were activated by treating with 10% glutaraldehyde (section 3.14.2.2) to couple the cutinases.

The effect of pH on cutinase coupling with glutaraldehyde activated MNP was investigated in the range of 6 to 9. As shown in Table 4.14, the best suited pH for ideal cutinase immobilization was found to be pH 7 with 58 and 56% immobilization for Cut1 and Cut2, respectively. In contrary to the chitosan immobilization, the acidic pH was found to be more favoring on cutinase coupling with MNP. Increase in the pH above 7 substantially decreased the coupling yield.

Table 4.14 Immobilization pH optimization for Cut1 and Cut2 on glutaraldehyde activated MNPs

	pH	Immobilization (%)	
		Cut1	Cut2
Variation of coupling pH	6	47 ± 1.2	41 ± 1.3
	6.5	51 ± 2	52 ± 1.7
	7	58 ± 2.1	56 ± 1.5
	7.5	52 ± 1	51 ± 1.2
	8	37 ± 1.2	43 ± 1.7
	8.5	33 ± 1	39 ± 2.2
	9	26 ± 1.5	27 ± 1.2

± correspond to the standard deviation of two determinations

The magnetic properties of the MNPs with and without cutinase immobilization were investigated by VSM. Fig. 4.38 shows the magnetization curves of MNPs with and without cutinase immobilization at room temperature. The specific saturation magnetization (σ_s) of MNP with cutinase immobilization was found to be $0.159 \text{ emu mg}^{-1}$, is comparable with the σ_s of the MNP without enzyme immobilization ($0.162 \text{ emu mg}^{-1}$). The result shows that the surface modification with silica and subsequent activation with glutaraldehyde and enzyme immobilization has little impact on the magnetic properties of the MNPs.

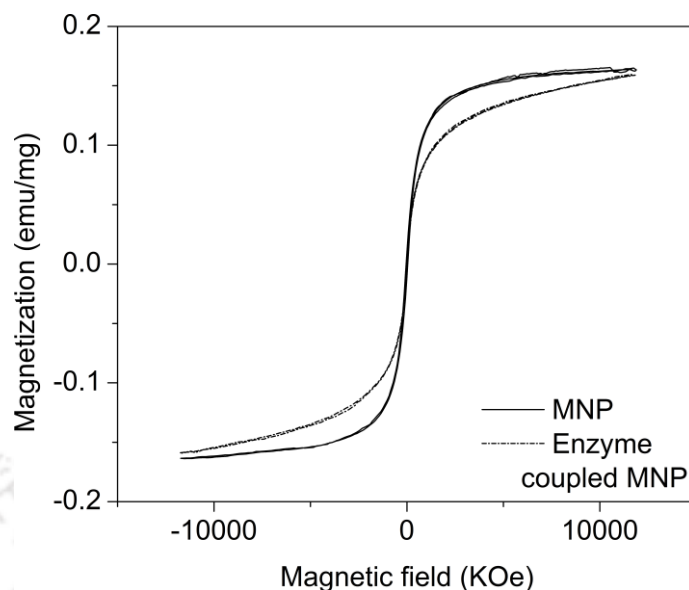


Fig. 4.38 The magnetic characteristic of MNP with and without immobilized enzyme as analysed by VSM

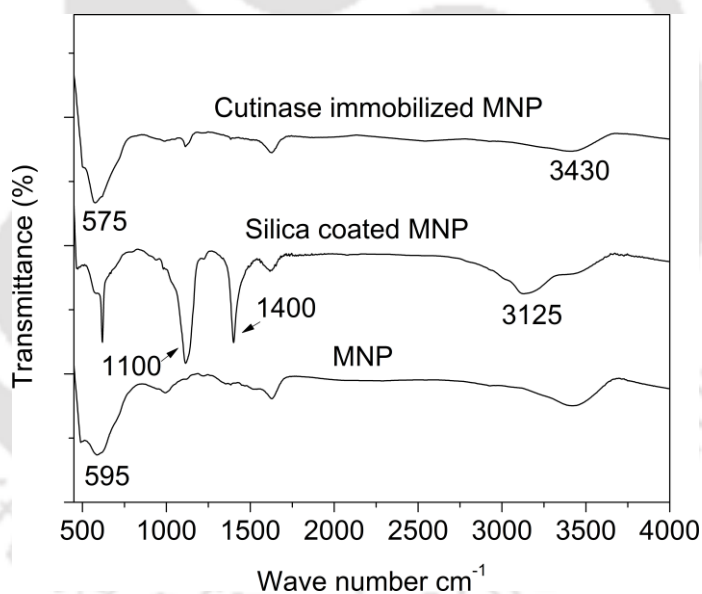


Fig 4.39 Comparison of FTIR spectra of MNP at different stages of immobilization

FTIR analysis of the MNP before and after silica coating and immobilization exhibited a characteristic FTIR peak in the region 575-595 cm^{-1} corresponding to the Fe-O vibration (Sen and Bruce, 2009) of magnetite (Fig. 4.39). Additional peaks at 1100 and 1400 cm^{-1} was observed after silica coating, which is a characteristic of Si-O vibration and stretching and

bending vibrations due to the presence of Si-O-Si bonding, respectively (Tran *et al.*, 2012). A peak in the range of 3125-3430 cm^{-1} is characteristic of C-H stretching vibrations (Socrates, 2001) and the variation in the peak at 3430 and 3125 cm^{-1} before and after immobilization would be due to the binding of cutinase.

4.11.2.2 Biochemical characterization of cutinases immobilized on MNPs

Various biochemical properties of the cutinases immobilized on MNPs were carried out as mentioned in the section 3.14.5

4.11.2.2.1 The pH and temperature optima

It was observed that free cutinase has broader pH optima as compare to immobilized cutinase (Fig. 4.40 A). There was a considerable decrease in the activity of immobilized cutinases throughout the range of pH studied in comparison to free cutinases. Though the relative activity in the alkaline pH was similar in comparison to free cutinases, the activity in the acidic pH was drastically decreased after immobilization for both Cut1 and Cut2. However, MNP immobilized Cut2 showed higher relative residual activity at acidic pH in comparison to MNP immobilized Cut1.

It was surprising to observe that the optimum functional temperature was dropped after immobilization for both the cutinases (Fig. 4.40 B). The temperature optima of the immobilized Cut1 was dropped to 45°C, whereas, immobilized Cut2 showed a drop in the temperature by 5°C in comparison to free cutinases which showed optimum activity at 55°C.

One possible reason for the reduction in activity for immobilized cutinase would be due to the inhibitory effect of MNP on the cutinases. As the Fe^{++} is inhibitory for the cutinases (Section

4.8.9 and Table 4.10) a trace amount of the unreacted Fe^{++} particles during the preparation of MNP would be interacting with the cutinases to hinder the activity after immobilization.

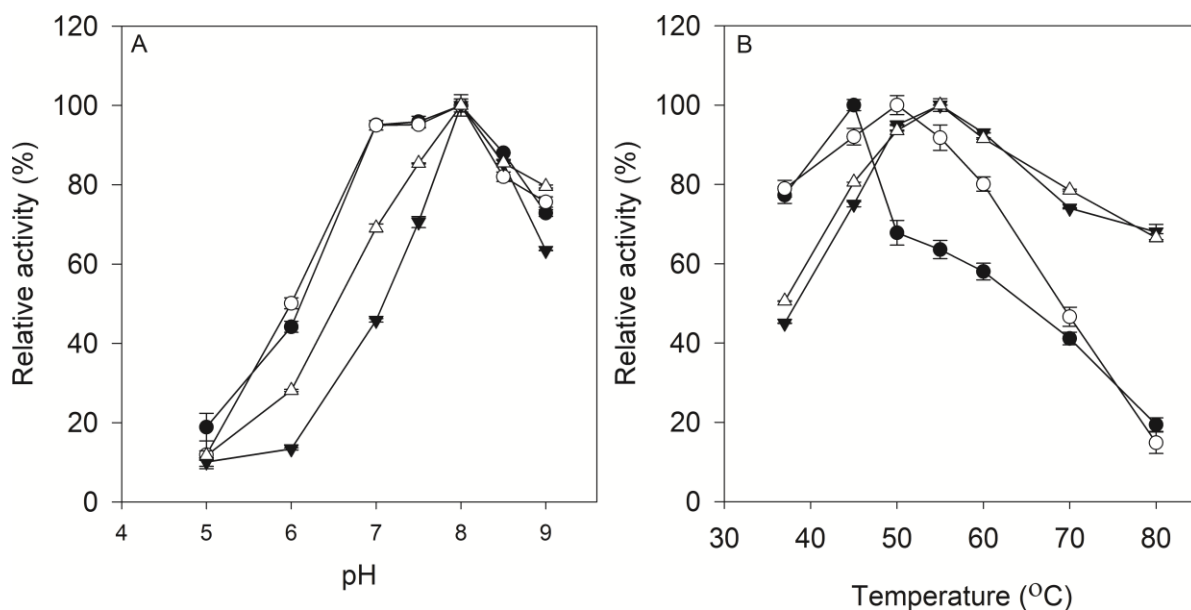


Fig. 4.40 (A) Effect of pH on activity of Cut1 (filled down-pointing triangle) and Cut2 (open up-pointing triangle) immobilized on MNP, in comparison to free Cut1 (filled circle) and Cut2 (open circle). (B) Effect of temperature on activity of Cut1 (filled circle) and Cut2 (open circle) immobilized on chitosan beads, in comparison to free Cut1 (filled down-pointing triangle) and Cut2 (open up-pointing triangle). Error bars correspond to the standard deviation of two replicates

4.11.2.2.2 The thermal stability

Thermal stability of the immobilized cutinase at 55°C showed a rapid deactivation, with loss of more than 50% activity within 6 h of incubation. There was a constant drop in the residual activity with time (Fig. 4.41), with almost 80% loss in the activity after 25 h, whereas the free cutinases were considerably much more stable even at 40 h incubation with retention of more than 40% residual activity.

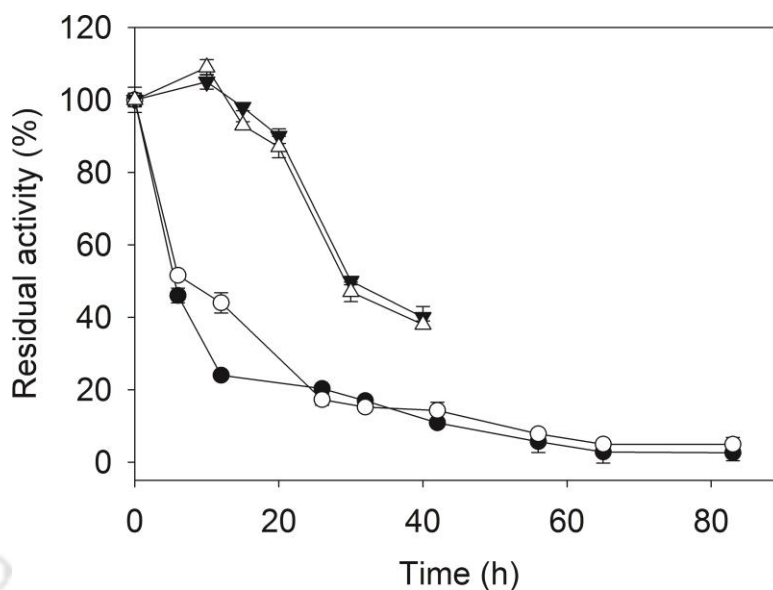


Fig. 4.41 Thermostability of Cut1 (filled circle) and Cut2 (open circle) immobilized on MNP, at 55°C in comparison to free Cut1 (filled down-pointing triangle) and Cut2 (open up-pointing triangle). Error bars correspond to the standard deviation of two replicates

4.11.2.2.3 Freeze-drying and its effect, Storage stability and reusability of immobilized cutinase

As shown in Fig. 4.42 A, both the immobilized Cut1 and Cut2 showed good stability after first freeze drying cycle with negligible loss of activity. However, the subsequent freeze drying showed a drastic decrease of more than 50% activity for both Cut1 and Cut2 indicating both the cutinases are highly susceptible to loss of activity upon freeze drying. Although both the cutinases retained more than 50% activity after 3 days storage at 4°C (Fig. 4.42 C), a trend of steep decrease in activity was observed for reusability of the immobilized Cut1 and Cut2 (Fig. 4.42 B) similar to freeze drying with more than 50% of the activity loss just after reusing for 4 times.

In summary, the biochemical investigation of the cutinases immobilized on MNP indicated that they are remarkably affected upon immobilization and MNP might not be an appropriate

immobilization support for *T. fusca* cutinase. There are no reports available on immobilization of cutinases on magnetic nanoparticles.

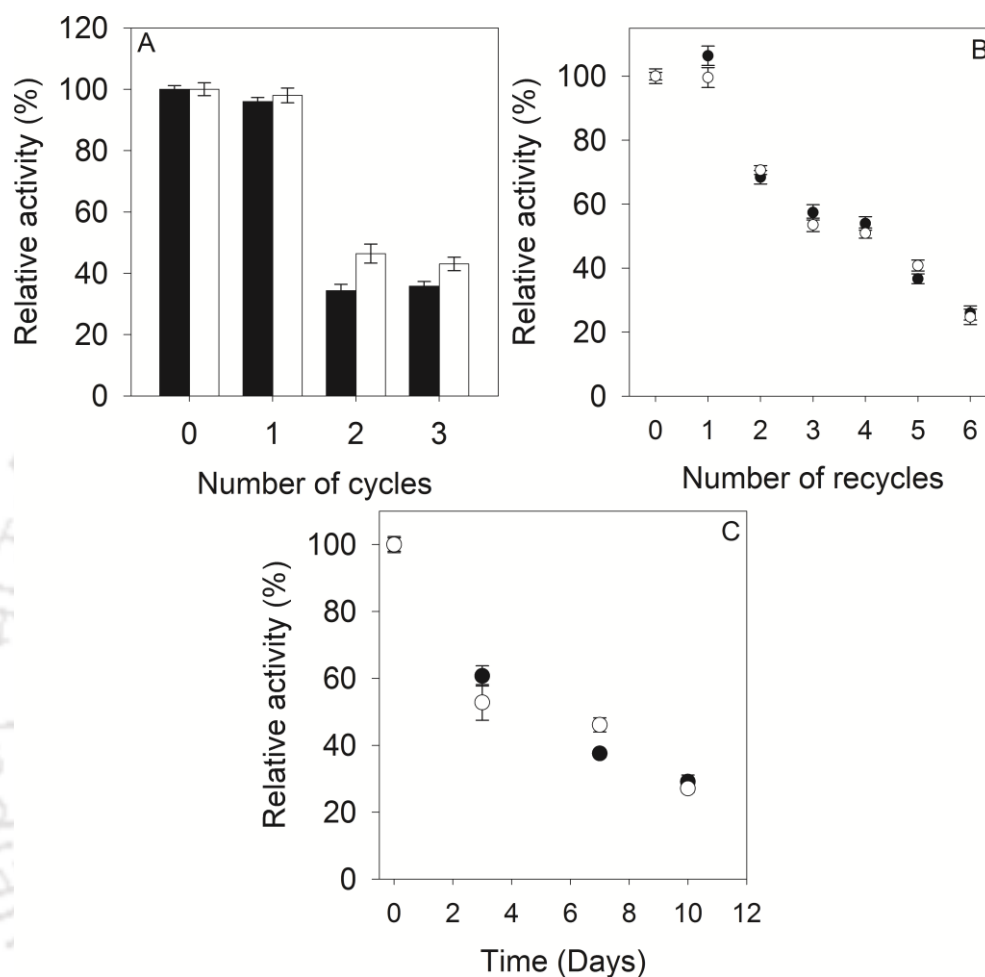


Fig. 4.42 (A) Effect of freeze drying on activity of MNP immobilized Cut1 (black bar) and Cut2 (white bars). (B) Reusability and (C) storage stability of MNP immobilized Cut1 (filled circle) and Cut2 (open circle). Error bars correspond to the standard deviation of two replicates

4.11.3 Adsorption on zeolite (NaY) and Celite-545

Various adsorption methods of immobilization had been studied with *F. solani* cutinase to enhance the activity and used to catalyze the alcoholysis reaction (Serralha *et al.*, 2004). Immobilization of *F. solani* cutinase on zeolite showed measurable enhancement in activity

and stability with increase in specific activity and higher tolerance to organic solvents (Goncalves *et al.*, 1996b, Goncalves *et al.*, 1995).

In the present study, the cutinase adsorption on Zeolite and Celite-545 was achieved according to the method described in section 3.14.3. Calculation of immobilization percentage (Eq. 3.11) showed that 81 and 85% of the Cut1 and Cut2, respectively was immobilized on the Zeolite, whereas Celite-545 showed highest immobilization among two adsorption supports studied, with 87 and 90% immobilization for Cut1 and Cut2, respectively.

4.11.3.1 Biochemical characterization of cutinases adsorbed on Zeolite and Celite-545

4.11.3.1.1 The pH and temperature optima

In order to determine the optimal levels of pH and temperature for the immobilized cutinases, experiments were performed according to the method described in the section 3.14.5.1.

It was observed that free cutinase has broader pH optima as compare to immobilized cutinase (Fig. 4.43 A and B). Free as well as immobilized cutinase showed highest activity at pH 8 and there was no change in the relative activity profile at alkaline pH (above pH 8) on immobilization with Zeolite or Celite-545 in comparison to free cutinase. However, immobilized Cut1 and Cut2 on both Zeolite and Celite-545 showed considerably lesser activity below pH 8 in comparison to free cutinases. In case of Cut1, the relative activity was decreased to 24% at pH 6 when immobilized on Zeolite whereas immobilization on Celite-545 retained its activity by 43%, which is almost equivalent to the relative activity of free cutinase (Fig 4.43 A). Nevertheless, Cut2 showed opposite behavior (Fig 4.43 B), with drop in the activity to 28% at pH 6 when immobilized on Celite-545 whereas immobilization on Zeolite showed retention of 46% relative activity. One possible reason for the differential

behavior of Cut1 and Cut2 immobilized on Zeolite or Celite-545 and reduction in the activity at acidic pH could be due to the difference in the hydrophobic surface area and surface electrostatic properties (Section 4.10.1).

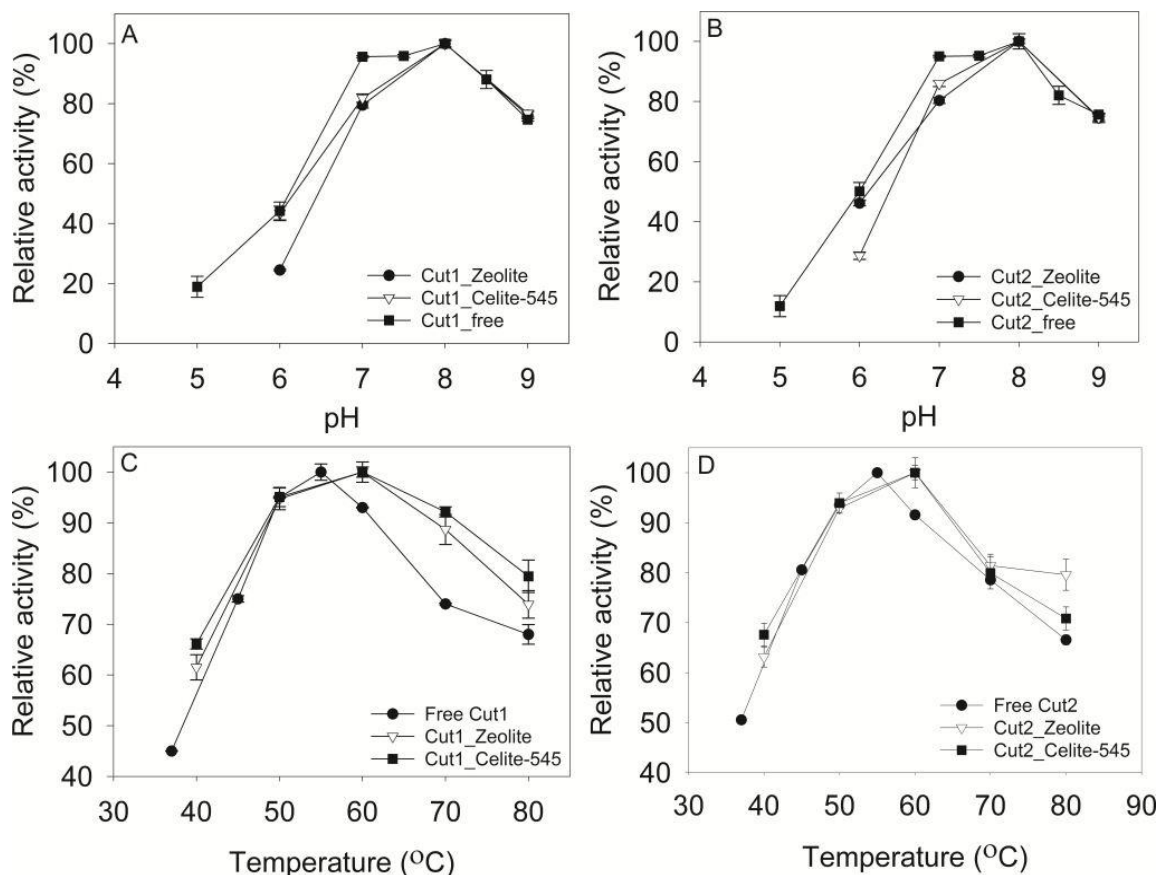


Fig. 4.43 (A) and (B) Effect of pH on activity of Cut1 (A) and Cut2 (B) immobilized on Zeolite and Celite-545 in comparison to free enzymes. (C) and (D) Effect of temperature on activity of Cut1 (C) and Cut2 (D) immobilized on Zeolite and Celite-545 in comparison to free enzymes. *Error bars* correspond to the standard deviation of two replicates

Immobilization on Zeolite and Celite-545 showed elevation in the temperature optima for both the cutinases in comparison to free cutinases (Fig 4.43 C and D). The temperature optima of the Zeolite or Celite-545 immobilized Cut1 and Cut2 were found to be 60°C, which is 5°C higher than the optimum temperature of the free cutinases. The operational stability of the immobilized Cut1 and Cut2 at temperature above 50°C was remarkably improved after

immobilization on Zeolite or Celite-545 in comparison to free cutinases, with no decrease in the activity at lower temperatures. Cut1 showed better operational stability at temperature above 60°C, with highest operational stability after immobilization on Celite-545. In contrast, Cut2 showed slightly better operational stability with immobilization on Zeolite.

4.11.3.1.2 The thermal stability

To determine the thermostability of immobilized cutinases, experiments were performed according to the method described in the section 3.14.5.2. Studies on the thermal stability of the immobilized cutinases showed a significant improvement in stability after immobilization on Zeolite and Celite-545 in comparison to free cutinases (Fig. 4.44 A and B). Free Cut1 and Cut2 showed a steep decrease in the residual activity after 20 h at 55°C, with almost loss of 60% activity at 40 h. However, Cut1 and Cut2 immobilized on Zeolite retained more than 53 and 51% activity, respectively even after 57 h and immobilization on Celite-545 showed highest thermostability with retention of 55 and 63% residual activity for Cut1 and Cut2, respectively after 57 h.

4.11.3.1.3 Freeze-drying and its effect

Experiments were performed to determine the performance of the immobilized cutinases after freeze drying according to the method described in the section 3.14.5.3. As shown in Fig. 4.45 A and B, Cut1 and Cut2 immobilized on Zeolite showed 23 and 26% loss in the activity, respectively after first freeze drying cycle, whereas the immobilization on Celite-545 showed 43 and 41% decrease in the activity, respectively for Cut1 and Cut2. Subsequent freeze drying cycles showed more than 50% loss in the activity for both the cutinases immobilized on Zeolite and Celite-545, indicating that the immobilized cutinases are not suitable for repeated

freeze drying. A possible reason would be the dissociation of the adsorbed cutinases upon freeze drying, which is decrease the relative activity of the immobilized enzyme.

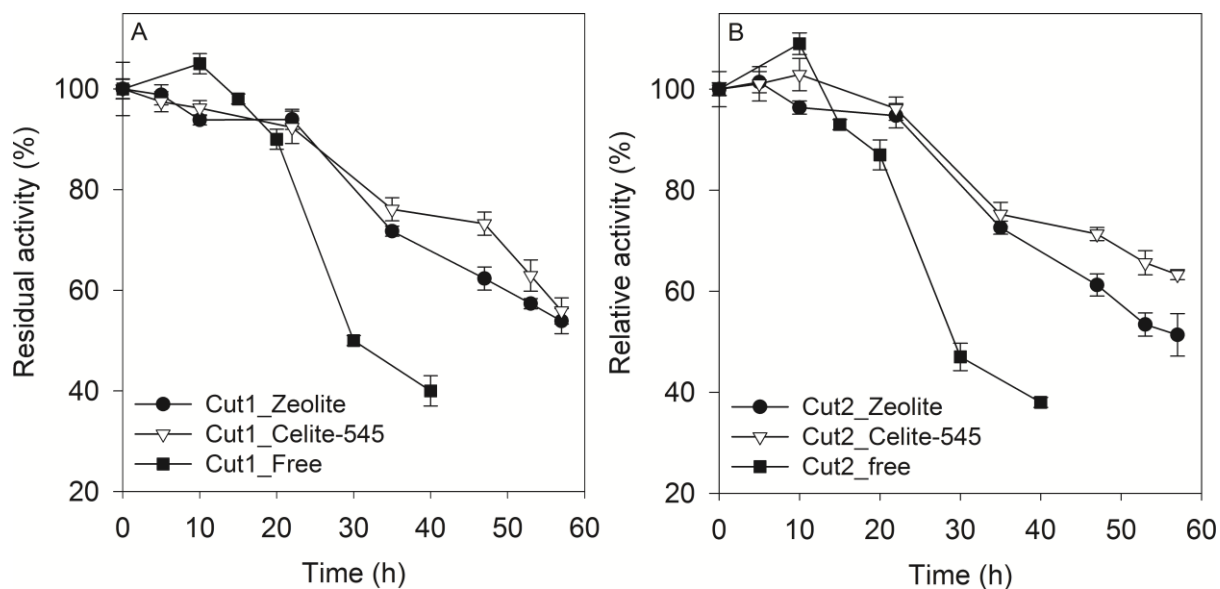


Fig. 4.44 Thermostability of Cut1 (A) and Cut2 (B) immobilized on Zeolite and Celite-545, at 55°C in comparison to free cutinase. *Error bars* correspond to the standard deviation of two replicates

4.11.3.1.4 Storage stability and reusability of the immobilized cutinases

Experiments were performed to evaluate the storage stability and reusability of the immobilized cutinases as per the method described in the section 3.14.5.4 and 3.14.5.5, respectively. Stability of the immobilized cutinases in 50 mM KPO₄ buffer (pH 8) at 4°C was analyzed by pNPB assay. Both the cutinases immobilized on Zeolite and Celite-545 showed good stability at 4°C, with loss in less than 25% activity after 7 days. However, there was more than 50% activity loss after 20 days storage for both the cutinases immobilized on Zeolite as well as Celite-545 due to the enzyme leaching. A trend of steep decrease in activity was observed for reusability of the Cut1 and Cut2 immobilized on Celite-545. However, both the cutinases showed relatively better retention of activity after immobilization on Zeolite (Fig. 4.45 C and D).

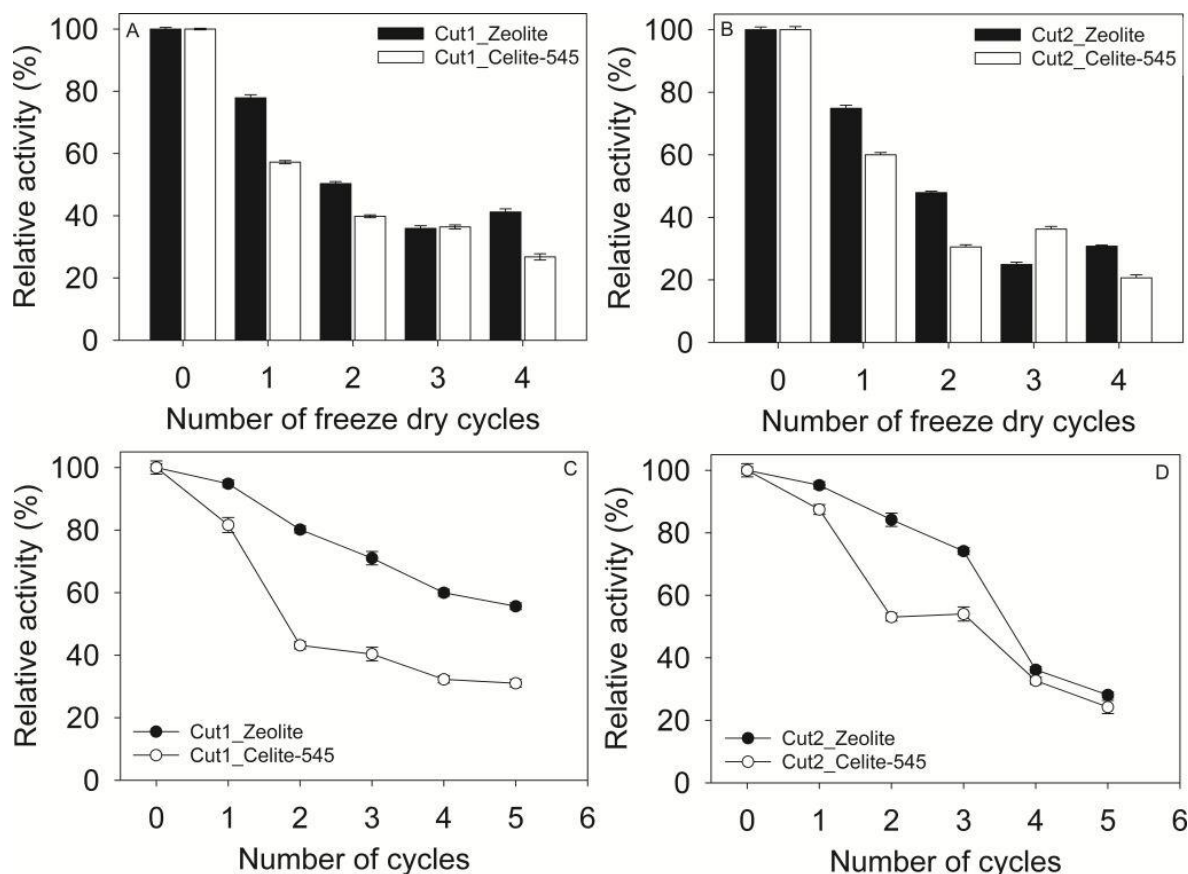


Fig. 4.45 Effect of freeze drying on activity of Cut1 (A) and Cut2 (B) immobilized on Zeolite and Celite-545. Reusability of Cut1 (C) and Cut2 (D) immobilized on Zeolite and Celite-545. Error bars correspond to the standard deviation of two replicates

4.12 Studies on applications of *T. fusca* cutinases in various esterification and transesterification reactions

Short chain alkyl esters are popularly being used in various industries including food, dairy, perfume, cosmetics and pharmaceuticals as an ingredient. Cutinase is known to have potential to catalyze the esterification and transesterification reactions for synthesis of short-chain alkyl esters. In this study, the ability of cutinase to catalyze esterification and transesterification reactions were evaluated.

4.12.1 Synthesis of isopropyl esters

Possible negative impacts of petroleum based solvents on the environment are a serious concern, which demands an environmentally acceptable alternative source. Biosolvents are one of such alternate that have an attractive industrial applications. Fatty acids alkyl esters like isopropyl oleate show an increasingly growing demand in various applications such as, cosmetic industries, food industries, pharmaceuticals (Eigtved *et al.*, 1988; Rattray, 1984), as a bio-solvents (Bouaid *et al.*, 2007) and as bio-lubricants for high precision machinery (Whitby, 2004). Though traditional synthetic methods with chemical catalysts such as strong mineral acids have been used for synthesis of isopropyl esters, the quality of the product was reported to be low due to undesirable side reactions (Lanzani *et al.*, 1989), which demands biocatalytic reactions as an alternative.

In the present investigation, the performance of *T. fusca* cutinase (free and immobilized) has been evaluated as a catalyst for the synthesis of isopropyl oleate from oleic acid and isopropanol (Fig. 4.46). The experiments were performed according the methods described in the section 3.15.

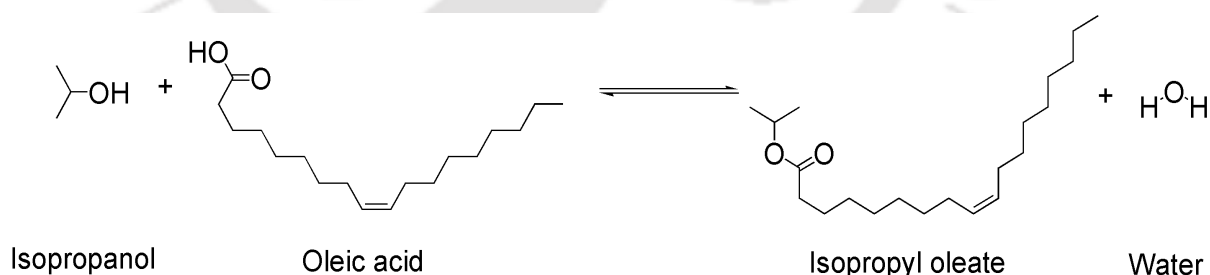


Fig. 4.46 Schematic representation of the esterification reaction of isopropyl oleate

4.12.1.1 Effect of initial alcohol to acid molar ratio on isopropyl oleate ester synthesis

The rate-determining step for an enzyme-catalyzed hydrolysis reaction in an aqueous emulsion system is acylation. But in the presence of excess water, the rate of deacylation will

be faster than the rate of acylation. So, the relative activity of the enzyme against different substrates will be dependent on the interaction between the substrate and the active site of the native enzyme (Lai and Connor, 1999). Whereas, in case of enzyme-catalyzed esterification reaction, which is reversible, where both substrates and water released as a by-product are in a single phase having equal opportunity to reach the enzyme active site. So, it is important to study the optimal molar ratio of the substrates to keep the reaction equilibrium towards the direction of ester formation.

The cutinase catalyzed esterification of isopropyl oleate was carried out at 50°C for 45 h using Cut1 and Cut2 with isopropanol/oleic acid molar ratio of 1:1, 3:1 and 5:1 using 0.5% (w/w) initial water content as described in the section 3.15. As shown in Fig. 4.47, both the cutinases showed different optimal molar ratio to achieve highest isopropyl oleate yield. Cut1 showed a maximum conversion of 29.7% at 1:1 molar ratio, whereas the highest conversion of 45.3% was achieved at 3:1 molar ratio for Cut2. In case of Cut1, further increase in the molar ratio from 1:1 resulted in considerable decrease in the conversion yield, whereas Cut2 showed 1.2 fold increase in the conversion yield when the molar ratio was increased from 1:1 to 1:3. Nevertheless, further increase in the molar ratio to 1:5 decreased the ester yield by 3.8% (Fig. 4.47). A similar effect of decrease in the conversion yield with increase in alcohol to oil ratio was also reported for *C. antarctica* lipase immobilized on an ion exchange resins (Bouaid *et al.*, 2007). It is evident from the Fig. 4.47 that both the cutinases reached highest conversion yield at around 25 h post incubation at 50°C and reached saturation at 35 h. Further increase in reaction time to 45 h did not increase the yield. This would probably be due to the inactivation of the cutinase. However, it was worth to note that the highest conversion yield by Cut1 was almost 15.6% lesser in comparison to catalysis by Cut2,

demonstrating Cut2 to be the better catalyst for isopropyl oleate synthesis. Moreover, the increase in the isopropanol ratio also decreased the yield to a considerable extent for Cut1, indicating that isopropanol is inhibitory to Cut1. Cut2 showed considerably better stability in isopropanol with tolerance up to 3:1 isopropanol/oleic acid ratio. These findings are in accordance with the study on effect of solvent on stability of Cut1 and Cut2 (Section 4.8.7), where the isopropanol (40% v/v) decreased the activity of Cut1 by 75% but only 33% for Cut2 (Table 4.8). Hence, all the further studies on esterification of isopropyl oleate were carried out only with Cut2 as a catalyst.

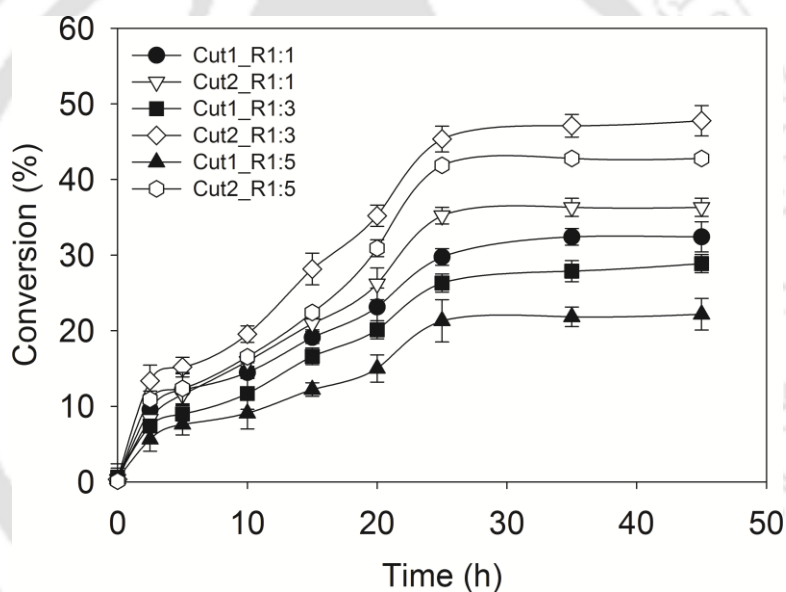


Fig. 4.47 Effect of oleic acid/isopropanol molar ratio (R) on the conversion of isopropyl oleate during esterification of isopropanol with oleic acid catalyzed by cutinase. Error bars correspond to the standard deviation of two replicates

4.12.1.2 Effect of initial water content on isopropyl oleate ester synthesis

Water plays a crucial role in esterification reactions carried out in non-conventional medium using biocatalysts (in this case, Cut1 or Cut2). On the one hand, water molecules are essential to maintain the enzymes activity and to control the thermodynamic equilibrium of the enzymatic reactions. On the other hand, as the esterification reaction is a reversible process

and the water is a by-product during the esterification reaction, generally, low water content favors the synthesis over hydrolysis during esterification reaction. Previously, several reports were available on the effect of water content in esterification reactions catalyzed by lipase or cutinase (Bezbradica *et al.*, 2007; De Barros *et al.*, 2009a; Dutta and Dasu, 2011; Lai and Connor, 1999). To study the effect of initial water content on the reaction rate, 0 to 5% of water (w/w) was added to the reaction media (Fig. 4.48). The reaction was carried out according to the method described in section 3.15 with alcohol/acid molar ratio of 3:1 at 50°C.

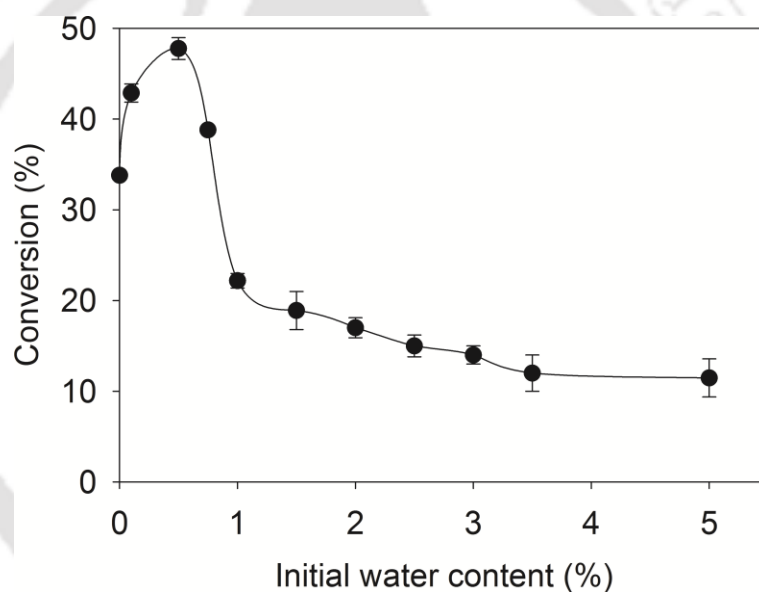


Fig. 4.48 Effect of initial water content (% w/w) on synthesis of isopropyl oleate in the reaction mixture of 2 ml of n-hexane containing 0.25 M oleic acid, 0.25 M isopropanol and 3 mg mL⁻¹ cutinase. *Error bars* correspond to the standard deviation of two replicates

The conversion yield was increased to 47.7% up to 0.5% water and then decreased slowly up to 1% water content. However, increasing the water content further, showed a sharp decrease in conversion yield (Fig. 4.48). This result agrees with the fact that a minimal quantity of water was necessary to maintain the activity of the enzyme, nevertheless, at higher water

concentrations, the reverse hydrolysis reaction was initiated and therefore decrease in net production of esters was observed.

4.12.1.3 Effect of temperature on isopropyl oleate ester synthesis

The effect of temperature on cutinase catalyzed esterification of isopropyl oleate was evaluated in the range of 30-60°C using lyophilized Cut2 as a catalyst with 0.5% initial water (w/w) content according to the method described in the section 3.15. Alcohol to acid ratio was taken as 3:1, which was the optimum ratio for highest conversion (%) by Cut2 as a catalyst (Section 4.12.1.1). The maximum molar conversion of 45.3% was observed at 50°C (Fig. 4.49), but more than 85% conversion (in comparison to optimum temperature; 50°C) was achieved in the range of 40-55°C. However, further increase or decrease in the temperature reduced the conversion yield.

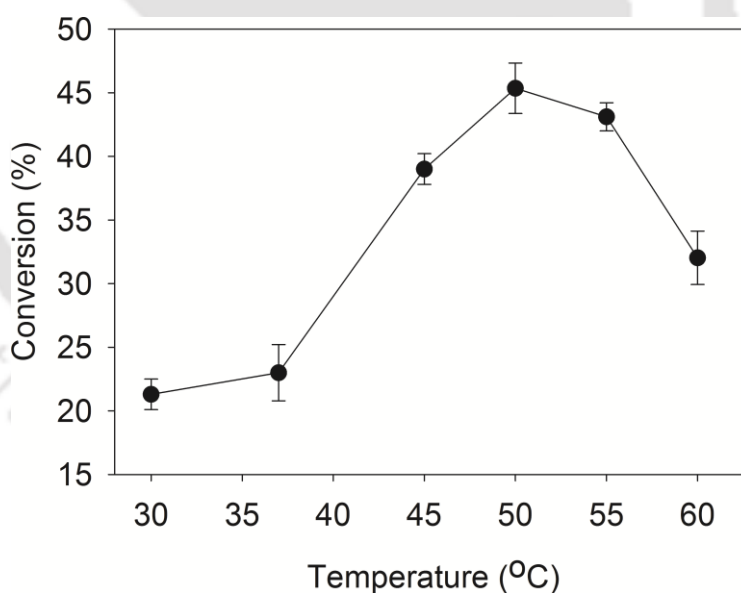


Fig. 4.49 Effect of temperature on the synthesis of isopropyl oleate esters in the reaction mixture of 2 mL of n-hexane containing 0.25 M oleic acid, 0.25 M isopropanol and 3 mg mL⁻¹ cutinase. *Error bars* correspond to the standard deviation of two replicates

4.12.1.4 Catalytic performance of immobilized cutinase on isopropyl oleate ester synthesis

Immobilized enzymes show many advantages over free catalyst in esterification and transesterification reactions, such as, high selectivity, no significant side reactions, and yields products of high purity. In addition, they are easily recoverable and reusable which reduces the production cost. Moreover, there are reports on improved catalysis by immobilization of cutinase from *F. solani* (Goncalves *et al.*, 1995; Goncalves *et al.*, 1996b; Serralha *et al.*, 2004).

In the present study, Cut2 immobilized on chitosan (Section 4.11.1), Zeolite (Section 4.11.3) and Celite-545 (Section 4.11.3) was evaluated as a catalyst in synthesis of isopropyl esters. Esterification was carried out at optimized parameters (3:1 molar ratio of isopropanol/oleic acid, 0.5% initial water content and 50°C reaction temperature) as described in the section 3.15. As depicted in the Fig. 4.50, there was a remarkable 16 ± 2.7 and $10 \pm 2.5\%$ increase in the conversion rate with immobilization on Celite-545 and Zeolite, respectively in comparison to free Cut2. Although the conversion rate was same as free enzyme till 5 h of reaction, there was a considerable elevation in the catalysis from 10 to 45 h, for Celite-545 and Zeolite immobilized cutinase (Fig. 4.50). Surprisingly, Cut2 immobilized on chitosan showed decrease in the ester yield compare to free Cut2. In addition, it was also observed that the morphology of the chitosan beads were shrunken after incubation for 10 h, which would probably due to the intolerance for organic reactants used in the reaction. This also would be the reason for lower catalysis by cutinase immobilized on chitosan.

To summarize the isopropyl ester synthesis using *T. fusca* cutinase as a biocatalyst, Cut1 was found to be an inappropriate catalyst for isopropyl oleate synthesis due to enzyme inhibition by isopropanol; however, Cut2 was comparatively more stable in isopropanol. Immobilization

on Celite-545 and Zeolite was found to be advantageous for achieving higher ester conversion yield and chitosan was found to be an inappropriate support for esterification reactions catalyzed by *T. fusca* cutinase.

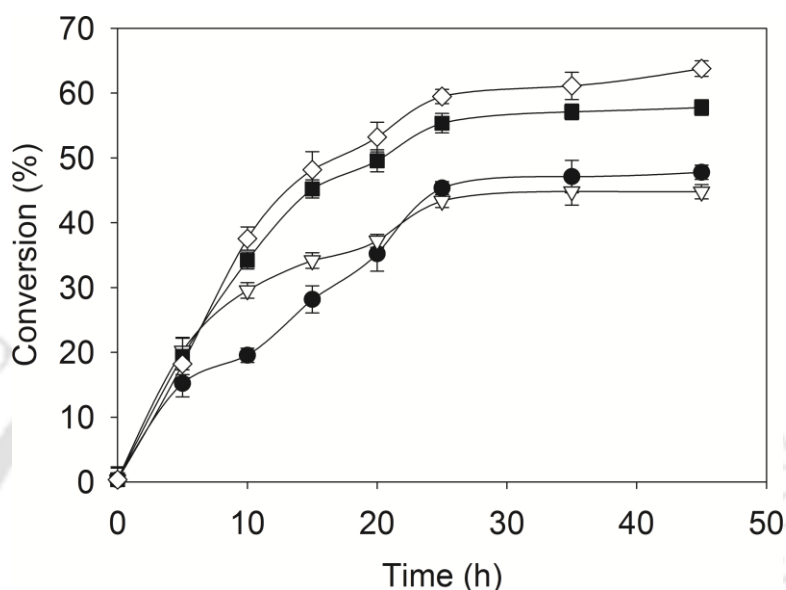


Fig. 4.50 Comparative study of the synthesis of isopropyl oleate esters by free Cut2 (Circle) and Cut2 immobilized on Chitosan (inverted triangle), Zeolite (square) and Celite-545 (diamond). Error bars correspond to the standard deviation of two replicates

4.12.2 Synthesis of geraniol esters

Terpenoid alcohol esters are organic compounds that have a great demand in the flavor and fragrance compounds in a variety of foods and beverages for creating fruity aromas, cosmetics, perfumes and pharmaceutical industries (Athawale *et al.*, 2002; Chaabouni *et al.*, 1996). Conventionally, these esters are obtained by chemical synthesis or extraction from natural sources. However, due to a number of shortcomings in the above said methods such as undesirable side products and high cost of production the biocatalytic synthesis as an alternative has gained the popularity. Geraniol is an important member of the terpenoid class, which is widely used in the form of esters in several industries such as perfume, aroma and cosmetic industries. For instance, geraniol acetate has an aroma of rose and used in perfume

and food industry. In the past decade, there are several works carried out to synthesize terpenoid alcohol esters, especially geraniol esters by direct esterification in organic medium catalyzed by lipases (Athawale *et al.*, 2002; Chaabouni *et al.*, 1996; Claon and Akoh, 1993, 1994; Langrand *et al.*, 1988, 1990; Nishio *et al.*, 1987; Oguntimein *et al.*, 1995). However, there are no reports available on geraniol ester synthesis catalyzed by cutinases.

In the present investigation, *T. fusca* cutinase (free and immobilized) has been evaluated as a catalyst for the synthesis of geraniol esters of various fatty acyl chain lengths (Fig. 4.51). The experiments were performed according to the methods described in the section 3.15.

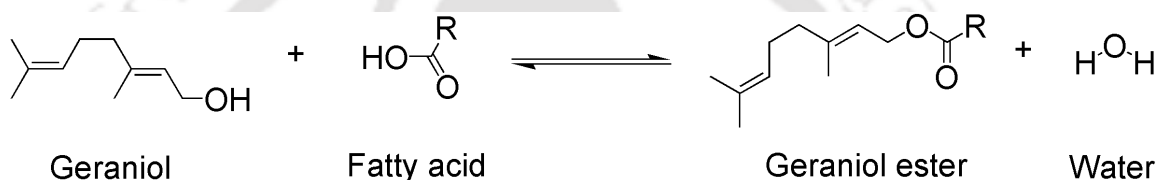


Fig. 4.51 Schematic representation of the esterification reaction of geraniol fatty acid ester (R stands for acetic acid, butyric acid, valeric acid and decanoic acid used in the present study)

4.12.2.1 Effect of initial water content on geraniol ester synthesis

Effect of initial water content on the cutinase catalyzed esterification of geraniol was evaluated using acetic acid as a model acyl donor (geraniol acetate synthesis). In the experiment (Section 3.15), 0 to 2% of water (w/w) was added to the reaction media (Fig. 4.52 A and B) and esterification yield was measured. The conversion was increased to 65% and 59.8% up to 0.5% water for Cut1 and Cut2, respectively at 15 h and then decreased sharply (Fig. 4.52 A). Cut1 showed slightly better catalytic yield at 0.1% as well as 0.5% initial water content with highest conversion yield of 78.8% and 69.8% for Cut1 and Cut2, respectively at 35 h. Further increase in the time (up to 45 h) showed almost saturation in the conversion (%) yield (Fig. 4.52 B). This result is in agreement with the experiments conducted with isopropyl

ester synthesis, where the presence of initial water content above 0.5% decreased the yield drastically (Section 4.12.1.2). This is also comparable with the result found by Chaabouni *et al.*, (1996) who reported that presence of 1% of water in the reaction mixture, leads to conversion yield of less than 50%. Sudhir *et al.*, (1995) who studied the effect of different amounts of water added to the reaction mixture (non-aqueous system) on the esterification of geraniol of palmarosa oil with n-butyric acid using immobilized lipase from *Mucor miehei* also observed a sharp decrease in the reaction yield with increase in the water content. This is probably due to the low solubility of geraniol in the water which reduces the contact of the geraniol with the enzyme and the possible reverse hydrolytic reaction as explained earlier (Section 4.12.1.2).

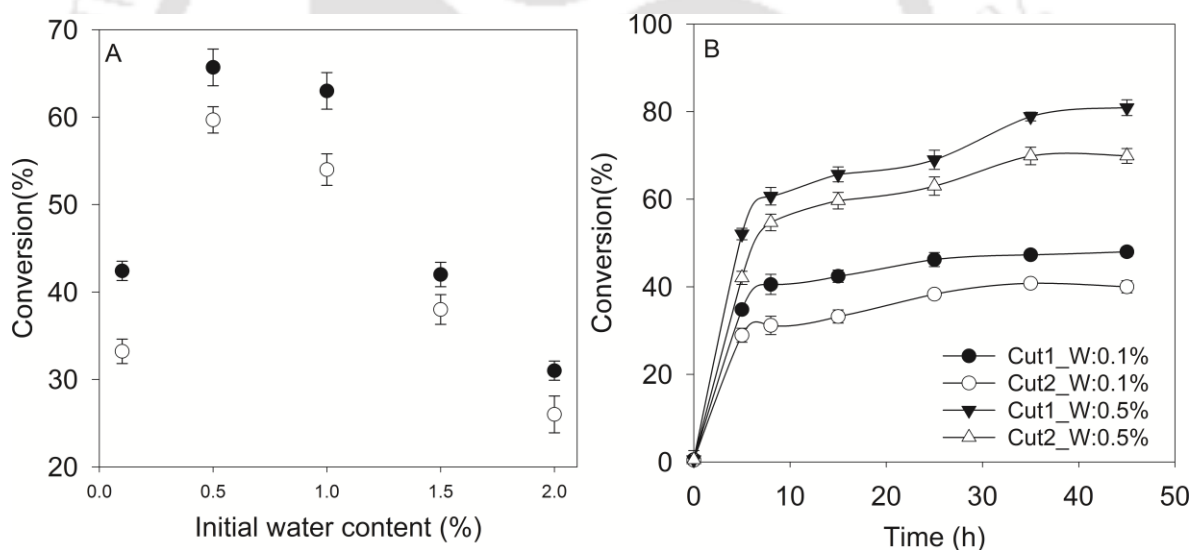


Fig. 4.52 (A) Effect of initial water content (% w/w) on synthesis of geraniol acetate catalyzed by Cut1 (filled circle) and Cut2 (empty circle). (B) Catalytic efficiency of Cut1 and Cut2 for synthesis of geraniol acetate at 0.1 and 0.5 (% w/w) initial water content. *Error bars* correspond to the standard deviation of two replicates

4.12.2.2 Effect of initial acid to alcohol molar ratio on geraniol ester synthesis

Effect of initial acid to alcohol molar ratio on the cutinase catalyzed esterification of geraniol was evaluated using butyric acid as a model acyl donor (geraniol butyrate synthesis). The reaction was carried out at 50°C for 45 h using Cut1 and Cut2 as a catalyst with butyric acid/geraniol molar ratio of 1:1, 3:1 and 5:1 using 0.5% (w/w) initial water content as described in the section 3.15. As shown in Fig. 4.53, both the cutinases showed optimal molar ratio of 3:1 to achieve highest geraniol butyrate yield. Cut1 and Cut2 showed a maximum conversion of 71.1 and 66.4% at this molar ratio at 45 h, indicating Cut1 to be a slightly better catalyst compare to Cut2. Stoichiometrically, the geraniol butyrate synthesis requires one mole of butyrate for each mole of geraniol. However, since the esterification of geraniol is a reversible reaction; excess butyrate is required to shift the equilibrium towards the direction of ester formation. Thus, both Cut1 and Cut2 showed a trend of decrease in conversion yield below the acid/alcohol molar ratio 3:1. The decrease in the conversion yield at 5:1 molar ratio would be due to the deactivation of the cutinases by high concentration of butyric acid as reported for *F. solani* cutinase (Carvalho, 1998; Pinto-Sousa *et al.*, 1994). As shown in the Fig. 4.53, both the cutinases reached highest conversion yield at approximately 25 h post incubation at 50°C and reached saturation at 35 h. Further increase in reaction time to 45 h did not increase the yield. This would probably be due to the denaturing of the cutinase.

4.12.2.3 Effect of acid chain length on the synthesis of geraniol esters

The selectivity of fatty acyl chain length in esterification generally depends on the native properties of the enzymes and mostly found to be similar to that of the hydrolytic reactions. In case of lipase/esterase/cutinase catalyzed esterification and transesterification reactions, an

acyl-enzyme intermediate was formed, so, the dependency on the fatty acids chain length would mostly due to the affinity between the fatty acid and enzyme itself.

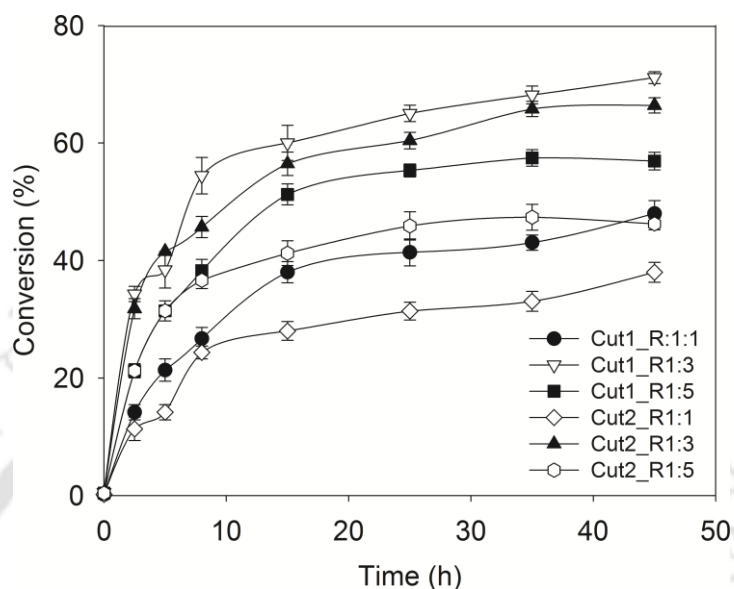


Fig. 4.53 Effect of geraniol/butyric acid molar ratio (R) on the conversion of geraniol butyrate during esterification of geraniol with butyric acid catalyzed by cutinase. Error bars correspond to the standard deviation of two replicates

As it was evident in the above study (Section 4.12.2.1 and 4.12.2.2) that both Cut1 and Cut2 of *T. fusca* showed a same optimum parameters and similar trend of catalytic mechanism in the optimization experiments. However, the catalytic efficiency of Cut1 was slightly better than Cut2. Thus, all the further experiments were performed with Cut1 as a biocatalyst.

To study the effect of acid chain length on geraniol ester synthesis, experiments were performed with fatty acids of chain length C2 to C10. As shown in Fig. 4.54, the molar conversion (%) of geraniol esters varies with the carbon chain length of the fatty acids. As described in the section 4.8.6, Cut1 showed higher hydrolytic activity in aqueous media with p-nitrophenyl butyrate and acetate than other long chain synthetic esters. In case of synthesis reaction in organic media by Cut1 as a catalyst, almost a similar trend of decrease in conversion rate with increase in fatty acid chain length was observed. The highest conversion

of 80.8% was observed for acetic acid, closely followed by butyric (68.1%) and valeric acid (57.4%) whereas conversion for decanoic acid was very low, probably due to the fact that long chain fatty acids react with the active site of cutinase and block the access of the alcohol to the intermediate acyl enzyme followed by formation of the product (De Barros *et al.*, 2009a). These results suggest that cutinase from *T. fusca* NRRL B-8184 had the highest affinity towards short chain fatty acids (C2–C6), which is in agreement with previous studies performed in organic solvent (De Barros *et al.*, 2009a) or reverse micellar systems (Cunnah *et al.*, 1996; Pinto-Sousa *et al.*, 1994; Sebastiao *et al.*, 1993) using recombinant *F. solani* cutinase and *P. cepacia* cutinase (Dutta and Dasu, 2011) for other esterification reactions.

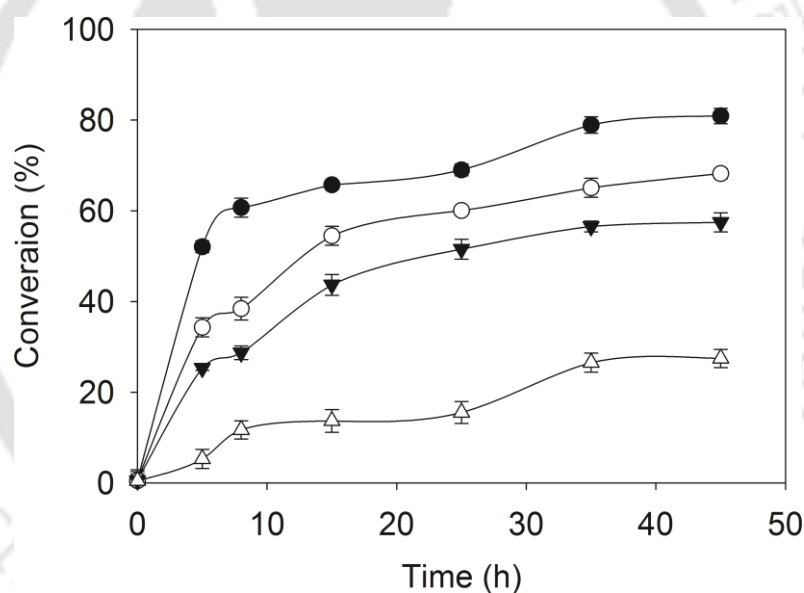


Fig. 4.54 Effect of acid chain length on the synthesis of geraniol esters at geraniol/fatty acid molar ratio, $R = 1:3$. Geraniol acetate (filled circle), geraniol butyrate (empty circle), geraniol valerate (inverted triangle) and geraniol decanoate (triangle). Error bars correspond to the standard deviation of two replicates

4.12.2.4 Catalytic performance of immobilized cutinase on geraniol ester synthesis

Catalytic efficiency of the immobilized Cut1 on synthesis of geraniol ester was evaluated using chitosan (Section 4.11.1), Zeolite (Section 4.11.3) and Celite-545 (Section 4.11.3) as an immobilization support. Esterification was carried out at optimized parameters (3:1 molar

ratio of acid/geraniol, 0.5% initial water content and 50°C reaction temperature) as described in the section 3.15.

As shown in the Fig. 4.55, there was an increase in the conversion rate with immobilization on Celite-545 and Zeolite, however, chitosan showed considerably lower yield in comparison to free cutinase (Fig. 4.55 A). Similar behavior was observed with isopropyl ester synthesis for the chitosan immobilized Cut2 (Section 4.12.1.4). Although the conversion rate was not enhanced remarkably after enzyme immobilization on Zeolite or Celite-545, there was an increase of 5, 3, 3 and 1% ester yield for geraniol acetate, butyrate, valerate and decanoate, respectively with Zeolite and 8, 9, 6 and 4% ester yield for geraniol acetate, butyrate, valerate and decanoate, respectively with Celite-545 immobilization (Fig. 4.55 B). Celite-545 was found to be a slightly better immobilization support for geraniol ester synthesis in comparison to Zeolite, which was also the case for isopropyl oleate synthesis (Section 4.12.1.4). Both Cut1 and Cut2 show different surface electrostatic properties. Since the mechanism of immobilization is based on adsorption, the protein surface electrostatic properties of the Cut1 favor the adsorption in better way on Celite-545, making it more stable compare to Cut2.

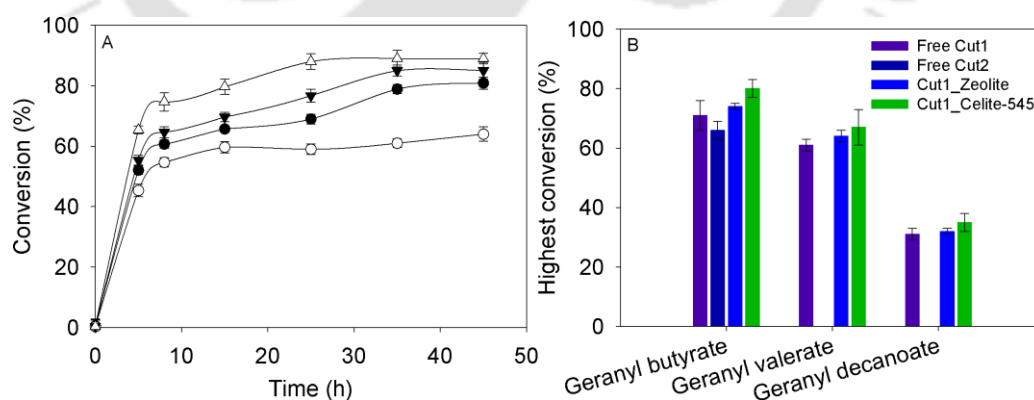


Fig. 4.55 (A) Comparative study of the synthesis of geraniol acetate ester by free Cut1 (filled circle) and Cut1 immobilized on Chitosan (empty circle), Zeolite (inverted triangle) and Celite-545 (triangle). (B) Comparative study of the synthesis of geraniol esters (highest molar conversion at 45 h) of various chain lengths by free and immobilized cutinase. *Error bars* correspond to the standard deviation of two replicates

To summarize the geraniol ester synthesis using *T. fusca* cutinase as a biocatalyst, both Cut1 and Cut2 from *T. fusca* was found to be an appropriate catalyst for geraniol ester synthesis with similar optimum catalytic parameters. However, Cut1 showed slightly better catalytic efficiency in comparison to Cut2. Increase in the acyl chain length showed decrease in the catalytic efficiency with highest molar conversion (%) for C2 to C4 acyl chains. Immobilization on Celite-545 and Zeolite was found to be beneficial for achieving better ester yield, although there was no remarkable increase in the molar conversion (%). Chitosan was found to be an inappropriate support for esterification reactions of geraniol catalyzed by *T. fusca* cutinase.

4.12.3 Assessment of cutinase as a catalyst for synthesis of various short chain alkyl esters

Cutinase has shown a great potential for esterification and transesterification reactions in different reaction media, where it showed selectivity toward the production of short chain carboxylic acid esters (Barros *et al.*, 2012). There are several reports on esterification and transesterification reactions catalyzed by *F. solani* cutinase (Barros *et al.*, 2012; Carvalho *et al.*, 1998a; Melo *et al.*, 1995a; Pinto-Sousa *et al.*, 1994; Sebastião *et al.*, 1993). Recently, *P. cepacia* cutinase was also evaluated as an effective catalyst for synthesis of short chain alkyl esters (Dutta and Dasu, 2011). However, no report exists on esterification and transesterification reactions catalyzed by *T. fusca* cutinase. Thus, the present section discusses the preliminary screening results of several esterification and transesterification reactions catalyzed by *T. fusca* cutinases to evaluate their potential as a biocatalyst.

Several esterification and transesterification reactions were performed according to the method described in the section 3.15. The schematic representation of the reaction performed is shown in Fig. 4.56.

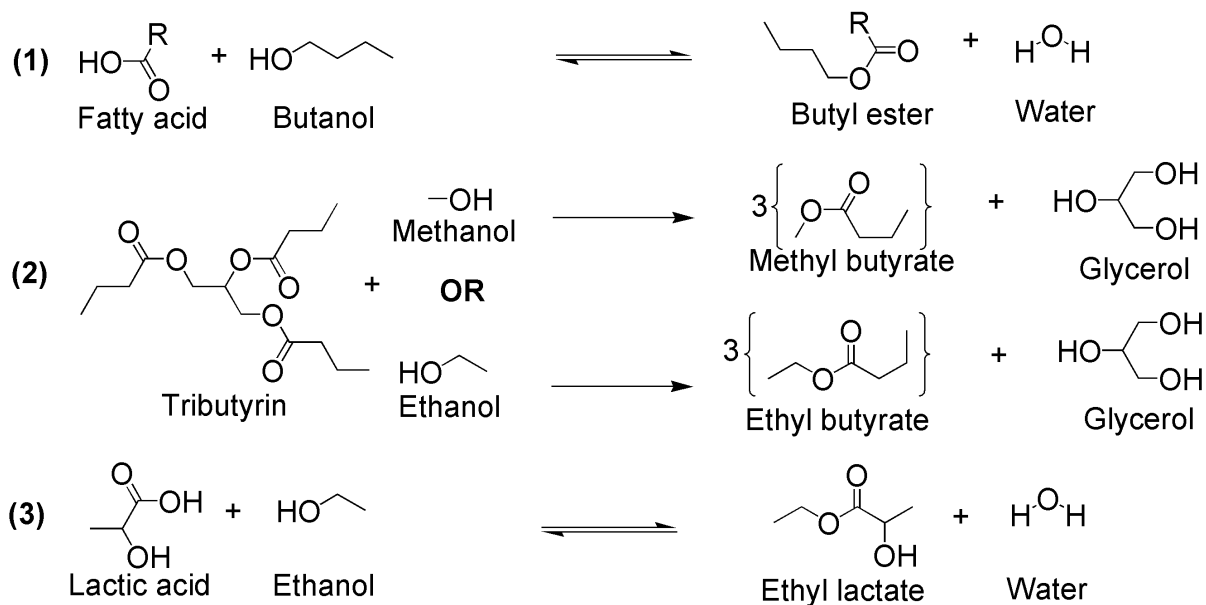


Fig. 4.56 Schematic representation of various esterification and transesterification reactions screened to evaluate the ability of *T. fusca* cutinase as a catalyst. R in the reaction (1) stands for butyric acid, valeric acid, octanoic acid and decanoic acid used in the present study

Butyl esters are employed in fruit-flavored products such as beverages, candies, jellies and jams, bakery products, wines and dairy products such as cultured butter, sour cream, yogurt and cheese (Barros *et al.*, 2012). As depicted in the Fig. 4.57, Cut1 was found to be a good catalyst for butyl ester synthesis with highest molar conversion of 89% at 15 h for butyl butyrate synthesis. However, Cut2 was found to be less efficient in catalysis with only 66% conversion for butyl butyrate. The reason would be inhibitory effect of butanol, as described earlier in the enzyme characterization (Section 4.8.7 and Fig 4.18) on Cut2, which is deactivating the enzyme faster. It is also evident from the Fig. 4.57 that there was an increase of 3% in the conversion of butyl butyrate after immobilization of Cut1 on Zeolite and Celite-545. However, Cut1 immobilized on Chitosan showed decrease in the ester conversion yield. Cut1 also showed a good catalytic efficiency for butyl valerate and butyl octanoate with 74 and 71% molar conversion. However, the catalytic efficiency was drastically affected for

butyl decanoate, which showed only 36% conversion. This would be due to increase in the acyl chain length, which was also observed for other esterification reaction. Dutta and Dasu (2011) achieved 94.6% molar conversion for butyl butyrate with *P. cepacia* cutinase. Recently, Barros *et al.* (2012) reported that esterification yield of 95% for C4-C6 esters with *F. solani* cutinase. Santos and Castro (2006) reported that 75% conversion at 41°C for the *Candida rugosa* lipase catalyzed reaction of butyric acid and butanol. The maximum of 95% conversion was also reported for *B. licheniformis* esterase catalyzed synthesis of ethyl caproate at 37°C in n-heptane (Macarie and Baratti, 2000).

The methyl and ethyl esters of oils are well appreciated to be used as bio-carburants, biosurfactants, biolubricants or biodiesel. Lipases are mostly used as a biocatalyst for transesterification of oils. However, cutinase also been reported to catalyze the transesterification of oils. Experiments were performed to evaluate the ability of *T. fusca* NRRL B-8184 cutinase to catalyze the transesterification of tributyrin. As shown in Fig 4.57 both Cut1 and Cut2 showed very low transesterification yield for fatty acid methyl (34 and 31% for Cut1 and Cut2, respectively) and ethyl esters (32 and 30% for Cut1 and Cut2, respectively), signifying the fact that cutinase from *T. fusca* might not be a good catalyst for transesterification reaction. Salis *et al.*, (2005) observed about 40% conversion of methyl ester during transesterification of triolein catalyzed by *P. cepacia* lipase. Several other studies also reported a similar low conversion yield for lipase and cutinase catalyzed transesterification of oil (Dutta *et al.*, 2013).

Lactic acid esters have a high value in formulation of biodegradable packaging materials, paints, grease removers and cleansers (Knez *et al.*, 2012). It is also increasingly used in food, cosmetic and pharmaceutical formulations due to their hygroscopic, emulsifying and

exfoliating properties (Knez *et al.*, 2012; Pirozzi and Greco, 2004). There are reports on enzyme catalyzed synthesis of lactic acid esters from lipases (From *et al.*, 1997; Knez *et al.*, 2012; Pirozzi and Greco, 2004). Biocatalytic synthesis of lactic acid esters offers an advantage of reduced amounts of by-products due to selectivity, lower energy consumption and simplified waste stream treatment (absence of acid catalysts) in comparison to conventional chemical synthesis (From *et al.*, 1997; Knez *et al.*, 2012). *T. fusca* cutinase was evaluated as a catalyst for synthesis of ethyl lactate. As shown in Fig 4.57, both the cutinases showed catalytic activity towards lactate ester synthesis. However the catalytic yield was very low. Cut2 showed slightly better catalytic yield with molar conversion of 21% in comparison to Cut1 (15%).

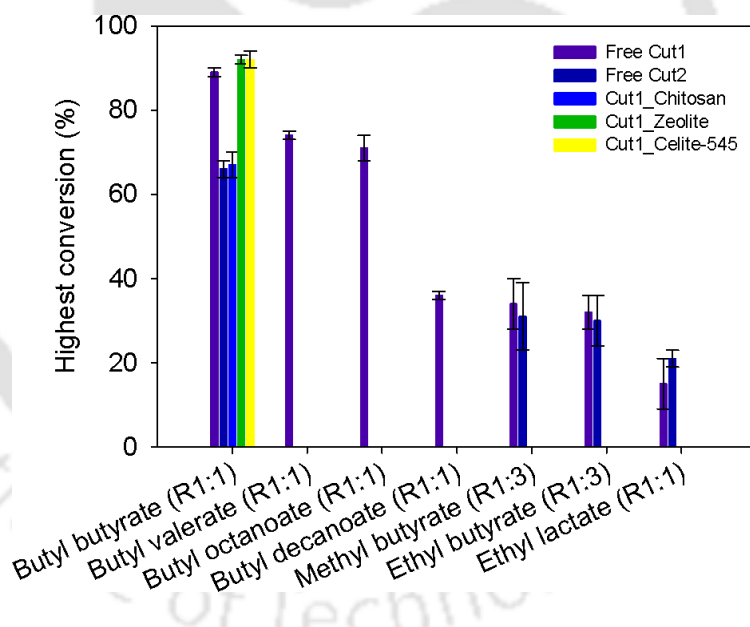
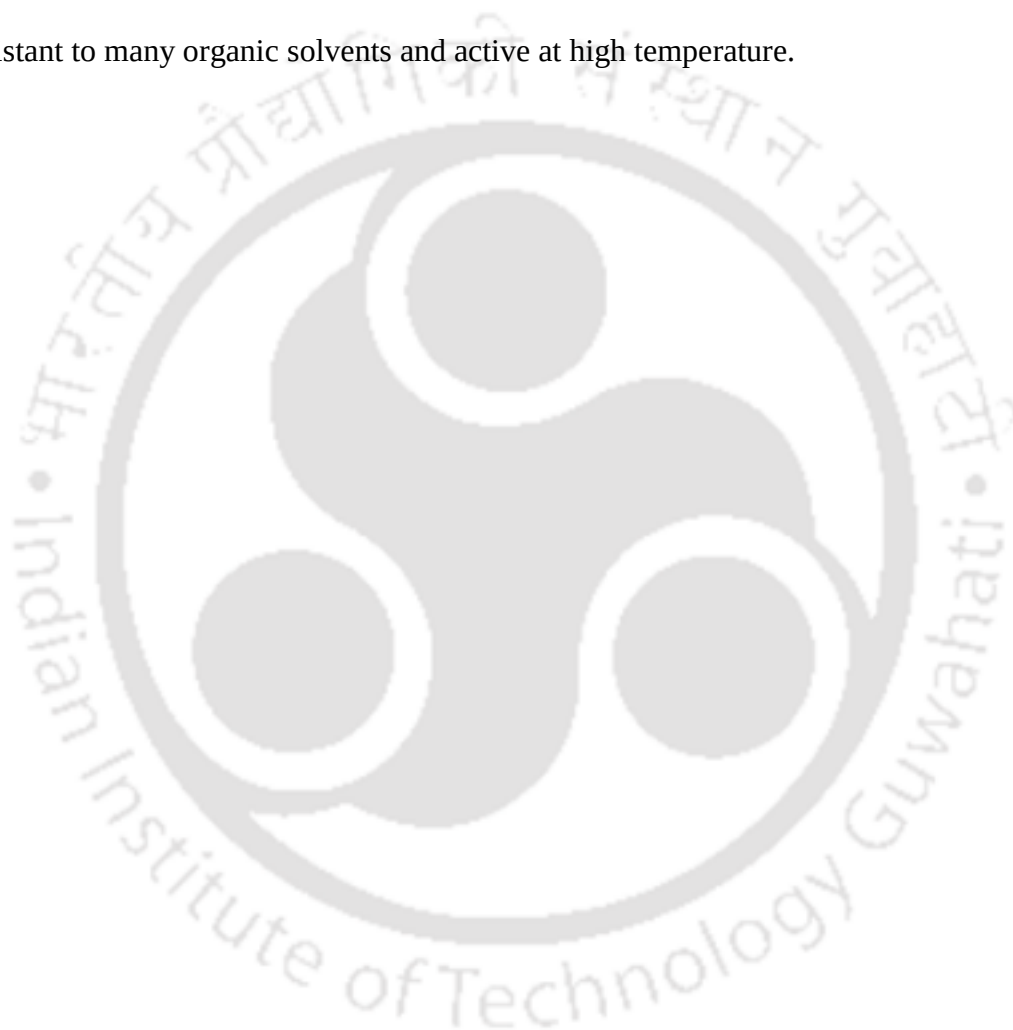


Fig 4.57 Evaluation of catalytic efficiency of *T. fusca* cutinase on various esterification and transesterification reactions as shown in Fig. 4.56. R is the corresponding fatty acid/alcohol molar ratio used in the study. Error bars correspond to the standard deviation of two replicates

There are reports where 75% yield was achieved for butyl lactate with *Candida antarctica* lipase B (CALB) in supercritical carbon dioxide (Knez *et al.*, 2012), 75% yield for ethyl lactate with commercial lipase, Novozym 435 (Zhihong *et al.*, 2009), 74% yield for ethyl

lactate with whole-cell biocatalyst displaying CALB on yeast (Inaba *et al.*, 2009) and 25% yield for ethyl lactate with immobilized commercial lipase, Novozym 43 (Sun *et al.*, 2010). In comparison to lipase, the catalytic efficiency of *T. fusca* cutinase towards lactic acid ester synthesis was very low. However, an effort to increase the catalytic efficiency by enzyme engineering and immobilization technology would be worth as the *T. fusca* cutinase is resistant to many organic solvents and active at high temperature.



CHAPTER 5

SUMMARY AND CONCLUSIONS

The present thesis was focused on the production, characterization and applications of a novel industrially important cutinases from *Thermobifida fusca* NRRL B-8184. This enzyme belongs to the family of hydrolases, specifically those acting on carboxylic ester bonds. Due to the importance of the potential applications of cutinase in various industrial process, this enzyme from various microbial and plant sources has been vastly studied. This chapter highlights the key findings of the present study on cloning, production characterization and applications of two cutinases from *T. fusca* NRRL B-8184.

- Two cutinase encoding genes, *cut1* and *cut2* from *Thermobifida fusca* NRRL B-8184 was cloned in to *E. coli* expression strain BL21 (DE3) under IPTG inducible vector pET22b(+). Sequencing of cloned genes, *cut1* and *cut2* showed 100% homology, respectively with *tfu_0882* and *tfu_0883* genes from *Thermobifida fusca* YX strain (Accession No. NC_007333) in the NCBI nucleotide data base.
- The Cut1 and Cut2 enzymes showed 93% homology among them at amino acid level.
- Optimum temperature, concentration of IPTG and cell concentration for the expression of the recombinant Cut1 and Cut2 was found to be 37°C, 0.1 mM and A_{600nm} 0.75, respectively without any inclusion body formation.
- Cloning and expression of *T. fusca* cutinase genes, Cut1 and Cut2 with and without C-terminal histidine tag showed no considerable difference in growth and activity of Cut1 and Cut2 with and without C-terminal His-tag.

- The medium for maximum cutinase production was developed after screening six different medium. Among the screened media, TB medium with 2 mM MgSO₄ and 1 mM NaCl was found to be the best production medium with increase in cutinase production by 1.1-fold higher than TB and ~2.5-fold higher than LB medium.
- Screening of glucose or glycerol as a carbon source showed that glycerol is the preferred substrate for recombinant cutinase production with higher cell growth and cutinase production compare to glucose.
- Statistical Taguchi's experimental designs were applied to maximize the production of recombinant cutinase, Cut1 and Cut2 of *T. fusca* NRRL B-8184 at shake flask level. The production of both the recombinant cutinase was enhanced by ~1.2 fold after Taguchi optimization of medium components with overall increase of ~11-folds, as compared with *T. fusca* NRRL B-8184 wild-type strain.
- Cellular localization of Cut1 and Cut2 showed most of the enzyme to be in periplasm (57 and 61% for Cut1 and Cut2 enzyme, respectively). Nearly 28% of the recombinant protein was seen in cytoplasm as soluble protein for both the enzymes. Respectively, 15 and 11% of Cut1 and Cut2 was secreted in to extracellular medium. No inclusion bodies were formed during production.
- A single step affinity purification of recombinant cutinases of *T. fusca* NRRL B-8184 was carried out using C-terminal His-tag. The molecular mass of the purified Cut1 and Cut2 were found to be ~30 kDa and ~29 kDa by SDS-PAGE, respectively. MALDI-TOF-MS analysis confirmed the molecular mass of Cut1 and Cut2 to be 30.1906 kDa and 29.6812 kDa, respectively. Isoelectric point as determined by 2-D electrophoresis for Cut1 and Cut2 were found to be 7.7 and 7.5, respectively.

- Purified Cut1 and Cut2 showed specific activity of 542.5 ± 2.7 and 643.4 ± 3.4 U mg⁻¹, respectively against pNPB and 0.218 ± 0.06 and 0.395 ± 0.17 U mg⁻¹ for Cut1 and Cut2, respectively with cutin analog substrate, p-NMSH.
- Studies on physical conditions, which influence the performance of purified cutinase, revealed that both the cutinases were active over a broad range of pH (6.8-9.0) and temperature (40-80°C). The enzyme showed stability at alkaline range of pH (pH 7.0–9.0). Maximum cutinase activity was obtained at pH of 8 and 55°C.
- Kinetic parameters, K_m , V_{max} and k_{cat} values for Cut1 and Cut2 was found to be, respectively, 131 and 89 $\mu\text{M L}^{-1}$ (K_m), 3.2 and 3.8 $\mu\text{M s}^{-1}$ (V_{max}), 178 and 253 s⁻¹ (k_{cat}) with the substrate pNPB.
- Thermodynamic parameters (K_d , $t_{1/2}$, ΔH , ΔS , ΔG) were determined to evaluate the probable mechanism of deactivation of recombinant cutinases.
- The recombinant cutinases of *T. fusca* NRRL B-8184 has the higher substrate specificity towards short chain fatty acid synthetic esters (C₂-C₆), though it also showed activity with longer chain esters (C₁₆). Cut1 showed better catalytic activity with longer chain esters compare to Cut2.
- Both Cut1 and Cut2 showed very good tolerance to most of the organic solvents studied. Benzene, hexyl acetate, isopropanol, dichloro methane was found to be more inhibitory for Cut1 compare to Cut2 and butanol and 1-hexanol was more inhibitory to Cut2 compare to Cut1.
- Both Cut1 and Cut2 showed enhanced activity in the presence of sodium taurodeoxycholate (anionic) detergent, no considerable effect on activity was observed

with deoxycholate, sodium deoxycholate and SDS. Triton X-100 and Twen-80 was found to be moderately inhibitory for Cut1.

- The activity of both the cutinases were enhanced by mono cations, Na^+ and K^+ , whereas it was moderately inhibited by some divalent cations (Pb^{2+} , Fe^{2+} , Zn^{2+}) and considerably inhibited by Ag^{2+} , Rb^+ , Cr^{2+} , Hg^{2+} , serine blocking reagent; phenyl methyl sulphonyl fluoride (PMSF) and histidine blocking reagent; Diethylpyrocarbonate (DEPC).
- Fluorescence and Far UV CD spectroscopy showed that Cut1 and Cut2 are stable at pH ranging from 6-9 at 25°C with no considerable change in the secondary structure and found to be α -helix in nature. Unfolding study with GdnHCl showed Cut2 to be more resistant to unfolding compare to Cut1. Both the cutinases found to be highly resistant to urea induced unfolding.
- Homology modeling of the Cut1 and Cut2 revealed that both the enzymes belong to α/β -hydrolases family with Ser-His-Asp catalytic triad. There is a disulfide bond present in both Cut1 (Cys²⁹⁹-Cys³¹⁷) and Cut2 (Cys²⁸¹-Cys²⁹⁹) in the C-terminal region. Exploration of the electrostatic surface of the cutinases showed that the surface electrostatic properties of the two cutinases were fairly dissimilar.
- Immobilization studies with various supports to enhance the activity and stability of cutinase showed that Chitosan, Celite-545 and Zeolite are the best among the studied supports for immobilization of cutinases. Immobilization on magnetic nanoparticle was found to be inappropriate support for both the cutinases with reduction in stability as well as activity.
- Cut1 and Cut2 were found to be efficiently catalyzing several esterification and transesterification reaction for synthesis of short-chain length fatty acid esters.

- Cutinase catalyzed esterification to synthesize isopropyl ester showed that Cut2 has a better catalytic efficiency for isopropyl esters synthesis with maximum molar conversion of 63% when immobilized on Celite-545. However, Cut1 was found to be significantly inhibited in presence of isopropanol. Catalytic efficiency in terms of molar conversion was enhanced by 16 and 10%, respectively after immobilization of Cut2 on Celite-545 and Zeolite. Nevertheless, chitosan was found to be an inappropriate immobilization support for *T. fusca* cutinase catalyzed esterification.
- Cutinase catalyzed esterification to synthesize geraniol ester showed both Cut1 and Cut2 to be an efficient catalyst. However, Cut1 showed a slightly better catalytic efficiency in comparison to Cut2. The maximum conversion of 88% obtained for geraniol acetate, followed by geraniol butyrate (80%) and geraniol valerate (67%) with Cut1 immobilized on Celite-545. Catalytic efficiency in terms of molar conversion was slightly enhanced after immobilization of Cut1 on Celite-545 and Zeolite. However, chitosan was found to be an inappropriate immobilization support for *T. fusca* cutinase catalyzed esterification.
- Study on evaluation of *T. fusca* cutinase as a catalyst for esterification of butyric acid ester revealed that Cut1 can efficiently synthesize butyric acid ester with molar conversion of 89% for butyl butyrate, followed by butyl valerate (74%) and butyl octanoate (71%). Immobilization of Cut1 on Zeolite or Celite-545 increased the ester yield of butyl butyrate to 92%. However, Cut2 and Cut1 immobilized on chitosan showed considerably lesser ester yield of 66 and 67%, respectively.
- Study on evaluation of *T. fusca* cutinase as a catalyst for esterification of lactic acid ester and transesterification of tributyrin with methanol (FAME) and ethanol (FAEE) showed less than 15 and 21% conversion efficiency from Cut1 and Cut2, respectively for ethyl

lactate and less than 35% conversion from Cut1 as well as Cut2 for FAME and FAEE, which makes them unsuitable catalyst for these esterification and transesterification reactions.

Future scope of the work

- Production optimization of recombinant cutinases at pilot scale fermenter levels in batch and fed-batch mode.
- Crystallization and structural analysis of Cut1 and Cut2 of *T. fusca* NRRL B-8184.
- Enhancing the substrate specificity of the *T. fusca* NRRL B-8184 cutinases by protein engineering for application in esterification and transesterification reactions.
- Evaluation of *T. fusca* NRRL B-8184 cutinase as a catalyst in synthesis of some pharmaceutically important stereo-specific compounds.
- Evaluation of *T. fusca* NRRL B-8184 cutinase as a catalyst in cotton scouring, detergent formulation and synthesis of several other industrially important products.

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A.1 Sample calculation for the estimation of cutinase activity

Preparation of standard plot for p-nitrophenol

A stock of 0.05 M of p-nitrophenol (p-NP) solution was prepared by dissolving 139.1 mg in 20 ml miliQ water. The solution was diluted to prepare final p-NP solution of 0.5 mM. Different amount of 0.5 mM p-NP was diluted with potassium phosphate buffer of pH 8 to the final volume of 1 mL. Experiments were performed for standard plot in triplicates and the absorbance was taken against a blank (only buffer) at 405 nm. The points were fitted with a linear regression model with the help of Microsoft Excel® software (Fig. A.1). One unit of cutinase (U) is defined as the amount of enzyme that releases 1 μMol of p-NP per min at 50°C.

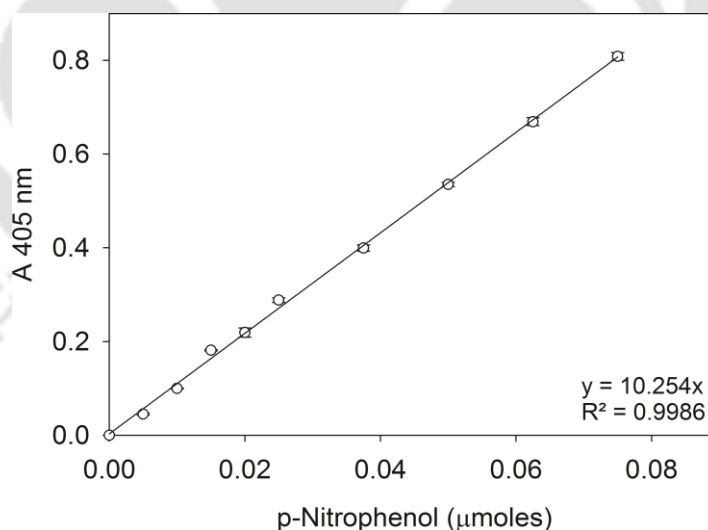


Fig. A.1 Standard curve drawn between known amount of p-NP and the corresponding absorbance measured at 405 nm

The molar extinction coefficient of p-NP varies with pH values. The equations for other pH values used in the experiments are given in Table A.1.

Table A.1 Standard plot equations of p-NP at different pHs

pH	Equation	R ²
6.00	$y = 1.45x$	0.952
6.50	$y = 3.98x$	0.93
7.00	$y = 4.417x$	0.96
7.50	$y = 10.05x$	0.981
8.00	$y = 10.25x$	0.998
8.50	$y = 10.7x$	0.949
9.05	$y = 15.03x$	0.987
9.50	$y = 17.75x$	0.932
10.00	$y = 18x$	0.926
10.50	$y = 18.28x$	0.938

The pNP-ester activity was calculated according to the formula,

$$\text{Activity (U mL}^{-1}\text{)} = \frac{A_{405 \text{ nm}} \times 1 \times \text{DF}}{\text{MEC} \times \text{EV}}$$

Where, $A_{405 \text{ nm}}$ is the absorbance of the sample at 405 nm, 1 is the volume (in milliliters) of assay, DF is the dilution factor, MEC is the micromolar extinction coefficient of p-nitrophenol at 405 nm and EV is the volume (in milliliter) of enzyme used.

A.2 Sample calculation for the estimation of protein

Preparation of standard plot for protein

Stock solution of 1 mg mL^{-1} protein (BSA) was prepared in 50 mM KPO_4 buffer (pH 8). The stock solution was appropriately diluted with same buffer to get standard solutions of various concentrations of protein (mg mL^{-1}) viz., 0.1 to 8 as shown in X-axis of Fig A.2. Experiments were performed for standard curve in triplicates and absorbance of the standard samples was measured at 595 nm against the appropriate blank as described for test samples of protein in section 3.4.2. The points were fitted with a linear regression model with the help of Microsoft Excel[®] software (Fig. A.2). Protein concentration in test sample was measured by Bradford's

method as described in section 3.4.2. Then concentration of protein (mg mL^{-1}) in the test sample was calculated with the help of data at A595 nm and the slope of standard curve (1 unit A595 nm = $11.641 \text{ mg mL}^{-1}$ of protein).

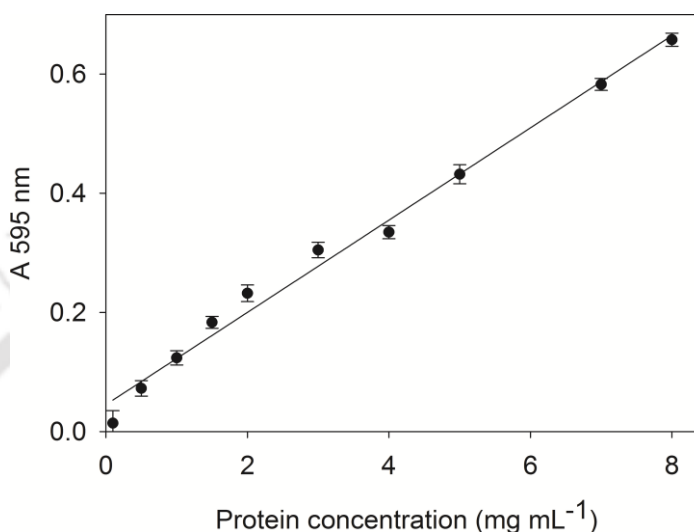


Fig. A.2 Standard curve drawn between known protein concentration and the absorbance measured at 595 nm

A.3 Sample calculation for the estimation of DCW

Preparation of standard plot for DCW

Experiments were performed for standard curve in triplicates and absorbance of the standard samples was measured at 600 nm against the blank (miliQ water) as described in the section 3.4.3. Different dilutions of cell samples were used for measuring cell OD (0.1-6.0) at 600 nm and corresponding DCW (g L^{-1}) was determined at 80°C for 24 h (Fig. A.3). DCW of the unknown sample was determined by measuring the OD of the culture broth at 600 nm using UV-visible spectrophotometer (1 unit OD at 600 nm = 0.5022 g L^{-1} DCW).

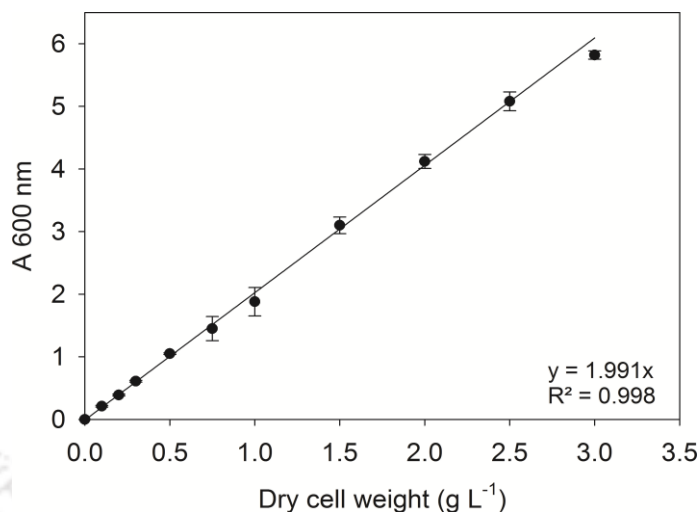


Fig. A.3 Standard curve drawn between dry cell weight of *E. coli* and the optical density measured at 600 nm

A.4 The method for preparation of pNMSH

The cutinase specific substrate, p-nitrophenyl (16-methyl sulphone ester) hexadecanoate (p-NMSH) was prepared using the method described by Degani *et al.*, (2006). One equivalent of 16-hydroxyhexadecanoic acid (3 g) mixed with two equivalent of methane sulfonyl chloride (1.7 mL) in dry dichloromethane. Subsequently, 5 mL of triethylamine was added and stirred for 1.5 h at -20°C under N₂ atmosphere. Then, 1.1 equivalents of p-nitrophenol (p-NP, 1.6 g) was added and stirred for another 6 h at the same temperature. The mixture was then left to reach room temperature under stirring. The disappearance of the reactant p-NP, was tested by thin layer chromatography (TLC). At the end of the reaction, 10% sodium bicarbonate (50 mL) was added to the mixture and the organic products were separated in a separating funnel with dichloromethane (3 × 30 mL). The organic phase was dried over sodium sulfate, filtered and concentrated to dryness in a rotary vacuum evaporator. The resulted powder was freed from byproducts using silica gel column with a mixture of dichloromethane (90%) and n-

hexane (10%) as a mobile phase. The substrate was characterized by ^1H NMR spectroscopy and stored in the amber bottle at -20°C until use.

A.5 The method for preparation of tomato cutin

Cutin was prepared from fresh tomato peels using method described by Walton and Kolattukudy (1972). Tomato peels collected by known quantity of fresh tomatoes were boiled, washed and dried properly. Then peels were boiled in oxalic acid (4 g L^{-1})/ammonium oxalate (16 g L^{-1}) buffer for 3-4 h. After digesting with enzymes [cellulase (sigma) and pectinase (Himedia)] for 18 h at 30°C , peels were subjected to extensive solvent extraction with methanol-chloroform in soxhlet apparatus. These peels were ground to powder (< 20 mesh) to get cutin.

A.6 The method for derivatization of fatty acids into fatty acid methyl esters (FAME)

After cutinase hydrolysis of the plant cutin the fatty acid monomers released was extracted by n-hexane as described in the section 3.4.1.3, and proceeded for derivatization into methyl ester before GC analysis (Section 3.4.6) as follows.

The fatty acid monomers extracted in n-Hexane was concentrated in a rotary vacuum evaporator to get a final volume of 5 mL. 2 mL BF_3 -methanol (10% w/w) was added to the above fatty acid mixture and heated at 60°C for 10 min and cooled to room temperature. 2 mL water and 2 mL n-hexane was added to the above methyl derivatized fatty acid ester (FAME) and mixed thoroughly to get the esters into the nonpolar solvent. The upper organic layer with FAME was carefully removed and dried by passing over anhydrous sodium sulfate bed. This FAME mixture was concentrated to ~ 0.5 mL in a rotary vacuum evaporator, filtered through 0.2 micron filter and used for GC analysis as described in the section 3.4.6.

A.7 Conversion of the machine CD units in millidegree (mdeg) to mean residue ellipticity (MRE, θ)

The machine CD units in millidegree (mdeg) obtained was converted to mean residue ellipticity (MRE, θ) for plotting the graph using the formula,

$$[\theta] = \frac{\theta_{\text{obs}} \text{MRW}}{10cl}$$

Where, θ_{obs} is the observed ellipticity in degrees, MRW is the mean residue weight, c is the concentration of the protein (grams per milliliter), and l is the path length in centimeters. The molecular weight (as determined by MALDI-TOF) 30.19 kDa and 29.68 kDa for Cut1 and Cut2, respectively was considered for calculation. MRW calculated with above said molecular weight for Cut1 and Cut2 is as mentioned in the Table A.2.

Table A.2 MRW values for the Cut1 and Cut2

Cutinase	Molecular weight (MW)	Total amino acids (AA)	MRW (MW/AA)
Cut1	30190 Da	319	94.64
Cut2	29681 Da	301	98.60

A.8 Sample calculation for the estimation of various esters by GC

Internal standard method was used for estimation of the ester concentrations. A known and constant quantity (1 mg mL^{-1}) of a compound that is not one of the analytes was added to the sample. The ratio of its peak area to the peak areas of the analytes was determined for analytes. The unknown concentrations of the analytes in sample was then calculated, using the area of the internal standard peak as a reference using the formula,

$$\text{Ester Conc.} = \frac{\text{Amount}_{\text{IS}} \times \text{Area}_{\text{SC}} \times \text{RRF}}{\text{Area}_{\text{IS}}}$$

Where, IS is the internal standard, SC is the specific compound of Interest (ester), Area is the GC peak area and RRF is the relative response factor to compensate for differences in the sensitivity of the detector to different analytes. RRF was calculated as,

$$RRF = \frac{\text{Peak area}_{IS}}{\text{Amount}_{IS}}$$

The detail method for GC analysis was described in the section 3.4.6. Table A.3 shows the internal standards used for various esters.

Table A.3 Internal standards used for various esters

Ester	Internal standard (1mg mL⁻¹)
Isopropyl oleate	Methyl Oleate
Geranyl acetate	Methyl acetate
Geranyl butyrate	Methyl butyrate
Geranyl valerate	Methyl octanoate
Geranyl decanoate	Methyl palmitate
Butyl butyrate	Methyl octanoate
Butyl valerate	Methyl octanoate
Butyl octanoate	Methyl palmitate
Butyl decanoate	Methyl palmitate
Methyl butyrate	Methyl acetate
Ethyl butyrate	Methyl acetate
Ethyl lactate	Methyl linoleate

A.9 Details of the bioinformatics tools used for homology modeling and validation of the model

Various web based online servers were used for the homology modeling and validation of the model (section 3.13). The Table A.4 gives the details on the various online servers used for the modeling study.

Table A.4 Various web based servers used for the modeling study

Server	Web address
ABPS	http://www.poissonboltzmann.org/apbs
CASTp	http://sts-fw.bioengr.uic.edu/castp/calculation.php
ERRAT	http://nihserver.mbi.ucla.edu/ERRAT/
ModFold	http://www.reading.ac.uk/bioinf/ModFOLD/ModFOLD_form_3_0.html
ModWeb	https://modbase.compbio.ucsf.edu/scgi/modweb.cgi
PDB2PQR	http://nbc-222.ucsd.edu/pdb2pqr_1.8/
PDBSum	http://www.ebi.ac.uk/thornton-srv/databases/pdbsum/Generate.html
QMEAN	http://swissmodel.expasy.org/qmean/cgi/index.cgi
RESPROX	http://www.resprox.ca/index.html
SAVER	http://nihserver.mbi.ucla.edu/SAVES_3/
VADAR	http://vadar.wishartlab.com/
VERIFY 3-D	http://nihserver.mbi.ucla.edu/Verify_3D/

A 10 Comparison of cutinase production achieved by different optimization stages in various microorganisms.

S. No	Cutinase gene source	Expression host	Optimization method	Cultivation method	Production achieved	Ref.
1	<i>F. oxysporum</i>	<i>F. oxysporum</i> ^W	RSM	Shake flask	22.68 U/ml	Pio and Macedo, 2007
2	<i>F. solanipisi</i>	<i>E. coli</i> Strain 0:17 ^R	None	Fed batch	280U/ml	Backlund et al., 2008
3	<i>F. solanipisi</i>	<i>S. cerevisiae</i> SU51 ^R	None	Batch	260 U/ml	Calado et al., 2004
4	<i>F. solinipisi</i>	<i>S. cerevasiae</i> SU50 ^R	None	Fed batch	840U/ml	Calado et al., 2003
5	<i>T. fusca</i> (tfu_0883)	<i>E. Coli</i> BL21 (DE3) ^R	None	Shake flask	149.2 U/ml	Chen et al., 2011
6	<i>T. fusca</i>	<i>E. coli</i> BL21 (DE3) ^R	CCD	Shake flask	150.93U/ml	Li et al., 2010
7	<i>T. fusca</i> WSH03-11	<i>T. fusca</i> WSH03-11 ^W	None	Fed batch	51.0 U/ml	He et al., 2009
8	<i>T. fusca</i> NRRL B-8184 (<i>cut1</i>)	<i>E. coli</i> BL21 (DE3) ^R	Taguchi	Shake flask	316 U/ml	Present study
9	<i>T. fusca</i> NRRL B-8184 (<i>cut2</i>)	<i>E. coli</i> BL21 (DE3) ^R	Taguchi	Shake flask	318 U/ml	Present study

LIST OF PUBLICATIONS

Published/Under Review in Referred International Journals

Hegde K and Dasu V V. (2013). Production Optimization and Characterization of Recombinant Cutinases from *Thermobifida fusca* sp. NRRL B-8184. *Applied Biochemistry and Biotechnology* 170: 654–675.

Hegde K and Dasu V V. (2013). Covalent immobilization of novel cutinases from *Thermobifida fusca* on glutaraldehyde activated chitosan beads. *Indian Journal of Biotechnology*. (Under review).

Hegde K and Dasu V V. (2013). Structural Stability and Unfolding Properties of Thermostable Bacterial cutinases: A Comparative Study of Homologous Enzymes. *Biochimica et Biophysica Acta-Proteins and Proteomics* (Under review).

Hegde K and Dasu V V. (2013). Evaluation of Zeolite and Celite as an immobilization support for cutinase from *T. fusca*. *The Journal of Agricultural and Food Chemistry* (Under review).

Hegde K and Dasu V V. (2013). pH dependent thermal deactivation study on cutinases from *T. fusca*. *Biochemistry (Moscow)* (Under review).

Under Preparation for Referred International Journals

Hegde K and Dasu V V. (2013). Evaluation of immobilized cutinase from *Thermobifida fusca* as a catalyst for synthesis of terpenoid esters (Under preparation).

Hegde K and Dasu V V. (2013). Evaluation of immobilized cutinase from *Thermobifida fusca* as a catalyst for synthesis of isopropyl esters (Under preparation).

Hegde K and Dasu V V. (2013). Studies on immobilization of novel cutinases from *Thermobifida fusca* on magnetic nanoparticles (Under preparation).

Patents

Dasu V V and **Hegde K**. (2012). Development of bioprocess for production of novel cutinases from *Thermobifida fusca* in *E. coli* BL21 (DE3) [Accession No. MTCC 5725 and Accession No. MTCC 5726] (Appl. No.1416/KOL/2012).

Hegde K and Dasu V V. (2013). Cutinase catalyzed synthesis of terpenoid esters (Under preparation).

Hegde K and Dasu V V. (2013). Cutinase as a catalyst for esterification and transesterification of fatty acids (Under preparation).

Published in International Conference Proceedings

Hegde K and Dasu V V. (2013). Structural stability and unfolding properties of thermostable cutinases: A comparative study of homologous enzymes. International Conference on Health, Environment and Industrial Biotechnology. November 21-23, MNIT, Allahabad (Accepted for oral presentation).

Hegde K and Dasu V V. (2013). Analysis of structural details of *T. fusca* cutinase based on homology modeling. International Conference on Structural and Functional Genomics. January 6-7, SASTRA University, Thanjavur, India (Poster presentation).

Dasu V V and **Hegde K.** (2012). Optimization of medium components and culture conditions for high level production of recombinant cutinase from *Thermobifida fusca* in *E. coli* BL21 (DE3). SIM Annual Meeting and Exhibition. August 12-16, Washington, DC, USA (Poster presentation).

Dasu V V and **Hegde K.** (2011). Purification and biochemical characterization of recombinant cutinase from *Thermobifida fusca*. SIM Annual meeting and exhibition, July 24-28, New Orleans, LA, USA (Poster presentation).

Hegde K and Dasu V V. (2010). Cloning, expression, purification and characterization of recombinant cutinase from *Thermobifida fusca*. International Conference on genomic sciences. November 12-14, Madurai kamaraj University, Madurai, TN, India (Poster presentation).

GenBank Submissions

NCBI GenBank Accession number JN129499.1_*Thermobifida fusca* strain NRRL B-8184 cutinase (*cut_1*) gene, complete cds.

NCBI GenBank Accession number JN129500.1_*Thermobifida fusca* strain NRRL B-8184 cutinase (*cut_2*) gene, complete cds.