PRODUCTION, PURIFICATION, CHARACTERIZATION AND APPLICATIONS OF CUTINASE FROM *PSEUDOMONAS CEPACIA* NRRL B 2320

A THESIS

submitted by

KASTURI DUTTA

for the award of the degree

of

DOCTOR OF PHILOSOPHY

DEPARTMENT OF BIOTECHNOLOGY

INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI

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Dedicated to my Parents

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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.

Date: ……. **Kasturi Dutta**

INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI

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CERTIFICATE

It is certified that the work described in this thesis entitled "**Production, purification, characterization and applications of cutinase from *Pseudomonas cepacia* NRRL B 2320**" by Ms. Kasturi Dutta 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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Date: …… **Kasturi Dutta**

ABSTRACT

Cutinase has some vital properties of lipase and esterase. Because of its unique nature, it has potential of being an industrially important enzyme. Some of the useful applications of cutinase include hydrolysis of fats and oils, esterification and transesterification reactions. It is used in the food industry for synthesis of flavors, petrochemical industry for synthesis of biodiesel and preparation of some house-hold detergents. At low water activities, transesterification of fats and oils or stereo selective esterification of alcohols can be achieved using cutinase. This enzyme is mainly produced by phytopathogenic fungi, but there are several bacteria, which are known to produce cutinase.

In this study, fifteen different microorganisms grown on medium containing Tween 80 or olive oil or cutin, were screened for their possible cutinase producing potential. Cutin, the essential substrate for the production of cutinase, was prepared from tomatoes and characterized. Among fifteen microorganisms, *Pseudomonas cepacia* NRRL B 2320, *Pseudomonas fluorescens* NRRL B 3178 and *Pseudomonas geniculata* NRRL B 1606 were capable of growing on the medium containing cutin as a sole source of carbon and their culture filtrates showed enzyme activity towards p-nitrophenyl butyrate (p-NPB). Cutinase activity was further confirmed by cutinase specific substrate, p-nitrophenyl (16 methyl sulphone ester) hexadecanoate (p-NMSH), where, *P. cepacia* NRRL B 2320 had shown highest activity of 40 U mg^{-1} and 41.2 $\times 10^{-2}$ U mg^{-1} towards p-NPB and p-NMSH, respectively. Hence, further studies had been carried out using *P. cepacia* NRRL B 2320. However, further attempts have been made to replace cutin with different oils, cutin monomer and other carbon sources for the production of cutinase.

The development of medium to enhance the production of cutinase from *P. cepacia* NRRL B 2320 was carried out by using Plackett-Burman and central composite design techniques. First, the medium components were screened using Plackett-Burman design, which suggested that cutin, peptone, KCl and $MgSO_4 \cdot 7H_2O$ have influenced the cutinase production significantly with very high confidence levels. While cutin, peptone and $MgSO_4 \cdot 7H_2O$ have shown a positive effect, KCl indicated a negative effect on the cutinase activity. The concentrations of these four medium components were optimized using 2^4 full factorial central composite design. An optimum combination of 10.06 g l^{-1} of cutin, 17.77 g l^{-1} of peptone, 0.635 g l^{-1} of KCl and 5.546 g l^{-1} of $MgSO_4 \cdot 7H_2O$ in the medium gave a maximum cutinase activity of 336 U ml^{-1} at 96 h of culture. An overall 2 fold increase in the production of cutinase was observed in the optimized medium. To estimate the biokinetic parameters, time course of cell growth and enzyme production data in shake flask and bioreactor (with or without pH control) were fitted to the existing kinetic models reported in the literature. These models suggested that the production of cutinase was growth associated in shake flask and mixed growth type in a batch bioreactor. Also an improvement in cutinase production was observed in batch bioreactor than in shake flask.

The production of cutinase was studied in shake flask by *P. cepacia* NRRL B 2320 for initial substrate concentration from 2 to 20 g l^{-1}. The growth and enzyme activity at different initial substrate concentrations were measured at different time intervals. The results obtained from the present study inferred that the presence of substrate inhibition kinetics. Therefore various substrate inhibition models were tested and biokinetic parameters were estimated using non-linear regression analysis. Webb model was found to be the best fitted model for growth of *P.*

cepacia NRRL B 2320. The highest volumetric enzyme activity was observed at an initial substrate concentration of 10 g l^{-1}.

An extracellular novel cutinase from *P. cepacia* NRRL B 2320 with high organic solvent and surfactant stability was purified to apparent homogeneity with purification fold and yield of 89.84 and 17.56%, respectively. The molecular mass of cutinase was found to be 26.25 kDa from MALDI-TOF-MS analysis. Optimum pH of purified cutinase for the hydrolysis of p-nitrophenyl butyrate was in the range of 7.0-8.5, and its optimum temperature was found to be 37ºC. The effect of assay conditions (enzyme and substrate amount (volume), pH and temperature) on the activity of purified cutinase was investigated. Central composite experimental design was applied to optimize chemical (enzyme and substrate volume) and physical (pH and temperature) parameters, separately. The optimal volume of enzyme and substrate were found to be 24 µl (1.4 µg) and 971 µl, respectively, for the maximum cutinase activity. The optimal pH and temperature for highest activity were found to be 7.9 and 36.5°C, respectively. An overall 1.42 fold increase in activity was observed after optimization of both assay and process conditions. The deactivation rate constants and the half life of cutinase were also estimated at different combinations of pH and temperature. At optimal pH of 6.0 and 7.0, the rate of enzyme deactivation was found to be the least. 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 40°C to 60°C at different pH (6.0-9.0). The fluorescence and CD spectra were obtained during the thermal deactivation of cutinase. A red shift in λ_{max} of about 10 nm observed from fluorescence spectra during thermal deactivation suggested that the exposure of hydrophobic group in polar solvent. From CD spectra, increase in ellipticity (θ) near 218 nm and decrease

in ellipticity (θ) near 200 nm implied that cutinase lost its α-helix structure and become more disordered at higher temperature. The native cutinase consists of 56.4% of α-helix and 23.2 % of β-sheet as determined by CD spectrum. The enzyme was able to show activity towards synthetic esters of chain length C_4-C_{16}. The activity of cutinase was activated by mono cations and various effectors including Na^+, K^+ and β-marceptoethanol, whereas it was moderately inhibited by various divalent cations and serine blocking reagent (phenyl methyl sulphonyl fluoride (PMSF)). Kinetic parameters, K_m, V_{max} and K_{cat} of purified cutinase from *P. cepacia* NRRL B 2320 were found to be 0.23 mM, 7.95 U μg^{-1} and 5.0×10^3 s^{-1}, respectively.

To evaluate the industrial applicability and performance of purified cutinase during esterification and transeseterification reactions, experiments were performed for synthesis of various esters. 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. Cutinase from *P. cepacia* NRRL B 2320 was found to be active in catalyzing the synthesis of alkyl esters in organic solvent. The optimal temperature range for the enzyme catalyzed synthesis was found to be from 35°C to 40°C. The maximum conversion (%) during synthesis of ester was obtained for butyric acid (C_4) and valeric acid (C_5) 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 (%) was decreased with increasing carbon chain length of alcohol used in the esterification reaction. The kinetic analysis for the synthesis of butyl butyrate by varying concentration of one substrate at a time (butanol or butyric acid), showed that Ping–Pong bi bi model with acid inhibition and influence of initial water is most suitable model for the prediction of the reaction kinetics.

The methyl esters of oils are used as bio-carburants, biosurfactants, biolubricants or biodiesel as an alternative to fossil/natural fuel. The shortage of natural oils invites the growing attention towards biodiesel production from vegetable oils. Cutinase from *P. cepacia* NRRL B 2320 was found to be active in catalyzing the synthesis of methyl esters by transesterification of tributyrin, triolein and soyabean oil in organic solvent. The optimal temperature range for the enzyme catalyzed synthesis was found to be from 35°C to 40°C. The maximum conversion (%) during synthesis of ester was obtained with 2.5 mg ml^{-1} of enzyme. The optimal methanol to oil ratio was found to be 1.5:1 with all three substrates. The kinetic analyses for the synthesis of methyl esters from tributyrin/triolein/soyabean oil have shown that Ping-Pong bi bi model with alcohol inhibition was the most suitable for the prediction of the reaction kinetics for all the cases.

The cutinase encoding genes in *P. cepacia* NRRL B 2320 were identified, cloned and expressed in *Escherichia coli* BL21 (DE3). The recombinant cutinase production under un-optimized condition was found to be higher than the cutinase production from *P. cepacia* NRRL B 2320 under optimized condition.

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ABBREVIATIONS AND NOTATIONS

Abbreviations

AFM	Atomic force microscopy
ANOVA	Analysis of variance
ARS	Agricultural research service
ATPS	Aqueous two-phase systems
CBB	Coomassie brilliant blue
CCD	Central composite design
CD	Circular dichorism
cDNA	Complementary dioxyribonucleic acid
DCW	Dry cell weight (g l^{-1})
DEAE	Diethylaminoethyl
DF	Degree of freedom
DO	Dissolved oxygen (%)
EDTA	Ethylenediaminetetraacetic acid
FT-IR	Fourier transform infrared
FID	Flame ionization detector
IPTG	Isopropyl β-D-1-thiogalactopyranoside
MALDI-TOF MS	Matrix assisted laser desorption ionization time-of-flight mass spectrometry
mRNA	Messenger ribose nucleic acid
MS	Mean square
NCIM	National collection of industrial microorganisms
NMR	Nuclear magnetic resonance
NRRL	Northern regional research laboratories

Abbreviations

OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PB	Plackett-Burman
PCR	Polymerase chain reaction
PEG	Polyethylene
PMSF	Phenyl methyl sulphonyl fluoride
p-NMSH	p-Nitrophenyl (16 methyl sulphone ester) hexadecanoate
p-NPB	p-Nitrophenyl butyrate
p-NPP	p-Nitrophenyl palmitate
PVDF	Polyvinylidene fluoride
rRNA	Ribosomal ribonucleic acid
RSM	Response surface methodology
RMSE	Root mean square error
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate- polyacrylamide gel electrophoresis
sp.	Species
SS	Sum of square
SSD	Sum of squares of the differences
SSF	Solid state fermentation
TCA	Tri-chloro acetic acid
TEA	Triethylamine
TFA	Trifluoroacetic acid
phoA	Signal peptide for alkaline phosphatase
TLC	Thin layer chromatography

Notations

°C	Degree centigrade
D	Dilution factor
E	Activation energy
g	Gram
g	Gravitational acceleration
h	Hour
ΔH*	Change in enthalpy
ΔS*	Change in entropy
ΔG*	Change in free energy
K_p	Protein partition coefficient
K_s	Saturation constant (g l^{-1})
K_{cut}	Cutinase partition coefficient
k_d	Deactivation constant (h^{-1})
pI	Isoelectric point
K_I	Inhibition constant (g l^{-1})
kb	Kilobase
k_{cat}	Turnover numbers (s^{-1})
kDa	Kilo dalton
K_m	Michaelis constant (mM)
K_{mA}	Michaelis constant for substrate A
K_{mB}	Michaelis constant for substrate B
K_{IA}	Inhibition constant due to substrate A
K_{IB}	Inhibition constant due to substrate B
M	Molar (mol l^{-1})
min	Minute
ml min^{-1}	Milliliter per minute
M_r	Molecular weight of enzyme (kDa)
P_0	Initial cutinase activity (U ml^{-1}),

Notations

P_{max}	Maximum cutinase activity (U ml^{-1})
P_r	Ratio between the initial volumetric rate of product formation and the initial product concentration
nm	Nanometer
R^2	Regression coefficient
rpm	Revolution per minute
s	Second
U	Unit of enzyme activity
U l^{-1} 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/v	Volume/volume
w/w	Weight/weight
Ve	Elution volume
V_{max}	Maximal reaction velocity (U μg^{-1})
V_o	Void volume
vvm	Volume of air per volume of medium per minute
w/v	Weight/volume
X	Biomass concentration (g of DCW l^{-1})
X_0	Initial biomass (g of DCW l^{-1})
X_{max}	Maximum biomass (g of DCW l^{-1})
Y	Predicated response

Greek letter

μ	Specific growth rate (h^{-1})
μ_{Expt}	Experimental specific growth rate (h^{-1})
µl	Microlitre
μ_m	Maximum specific growth rate (h^{-1})

Notations

µM	Micromolar (µmol l^{-1})
µmol	Micromoles
pM	Picomolar
μ_{Pred}	Theoretical specific growth rate (h^{-1})
α	Growth-associated constant for cutinase production (U mg^{-1})
β	Non growth-associated constant (U mg^{-1} h^{-1})
β_i	Linear effect
β_{ii}	Square effect
β_{ij}	Interaction effect
γ_s	Initial substrate concentration (g l^{-1})
γ_s^*	Critical substrate concentration (g l^{-1})
κ	Boltzmann constant

CHAPTER 1
INTRODUCTION

1.1 Generalities

Cutinases (3.1.1.74) are hydrolytic enzymes, the smallest member of α/β hydrolase family (Longhi *et al.*, 1999), which degrade cutin. Cutin is the cuticular polymer of higher plants that composed of hydroxy and epoxy fatty acid polyesters (Carvalho *et al.*, 1999; Purdy and Kolattukudy, 1975). Cutinases have serine in its active site as evident from its crystal structure and this serine, unlike lipases, is not buried under an amphipathic loop (Longhi *et al.*, 1999). Hence, it is accessible to solvent and substrate even in the absence of any interfacial phase. The fatty acids present in cutin are usually of n-C_{16} and n-C_{18} which contain one or more (up to three) hydroxyl groups. The cuticle acts as a protecting layer in plant which prevents desiccation as well as microbial and insect attack (Fett *et al.*, 1992a). 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 as efficiently as pancreatic lipases (Mannesse *et al.*, 1995a). Binding of a non-hydrolyzable substrate analogue to cutinase did not affect the structural arrangements significantly. This feature of cutinase was not observed in any 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. Cutinases are active on both soluble and emulsified triglycerides (Verger and Haas, 1976).

Cutinases have potential use in dairy industry for hydrolysis of milk fat, house hold detergents, oleochemical industry, synthesis of structured triglycerides polymers and surfactants (Dekoster *et al*., 1995), synthesis of ingredients for personal-care products, pharmaceuticals and agrochemicals containing one or more chiral centers (Carvalho *et al*., 1998a; Melo *et al*., 2003). At low water activities, transesterification of fats and oils or stereo selective esterification of alcohols can be achieved using cutinase. Some of these processes are already applied in industry (Carvalho *et al*., 1998a), while others are still under evaluation at research level. Cutinase has also been used for ester synthesis (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. Methyl and ethyl esters of long-chain acids have been used to enrich diesel fuels. 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 shortcomings of lipases are their relatively large size and instability under industrial process conditions. Cutinase has found to be potent catalyst for biodiesel production. Biodiesel is the methyl or ethyl ester of fatty acid produced by transesterification from virgin or used vegetable oils (both edible and non-edible). 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. 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).

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). Microbial organisms 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 studied till date (Fett *et al.*, 1992b; Lin and Kolattukudy 1978; Pio and Macedo, 2007; Rispoli and Shah, 2007). 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).

1.2 Screening of efficient cutinase producing bacteria

Mostly, phytopathogenic fungi are reported to be capable of producing cutinases. 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 on bacteria that appear capable of cutin hydrolysis (Chen *et al*., 2008; Fett *et al*., 1992a; Fett *et al*., 1999; Sebastian and Kolattukudy, 1988). Bacterial cutinases are different from fungal cutinase in respect of their thermal and high pH stability. It has been observed by Sebastian *et al.,* (1987) that bacterial cutinase is

stable even at 70°C, whereas fungal cutinases looses its activity at 45°C. Previously few studies have been done on *Pseudomonas sp* for the production of cutinase (Sebastian and Kolattukudy 1988; Sebastian *et al.,* 1987) using apple cutin as substrate. There might be many more cutinase producing microorganisms as is evident from the study of Belbahri *et al*., (2008) reporting the inter domain gene transfer of cutinases in terms of virulence factors in eukaryotic fungi and prokaryotic bacterial lineages. There are many cutinase producing bacteria isolated and screened till now and we can expect many more from the horizontal gene transfer (Belbahri *et al*., 2008). Thus we can have a favorable species, which may produce more active and stable cutinase.

1.3 Optimization of process parameters for enhanced production of cutinase

Process optimization plays a vital role in industrial production processes in which even small improvements would be decisive for commercial success. In any bioprocess, the improvement in productivity of any metabolite could be achieved through manipulation of nutritional and physical parameters screening and evaluation of nutritional requirements for microorganism is an important step for bioprocess development. Optimization studies involving one-factor-at-a-time approach is tedious, tends to overlook the effects of interaction among the factors and might lead to misinterpretation of results. Statistical methodologies are generally preferred due to their several advantages over the conventional methods (Dasu and Panda 2000; Kumar *et al*., 2009) and statistically designed experiments minimize the error in determining the effect of parameters in an economical manner (Dasu and Panda 2002; Sharma and Satyanarayana, 2006). The Plackett–Burman (PB) factorial designs allow for the screening of main factors from a large number of process variables, and these designs are quite useful in

preliminary studies in which the principal objective is to select variables that can be fixed or eliminated for further optimization processes. In addition, response surface methodology (RSM) is an efficient strategic experimental tool by which the optimal conditions of a multivariable system would be determined (Dasu and Panda 2002; Kumar *et al*., 2009).

1.4 Kinetic modeling of cutinase

Mathematical modeling is necessary to predict the results of industrial fermentations and to choose optimized conditions. Additionally, it helps in understanding the complex mechanisms of growth and product formation of the microorganism (Zinn *et al.,* 2004). Kinetic studies would allow the prediction of fermentation rate, product yield and the control of the fermentation process. As the cutinase production system is of a complex nature, the production of cutinase in a batch system from bacteria is dependent on many factors. Complete quantitative description of its kinetic behavior requires mathematical expressions describing the time course of each important variable (growth kinetics and enzyme production). Several reports (Fett *et al*., 1992a; Fett *et al*., 1992b; Fett *et al*., 1999; Gindro and Pezet, 1999) are available on the production of cutinase using cutin as carbon source. However, kinetics of microbial production of cutinase has not been studied yet, which is essential to evaluate, predict and optimize the enzyme production in any bioprocess system. Microbial production and specificity of enzymes have been shown to be dependent on the nature and concentration of substrate.

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). Chromatographic techniques are widely used as high performance purification steps in biotechnology (Kumar *et al*., 2011; Martin *et al*., 2002). Each enzyme requires specific strategy for purification. Therefore, it becomes necessary to develop strategies for the purification of individual enzyme. Several reports are available on physical/biochemical/molecular properties, such as optimum pH, temperature, metal ion requirement, substrate specificity, inhibition pattern and molecular weight of cutinase produced mostly by fungal species (Koller and Parker, 1989; Purdy and Kolattukudy, 1975; Soliday and Kolattukudy, 1976; Trail and Koller 1990; Trail and Koller 1993; Wang *et al*., 2000). Though quite a few studies have been done on cutinase production from bacterial sources (Fett *et al*., 1992a; Fett *et al*., 1999; Fett *et al*., 1992b; Fett *et al*., 2000; Lin and Kolattukudy, 1980), the enzyme was purified and characterized only from two bacterial sources *Thermobifida fusca* (Chen *et al*., 2008) and *Pseudomonas putida* (Sebastian and Kolattukudy, 1988). 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.

1.6 Esterification reactions catalyzed by cutinase

Esters of short chain fatty acids and alcohols are important components of natural aromas used in the food industry. They are employed in fruit-flavored products like beverages, candies, jam, jelly, baked food, wines, butter, cream, yoghurt, cheese etc. Until now, lipases and esterases are used for the production of a wide range of ester products in non-conventional media. But 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 chain length of substrate is more than two (Macarie and Baratti, 2000; Torres *et al*., 2009). Recently, few reports also available where cutinases (from *Fusarium solani pisi*) (Cunnah *et al*., 1996; De Barros *et al*., 2009a; De Barros *et al*., 2010a; 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 enzyme intermediate between lipase and esterase, which are able to hydrolyze cutin polymer, soluble esters and emulsified triacylglycerol. Cutinases mostly belong to the group of serine-hydrolase family, which contains serine group in its active site. As the reaction is reversible, the enzymes can also catalyze the formation of alcohols and fatty acids from esters. 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. This reversible reaction was

previously shown to follow a Ping-Pong bi–bi mechanism by several authors (Carvalho *et al*., 1999; Serralha *et al*., 2001).

1.7 Transesterification reactions catalyzed by cutinase

Fatty acid esters of alcohols are 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). Cutinase has found to be potent catalyst for biodiesel production. Biodiesel is the methyl or ethyl ester of fatty acid produced by transesterfication from virgin or used vegetable oils (both edible and non-edible) and animal fat. 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. Lipase was widely used for biodiesel production from vegetable oils (Pizarro and Park, 2003; Royon *et al.,* 2007; Shah and Gupta, 2007). Cutinase have been proved to be a very promising catalyst. Cutinase is a hydrolytic enzyme with lypolytic activity and was observed to efficiently catalyze the triglyceride hydrolysis and transesterification reaction in homogeneous as well as reverse micellar medium. 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 Objectives and scope

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 cutinase from *P. cepacia* NRRL B 2320. In order to establish its potential applications in industries, the following objectives have envisaged.

- Isolation, screening and selection of microorganisms producing cutinase.
- Effect of different carbon sources on the production of cutinase from *P. cepacia* NRRL B 2320.
- Development of medium for enhanced production of cutinase from *P. cepacia* NRRL B 2320.
- Growth kinetics of *P. cepacia* NRRL B 2320 under substrate inhibition during cutinase production.
- Purification of cutinase from *P. cepacia* NRRL B 2320.
- Characterization of cutinase from *P. cepacia* NRRL B 2320.
- Applications of cutinase.
    - Esterification reactions catalyzed by cutinase.
    - Transesterification reactions catalyzed by Cutinase.
- Identification, cloning and expression of cutinase encoding genes from *P. cepacia* NRRL B 2320.

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 preparation of cutin and

cutinase specific substrate, screening of microorganisms, optimization of medium components in shake flask and kinetic modeling of cutinase, purification, characterization and applications of cutinase from *P. cepacia* NRRL B 2320 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 optimization, purification, characterization, and applications of cutinase are presented and thoroughly discussed. This chapter addresses screening of microorganisms, optimization of medium components for higher cutinase production, purification and characterization of novel cutinase from *P. cepacia* NRRL B 2320. Finally, the chapter emphasizes on some esterification and transesterification reactions catalyzed by cutinase from *P. cepacia* NRRL B 2320. **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

Cutinase (3.1.1.74) is the smallest member of α/β hydrolase family (Longhi *et al.*, 1999) that degrades cutin. Cutin is the cuticular polymer of higher plants, composed of hydroxy and epoxy fatty acid polyesters (Fig. 2.1) (Carvalho *et al*., 1999; Purdy and Kolattukudy, 1975). The fatty acids of cutin are usually n-C_{16} and n-C_{18} and contain one to three hydroxyl groups. Ester bonds predominate in cutin, although presence of peroxide bridges and ether linkages has also been reported. Cutin is embedded in and covered by a coating of cuticular waxes typically composed of chloroform/methanol-soluble lipids such as hydrocarbons and wax esters. The cuticle is thought to protect plant from desiccation as well as microbial and insect attack (Fett *et al.*, 1992a). The enzymatic hydrolysis of cutin is one of the first steps of infection. Cutinase has found to be associated with the ability of fungal pathogens to penetrate into their hosts (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). Reports available on disruption of several cutinase genes did not confirm involvement of respective cutinases in plant infection (Crowhurst *et al.*, 1997; Köller *et al.*, 1995; Stahl *et al.*, 1994; Sweigard *et al.*, 1992; van der Vlugt-Bergmans *et al.*, 1997; van Kan *et al.*, 1997; Yao and Köller 1995). However, the release of other cutinolytic esterases from germinating spores has been reported for 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) has been suggested. Cutinase is also member of serine-hydrolase group of enzyme. It is evident from the crystal structure of cutinase that this serine is not buried under an amphipathic loop (Longhi *et al.*, 1999). Hence, it is accessible to solvent and substrate even in absence of any interfacial phase. Cutinase has ability to hydrolyse a variety of synthetic esters and show activity on short- and long-chain emulsified triacylglycerols (Mannesse *et al.*, 1995a).

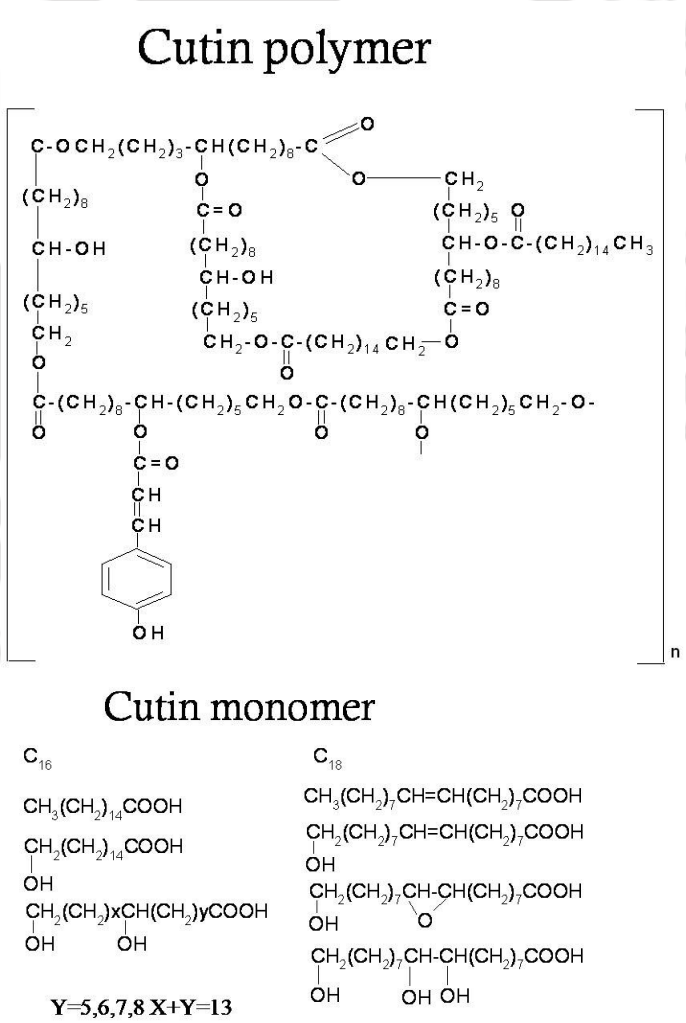


Fig. 2.1 Structure of cutin.

2.2 Cutinases, lipases and esterases

Hydrolases group of enzyme usually showed very wide substrate specificity. They can hydrolyse peptides, amides and halides in addition to esters and triglycerides. The fact that enzymes which show esterase activity are also able to hydrolyse non-ester bonds raises interesting questions on the terminology and classification of these enzymes (Junge and Kirsch, 1973). Previously, these enzymes have been classified according to their known substrate specificity. Esterases are group of enzyme that hydrolyses ester linkages by the addition of a water molecule. Generally, an esterase is specific for either the alcohol or the acid moiety of the substrate, but not for both. 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. This enzyme 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. Besides the above mentioned esterases, 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 the physical state of the substrate is most likely a contributing factor towards the substrate specificity. Long-chain fatty acids are typically insoluble or at least poorly soluble (emulsions). Thus the lipase has capacity to identify an insoluble or heavily 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. The number of esterases and lipases that have been studied has increased tremendously 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 triacylglycerole lipases contain all fungal, bacterial and pancreatic lipases. The esterases are split into groups such as the cutinase group, acetylcholineesterase 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. Such hydrophobic patches could be involved in the docking to the lipid-water interface. Esterases (and a few lipases) do not display a lid structure. Prominent case of this is cutinase from *Fusarium solani pisi* (Longhi *et al*., 1997). The absence of any significant structural rearrangements upon binding to a non-hydrolyzable substrate analogue represents an important feature of cutinase, which is probably shared only by *Candida antarctica* lipase B (Longhi *et al*., 1996). Contrary 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). All lipases investigated so far vary widely in size and amino acid sequence. However, they 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 (Longhi *et al.*, 1997). Lipases have catalytic machinery similar to those present in serine proteases. They are characterized by the triad Ser, His, Asp (Glu) 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).

2.2.1 Cutinase structure

The three dimensional structure of *F. solani f. pisi* cutinase cloned and expressed in *E. coli* was solved at 1.6 Å resolution (Martinez *et al.*, 1992) and the resolution was recently extended at 1.0 Å (Jelsch *et al.*, 1998; Longhi *et al.*, 1997). Cutinase is a 197-residue protein in a compact one domain molecule 45×30×30 Å3 in size. Cutinases have a molecular weight around 22,000 daltons with highly conserved stretches, which include four invariant cysteines, forming two disulfide bridges. *F. solani f. pisi* cutinase has an isoelectric point of 7.8 (Petersen *et al.*, 1997; Egmond *et al.*, 1996). Cutinase is also an α/β hydrolase and has a central β-sheet consisting of five parallel strands covered by two and three helices on either side of the sheet. Cutinase belongs to the class of the serine esterases. 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. The catalytic triad Ser120, Asp175 and His188, is accessible to the solvent and it is located at one extremity of the protein ellipsoid, and is surrounded by the loop 80-87 and by the more hydrophobic loop 180-188 (Jelsch *et al.*, 1998). To date, around 40 X-ray structures of cutinase and its mutants and inhibitor conjugates have been solved (Jelsch *et al.*, 1998; Longhi *et al.*, 1996; Longhi *et al.*, 1997; Martinez *et al.*, 1992; Martinez *et al.*, 1993; Martinez *et al.*, 1994; Nicolas *et al.*, 1996; Prangé *et al.*, 1998). Lipase activity is greatly enhanced at lipid-water interfaces. They

do not hydrolyse substrates under the critical micellar concentration (cmc) and display a high activity beyond it. This phenomenon of interfacial activation is necessary for lipases to display their activity due to the existence of a hydrophobic lid (or flap) in their structures, which covers the catalytic site. The conformational change of the lid is thought to be closely related to the interfacial activation phenomenon process (Martinez *et al.*, 1994). The rearrangement of the lid results in a large increase in the hydrophobic surface stabilized by interaction with the interface.

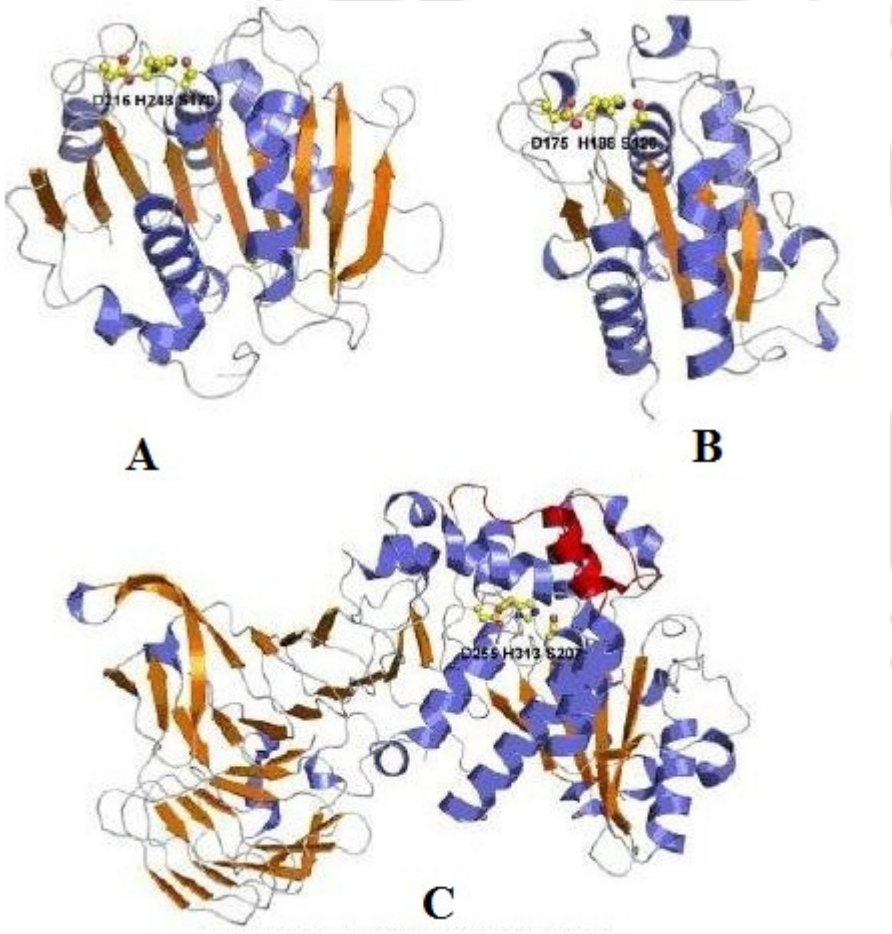


Fig. 2.2 The structure of cutinase from (A) *T. fusca*, (B) *F. solani pisi*, and (C) lipase from *Pseudomonas sp*

Also, a consequence of the movement of the lid in lipases is the formation of the oxyanion hole during the nucleophilic attack on the scissile bond of the substrate (Martinez *et al*., 1994). Cutinases differ from classical lipases, as they do not exhibit interfacial activation. 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. 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 is that the oxyanion hole is preformed in cutinase instead of being induced by ligand binding and seems to be stabilized by cutinase Ser42 side chain (Nicolas *et al.*, 1996).

2.3 Production of cutinase

There are reports in the literature on the production of cutinase from microbial sources (Fett *et al.*, 1992a; Fett *et al.*, 1992b; Fett *et al.*, 1999; Fett *et al.*, 2000; Kim *et al.*, 2003; Lin and Kolattukudy, 1980; Purdy and Kolattukudy, 1975; Pio and Macedo, 2007; Sebastian and Kolattukudy, 1988; Sebastian *et al.*, 1987) and from plant source (Maiti *et al.*, 1979). 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. Cutinase producing microorganisms include fungi, bacteria and actinomycetes.

2.3.1 Isolation and screening of cutinase producing microorganisms

Some cutinase producing microorganisms studied vividly are given in the Table 2.1. As it is evident from the study of Belbahri *et al*., (2008), the inter domain gene transfer of cutinases in terms of virulence factors in eukaryotic fungi and prokaryotic bacterial lineages. There are many cutinase producing bacteria isolated and screened till now and we can expect many more from the horizontal gene transfer (Belbahri *et al.*, 2008). Thus we can have a favorable species, which may produce more active and stable cutinase. Though microbial cutinases are mostly produced by submerged culture (Fett *et al.*, 1992; Fett *et al.*, 1999; Fett *et al.*, 2000; Ferreira *et al.*, 2004; Macedo and Pio, 2005) solid state fermentation has also been reported (Macedo and Fraga, 2007). The production of cutinase is influenced by the type and concentration of carbon and nitrogen sources, culture pH and temperature, and dissolved oxygen concentration (Du *et al.*, 2007). Cutin was used as a carbon source 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).

2.3.2 Effect of nutrients

Cutin hydrolysate induced the secretion of an extracellular cutinase by *F. solani f. sp. pisi* (Lin and Kolattukudy, 1978). The rate of cutinase production depends on the amount of cutin hydrolysate added to the medium containing glucose and saturation was achieved at 80 µg/ml of cutin.

Table 2.1 Cutinase Producing micro-organisms.

Source	Genus	Species	Reference
Bacteria	*Streptomyces*	*Scabies*	Lin and Kolattukudy, 1980
		acidiscabies	Fett *et al*., 1992a
		badius	Fett *et al*., 1992a
	Pseudomonas	*Syringae pvar tomato*	Bashan *et al*., 1985
		putida	Sebastian and Kolattukudy, 1988
		putida	sebastian *et al*., 1987
		mendocina	Kim *et al*., 2003
		aeruginosa	Fett *et al*., 1992b
	Thermomonospora	*fusca*	Fett *et al*., 1999
	Thermoactinomyces	*vulgaris*	Fett *et al*., 2000
	Thermobifida	*fusca*	Chen *et al*., 2008
Fungi	*Fusarium*	*Solani pisi*	Purdy and Kolattukudy, 1975
		oxysporium	Pio and Macedo, 2007
		roseum culmorum	Soliday and Kolattukudy, 1976
	Colletotrichum	*kahawae*	Chen *et al*., 2007
		gloeosporioides	Chen *et al*., 2007
	Monilinia	*fructicola*	Wang *et al*., 2002
	Venturia	*inaequalis*	Trail and Koller, 1990
	Alternaria	*brassicicola*	Trail and Koller, 1993
Pollen	*(Nasturtium pollen)*		
	Tropaeolum	*majus*	Maiti *et al*.,1979

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. It was also observed 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 (Lin and Kolattukudy, 1978). Experiments with derivatives and analogues of ω-hydroxy C_{16} 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*-C_{16} 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 μMol min^{-1} ml^{-1}) was observed by Pio and Macedo (2007) after 48 hour 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 *Colletotrichum 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 Purification of cutinases

The purification of cutinases from 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). Two types of cutinase (cutinases A and B) were purified to homogeneity by Trail and Koller (1993) from *Alternaria brassicicola*. Affinity chromatography is a very efficient technique for purifying an enzyme. In this method suitable ligand is necessary, which should have high specificity but weakly bound to the enzyme, so that it can be easily eluted.

2.4.1 Purification by affinity chromatography

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). TFKs are highly polarized inhibitors of serine esterases and proteases (Brodbeck *et al.*, 1979; Hammock *et al.*, 1984). 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. Previously, TFK had been shown to inhibit juvenile hormone esterases. Since, TFKs are slowly reversible covalent inhibitors of serine esterases (Thomas *et al.*, 1990), the proteins bound to the affinity gels usually would be eluted by competition with another soluble TFK inhibitor (Hanzlik *et al.*, 1987; Shiotsuki *et al.*, 1994; Thomas *et al.*, 1990). Cutinase can also be purified by aqueous two-phase system, depending on its partition coefficient in the two phases.

2.4.2 Purification by aqueous two-phase system

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. The partition coefficients in most of the experiments close to or below one and the yield for the top phase were low. Cutinase partition behavior seems to be influenced minimally by changing PEG average molecular mass (from 1000 to 8000). It was observed that the increase in partition coefficient with the system pH in ATPSs with HPS, whose impurities change the electrostatic interactions in the system. At pH 8.0, K_p (protein partition coefficient defined as ratio of protein top and bottom phase concentration) and K_{cut} (cutinase partition coefficient defined as ratio between cutinase top and bottom phase activity/ml) are generally higher than the other pH values tested. So, there was stronger cutinase attraction to the top, PEG-rich and more hydrophobic phase occurred at a pH near its pI. The reported highest values for the cutinase partition coefficient occurred at pH 4.0, with ammonium sulphate in systems with HPS. At

pH 8.0, K_{cut} showed little alteration with the different salt types and concentrations, because at this pH cutinase is near its pI. Hence, it was less influenced by electrostatic changes produced by the salts (Almeida *et al.*, 1998). 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. 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). The pI of *F. solani pisi* cutinase has been determined by different methods, with consistent results. Egmond *et al*., (1996) obtained a value of 7.8 and Goncalves *et al*., (1996b) a value of 7.9, and these data were supported by the titration of protein residues results (Petersen *et al*., 1997). 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. Low ionic strength favors the extraction process. 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 protein and cutinase overall yields for the global extraction and back extraction processes were 38% and 45%, respectively (Carneiro-da-Cunha *et al*., 1994b). 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. The potentiality of the extraction process seems to be limited by the amount of extractant used for the selected experimental conditions. The optimal conditions for the direct extraction of cutinase in the presence of cell debris led to a purification factor of 1.6 and yields of protein and activity of 14% and 21.8%, respectively (Carneiro-da- Cunha *et al.*, 1996c). 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 minutes (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.

Table 2.2 Fold purification and recovery of cutinase using different purification methods

Method used	% Recovery	Fold purification	Reference
acetone precipitation	78	3	Sebastian and Kolattukudy, 1988
DEAE 52	50	8	
QAE-Sephadex	9	78	
Sepharose-6B	6	99	
Sepharose G-100	3	207	
TFK affinity Chromatography	-----	-----	Wang *et al*., 2000
Fraction eluted with Phosphate	16.2	2.6	
Fraction eluted with acetate	6.5	3	
Fraction eluted with ligand	65.6	14.2	
Ammonium sulphate precipitate	-----	1	Chen *et al*., 2007
Supernatant of acetic acid precipitate	-----	5.2	
DEAE (pH 8.5, major peak of void volume)	-----	15.4	
Sulfopropyl (pH 7.6, void volume)	-----	21.2	
DEAE (pH 9.5, void volume)	-----	6.1	
Mono-Q (pH 9.5, void volume)	-----	0.9	
Ammonium sulphate precipitate	-----	1	
Supernatant of acetic acid precipitate	-----	2.9	
DEAE (pH 8.5, major peak of void volume)	-----	7.1	
Sulfopropyl (pH 7.6, void volume)	-----	12.4	
DEAE (pH 9.5, void volume)	-----	4.7	
Mono-Q (pH 9.5, void volume)	-----	0.8	
Ammonium sulphate precipitate	42.3	2.1	Chen *et al*., 2008
Phenyl HP Sepharose FF	12.4	6.1	
DEAE Sepharose	9.3	7.6	

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.

2.4.3 Purification by 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 (Table 2.2). 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 (Table 2.2). The purification fold and recovery of cutinase obtained by different techniques have been shown in Table 2.2.

2.5 Characterization of cutinase

In order to optimize the enzyme performance under process conditions 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 and specificity *etc*.

2.5.1 Molecular properties

Molecular weight of *Pseudomonas putida* cutinase was reported to be 30 kDa (Sebastian and Kolattukudy, 1988), where as that of fungal cutinase was around 22-26 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 (Chen *et al.*, 2007). Cutinase is the smallest member of the α/β hydrolase fold family. The primary structure of cutinase has been determined from the nucleotide sequence of cloned cDNA by Soliday *et al.,* (1984). 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 *Thermobifida 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 that 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. Given their high sequence identity, it is not surprising that Tfu 0882 and Tfu 0883 have similar physical properties. Catalytically, both Tfu 0882 and Tfu 0883 are relatively versatile in utilizing both insoluble triglycerides (triolein) and soluble esters (*p*NPB) as substrate in addition to cutin. Moreover, the two enzymes share similar temperature and pH dependence profiles and thermostability. Therefore they may be defined as cutinase isoenzymes in *T. fusca*. Although, *Tfu 0882* and *Tfu 0883* are sequential in the genome, likely due to a gene duplication event, they do not appear to be in an operon as determined by the operon prediction tool VIMSS (www.microbesonline.org/operons) (Price *et al.*, 2005). Moreover, there is no synergism between the two enzymes in cutin degradation. Thus, further studies are needed to understand why there are two sequential genes for *T. fusca* cutinase. Sequence analysis suggested that *T. fusca* cutinases belong to the α/β-hydrolase fold superfamily (Nardini and Dijkstra, 1999). Enzymes in this superfamily exhibit a wide variety of hydrolytic activities as discussed previously. However, they all adopt a conserved three-dimensional fold and are believed to have evolved from a common ancestor (Nardini and Dijkstra, 1999). As expected, the homology model of Tfu 0883 displays a canonical α/β -hydrolase fold with a Ser170-His248-Asp216 triad and a preformed oxyanion hole, suggesting a classic serine hydrolase mechanism involving two tetrahedral transition states and an acyl-enzyme intermediate (Dodson and Wlodawer, 1998). Indeed this mechanism was supported by PMSF-

mediated irreversible inhibition of Tfu 0882 and Tfu 0883 as well as site-directed mutagenesis of the catalytic serine (to alanine) in both enzymes. A notable feature of the *T. fusca* cutinase model was that the enzyme does not contain a lid insertion commonly observed in true lipases (Angkawidjaja *et al.*, 2007; Brzozowski *et al.*, 2000; Brzozowski *et al.*, 1991; Lang *et al.*, 1996), and exposes its nucleophilic serine to the solvent. In lipases, the lid insertion was reported to be involved in the interfacial activation in which it undergoes conformational change in response to adsorption at the oil-water interface (Jaeger *et al.*, 1994). The absence of such a lid insertion suggests that the *T. fusca* cutinases should belong to a class of α/β-hydrolases different from the classic lipases. On the other hand, the open active site of *T. fusca* cutinase readily explains the ability of the enzyme to accommodate large substrates like cutin. Further studies, especially x-ray crystallography studies of ligand bound cutinase may reveal the structural basis of substrate recognition. Comparative study on biochemical characterization of bacterial and fungal cutinases indicated that they have similar substrate specificity and catalytic properties except that *T. fusca* cutinases demonstrate remarkably greater thermo-stability. This unique feature may render *T. fusca* cutinases practically more amenable for industrial applications. Although both *T. fusca* and *F. solani pisi* cutinases belong to the α/β -hydrolases superfamily and contain an open active site, the bacterial enzymes have significantly longer sequences and demonstrate no sequence similarity to the fungal enzyme. Moreover, the fungal cutinase contains neither the two N-terminal β strands of the canonical α/β - hydrolase fold nor the unique C-terminal extension of Tfu 0883. Thus, the bacterial and fungal enzymes must have undergone extensive evolutionary differentiation and are suggested to be classified into prokaryotic and eukaryotic cutinase subfamilies, respectively.

2.5.2 Substrate specificity

The bacterial cutinase catalyses hydrolysis of p-nitrophenyl esters of C_4 to C_{16} fatty acids. A general trend of increasing K_m, and decreasing V_{max} was observed as the chain length of acyl group was increased (Sebastian and Kolattukudy, 1988). However, these changes were not as large as those observed with some fungal cutinases. As the chain length of the acyl moiety increased from C_2 to C_{12}, the V_{max} of many fungal cutinases decreased from 200 to 1000-fold (Sebastian and Kolattukudy, 1988). Cutinase hydrolyses 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. Hydrolysis of lipid by cutinase from *F. solani pisi* was investigated with the oil-drop tensiometer technique by Flipsen *et al.*, (1996). 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 min^{-1}, K_m=395 mM and K_{cat}/K_m=4.1 $min^{-1}mM^{-1}$. 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 mM and K_{cat}/K_m=8.2 $min^{-1}mM^{-1}$ was observed. These results were in accordance with others obtained on the selectivity and specificity of cutinase (Mannesse *et al.*, 1995a). 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. 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 k_{cat}=6.51 min^{-1} and K_m=35.6 mM (Gonçalves *et al.*, 1995). The apparent specific constant (k_{cat}/K_m) was 0.183 $min^{-1}M^{-1}$. 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). Chen *et al.*, (2010) studied the kinetic constants of Tfu 0882, Tfu 0883 and FspC, and observed that the results showed that all three cutinases showed Michaelis–Menten kinetics with FspC exhibiting the highest affinity (lowest K_m value) for the substrate. In addition, among the three enzymes, FspC exhibited the highest catalytic efficiency with a K_{cat}/K_m of 3.214 s^{-1} M^{-1}. Interestingly, the catalytic efficiency (K_{cat}/K_m) of Tfu 0883 was twice as much as that of Tfu 0882 even though they showed a 93% identity in amino acid sequences and an almost identical structure of their active sites.

2.5.3 Effect of inhibitors

Active serine directed reagents such as di-isopropylfluorophosphate and (O,O)-diethyl-(3,5,6-trichloro)-2-pyridylphosphorothioate, which does not have any effect on pollen cutinase but inhibits bacterial and fungal cutinase activity. 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). Chen *et al.*, (2010) studied the effect of different metal ions on recombinant cutinase activity from *T. fusca* and *F. solani pisi.* They observed that EDTA (1 mM or 10 mM) did not affect activities of cutinases, suggesting that the cutinases did not require divalent cations for their activity. They also incubated cutinases in presence of 1mM of divalent metal ions, Mn, Co, Ni, Mg, Ba, Cu, or Ca. The authors observed that these metal ions did not exhibit a significant effect on the enzyme activity, whereas Zn, Fe, or Pb showed a medium inhibitory effect. However, Cr inhibited most enzyme activity, whereas Hg completely inactivated the cutinases. Application of industrial enzymes often involves relatively harsh conditions such as the presence of surfactants and organic solvents. Chen *et al*., (2010) also tested activity of the cutinases in the presence of the nonionic surfactants, Triton X-100, Tween 20, and the anionic surfactants SDS and Sodium tauro-deoxycholate (TDOC). They found that, at concentrations of 1 mM and 10 mM, Triton X-100 and Tween 20 inhibited both cutinases from *T. fusca* but did not significantly reduce FspC activity. They also observed that SDS inhibited all three enzymes but TDOC, on the other hand, stimulated FspC 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 reported that Tfu 0882 appeared to be fairly stable in the presence of this surfactant with 71.43% activity remaining at 100 mM. Tfu 0883 was slightly stimulated at TDOC concentrations between 1 mM and 50 mM and fully retained its activity at 100 mM. FspC was significantly stimulated by TDOC with the highest stimulatory effect at 10mM and almost full activity remains at 100 mM. TDOC is an anionic surfactant with a bulky side chain, which may bind to the hydrophobic sites of proteins and metal ion requirement. However, they differ significantly in their sensitivity to surfactants and dramatically in their sensitivity to organic solvents. Considering their versatile hydrolytic activity, good tolerance to surfactants, superior stability in organic solvents, and superior thermostability of *T. fusca* cutinases may have promising applications in related industries preventing their aggregation and rendering them more stable (Creveld *et al.*, 2001). FspC is less stable than Tfu 0882 and Tfu 0883 in solution, so that TDOC showed more effective in stimulating FspC. In addition, 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. Cutinases have been reported to show esterification and transesterification activity and have potential for use in the production of biodiesel (Cunnah *et al.*, 1996; Serralha *et al.*, 1998). Such applications often involve the use of organic solvents, therefore it is important to evaluate their stability in organic solvents. Chen *et al*., (2010) also

studied the solvent tolerance of the cuinases. 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, 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.

2.5.4 Effect of pH and cutin concentration on cutinase function

Optimum pH for *Venturia inaequalis* cutinase is 5 to 6 (Ko1ler *et al.*, 1989) whereas other cutinases from *F. soloni f. pisi* (Soliday and Kolattukudy, 1976) and *F. roseumculmorum* shows their optimal activity at pH 10 (Soliday and Kolattukudy 1976). 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 hour incubation time for pollen cutinase (Maiti *et al.*, 1979).

2.6 Strategies for improvement of cutinase activity and stability

Activity and stability of cutinase can be improved by several strategies such as production of recombinant cutinase and immobilizing the cutinase.

2.6.1 Genetic engineering strategy

Cutinase gene from *F. solani f. pisi* and *C. capsici* ATCC 48574 was cloned into *Escherichia coli KH 802* and sequenced. The nucleotide sequence of cutinase gene completely matched with the cDNA induced by cutin (Soliday *et al.*, 1984). High level production of recombinant cutinase was reported in *E. coli* WK6 (Lauwereys *et al.*, 1991) and *Pichia 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. Cutinase has been cloned and expressed in heterologous hosts. A *F. solani f. pisi* cDNA library containing the cutinase gene was constructed, and transferred to the plasmid pUC19. The cloned gene was expressed behind the signal peptide for alkaline phosphatase (*phoA*) in order to direct the cutinase to the *E. coli* periplasm region. The fusion of the sequences for *phoA* and cutinase was carried out by single oligonucleotide-directed mutagenesis using the pMa/c type of plasmids. In the resultant pMa/c5-CUF vector the *phoA*/cutinase gene fusion is under the transcriptional control of the IPTG-inducible Ptac promoter (Lauwereys *et al.*, 1991). *E. coli* WK6 was used as the host for expression. High levels of cutinase have been synthesized and due to its periplasmic location the recombinant cutinase can be easily purified in large quantities (Lauwereys *et al.*, 1991). More recently, 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. The primary structure of cutinase from *F. solani f. pisi* was determined from the nucleotide sequenced of cloned cDNA (Soliday *et al.*, 1984). Blot analysis showed that cutinase mRNA contained 1050 nucleotides. A peptide isolated from a trypsin digest of cutinase was sequenced and the amino acid sequences as well as the initiation and termination codons were used to identify the coding region of cDNA (Soliday *et al*., 1984). 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). A 2,818-basepair (bp) segment was sequenced, revealing a 690-nucleotide open reading frame that was identical to that found in the cutinase cDNA with a single 51-bp intron. Due to the importance of the potential applications of cutinase, this enzyme has been cloned and expressed in heterologous hosts. A *F. solani f. pisi* cDNA library was constructed (Lauwereys *et al.*, 1991) based on the work of Soliday *et al*., (1989). It has been proposed that glycine at position 32 in the cutinase primary translation product corresponds to the N-terminal residue of mature cutinase. The fusion of the *phoA* signal to Gly-32 resulted in a typical processing site; therefore, to compose a probable prokaryotic cleavage site, the first 16 residues of pre-cutinase were substituted by the *phoA* signal and at the same time, Leu-17 was replaced by an Ala. This plasmid combines a high copy number (100 to 200 per cell) with a high mitotic stability under nonselective conditions (Lopes *et al.*, 1989). Transformants were obtained in 5 days and were grown in a fed-batch culture with the accumulation of the

extracellular cutinase in the fermentation medium (Longhi *et al.*, 1996). Further studies involving *Aspergilli* strains and *S. cerevisiae* harboring other plasmids have also been reported. A copy of the cutinase cDNA from *F. solani f.* sp. *Pisi* was constructed starting from the synthetic oligonucleotide. Sequencing was followed by cloning into the plasmid pUC9, resulting in the cutinase vector plasmid achieved in *S. cerevisiae* SU50 and *A. awamori* using expression vector pUR7320 and plasmid pUR7382 (van Gemeren *et al.*, 1995). The efficiencies of secretion of the wild-type (CY000) and hydrophobic mutant (CY028) cutinase 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). 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 *p*NPB hydrolyzing activity in the culture supernatant of

transformed *E. coli* cells harboring plasmid pET20b-Tfu 0883 was 180 units ml^{-1}, which was 782-fold higher than that of un-transformed *E. coli* cells and 9.5-fold higher than that of cutin-induced *T. fusca* cells. The *p*NPB hydrolyzing activity in the culture supernatant of transformed *E. coli* cells harboring pET20b-Tfu 0882 plasmid was 69 units ml^{-1}, which was 300-fold higher than that of un-transformed *E. coli* cells and 3.6-fold higher than that of cutin-induced *T. fusca* cells. They had purified recombinant enzymes to homogeneity in a single step by nickel affinity chromatography and exhibited a specific activity of 458 units mg^{-1} for Tfu 0883 and 223 units mg^{-1} for Tfu 0882 (Chen *et al*., 2008).

2.6.2 Immobilization strategy

To enhance the activity further, *F. solani pisi* recombinant cutinase was immobilized on sodium form of zeolite Y 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-NH_2 and Biosil –dextran-NH_2 retains almost 97%

and 58% of initial activity, respectively after 50 days at 4°C (Goncalves *et al.*, 1999, Goncalves *et al.*, 1996a). The stability of bacterial cutinase (*Pseudomonas spp*) was measured by incubating the extracellular fluid at different temperatures for different periods and remaining enzyme activity was measured at 30°C. The enzyme was completely stable at 60°C for 1 h, and retained 85% of the activity after 1 h at 70°C. Fungal cutinases (*F. solani pisi,f. roseum culmorum, C. gloeosporoides and F. roseum sambucinum)* are unstable above 45°C and lost more than 80% of the activity in 1 h at 60°C (Sebastian *et al.*, 1987). The optimum pH of the cutinase varies between wide ranges 8.5 to 10.5 (Sebastian *et al.*, 1987). 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 was very low, 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).

2.7 Cutinase function

The definition 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). A few studies aimed to elucidate whether cutinase can be considered as a member of the family of lipases or rather as an esterase (Egmond and van Bemmel, 1997). Time resolved fluorescence was used to observe directly the formation of enzyme-lipid aggregates, leading to the conclusion that cutinase behaves like

a lipase rather than an esterase. Even below the CMC of micellar substrates, kinetically relevant cutinase complexes may be formed. This also explains the exposed active site of serine, which behave like interfacial activated lipases. A lipase stereo and regioselectivity towards tricylglycerols and diacylglycerols study, using a monolayer technique was undertaken to complement the structural data (Rogalska *et al.*, 1997). The results obtained with prochiral triacylglycerols showed that *F. solani* cutinase and mammalian gastric lipases display a distinct stereo preference for position sn-3, while *Rhizomucor miehei* and *Pseudomonas sp*. lipases show some preference for position sn-1. Triacylglycerol analogues have been used to study the substrate specificity and selectivity of cutinase and its mutants (Egmond and van Bemmel, 1997; Mannesse *et al.*, 1995a; Mannesse *et al.*, 1995b; Mannesse *et al.*, 1997; Rogalska *et al.*, 1997; Zandonella *et al.*, 1995). Triglyceride analogues were synthesized replacing one of the primary acyl ester functions by an alkyl group, while the secondary acyl ester bond was replaced by an acyl amino bond (Mannesse *et al.*, 1995a). These synthetic triglycerides contain only one hydrolyzable ester bond and they are suited to study the influence of the chain length at the 1-, 2-, and 3-position on lipase activity and on stereoselectivity. It was found that cutinase is very sensitive to the length and distribution of the acyl chains, being highly specific for hydrolysis of the sn-3 acyl chain of triacylglycerols being the highest activities obtained for chain lengths of about five carbon atoms (Egmond and van Bemmel, 1997; Mannesse *et al.*, 1995a). This indicates that the binding cleft of cutinase, that accommodates the chain to be split off, must be relatively short. Regarding enantioselectivity, the enzyme preferentially hydrolyses the (R)-enantiomers (Mannesse *et al*., 1995a). Some important reactions catalysed by cutinase are given in the Table 2.3.

2.8 Industrially important applications

The ability of cutinases to hydrolyse a varity of short and long chain esters and its stability and activity in presence of oxidizing agents, detergents and protease makes it industrially very important especially for detergent industry, pharmaceutical industry, food industry, agriculture and many more. 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; Poulose and Kolattukudy, 1995). Cutinases also have been suggested for use with surfactants in detergents (Kolattukudy and Poulose, 1988). 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 higher than that of any lipase reported so far. 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.

2.8.1 Cutinase in detergent and laundry industry

Cutinases have been evaluated as a lipolytic enzyme in laundry and dishwashing detergent formulations (Egmond and van Bemmel, 1997; Okkels, 1997a; Uniliver, 1994a). They have stability oxidatively such as in H_2O_2. 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 Poulose, 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 Bemmel, 1997).

2.8.2 Cutinase in production of biodiesel

Cutinase has found to be potent catalyst for biodiesel production. Biodiesel is the methyl or ethyl ester of fatty acid produced by transesterfication from virgin or used vegetable oils (both edible and non-edible) and animal fat. 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. Lipase was widely used for biodiesel production from vegetable oils (Pizarro and Park, 2003; Royon *et al*., 2007; Shah and Gupta, 2007). 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). The advantage of using cutinase for biodiesel production over lipase is that it can hydrolyse both soluble and emulsified triacylglycerol and does not require interfacial activation (Martinez *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/ g of enzyme.

2.8.3 Cutinase in ester synthesis

Cutinase has also been used for ester synthesis (Sebastião *et al*., 1993; Sebastião *et al*., 1992). The esters produced from short-chain fatty acids have applications as flavoring agents in food industry. Methyl and ethyl esters of long-chain acids have been used to enrich diesel fuels. 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-nitrophenylbutyrate, and triglycerides, such as triolein and tributyrin.

Table 2.3 Reported biocatalytic applications of cutinase.

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

Type of reaction		Substrates	Enzymatic Preparation	Reference
Synthesis	Esterification	Oleic acid+hexanol	Reversed micelles of AOT/isooctane	Sebastião *et al*., 1993,Sebastião et al., 1992
		Caprylic acid+butanol	Substrates in organic media	Sarazin et al., 1992, Sarazin *et al*., 1995
		Butyric acid+2-butanol	Phosphatidylcholine/ isooctane reversed micelles	Pinto-Sousa *et al*., 1994
		Oleic acid+glycerol	Organic solvents	Melo *et al*., 1995a
		Hexanoic acid+hexanol	CTAB reversed micelles	Cunnah *et al*., 1996
			Immobilization onto Accurel EP 100 in SC CO_2	Sereti et al., 1997
		Lauric acid+pentanol	Reversed micelles of AOT/isooctane	Papadimitriou *et al*., 1996
		Short chain fatty acid (C4-C18) + ethanol	Lyophilized enzyme in organic media	De Barros *et al* 2009a
		Caproic acid+ ethanol	Lyophilized enzyme in organic media	De Barros *et al*., 2010a
				De Barros *et al*., 2010b
		Methyl propionate+propanol	Gas/solid system	Lamare and Legoy, 1995 ,Lamare *et al*., 1997
	Transestrification	Butyl acetate+hexanol	Reversed micelles of AOT/isooctane	Carvalho *et al*., 1997a; Carvalho *et al*., 1998b
			Reversed micelles of CTAB/isooctane	Cunnah *et al*., 1996

Type of reaction		Substrates	Enzymatic Preparation	Reference
			Immobilization onto zeolites	Serralha *et al*., 1998
		Methanol + Triolien	Reversed micelles of AOT/isooctane	Badenes *et al*., 2010a
			Reversed micelles of AOT/isooctane	Badenes *et al*., 2010b
			Reversed micelles of AOT/isooctane	Badenes *et al*., 2011a

Unlike a specific esterase, cutinases can also catalyse the rapid hydrolysis of long-chain triglycerides, such as triolein, in emulsion form. The substrate dependence profile of the catalytic activity of cutinases on tributyrine showed (Longhi *et al*., 1999) that its enzymatic activity was not influenced by the formation of an interface. At the substrate concentration where aggregates of tributyrine are formed, no appreciable increase in enzyme activity could be noticed, quite in contrast to what is known for lipases (Fleet *et al*., 1983; Verger *et al*., 1976). 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*., 1993; Sebastião *et al*., 1992) 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*., 1993; Sebastião *et al*., 1992). 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. This reaction was used as a model system to evaluate the reactants solvation effects on cutinase, namely solvation of reactants. The results showed that the optimum water activity decreased with increasing polarity of the solvent. Substrate solvation was found to be most strongly affected by water in acetonitrile. To relate to solvation, kinetic parameters were determined at two water activities in each solvent. A Ping-Pong model with competitive inhibition by hexanol and butyric acid was developed. The Michaelis constants for hexanol in hexane at two a_w values of 0.44 and 0.69 were 299 and 231 mM, respectively (Carvalho *et al*., 1998a). These values are higher than the K_m=86 mM, obtained for hexanol in the reaction catalysed in AOT reversed micelles (Sebastião *et al*., 1993). This may result from higher diffusion effects present in the supported enzyme when compared with the microencapsulated cutinase. The esterification of hexanoic acid with hexanol has also been performed in some studies (Cunnah *et al*., 1996; Sereti *et al*., 1997). In supercritical CO_2 (Sereti *et al*., 1997), this reaction was very slow when compared with a CTAB reversed micellar system (Cunnah *et al*., 1996). The initial reaction rate in supercritical CO_2 is considerably lower (around 1.1 nmol min^{-1} mg^{-1} protein) than that in CTAB reversed

micelles, (14 mmol min^{-1} mg^{-1} protein). Also 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 hours. The ester synthesis of butyric acid and 2-butanol was carried out by cutinase microencapsulated in nonionic surfactant, phosphotidylcholine (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. The apparent Michaelis constants obtained for 2-butanol and butyric acid were 47.2 and 38.8 mM, respectively. 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 w_o=9. Up to w_o 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 (C_4) and valeric acid (C_5) as compared with the shorter or longer chain length acid. De Barros *et al*., (2009b) observed that cutinase has substrate specificity towards short- chain fatty acids (C_4-C_5) for synthesis of ethyl esters by esterification in iso-octane, where as the specificity shifted to C_{10}-C_{18} when cutinase was used in miniemulsion system. De Barros *et al*., (2010b) obtained higher stability of cutinase, when esterification reactions were carried out in fed batch mode using consecutive feeding pulse of substrate (alcohol).

2.8.4 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 (Poulose and Boston, 1994).

2.8.5 Role of cutinase in biodegradation

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. It is characterized by a relatively inefficient utilization of its raw materials resulting in high amounts of liquid and solid wastes to be released into rivers and water streams. Fats must be

removed from the hides, because they hinder the access of the chemicals used in leather processing towards the skins, as well as lead to the formation of stains of insoluble chromium soaps (Christner, 1992). However, since the waste fats block factories conduits, a treatment of these wastes is required. 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 for degrading many toxic substances. Cutinase has also been found to degrade malathion. Malathion is an organophosphate insecticide and acaricide that have been used for the control of insects on field crops, fruits, vegetable, live stock 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). Fungal cutinase can degrade malathion much faster than yeast esterase with high stability. 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 including dipropyl phthalate (DPrP) and dimethyl phthalte (DMP) were classified as priority pollutants by the United States Environmental Protection Agency (Sung *et al*., 2003). Cutinase had been reported to degrade DPrP (Kim *et al*., 2005).

2.8.6 Cutinase in textile 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 crosslinked isopeptide via amide bonds, which makes the wool surface highly hydrophobic and the enzymatic degradation to the cuticle cells restrictedly. Some native proteases can penetrate through the intercellular cement and cause unacceptable fiber damages (Evans and Lanczki, 1997; Hollfelder *et al*., 2000; Negri *et al*., 1993). Chemical or physical treatments, such as alkali, oxidation, chlorination, or plasma treatments can dislodge some fatty acids bonds in the wool surface, break some disulphide crosslinks and provide polar functional groups. These pretreatments might help the accessibility of the enzyme to wool substrate during the following protease treatment (Cardamone *et al*., 2004; Kan *et al*., 2006). 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) achieved 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. Since 1991, several papers have addressed the treatment of wool fibers with lipase. El-Sayed *et al*., (2001) reported that the

lipase pretreatment in the shrink-resist process of wool fabric could help to improve the wettability of the fibers and enhance shrink resistance about 2–3%. Hutchinson *et al*., (2007) studied the activities of thioesterase and several lipases with the thioester substratemimic. Although the conversion of the substrate reached 90% under the optimal condition, there was no observable change in the wettability of the wool fabric. Monlleó *et al*., (1994) also published a paper about the lipases treatment of wool fibers and they found that none of the commercial lipases changes the surface of wool significantly by using microscopic examination and wettability test. 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). Eberl *et al*., (2008) reported that the treatment of poly(trimethylene terephthalate) (PTT) fabrics with cutinase improved the dyeability with a significant increase in *K/S* value. More 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. The weight loss of the sample treated with cutinase was similar to that of the fabric treated with hydrogen peroxide. The encouraging shrink-resistance and weakened fiber damage were also achieved after the combination of cutinase and protease treatments by Wang *et al*., (2009). 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. Using X-ray photoelectron spectroscopy (XPS), a loss of hydrocarbon from the fiber surface was detected after cutinase pretreatment. The combination of cutinase and protease treatment improved the dyeability of the wool fabrics mainly due to the enhancement of the wettability and the uniform removal of outer cuticle during the protease treatment. To date, bio-scouring or bio-preparation has become a promising eco-friendly alternative to chemical scouring in cotton preparations. Cotton seed coat fragments are the impurities on the cotton fabric most resistant to degrade. It is difficult to remove them from the surface of cotton fabric in bio-scouring process, which is the largest obstacle to the commercialization of enzymatic treatment of cotton fabric. Several works have been done in order to improve the degradation of cotton seed coat. Enzymes such as cellulase, pectinase, hemicellulase, etc. and chelating agent such as EDTA were extensively studied on the degradation of cotton seed coat and some positive results were published (Csiszár *et al*., 1998; Csiszár *et al*., 2001a; Csiszár *et al*., 2001b; Csiszár *et al*., 2006; Csiszár *et al*., 2007). The epidermal layer of cotton seed coat is covered with cuticle (Fryxell, 1963; Himmelsbach *et al*., 2003; Kolattukudy, 1980). Cutins and waxes are the main components of the cuticle in primary plant surface. Some amorphous waxy and most of the carbohydrate materials are embedded in the matrix of cutins. Cuticle contributes significantly to the hydrophobia of the cotton seed coat and hinders the performance of enzymes targeted for their substrates (Gevens and Nicholson, 2000), indicating that the destabilization of cuticle may be improved the enzymatic degradation of cotton seed coat. Cutinase hydrolysed the cuticle of cotton fiber (Agrawal *et al*., 2007; Agrawal *et al*., 2008; Degani *et al*., 2002). However, the effect of cutinase on the degradation of cotton seed coat has never been reported. Cutinases display hydrolytic activity toward a broad variety of esters, from soluble synthetic esters (e.g., p-

nitrophenyl esters) to insoluble long-chain triglycerides, including the ability to degrade 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). Therefore, it is expected that cutinase can improve the degradation of cotton seed coat by hydrolyzing the cutin in cotton seed coat, although there are some differences in the composition of cuticle between cotton fiber and cotton seed coat (Ryser and Holloway, 1985). Yan *et al*., (2009) has reported that Cutinase was capable of degrading the cotton seed coat. They used Fourier transform infrared (FT-IR) microspectroscopy to study the changes of chemical compositions in cotton seed coat epidermal layer and gas chromatography/mass spectrometry (GC/MS) to analyse cutinase depolymerization of cotton seed coat. Based on their study, they confirmed the ability of cutinase to degrade aliphatic components in cotton seed coat. They also observe positive effect of cutinase on degradation of cotton seed coat with the combination of alkaline pectinase or xylanase. 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. In the textile industry, enzymatic processing has been used extensively in various stages of the fabric production cycle, from desizing to finishing. Diverse enzymes were primarily used to improve the softness and resiliency of cotton fabrics (Ibrahim *et al*., 2005). However, these enzymatic treatments hydrolysed cellulose in the cotton fibers causing excessive weight loss. As a result, the dyeing properties and moisture regain of cotton fabrics decreased (Lee *et al*., 2009). To prevent these problems, studies have been conducted on the hydrolysis of the non-cellulose components of cotton during the scouring process (Lee *et al.,* 2009). 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. Lipase hydrolyses waxes and fats, while pectinase hydrolyses pectin (Lee *et al.,* 2009). 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 50ºC, 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 hydrolysed. Cutinase treatment did not affect to decrease of the moisture regain or the *K/S* value. In addition, Lee *et al*., (2009) also reported that Triton X-100 and calcium chloride were effective auxiliaries for cutinase treatment with optimum concentrations of 0.5% and 70 mM, respectively. Moreover, cutinase treatment did not decrease the tensile strength of the cotton fabric.

CHAPTER 3
MATERIALS AND METHODS

3.1 Chemicals and reagents

Chemicals and reagents used in the medium development study were of analytical grade and obtained from HiMedia, India. Most of the chemicals used in the study of specificity (p-nitrophenyl butyrate, p-nitrophenyl palmitate, p-nitrophenyl caproate, p-nitrophenyl valerate, p-nitrophenyl laurate), effectors (EDTA, SDS, Triton X-100), chromatographic matrixes (Sephadex G-100 and Sephacryl S-300), esterification and transesterification activity (Appendix A.1) were procured from Sigma-Aldrich (Bangalore, India). The CM-650 TOYOPEARL® procured from Tosho Corporation, Japan. Chemicals and markers for SDS PAGE were obtained from Bangalore Genei, India. All other chemicals used in protein analysis were of analytical grade and of the highest purity available in the local market.

3.2 Microorganisms

Different *Pseudomonas* strains used for this study are *P. syringae pathovar lachrymans* van Hall 1902 NRRL B 799, *P. cepacia* NRRL B 2320 (also known as *Burkholderia cepacia*), *P. fluorescens* NRRL B 14678, NRRL B 10, NRRL B 11, NRRL B 253, NRRL B 1603, NRRL B 3178, NRRL B 2641, *P. geniculata* NRRL B 1606, NRRL B 1612, NRRL B 1888, and NRRL B 1890 procured from Agricultural Research Service (ARS-Culture collection), USDA, Peoria, USA and *P. cepacia* NCIM 5029 was procured from National Collection of Industrial Microorganisms, Pune, India. Four strains were isolated from rotten tomatoes. *E.coli* DH 5α, *E.coli* BL 21 (DE3) and pET22 (b)+ were procured from Novagen Inc. USA.

All *Pseudomonas species* and isolated strains were grown on nutrient agar medium at 28°C and maintained at 4±1°C. *E. coli* strains were grown on Luria bertani agar medium at 37°C and maintained at 4±1°C.

3.3 Isolation of microorganisms from rotten tomatoes

The fresh tomatoes were kept at 37ºC for two days and the outer part of the tomatoes were washed with autoclaved distilled water, and then spread on the solid medium containing 1% Tween 80. The microorganisms grown on medium containing Tween 80 were again cultured on medium containing olive oil. Finally, four microorganisms were isolated which were able to grow in the presence of Tween 80 as well as olive oil.

3.4 Preparation of 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 hours. 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. Cutin was dried at 60-80 ºC in hot air oven. These peels were ground to powder (< 20 mesh) to get cutin. This cutin was further characterized by FT-IR and AFM studies.

3.5 Preparation of cutinase specific substrate

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. Thereafter, 5 ml of triethylamine was added and stirred for 1.5 h at -20°C under N_2 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 above 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 hexane (10%) as a mobile phase and stored in the darkness at -20°C until use. The substrate was characterized by H^1NMR spectroscopy.

3.6 Cultivation medium and culture conditions

The production of cutinase was performed in the modified basal semisynthetic medium containing (g l^{-1}): carbon source 4.0, beef extract 3.0, peptone 15.0, urea 6.0, KH_2PO_4 2.0, KCl 0.5, $MgSO_4 \cdot 7H_2O$ 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 28ºC and 180 rpm in an orbital shaking incubator for 10-12 h (Optical density (OD) at 600nm = 0.6~0.8). A 2% of inoculum from the above seed culture was added to 50 ml of the medium in 250 ml Erlenmeyer flasks and incubated in an orbital shaking incubator at 28°C and 180 rpm. Samples were withdrawn at regular intervals to determine cell growth and cutnase activity.

3.7 Analytical methods

3.7.1 Assay of cutinase

The activity was measured by following the hydrolysis of p-nitrophenyl butyrate (p-NPB) (Sigma) as substrate. An aliquot of (0.020 ml) culture supernatant was added to 0.98 ml of reaction mixture, which was prepared by adding 1 ml of 23 mM pNPB in tetrahydrofuran to 40 ml of 50 mM potassium phosphate buffer containing 11.5 mM of sodium deoxycholate. The reaction was monitored for 15 minutes at 37ºC and absorbance of released p-nitrophenol measured at 410 nm. Activity with cutinase specific substrate, p-nitrophenyl (16 methyl sulphone ester) hexadecanoate (p-NMSH) was measured by method described by Degani *et al*., (2006). An aliquot of (0.1 ml) culture supernatant was added to 0.9 ml of reaction mixture, which was prepared by adding 1 ml of 23 mM pNMSH in tetrahydrofuran to 40 ml of 50 mM potassium phosphate buffer of pH 8. The enzyme substrate mixture was incubated for 4 h at 37ºC and absorbance of released p-nitrophenol measured as above. One enzyme unit is defined as the amount of enzyme required to release one µMol of p-nitrophenol min^{-1} under assay conditions. The method for preparation of p-nitrophenol standard curve was described in appendix A.2.

3.7.2 Protein determination

The total protein content of the samples was determined according to the method described by Lowry *et al.,* (1951). The protein assay mixture consisted of 200 µl of diluted crude extract of enzyme (5 times) and 1 ml of freshly prepared complex forming reagent (2% Na_2CO_3 in 0.1 N NaOH : 1.0% $CuSO_4.5H_2O$: 2% potassium sodium tartarate ≡ 100:1:1). The contents in the sample were vortexed and incubated at room temperature for 15 min. Then 100 µl of freshly prepared diluted Folin reagent with distilled water (1:1) was added

and vortexed and kept in the dark at room temperature for 30 min. Blue color was developed and OD at 660 nm was measured against the blank (no crude enzyme, 200 µl distilled water). The protein concentration in the reaction was determined based on a standard curve (appendix A.3) obtained with bovine serum albumin (Sigma) as standard.

3.7.3 Dry cell weight (DCW)

Due to the presence of cutin, cell dry-weight concentration could not be measured directly and therefore, intracellular protein concentration was measured (Agarwal *et al*., 2009). For standard curve of DCW, cells were centrifuged at 10,000*g* for 10 min at 4±1ºC and separated from the supernatant. The pellet was resuspended with same volume of distilled water and centrifuged at 10,000*g* for 10 min at 4±1ºC, washed twice with distilled water and supernatant free cells were used to determine the DCW. Different dilutions of cell samples were used for measuring intracellular protein concentration**.** For measuring intracellular protein, cells were washed as above and subjected to lysis by dissolving the cell samples in 1ml of 0.4 N NaOH. Then the samples were heated at 100ºC for 20 min. After cooling down the sample at room temperature, the protein concentration was measured using method described in section 3.7.2. DCW of the unknown sample was determined by measuring the intracellular protein concentration and compared with standard curve (appendix A.4) between dry cell weight and intracellular protein (Cell dry weight in g l^{-1} = Intracellular protein in g l^{-1} × 19.03).

3.7.4 Estimation of alkyl ester

The ester formation was estimated by consumption of fatty acid. To estimate the fatty acid consumption, 300 µl of the mixture (for butyric acid) or 100 µl (for all other acids) sample

were transferred to 2 ml micro centrifuge tube and centrifuged at 10,000*g* for 1 min to remove the suspended enzyme particles. The progress of reaction was determined by measuring the decreasing profile of acids by titration with 0.01 N NaOH using phenolphthanein as indicator and also with Lowry and Tinsley method (1976). According to Lowry and Tinsley method, the samples were diluted (7:3 for butyric acid or 9:1 for other) in isooctane making the total volume of 1 ml. Cupric acetate aqueous solution (0.2 ml) containing pyridine (5%, w/v, pH 6.0) was then added into the tube and the solutions were vigorously mixed for 1 min using a vortex mixture. After centrifugation at 5000*g* for 5 min, the upper organic phase was measured by a UV/visible spectrophotometer (Cary 100, Varian) at 715 nm. The amount of fatty acid consumed was calculated using the standard curve for that fatty acid (Appendix A.5).

3.7.5 GC analysis of esters

Synthesis of fatty acid ester was analyzed by gas chromatograph (Varian 390). The diluted aliquots of the reaction mixture were injected into CPSIL 8CB column and compounds were detected by FID. The injector and detector temperature were set at 250°C. The program (temperature and time) and retention time (t_R) used for different esters are given below.

Butyl butyrate: 150°C for (0.5min) -15 °C/min-250°C (10min); t_R 2.42min

Butyl valerate: 150°C for (0.5min) -15 °C/min-250°C (10min); t_R 3.14 min

Butyl hexanoate: 150°C for (0.5min) -15 °C/min-250°C (10min); t_R 4.38 min

Butyl octanoate: 150°C for (0.5min) -30 °C/min-250°C (10min); t_R 6.65 min

Butyl decanoate: 150°C for (0.5min) -30 °C/min-270°C (10min); t_R 15.5 min

Butyl Palmitate: 150°C for (0.5min) -30 °C/min-300°C (10min); t_R 20.3 min

Ethyl butyrate: 100°C for (0.5min) -15 °C/min-150°C (10min); t_R 1.36min

Ethyl valerate: 100°C for (0.5min) -15 °C/min-150°C (10min); t_R 2.55 min

Ethyl hexanoate: 50°C for (0.5min) -30°C/min-160°C (10min); t_R 2.15 min

Pentyl butyrate: 150°C for (0.5min) -30 °C/min-250°C (10min); t_R 3.58 min

Hexyl butyrate: 150°C for (0.5min) -30 °C/min-250°C (10min); t_R 4.18 min

Octyl Butyrate: 150°C for (0.5min) -30 °C/min-250°C (10min); t_R 5.43

Decyl butyrate: 100°C for (0.5min) -30 °C/min-150°C (10min); t_R 2.20 min

Methyl butyrate: 150°C for (0.5min) -15 °C/min-250°C (10min); tR 1.69min

Methyl palmitate: 150°C for (0.5min) -30 °C/min-250°C (10min); tR 3.14 min

Methyl oleate: 150°C for (0.5min) -30 °C/min-250°C (10min); tR 4.88 min

Methyl linoleate: 150°C for (0.5min) -30 °C/min-250°C (10min); tR 2.67 min

3.8 Screening and selection of the most efficient cutinase producer

The microorganisms were primarily screened for lipase activity by plate technique. The composition of medium used in g l^{-1}: meat extract 3.0, polyoxyethylene-sorbitan mono laurate (Tween 20) 5.0, $CaCl_2.2H_2O$ 0.4, agar 15.0. The plates were incubated at 28ºC for 48 h. In order to enhance the contrast, 5 ml of Lugols iodine solution was added to each plate and removed after 1 min (Castro *et al*., 1992).

In order to select the most efficient cutinase producer among the microorganisms grown on above plates, experiments were performed with different media containing glucose (6 g l^{-1}) or glucose (6 g l^{-1}) with Tween 80 (10 g l^{-1}) or glucose (6 g l^{-1}) with olive oil (10 g l^{-1}) or glucose (6 g l^{-1}) with cutin (4 g l^{-1}) or cutin (4 g l^{-1}).

3.9 Effect of various carbon sources on the production of cutinase from *P. cepacia* NRRL B 2320

In order to select the most suitable substrate(s) for the maximum production of cutinase from *P. cepacia* NRRL B 2320, experiments were conducted with various carbon sources (lactose, maltose, fructose, galactose, sucrose, xylose and starch) with or without cutin. The medium contained basal semisynthetic medium (carbon source (4.0 g l^{-1}), with or without tomato cutin (4.0 g l^{-1})). The effect of oleic acid, 16-hydroxyhexadecanoic acid and different oils (mustards oil, soyabean oil, sunflower oil) was also studied.

3.10 Optimization of chemical parameters for enhanced production of cutinase from *P. cepacia* NRRL B 2320

3.10.1 Screening of significantly influencing medium components by Plackett-Burman experimental design

To maximize the production of cutinase, Plackett-Burman experimental design was applied to screen the significantly influencing medium components (Plackett and Burman, 1946). A total of seven parameters *viz.,* cutin, peptone, beef extract, urea, KH_2PO_4, KCl and $MgSO_4 \cdot 7H_2O$ have been considered for screening experiment. Each variable is represented at two levels, i.e., a high (+) and low (-). According to Plackett–Burman experimental design a total of 12 experiments were performed. The levels of variables and design matrix in the coded levels and real values are shown in the Table 3.1. Plackett–Burman experimental design is based on the first order polynomial model.

$$Y = \beta_0 + \sum \beta_i X_i \qquad 3.1$$

where, Y is the response (enzyme activity), β_o is the model intercept, β_i is the linear coefficient, and x_i is the level of the independent variable. The significance of each variable was determined using Student's t-test. All experiments were conducted in duplicates and averages of the results were taken as the response.

Table 3.1 Plackett-Burman design matrix (coded units and real values in parenthesis) for screening of medium components

Run Order	Coded and uncoded values						
	Cutin X_1	***Peptone*** X_2	***Beef Extract*** X_3	***Urea*** X_4	KH_2PO_4 X_5	***KCl*** X_6	$MgSO_4.7H_2O$ X_7
1	1(10)	-1(2)	1(7)	-1(1)	-1(0.5)	-1(0.1)	1(8.5)
2	1(10)	1(20)	-1(1)	1(9)	-1(0.5)	-1(0.1)	-1(0.5)
3	-1(2)	1(20)	1(7)	-1(1)	1(5.5)	-1(0.1)	-1(0.5)
4	1(10)	-1(2)	1(7)	1(9)	-1(0.5)	1(1.1)	-1(0.5)
5	1(10)	1(20)	-1(1)	1(9)	1(5.5)	-1(0.1)	1(8.5)
6	1(10)	1(20)	1(7)	-1(1)	1(5.5)	1(1.1)	-1(0.5)
7	-1(2)	1(20)	1(7)	1(9)	-1(0.5)	1(1.1)	1(8.5)
8	-1(2)	-1(2)	1(7)	1(9)	1(5.5)	-1(0.1)	1(8.5)
9	-1(2)	-1(2)	-1(1)	1(9)	1(5.5)	1(1.1)	-1(0.5)
10	1(10)	-1(2)	-1(1)	-1(1)	1(5.5)	1(1.1)	1(8.5)
11	-1(2)	1(20)	-1(1)	-1(1)	-1(0.5)	1(1.1)	1(8.5)
12	-1(2)	-1(2)	-1(1)	-1(1)	-1(0.5)	-1(0.1)	-1(0.5)
13(Ct. Pt.)	0(6)	0(11)	0(4)	0(5)	0(3)	0(0.6)	0(4.5)

X_1-X_7 are mentioned in g l^{-1}

3.10.2 Optimization of screened medium components for enhanced production of cutinase from P. cepacia NRRL B 2320

The central composite design (Khuri and Cornell, 1987) has been applied to optimize the levels and explain the combined effect of the significantly influencing medium constituents, *viz.* cutin, peptone, KCl and $MgSO_4 \cdot 7H_2O$ on the production of cutinase from *P. cepacia* NRRL B 2320. Each variable (medium component) was assessed at five coded levels (-2, -1,

0, +1, and +2) given in Table 3.2 with 30 ($=2^k + 2k + 6$) treatment combinations, where, k is the number of independent variables (Araujo and Brereton, 1996). Twenty four experiments were augmented with six replications at the center points to evaluate the pure error. The minimum and maximum ranges of the variables were used and the experimental plan with regard to their values in actual and coded form is provided in Table 3.3.

Table 3.2 Ranges and levels of the independent variables used in the optimization of screened medium components by central composite design of experiment

Variables	Symbol coded	Range and levels				
		-2(-α)	-1	0	+1	+2(+α)
Cutin	X_1	2.00	5.50	9.00	12.50	16.00
Peptone	X_2	2.00	7.50	13.00	18.50	24.00
KCl	X_6	0.10	0.35	0.60	0.85	1.10
MgSO4, 7H2O	X_7	1.00	3.25	5.50	7.75	10.00

The quadratic model for predicting the optimal levels was expressed according to the equation 3.2.

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i} \sum_{j} \beta_{ij} X_i X_j \qquad 3.2$$

where, Y is the predicted response, k is the number of variables, X_i *and* X_j are independent variables, β_0 is the offset term, β_i is the i^{th} linear coefficient, β_{ii} is the i^{th} quadratic coefficient and β_{ij} is the ij^{th} interaction coefficient. The statistical software package, MINITAB® Release 15.1, PA, USA was used for the regression analysis of the experimental data, and also to plot the response surface graphs.

Table 3.3 A 2^4 full-factorial central composite experimental design matrix in coded units and real values (parenthesis).

Run Order	Experimental values			
	X_1 *(Cutin)*	X_2 *(Peptone)*	X_6 *(KCl)*	X_7 *($MgSO_4.7H_2O$)*
1	-1(5.5)	-1(7.5)	-1(0.35)	-1(3.25
2	1(12.5)	-1(7.5)	-1(0.35)	-1(3.25)
3	-1(5.5)	1(18.5)	-1(0.35)	-1(3.25)
4	1(12.5)	1(18.5)	-1(0.35)	-1(3.25)
5	-1(5.5)	-1(7.5)	1(0.85)	-1(3.25)
6	1(12.5)	-1(7.5)	1(0.85)	-1(3.25)
7	-1(5.5)	1(18.5)	1(0.85)	-1(3.25)
8	1(12.5)	1(18.5)	1(0.85)	-1(3.25)
9	-1(5.5)	-1(7.5)	-1(0.35)	1(7.75)
10	1(12.5)	-1(7.5)	-1(0.35)	1(7.75)
11	-1(5.5)	1(18.5)	-1(0.35)	1(7.75)
12	1(12.5)	1(18.5)	-1(0.35)	1(7.75)
13	-1(5.5)	-1(7.5)	1(0.85)	1(7.75)
14	1(12.5)	-1(7.5)	1(0.85)	1(7.75)
15	-1(5.5)	1(18.5)	1(0.85)	1(7.75)
16	1(12.5)	1(18.5)	1(0.85)	1(7.75)
17	0(9.0)	0(13.0)	0(0.60)	0(5.50)
18	0(9.0)	0(13.0)	0(0.60)	0(5.50)
19	0(9.0)	0(13.0)	0(0.60)	0(5.50)
20	0(9.0)	0(13.0)	0(0.60)	0(5.50)
21	-2(2.0)	0(13.0)	0(0.60)	0(5.50)
22	2(16.0)	0(13.0)	0(0.60)	0(5.50)
23	0(9.0)	-2(2.0)	0(0.60)	0(5.50)
24	0(9.0)	2(24.0)	0(0.60)	0(5.50)
25	0(9.0)	0(13.0)	-2(0.10)	0(5.50)
26	0(9.0)	0(13.0)	2(1.10)	0(5.50)
27	0(9.0)	0(13.0)	0(0.60)	-2(1.00)
28	0(9.0)	0(13.0)	0(0.60)	2(10.0)
29	0(9.0)	0(13.0)	0(0.60)	0(5.50)
30	0(9.0)	0(13.0)	0(0.60)	0(5.50)

3.10.3 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 significant variables and at middle level of other medium components both in shake flask and 3L bioreactor (Applikon). The medium was inoculated with 2% seed culture and the bioreactor was operated at 28°C, 200 rpm and 2 vvm with or without pH control. All experiments in shake flask were conducted in duplicates and averages of the results were taken as response.

3.11 Unstructured model prediction

To estimate the biokinetic constants involved in the microbial growth and cutinase production process, various unstructured models reported in the literature were used to fit the experimental data (obtained from experiments performed in shake flask and bioreactor as described in the section 3.10.3) on cutinase production and biomass growth of *P. cepacia* NRRL B 2320. The equations used for growth and production kinetics are given below.

Logistic model for growth (Mercier *et al*., 1992)

$$X = \frac{X_0 X_{max} e^{\mu t}}{X_{max} - X_0 + X_0 e^{\mu t}} \qquad 3.3$$

where, X is cell biomass (g l^{-1}), X_0 is initial biomass (g l^{-1}), X_{max} is the maximum biomass in (g l^{-1}), t is time (h) and μ is specific growth rate (h^{-1}).

Logistic Model for production (Mercier *et al*., 1992)

$$P = \frac{P_0 P_{max} e^{P_r t}}{P_{max} - P_0 + P_0 e^{P_r t}} \qquad 3.4$$

where, P is the cutinase activity (U ml^{-1}), P_0 is the initial cutinase activity (U ml^{-1}), P_{max} is the maximum cutinase activity (U ml^{-1}) and P_r is ratio between the initial volumetric rate of product formation and the initial product concentration P_0.

Luedeking-Piret Model for Production (Luedeking and Pirate, 1959)

$$P = P_0 + \alpha X_0 \left[\frac{e^{\mu_0 t}}{1 - \left(\frac{X_0}{X_{max}} \right) \left(1 - e^{\mu_0 t} \right)} - 1 \right] + \beta \frac{X_{max}}{\mu_0} \ln \left[1 - \frac{X_0}{X_{max}} (1 - e^{\mu_0 t}) \right] \qquad 3.5$$

where, P is the cutinase activity (U ml^{-1}), P_0 is the initial cutinase activity (U ml^{-1}), X is cell biomass (g l^{-1}), X_0 is initial biomass (g l^{-1}), X_{max} is the maximum biomass in (g l^{-1}) and μ_0 is specific growth rate (h^{-1}), and α is growth associated term (UgX^{-1}) and β is non growth associated term ($UgX^{-1}h^{-1}$).

3.12 Modeling of growth of *P. cepacia* NRRL B 2320 under substrate inhibition during production of cutinase

Shake flask studies were carried out in 250 ml Erlenmeyer flask containing 50 ml of the optimized medium with different cutin concentration, 2-20 g l^{-1} was inoculated with 2% of seed culture (OD = 0.6 at 600 nm) and incubated at 28°C and 180 rpm in an orbital shaker.

The initial pH of the medium was adjusted to 7.0, however it was neither maintained nor monitored during the fermentation process.

3.12.1 Mathematical models

Usually at high substrate concentration inhibition of growth occurs. It is primarily occurred when more than one substrate molecule binds to an active site, and/or often by different parts of the substrate molecules binding to different sub-sites within the substrate binding site. If the resultant complex is inactive, this type of inhibition causes a reduction in the rate of reaction. Different substrate inhibition models used to explain cell growth kinetics are presented in Table 3.4. Monod's model is the basic model proposed to explain microbial growth at low substrate concentration, where specific growth rate proportionately varies with initial substrate concentration. At high substrate concentration, a constant and maximum specific growth rate is reached. Andrew's model explains the inhibitory effects of substrate at higher concentrations. It reduces to the conventional Monod's equation when inhibition constant becomes very high. Luong's model is a generalisation of Monod's kinetics to account for substrate inhibition on growth. The Luong's model includes an extra term for substrate concentration above which growth is completely inhibited. As given in the model equation, the relation between specific growth rate and initial substrate concentration could be linear ($n = 1$), non-linear (concavity upward) ($n > 1$) or a concavity downward ($n < 1$) depending on the value of constant parameter (n). Han and Levenspiel model is extension of the Monod's model to account for cell, product and substrate inhibition, and also capable of explaining the type of inhibition as competitive, non-competitive or uncompetitive depending

upon the values of two constant parameters (*n*, *m*). This model assumes a value for critical inhibitory concentration of substrate above which growth completely ceases.

Table 3.4 Different models used in kinetic study

Model	Equation	Reference
Andrew's	$\mu = \frac{\mu_{max}\gamma_s}{(K_s+\gamma_s)\left(1+\frac{\gamma_s}{K_I}\right)}$	Andrews 1968
Luong's	$\mu = \frac{\mu_{max}\gamma_s}{(K_s+\gamma_s)}\left[1-\frac{\gamma_s}{\gamma_s^*}\right]^n$	Luong's 1987
Han-Levelspiel	$r = k\left[\left(1-\frac{\gamma_s}{\gamma_s^*}\right)^n\right]\frac{\gamma_s C}{\gamma_s + K_M\left[\left(1-\frac{\gamma_s}{\gamma_s^*}\right)^m\right]}$	Han-levenspiel 1988
Haldane	$\mu = \frac{\mu_{max}\gamma_s}{K_s+\gamma_s+\frac{\gamma_s^*}{K_I}}$	Haldane 1965
Moser	$\mu = \frac{\mu_{max}\gamma_s^n}{K_s+\gamma_s^n}$	Moser 1985
Aiba	$\mu = \frac{\mu_{max}\gamma_s}{K_s+\gamma_s}\exp\left(\frac{-\gamma_s}{K_I}\right)$	Aiba 1968
Yano	$\mu = \frac{\mu_{max}\gamma_s}{K_s+\gamma_s+\frac{\gamma_s^2}{K_I}\left(1+\frac{\gamma_s}{K}\right)}$	Yano 1966
Edward	$\mu = \mu_{max}\gamma_s\left[\exp\left(\frac{-\gamma_s}{K_I}\right)-\exp\left(\frac{-\gamma_s}{K_s}\right)\right]$	Edwards' 1970
Webb	$\mu = \frac{\mu_{max}\gamma_s\left(1+\frac{\gamma_s}{K}\right)}{\gamma_s+K_s+\frac{\gamma_s^2}{K_I}}$	Webb 1963

The Haldane growth model is widely accepted due to its mathematical simplicity and wide acceptance for representing the growth kinetics of inhibitory substrates, incorporating both substrate affinity constant and the substrate-inhibition constant. The kinetics follows simple Monod's model when the inhibition constant is infinitely large. Moser model is a modified Monod equation with power function of substrate concentration. The value of the power determines the degree of inhibition. However, it does not indicate any critical substrate concentration or inhibition constant. The Aiba growth inhibition model was originally proposed for product inhibition in alcohol fermentation, where specific growth rate decreases as the product concentration increases. Exponential term to take care of the product inhibition could be well replaced with substrate concentration. Though, Aiba's exponential model has been widely used to analyze product inhibition, it fails to give the critical value of inhibitory substrate/product concentration. Yano model was originally proposed for the kinetics of amylase production at high sugar concentration. Edward model gives the protective diffusional-limitation of high and inhibitory substrate concentrations. Webb model is the modified Yano model where $(1+\gamma_s/K)$ term is present in numerator rather than denominator.

3.13. Isolation and purification of cutinase produced from *P. cepacia* NRRL B 2320

3.13.1 Enzyme production for purification

The production of cutinase was performed in the optimized semisynthetic medium containing (g l^{-1}): beef extract 4.0, peptone 17.77, urea 5.0, KH_2PO_4 3.0, KCl 0.635, $MgSO_4.7H_2O$ 5.546 and cutin 10.06. Initial pH of the medium was maintained at 7. A 2% of inoculum from the seed culture (section 3.6) was added to 200 ml of the medium in 1000 ml Erlenmeyer flasks.

The flasks were incubated in a shaking incubator at 28°C and 180 rpm. After 96 h fermentation the cells were centrifuged at 10,000g for 10 min at 4±1 °C.

3.13.2 Isolation and purification

All isolation and purification steps were carried out at 0-4°C unless otherwise indicated and all chromatographic runs in the study were monitored for protein at 280 nm. Isolation and purification steps are summarized in the following flowchart (Fig. 3.1).

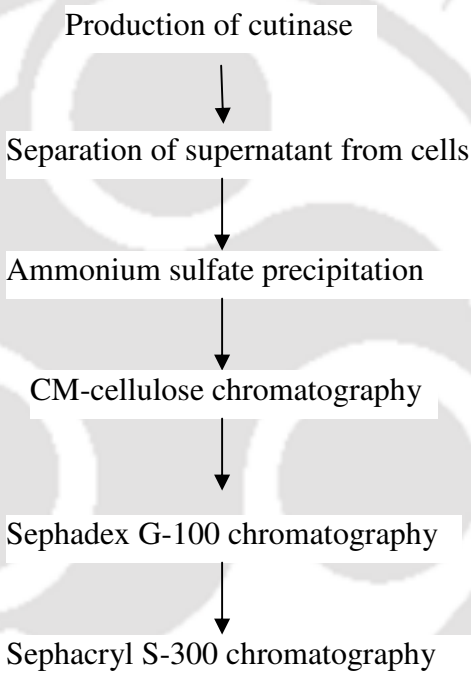


Fig. 3.1. Flow chart of the isolation and purification steps involved in downstream of extracellular cutinase from *P. cepacia* NRRL B 2320

3.13.3 Ammonium sulfate precipitation

Finely powdered ammonium sulfate (20 to 80%) was added to the clear supernatant obtained after centrifugation with constant stirring and incubated for overnight. The maximum cutinase activity was observed with the fraction precipitated at 80% saturation. The precipitate was collected by centrifugation at 10,000*g* for 30 min and dissolved in minimal amount of 20 mM Tris HCl buffer (pH 8.0) and dialyzed against the same buffer for 24 h.

3.13.4 CM cellulose chromatography

Dialyzed ammonium sulphate fraction was applied to a 2 × 50 cm CM-650 TOYOPEARL® column (Tosho Corporation, Tokyo, Japan). This column served as a weak cation exchanger for biomolecule purification. The column was pre-equilibrated with potassium phosphate buffer (20 mM, pH 6.5) at a flow rate of 1 ml min^{-1}. The column was washed with 2 column volume of the above buffer and the adsorbed protein was eluted using linear gradient of NaCl (0-500 mM). Active fractions obtained after the ion exchange chromatography were pooled separately, dialyzed and concentrated.

3.13.5 Sephadex G-100 chromatography

The concentrated fractions from above step were further purified on gel filtration chromatography using Sephadex G-100 column pre-equilibrated and eluted with Tris HCl buffer (20 mM, pH 8.0). The protein elution was done with the same buffer at a flow rate of 0.2 ml min^{-1}. The protein concentration was monitored at 280 nm and assayed for cutinase activity. The active fractions were pooled, concentrated and dialyzed against the Tris HCl buffer (20 mM, pH 8.0). Active fractions obtained after the gel filtration chromatography were pooled and used for next purification step.

3.13.6 Sephacryl S-300 chromatography

The Sephadex G-100 concentrated fractions were further purified on another gel filtration chromatography using Sephacryl S-300 pre-equilibrated and eluted with Tris HCl buffer (20 mM, pH 8.0). The protein elution was done with the same buffer at a flow rate of 0.2 ml min^{-1}. The protein concentration was monitored at 280 nm and assayed for cutinase activity. The active fractions were pooled, concentrated and dialyzed against the Tris HCl buffer (20

mM, pH 8.0) for 24 h. This concentrated fraction was stored at -20°C till further used for characterization.

3.13.7 Electrophoretic analysis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of the previously purified enzyme fractions were 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.

3.13.8 MALDI-TOF-MS analysis

The intact molecular mass was also 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 µl of the sample was spotted on plate, dried at room temperature and analyzed.

3.14 Characterization of purified cutinase from *P. cepacia* NRRL B 2320

3.14.1 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 5.5-10. For pH stability studies, the enzyme preparations were incubated in a pH range of 5.5-10 for 24 h at 25°C in the absence of substrate and residual activity was determined. The buffers, potassium phosphate (50 mM, pH 5.5-7.5), Tris HC1 (50 mM, pH 8.0-9.5) and sodium carbonate/bicarbonate (50 mM, pH 10) were used for this study.

3.14.2 Effect of temperature on activity of purified enzyme

The optimum temperature range for enzyme activity was determined by incubating the assay mixture at different temperatures ranging from 30 to 80°C.

3.14.3 Optimization of combined effect of substrate and enzyme amount on the performance of cutinase under assay conditions

The central composite design was applied to optimize the levels and explain the combined effect of amount of substrate and enzyme on the performance of purified cutinase under assay conditions. The concentration of substrate used was 0.57 mM. Each variable (pH and temperature) was assessed at five coded levels (-α, - 1, 0, +1, and +α). The minimum and maximum ranges of the variables were used and the full experimental plan with regard to their values in actual and coded form is provided in Table 3.5 and Table 3.6, respectively.

Table 3.5 Experimental ranges and levels of amount of substrate and enzyme (µl) for optimization of these levels using central composite design of experiment

Variables	Range and levels				
	-α(1.414)	**-1**	**0**	**+1**	**+α(1.414)**
Substrate volume (µl)	810.3	860	980	1100	1149.7
Enzyme volume (µl)	8.3	12	40	30	33.7

Table 3.6 Experimental design at various combinations of amount of substrate and enzyme

Run Order	Uncoded and coded values					
	Volume of substrate X_1 (µl)			*Volume of Enzyme used X_2*		
	Coded value	*Actual value*		*Coded value*	*Actual value*	
		µl	*µg*		*µl*	*µg*
1	-1	860	67.0	-1	12	0.691
2	+1	1100	85.69	-1	12	0.69
3	-1	860	67.0	+1	30	1.72
4	+1	1100	85.69	+1	30	1.72
5	0	980	76.34	0	21	1.21
6	0	980	76.34	0	21	1.21
7	0	980	76.34	0	21	1.216
8	-1.414	810.3	63.11	0	21	1.21
9	+1.414	1149.7	89.56	0	21	1.216
10	0	980	76.34	+1.414	8.3	0.47
11	0	980	76.34	-1.414	33.7	1.94
12	0	980	76.34	0	21	1.21
13	0	980	76.34	0	21	1.21
14	0	980	76.34	0	21	1.21

According to this design, the total number of treatment combinations was 14 ($=2^k + 2k + 6$). Where, k is the number of independent variables (Araujo and Brereton, 1996). CCD with four axial points (α = 1.414) and six replicates at the center point with a total number of 14 experiments were employed to evaluate the error. All variables were taken at a central coded value, which was considered as zero. The quadratic model for predicting the optimal levels was expressed according to the eq. 3.2. The statistical software package, MINITAB® Release 15.1, PA, USA was used for the regression analysis of experimental data and also to plot the response surface graphs. All experiments were performed in triplicates and averages of the results were taken as the response.

3.14.4 Effect of pH and temperature on the performance of cutinase under assay conditions

The combined effect of pH and temperature on cutinase activity was optimized under assay conditions. Each variable was assessed at five coded levels (-1.414, - 1, 0, +1, and +1.414) with 14 ($=2^k + 2k + 6$) treatment combinations, where, k is the number of independent variables. Fourteen experiments were augmented with six replications at the center points to evaluate the pure error. The minimum and maximum ranges of the variables were used and the full experimental plan with regard to their values in actual and coded forms is provided in Table 3.7 and 3.8, respectively. The quadratic model for predicting the optimal levels was expressed according to the equation 3.2. The statistical software package, MINITAB® Release 15.1, PA, USA was used for experimental design, regression analysis of the experimental data, and also to plot the response surface graphs.

Table 3.7 Ranges and levels of pH and temperature for optimization of their levels by central composite design of experiment

Variables	Range and levels				
	-α (1.141)	**-1**	**0**	**+1**	**+α (1.414)**
pH	6.65	8.0	7.85	8.7	9.05
Temperature	34.7	37	42.5	48	50.2

Table 3.8 Central composite experimental design at various combinations of pH and temperature

Run Order	Experimental values			
	pH X_1		***Temperature*** (°C) X_2	
	Coded value	***Actual value***	***Coded value***	***Actual value***
1	-1	7	-1	37
2	+1	8.7	-1	37
3	-1	7	+1	48
4	+1	8.7	+1	48
5	0	7.85	0	42.5
6	0	7.85	0	42.5
7	0	7.85	0	42.5
8	-1.414	6.64	0	42.5
9	+1.414	9.05	0	42.5
10	0	7.85	+1.414	34.7
11	0	7.85	-1.414	50.2
12	0	7.85	0	42.5
13	0	7.85	0	42.5
14	0	7.85	0	42.5

3.14.5 Studies on the thermal deactivation kinetics of cutinase

In order to study the thermal stability of cutinase, the enzyme was incubated at five different temperatures at 5°C intervals between 40°C and 60°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 3.9 and aliquots of samples were collected at different intervals of time and were assayed for the residual enzyme activity as described in section 3.7.1.

Table 3.9 Experimental condition adopted to study the deactivation of purified cutinase at different pH and temperature

pH	Temperature (ºC)
6	40
	45
	50
	55
	60
7	40
	45
	50
	55
	60
8	40
	45
	50
	55
	60
9	40
	45
	50
	55

3.14.6 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.

$$\frac{dE}{dt} = -k_d E \qquad 3.6$$

so that,

$$\ln\left[\frac{E_t}{E_0}\right] = -k_d t \qquad 3.7$$

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 loose half of its initial activity and can be expressed by the following equation.

$$t_{\frac{1}{2}} = \frac{\ln 2}{k_d} \qquad 3.8$$

3.14.7 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.

$$k_d = \frac{\kappa T}{h} . e^{\frac{\Delta S^*}{R}} . e^{\frac{-\Delta H}{RT}} \quad 3.9$$

or

$$\ln\left(\frac{k_d}{T}\right) = \ln\left(\frac{\kappa}{h}\right) + \left(\frac{\Delta S^*}{R}\right) - \left(\frac{-\Delta H^*}{R}\right)\left(\frac{1}{T}\right) \quad 3.10$$

where, k_d is enzyme deactivation rate constant (h^{-1}); κ is Boltzmann constant (1.38×10^{-23} $J\cdot K^{-1}$); h is Plank's constant ($6.626\times10^{-34}J\cdot s$) ΔH^* is change in enthalpy ($J\cdot mol^{-1}$); ΔS^* is change in entropy ($J\cdot mol^{-1}\cdot K^{-1}$); R is gas constant (8.314 $J\cdot M^{-1}\cdot K^{-1}$) 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.

$$\Delta G^* = \Delta H^* - T\Delta S^* \quad 3.11$$

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

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

$$k_d = k_0 \exp\left(-\frac{E_A}{RT}\right) \quad 3.12$$

or

$$\ln k_d = \ln k_0 - \left(\frac{E_A}{R}\right)\left(\frac{1}{T}\right) \quad 3.13$$

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.

3.14.8 Fluorescence spectroscopy

The enzyme (0.07 mg ml^{-1}) dissolved in buffers at different pH (6 to 9) was heated at different temperature (40°C, 50°C, 60°C and 70°C) for 15 min. The change in environment of tryptophan residue present in the protein was analyzed using fluorescence spectroscopy. The excitation was done at 295 nm and emission was measured from 315 nm to 450 nm.

3.14.9 Circular dichorism (CD) spectroscopy

Similarly as fluorescence analysis, 0.07 mg ml^{-1} protein was heated at different combination of temperature and pH for 15 min and the change in its secondary structure was monitored using CD spectrophotometer (JASCO). The CD spectrum was obtained from 190 to 250 nm range.

3.14.10 Effect of various metal ions and effectors on cutinase activity

The enzyme activity was measured in the presence of different effectors (metal ions, β-Marceptoethanol, EDTA). The purified enzyme was incubated with various modifiers at 37°C for 5 min and the residual activities were then measured. 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.

3.14.11 Effect of various surfactants on cutinase activity

In the surfactant stability test, the enzyme was incubated with 1mM and 10 mM solution of different surfactants at 30°C for 4 h. The remaining activity was measured with pNPB as substrate and expressed as relative activity as compared to surfactant free controls.

3.14.12 Effect of various solvents on cutinase stability

In the organic solvent stability test, the enzyme was incubated in 75% of different organic solvents at 30°C for 4 h. The remaining activity was measured with pNPB as substrate and expressed as relative activity compared to solvent free controls.

3.14.13 Substrate specificity

The cutinase activity was monitored using p-nitrophenyl valerate, caproate, decanoate, laurate and palmitate. The activity was expressed as relative activity as compared to activity with p-nitrophenyl butyrate.

3.14.14 Kinetic analysis of cutinase

Kinetic studies were performed with pNPB (0.05–2mM) as substrate using the continuous spectrophotometric assay as described above. 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 (26.5 kDa) of enzyme detected by SDS-PAGE analysis.

3.14.15 Inactivation of cutinase

Inactivation of cutinase was studied by incubating the enzyme with 0.5 mM PMSF at different interval of time at 37 °C. The residual activity was determined immediately using pNPB as substrate.

3.15 Esterification reactions catalyzed by cutinase from *P. cepacia* NRRL B 2320

In the standard protocol, ester synthesis was carried out in screw-capped test tubes as bioreactors. Unless otherwise specified, 0.1mM of enzyme in lyophilized powder form (calculated on the basis of molecular mass of enzyme as obtained from SDS PAGE was 26.5 kDa) was added to 2.5 ml of isooctane containing 0.25 M fatty acid and 0.25 M alcohol. All reagents are previously dried over 4 Å molecular sieves. The tubes were kept in a horizontal shaker at 37°C. At regular intervals, 300 µl of the mixture (for butyric acid) or 100 µl (for all other acids) were transferred to 2 ml micro centrifuge tube and centrifuged at 10,000*g* for 1 min to remove the suspended enzyme particles. The progress of reaction was monitored by the methods as described in sections 3.7.4 and 3.7.5.

3.15.1 Kinetic study for butyl butyrate synthesis

The experiment for kinetic study for synthesis of butyl butyrate by esterification of butyric acid and butanol catalyzed by cutinase has shown in Table 3.10. Originally, Michelis–Menton equation was derived for kinetics of single substrate reaction. However, a reaction involving two substrates may also thought to obey the Michelis-Menton kinetics, if the reaction rate depends on the concentration of both the substrates, so that if one substrate concentration

varied while other maintaining constant, the reaction behaves like a single substrate reaction obeying Michelis-Menton kinetics.

Table 3.10 Experimental plan for kinetic study of esterification of butyric acid and butanol catalyzed by cutinase

Concentration of butyric acid (A) (M)	Concentration of butanol (B) (M)
0.05-0.5	0.05
0.05-0.5	0.1
0.05-0.5	0.25
0.05-0.5	0.5
0.05	0.05-0.5
0.1	0.05-0.5
0.25	0.05-0.5
0.5	0.05-0.5

Previously, lipase or cutinase catalyzed esterification reactions have been described by Ping-Pong bi bi kinetic model equation 3.14.

$$v = \frac{V_{\max}}{1 + \frac{K_{mA}}{[A]} + \frac{K_{mB}}{[B]}} \qquad 3.14$$

where [A] and [B] are the initial concentrations of butyric acid and butanol respectively, v is the initial reaction rate, V_{max} is the maximum reaction rate, K_{mA} and K_{mB} are the Ping-Pong constants for the butyric acid (A) and the butanol (B), respectively.

The competitive inhibition by alcohol (Janseen *et al*., 1999) or acid (Maury *et al*., 2005; Lima *et al*., 1996) or both alcohol and acid leads to the use of following modified Michelis-Menton equations.

$$v = \frac{V_{\max}}{1 + \frac{K_{mA}}{[A]}\left(1 + \frac{[B]}{K_{IB}}\right) + \frac{K_{mB}}{[B]}} \qquad 3.15$$

where, K_{IB} is the inhibition constant for butanol

$$v = \frac{V_{max}}{1 + \frac{K_{mb}}{[B]}\left(1 + \frac{[A]}{K_{IA}}\right) + \frac{K_{mA}}{[A]}} \quad 3.16$$

where, K_{IA} is the inhibition constant for butyric acid,

$$v = \frac{V_{max}}{1 + \frac{K_{MA}}{[A]}\left(1 + \frac{[B]}{K_{IB}}\right) + \frac{K_{MB}}{[B]}\left(1 + \frac{[A]}{K_{IA}}\right)} \quad 3.17$$

The presence of water (one of the product) at the start of the reaction modifies the eq. 3.15, 3.16 and 3.17 to the following equations (eq. 3.18, 3.19 and 3.20) (Janseen *et al*., 1999).

$$v = \frac{V_{max}}{1 + \left(\frac{K_A}{[A]} + \frac{K_{AB}}{[A]\bullet[B]}\right)\left(1 + \frac{[B]}{K_{IB}}\right) + \frac{K_{BP}}{[B]}} \quad 3.18$$

$$v = \frac{V_{max}}{1 + \left(\frac{K_A}{[A]} + \frac{K_{AB}}{[A]\bullet[B]}\right) + \frac{K_{BP}}{[B]}\left(1 + \frac{[A]}{K_{IA}}\right)} \quad 3.19$$

$$v = \frac{V_{max}}{1 + \left(\frac{K_A}{[A]} + \frac{K_{AB}}{[A]\bullet[B]}\right)\left(1 + \frac{[B]}{K_{IB}}\right) + \frac{K_{BP}}{[B]}\left(1 + \frac{[A]}{K_{IA}}\right)} \quad 3.20$$

where, K_{AB} is the constant term contributing forward and reverse reaction of esterification, K_{BP} is the modified K_{MB} due to water inhibition. Microsoft 2003 excel solver used to solve the equations.

3.16 Transesterification reactions catalyzed by cutinase

Transesterification reaction was carried out at 37°C in screw-capped vials placed inside a shaking incubator. The initial reaction mixture consisted of methanol to oil molar ratio of 1:1, lyophilized powdered enzyme and along with the respective controls (samples without enzyme) incubated in a horizontal shaker at 37°C. The effect of enzyme concentration, temperature and molar ratio on the conversion of methyl esters of tributyrin, triolein and soyabean oil was carried out using cutinase as catalyst. The catalytic activity of cutinase was also compared with the commercially available lipase. At regular intervals, samples were withdrawn and analyzed by method as described in section 3.7.5.

3.16.1 Kinetic study for transesterification reaction of tributyrin, triolein and soyabean oil

Previously, lipase catalyzed transerification reactions have been described by Ping-Pong bi bi kinetic model equation 3.14 (Paiva *et al*., 2000). The competitive inhibition by alcohol (Dossat *et al*., 2002) or triglyceride or both alcohol and triglyceride leads to the use of modified Michaelis–Menten equations eq. 3.15, 3.16 and 3.17.

Table 3.11 Experimental plan for kinetic study of transesterification of tributyrin, triolein and soyabean oil catalyzed by cutinase

Concentration of tributyrin/ triolein/ soyabean oil (A) (M)	**Concentration of methanol (B) (M)**
0.05-0.5	0.05
0.05-0.5	0.1
0.05-0.5	0.2
0.05	0.05-0.5
0.1	0.05-0.5
0.2	0.05-0.5

The experiments for kinetic analysis of tributyrin, triolein and soyabean oil were performed according to the plan shown in Table 3.11.

3.17 Identification, cloning and expression of cutinase encoding genes from *P. cepacia* NRRL B 2320

3.17.1 Primer design

The primers used in this study were chosen on the basis of phylogenetic relationship (Fig 3.2) of *P. cepacia* with the microorganisms reported to produce cutinase. The nearest microorganism with available gene sequence was *T. fusca*. The primers used are given in Table 3.12.

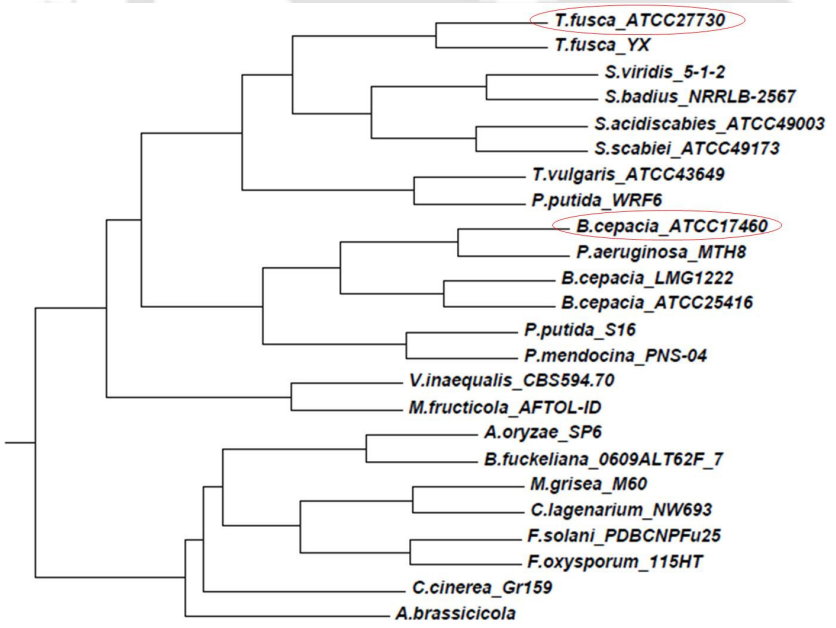


Fig. 3.2 Construction of Phylogenetic tree based on 16s and 18s rRNA of Prokaryotes and Eukaryotes, respectively of various cutinase producing organisms reported in literature. *T. fusca* (circled) used for designing primers and *P. cepacia* (or *B. cepacia*) (circled) used as source of organism for PCR amplification of cutinase genes.

3.17.2 Preparation of genomic DNA from P. cepacia NRRL B 2320

Genomic DNA was prepared using Sigma genomic DNA preparation kit (Cat No. NA2100-1KT) as per the manufacturer's instructions from 2-3 ml cultxxure of *P. cepacia* NRRL B 2320 grown at 28°C and 180 rpm for 12 h.

Table 3.12 Primer sequences used for PCR amplification

Primer Name	Sequence
Cut_1F	5'-ggaattcggatcca*atg*cccccgcatgcggcgcg-3'
Cut_1R	5'-gaagcttctcgaggaaggggcaggtggagcggta-3'
Cut_2F	5'-ggaattcggatcca*atg*gctgtgatgacccccg-3'
Cut_2R	5'-gaagcttctcgaggaacgggcaggtggagcggt-3'

The restriction site used for forward primer was Bam HI, and for reverse primers was Xho I.

3.17.3 PCR

All diagnostic PCR reactions (including PCR for checking of integrants, PCRs for quick check of plasmids and confirmation of clones) were set up as given in Table 3.13 with NEB taq (Cat No. M0273L) and preparative PCRs for amplification of insert to clone into the pET vector were set up as follows with high fidelity taq from NEB (Cat No. M0530L).

Table 3.13 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 (3 U/µl)	0.20 µl
10pM/µl Forward primer	0.20 µl
10pM/µl Reverse primer	0.20 µl
water	To make up to volume
Total	20 µl

Cycling conditions followed for all diagnostic PCR and for all preparative PCR are shown in Table 3.14 and Table 3.15, respectively.

Table 3.14 Condition for diagnostic PCR for cloning of cutinase gene

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

Table 3.15 Condition 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 2-4: 20 cycles
5	72°C-10 min
6	4°C

3.17.4 Double digestion of pET22b (+) and amplicon with XhoI & BamHI

The DNA was double digested for 4 hours with Xho I and Bam HI using manufacturer's instruction (NEB http://www.neb.com/nebecomm/DoubleDigestCalculator.asp) in 50 µl reaction volume and purified by gel extraction.

3.17.5 Ligation

Ligations were carried out using instant ligation kit from NEB (Cat No. M0202L). The ligation reaction was set up in the molar ratio 1:3 (vector: insert) in final volume of 20 µl as per the manufacturer's instruction (NEB http://www.neb.com/nebecomm/products/

productM0202.asp). Appropriate amount of ligation mix (2 µl and 3 µl) was used for transformation in *E. coli* DH5α and BL 21 (DE3).

3.17.6 Preparation of competent cells of E. coli DH5α and BL 21 (DE3)

E. coli DH5α and BL 21 (DE3) competent cells were prepared using 25 ml culture by calcium chloride method as per the standard protocol (Sambrook and Russell, 2001) and immediately used for transformation. The *E. coli* (DH5α or BL 21 (DE3)) culture grown in Luria-bertani (LB) medium for 12 h inoculated again in fresh LB medium and incubated at 37°C, 220 rpm. After 2 h (OD_{600} ~0.4) the culture was centrifuged at 5000*g* and the supernatant was discarded. The cell pellet was suspended in the solution of calcium chloride and magnesium chloride (20 mM and 80 mM). After 10 min of incubation at 0-4°C, the cells were centrifuged at 4000*g*. The cell pellets were separated from supernatant and resuspended in 100 mM calcium chloride solution, and cells were kept in ice for 2 h before transformation.

3.17.7 Transformation of cutinase encoding genes in E. coli

Both *E. coli* DH5α and *E. coli* BL21 (DE3) were transformed as follows. 2-3 µl of ligation mix added to the competent cells prepared by $CaCl_2$ 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 was added to the cells and mixed by gentle tapping of the tube and the whole volume plated on LB Agar plate with ampicillin (100 µg ml^{-1}) and incubated for 12-14 hours at 37°C.

3.17.8 Screening of E. coli recombinants clones

All the colonies growing on the ampicillin plates after transformation in DH5α were streaked on LB agar plate containing ampicilin (100 µg 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 sequencing and restriction digestion analysis.

3.17.9 Expression of cutinase encoding genes in E. coli BL 21 (DE3)

Cutinase construct in *E. coli* BL21 (DE3) were inoculated in 50 ml LB medium in 250 ml Erlenmeyer flask with ampicillin (100 µg ml^{-1}) and incubated at 37°C and 200 rpm. Culture was induced with 0.6 mM IPTG (at A_{600} nm ~ 0.75), 5 ml aliquot was taken in sterile condition at regular time interval and centrifuged at 8000*g* 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 10,000*g*. The supernatant was used as crude enzyme source for analysis by pNPB assay and SDS-PAGE.

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 is a link between lipase and esterase, and has immense potential in different industries as discussed in chapter 2. So, following studies on production, purification, characterization and applications of cutinase have been carried out.

4.1 Characterization of cutin

The cutin was prepared from tomatoes as described in the section 3.4 and characterized using AFM and FT-IR studies. AFM was used successfully to image the surface morphology and elucidate structure details of amorphous and crystalline polymers, and phase separated macromolecular systems (Benitez *et al.,* 2004). The topography of prepared cutin at different stages of preparation was obtained using AFM. The gradual removal of big agglomerates as observed from Fig. 4.1a, 4.1b and 4.1c was evidence of the removal of other unwanted substances (pectin, cellulose and waxes) present in the tomato peels. Cutin isolated from tomato fruits mainly formed by esterification of secondary hydroxyl groups of the dihydroxy fatty acids monomers. Previously, FT-IR spectroscopy was used to characterize (in situ) the functional chemical groups of isolated cuticles and their interactions with exogenous chemicals at the cuticular level (Benitez *et al.,* 2004). In the present investigation, experiments have been performed to obtain FT-IR of cutin (Fig. 4.2), and absorption around 1630 and 1500 cm^{-1} were due to the stretching of C=C bonds and stretching of benzenoid ring, respectively.

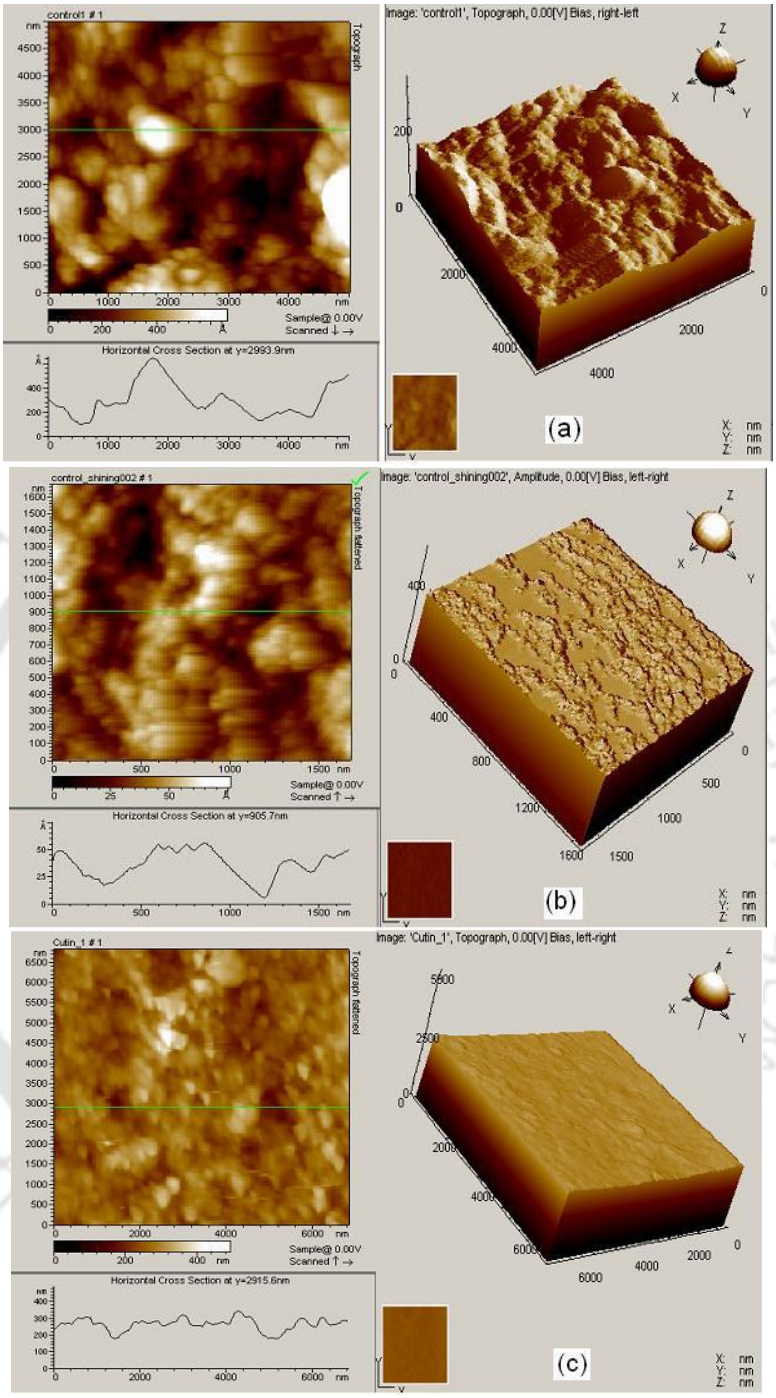


Fig. 4.1 AFM image of (a) untreated tomato peels (b) enzyme treated tomato peels and (c) cutin.

Absorption band at 1730 cm^{-1} was due to the C–O stretching vibration of the carbonyl group of the ester bond, which was the link between the different hydroxy fatty acids to form the cutin cross-linking.

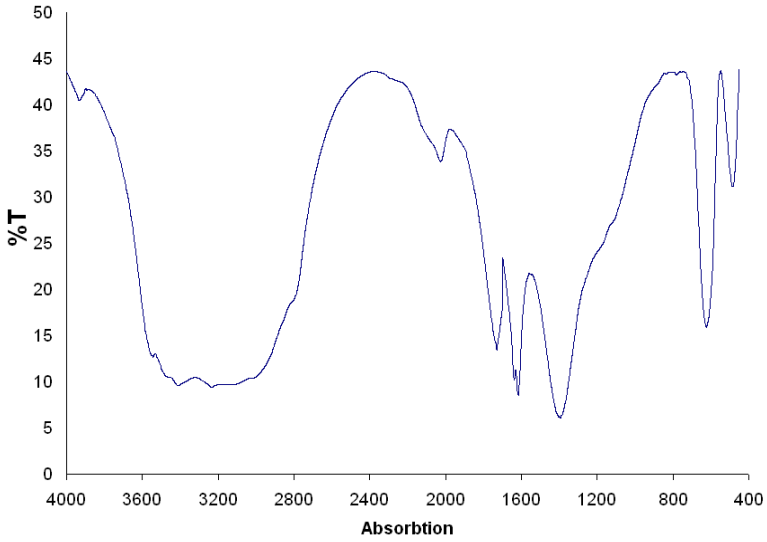


Fig. 4.2 FT-IR spectrum of cutin prepared from tomato peels.

4.2 Characterization of p-nitrophenyl (16 methyl sulphone ester) hexadecanoate (p-NMSH)

The cutinase specific substrate, p-NMSH was prepared according to the method described in the section 3.5 and characterized by H^1NMR and its spectrum is shown in Fig. 4.3.

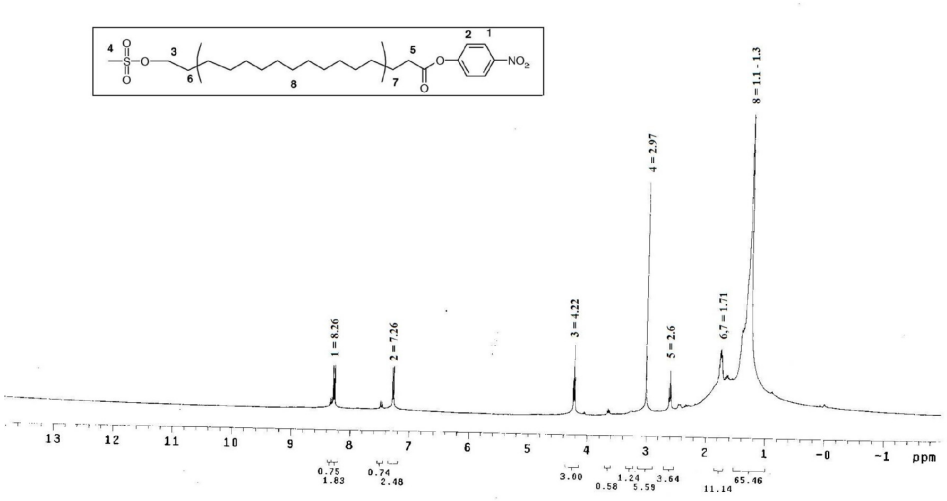


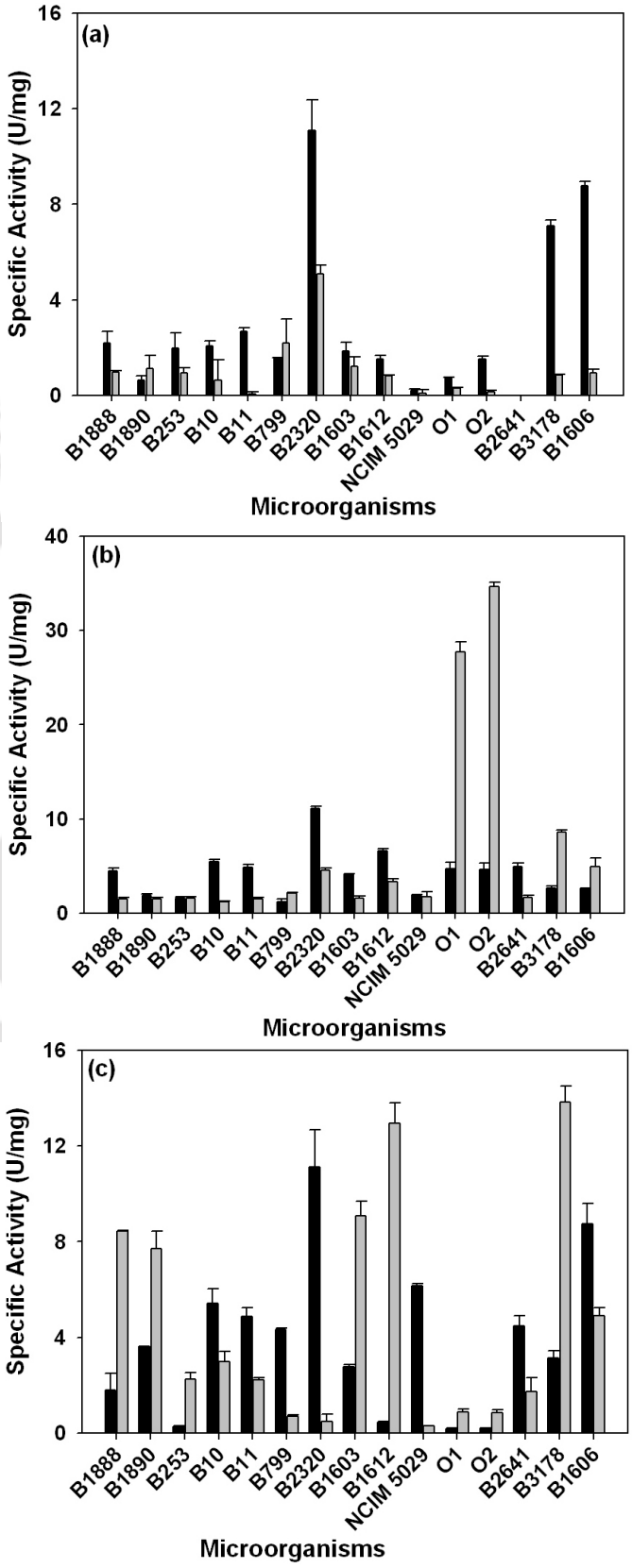
Fig. 4.3 NMR spectrum of p-nitrophenyl (16 methyl sulphone ester) hexadecanoate.

The observed H^1NMR spectrum (Fig. 4.3) of the synthesized p-NMSH was comparable with the previously reported spectrum (Degani *et al*., 2006). The sharp peak between 1.1-1.3 ppm was due to the presence of a long chain fatty acid. At the one end of long chain fatty acid nitrophenol group was attached giving peaks at 8.26 ppm and 7.26 ppm. The existence of sulphonyl group at other end was confirmed by peaks at 4.22 ppm and 2.97 ppm.

4.3 Screening of microorganisms using different media

For screening and selection of potential cutinase producer, bacteria were grown on different plates containing primary screening media (section 3.8) which composed of meat extract, Tween 20, $CaCl_2$ and agar. A clear zone was observed around the colonies of bacteria producing lipase/esterase/cutinase. The clear zone formed is due to the precipitation of calcium laurate around each colony of the strains producing lipase/esterase/cutinase. Among the tested bacteria, most of the procured strains (except *P. fluorescens* NRRL B14678) and two isolated bacteria showed clear zone around their colonies. All of these bacteria have ability to produce lipase/esterase/cutinase. To distinguish between lipase/esterase and cutinase producer, the selected bacteria were then grown on different fermentation media containing either glucose or glucose with Tween 80 or glucose with olive oil or glucose with cutin or cutin as carbon source (section 3.8). The effect of Tween 80 and olive oil on p-NPBase and p-NPPase activity of different microorganisms have been studied. As cutinase is an intermediate between lipase and esterase, initially experiments were performed to select the efficient lipase and esterase producer and then the cutinase producer. It was found that the affinity towards the substrates (p-NPB and p-NPP) varies with the inducers used in the media. Among the different fermentation media used, when all bacteria were grown on medium containing

glucose showed relatively higher activity with p-NPB than p-NPP (Fig. 4.4a). The highest production of p-NPBase (esterase/cutinase) was observed to be 11.08 U mg^{-1}, 8.77 U mg^{-1} and 7.09 U mg^{-1} from *P. cepacia* NRRL B 2320, *P. geniculata* NRRL B 1606 and *P. fluorescens* NRRL B 3178, respectively. Similarly, when they were grown on medium containing glucose with Tween 80 (Polyoxyethylenesorbitan monooleate) most of them showed higher activity of p-NPBase (Fig. 4.4b). It was observed that the glucose alone and glucose with Tween 80 have induced the esterases from ten microorganisms, which were having higher affinity for short-chain fatty acid esters (Fig. 4.4a and 4.4b). In these microorganisms, Tween 80 induces the esterase production rather than lipase. Surprisingly, four microorganisms *viz.,* two isolated strains (O1 and O2), *P. fluorescens* NRRL B 3178 and *P. geniculata* NRRL B 1606 have shown higher activity with p-NPP when Tween 80 was used as inducer in the medium. The highest p-NPPase (lipase) activity was found to be 34.64 U mg^{-1} and 27.72 U mg^{-1} for two isolates O1 and O2, respectively in the medium containing Tween 80. In these four microorganisms, lipase production was induced by Tween 80 rather than esterase. In the literature, reports are available on the production of lipase using Tween 80 as an inducer (Li *et al*., 2001). Tween 80 (Li *et al*., 2001) and olive oil (Ciafardini *et al*., 2006) induced lipase/esterase production from different microorganisms. The probable dual role of Tween 80 to induce lipase from some microrganisms and esterase from others due to the presence of ester bond with fatty acid of C_{12} carbon chain length in it. The lipase was induced due to its long chain fatty acid part of the synthetic ester (Tween 80), whereas esterase was induced when microorganisms needed to hydrolyse the ester bond present in Tween 80.



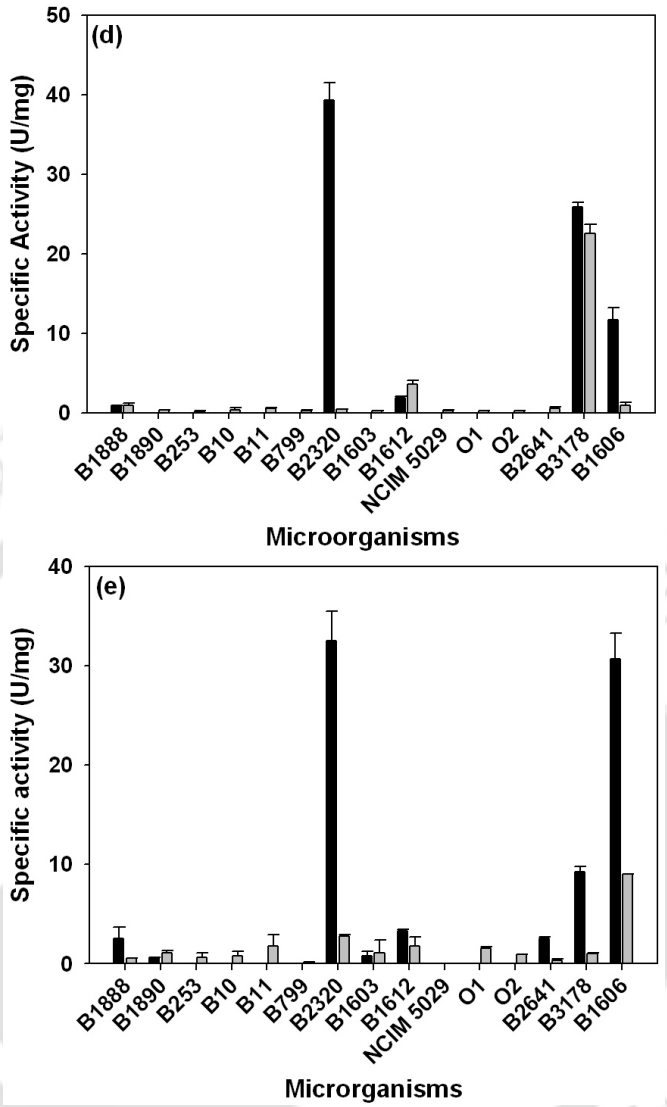


Fig. 4.4 Screening of microorganisms using media containing different carbon sources (a) glucose, (b) glucose + Tween 80, (c) glucose + olive oil, (d) cutin and (e) glucose + cutin (other components used were as given in the section 3.6). Microorganisms were grown at 28°C and 180 RPM, and enzyme assay was performed with pNPB (■) and p-NPP (■) at 37°C.

Though, Tween 80 induced p-NPP hydrolase activity (lipase) of two isolates, olive oil or cutin (Fig. 4.4c and 4.4d) did not show any induction of either of p-NPBase (esterase or/ and cutinase) or p-NPPase (lipase) activity of these two isolates. This is due to the fact that Tween 80 is a synthetic ester of oleic acid, whereas olive oil is the mixture of different fatty

acids with high content of oleic acid, which could not be utilized by these isolates. Also, cutin is the polymer of long chain hydroxy and epoxy fatty acids. Hence, cutin or olive oil failed to induce the enzymes from the isolates. Interestingly, *P. cepacia* NRRL B 2320, *P. fluorescens* NRRL B 3178 and *P. geniculata* NRRL B 1606 have shown p-NPBase (cutinase) activity in the medium containing cutin as an inducer (Fig. 4.4e). Among these, *P. cepacia* NRRL B 2320 was observed to show highest cutinase (p-NPBase) activity of 40 U mg^{-1} and 32 U mg^{-1} when grown on medium containing cutin and cutin with glucose, respectively (Fig. 4.4d and 4.4e). *P. fluorescens* NRRL B 3178 and *P. geniculata* NRRL B 1606 have shown cutinase (p-NPBase) activity of 25.9 U mg^{-1} and 11.68 U mg^{-1}, respectively in presence of cutin. However, cutin was unable to induced p-NPPase activity in *P. geniculata* NRRL B 1606 and *P. cepacia* NRRL B 2320, but enhanced p-NPPase activity from *P. fluorescens* NRRL B 3178. This was due to the presence of some long chain fatty acids along with hydroxy or dihydroxy fatty acids in the cutin, which induced some other lipases along with cutinase. Glucose has shown diverse effect on the cutinase production in the medium containing glucose and cutin for these three microorganisms. In presence of glucose and cutin, the p-NPBase activity (cutinase) of *P. cepacia* NRRL B 2320 and *P. fluorescens* NRRL B 3178 was repressed (Fig. 4.4e), whereas induction of the same was observed in *P. geniculata* NRRL B 1606. This was due to the presence or absence of catabolic repressor or inducer in different microorganisms. The lack of catabolic repression was also reported in the *P. putida* (Sebastian *et al*., 1987). This was first observation where glucose acted as repressor for some bacteria and inducer for other bacteria for the production of same enzyme. The cutinase activity of these three microorganisms was further confirmed by using cutinase specific substrate, p-NMSH. Among these three microorganisms, *P. cepacia* NRRL B 2320 was found

to be the best producer of cutinase (Fig. 4.5). *P. cepacia* NRRL B 2320 have shown p-NPBase, p-NPPase and p-NMSHase (cutinase) activity when grown on medium containing cutin. Degani *et al*., (2006) showed that p-NMSH was a highly specific substrate for bacterial cutinase. Purdy and Kolattukudy (1975) concluded that the non-specific esterase hydrolyses only soluble substrates, whereas cutinases prefer insoluble substrates. As p-NMSH is indeed insoluble and this could be one of the reasons for the lack of reactivity with nonspecific esterases and specificity towards cutinase. Lipases were also not able to hydrolyse p-NMSH, because lipases require interfacial activation for its activity where as cutinases can hydrolyse p-NMSH without interfacial activation.

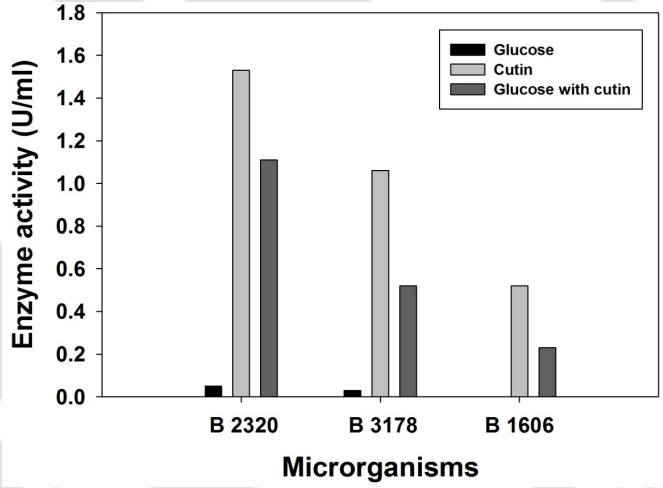


Fig. 4.5 Activity with p-NMSH of *P. cepacia* NRRL B 2320, *P. geniculata* NRRL B 1606 and *P. fluorescens* NRRL B 3178 in glucose, cutin and glucose with cutin containing media (other components used were as given in the section 3.6). Microorganisms were grown at 28°C and 180 RPM.

4.4 Effect of carbon sources/inducers on the production of cutinase

The effect of different inducers and carbon sources on the production of cutinase from *P. cepacia* NRRL B 2320 is presented in Table. 4.1. Experiments were performed according to

the method described in the section 3.9. It was observed that the oils *viz*., mustard, sunflower and soybean oil induced p-NPBase activity much higher than p-NMSH hydrolase activity.

Table 4.1 Specific Activity towards p-NPB, p-NPP and p-NMSH of *P. cepacia* NRRL B 2320 grown on media containing different inducers and carbon sources (other components used were as given in the section 3.6). Cultures were incubated at 28°C and 180 RPM

Carbon sources or inducers	Production of cutinase		
	p-NPBase ($U\ mg^{-1}$)	p-NPPase ($U\ mg^{-1}$)	p-NMSHase ($U\ mg^{-1} \times 10^{-2}$)
Mastard Oil	45.79±1.7	2.20±.043	13.6±0.49
Oleic acid	1.18±.0.35	0.41±0.022	3.1±.0.07
Sunflower oil	42.34±2.63	1.42±0.89	10.4±0.42
Soybean oil	46.87±1.10	1.45±0.66	11.0±0.84
16-HDA	9.74±2.59	0	5.8±0.21
Lactose	12.48±1.84	0.39±0.27	2.01±0.03
Lactose+cutin	36.09±0.85	0.21±0.13	28.51±0.12
Fructose	10.26±0.96	1.30±0.52	3.32±0.14
Fructose+cutin	43.82±4.71	0.62±0.16	12.09±0.00
Maltose	12.42±1.96	0.87±0.35	2.19±0.11
Maltose+cutin	31.29±2.37	1.36±0.21	8.05±0.49
Sucrose	5.38±0.80	2.64±0.89	3.30±0.13
Sucrose+cutin	53.75±0.78	1.76±0.12	16.61±1.23
Xylose	8.03±0.37	0.88±0.82	1.87±0.30
Xylose+cuitn	35.23±0.82	0.51±0.21	15.02±2.48
Galactose	17.58±1.25	0.48±0.12	6.37±0.93
Galactose+Cutin	42.40±2.35	1.27±0.05	12.81±0.40
Starch	15.70±3.22	0.95±0.00	2.95±0.07
Starch+cutin	47.94±4.21	1.74±0.75	40.83±1.66
Glucose	11.08±1.29	5.08±0.36	1.0±0.03
Cutin	40.15±2.17	0.41±0.01	41.2±0.26
Glucose+Cutin	32.54±2.93	2.76±0.19	13.90±0.29

*All experiments are performed in duplicates

But, cutin monomer, 16-hydroxyhexadecanoic acid was unable to induce esterase or cutinase production from *P. cepacia* NRRL B 2320. In the presence of 16-hydroxyhexadecanoic acid, *P. cepacia* NRRL B 2320 have shown 9.74 U mg^{-1} and $5.8x10^{-2}$ U mg^{-1} activity towards p-NPB and p-NMSH, respectively.It was also observed that the production of cutinase was much lower in the medium containing glucose or other carbohydrate as a sole source of carbon than cutin in the medium. The monomer of cutin, 16-hydroxyhexadecanoic acid, was also unable to induce cutinase production. This may be due to the polymeric structure of cutin, which is absent in 16-hydroxyhexadecanoic acid. In case of other carbon sources, the production of cutinase was found to be very less in the medium without cutin. The cutinase activity towards p-NMSH was found to be 41.2 x 10^{-2} U mg^{-1} from *P. cepacia* NRRL B 2320 in the medium containing cutin as a sole source of carbon, whereas in the medium containing other carbon sources (*viz*., glucose, lactose, fructose, maltose, sucrose, xylose, galactose, starch), the activity towards p-NMSH was < $7x10^{-2}$ U mg^{-1}. Hence, the production/induction of cuinase was favored in the presence of cutin as essential carbon source. Though cutin along with other carbon sources found to induce the p-NPBase activity appreciably in some cases, but unable to induce enzyme catalyzing hydrolysis of p-NMSH further. This was due to the presence of carbon sources other than cutin which would enhance some esterases but fail to induce the cutinase production any more.

4.5 Development of medium for enhanced production of cutinase from *P. cepacia* NRRL B 2320

The optimization of medium for enhanced production of cutinase from *P. cepacia* NRRL B 2320 was performed in two step method. Screening of medium components significantly

influencing the cutinase production was carried out using Plackett- Burman experimental design and optimization of screened components was performed using central composite design.

4.5.1 Screening of significantly influencing medium components

To evaluate the effect of most significantly influencing medium components on the production of cutinase, experiments were conducted in 12 runs in duplicate as per the experimental design given in the Table 3.1 (in the section 3.10.1). The design matrix selected for the screening of significant variables for cutinase production and the corresponding responses are shown in the Table 4.2. The production of cutinase from *P. cepacia* NRRL B 2320 was varied from 30 to 293.5 U ml^{-1} inferring that the strong influence of media components on the production. This variation reflects the significance of medium optimization to achieve higher production. To assess the significance of each individual factor on the cutinase activity, a student's *t*-test was performed and results are presented in the Table 4.3. Generally, a large *t* value and lesser *p* value indicate a high significance of the corresponding model term. Factors evidencing *p*-values of less than 0.04 (Kumar *et al*., 2009) were considered to have significant effects on the response, and were therefore selected for further optimization study. The lower probability values indicate that the factors were relatively more significant on the production of cutinase. A positive sign indicates that at higher level of variables setting, results in a higher response than the lower level variable setting. Alternatively, a negative sign indicates that the lower level of variable setting results in a higher response than the high level variable setting (Kumar *et al*., 2009).

Table 4.2 Plackett–Burman design matrix with coded and actual values along with the observed and predicted cutinase production from *P. cepacia* NRRL B 2320

Run Order	Levels of variables							Enzyme Activity (U ml^{-1})		Specific activity (U mg^{-1})	Cell Growth (g l^{-1})
	Cutin X_1	*Peptone* X_2	*Beef Extract* X_3	*Urea* X_4	KH_2PO_4 X_5	*KCl* X_6	$MgSO_4.7H_2O$ X_7	Observed[a]	Predicted		
1	1(10)	-1(2)	1(7)	-1(1)	-1(0.5)	-1(0.1)	1(8.5)	162.98±1.91	168.37	61.36	1.97
2	1(10)	1(20)	-1(1)	1(9)	-1(0.5)	-1(0.1)	-1(0.5)	192.35±0.83	200.75	40.96	1.52
3	-1(2)	1(20)	1(7)	-1(1)	1(5.5)	-1(0.1)	-1(0.5)	164.38±5.87	167.44	32.15	0.74
4	1(10)	-1(2)	1(7)	1(9)	-1(0.5)	1(1.1)	-1(0.5)	57.20±1.82	61.92	22.80	1.97
5	1(10)	1(20)	-1(1)	1(9)	1(5.5)	-1(0.1)	1(8.5)	289.90±5.09	281.49	67.20	2.14
6	1(10)	1(20)	1(7)	-1(1)	1(5.5)	1(1.1)	-1(0.5)	235.47±4.26	202.40	44.32	0.73
7	-1(2)	1(20)	1(7)	1(9)	-1(0.5)	1(1.1)	1(8.5)	194.64±6.19	204.45	37.37	1.86
8	-1(2)	-1(2)	1(7)	1(9)	1(5.5)	-1(0.1)	1(8.5)	157.65±1.90	137.71	69.32	1.59
9	-1(2)	-1(2)	-1(1)	1(9)	1(5.5)	1(1.1)	-1(0.5)	26.73±4.63	32.11	25.24	0.40
10	1(10)	-1(2)	-1(1)	-1(1)	1(5.5)	1(1.1)	1(8.5)	120.58±0.03	143.52	88.48	1.36
11	-1(2)	1(20)	-1(1)	-1(1)	-1(0.5)	1(1.1)	1(8.5)	205.12±1.61	205.30	51.48	0.95
12	-1(2)	-1(2)	-1(1)	-1(1)	-1(0.5)	-1(0.1)	-1(0.5)	76.35±2.98	75.81	55.32	0.77
13(Ct. Pt.)	0(6)	0(11)	0(4)	0(5)	0(3)	0(0.6)	0(4.5)	269.70±1.98	264.69	80.08	1.52

[a]The observed values of cutinase activity were the mean values of duplicates with standard deviation (Mean ± S.D.)

*Values in parentheses are actual level,

X_1-X_7 in g l^{-1}

The significant medium components screened by Plackett-Burman design are cutin, peptone, $MgSO_4 \cdot 7H_2O$ (with positive effect) and KCl (with negative effect). Though KCl and urea have shown negative effect on the production of cutinase in the selected range, but they are necessary for maximum cutinase production. The necessity of presence was checked by removing these components from the media, which results in reduced enzyme production.

Neglecting the terms, which were insignificant, the model equation for cutinase enzyme activity is:

$$Y_{\text{enzyme activity}} = 30.770 + 4.867\ X_1 + 6.299\ X_2 - 33.978\ X_6 + 7.883\ X_7 \quad (4.1)$$

where, X_1, X_2, X_6 *and* X_7 are the cutin, peptone, KCl and $MgSO_4 \cdot 7H_2O$, respectively.

Pareto chart (Fig. 4.6) shows that the ranking of variables according to the absolute values of standardized effect, important in the design of the experiment for optimization and it is a convenient way to view the results of a Plackett-Burman experimental design. The reference line (2.11) indicates that effects were significant with α value of 0.05. The variables effects, which extend past the line were known to be significant at particular α. The standardized effects were the *t* statistics shown in Fig. 4.6.

Table 4.3 Statistical analysis of Plackett–Burman design showing effect, coefficient values, *t* and *p*-value for each variable

Variable	Symbol code	Effect	Coefficient	*t*-Stat	*p*-value
Constant			30.770	36.82	0.000
Cutin	X_1	38.94	4.867	4.57	0.000[a]
Peptone	X_2	113.46	6.299	13.30	0.000[a]
Beef Extract	X_3	10.22	1.702	1.20	0.247[b]
Urea	X_4	-7.73	-0.966	-0.91	0.377[b]
KH2PO4	X_5	17.68	3.535	2.07	0.054[b]
KCl	X_6	-33.98	-33.978	-3.99	0.001[a]
MgSO4	X_7	63.07	7.883	7.40	0.000[a]
Ct pt			112.753	7.34	0.000[a]

[a] significant at $p<0.04$, [b] Non-significant; R^2 =94.52%, R^2_{adj} = 91.9

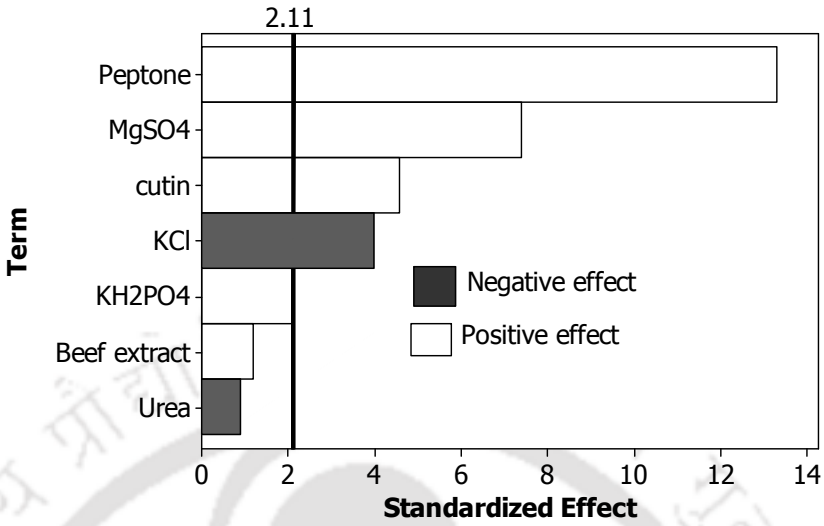


Fig. 4.6 Pareto chart of standardized effects of the factors on cutinase production.

The *t* statistics were calculated by dividing each coefficient by its standard error. The variables cutin, peptone, KCl and $MgSO_4 \cdot 7H_2O$ were influencing the production of cutinase very significantly (Fig. 4.6). The effect of other insignificant variables were not included in the next optimization experiment, but used in all experiments at their middle level (centre point). Previously, experiments were carried out to observe the effect of different carbon sources on the cutinase production (section 4.4), but no other carbon source except cutin was able to induce the cutinase. Among the seven media components, peptone, cutin, KCl and $MgSO_4 \cdot 7H_2O$ were found to be significant with high (>99%) confidence level. The major factor for expression of cutinase is the carbon source, since cutinases are inducible enzymes and thus produced in the presence of cutin as an essential carbon source. The nitrogen source in the medium also regulates the growth and fermentation process for cutinase production. Previously, cutinase production was reported from *P. putida* co-cultured with nitrogen fixing bacteria *clostridium* (Sebastian and Kolattukudy, 1988). Although, except peptone other

nitrogen sources have not shown much significant influence on the production of cutinase, KCl and $MgSO_4 \cdot 7H_2O$ were also shown impact on the cutinase production. Increment of KCl concentration resulted in decrease in cutinase production, where as increment in $MgSO_4 \cdot 7H_2O$ lead to higher cutinase activity. Rispoli and Shah (2007) and Pio and Macedo (2007), reported that $MgSO_4 \cdot 7H_2O$ had minimal effect on cutinase production from *C. lindemuthianum* and *F. oxysporium*, respectively, which is contradictory to the present investigation. This may be due to the fact that cutinases from bacterial and fungal source differed widely in respect of their pH and temperature stability and salt tolerance (Sebastian and Kolattukudy, 1988).

4.5.2 Optimization of screened medium constituents for enhanced cutinase production

The screened medium constituents (cutin, peptone, KCl, $MgSO_4 \cdot 7H_2O$) were optimized using central composite experimental design (CCD). The experiments were conducted as per the experimental design given in Table 3.3, (section 3.10.2). The design matrix and the corresponding results of CCD experiments to determine the effects of four independent variables (cutin, peptone, KCl, $MgSO_4 \cdot 7H_2O$) are shown in Table 4.4, along with the mean predicted values. Using the experimental results of CCD, regression model equations were developed for predicting the maximum cutinase activity given by equation (4.2).

$$Y_{\text{specific activity}} = -537.287 + 41.197X_1 + 36.027X_2 + 522.274X_6 + 62.065X_7 - 2.277X_1^2 - 1.239X_2^2 - 333.791X_6^2 - 4.276X_7^2 + 0.507X_1X_2 - 3.093X_1X_6 - 0.399X_1X_7 + 3.006X_2X_6 + 0.188X_2X_7 - 21.436X_6X_7 \quad (4.2)$$

where, X_1, X_2, X_6 *and* X_7 are the cutin, peptone, KCl and $MgSO_4 \cdot 7H_2O$, respectively.

The results were analyzed in the form of analysis of variance (ANOVA), which is a statistical technique that subdivides the total variation in a set of data into component parts associated with specific sources of variation for the purpose of testing hypotheses on the parameters of the model. Table 4.5 represents the ANOVA for cutinase activity. The mean sum of squares (MS) of the model term is obtained from the ratio of sum of squares (SS) and degrees of freedom (df). The Fisher's *F* value is calculated by dividing the MS owing to the model by the MS owing to error. Table 4.5 also shows a term for error, the MS value, which indicates that the amount of variation in the response data is low. The high Fisher's *F* value (91.51) indicates that the most of the variation in the response can be explained by the model equation. The associated *p* value is used to judge, whether *F* is large enough to indicate statistical significance or not. A *p* value < 0.04 is considered to be statistically significant with high confidence level (> 96%). Overall, the regression model for the cutinase activity was highly significant ($p<0.0001$), which indicates that the second-order polynomial model was adequate in representing the actual relationship among the response (cutinase activity) and the variables. This inference was also confirmed by high coefficient of regression ($R^2 = 96.83\%$). To determine the significance of the regression coefficient of the factors, the results were subjected to a student's *t*-test. The coefficients, *t*-value and *p*-value of different variable, their square terms and interaction terms are given in Table 4.6. The linear and quadratic terms of all the four components are found to be highly significant from their low *p*- values (<0.0001). Among the interaction effects, the coefficient term between cutin-peptone and those between KCl-$MgSO_4 \cdot 7H_2O$ have also shown high significance ($p<0.001$) for the production of cutinase. Such an observation in significance of interaction effects between the variables would have been lost, if the experiments were carried out by conventional methods.

Table 4.4 A 2^4 full-factorial central composite design matrix of four variables (in coded and actual values) with experimental and predicted values of cutinase production from *P. cepacia* NRRL B 2320

Run Order	Variables in coded and actual values				Enzyme Activity (U ml^{-1})		Specific Activity (U mg^{-1})	Cell Growth (g l^{-1})
	X_1 *(Cutin)*	X_2 *(Peptone)*	X_6 *(KCl)*	X_7 *($MgSO_4.7H_2O$)*	Observed[a]	Predicted		
1	-1(5.5)	-1(7.5)	-1(0.35)	-1(3.25	128.968±1.79	117.233	36.65	1.74
2	1(12.5)	-1(7.5)	-1(0.35)	-1(3.25)	121.395±1.68	128.680	29.97	1.87
3	-1(5.5)	1(18.5)	-1(0.35)	-1(3.25)	185.802±2.58	208.125	38.46	1.12
4	1(12.5)	1(18.5)	-1(0.35)	-1(3.25)	279.457±3.87	258.627	59.42	2.26
5	-1(5.5)	-1(7.5)	1(0.85)	-1(3.25)	143.534±1.99	146.027	45.82	1.58
6	1(12.5)	-1(7.5)	1(0.85)	-1(3.25)	129.470±1.79	146.650	45.32	2.02
7	-1(5.5)	1(18.5)	1(0.85)	-1(3.25)	256.507±3.56	253.450	55.18	1.42
8	1(12.5)	1(18.5)	1(0.85)	-1(3.25)	313.009±0.61	293.129	60.52	1.82
9	-1(5.5)	-1(7.5)	-1(0.35)	1(7.75)	127.770±1.77	147.546	41.51	1.41
10	1(12.5)	-1(7.5)	-1(0.35)	1(7.75)	141.177±1.96	146.422	31.43	1.52
11	-1(5.5)	1(18.5)	-1(0.35)	1(7.75)	262.727±3.64	247.736	60.23	2.62
12	1(12.5)	1(18.5)	-1(0.35)	1(7.75)	288.266±4.00	285.668	55.04	3.11
13	-1(5.5)	-1(7.5)	1(0.85)	1(7.75)	105.091±1.46	128.109	40.21	1.97
14	1(12.5)	-1(7.5)	1(0.85)	1(7.75)	138.589±1.92	116.161	65.70	1.88
15	-1(5.5)	1(18.5)	1(0.85)	1(7.75)	252.218±3.50	244.829	67.51	2.88
16	1(12.5)	1(18.5)	1(0.85)	1(7.75)	258.014±3.58	271.938	70.19	3.97
17	0(9.0)	0(13.0)	0(0.60)	0(5.50)	302.498±3.55	304.158	83.41	2.29
18	0(9.0)	0(13.0)	0(0.60)	0(5.50)	301.823±3.00	304.158	80.92	1.99
19	0(9.0)	0(13.0)	0(0.60)	0(5.50)	302.823±4.17	304.158	82.83	2.11
20	0(9.0)	0(13.0)	0(0.60)	0(5.50)	300.823±4.00	304.158	85.45	2.21
21	-2(2.0)	0(13.0)	0(0.60)	0(5.50)	183.600±2.55	169.424	60.90	2.28
22	2(16.0)	0(13.0)	0(0.60)	0(5.50)	195.886±2.72	207.979	48.37	2.51
23	0(9.0)	-2(2.0)	0(0.60)	0(5.50)	46.387±0.64	27.013	29.17	1.37
24	0(9.0)	2(24.0)	0(0.60)	0(5.50)	256.391±3.55	273.681	55.54	2.64
25	0(9.0)	0(13.0)	-2(0.10)	0(5.50)	210.491±2.92	209.296	69.21	1.72
26	0(9.0)	0(13.0)	2(1.10)	0(5.50)	225.250±3.12	224.361	66.57	3.23
27	0(9.0)	0(13.0)	0(0.60)	-2(1.00)	204.966±2.84	209.119	50.40	1.30
28	0(9.0)	0(13.0)	0(0.60)	2(10.0)	224.477±3.11	218.241	63.43	2.97
29	0(9.0)	0(13.0)	0(0.60)	0(5.50)	296.109±4.11	300.276	82.78	2.13
30	0(9.0)	0(13.0)	0(0.60)	0(5.50)	298.109±1.28	300.276	75.65	2.27

[a]The observed values of cutinase activity were the mean values of duplicates with standard deviation (Mean ± S.D.), * Values in the parentheses are actual level.

Table 4.5 Analysis of variance (ANOVA) for quadratic model

Source	DF	SS	MS	*F*-value	*p*-value
Model	14	319967	22854.8	91.51	<0.0001
Residual (error)	42	10489	249.7	-	-
Lack-of-Fit	34	9784	287.8	3.27	0.041
Pure Error	8	705	88.1	-	-
Total	59	330657			

R^2 = 96.83%; Adj R^2 = 95.54%;

SS, sum of squares; DF, degrees of freedom; MS, mean square.

Table 4.6 Model coefficient estimated by multiple linear regressions

Model term	Parameter estimate	Standard error	Computed *t*-value	*p*-value
Intercept	-537.287	50.0473	-10.736	0.000
X_1	41.197	4.6141	8.929	0.000
X_2	36.027	2.8755	12.529	0.000
X_6	522.274	63.4895	8.226	0.000
X_7	62.065	7.0857	8.759	0.000
X_1^2	-2.277	0.1742	-13.073	0.000
X_2^2	-1.239	0.0705	-17.567	0.000
X_6^2	-333.790	34.1387	-9.778	0.000
X_7^2	-4.276	0.4215	-10.146	0.000
X_1X_2	0.507	0.1451	3.495	0.001
X_1X_6	-3.093	3.1927	-0.969	0.338
X_1X_7	-0.399	0.3547	-1.125	0.267
X_2X_6	3.006	2.0317	1.479	0.147
X_2X_7	0.188	0.2257	0.832	0.410
X_6X_7	-21.436	4.9664	-4.316	0.000

In order to determine the optimal levels of each variable for maximum cutinase production, three-dimensional response surface plots were constructed by plotting the response (cutinase activity) on the Z-axis against any two independent variables, while maintaining other variables at their optimal levels. As shown in Fig. 4.7a, a curvature in the response surface indicates that the lower and higher values of both cutin and peptone did not result in higher response.

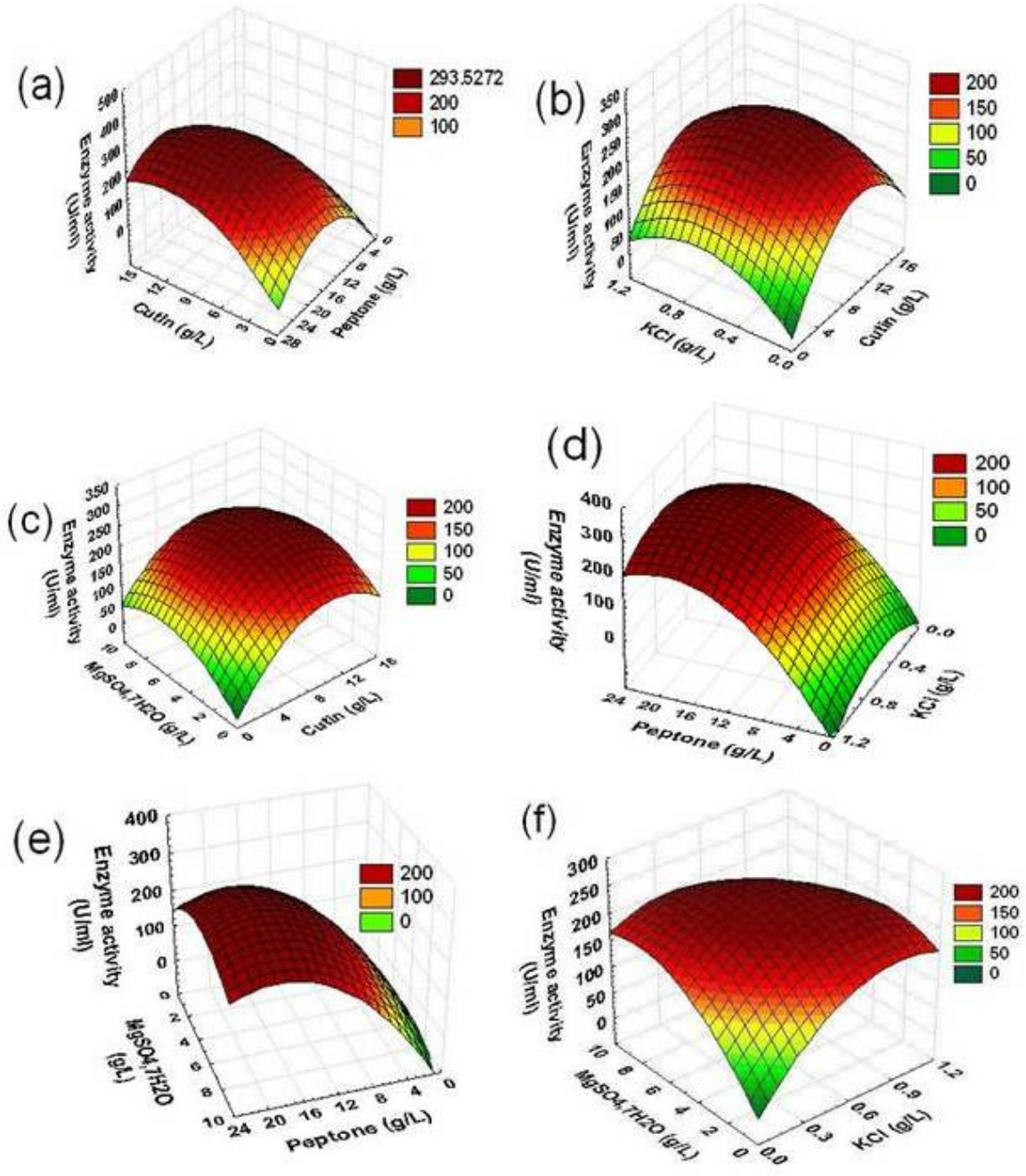


Fig. 4.7 Three-dimensional response surface plot for cutinase production showing the interactive effects of (a) cutin and peptone, (b) cutin and KCl, (c) cutin and $MgSO_4.7H_2O$, (d) peptone and KCl, (e) peptone and $MgSO_4.7H_2O$ and (f) KCl and $MgSO_4.7H_2O$.

The increment of cutin concentration from 2 to 10 g l^{-1} and peptone concentration from 2 to 18 g l^{-1} increased the cutinase activity but further increment in the concentration of both the components repressed the cutinase activity. A similar profile was observed in Fig. 4.7b (with cutin and KCl concentration) and Fig. 4.7c (with cutin and $MgSO_4 \cdot 7H_2O$ concentration), where cutinase activity increased with increasing KCl concentration up to 0.63 g l^{-1} and $MgSO_4 \cdot 7H_2O$ concentration up to 5 to 6 g l^{-1}. The cutinase production was also significantly influenced by peptone concentration in the medium and reached maximum at 17.77 g l^{-1}. From the Fig. 4.7d and Fig. 4.7e a steep increase in cutinase activity was observed with increasing concentration of peptone from 2 to 17.77 g l^{-1}. Further increase in peptone concentration was unable to enhance the activity any more. Again the interaction of KCl and $MgSO_4 \cdot 7H_2O$ (Fig. 4.7f) was very prominent with $p<0.001$. Similar effect was also observed for KCl and $MgSO_4 \cdot 7H_2O$. The experimental data were fitted into the aforementioned equation (4.2), and the optimum levels of each variable were determined to be as follows: cutin: 10.06 g l^{-1}, peptone: 17.77 g l^{-1}, KCl: 0.635 g l^{-1} and $MgSO_4.7H_2O$: 5.546 g l^{-1}. However, very few studies deal with statistical optimization of the process for cutinase production. There is no report available in the literature on the optimization of medium components for the production of cutinase using tomato cutin as a source of carbon from *P. cepacia* NRRL B 2320. An overall 2 fold increase in cutinase production was achieved in the optimized medium as compared with the un-optimized basal medium, which reflects the necessity and value of optimization process.

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

To verify validity of the model, experiments were carried out at optimal levels of significantly influenced medium components with other medium components at middle level and

compared with the calculated data from the model. The estimated enzyme activity of cutinase was found to be 336.76 U ml^{-1}, where as the predicted value from the polynomial model was 330.96 U ml^{-1}. The verification revealed a high degree of accuracy of the model of more than 98.73%, which is an evidence for the model validation under the investigated conditions. This value was also found to be 4.39% higher than the maximum measured cutinase activity observed in the CCD of experiments shown in Table 4.4. The estimated enzyme activity towards p-NMSH (cutinase specific substrate) was found to be 3.12 U ml^{-1}. After optimization the activity of cutinase was increased by two fold.

4.6 Unstructured model prediction

The cutinase production and growth of *P. cepacia* NRRL B 2320 was studied in shake flask and bioreactor (with or without pH control) as described in the section 3.10.3 using optimized medium. The profiles for cutinase production, and cell growth in shake flask and bioreactor (under uncontrolled and controlled pH) were illustrated in the Fig. 4.8, 4.9 and 4.10, respectively. From the profile, it was observed that the cutinase production increased with increasing growth up to 96 h of culture and maintained after this. Biokinetic parameters involved in the process were estimated using the models mentioned in equations 3.3, 3.4 and 3.5 described in material and methods (section 3.11). These models are essentially unstructured logistic models, which describes the kinetics of cell growth and product accumulation (Luedeking and Pirate, 1959; Mercier *et al.*, 1992). In this investigation, nonlinear regression using the least-square method was used employing Microsoft Excel Solver 2003 for fitting of experimental data with the models.

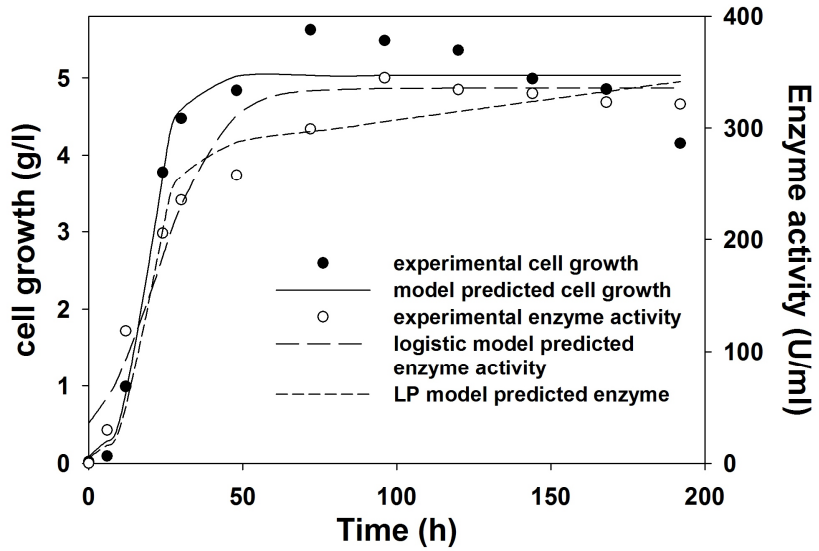


Fig. 4.8 Experimental and model predicted growth and production kinetics during cutinase production in shake flask in optimized medium, at 28°C, 180 RPM.

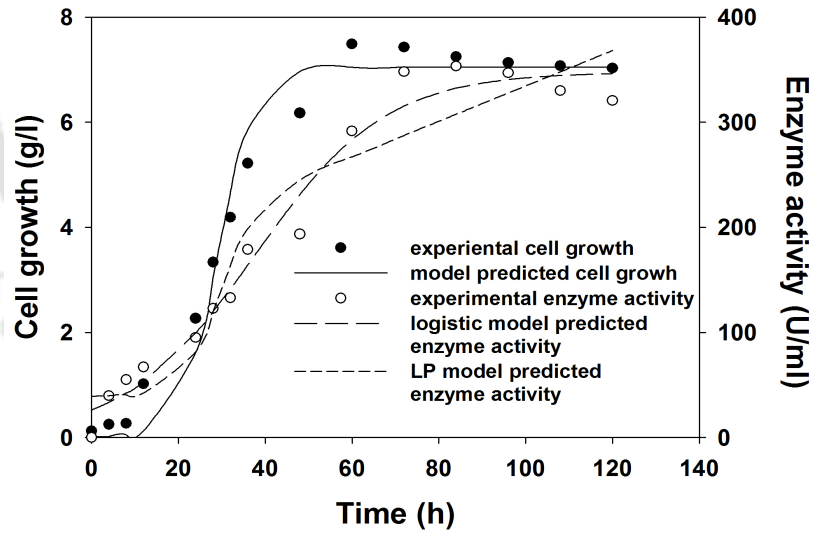


Fig. 4.9 Experimental and model predicted growth and production kinetics during cutinase production in bioreactor in optimized medium at uncontrolled pH, 2 vvm aeration and 200 RPM.

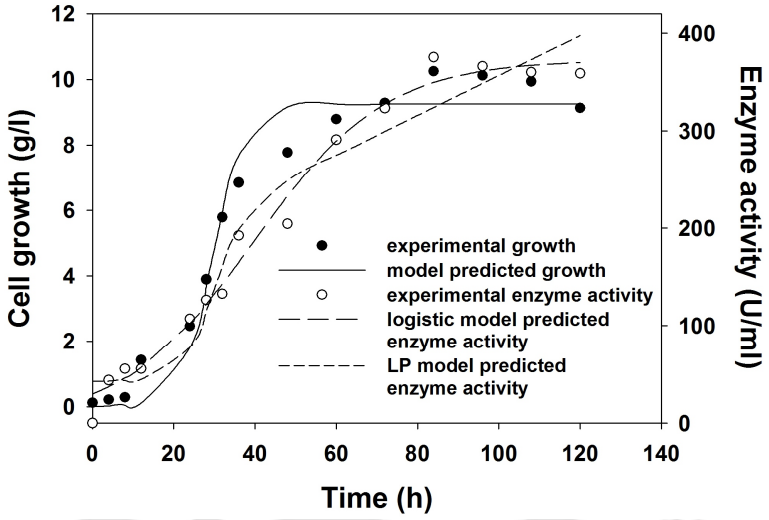


Fig. 4.10 Experimental and model predicted growth and production kinetics during cutinase production in bioreactor in optimized medium at controlled pH 7, 2 vvm aeration and 200 RPM.

The estimated kinetic parameters values obtained from these models are mentioned in Table 4.7, which also shows that the determination coefficient (R^2) values obtained by fitting the various models to the experimental data were found to be very high (≥0.93), thus revealing good precision of the models. Using the logistic model, μ, X_o and X_{max} were obtained for growth kinetic, and P_r, P_o and P_{max} were obtained from enzyme production kinetic profile in shake flask as well as bioreactor with or without pH control. Using Luedeking-piret (LP) model, α and β values were predicted. These α and β values are growth and non-growth associated parameters during enzyme production. A much higher α value than β value was observed in shake flask, which predicted that cutinase production was more growth associated than non-growth associated in shake flask. Since, the magnitude of the growth-associated parameter α was much greater than the magnitude of non-growth associated parameter β in the product formation model, so the production of cutinase mostly occurred during logarithmic growth phase.

Table 4.7 Parameters estimated by Logistic and Luedeking-Pirate (LP) model equation

Model	**Shake flask/ Bioreactor**	**Parameter estimated**	**R^2**
Logistic Model (eq. 3.3)	Shake flask	μ=0.213 h^{-1}, X_{max}=5.03 g l^{-1}, X_0= 0.08 gl^{-1}	0.97
Logistic Model (eq. 3.4)		P_r=0.097 U $ml^{-1}h^{-1}$, P_{max}=336 U ml^{-1}, P_0=35.53 Uml^{-1}	0.95
LP Model (eq. 3.5)		P_0=0, α=55.05 UgX^{-1}, β = 0.016 U$gX^{-1}h^{-1}$	0.95
Logistic model (eq. 3.3)	Bioreactor at uncontrolled pH	μ=0.234 h^{-1}, X_{max}=7.05 g l^{-1}, X_0= 0.008 gl^{-1}	0.97
Logistic model (eq. 3.4)		P_r=0.066 U $ml^{-1}h^{-1}$, P_{max}=347.73 U ml^{-1}, P_0=26.17 Uml^{-1}	0.97
LP Model (eq. 3.5)		P_0=39.32, α=24.99 UgX^{-1}, β = 0.24 U$gX^{-1}h^{-1}$	0.93
Logistic model(eq. 3.3)	Bioreactor at controlled pH 7	μ=0.25 h^{-1}, X_{max}=9.25 g l^{-1}, X_0= 0.005 gl^{-1}	0.97
Logistic model (eq. 3.4)		P_r=0.062 U $ml^{-1}h^{-1}$, P_{max}=372.80 U ml^{-1}, P_0=29.65 U ml^{-1}	0.98
LP Model (eq. 3.5)		P_0=42.72, α=18.43 UgX^{-1}, β = 0.22 U$gX^{-1}h^{-1}$	0.96

A Few reports are available on production kinetics of lipase (Rajendran and Thangavelu, 2009) and xylanase (Rajendran *et al*., 2008), where production was found to be mostly growth associated. It was also observed from Table 4.7 that in bioreactor (with or without pH control) α value decreased and β value increased, suggesting that cutinase production was mixed growth type in the bioreactors. This may be due to better controlled condition in the bioreactor than shake flask; also, the mass transfer of oxygen and other nutrients was facilitated in bioreactor. The coefficient of determination, R^2, is a measure of the strength of the linear relationship between the experimental and predicted values. The logistic model predicted the growth kinetics with an R^2 value of 0.97 in shake flask and bioreactors. For shake flask study, cutinase production kinetics was predicted by logistic and LP model with same R^2 value of 0.95. But in case of cutinase production under uncontrolled pH, the kinetics

for production was predicted with R^2 of 0.97 by logistic model, whereas the same was predicted with R^2 of 0.93 by LP model. It was observed that the logistic model predicted the production kinetics with R^2 of 0.98 on the production of cutinase under controlled pH of 7, and LP model showed R^2 of 0.96. The maximum cutinase production increased from shake flask (336.76 U ml^{-1}) to bioreactor and in bioreactor the cutinase production increased when pH was controlled at 7 (372.8 U ml^{-1}), than the uncontrolled pH (344.4 U ml^{-1}) (Table 4.7).

4.7 Growth kinetics of *P. cepacia* NRRL B 2320 under substrate inhibition during cutinase production

4.7.1 Growth profile at different initial substrate concentrations

To obtain the growth profile at different cutin concentrations, experiments were conducted according to the experimental procedure given in the section 3.12. The growth profile of *P. cepacia* NRRL B 2320 at different initial substrate concentrations was shown in Fig. 4.11. At the initial cutin concentration of 2 g l^{-1}, the growth of *P. cepacia* NRRL B 2320 was observed to be very low. It increases upon increasing the substrate concentration and the maximum cell growth was observed at 96 h at initial cutin concentration of 8 g l^{-1}. Further increase of the cutin concentration leads to inhibition of cell growth. So, the substrate inhibition was observed when substrate concentration increases more than 8 g l^{-1}. At very low initial substrate concentrations, the effect of nutrient was negligible on gross measures of metabolic activity, such as specific growth rate, respiration rate and rate of protein/enzyme synthesis, etc. These physiological parameters could be increased by means of stimulation of the metabolism of the micro-organisms by increasing the nutrient concentration. Eventually a concentration was reached at which further increase in nutrient concentration do not increase

the physiological parameter. At this point either some other environmental factor is in limiting supply or else the cells themselves have reached their own limit for the present cultural conditions. Further increase in the concentration of the chosen nutrient or substrate will eventually cause the physiological parameter to decrease (Edwards, 1970).

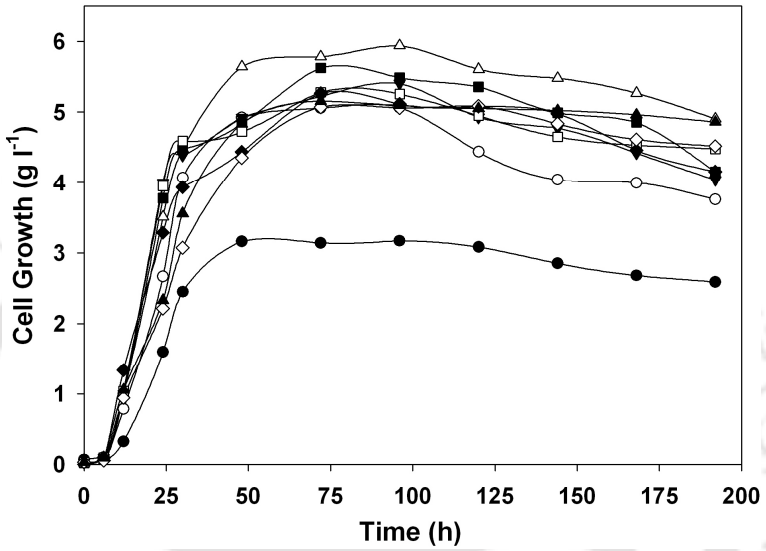


Fig. 4.11 Growth profile of *P. cepacia* NRRL B 2320 at different initial substrate (cutin) concentration, (—●— 2g l^{-1},—○— 4 g l^{-1}, —▼— 6 g l^{-1}, —Δ— 8 g l^{-1}, —■— 10 g l^{-1}, —□— 12 g l^{-1}, —♦— 14 g l^{-1}, —◊— 16 g l^{-1}, —▲—20g l^{-1}).

4.7.2 Effect of initial substrate concentration on specific growth rate

The cell growth increased with increasing initial concentration of cutin. For all initial cutin concentrations in shake flask, there was a lag of about 4 h, but the time to reach stationary phase did not vary with increasing initial cutin concentration. The specific growth rate as a function of initial substrate concentration was presented in Fig. 4.12. It was observed that the specific growth rate (μ) has a continuously decreasing trend above initial substrate concentration (γ_s) of 10 g l^{-1}, indicating the possibility of substrate inhibition kinetics.

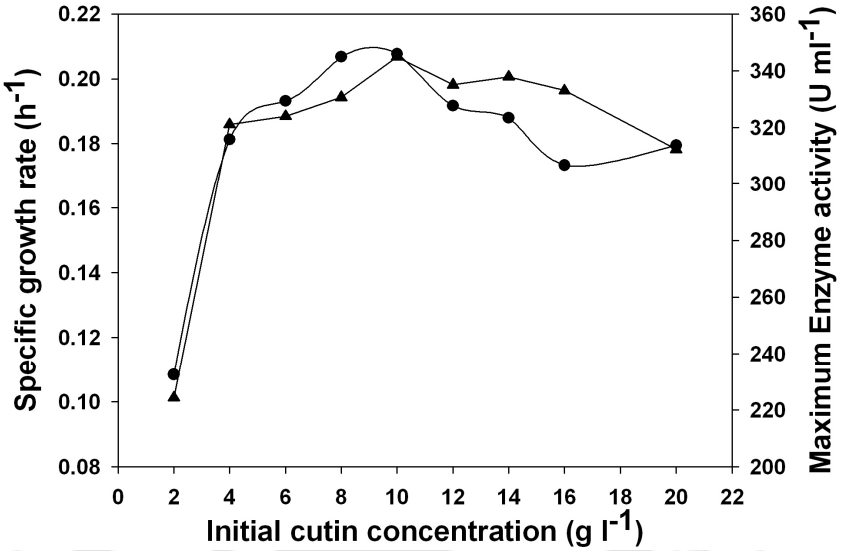


Fig. 4.12 Specific growth rate (—●—) and maximum enzyme (—▲—) activity as a function of initial substrate (cutin) concentration**.**

4.7.3 Enzyme activity at different initial substrate concentrations

Extracellular enzyme activity was measured at different initial substrate concentrations and the results are presented in Fig. 4.13.

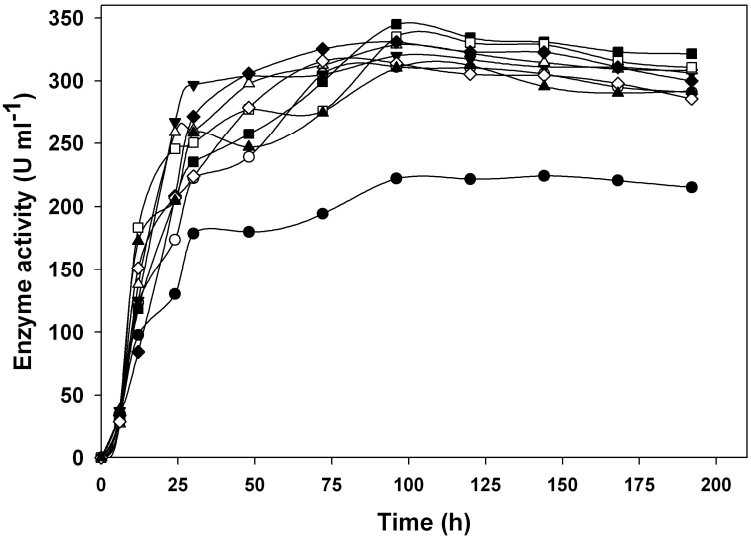


Fig. 4.13 Enzyme activity profile obtained at different initial substrate (cutin) concentration, (—●— 2g l^{-1},—○— 4 g l^{-1}, —▼— 6 g l^{-1}, —Δ— 8 g l^{-1}, —■— 10 g l^{-1}, —□— 12 g l^{-1}, —♦— 14 g l^{-1}, —◊— 16 g l^{-1}, —▲—20g l^{-1}).

The highest enzyme activity was observed at initial substrate concentration (γ_s) of 10 g l^{-1}. Minimum enzyme activity of 221 U ml^{-1} was observed for 2 g l^{-1} of cutin. Upon increasing the cutin concentration, the enzyme activity increases and reached maximum (344 U ml^{-1}) at 10 g l^{-1}. Further increase in the cutin concentration causes the decrease in enzyme activity due to the substrate inhibition at higher concentration of cutin. At higher concentration of cutin, the mass and oxygen transfer in the media had hindered due to insoluble nature of cutin, as a result cell growth as well as the enzyme production decreased. It was observed that the cutin concentration above 10 g l^{-1} was responsible for attenuation of specific growth rate and enzyme production as well. As cutinase production was found to be mostly growth associated, so the production was also inhibited when growth started to decrease.

4.7.4 Mathematical modeling of growth kinetics of P. cepacia NRRL B 2320

The growth kinetics of *P. cepacia* NRRL B 2320 was modeled using the substrate inhibition models (equations are given in Table 3.4 in the section 3.12.1) and the parameters estimated are presented in Table 4.8. The variation of experimental specific growth rate as a function of initial substrate concentration and the fitted curves are presented in Fig. 4.14. Parity plot showing estimated specific growth rate by different models that fit to the entire data versus experimental specific growth rate is shown in Fig. 4.15. It was observed that Webb model has highest R^2 value of 0.933. Andrew's model is also showed good fit to the experimental data with R^2 of 0.92. The root mean square (RMS) error between experimental and model predicted values was found to be very less, (0.0096) for Andrew's model and 0.0097 for Webb model.

Table 4.8 Estimated biokinetic parameters from different models

Model	Parameters	R^2	RMSE
Andrew's	μ_{max}=0.72, K_s= 7.94, K_I =10	0.92	0.0096
Luong's	μ_{max}=0.44, K_s=4.87, γ_s^*=50256, n=1894	0.90	0.0113
Han-Levelspiel	μ_{max}=0.136, k=478.1, K_m=1061.98, γ_s^*=8002.22, n=834.5, m=6	0.73	0.0250
Haldane	μ_{max}=0.21, Ks=1.105, K_I =5364.48	0.569	0.022
Moser	μ_{max}=0.1913, K_s=15.75, n=4.35	0.855	0.013
Aiba	μ_{max}=0.44, K_s= 4.88, K_i=26.52	0.909	0.0102
Yano	μ_{max}=0.208, K_s= 1.79, K_I=10062.31, K=2.269	0.836	0.0151
Edward	μ_{max}=0.061, K_s= 0.051, K_i=9.49	0.731	0.0177
Webb	μ_{max}=0.518, K_s= 6.627, K_I=11.28, K=26487.93	0.933	0.0097

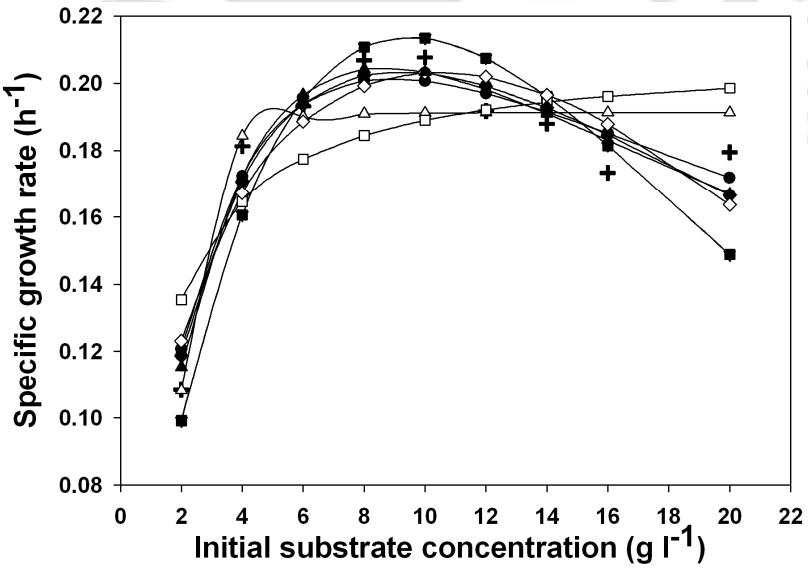


Fig. 4.14 Specific growth rate as a function of initial substrate (cutin) concentration (✚ Experimental data, —●— Andrews model,—○— Luongs' model, —▼— Han-Levenspiel model, —Δ— Moser model, —■— Edward model, —□— Haldane model, —♦— Aiba Model, —◊— Yano model, —▲—Webb model).

Andrew's model explains the inhibitory effects of substrate at higher concentrations. It reduces to the conventional Monod's equation when inhibition constant becomes very high. In the case of Webb model, larger K_I value indicates that the culture is less sensitive to substrate inhibition. The value of biokinetic parameters (μ_{max}, K_s and K_I) obtained by fitting the data to these models are also found to be comparable. The μ_{max} predicted by the Andrew's model was 0.72 h^{-1}, followed by the Webb model 0.518 h^{-1}. These values are greater than the μ_{max} predicted by all other models. Luong's and Aiba models were also showed reasonably good fit to the experimental data with R^2 value of 0.90, RMSE of 0.011 and 0.010, respectively, and the μ_{max} predicted by these models are 0.44. According to Andrews model, the substrate inhibition starts at 10 g l^{-1} and the same occurs at 11.28 g l^{-1} for Webb model. When cutin concentration increases above 12 g l^{-1} the cell growth as well as enzyme production starts decreasing (Fig. 4.12). According to Webb model, the inhibition of cellular growth and enzyme production occur either due to inhibition of transport mechanism or inhibition of the series of reactions involved in the oxidative metabolism of the substrate, followed by conversion of the resultant reducing power to high energy phosphate and sulfate structures using an electron transport chain. Previous reports are available on kinetic modeling of growth of microorganism during the production of cellulase (Agarwal *et al.*, 2009) utilizing insoluble substrate, cellulose. The best model for growth of *Cellulomonas cellulans* was Han Levenspiel model with R^2 of 0.949 and μ_{max} of 0.16. Theodore and Panda (1999) observed that the μ_{max} of 0.2105 h^{-1}, and K_s of 8.42 g l^{-1} for the growth of *Trichoderma harzianum* during production of β-1,3 glucanase with Luong's model. Agarrey and Solomon (2008) studied the degradation of phenol by *P. fluorescence*. They observed that the experimental data were good fit to Haldane model with maximum specific degradation rate of

0.357 h^{-1}, and K_s of 50 mg l^{-1}. It was observed that different models applicable for different systems.

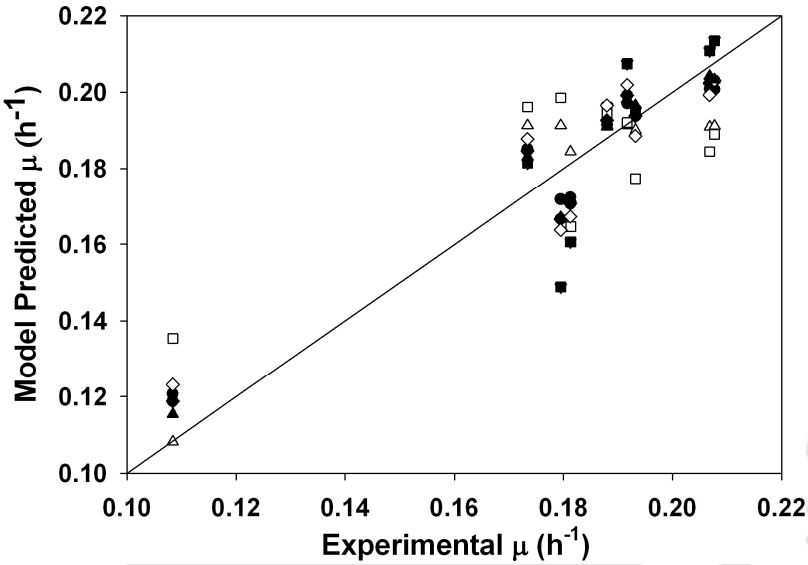


Fig. 4.15 Parity plot for prediction of specific growth rate by various substrate inhibition models (● Andrews model, ○ Luongs' model, ▼ Han-Levenspiel model, Δ Moser model, ■ Edward model, □ Haldane model, ♦ Aiba Model, ◊ Yano model, ▲Webb model).

4.8 Purification of cutinase

Purification of cutinase was carried out as per the strategy given in Fig. 3.1 and method described in the section 3.12.2. The precipitation of cutinase was performed with 20-80% of ammonium sulfate saturation, where the maximum enzyme activity was obtained at 80%. So, the crude extracellular protein was precipitated by the addition of ammonium sulfate (80% saturation) for further study. The precipitated enzyme was dissolved in minimal volume of Tris HCl buffer (20 mM, pH 8.0) followed by dialysis. The results of purification procedure of cutinase from *P. cepacia* NRRL B 2320 are summarized in Table 4.9. The purification of

cutinase was involved ammonium sulfate precipitation followed by cation exchange chromatography using CM cellulose column and followed by two gel filtration steps (Sephadex G-100 and Sephacryl S-300) (Fig. 4.16). The purified cutinase was found to be stable at pH 8.0. Hence, Tris HCl buffer system (20 mM, pH 8.0) was used during all purification steps. The retention of cutinase on CM cellulose column was dependent on pH of the buffer used. The adsorbed cutinase from CM cellulose column was eluted with gradient of 0-500 mM NaCl.

Table 4.9 Summary of steps employed in purification of cutinase from *P. cepacia* NRRL B 2320

Purification Step	Volume (ml)	Enzyme (U)	Protein (mg)	Specific activity (U/mg)	Purification Fold	Yield (%)
Crude	1000	191300	3207.58	59.64	---	100.00
$(NH_4)_2SO_4$ precipitation	200	121833.34	219.35	555.42	9.31	63.69
CM-cellulose column	60	75454.55	57.90	1303.14	21.86	39.44
Sephadex G-100	12	55274.24	17.75	3114.50	52.21	28.89
Sephacryl S-300	3.5	33592.28	6.27	5359.15	89.84	17.56

The purified cutinase from *P. cepacia* NRRL B 2320 could be achieved in four steps. The enzyme was purified approximately 89.84 fold with an overall yield of 17.56% and specific activity of 5359.15 U mg^{-1} and found to be homogeneous, as evident from SDS-PAGE (Fig. 4.16). The molecular mass of cutinase was found to be 26.5 kDa by SDS-PAGE analysis. The molecular mass of the protein was also confirmed by MALDI-TOF-MS analysis. The MALDI-TOF analysis predicted that the molecular mass of the isolated cutinase was a monomer of size 26.25 kDa (Fig. 4.17). The protein was purified to homogeneity using

Sephacryl S-300 chromatography with 17.56% yield. This is the first report on the purification of cutinase from *P. cepacia* NRRL B 2320.

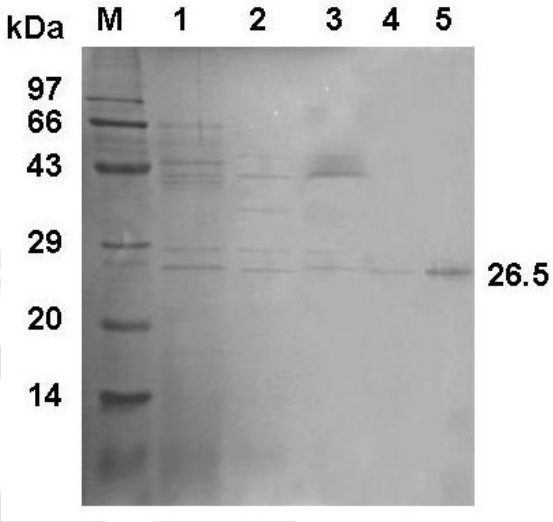


Fig. 4.16 SDS PAGE cutinase from *P. cepacia* NRRL B 2320 at different stages of purification. Lane M: Protein molecular marker; Lane 1: Ammonium sulfate precipitated fraction; Lane 2: CM cellulose fraction; Lane 3: Sephadex G-100 fraction; Lane 4 & 5: Sephacryl S-300 fraction.

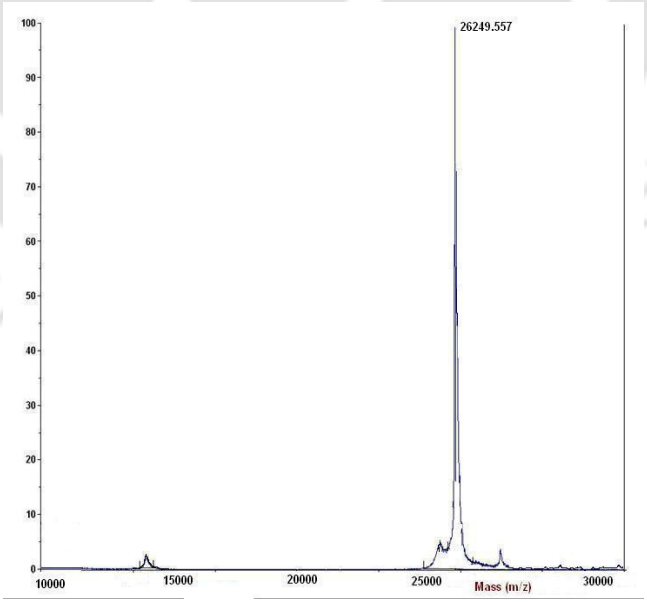


Fig. 4.17 MALDI-TOF-MS analysis of purified cutinase.

All other contaminating proteins could be successfully removed in the final step of gel filtration chromatography using Sephacryl S-300 matrix. The enzyme mostly purified in ion exchange step, the remaining proteins were removed in Sephadex G-100 and Sephacryl S-300. As seen from the Fig. 4.16, after ion exchange the removal of all other contaminating protein could not be achieved in Sephadex G-100, but fold purification increased from 21.86 to 52.21. Finally, the protein have purified completely by Sephacryl S-300 with 89.84 fold purification. Sebastian and Kolattukudy (1988) purified the cutinase from *P. putida* in five steps, *viz*., acetone precipitation, two ion exchange chromatography (DEAE and QAE) and two gel filtration chromatography (Sepharose 6B and Sephadex G-100). They could able to 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 the cutinase from *Botrytis cinera* in four steps with 2.9% yield and 66.5 purification fold. The molecular mass of the purified cutinase was 26.5 as calculated from SDS–PAGE molecular markers. The molecular mass of the cutinase isolated from *P. cepacia* NRRL B 2320 was very close to previously reported cutinase from other bacterial sources. The molecular mass of cutinases isolated from *P. putida* and *T. fusca* (Chen *et al*., 2008) were found to be 30 kDa and 29 kDa, respectively. The molecular mass of cutinases isolated from *F. solani pisi* and *C. kahawae* were found to be 21 and 22.1, 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).

4.9 Characterization of cutinase

Any enzyme needs to be characterized prior to be used in any industrial purpose, because the optimum condition for maximum enzyme activity, 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.

4.9.1 Effect of pH and temperature on activity and pH stability of purified cutinase

The purified cutinase of *P. cepacia* NRRL B 2320 was active over a broad range of pH (7.5-9.0) with at an optimum pH of 8 to 8.5 (Fig. 4.18). To study the effect of pH and temperature on the performance of cutinase, experiments were performed according to the method described in the section 3.14.1 and 3.14.2 Maximum cutinase activity was achieved at pH 8.0 and 40ºC, and decreased significantly (50%) when the pH was decreased to 6.5 (Fig. 4.18 and 4.19). The enzyme showed stability at alkaline pH range (pH 8.0–10.0) as it retained 90% of its original activity after incubation for 24 h (Fig. 4.18). Majority of the purified enzyme exhibited maximum activity at a temperature of 37ºC. The activity decreased sharply above and below the optimum temperature range (35-45ºC) with almost 65% loss of its original activity at 60ºC (Fig. 4.19). No significant enzyme activity was lost when the purified enzyme was pre-incubated at 40ºC for 60 min, beyond this temperature the enzyme became increasingly unstable. The cutinase hydrolytic activity was stable in broad pH range. Previously, it was also reported that bacterial cutinases are more stable in high pH than fungal cutinase. The pH stability of the cutinase from *P. cepacia* NRRL B 2320 is similar to that of the cutinase from *P. putida*. Though the cutinase showed optimum activity in the temperature range of 35-45ºC, but it showed somewhat lesser activity at 60ºC as well.

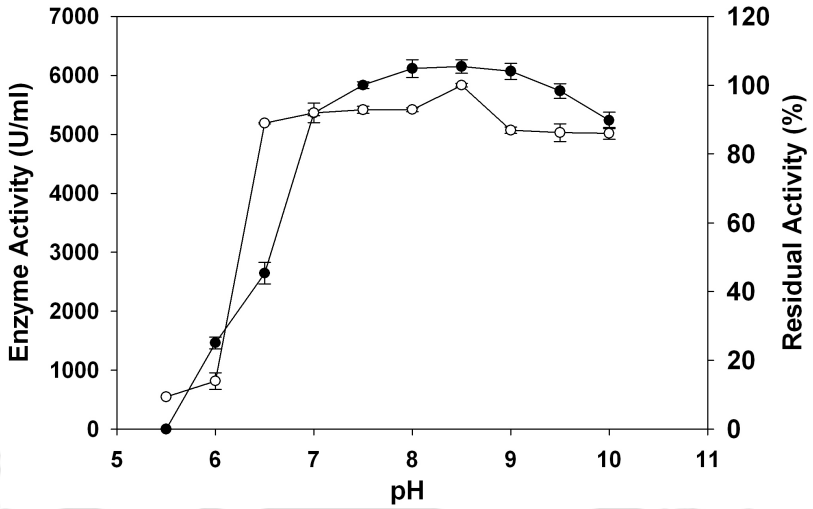


Fig. 4.18 Effect of pH on the activity and stability, enzyme activity (●), residual activity (○) of cutinase purified from *P. cepacia* NRRL B 2320. For activity, pH of assay mixture was adjusted to different pH values (5.5-10), and for stability, enzyme incubated at different pH values (5.5-10), assay was performed at pH 7.

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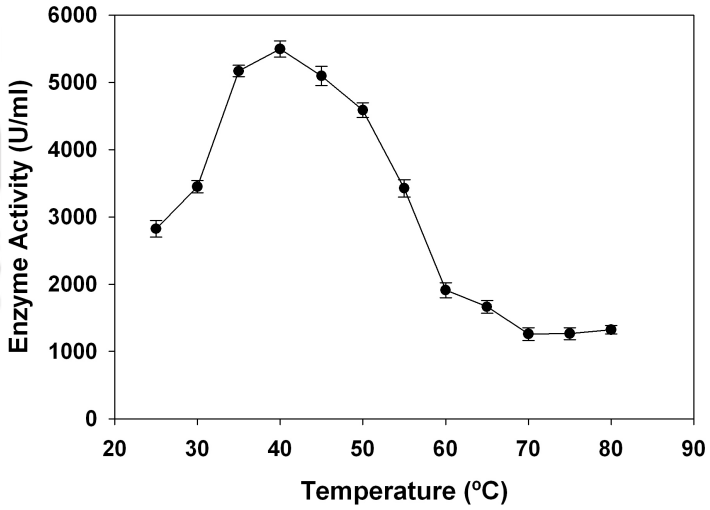


Fig. 4.19 Effect of temperature on the activity of cutinase purified from *P. cepacia* NRRL B 2320. The assay was performed at different temperatures (25°C-80°C) and pH 7.

4.9.2 Optimization of combined effect of substrate and enzyme amount on the performance of cutinase under assay conditions

In order to find out the appropriate proportion of cutinase and substrate (volume/amount) at which maximum amount of product released during hydrolysis by the enzyme, the experiments were performed as per the experimental design given in the Table 3.6 (section 3.14.3). The experimental design matrix and the corresponding results of CCD experiments to determine the effects of enzyme and substrate volume are shown in the Table 4.10, along with the mean predicted values. Using the experimental results of CCD, regression model equation was developed for predicting the maximum cutinase activity given by equation 4.3.

$$Y_{EnzymeActivity} = -70786.1 + 144.2X_1 + 605.1X_2 - 0.1X_1^2 - 5.2X_2^2 - 0.4X_1X_2 \quad (4.3)$$

where, X_1 is volume of substrate (µl) and X_2 is volume of enzyme (µl).

According to the ANOVA of the quadratic regression model (Table 4.11), the model was highly significant, as manifested from the Fisher, *F* test (mean square regression: mean square residual is 79.72) with a very low probability value ($p_{model}>F$ is 0.0001). The *F* value is the ratio of the mean square due to regression to the mean square due to error. In general, the calculated *F* value should be several times greater than the tabulated value for good model. If the *F* value is greater than tabulated *F* value, the null hypothesis is rejected at α level of significance and infers that the variation accounted for the model is significantly greater than the unexplained variation. In this case, the null hypothesis was rejected at $p<0.0001$ level of significance for cutinase activity. This shows that the squared regression was significant at the level of 98.28% for cutinase activity. This indicates that the combined effects of all the independent variables significantly contributed to maximize the cutinase activity.

Table 4.10 Experimental design and results for the activity of cutinase from *P. cepacia* NRRL B 2320 at various combinations of substrate (0.57 mM) and enzyme amount

Run order	Experimental values						Enzyme Activity(U ml^{-1})	
	Amount of substrate X_1			*Amount of Enzyme* X_2			*Observed*[a]	*Predicted*
	Coded value	*Actual value*		*Coded value*	*Actual value*			
		µl	*µg*		*µl*	*µg*		
1	-1	860	67.0	-1	12	0.691	4393.03±10	4463.67
2	+1	1100	85.69	-1	12	0.69	5433.63±4	5277.11
3	-1	860	67.0	+1	30	1.72	5589.59±15	5810.23
4	+1	1100	85.69	+1	30	1.72	5063.13±16	5056.60
5	0	980	76.34	0	21	1.21	6623.33±21	6575.27
6	0	980	76.34	0	21	1.21	6620.11±8	6575.27
7	0	980	76.34	0	21	1.216	6610.55±6	6575.27
8	-1.414	810.3	63.11	0	21	1.21	4836.07±18	4643.39
9	+1.414	1149.7	89.56	0	21	1.216	4557.11±12	4685.69
10	0	980	76.34	+1.414	8.3	0.47	5355.26±9	5429.28
11	0	980	76.34	-1.414	33.7	1.94	6363.62±14	6225.50
12	0	980	76.34	0	21	1.21	6630.33±17	6669.33
13	0	980	76.34	0	21	1.21	6615±8	6669.33
14	0	980	76.34	0	21	1.21	6634.44±5	6669.33

[a]The observed values of cutinase activity were the mean values of duplicates with standard deviation (Mean ± S.D.)

The goodness of the model was checked by coefficient of determination, R^2, implies that the sample variation of 98.28% for cutinase activity is attributed to the chemical parameters and also only 1.72% of the total variation was not explained by the model. The Student's *t* distribution and the corresponding *p* values, along with estimated parameters are shown in Table 4.12. The *p* values of all linear and quadratic relationships between assay parameters and cutinase activity suggest that they are highly significant ($p<0.0004$). However, the interaction effect between volume of substrate and enzyme is lower than linear effect ($p<0.001$), but accounted appreciably.

Table 4.11 Analysis of variance (ANOVA) for cutinase activity: effect of substrate and enzyme amount

Source	DF[a]	SS[b]	MS[c]	*F*	*p*
Regression	5	9548094	1909619	79.72	0.0001
Linear	2	635778	4199794	175.33	0.0001
Square	2	8298388	4149194	173.22	0.0004
Interaction	1	613928	613928	25.63	0.001
Residual error	7	167677	23954		
Lack-of-fit	3	167378	55793	748.01	0.0005
Pure error	4	298	75		
Total	13	9746738			

[a]Degree of freedom, [b]Sum of square, [c]Mean sum of square

Table 4.12 Model coefficient estimated by multiple linear regressions

Term	Coefficient	SE Coefficient	*t* value	*p* value
Constant	-70768.1	4100.31	-17.259	0.0001
Substrate volume	144.2	7.91	18.228	0.0001
Enzyme volume	605.1	76.42	7.918	0.0004
Substrate volume*substrate volume	-0.1	0.00	-17.600	0.0001
Enzyme volume*enzyme volume	-5.2	0.70	-7.391	0.0004
Substrate volume*enzyme volume	-0.4	0.07	-5.063	0.001

The 2D contour plot shown in Fig. 4.20 explains the behavior of the system and enzymatic activity over independent variables, volume (amount) of substrate and enzyme. The enzyme activity was higher at a range of substrate volume (900-1000 µl) and enzyme volume (20-30 µl). The equation 3.2 was optimized and solved by MINITAB optimizer. The optimum levels of substrate and enzyme volumes were found to be 971 µl and 24 µl (1.4 µg), respectively.

The experiments were performed at optimal levels of variables to verify the optimal conditions. These conditions have showed higher enzyme activity (6680.56 U ml^{-1}) as compared to un-optimized levels of substrate (980 µl) and enzyme (20 µl) volume for the analysis of enzyme activity. After optimization, cutinase activity was increased by 500 U ml^{-1} in the purified sample.

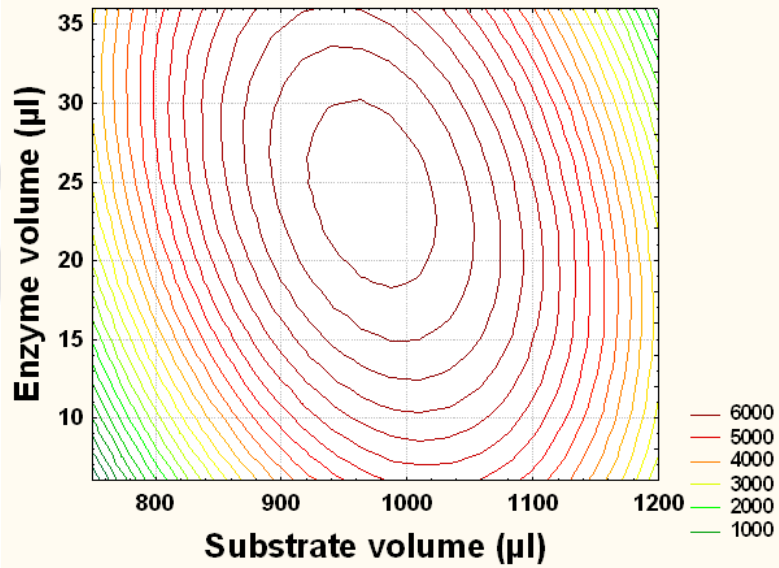


Fig. 4.20 2D contour plot of cutinase activity at different levels of substrate and enzyme volume.

4.9.3 Optimization of combined effect of pH and temperature on the performance of cutinase under assay conditions

The most important physical factors, which influence the enzymatic reaction rate, are pH of the assay medium and incubation temperature of enzyme with the substrate. Each enzyme has a characteristic pH and temperature optima beyond which the reduction in activity was observed. Therefore, optimum temperature and pH for enzyme-substrate system for cutinase was determined using statistical experimental design. According to the design matrix shown in Table 3.8 (section 3.14.4), experiments were performed at various combinations of pH

and temperature. The corresponding results of observed and predicted enzyme activity at each combination are given in the Table 4.13.

Table 4.13 Experimental design and results for the activity of cutinase from *P. cepacia* NRRL B 2320 at various combinations of pH and temperature

Run Order	Experimental values				Enzyme Activity (U ml^{-1})	
	pH X_1		*Temperature* (°C) X_2		*Observed*[a]	*Predicted*
	Coded value	*Actual value*	*Coded value*	*Actual value*		
1	-1	7	-1	37	6680.46±18	6121.40
2	+1	8.7	-1	37	7150±15	6723.10
3	-1	7	+1	48	6318.18±20	6254.19
4	+1	8.7	+1	48	2104.17±8	2118.90
5	0	7.85	0	42.5	8309.68±14	8633.35
6	0	7.85	0	42.5	8225.81±25	8633.35
7	0	7.85	0	42.5	8382.80±10	8633.35
8	-1.414	6.64	0	42.5	3151.51±5.6	3452.62
9	+1.414	9.05	0	42.5	850.89±8.6	1040.66
10	0	7.85	+1.414	34.7	7813.98±28	8371.7
11	0	7.85	-1.414	50.2	5363.44±26	5296.60
12	0	7.85	0	42.5	8225.81±31	7899.99
13	0	7.85	0	42.5	8227.96±15	7899.99
14	0	7.85	0	42.5	8227.96±18.5	7899.99

[a]The observed values of cutinase activity were the mean values of duplicates with standard deviation (Mean ± S.D.)

Using the experimental results of CCD, regression model equation was developed to predict the maximum cutinase activity given by the equation 4.4.

$$Y_{EnzymeActivity} = -329957 + 70800X_1 + 3231X_2 - 389X_1^2 - 17X_2^2 - 250X_1X_2 \quad (4.4)$$

where, X_1= pH, and X_2 = temperature.

Table 4.14 Analysis of variance (ANOVA) for cutinase activity: effect of pH and temperature

Source	DF[a]	SS[b]	MS[c]	*F*	*p*
Regression	5	80686110	16137222	65.42	0.0001
Residual Error	7	1726702	246672		
Lack-of-Fit	3	1714356	571452	185.16	0.0005
Pure Error	4	12345	3086		
Total	13	84426470			

[a]Degree of freedom, [b]Sum of square, [c]Mean sum of square

The results were analyzed using the ANOVA as appropriate to the experimental design used (Table 4.14). According to the ANOVA of the quadratic regression model, the model was highly significant, as is evident from the Fisher *F* test (mean square regression: mean square residual was 65.42) with a very low probability value ($p_{model} > F$ is 0.0001). This indicates that the combined effects of pH and temperature significantly attributed for maximizing the cutinase activity. The goodness of the model was confirmed by coefficient of determination, R^2, which implied that the model can explain the variation of 97.95%. The Student's *t* distribution, *p* values, and the estimated parameters are shown in Table 4.15. The *p* values (<0.02) of all linear, quadratic and interaction relationships among process parameters under assay condition and cutinase activity are highly significant. The much higher coefficient of pH than temperature suggests that pH is having greater effect on the assay condition than temperature within the experimental range. The 2D contour plot has elucidated to know the behavior of the system and enzymatic activity over independent variables, pH and temperature (Fig. 4.21). The elliptical shape of the plot suggests that the interaction among the two parameters is highly significant.

Table 4.15 Model coefficient estimated by multiple linear regressions

Term	Coefficient	SE Coefficient	*t* value	*p* value
Constant	-329957	26474.0	-12.463	0.0001
pH	70800	4573.0	15.482	0.0001
Temperature	3231	662.3	4.878	0.002
pH*pH	-3897	253.0	-15.406	0.0004
Temperature*Temperature	-17	6.0	-2.856	0.024
pH*Temperature	-250	53.1	-4.715	0.002

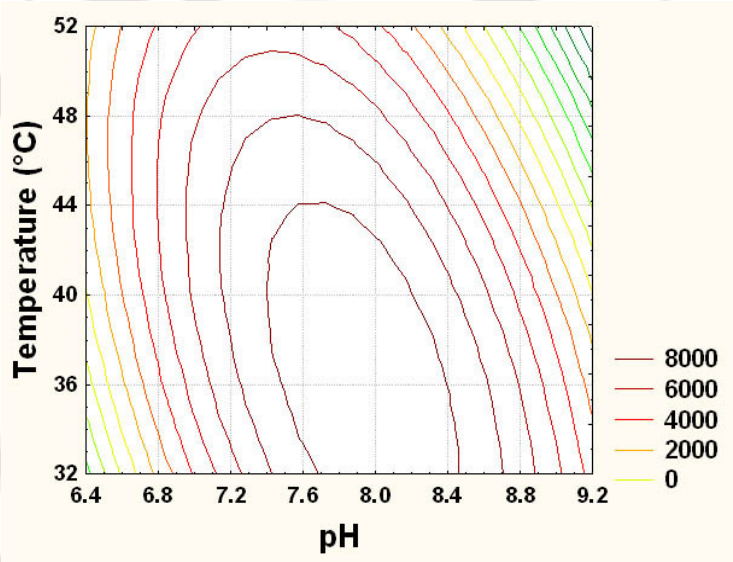


Fig. 4.21 2D contour plot of cutinase activity at different levels of pH and temperature under assay conditions.

The enzyme activity was higher at a range of pH 7.5-8.4 and temperature 35-40°C. By solving the equation 3.2 using MINITAB optimizer, the optimum levels of pH and temperature were found to be 7.9 and 36.5°C, respectively. In order to validate the optimal conditions, the experiments were performed at optimal levels of variables. Higher enzyme activity (8835.50 Uml^{-1}) was observed under optimum condition as compared to un-optimized physical parameters. An overall 1.42 fold increase in activity was observed after optimization of both assay and process conditions.

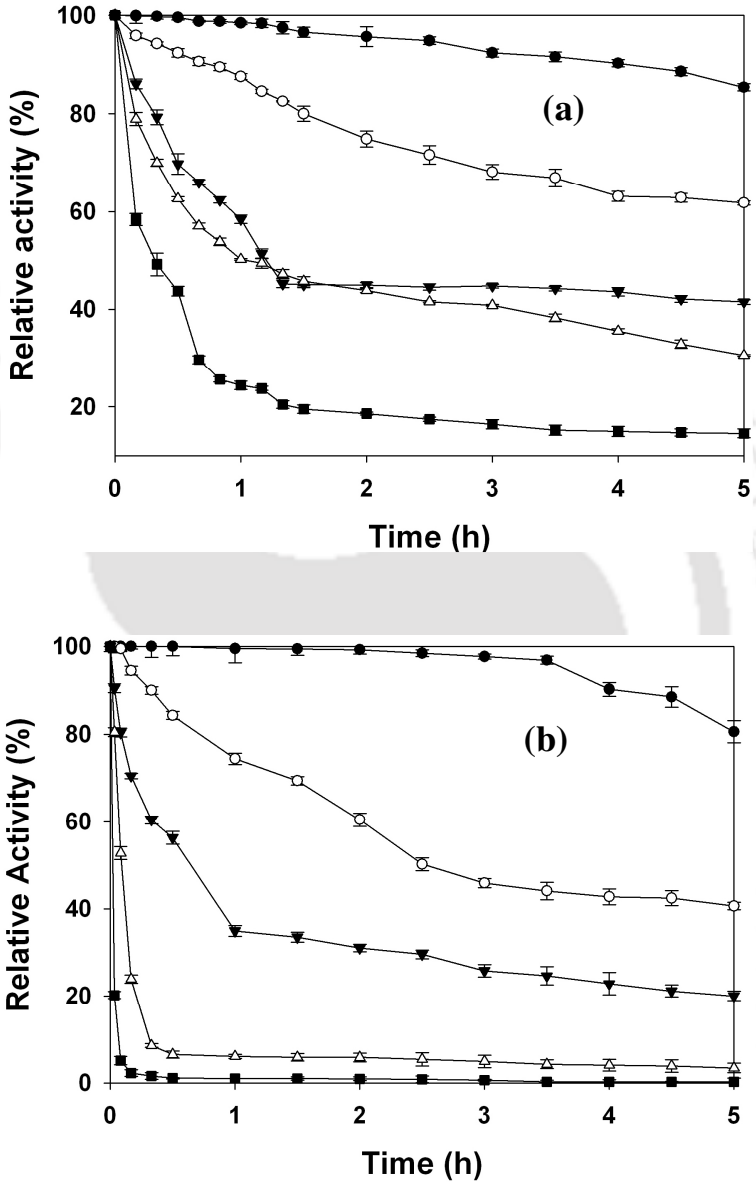
4.9.4 Deactivation kinetics of cutinase

The deactivation rate is proportional to the active enzyme concentration, and k_d (deactivation rate constant is the proportional constant). The deactivation process was modeled as first-order kinetics and the deactivation rate constant was evaluated. The experiments for thermal deactivation of cutinase were carried out as per the method described in the sections 3.14.5 and 3.14.6 (Table 3.9). The effect of temperature on deactivation has been studied and the results are shown in Fig. 4.22. The minimum value of k_d observed for cutinase was found to be 0.024 h^{-1} (Table 4.16). The above mentioned minimum deactivation constant was found at 40°C and of pH 6.0 and pH 7.0. The deactivation process was found to be faster at higher pH (pH 8.0, pH 9.0) than lower pH (pH 6.0, pH 7.0) for cutinase. This depicts the fact that the enzyme was more stable at pH 6.0 and 7.0 at higher temperature. Although at 40°C, the enzyme was stable for more than 20 h at pH 8.0 and 1 h at pH of 9.0. 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 indicated that the optimum pH and temperature lie near that of the growth condition.

4.9.5 Estimation of thermodynamic parameters

The thermodynamic parameters were estimated according to the method described in the section 3.14.7. The change in enthalpy and entropy for purified cutinase from *P. cepacia* NRRL B 2320 were calculated by transition state theory (eq. 3.10) and the results were shown in Table. 4.17.

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



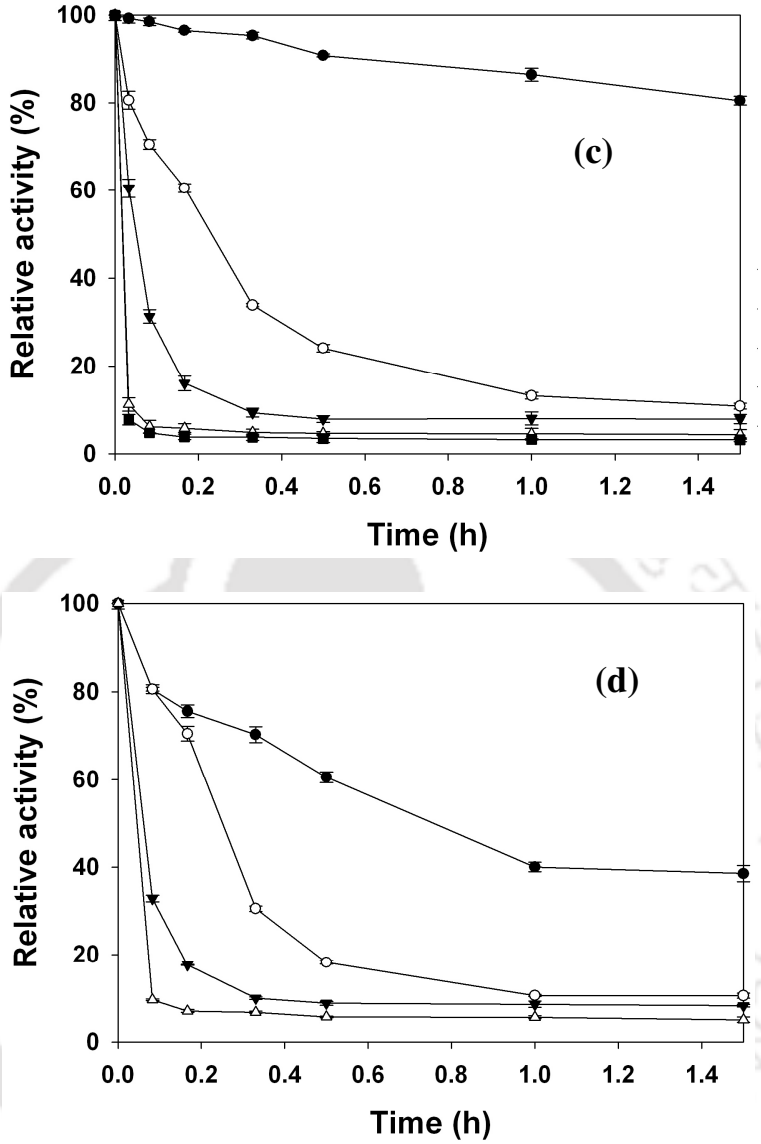


Fig. 4.22 Deactivation kinetics of cutinase at (a) pH 6.0, (b) pH 7.0, (c) pH 8.0 and (d) pH 9.0 at temperatures —●— 40°C, —○— 45°C, —▼— 50°C, —Δ— 55°C, —■— 60°C.

Increase in entropy and enthalpy values was observed when pH increases from 6.0 to 7.0. Beyond pH 7.0, there was decrease in both the entropy and enthalpy. Positive values of ΔH* would expect for the breaking of hydrogen bonds as well as for the exposure of hydrophobic groups from the interior of the native protein during the deactivation process. Such events would raise the energy of the protein–water solution.

Table 4.16 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)	K_d (h^{-1})	$t_{1/2}$ (h)
6.0	40	0.024	28.63
	45	0.112	6.18
	50	.5871	1.18
	55	.8401	0.824
	60	1.85	0.372
7.0	40	.024	28.87
	45	.282	2.45
	50	1.22	0.547
	55	7.704	0.089
	60	26.63	0.026
8.0	40	.03	23.1
	45	1.74	0.398
	50	8.59	0.080
	55	18.61	0.037
	60	28.91	0.024
9.0	40	.6026	1.15
	45	1.79	0.385
	50	9.32	0.074
	55	18.35	0.037

Positive value of ΔS* indicates that the protein solution has become more disordered as it deactivated by temperature (Garret and Grisham, 1999). The increase in ΔS* demonstrates an increase in the number of protein molecules in a transition activated state, which in turn, gives lower values of ΔG*. The values of ΔG* (calculated from eq. 3.11) are given in Table 4.17 for the enzyme. The decrease in entropy and enthalpy values was observed with increasing pH. Probably, at higher pH, the stable three dimensional structure of active site of the enzyme gets altered, resulted in decrease in residual activity. To gain a deeper insight into the mechanism and specificity of cutinase, the temperature-dependence of the catalytic activity was investigated. The

temperature dependency of first-order deactivation rate constant was studied by Arrhenius equation (eq. 3.13). The activation energy E_A and frequency factor k_0 were estimated from equation 3.13 and they are shown in Table 4.17. It was found that the maximum activation energy at optimum pH and further increase in pH, results in decrease of activation energy.

Table 4.17 Estimated thermodynamic parameters during thermal deactivation of purified cutinase from *P. cepacia* NRRL B 2320

pH	ΔH* (KJ mol^{-1})	ΔS* (J mol^{-1} K^{-1})	E_A (KJ mol^{-1})	k_0 (h^{-1})	ΔG* (KJ mol^{-1})
6.0	183.26	244.04	185.95	3.65×10^{29}	106.88-102.00[a]
7.0	298.18	610.65	300.86	5.34×10^{48}	107.04-94.83[a]
8.0	278.66	558.62	281.34	1×10^{46}	103.82-92.64[a]
9.0	199.87	321.05	202.53	3.85×10^{33}	99.38-94.56[b]

R^2 of plot of ln(k_d/T) versus 1/T at pH 6 0.95, pH 7 0.989, pH 8 0.892, pH 9 0978; R^2 of plot of ln(k_d) versus 1/T for pH 6 0.95, pH 7 0.99, pH 8 0.96, pH 9 0.98; [a]The temperature range is 40-60°C; [b]The temperature range is 40-55°C.

Kapat and Panda (1997), and Naidu and Panda, (2003) were also reported the similar observation on temperature dependency of deactivation rate constant for thermal deactivation of chitinase from *Trichoderma harzianum* and pectolytic enzymes from *Aspergillus niger*, respectively. For cutinase, the deactivation energy was highest at pH 7 suggesting that cutinase require more amount of energy to deactivate at pH 7 than other pH values (pH 6, 8 and 9). This is in agreement with the result that the cutinase was more stable at pH 6.0 and pH 7.0 at 40°C rather than pH 8.0 and pH 9.0.

4.9.6 Fluorescence spectroscopy

In order to investigate the conformational change in protein during thermal deactivation, tryptophan proved to be an important intrinsic fluorescent probe (amino acid), which was used to estimate the nature of microenvironment of the residue. From the fluorescence spectra

(obtained as illustrated in section 3.14.8) as shown in Fig. 4.23, a red shift of λ_{max}≈10 nm, and decrease in intensity was observed with increase in temperature for all four pH levels.

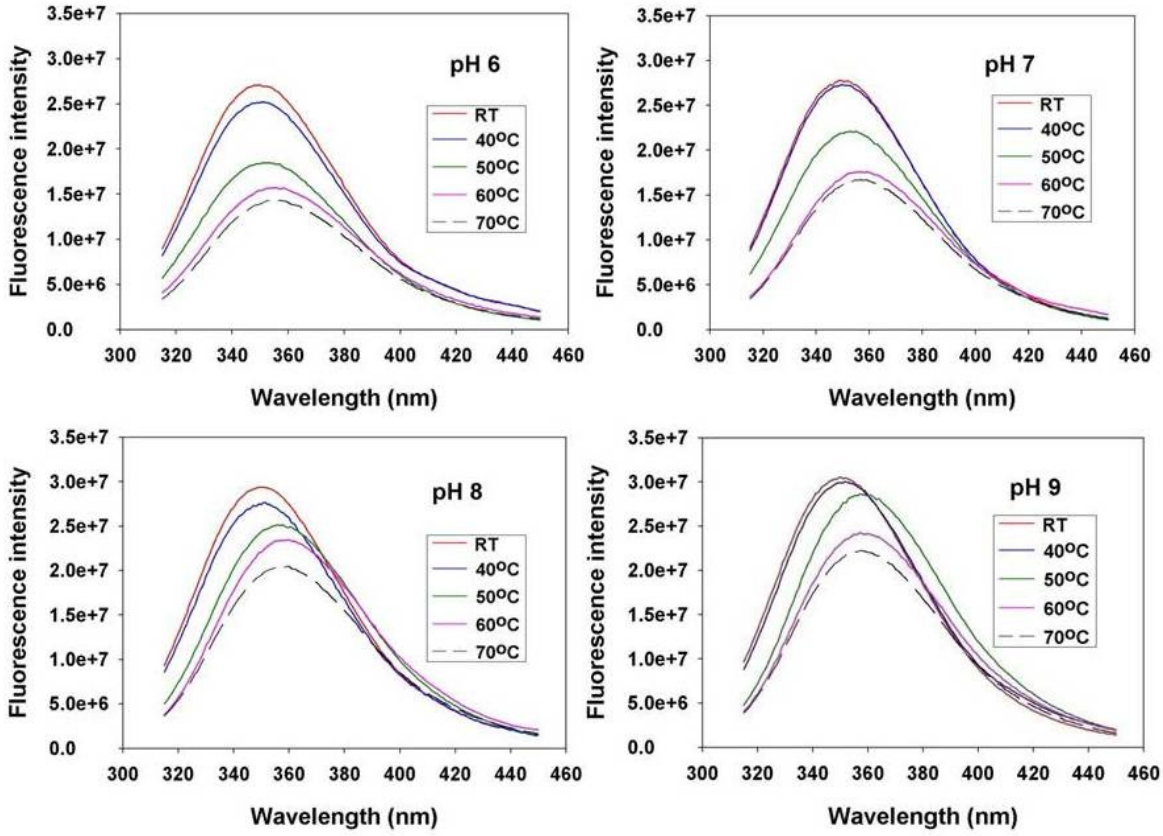


Fig. 4.23 The fluorescence spectra of cutinase enzyme at different pH and temperature. The enzyme (0.07 mg ml^{-1}) at different pH values (6.0 to 9.0) was heated at different temperature (40-70°C) for 15 min. The excitation was done at 295 nm and emission was measured from 315 nm to 450 nm**.**

At room temperature and 40°C, there was no change in spectra, which suggest that protein retains its native form at these temperatures, whereas the further increase in temperature results in gradual decrease in intensity and red shift in wavelength. This is due to the exposure of the tryptophan to an aqueous environment as opposed to a hydrophobic protein interior at higher temperature. The decrease in intensity suggests that there was a conformational change

in protein to make one or more tryptophan residue(s) available to polar solvent or bring them near charged groups. Hence, the florescence spectral analysis and thermodynamic parameters proves the exposure phenomena of hydrophobic residue (tryptophan) in aqueous environment. Previously, it was also observed that the spectrum shifts significantly towards longer wavelengths under unfavorable condition, and there was an overall decrease in the fluorescence intensity (Royer *et al.,* 1993). Kazakov *et al.,* (2009) was also observed similar kind of change in fluorescence spectrum, red shift in λ_{max} and decrease in intensity when protein (Hsp 22) undergoes thermally induced unfolding.

4.9.7 CD spectroscopy

The disorderedness of deactivated protein at higher temperature was further confirmed by circular dichorism spectral (CD) analysis (Fig. 4.24). The simplest method of extracting secondary structure content from CD data is to assume that a spectrum is a linear combination of CD spectra of each contributing secondary structure type (e.g. pure α-helix, pure β, pure turn, etc.) weighted by its abundance in the polypeptide conformation (Efimova *et al.,* 2005). From the CD spectra obtained under the conditions described in the section 3.14.9, it was observed that there was increase in ellipticity θ (mdeg) from 208 to 220 nm as temperature increases from room temperature to 70°C. The increase in ellipticity value shows that there was loss of α-helixes (208 nm and 220 nm) and β-sheet structures (218 nm) at higher temperature. The minimum ellipticity was observed at room temperature and the change in ellipticity was very less when temperature increases up to 50°C. But at 60°C and 70°C, the prominent increase in ellipticity was observed. This observation suggests that enzyme retains its secondary structure at 40°C and 50°C, but looses the same when incubated at 60°C and

above. Hence, the protein lost its secondary structure above 60°C. Also, there was decrease in ellipticity with increasing temperature near 200 nm, which reveals that the raise in temperature results in disorderedness of the protein.

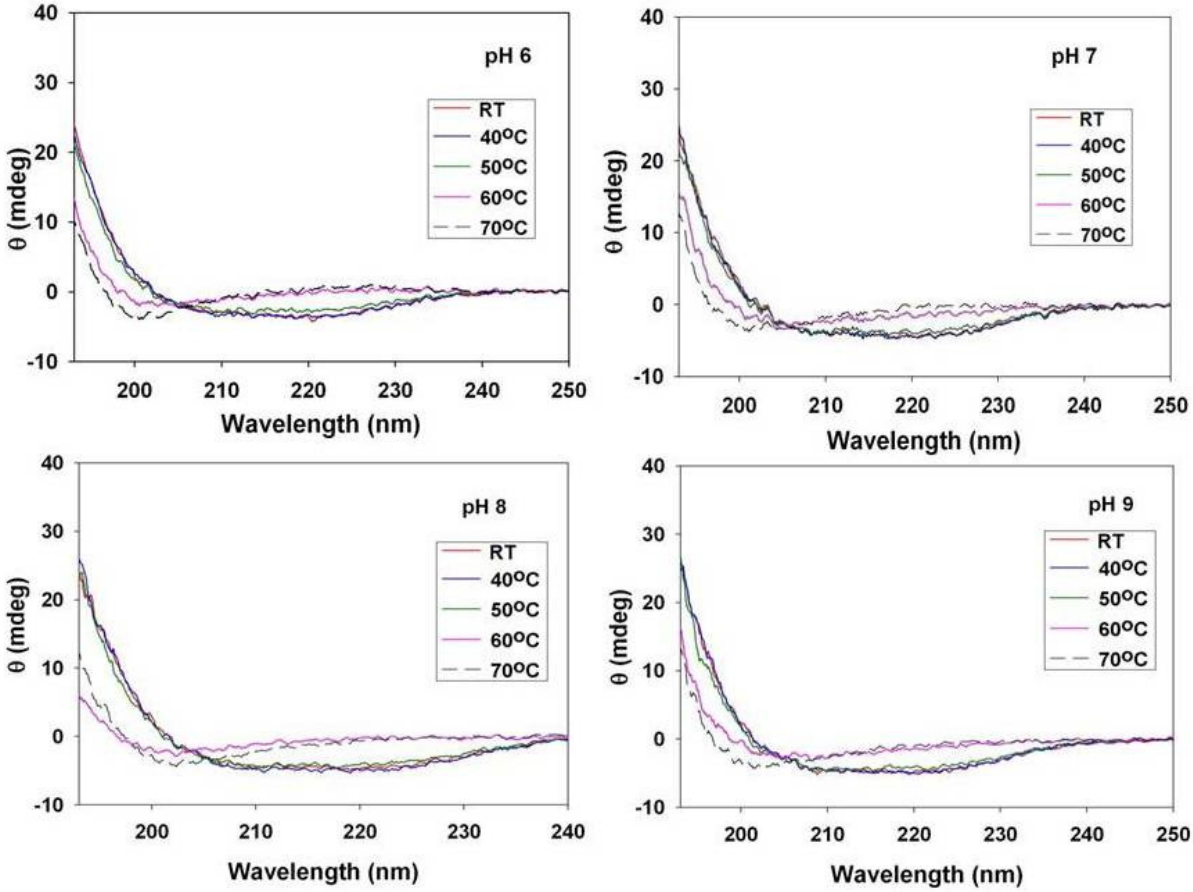


Fig. 4.24 The CD spectra of cutinase enzyme at different pH and temperature. 0.07 mg ml^{-1} protein in different pH values (6.0 to 9.0) was heated at different temperatures (40-70°C) for 15 min. The CD spectrum was obtained from 190 to 250 nm range.

This trend was similar for all four pH levels. Vermeer and Norde (2000) had studied thermal deactivation of immunoglobulin and found that the protein secondary structure stable at temperature below 55°C with maximum negative ellipticity at 217 nm, but it gradually shifts towards lower wavelength as temperature increases from 55°C to 75°C. Wu *et al.,* (2008) reported that the minimum ellipticity shift from 220 nm to 200 nm for arecheal DNA RNA

binding protein, SSh10 due to loss of α-helical structures and increase in random coil upon increase in temperature from 10°C to 90°C. Previously, Melo *et al.,* (2001) also observed that the loss of α-helix structure upon unfolding of cutinase at higher temperatures and in presence of guanidine hydrochloride.

4.9.8 Effect of different metal ions and effectors

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 3.14.10. 1mM of divalent metal ions, Mn^{++}, Ni^{++}, or Ca^{++} did not exhibit any significant effect on the enzyme activity, whereas Zn^{++}, Fe^{++}, or Cu^{++} showed little extent of inhibitory effect on the enzyme. Some of the divalent metal ions (Mg^{++}, Ca^{++} and Co^{++}) and monovalent metal ions (K^{+} and Na^{+}) were found to enhance the enzyme activity when present in reaction mixture at the concentration of 1mM. Inhibitory effect of Zn^{++} and Hg^{++} suggest that the presence of sulfhydryl group in the vicinity of active site of the enzyme. EDTA (1mM or 10mM) did not affect the activity of cutinase (Table 4.18), suggesting that the divalent ions were not essential for cutinase activity. 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). The enzyme was found to be stable in the presence of β-marcaptoethanol in assay mixture. So, there was no disulphide bond present near the active sites which cause the loss in enzyme activity.

Table 4.18 Effect of different metal ions and effectors on cutinase activity. The enzyme incubated in the buffer containing different metal ions and effectors at 37°C for 5 min

Metal ions or effectors	Concentration	Relative activity (%)
Control	---	100±0.50
KCl	1mM	106.56±1.67
	10mM	105.92±1.49
NaCl	1mM	138.01±1.09
	10mM	104.79±0.50
$MgCl_2$	1mM	120.50±1.88
	10mM	108.51±.86
$CaCl_2$	1mM	122.71±2.76
	10mM	66.44±0.52
$CoCl_2$	1mM	133.44±1.53
	10mM	41.98±2.45
$MnCl_2$	1mM	97.08±1.15
	10mM	44.67±2.03
$NiCl_2$	1mM	99.63±3.28
	10mM	25.03±1.39
$HgCl_2$	1mM	N.D.
	10mM	N.D.
$ZnSO_4$	1mM	82.15±3.56
	10mM	23.57±2.36
$FeSO_4$	1mM	80.22±0.98
	10mM	20.97±0.53
$CuSO_4$	1mM	15.41±0.71
	10mM	4.46±1.60
EDTA	1mM	108.07±1.76
	10mM	105.93±1.09
β-marcaptoethanol	1mM	94±0.25
	10mM	91.72±0.54

4.9.9 Effect of different surfactants

Most of the enzymes of industrial application often need to go through to relatively harsh conditions such as the presence of surfactants and organic solvents. The activity of the cutinase was tested (as described in the section 3.14.11) in the presence of nonionic

surfactants (Triton X-100 and Tween 80) and the anionic surfactants (SDS and sodium deoxycholate) (Table 4.19). Triton X-100 and Tween 80 did not affect the enzyme activity at a concentration of 1mM or 10mM in the reaction mixture.

Table 4.19. Effect of different surfactants on cutinase activity. The enzyme incubated with the surfactant at 30°C for 4 h

Surfactant	Concentration	Relative activity (%)
SDS	1 mM	89.76±0.88
	10 mM	71.06±1.88
Tween 80	1 mM	99.14±0.21
	10 mM	97.80±0.33
Triton X-100	1 mM	96.11±0.71
	10 mM	93.47±0.16
Sodium deoxycholate	1 mM	107.50±0.08
	10 mM	106.67±0.67

Whereas, sodium deoxycholate slightly enhance the cuinase activity at both 1mM and 10 mM. In the presence of 1mM or 10mM of SDS, the activity was slightly decreased. Similar kind of observation was also reported by Chen *et al*., (2010) for cutinase from *T. fusca*. The enzyme was also very stable in the presence of surfactants like Tween 80 and Triton X-100 etc. This characteristic of the enzyme made it more suitable for industrial use, because cutinase has application in detergent and laundry industry where it has to undergo many harsh conditions. Also, the enzyme is more suitable for any kind of detergent formulations.

4.9.10 Effect of different solvents

Cutinase has been reported to be successfully used as catalyst in esterification and transeseterification reactions. It has also potential to use in the production of biodiesel. These reactions often involve organic solvents, so it is important to evaluate the stability of the enzyme in organic solvents.

Table 4.20 Effect of different surfactants on cutinase activity. Cutinase was incubated in 75% solvent for 4 h

Organic solvents	**Relative activity %**
control	100±1.2
methanol	9.65±0.025
Ethanol	2.66±0.068
Isopropanol	N.D.
Butanol	103.70±1.36
Dioxan	77.44±0.83
Isoamyl Alcohol	96.42±2.38
DMSO	50.90±1.92
n-Hexane	81.55±2.29
Acetone	8.10±0.05
DCM	22.19±0.911
Chloroform	100±1.9
Isooctane	96.5±1.41

It was observed that cutinase from *P. cepacia* NRRL B 2320 showed excellent stability in the presence of butanol, chloroform, isoamyl alcohol, isooctane and n-hexane, where as it was moderately stable in dioxane, dimethyl sulfoxide and dichloromethane. The enzyme was less stable in methanol, ethanol, and acetone. The stability of the enzyme in organic solvents makes it more attractive for the synthetic (esterification and transesterification) reactions.

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. So, the enzyme must have stability at minimum water concentration and organic solvents (De Barros *et al*., 2009a) for synthesis of esters. Thus the enzyme purified from *P. ceapcia* NRRL B2320 has potential to be used in several industries.

4.9.11 Substrate specificity

The specificity of the enzyme towards various chain length esters was investigated using p-nitrophenyl-fatty acyl esters (Table 4.21). The enzyme showed highest specificity towards short-chain fatty acid esters.

Table 4.21 Specificity of the cutinases towards the acyl chain length of different esters

Substrate	**% Activity**
p-nitrophenyl butyrate (C_4)	100±2
p-nitrophenyl valerate (C_5)	96.25±1.5
p-nitrophenyl caprylate (C_8)	85.36±2.1
p-nitrophenyl laurate (C_{12})	81.41±1.56
p-nitrophenyl palmitate (C_{16})	50.13±1.88

The hydrolytic activity was found to be highest in case of esters of C_4-C_6 fatty acid. Further increase in the chain length of fatty acid causes decrease in the activity. The enzyme showed significantly higher activity towards p-nitrophenyl butyrate than p-nitrophenyl palmitate. The cutinase isolated from *P. ceapcia* NRRL B 2320 has the substrate specificity towards short chain fatty acid synthetic esters (C_4-C_6), though it showed activity with longer chain esters

(C_{16}) too. The cutinase from *P. cepacia* NRRL B 2320 and *P. putida* showed almost similar K_m values for pNPB, which reveals that both the enzymes have similar kind of substrate specificity.

4.9.12 Kinetic Analysis

The kinetic parameters for the cutinase were determined using common substrate, pNPB. The values of K_m and V_{max} of cutinase were determined by steady state kinetic analysis as described in the section 3.14.14 (Fig. 4.25).

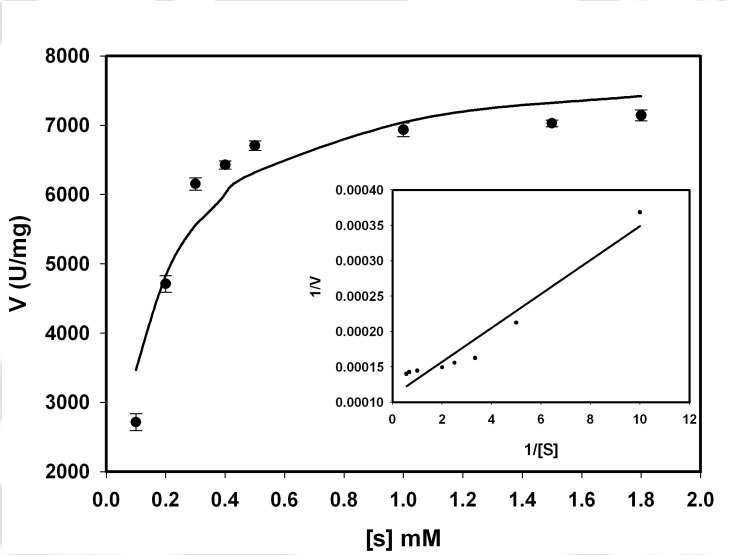


Fig. 4.25 Plot of the reaction velocities (*V*) *vs*. substrate concentration (S: 0.05-2.0 mM) fitted to the Michaelis–Menten equation (R^2=0.98) and determination of K_m and V_{max} of purified cutinase for p-NPB hydrolysis by non-linear regression analysis of experimental steady-state data. (*Inset*) The corresponding Lineweaver-Burk plot for cutinase catalyzed reaction.

Plots of the reaction velocities versus substrate concentration (0.05-2.0 mM) displayed typical hyperbolic saturation curves, which were fitted to the Michaelis–Menten equation (R^2=0.96), yielding the kinetic constants. The K_m, V_{max} and K_{cat} values of purified cutinase were found to be 0.23 mM, 7.955 U μg^{-1} and 5.0×10^3 s^{-1}, respectively. The K_m for other cutinases from *T. fusca* and *F. solani pisi* was observed to be 0.673 and 0.272, respectively (Chen *et al*., 2010).

Previously, Sebastian and Kolattukudy (1988) also reported that the K_m of 0.27 mM for *P. putida* cutinase. Substrate specificity of the cutinase from *P. cepacia* NRRL B 2320 was similar to that of *P. putida* and higher than *T. fusca* cutinase.

4.9.13 Inhibition of cutinase by PMSF

To investigate whether the enzyme indeed utilizes a serine-based catalytic triad, the assay mixture incubated with phenyl methyl sulphonyl fluoride (PMSF), serine hydrolase inhibitor as illustrated in the section 3.14.15.

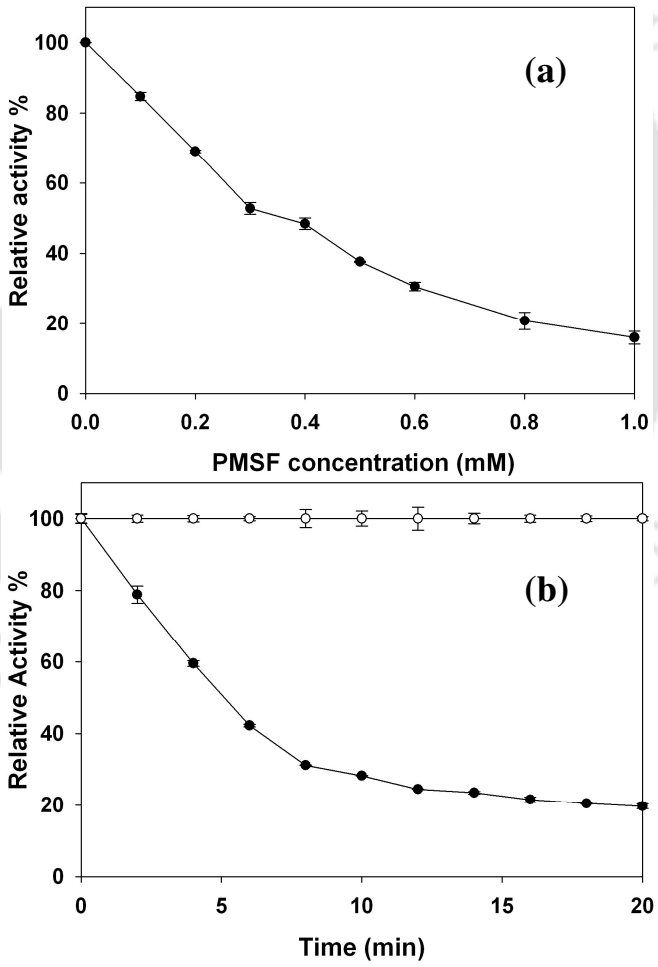


Fig. 4.26 Inactivation of cutinase enzyme by PMSF, (a) the relative activity profile of cutinase at different concentration of PMSF, (b) time course of cutinase inactivation by 0.5 mM PMSF, ○ control, ● with PMSF.

As shown in Fig. 4.26, more than 80% of cutinase activity was reduced by PMSF (0.5 mM) after 15 min with a half life of nearly 5 min. This observation indicates that the irreversible modification of catalytic serine of the enzyme. Also 50% of inhibition was observed by 0.3 and 0.4 mM PMSF after 10 min of incubation (4.26a). In the earlier reports, it was stated that cutinases from different sources like *F. solani pisi, P. pudita, T. fusca* fall under the group of serine hydrolase. The inhibition of the cutinase enzyme by PMSF confirms that cutinase has serine group in its active site, and thereby supports the fact that cutinase from *P. cepacia* NRRL B 2320 also under the serine hydrolase group.

4.9.14 Secondary structure determination using CD spectrum

The CD spectrum of the cutinase in a buffer of pH 7 at room temperature was obtained. Analyzing the CD spectrum (Fig. 4.27) by DICHROWEB software (Provencher and Glockner, 1981; Whitmore and Wallace, 2008), the percentage of secondary structures was determined.

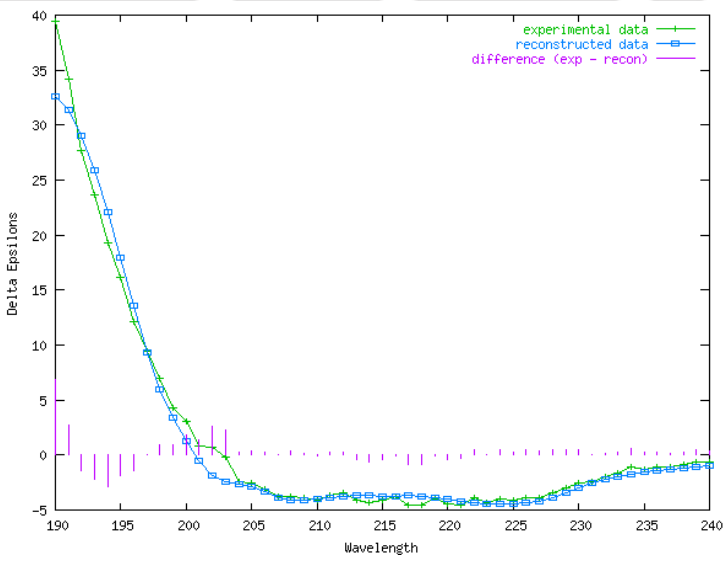


Fig. 4.27 CD spectrum of cutinase at room temperature and pH 7.

It was observed that the native protein contains 56.4% of α-helix, 23.2% β-sheet and rest 20% random coil. Fig. 4.27 was obtained from the DICHROWEB software, which showed the actual CD spectrum and reconstructed spectrum by software. The difference between the actual and reconstructed data was found to be very low. Hence, the predicted secondary structures by the software were taken for analysis.

4.10 Esterification reactions catalyzed by cutinase

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. Cutinase is known to have potential to catalyze the reaction called esterification for synthesis of short-chain alkyl esters. In this study, the ability of cutinase to catalyze esterification reactions was evaluated.

4.10.1 Effect of temperature on ester synthesis

The effect of temperature on cutinase activity was evaluated in the range of 25–50°C, for butyric and valeric acid, with butanol as described in the section 3.15. The conversion (%) was the maximum in the temperature range of 35-40°C (Fig. 4.28) and above 40°C, decrease in conversion was observed. The optimum temperature for the esterification reaction was found to be at 37°C. The highest conversion of 94.6% and 87.5% was achieved at 37 ºC after 12 h of incubation for butyric acid and valeric acid, respectively. At higher temperatures, the conversion was decreased. For the hydrolysis of synthetic ester, cutinase showed its maximum activity at the range of 35-45°C (section 4.9.1). Santos and Castro (2006) have achieved 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).

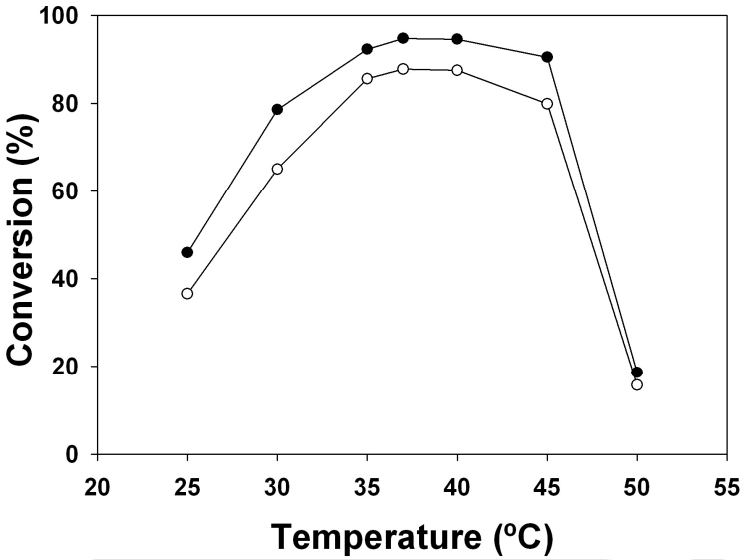


Fig. 4.28 Effect of temperature on the synthesis of butyl esters in the reaction mixture of 2.5 ml of isooctane containing 0.25 M fatty acid and 0.25 M butanol and 0.1mM cutinase. Butyl butyrate (—●—), butyl valerate (—○—).

4.10.2 Effect of water content

Water plays a vital role to carry out esterification reactions in non-conventional medium. Water molecules maintain the three-dimensional tertiary conformation of enzymes to retain their activity and it also controls the thermodynamic equilibrium of the enzymatic reactions. However, water is a second product during the reaction of esterification. Generally, low water content favours 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; Lai and Connor, 1999).

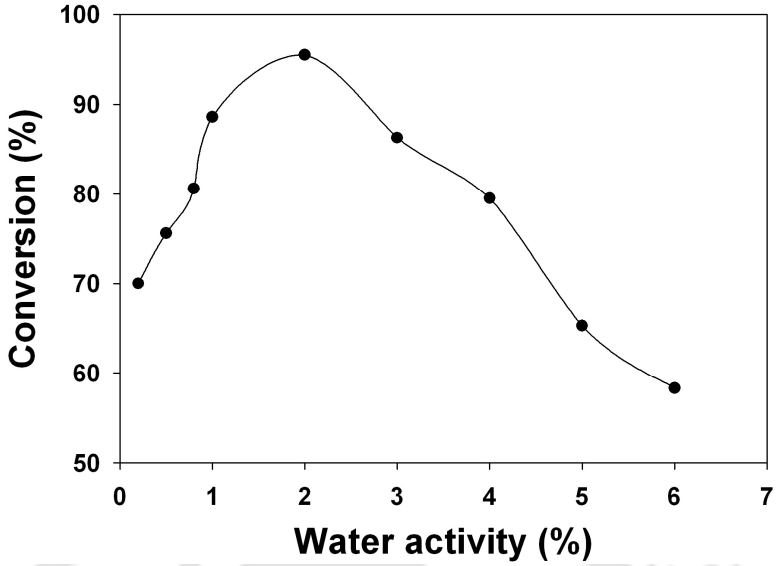


Fig. 4.29 Effect of water activity (% w/w) on synthesis of butyl butyrate in the reaction mixture of 2.5 ml of isooctane containing 0.25 M fatty acid and 0.25 M butanol and 0.1 mM cutinase.

To study the effect of water content on the reaction rate, 0–6% of water (w/w) was added to the reaction media (Fig. 4.29). The conversion was increased to 95% up to 2% water and then decreased sharply. This result agrees with the fact that a minimal quantity of water was necessary to maintain the active conformation of the enzyme. At higher water concentrations, the reverse hydrolysis reaction was initiated and therefore decrease in net production of esters was observed. Stimulation of enzyme activity in the presence of a limited concentration of water has also been reported for other lipases (Lai and Connor, 1999; Macarie and Baratti, 2000) and cutinases (De Barros *et al*., 2009a) as well.

4.10.3 Effect of alcohol chain length

The conversion (%) for the synthesis of alkyl butyrate catalyzed by *P. cepacia* NRRL B 2320*cutinase* (PCC) at 37°C was decreased with the increasing chain length of alcohol (C_4-C_{10}) (Fig. 4.30). It was found that PCC showed the highest conversion for butanol among the

alcohols tested. For a particular fatty acid, the synthesis of esters will be dependent on its accessibility to the active site, whereas the selection of alcohols for the enzyme-catalyzed synthesis will be more dependent on the region that surrounds the active site. As acyl-enzyme intermediates are not formed by alcohols during the acylation process, the net synthesis of an ester was determined by the diffusion of alcohol molecules into the active site of an enzyme (Lai and Connor, 1999).

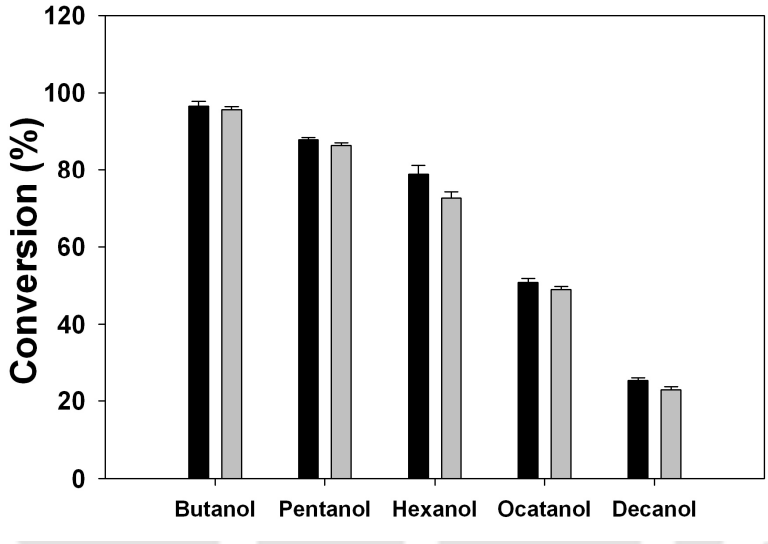


Fig. 4.30 Effect of alcohol chain length on synthesis of alkyl butyrate. Alcohol/acid molar ratio, R = 1 (alcohol 0.1 M and butyric acid 0.1M) (■), and R = 2 (alcohol 0.2 M and butyric acid 0.1 M) (■).

Small chain alcohols have the advantage over long chain bulky alcohols, as they are capable of diffusing into the active site of the enzyme more readily. So, decrease in reactivity was observed with increase of alcohol chain length. Microencapsulated cutinase in AOT reverse micelle also showed preference for alcohols chain length C_5-C_6 (Sebastiao *et al*., 1993) reflecting both the intrinsic selectivity of the enzyme and the different accessibility of the alcohol substrates to the cutinase active site (Carvalho *et al*., 1998a). Sarazin *et al*., (1992, 1995) were achieved 80% conversion for the esterification of caprylic acid with butanol with

lyophilized cutinase. Previously, similar trend have been found for lipases from other sources, e.g., *B. licheniformis*, *P. fluorescence*, *M. miehei, Aspergillus*, *C. rugosa*, and *Rhizopus arrhizus* (Lai and Connor, 1999; Langrand *et al*., 1990) with maximum activity being seen for C_4–C_6 alcohols.

4.10.4 Effect of acid chain length on the synthesis of butyl esters

The selectivity of fatty acid chain-length usually depends on the native properties of the enzymes and mostly found to be similar to that of the hydrolysis reactions. As in case of lipase/esterase-catalyzed synthesis reactions an acyl-enzyme intermediate was formed. So, dependency on the fatty acids chain length would mostly due to the affinity between the fatty acid and enzyme itself.

Fig. 4.31 shows that the conversion (%) of butyl esters varies with the carbon chain length of the fatty acids. Experiments were conducted for fatty acids of chain length C_4 to C_{16}. Cutinase showed higher hydrolytic activity in aqueous media with p-nitrophenyl butyrate and valerate than other long chain synthetic esters as described in the section 4.9.11. In case of synthesis reaction in organic media by PCC, almost a similar trend was observed. The decrease in conversion was observed with increase in fatty acid chain length. The highest conversion was observed for butyric acid and then valeric acid, whereas conversion for octanoic acid and decanoic acid was low. The minimum conversion was observed for the palmitic acid due to the long chain fatty acids react with the active site of cutinase and probably 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 *P. ceapcia* NRRL B 2320 had the highest affinity towards short chain fatty acids (C_4–C_6), 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. Lipase from *P. fluorescens* also showed similar kind of affinity towards short-chain length fatty acid (butyric acid) (Welsh *et al*., 1989).

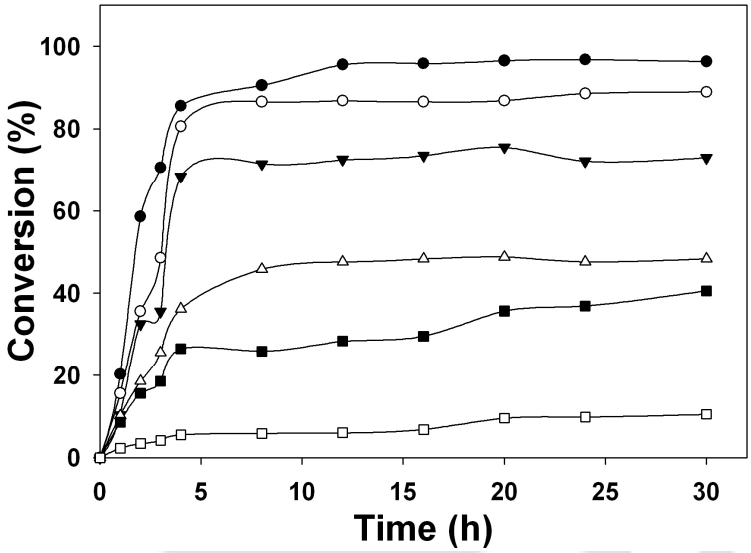


Fig. 4.31 Effect of acid chain length on synthesis of butyl esters. Alcohol/acid molar ratio, R = 1 ([butanol] = 0.1 M). Butyl butyrate (—●—), butyl valerate, (—○—), butyl caproate (—▼—), butyl octanoate (—Δ—), butyl decanoate (—■—), butyl palmitate (—□—).

4.10.5 Synthesis of ethyl esters

In this study, experiments were conducted to check the ability of *P. cepacia* NRRL B 2320 cutinase to synthesize ethyl esters. Fig. 4.32, showed that the conversion of ethyl esters of butyric acid, valeric acid and caproic acid. Similar kind of profile was observed for ethyl esters as observed before for butyl esters. The maximum conversion (94%) was obtained for ethyl butyrate, followed by ethyl valerate (86%) and ethyl caproate (65%). Previously, De Barros *et al*., (2009a) was also studied the synthesis of ethyl esters for these three fatty acids catalyzed by recombinant *F. solani pisi* cutinase.

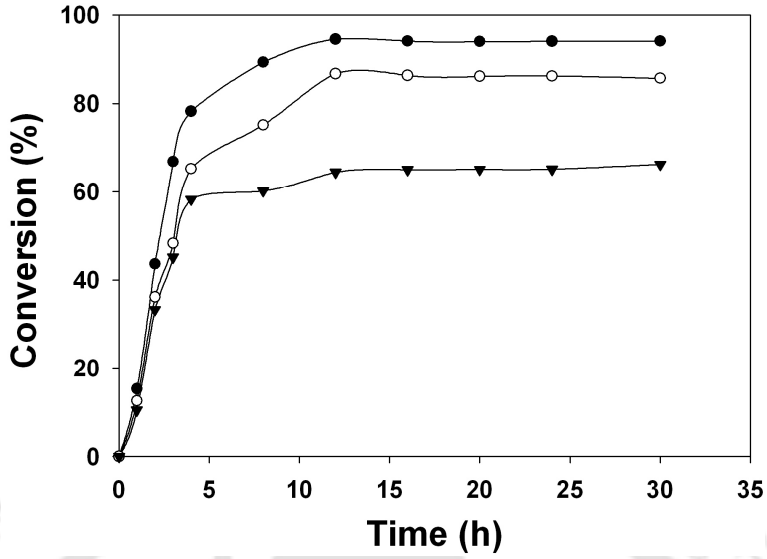


Fig. 4.32 Effect of acid chain length on the synthesis of ethyl esters. Alcohol/acid molar ratio, R = 1 ([ethanol] = 0.1 M). Ethyl butyrate (—●—), ethyl valerate (—○—) and ethyl caproate (—▼—).

They have achieved the maximum conversion for valeric acid (95%) followed by butyric acid (84%) and caproic acid (62%), though they found the rate of ester synthesis is higher for butyric acid than that of valeric acid. The specificity for cutinase from two different sources may slightly vary with the fatty acid chain length, but both enzymes prefer short chain length fatty acid (C_4-C_6) for esterification reaction. In the present study, it was observed that the maximum conversion obtained (94.6%) for butyric acid, followed by valeric acid (87.5%) and caproic acid (72.5%) for the synthesis of butyl ester. The efficiency of *P. cepacia* NRRL B 2320 cutinase is similar for the synthesis of both butyl and ethyl esters.

4.10.6. Effect of substrate concentration and kinetics for synthesis of butyl butyrate

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, where both substrates are soluble in a single phase having equal opportunity to reach the enzyme active site, and thus the kinetics of the reaction will be more complex than those of the hydrolysis reaction.

The experiments for kinetic study were performed according to the experimental plan given in Table 3.10 (section 3.15.1). The effect of different concentrations of butyric acid (Fig. 4.33) or butanol (Fig. 4.34) on the initial rates of butyl butyrate synthesis was studied in the range of 0.05–0.5 M for both the substrates. The effect of increasing butyric acid concentration on initial reaction rate at four different concentrations of butanol (0.05M, 0.1M, 0.25M and 0.5M) is shown in Fig. 4.33. Fig. 4.34 shows the effect of increasing butanol concentration on initial rate of synthesis of butyl butyrate at four different concentrations of butyric acid (0.05M, 0.1M, 0.25M and 0.5M). From Fig. 4.33 and 4.34, it was observed that the rate of reaction increases with the increase in substrate concentration up to 0.3 M. When the substrate concentrations increases further (>0.3 M), the initial reaction rate was decreased. The loss of activity at high alcohol concentrations might be due to its dehydrating effect on the surface of the enzyme in organic media, which inhibits the cutinase activity. On the other hand, decrease in reaction rate at high acid concentration may be due to the change in catalytic environment at higher concentration of acids. It was previously found that cutinase showed very little hydrolysis activity at pH less than 5.5, as increase in acid concentration lowered pH of the organic media, and hence decrease in esterification activity too. The esterification using other

lipase or cutinase (recombinant *F. solani* cutinase) were also showed similar trend at different substrate concentrations (De Barros *et al*., 2009a).

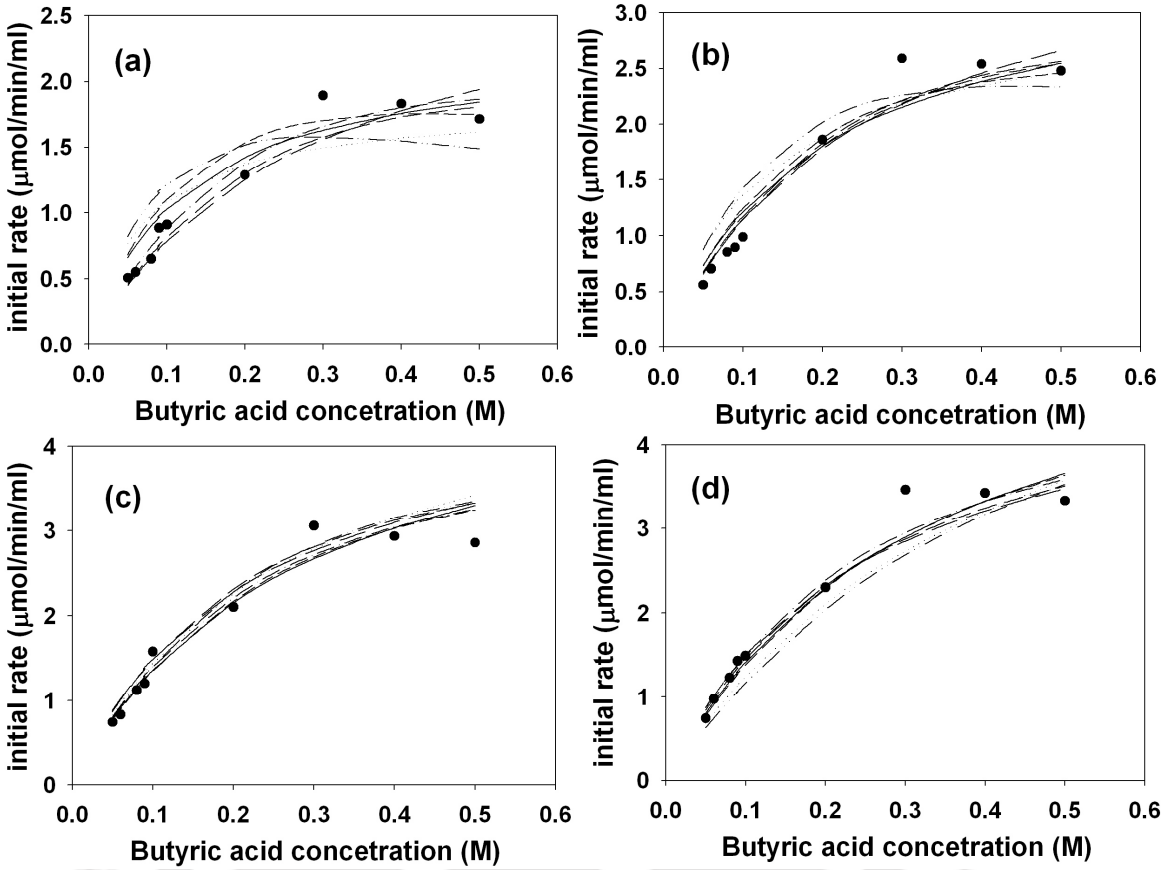


Fig. 4.33 The initial reaction rates for synthesis of butyl butyrate as a function of the butyric acid concentration at the fixed concentrations of butanol. The experimental data were fitted to Ping Pong kinetic model equations (Eq. 3.14-3.20) and the lines shown were calculated using the parameters presented in Table 1. (a) 0.05 M butanol, (b) 0.1 M butanol (c) 0.25 M butanol (d) 0.5 M butanol. Eq. 3.14(——), eq. 3.15 (·····), eq. 3.16 (------), eq. 3.17 (–••–••–), eq. 3.18 (— — —), eq. 3.19 (—•—•—), eq. 3.20 (– – – –).

For kinetic study of butyl butyrate synthesis, the effect of concentrations of both substrates on the rate of reaction was investigated. To estimate the kinetic parameters, the experimental data were fitted to the model equations 3.14-3.20 demonstrated in the section 3.15.1. The kinetic parameters obtained by fitting the data to these equations are given in the Table 4.22. The

variation of experimental initial reaction rate as a function of initial substrate concentration and the fitted curves are presented in Fig. 4.33 and Fig. 4.34 for butyric acid and butanol, respectively.

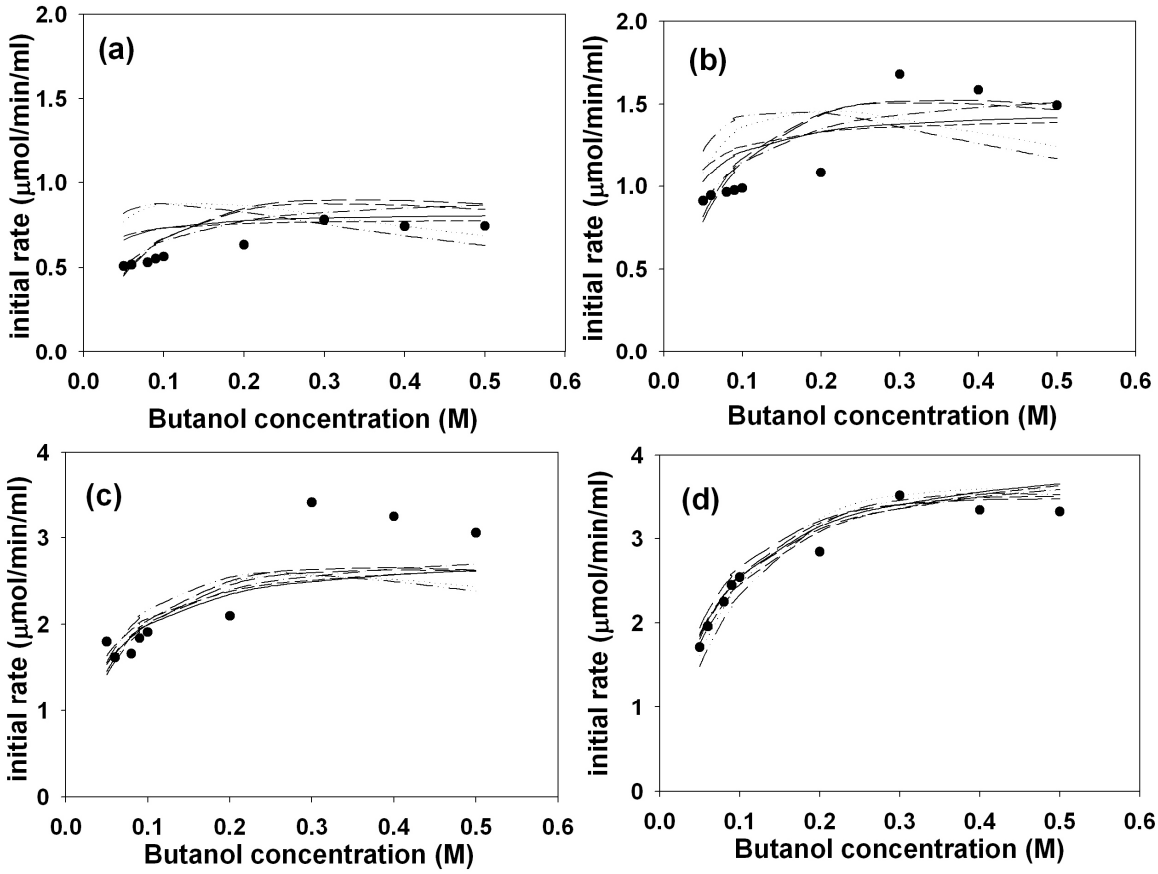


Fig. 4.34 The initial reaction rates for synthesis of butyl butyrate as a function of the butanol concentration at the fixed concentrations of butyric acid. The experimental data were fitted to Ping Pong kinetic model equations (Eq. 3.14-3.20). The lines shown were calculated using the parameters presented in Table 1. (a) 0.05 M butyric acid, (b) 0.1 M butyric acid (c) 0.25 M butyric acid (d) 0.5 M butyric acid. eq. 3.14(——), eq. 3.15 (·····), eq. 3.16 (------), eq. 3.17 (–••–••–), eq. 3.18 (— — —), eq. 3.19 (—•—•—), eq. 3.20 (– – – –).

The model equation 3.14 gives the simple Michaelis-Menten equation for two substrate Ping–Pong bi bi kinetic model. But this model may not predict the kinetics of the reaction appropriately after certain concentration of substrates, where increasing concentration of one

or both substrates has inhibitory effect on reaction rate. So, the introduction of one or more extra terms may be helpful for proper prediction of the reaction kinetics with inhibitory effect of one or more substrates. K_{IB} and K_{IA} are inhibition constant for butanol and butyric acid, respectively. The eq. 3.15 and 3.16 are obtained when inhibition due to butanol or butyric acid are considered, where as eq. 3.17 has both K_{IA} and K_{IB} by considering inhibition due to the both substrates. From the results obtained by fitting the experimental data to the above mentioned four equations, it was observed that kinetic parameters does not vary much for the other three equations than the classical Michaelis-Menten equation. But the decrease in R^2 value is due to incorporation of one or more extra terms. The data obtained in the present study was well fitted with the model eq. 3.16 (with acid inhibition term) with high R^2 value of 94.6% as compared to other equations (3.15 and 3.17).

Table 4.22 Estimated kinetic constants for synthesis of butyl butyrate using different kinetic models

	V_{max} (µmol/min/ml)	K_{mA} (M)	K_{mB} or K_{BP} (M)	K_{IA} (M)	K_{IB} (M)	K_{AB} (M^2)	R^2 (%)
Eq. 3.14	7.40	0.40	0.11	----	---	---	94.7
Eq. 3.15	9.60	0.34	0.21	----	0.57	---	90.4
Eq. 3.16	7.86	0.45	0.07	0.58	----	----	94.6
Eq. 3.17	11.7	0.45	0.16	0.58	0.55	---	88.5
Eq. 3.18	5.64	0.11	0.04	----	0.58	0.02	95.0
Eq. 3.19	6.27	0.28	0.03	0.35	---	0.01	96.6
Eq. 3.20	6.13	0.13	0.03	0.35	0.58	0.02	95.5

In the esterification reaction, water is one of the products that were also present at the start of the reaction, because minimum amount of water is required for the enzyme activity. The incorporation of effect of water, converts eq. 3.16 to 3.18, which contains an extra term K_{AB} and modified K_{MB} to K_{BP}. The influence of water for all three above mentioned cases of alcohol, acid and both alcohol and acid inhibition was investigated. The modified model

equations are given by eq. 3.18, 3.19 and 3.20 (section 3.15.1). It has been clearly observed from the kinetic parameters and the regression coefficients that incorporation of the effect of water improves the fit for all three conditions (Table 4.22).

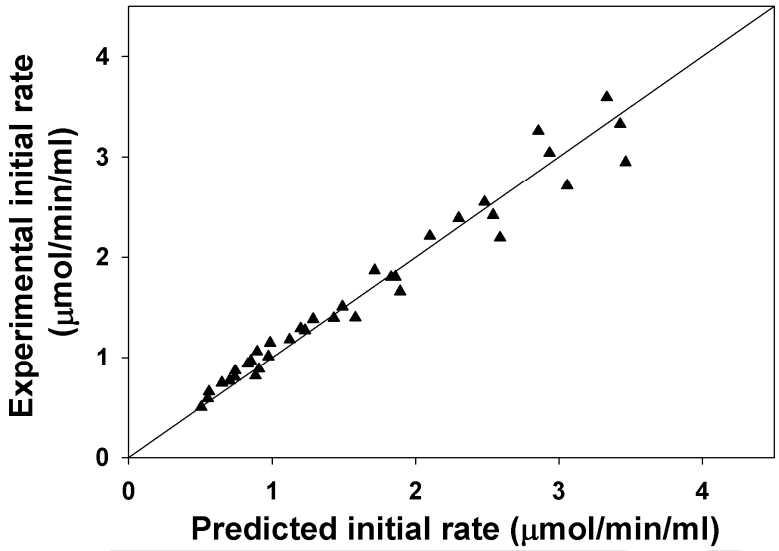


Fig. 4.35 Parity plot for predictions of initial reaction rate for best fitted model (model equation 3.19).

Parity plot (Fig. 4.35) showing estimated initial reaction rate by different models that fit to the entire data versus experimental initial reaction rate. The best fit was obtained for the reaction with eq. 3.19 (R^2 = 96.6%). This depicts that the inhibition by butyric acid in the experimental range is more prominent than other substrate. Pinto-Sousa *et al*., (1994) had also studied the kinetics of esterification reaction of butyric acid and 2-butanol catalyzed by microencapsulated cutinase in nonionic surfactant, phosphotidylcholine. They have also observed that the substrate inhibition by 2-butanol and butyric acid above the concentration of 0.5M and 0.2M, respectively.

4.11 Transesterification reactions catalyzed by cutinase

The methyl esters of oils are well appreciated to be used as bio-carburants, biosurfactants, biolubricants or biodiesel as an alternative to fossil/natural fuel. The biodiesel can be used either blended with or without blending with the natural diesel. Lipases are mostly used for transesterification of oils. Cutinase having lipolytic activity also catalyze the transesterification of oils. In this study, experiments were performed to evaluate the ability of cutinase to catalyze the transesterification of tributyrin, triolein and soyabean oil.

4.11.1 Effect of enzyme concentration

Experiments were carried out as illustrated in the section 3.16. Fig. 4.36 shows that the effect of cutinase concentration on the conversion (%) of methyl ester of tributyrin and triolein. The conversion (%) has increased with increasing concentration of cutinase from 0.5 mg ml^{-1} to 2.5 mg ml^{-1} of reaction mixture. Further increase of cutinase concentration did not show any significant increase in methyl ester conversion for both tributyrin and triolein. The maximum conversion achieved for methyl butyrate and methyl oleate was found to be 65% and 30%, respectively. Cutinase from *P. cepacia* NRRL B 2320 have shown higher affinity towards shorter chain length substrate, so the conversion (%) has decreased with increase in fatty acid chain length. In this study, reactions were carried out with 1-3% cutinase (w/w of oil). Xie and Ma (2010) reported that 45% conversion was observed for transesterification of oil with methanol using 40% lipase (w/w). Tamalampudi *et al*., (2008), and Hernandez-Martin and Otero (2008) achieved 75% conversion for methanolysis of oil with 10% (w/w of oil) and 50% (w/w of oil) of lipase, respectively. Badenes *et al*., (2010b) observed that 70% of conversion during the transesterification of triolein with 1-2% cutinase (w/w of oil).

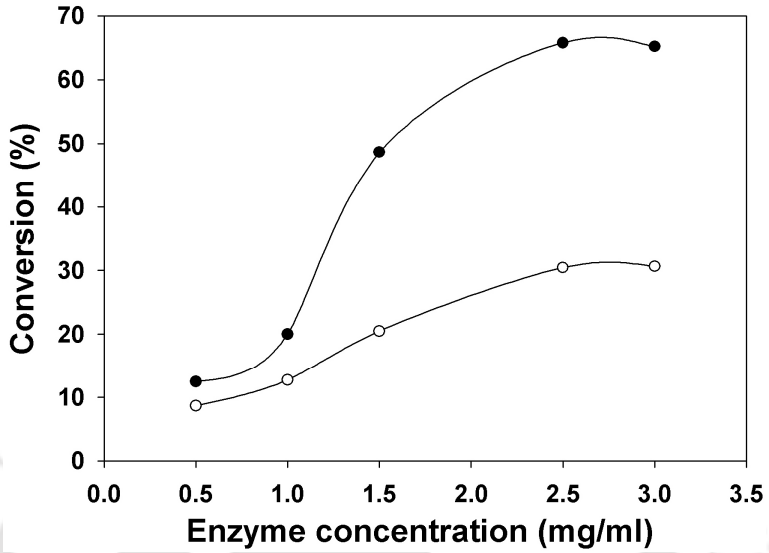


Fig. 4.36 Effect of enzyme concentration on conversion of methyl butyrate (—•—) and methyl oleate (—○—) during cutinase catalyzed transesterification of tributyrin and triolein.

4.11.2 Effect of temperature on methyl ester synthesis

The effect of temperature on cutinase and commercial lipase (Sigma) activity was evaluated in the range of 25–50°C, for transesterification reaction of tributyrin and triolein with methanol (Fig. 4.37). It was observed that the stability of cutinase was slightly better than that of the lipase. The maximum conversion was achieved at 37°C for cutinase, but it did not decrease much up to 45°C. Whereas, in the case of lipase, the maximum conversion obtained at 35°C, but it decreased when temperature increased above 40°C. The methyl ester conversion was higher for transesterfication of tributyrin than triolein with cutinase, whereas reverse was true for the lipase due to the affinity of cutinase towards short-chain length fatty acids was more and lipase was active on longer chain length fatty acids. The conversion during the transesterification of triolein by cutinase was almost comparable to the commercial lipase.

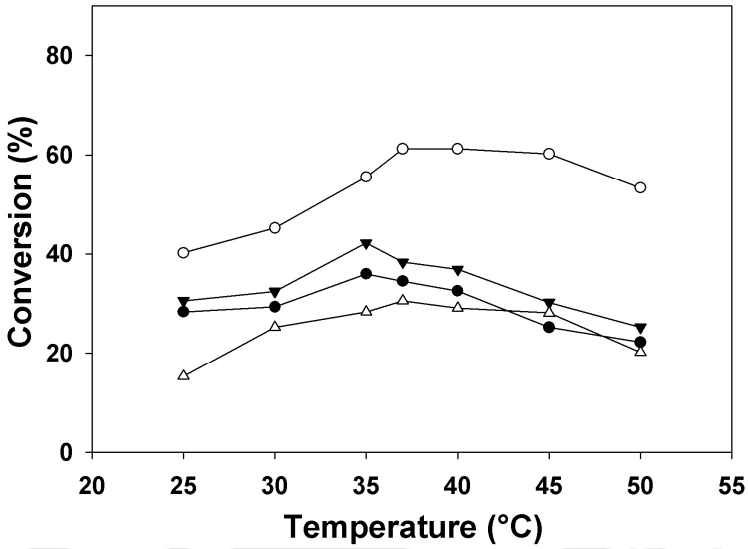


Fig. 4.37 Effect of temperature on the conversion of methyl butyrate by lipase (—●—) and cutinase (—○—), and methyl oleate by lipase (—▼—) and cutinase (—Δ—).

The cutinase from *F. solani pisi* was showed optimum transesterification activity at 30 °C (Badenes *et al*., 2010b).

4.11.3 Effect of substrate molar ratio

Molar ratio of methanol to oil is one of the important parameters affecting the enzymatic transesterification reaction. Fig. 4.38 and 4.39 have shown that the change of methyl butyrate/oleate conversion with time as a function of molar ratio of methanol/oil. The enzymatic transesterification reaction was carried out at 37°C for 30 h, with methanol/oil molar ratio of 0.5:1, 1:1, 1.5:1, 2:1 and 3:1. As shown in Fig. 4.38, the conversion to methyl butyrate increased with increase in molar ratio of methanol/tributyrin from 0.5:1 and reached maximum value at a molar ratio of 1.5:1. Further increase in molar ratio from 1.5:1 to 3:1 resulted decrease in conversion to some degree.

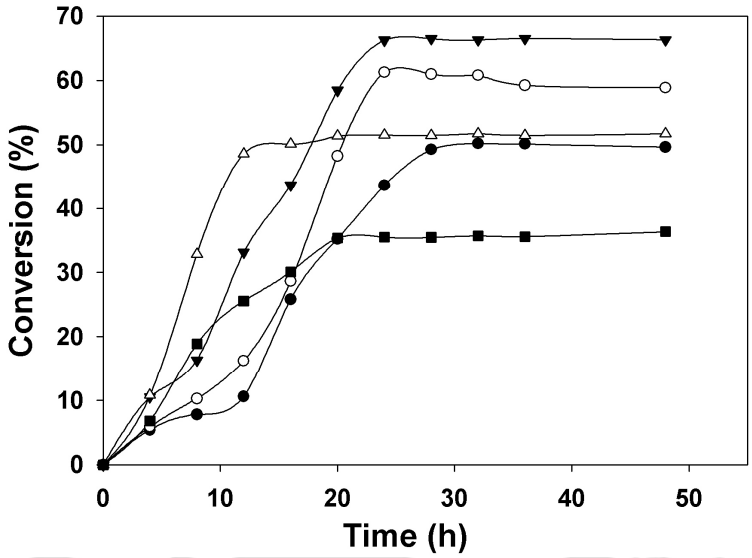


Fig. 4.38 Effect of molar ratio (methanol/tributyrin —●—0.5:1, —○— 1:1, —▼—1.5:1, —Δ— 2:1, —■— 3:1) on the conversion of methyl butyrate during transesterification of tributyrin with methanol catalyzed by cutinase.

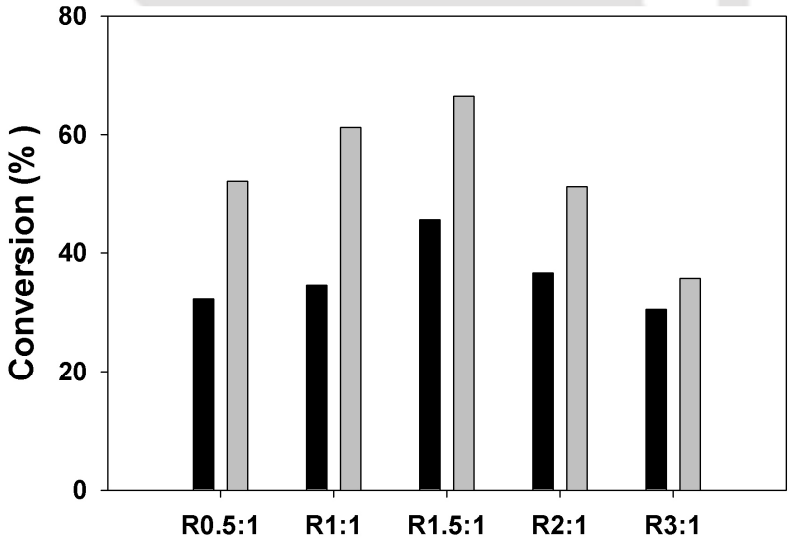


Fig. 4.39 Effect of molar ratio (methanol/tributyrin) on conversion of methyl butyrate during transesterification of tributyrin with methanol catalyzed by lipase (■) and cutinase (■).

A similar trend was observed during the transesterification reaction of triolein and methanol. A maximum of 36% conversion of methyl oleate was achieved after 24 h with methanol/triolein ratio of 1.5:1. Salis *et al*., (2005) observed about 40% conversion of methyl ester during transesterification of triolein catalyzed by *P. cepacia* lipase.

In order to compare the performance of cutinse with commercial lipase during transesterification reactions, experiments were also carried out with commercial lipase. Transesterification activity of lipase was also decreased when methanol/oil ratio increases beyond 1.5:1 (Fig. 4.39 and 4.41). In this experiment, the efficiency of lipase was less than cutinase for the transesterification of tributyrin.

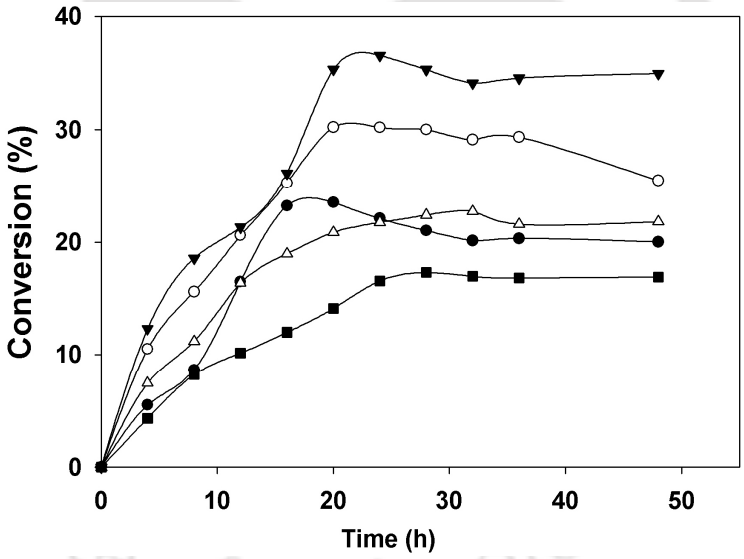


Fig. 4.40 Effect of molar ratio (methanol/triolein —●—0.5:1, —○— 1:1, —▼—1.5:1, —Δ— 2:1, —■— 3:1) on the conversion of methyl oleate during transesterification of triolein with methanol catalyzed by cutinase.

Increase in molar ratio of methanol/oil beyond 1.5:1 decreased the conversion to methyl esters catalyzed by lipases/cutinases was also reported by other authors (Shimada *et al*., 2002; Badenes *et al*., 2010b). It is clear that any molar ratio of methanol to oil above 1.5:1 could

lead to the deactivation of the enzyme. Owing to the low solubility of methanol in the oil; excessive methanol might decrease the enzyme activity by the contact with insoluble methanol, which exists as drops in the oil (Watanabe *et al*., 2009).

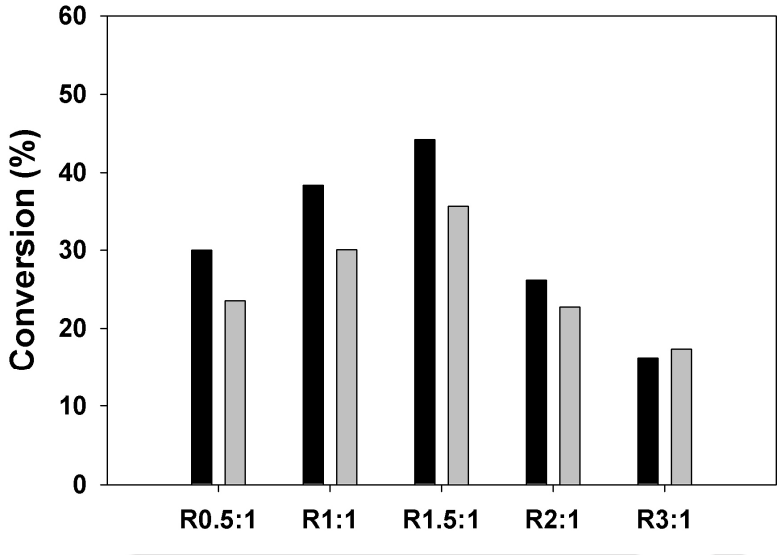


Fig. 4.41 Effect of molar ratio (methanol/triolein) on conversion of methyl oleate during transesterification of triolein with methanol catalyzed by lipase (■) and cutinase (■).

4.11.4. Kinetic study of synthesis of methyl ester of tributyrin and triolein

To study the kinetics of transeseterification reactions catalyzed by cutinase, experiments were performed according to the experimental plan given in the Table 3.11 (section 3.16.1). The effect of different concentrations of tributyrin (Fig. 4.42a) or methanol (Fig. 4.42b) on the initial rates of methyl butyrate synthesis was studied in the range of 0.05–0.5 M for both the substrates. The effect of increasing tributyrin concentration on initial reaction rate at three different concentrations of methanol (0.05M, 0.1M, 0.2M) is shown in Fig. 4.42a. The effect of methanol concentration on initial reaction at three different concentrations of tributyrin (0.05M, 0.1M, and 0.2M) is shown in Fig. 4.42b. Different kinetic model equations, 3.14,

3.15, 3.16 and 3.17 were used to fit the experimental data for both the cases. The kinetic parameters estimated using these equations are given in the Table 4.23.

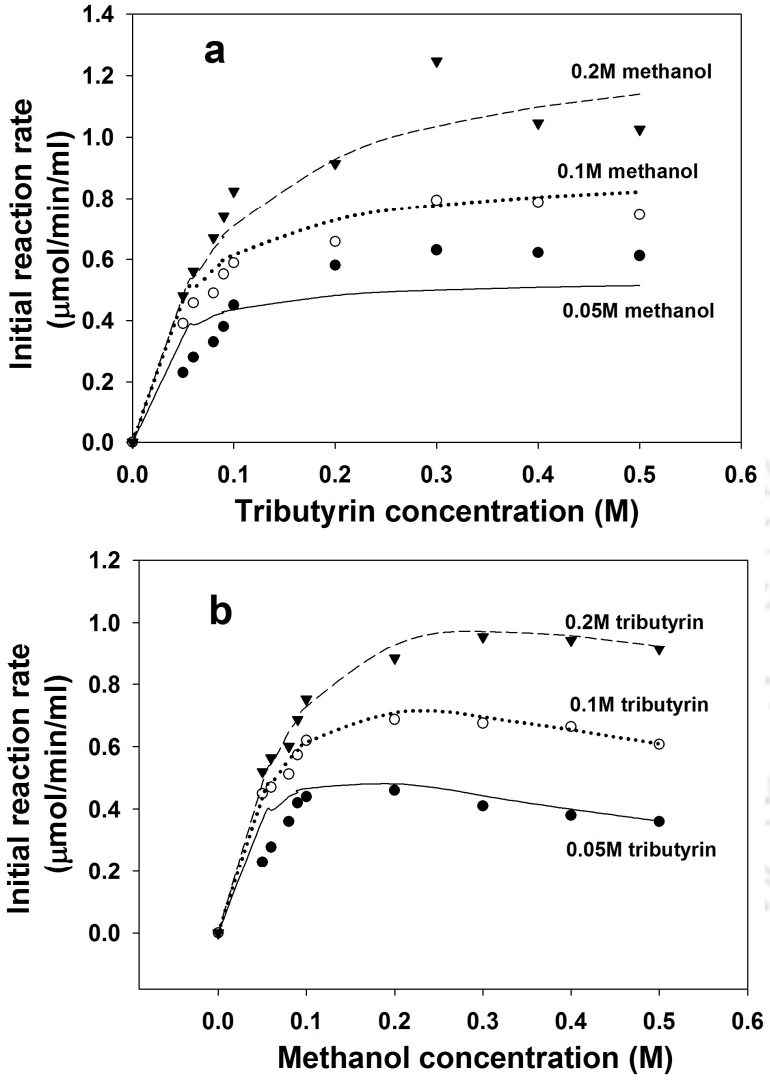


Fig. 4.42 Initial reaction rate as a function of (a) tributyrin or (b) methanol concentrations. The experimental data are fitted to a Ping-Pong kinetic model with alcohol inhibition eq. 3.15. The parameter values in Table 4.23 are used to calculate the lines shown in the figure.

Eq. 3.14 is basic Michaelis-Menten equation for two substrates, Ping–Pong bi bi kinetic model. But, this model may not predict the kinetics of the reaction appropriately after certain concentration of substrates, where increasing concentration of one or both substrates have

inhibitory effect on reaction rate. So, the introduction of one or more extra terms may be useful for proper prediction of the reaction kinetics with inhibitory effect of one or more substrates. K_{IB} and K_{IA} are inhibition constants for methanol and tributyrin/triolein, respectively. The eq. 3.15 and 3.16 are obtained when inhibition due to methanol or tributyrin/triolein are considered, where as eq. 3.17 has both K_{IA} and K_{IB} considering the inhibition by both the substrates. By fitting the experimental data to the above mentioned four equations, it was observed that the kinetic parameters did not vary much for the other three equations than the classical Michaelis-Menten equation

Table 4.23 Estimated kinetic constants for the *P. cepacia* NRRL B 2320 cutinase-catalyzed transesterification of tributyrin (A) with methanol (B) using different kinetic models

	V_{max} (µmol/min/ml)	K_{mA} (M)	K_{mB} (M)	K_{IA} (M)	K_{IB} (M)	R^2
Eq. 3.14	2.237	0.141	0.138	---	---	0.904
Eq. 3.15	2.678	0.097	0.198	---	0.24	0.918
Eq. 3.16	2.655	0.206	0.118	0.80	----	0.896
Eq. 3.17	3.729	0.216	0.176	0.55	0.35	0.838

The lines shown in the Fig. 4.42a and 4.42b were obtained using the values obtained from eq. 3.15 in Table 4.23. There was strong alcohol inhibition during transesterification reaction of tributyrin within the experimental ranges of methanol and tributyrin (Fig. 4.42). It could also be observed that the eq. 3.15 has the highest R^2 (0.918) among the all tested models. Similar experiments were conducted for synthesis of methyl oleate by transesterification of triolein with methanol (shown in Fig. 4.43a and 4.43b). The experimental data were fitted to the eq. 3.14, 3.15, 3.16 and 3.17 and the estimated kinetic parameters were presented in Table 4.24.

The lines shown in the Fig. 4.43a and 4.43b were calculated using the parameters obtained from eq 3.15, in Table 4.24.

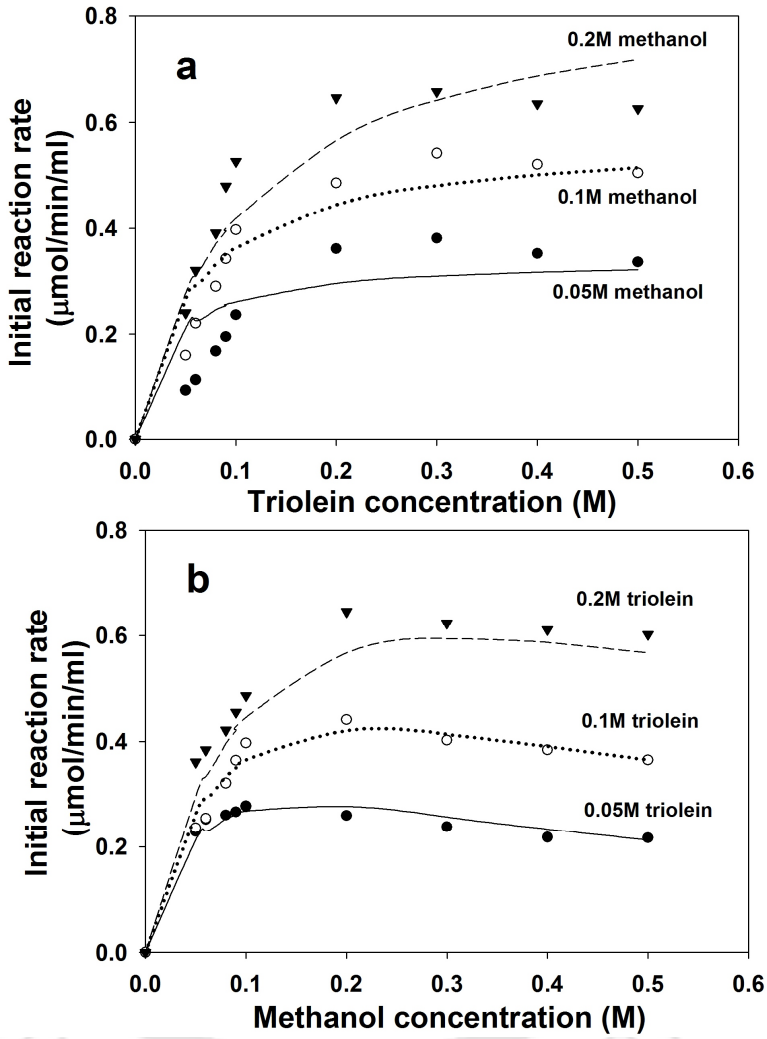


Fig. 4.43 Initial reaction rate as a function of (a) triolein or (b) methanol concentrations. The experimental data are fitted to a Ping-Pong kinetic model with alcohol inhibition eq. 3.15. The parameter values in Table 4.24 are used to calculate the lines shown in the figure.

The decreasing initial reaction rate with increasing methanol concentration during the transestrification of triolein catalyzed by *P. cepacia* NRRL B 2320 cutinase was due to the strong alcohol inhibition, and eq. 3.15 was found to be the best fitted model in this case also.

Previously, Badenes *et al*., (2010b) also reported that the similar kind of alcohol inhibition during the transesterification reaction of triolein with methanol catalyzed by *F. solani* cutinase. They observed that the decrease in methyl ester conversion from 77% to 20% at 24 h, when methanol concentration increased from 390 mM to 590 mM. Several reports are also available on the inhibition effect of alcohol on transesterification reaction catalyzed by lipase (Badenes *et al*., 2010b).

Table 4.24 Estimated kinetic constants for the *P. cepacia* NRRL B 2320 cutinase-catalyzed transesterification of triolein (A) with methanol (B) using different kinetic models

	V_{max} (µmol/min/ml)	K_{mA} (M)	K_{mB} (M)	K_{IA} (M)	K_{IB} (M)	R^2
Eq. 3.14	1.528	0.179	0.156	-----	----	0.881
Eq. 3.15	1.838	0.137	0.222	-----	0.31	0.897
Eq. 3.16	1.841	0.28	0.099	0.414	----	0.852
Eq. 3.17	2.464	0.235	0.204	0.81	0.35	0.83

4.11.5. Transesterification of soyabean oil catalyzed by cutinase

Experiments were carried out for transesterification of soyabean oil catalyzed by cutinase from *P. cepacia* NRRL B 2320. The soyabean oil used in this study has the following composition as given by the manufacturer; *viz*., palmitic acid 11%, stearic acid 4%, oleic acid 24%, linoleic acid 54% and linolenin acid 7%. The average molecular weight calculated as 879.87g mol^{-1}. The enzymatic transesterification reaction was carried out at 37°C, with methanol/oil molar ratio of 0.5:1, 1:1, 1.5:1, 2:1 and 3:1 (Fig. 4.44). It was observed that the increase in molar ratio from 0.5:1 to 1.5:1, the maximum conversion was increased from 22.45% to 32.66% at 24 h, but further increase in the molar ratio resulted in reduction of conversion to 10% due to inhibition of the enzyme by methanol.

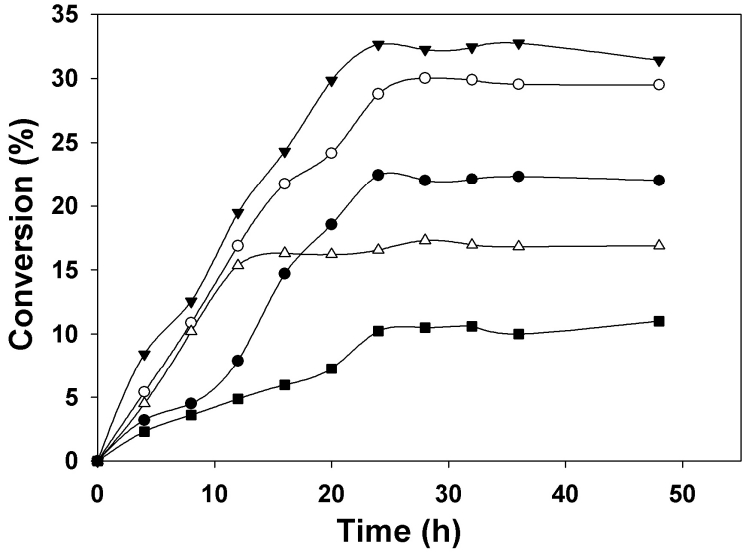


Fig. 4.44 Effect of molar ratio (methanol/soyabean oil —●—0.5:1, —○— 1:1, —▼—1.5:1, —Δ— 2:1, —■— 3:1) on the conversion of methyl ester of oil during transesterification of soyabean oil with methanol catalyzed by cutinase.

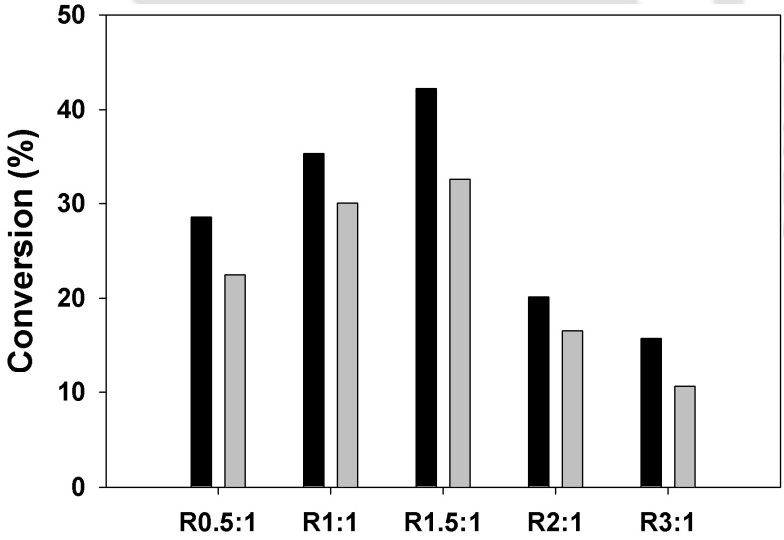


Fig. 4.45 Effect of molar ratio (methanol/soyabean oil) on conversion of methyl ester during transesterification of soyabean oil with methanol catalyzed by lipase (■) and cutinase (■).

The reaction efficiency was also compared with commercial lipase (Fig. 4.45). The conversion (%) of methyl esters of soyabean oil by cutinase was comparable with the commercially available lipase.

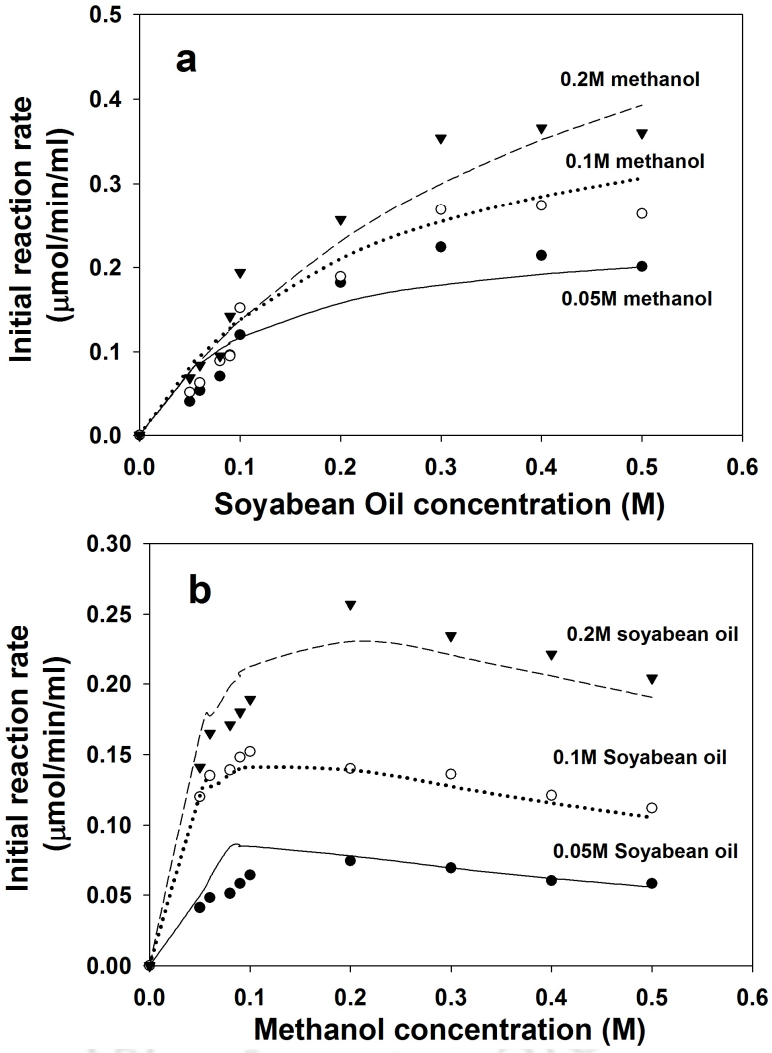


Fig. 4.46 Initial reaction rate as a function of (a) soyabean oil or (b) methanol concentrations. The experimental data are fitted to a Ping-Pong kinetic model with alcohol inhibition eq. 3.15. The parameter values in Table 4.25 are used to calculate the lines shown in the figure.

Xie and Ma (2010) reported that 45% conversion of methyl esters during transesterification of soyabean oil catalyzed by lipase with 1.5:1 methanol/soyabean oil ratio. The increase in

methanol/ soyabean oil ratio to 3:1 resulted in the decrease of conversion to 5%. Yang *et al*., (2009) observed that the conversion of 40% and 20% methyl ester after 24 h incubation during transesterification catalyzed by recombinant *P. fluorescence* lipase 26-2 and lipase AK, respectively. The kinetics of transesterification catalyzed by *P. cepacia* NRRL B 2320 cutinase was studied with the different concentrations of soyabean oil and methanol. The kinetic parameters calculated using the eq. 3.14, 3.15, 3.16 and 3.17 are shown in the Table 4.25. Fig. 4.46 shows the variation in initial reaction rate as the function of substrate (soyabean oil or methanol) concentration. The strong methanol inhibition was observed in this case also. The best result (on the basis of R^2 0.931) was obtained when experimental data were fitted to eq. 3.15. The lines shown in Fig. 4.46a and 4.46b were obtained by using the parameters given in Table 4.25 (eq. 3.15).

Table 4.25 Estimated kinetic constants for the *P. cepacia* NRRL B 2320 cutinase-catalyzed transesterification of soyabean oil (A) with methanol (B) using different kinetic models

	V_{max} (µmol/min/ml)	K_{mA} (M)	K_{mB} (M)	K_{IA} (M)	K_{IB} (M)	R^2
Eq. 3.14	1.331	0.723	0.191	----	----	0.902
Eq. 3.15	1.501	0.546	0.253	----	0.351	0.931
Eq. 3.16	1.60	0.928	0.185	1.4	----	0.911
Eq. 3.17	1.5	0.632	0.133	0.55	0.41	0.907

4.12 Identification, cloning and expression of cutinase encoding genes from *P. cepacia* NRRL B 2320

In the present study, the genes encoding cutinase in *P. cepacia* NRRL B 2320 were identified, cloned and expressed in *E. coli* BL21 (DE3).

4.12.1 Cloning and identification of cutinase encoding genes in P. cepacia NRRL B 2320

The cutinase encoding genes in *P. cepacia* NRRL B 2320 were amplified using the primers given in Table 3.12, section 3.17.1 by PCR. The amplified genes were found to be of 1 kb as shown in Fig. 4.47.

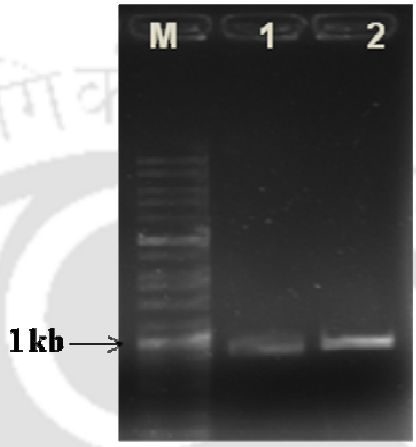


Fig. 4.47 PCR amplification of genomic DNA of *P. cepacia* NRRL B 2320. Lane M: 1 kb marker, Lane 1: *cut_1,* Lane 2: *cut_2.*

The genes obtained from PCR were cloned in pET22b (+) and transformed to *E.coli* DH-5α. The positive clones were selected and confirmed by PCR reaction of plasmid isolated from them. The genes obtained from the clones were then sequenced and the BLAST-n result of the sequence of both the genes showed 100% homology with Gene ID: 3579571 and ID: 3579437 of *T. fusca* cutinase. The BLAST-n result of the genes *cut_1* and *cut_2* are shown in Fig. 4.48 and 4.49, respectively. The similarity in the cutinase genes in two different bacteria was due to horizontal gene transfer as studied by Belbahri *et al*., (2008).

Score = 1768 bits (957), Expect = 0.0, GENE ID: 3579571
Identities = 957/957 (100%), Gaps = 0/957 (0%)
Strand=Plus/Minus

```
Query  1        ATGCCCCCGCATGCGGCGCGGCCCGGCCCTGCACAGAACCGAAGAGGACGTGCAATGGCT  60
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1041911  ATGCCCCCGCATGCGGCGCGGCCCGGCCCTGCACAGAACCGAAGAGGACGTGCAATGGCT  1041852

Query  61       GTGATTACCCCCCGCCGGGAGCGCTCTTCCCTGCTCTCCCGGGCACTGCGCTTCACCGCC  120
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1041851  GTGATTACCCCCCGCCGGGAGCGCTCTTCCCTGCTCTCCCGGGCACTGCGCTTCACCGCC  1041792

Query  121      GCGGCTGCCACAGCGCTTGTGACCGCGGTCAGCCTGGCCGCCCCCGCTCATGCCGCCAAC  180
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1041791  GCGGCTGCCACAGCGCTTGTGACCGCGGTCAGCCTGGCCGCCCCCGCTCATGCCGCCAAC  1041732

Query  181      CCCTACGAGCGCGGCCCCAACCCGACCGACGCCCTGCTCGAAGCCCGCAGCGGCCCCTTC  240
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1041731  CCCTACGAGCGCGGCCCCAACCCGACCGACGCCCTGCTCGAAGCCCGCAGCGGCCCCTTC  1041672

Query  241      TCCGTGAGTGAAGAACGGGCCTCCCGCTTCGGTGCTGACGGTTTCGGCGGCGGCACCATC  300
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1041671  TCCGTGAGTGAAGAACGGGCCTCCCGCTTCGGTGCTGACGGTTTCGGCGGCGGCACCATC  1041612

Query  301      TACTACCCGCGGGAGAACAACACCTACGGTGCCGTGGCGATCTCCCCCGGCTACACCGGC  360
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1041611  TACTACCCGCGGGAGAACAACACCTACGGTGCCGTGGCGATCTCCCCCGGCTACACCGGC  1041552

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Query  421      ACCATCGACACCAACACCACCCTCGACCAGCCGGACAGCCGGGCCCGCCAGCTCAACGCC  480
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Sbjct  1041491  ACCATCGACACCAACACCACCCTCGACCAGCCGGACAGCCGGGCCCGCCAGCTCAACGCC  1041432

Query  481      GCGCTGGACTACATGATCAACGACGCCTCGTCCGCGGTGCGCAGCCGGATCGACAGCAGC  540
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Sbjct  1041431  GCGCTGGACTACATGATCAACGACGCCTCGTCCGCGGTGCGCAGCCGGATCGACAGCAGC  1041372

Query  541      CGACTGGCGGTCATGGGCCACTCCATGGGCGGCGGCGGCACCCTGCGTCTGGCCTCCCAG  600
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1041371  CGACTGGCGGTCATGGGCCACTCCATGGGCGGCGGCGGCACCCTGCGTCTGGCCTCCCAG  1041312

Query  601      CGTCCCGACCTGAAGGCCGCCATCCCGCTCACCCCGTGGCACCTCAACAAGAACTGGAGC  660
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Sbjct  1041311  CGTCCCGACCTGAAGGCCGCCATCCCGCTCACCCCGTGGCACCTCAACAAGAACTGGAGC  1041252

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Query  721      ACCCACGCCCGGCCCTTCTACAACAGCCTCCCGACCTCGATCAGCAAGGCCTACCTGGAG  780
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Query  901      GGACCGCGCGACGGACTCTTCGGCGAGGTCGAAGAGTACCGCTCCACCTGCCCCTTC  957
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Sbjct  1041011  GGACCGCGCGACGGACTCTTCGGCGAGGTCGAAGAGTACCGCTCCACCTGCCCCTTC  1040955
```

Fig. 4.48 BLATn for *cut_1* gene.

Score = 1668 bits (903), Expect = 0.0, GENE ID: 3579437
Identities = 903/903 (100%), Gaps = 0/903 (0%)
Strand=Plus/Minus

```
Query  1        ATGGCTGTGATGACCCCCCGCCGGGAGCGCTCTTCCCTGCTCTCCCGAGCTCTGCAAGTG  60
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Sbjct  1043262  ACGGCTGCGGCTGCCACAGCGCTTGTGACCGCGGTCAGCCTGGCCGCCCCCGCTCATGCC  1043203

Query  121      GCCAACCCCTACGAGCGCGGCCCCAACCCGACCGACGCCCTGCTCGAAGCCAGCAGCGGC  180
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Sbjct  1043202  GCCAACCCCTACGAGCGCGGCCCCAACCCGACCGACGCCCTGCTCGAAGCCAGCAGCGGC  1043143

Query  181      CCCTTCTCCGTCAGCGAGGAGAACGTCTCCCGGTTGAGCGCCAGCGGCTTCGGCGGCGGC  240
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Sbjct  1043142  CCCTTCTCCGTCAGCGAGGAGAACGTCTCCCGGTTGAGCGCCAGCGGCTTCGGCGGCGGC  1043083

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Sbjct  1043082  ACCATCTACTACCCGCGGGAGAACAACACCTACGGTGCGGTGGCGATCTCCCCCGGCTAC  1043023

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Sbjct  1043022  ACCGGCACTGAGGCTTCCATCGCCTGGCTGGGCGAGCGCATCGCCTCCCACGGCTTCGTC  1042963

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Sbjct  1042962  GTCATCACCATCGACACCATCACCACCCTCGACCAGCCGGACAGCCGGGCAGAGCAGCTC  1042903

Query  421      AACGCCGCGCTGAACCACATGATCAACCGGGCGTCCTCCACGGTGCGCAGCCGGATCGAT  480
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Sbjct  1042902  AACGCCGCGCTGAACCACATGATCAACCGGGCGTCCTCCACGGTGCGCAGCCGGATCGAT  1042843

Query  481      AGCAGCCGACTGGCGGTCATGGGCCACTCCATGGGCGGCGGCGGCACCCTGCGTCTGGCC  540
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1042842  AGCAGCCGACTGGCGGTCATGGGCCACTCCATGGGCGGCGGCGGCACCCTGCGTCTGGCC  1042783

Query  541      TCCCAGCGTCCCGACCTGAAGGCCGCCATCCCGCTCACCCCGTGGCACCTCAACAAGAAC  600
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1042782  TCCCAGCGTCCCGACCTGAAGGCCGCCATCCCGCTCACCCCGTGGCACCTCAACAAGAAC  1042723

Query  601      TGGAGCAGCGTCACCGTGCCGACGCTGATCATCGGGGCCGACCTCGACACGATCGCGCCG  660
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1042722  TGGAGCAGCGTCACCGTGCCGACGCTGATCATCGGGGCCGACCTCGACACGATCGCGCCG  1042663

Query  661      GTCGCCACGCACGCGAAACCGTTCTACAACAGCCTGCCGAGCTCCATCAGCAAGGCCTAC  720
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Sbjct  1042662  GTCGCCACGCACGCGAAACCGTTCTACAACAGCCTGCCGAGCTCCATCAGCAAGGCCTAC  1042603

Query  721      CTGGAGCTGGACGGCGCAACCCACTTCGCCCCGAACATCCCCAACAAGATCATCGGCAAG  780
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Sbjct  1042602  CTGGAGCTGGACGGCGCAACCCACTTCGCCCCGAACATCCCCAACAAGATCATCGGCAAG  1042543

Query  781      TACAGCGTCGCCTGGCTCAAGCGGTTCGTCGACAACGACACCCGCTACACCCAGTTCCTC  840
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Sbjct  1042542  TACAGCGTCGCCTGGCTCAAGCGGTTCGTCGACAACGACACCCGCTACACCCAGTTCCTC  1042483

Query  841      TGCCCCGGACCGCGCGACGGACTCTTCGGCGAGGTCGAAGAGTACCGCTCCACCTGCCCG  900
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Sbjct  1042482  TGCCCCGGACCGCGCGACGGACTCTTCGGCGAGGTCGAAGAGTACCGCTCCACCTGCCCG  1042423

Query  901      TTC  903
                |||
Sbjct  1042422  TTC  1042420
```

Fig. 4.49 BLATn for *cut_2* gene.

4.12.2 Expression of cutinase encoding genes in E. coli BL 21(DE3)

The cutinase genes (*cut_1* and *cut_2*) were expressed in *E. coli* BL 21 (DE3) as per the method described in the section 3.17. A high cutinase producing recombinant strains were obtained.

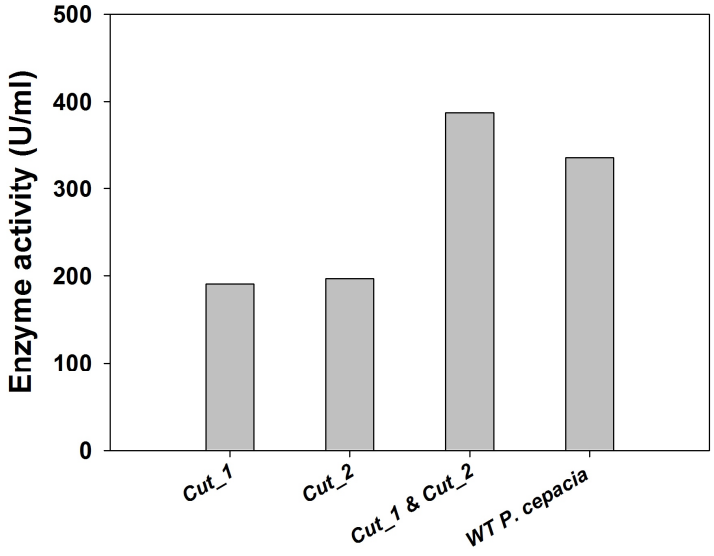

Fig. 4.50 Comparison of wild type cutinase production in *P. cepacia* NRRL B 2320 with expression of recombinant cutinases in *E.coli* BL21 (DE3).

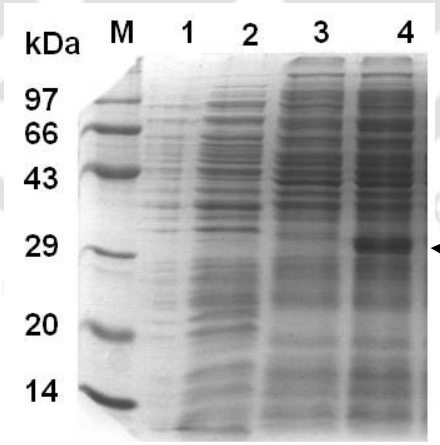

Fig. 4.51 Expression of Cut_1 protein, Lane M: marker, Lane 1: *E. coli* BL21 without vector; Lane 2: *E. coli* BL21 with pET22b (+); Lane 3: uninduced *E. coli* BL21 with construct; Lane 4: *E. coli* BL 21 with construct after 6 h induction with IPTG.

The production of cutinase (activity was measured using p-NPB as substrate) from wild *P. cepacia* NRRL B 2320 was found to be 336.4 U ml^{-1} in optimized medium whereas the expression by both recombinants cutinases (*cut_1* and *cut_2*) was found to be 387.46 U ml^{-1} in un-optimized medium (Fig. 4.50). An Increase in cutinase activity of about 50 U ml^{-1} was observed for recombinant cutinase production in un-optimized medium than cutinase production from *P. cepacia* NRRL B 2320. The expression of cutinase was also confirmed from SDS PAGE (Fig. 4.51 and 4.52). This is the first report on identification of cutinase genes in *P. cepacia* NRRL B 2320.

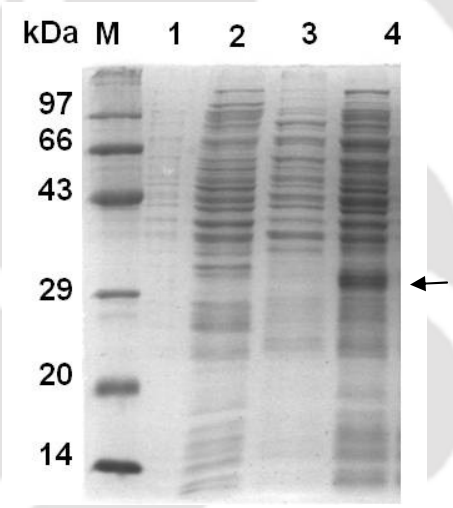

Fig. 4.52 Expression of Cut_2 protein, Lane M: marker, Lane 1: *E. coli* BL21 without vector; Lane 2: *E. coli* BL21 with pET22b (+); Lane 3: uninduced *E. coli* BL21 with construct; Lane 4: *E. coli* BL 21 with construct after 6 h induction with IPTG.

Expression of the recombinant cutinase in TB media (Fig. 4.50, Fig. 4.51 and Fig. 4.52) showed higher level of expression as compare to yield of wild type cutinase. The Fig. 4.51 and 4.52 shows the protein band near 29 kDa (lane 4) was absent in all control experiments (lane 1, 2 and 3, which were *E. coli* BL21 (DE3) without vector, *E. coli* BL21 (DE3) with pET22b (+), uninduced *E. coli* BL21 (DE3) with construct, respectively) suggesting that the cutinase expression was due the expressed gene in the *E. coli* BL21 (DE3).

CHAPTER 5
SUMMARY AND CONCLUSIONS

- A new cutinase producing strain, *P. cepacia* NRRL B 2320 was screened by cutinase specific substrate, p-nitrophenyl (16 methyl sulphone ester) hexadecanoate (p-NMSH) using tomato cutin as a source of carbon.
- The medium for maximum cutinase production was developed and about 2 fold increase in the activity was achieved. The unstructured kinetic models, such as logistic model for growth and enzyme production, and Luedeking-Pirate model for enzyme production have been proved to be accurately evaluating the fermentation kinetic parameters with good accuracy both in shake flask and bioreactors.
- From the Luedeking-Pirate model a much higher α value than β value suggested that the production of cutinase was mostly growth associated in shake flask. A decrease in α value and increase β value was observed when cutinase production was performed in batch bioreactor with or without pH control. This suggested that cutinase production was mixed growth type in the batch bioreactors.
- The kinetic study of the growth of *P. cepacia* NRRL B 2320 during production of cutinase on insoluble substrate, cutin showed substrate inhibition at a concentration above 10 g l^{-1}. Webb model was found to be the best fitted model for growth of *P. cepacia* NRRL B 2320. The highest volumetric enzyme activity was observed at initial substrate concentration of 10 g l^{-1}.

- A four step purification process was developed and achieved a native cutinase from *P. cepacia* NRRL B 2320. The molecular mass of cutinase was found to be 26.25 kDa from the MALDI-TOF-MS analysis.
- Studies on physical conditions, which influence the performance of purified cutinase, revealed that the purified cutinase was active over a broad range of pH (7.0-9.0) and temperature (35-60ºC). The enzyme showed stability at alkaline range of pH (pH 7.0–10.0). Maximum cutinase activity was obtained at pH of 8 and 37ºC.
- The optimal volume of enzyme and substrate were found to be 24.2 µl (1.39 µg) and 971.42 µl, respectively, for maximum enzyme activity. The optimal pH and temperature for highest activity were found to be 7.9 and 36.5°C, respectively.
- Thermodynamic parameters (K_d, $t_{1/2}$, ΔH, ΔS, ΔG and E_a) were determined to evaluate the probable deactivation mechanism of this enzyme. The estimated thermodynamic parameters i.e. positive values of entropy of cutinase deactivation, the red shift observed in fluorescence spectra, and change in ellipticity in CD spectra suggested that the deactivation of enzyme was due to the disorder-ness (change in secondary structure) of the protein molecules at the higher temperature.
- The activity of cutinase was enhanced by mono cations and various effectors including Na^+, K^+ and β-marceptoethanol, whereas it was moderately inhibited by some divalent cations (Cu^{2+}, Fe^{2+}, Zn^{2+}) and completely inhibited by Hg^{2+} and serine blocking reagent, phenyl methyl sulphonyl fluoride (PMSF).
- The cutinase isolated from *P. cepacia* NRRL B 2320 has the substrate specificity towards short chain fatty acid synthetic esters (C_4-C_6), though it also showed activity with longer chain esters (C_{16}).

- Kinetic parameters, K_m, V_{max} and K_{cat} of purified cutinase from *P. cepacia* NRRL B 2320 were found to be 0.23 mM and 7.95 U/µg and 5.0×10^3 s^{-1}, respectively.
- Cutinase from *P. cepacia* NRRL B 2320 was found to be efficiently catalyzing reactions for synthesis of short-chain length fatty acid esters. The maximum conversion (94%) obtained for ethyl butyrate, followed by ethyl valerate (86%) and ethyl caproate (65%). The highest conversion of 94.6% and 87.5% was achieved after 12 h of incubation for butyl butyrate and valerate, respectively.
- The kinetic parameters for the synthesis of butyl butyrate were also estimated using simple and modified Ping-Pong –bi bi kinetic models. Among the tested models, Ping-Pong model with acid inhibition and water influence was found to the best fitted model for the experimental data.
- Cutinase from *P. cepacia* NRRL B 2320 was found to be efficient in catalyzing transesterification reactions for the synthesis of methyl esters of oils. The optimum conversion could be achieved with 2.5 mg ml^{-1} of protein. The maximum conversions of methyl butyrate and oleate were achieved at 37°C, after 24 h of transesterification reaction of tributyrin and triolein, respectively. The optimum molar ratio for methanol to tributyrin/triolein was found to be 1.5:1.
- The kinetic parameters for the synthesis of methyl esters by transesterification of tributyrin, triolein and soyabean oil were also estimated using simple and modified Ping-Pong –bi bi kinetic models. Among the tested models, Ping-Pong model with alcohol inhibition was found to be the best fitted model for the experimental data in all three cases. The V_{max} for transesterification of tributyrin, triolein and soyabean oil was found to be 2.678, 1.838 and 1.5 µmol min^{-1} ml^{-1}, respectively with the alcohol inhibition model.

- The cutinase encoding genes in *P. cepacia* NRRL B 2320 was identified, cloned and expressed in *E. coli* BL21 (DE3). The recombinant cutinase production under un-optimized condition was found to be higher than the cutinase production from wild strain under optimized condition.

Future scope of work

- Optimization of recombinant cutinase production.
- Characterization of recombinant cutinase.
- Structural study of the cutinase from *P. cepacia* NRRL B 2320.
- Synthesis of some pharmaceutically important stereo-specific compounds using cutinase.
- Use of cutinase in cotton scouring, detergent formulation and synthesis of several other industrially important products.

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APPENDIX

A.1 Reagents used in esterification and transeseterification reactions

The chemicals used in esterification reactions were butanol, ethanol, pentanol, hexanol, octanol, decanol, butyric acid, valeric acid (pentanoic acid), hexanoic acid (caproic acid), octanoic acid (caprylic acid), decanoic acid and palmitic acid. The standards used in esterification reactions were butyl butyrate, butyl valerate, butyl hexanoate, butyl octanoate, butyl decanoate, butyl palmitate, ethyl butyrate, ethyl valerate, ethyl hexanoate. The reagents used in transeseterification reactions were tributyrin, triolein and methanol. The standards used in transeseterification reactions were methyl butyrate, methyl oleate, methyl palmitate, methyl linoleate.

A.2 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 (10 – 50 µl) was diluted with potassium phosphate buffer of pH 7 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 410 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 liberates 1 µMol of p-NP per min at 37°C.

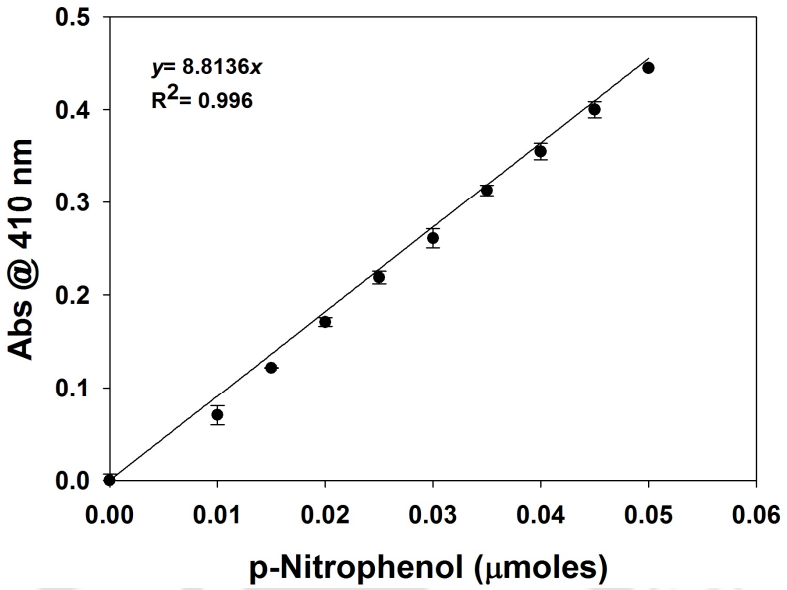

Fig. A.1 Standard curve drawn between known amount of p-NP and the corresponding absorbance measured at 410 nm.

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	$y = 1.45x$	0.962
6.50	$y = 3.98x$	0.98
6.64	$y = 4.417x$	0.98
7.50	$y = 14.15x$	0.991
7.85	$y = 15.5x$	0.994
8.00	$y = 15.99x$	0.992
8.50	$y = 16.5x$	0.989
8.70	$y = 16.88x$	0.999
9.05	$y = 17.07x$	0.997
9.50	$y = 17.75x$	0.995
10.00	$y = 18x$	0.992
10.50	$y = 18.28x$	0.998

A.3 Sample calculation for the estimation of protein

Preparation of standard plot for protein

Stock solution of 0.1 mg ml^{-1} protein (BSA) was prepared in miliQ water. The stock solution was appropriately diluted with same water to get standard solutions of various concentrations of protein (mg ml^{-1}) *viz*., 0.01 to 0.1 of interval 0.01 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 660 nm against the appropriate blank as described for test samples of protein in section 3.6.2. 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 Lowry method as described in section 3.6.2. Then concentration of protein (mg ml^{-1}) in the test sample was calculated with the help of data at OD 660 nm and the slope of standard curve (1 unit OD at 660 nm = 0.434 mg ml^{-1} of protein).

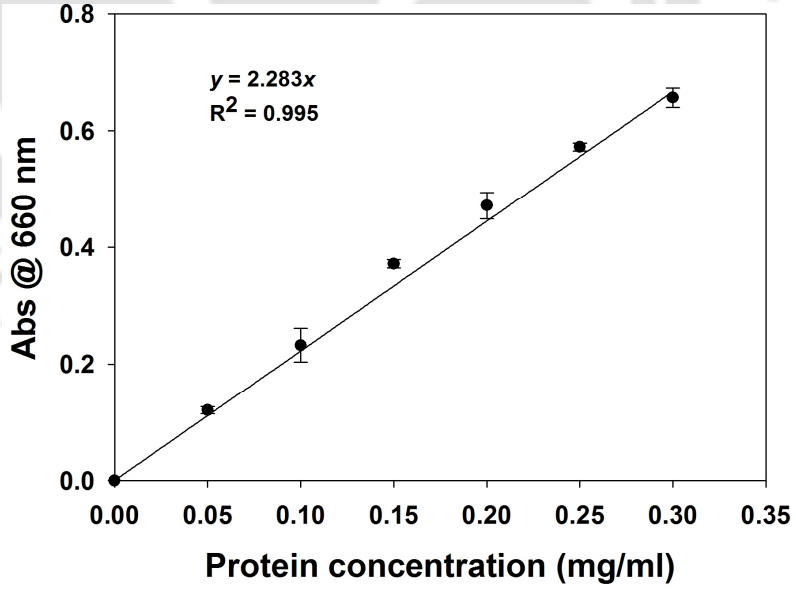

Fig. A.2 Standard curve drawn between known protein concentration and the absorbance measured at 660 nm.

A.4 Sample calculation for the estimation of DCW

Preparation of standard plot for DCW

Experiments were performed for standard curve in triplicates and intracellular protein concentration of the microorganisms grown in glucose containing media was measured using the method described in section 3.7.3. Different dilutions of cell samples were used for measuring intracellular protein and corresponding DCW (g l^{-1}) determined at 105°C for 24 h (Fig. A.3). DCW of the unknown samples were determined by measuring the intracellular protein of the culture and compared with standard curve between protein concentration vs. DCW (dry cell weight (g l^{-1}) = intracellular protein (g l^{-1}) x 19.03).

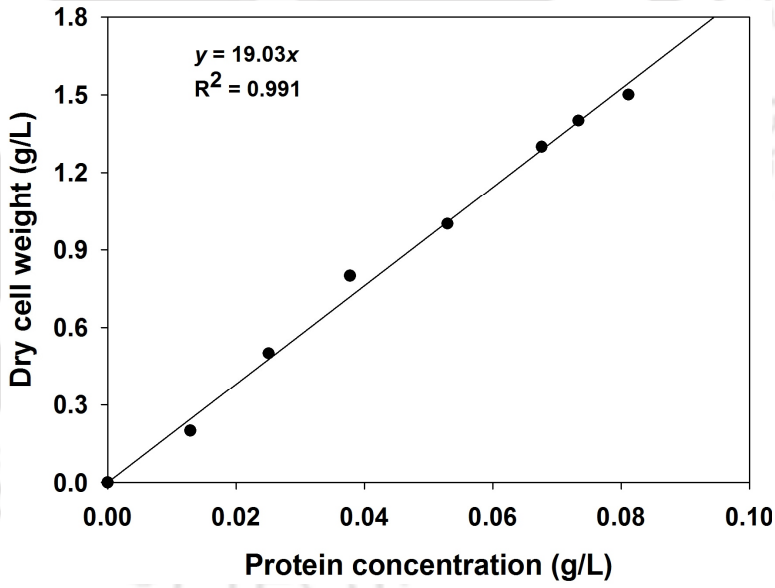

Fig. A.3 Standard curve drawn between cell dry weight of *P. cepacia* NRRL B 2320 and the intracellular protein

A.5 Sample calculation for the estimation of free fatty acid content

Preparation of standard plot for butyric acid

Experiments were performed for standard curve in triplicates for preparation of standard curve of butyric acid. Different concentration of butyric acid (50 to 400 µmoles ml^{-1}) in isooctane was prepared. Now, experiments were performed as the method described in the section 3.7.4. The absorbance of the samples was measured at 715 nm against a blank. Standard curve was prepared (Fig A.4.) using concentration of butyric acid verses OD. Using above method, the standard plot for all other fatty acids used in the experiment was prepared. The standard plot equations and the R^2 values were given in Table A.2.

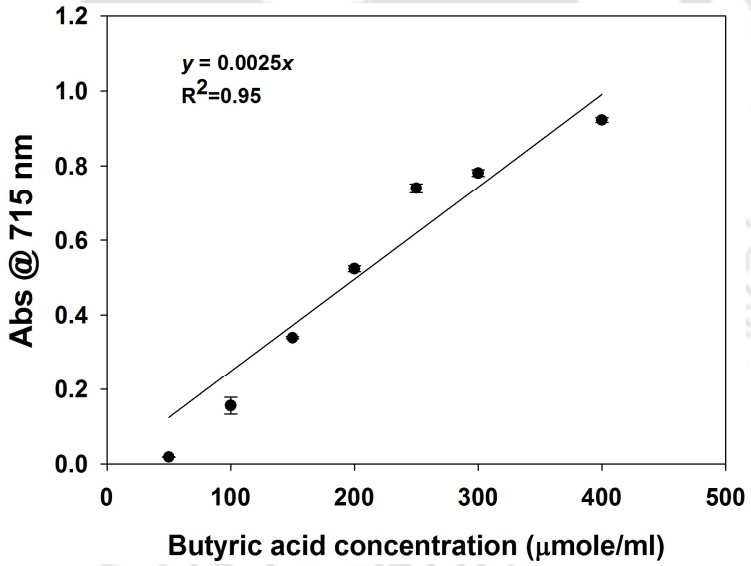

Fig. A.4 Standard curve drawn between known concentration of butyric acid and absorbance measured at 715 nm

Table A.2. Standard plot equations of different fatty acids

Fatty acid (chain length)	Equation	R^2
Valeric acid (C5)	$y= 0.02x$	0.97
Caproic acid (C6)	$y= 0.085x$	0.99
Caprylic acid (C8)	$y = 0.98x$	0.985
Decanoic acid (C10)	$y =0.108x$	0.98
Palmitic acid (C16)	$y = 0.12x$	0.99

A.6 Sample calculation for the estimation of methyl esters by GC

Preparation of standard plot for methyl butyrate

Experiments were performed for standard curve in triplicates and injected the known amount of methyl butyrate into the GC column. The detail method for GC described in the section 3.7.5. The methyl butyrate concentrations in the samples were determined based on a standard curve obtained with methyl butyrate concentration (100-700 µM) *vs*. peak area as standard (Fig A.5).

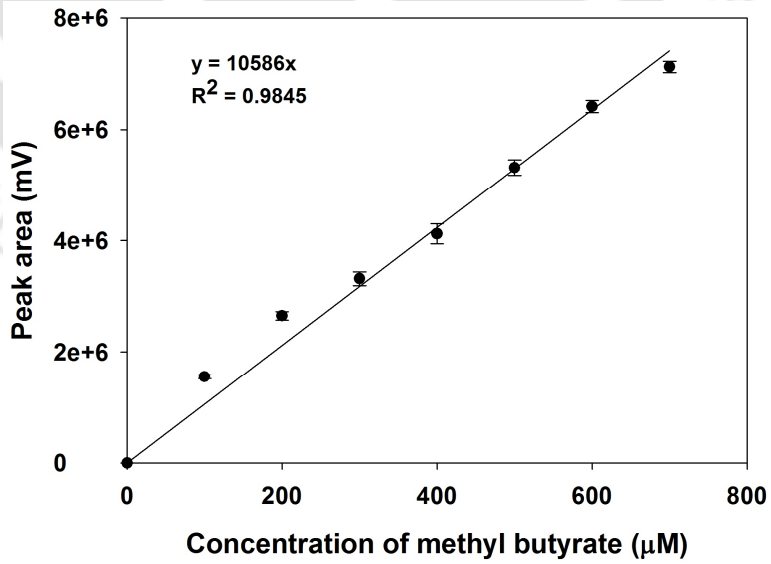

Fig. A.5 Standard curve drawn between known concentration of methyl butyrate and peak area

Methyl butyrate sample was diluted in the range of 100-700 µM in n-hexane. The points were fitted with a linear regression model using Microsoft Excel® software. Using above method the standard plot for all other methyl esters used in the experiment was prepared. The standard plot equations and the R^2 values were given in Table A.3.

Table A.3 Standard plot equations of different fatty acid methyl esters

Methyl esters	**Equation**	**R^2**
Methyl Oleate	$y = 11810x$	0.988
Methyl palmitate	$y = 10101x$	0.996
Methyl linoleate	$y = 9928.3x$	0.988

LIST OF PUBLICATIONS

Published in Referred International Journals

Dutta K, Sen S, Venkata Dasu V. 2009. Production, characterization and applications of microbial cutinases. *Process Biochemistry* **44**: 127-134.

Dutta K, Venkata Dasu V. 2011. Synthesis of short chain alkyl esters using cutinase from *Burkholderia cepacia* NRRL B 2320. *Journal of Molecular Catalysis B: Enzymatic* **72**:150-156.

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Dutta K, Venkata Dasu V. 2011. Development of media for enhanced production of cutinase from *Pseudomonas sp* and its kinetic modelling**.** *Food and Bioprocess Technology* (under review).

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