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Novel cutinase from *Pseudomonas cepacia* NRRL B 2320: Purification, characterization and identification of cutinase encoding genes

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An extracellular cutinase from *Pseudomonas cepacia* NRRL B 2320 was purified to apparent homogeneity. Upon biochemical characterization, the purified cutinase was found to be tolerant to organic solvents and surfactants under assay conditions. The molecular mass of cutinase was found to be 26.25 kDa by MALDI-TOF-MS analysis. The enzyme was able to show activity towards synthetic esters of chain length C₄–C₁₆. The activity of cutinase was enhanced by mono cations and various effectors, whereas it was moderately inhibited by various divalent cations and serine blocking reagent, phenyl methyl sulphonyl fluoride (PMSF). 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 exposure of hydrophobic amino acid to an aqueous environment and change in secondary structure of cutinase was observed from thermodynamic parameters (ΔH^* , ΔS^*), fluorescence and circular dichroism spectra during the deactivation process. Two cutinase encoding genes were identified in *P. cepacia*, cloned and expressed in *E. coli* BL21 (DE3).

Key Words—cloning PMSF; cutinase; kinetic constants; *Pseudomonas cepacia*; purification

Introduction

Cutinases (3.1.1.74) are hydrolytic enzymes, the smallest members of the α/β hydrolase family (Dutta et al., 2009) that degrade cutin. Cutin is the cuticular polymer of higher plants, composed of hydroxy and epoxy fatty acid polyester (Carvalho et al., 1999; Purdy and Kolattukudy, 1975). Cutinase, being a versatile enzyme, has several properties which can be used in various industries. In addition to hydrolysis, it can also

catalyze esterification and transesterification reactions, which makes it a highly potential industrial enzyme. Cutinase has potential use in the dairy industry for hydrolysis of milk fat, house hold detergents, the oleochemical industry, synthesis of structured triglycerides, polymers and surfactants (Dekoster et al., 1995), synthesis of ingredients for personal-care products, synthesis of pharmaceuticals and agrochemicals containing one or more chiral centers (Carvalho et al., 1998; Melo et al., 2003).

Downstream processing of biomolecules obtained from fermentation broths is a fundamental step in process biotechnology, since it often contributes to the major manufacturing cost. The economic viability of a biochemical process depends not only on the improvements achieved in the production but also on innovations and optimization of downstream processes

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(Haki and Rakshit, 2003; Wheelwright, 1987). Chromatographic techniques are widely used as high performance purification steps in biotechnology (Kumar et al., 2011). Therefore, it becomes necessary to develop strategies for the purification of individual enzymes. Source dependent variation in optimum pH, temperature, metal ion requirement, substrate specificity, inhibition pattern and molecular mass of cutinase from a fungal source has been studied (Purdy and Kolattukudy, 1975; Rispoli and Shah, 2007; Soliday and Kolattukudy, 1976; 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., 1992, 1999, 2000; Lin and Kolattukudy, 1980), the enzyme was purified and characterized only from two sources, *Thermobifida fusca* (Chen et al., 2008) and *Pseudomonas putida* (Sebastian and Kolattukudy, 1988).

In our previous study, different microorganisms were screened for selecting high cutinase producers. Among the tested microorganisms, *Pseudomonas cepacia* NRRL B 2320 was found to be the best cutinase producer. In the present study, attempts have been made for the purification, subsequent systematic biochemical and biophysical characterization, identification, cloning and expression of cutinase from *Pseudomonas cepacia*.

Materials and Methods

Bacterial strain, plasmid and growth conditions.

The bacterium used throughout the study, *Pseudomonas cepacia* NRRL B 2320 (also known as *Burkholderia cepacia*), was procured from Agricultural Research Service (ARS-Culture collection), USDA, Peoria, IL. The organism was grown on nutrient agar medium at 28°C. The organism was sub-cultured every month and maintained at $4 \pm 1^\circ\text{C}$. *E. coli* DH5 α was obtained from MTCC, Chandigarh, India. *E. coli* BL21 (DE3) and pET22b (+) vector was procured from Novagen, Inc., USA. Both *E. coli* DH5 α and *E. coli* BL21 (DE3) were grown in Luria-Bertani (LB) medium at 37°C.

Enzyme production. The following medium was used for the production of cutinase (g L^{-1}): beef extract 4.0, peptone 17.77, urea 5.0, KH_2PO_4 3.0, KCl 0.6354, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 5.55 and cutin 10.06. Initial pH of the medium was maintained at 7. A 2% of inoculum from the above seed culture was added to 200 ml of the medium in 1,000 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 $10,000 \times g$ for 10 min at $4 \pm 1^\circ\text{C}$.

Enzyme purification and quantification. All purification steps were carried out at 0–4°C unless otherwise indicated. All chromatographic runs were monitored for protein at 280 nm.

Ammonium sulphate precipitation. Finely powdered ammonium sulfate was added (20–80% saturation) to the clear supernatant obtained after centrifugation with constant stirring and incubated overnight. The precipitate was collected by centrifugation at $10,000 \times g$ 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.

CM cellulose chromatography. The dialyzed ammonium sulphate fraction was applied to a 2 cm $\times$ 50 cm CM-650 TOYOPEARL® column (Tosho Corporation, Tokyo, Japan) pre-equilibrated with potassium phosphate buffer (20 mM, pH 6.5) at a flow rate of 1 ml min^{-1} . Protein was eluted using a linear gradient of NaCl (0–500 mM). Active fractions were pooled, dialyzed and concentrated.

Sephadex G-100 chromatography. The concentrated fractions from the above step were further purified on gel filtration chromatography using Sephadex G-100 pre-equilibrated and eluted with Tris HCl buffer (20 mM, pH 8.0). Active fractions obtained after the gel filtration chromatography were pooled separately, dialyzed and concentrated.

Sephacryl S-300 chromatography. The Sephadex G-100 concentrated fractions were further purified on another gel filtration chromatograph using Sephacryl S-300 pre-equilibrated and eluted with Tris HCl buffer (20 mM, pH 8.0). The active fractions were pooled, concentrated and dialyzed against the Tris HCl buffer (20 mM, pH 8.0). This concentrated fraction was stored at -20°C till further use for characterization.

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

Cutinase assay using *p*-nitrophenyl butyrate (*p*-NPB) as substrate. The activity was measured by following the hydrolysis of *p*-nitrophenyl butyrate (*p*-NPB) (Sig-

ma) as substrate as described by Dutta and Dasu (2011). The reaction was monitored for 15 min at 37°C and absorbance of released *p*-nitrophenol measured at 410 nm. One enzyme unit is defined as the amount of enzyme required to release 1 µmol of *p*-nitrophenol min⁻¹ under assay conditions.

Cutinase assay using *p*-nitrophenyl (16 methyl sulphone ester) hexadecanoate (*p*-NMSH) as substrate. The cutinase production from *P. cepacia* NRRL B 2320 was confirmed using a cutinase-specific substrate, *p*-nitrophenyl (16 methyl sulphone ester) hexadecanoate (*p*-NMSH), which was prepared in our laboratory according to the method described by Degani et al. (2006). Assay was performed using the method described in the enzyme assay, with the change in incubation period for 1 h instead of 5 min at 55°C. One unit of enzyme activity is defined as release of 1 µmol of *p*-nitrophenol per min.

Effect of pH and temperature on activity and stability of purified enzyme. The optimum pH for cutinase activity was determined under the assay condition over a pH range of 5.5–10. For pH stability studies, the enzyme preparations were incubated at pH of 5.5–10 for 24 h at 25°C in the absence of substrate and residual activity was determined. The optimum temperature range for enzyme activity was determined by incubating the assay mixture at different temperatures ranging from 30 to 80°C. The buffers, potassium phosphate (50 mM, pH 5.5–7.5), Tris HCl (50 mM, pH 8.0–9.5) and sodium carbonate/bicarbonate (50 mM, pH 10–11) were used for this study.

Effect of various effectors on cutinase activity. Various effectors like metal ions, organic solvents and surfactants may either positively or negatively influence the cutinase catalyzed reaction considerably (Carvalho et al., 1999; Chen et al., 2010). Moreover, a prior knowledge of the tolerability of organic solvents and surfactants would be adventitious in industrial applications of cutinase like, esterification and transesterification reactions, where organic solvents are preferred over other media for catalysis and use in the detergent industry (Carvalho et al., 1998, 1999; Dutta and Dasu, 2011). The enzyme activity was determined in the presence of different effectors (metal ions, 2-mercaptoethanol, EDTA). The purified enzyme was incubated with various modifiers at 37°C for 5 min, and the residual activities were then measured. In the organic solvent stability test, the enzyme was incubated in 75% of different organic solvents at 30°C for 4 h. In the

surfactant stability test, the enzyme was incubated with 1 mM and 10 mM solution of different surfactants at 37°C for 5 min. The remaining activity was measured with *p*-NPB as the substrate and expressed as relative activity compared to surfactant-free controls.

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

Kinetic analysis of cutinase. Kinetic studies were performed with *p*-NPB (0.05–2 mM) as the 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 was calculated from a double-reciprocal plot ($1/v$ vs. $1/[p\text{-NPB}]$) of the initial rate data. Results are the average of triplicate assays. k_{cat} was calculated on the basis of the molecular mass (26.5 kDa) of the enzyme detected by SDS-PAGE.

Optimization of physical and chemical parameters for cutinase assay. Central composite design (CCD), a statistical design, was used for optimization of reaction parameters. The parameters were divided into two categories, viz. chemical and physical. The chemical parameters considered for optimization were substrate and enzyme volume. On the other hand, pH and temperature, the most influential physical parameters on the enzyme activity were selected.

Fourteen experiments were augmented with six replications at the center points to evaluate the pure error. The quadratic model for predicting the optimal levels was expressed according to Eq. (1).

$$Y = \beta_0 + \sum_{i=1}^k \beta_i X_i + \sum_{i=1}^k \beta_{ii} X_i^2 + \sum_i \sum_j \beta_{ij} X_i X_j \quad (1)$$

where Y is the predicted response, k is the number of factor 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 experimental design, regression analysis of the experimental data, and also to plot the response surface graphs.

Estimation of deactivation rate constant. The following first order expression was used to account for the zero activity at a particular temperature and at a

specified incubation time:

$$\ln \left[\frac{E_t}{E_0} \right] = -k_d t \quad (2)$$

where k_d is the 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 $\ln(E_t/E_0)$ versus t at a particular temperature.

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

$$t_{1/2} = \frac{\ln 2}{k_d} \quad (3)$$

Estimation of thermodynamic parameters for cutinase deactivation. In order to obtain the change in enthalpies (ΔH^*) and change in entropies (ΔS^*) during the enzyme deactivation process, it is necessary to make use of the theory of absolute reaction rates. The deactivation constant is expressed by the following equation:

$$\ln \left(\frac{k_d}{T} \right) = \left(\frac{\kappa}{h} \right) + \left(\frac{\Delta S^*}{R} \right) - \left(\frac{-\Delta H^*}{R} \right) \left(\frac{1}{T} \right) \quad (4)$$

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

$$\Delta G^* = \Delta H^* - T\Delta S^* \quad (5)$$

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

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

$$\ln k_d = \ln k_0 - \left(\frac{E_A}{R} \right) \left(\frac{1}{T} \right) \quad (6)$$

where E_A is activation energy (J mol^{-1}) and k_0 is the 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.

Fluorescence and CD spectroscopy. The enzyme

(0.07 mg ml^{-1}) at different pH values (6.0 to 9.0) was incubated at different temperatures (40°C , 50°C , 60°C and 70°C) for 15 min. The change in environment of tryptophan residue present in the protein and secondary structures was analyzed using fluorescence and CD spectroscopy, respectively. The excitation was done at 295 nm and emission was measured from 315 to 450 nm. The CD spectra of the above samples were obtained from 190 to 250 nm.

Identification, cloning and expression of cutinase-encoding genes. Since, there is not sufficient genome sequence information available on *P. cepacia*, we developed a phylogenetic relationship (Fig. 1) of the organisms producing cutinase based on the available literature reports and cutinase sequences in the database. The cutinase-producing bacteria showing the nearest phylogenetic relationship with *P. cepacia* NRRL B 2320 was selected for designing primers for amplification of the gene. Two sets of primers, Set 1 and Set 2, respectively, for NCBI Gene YP_288943 and YP_288944 were designed for the available cutinase genes of *Thermobifida* sp. (Accession no. CP000088.1). The first set of primers was used for amplification of gene with the forward primer 5'-GGAATTCGGATC CAATGCCCCCGCATGCGGCGCG-3' (*Bam*HI sites underlined) and reverse primer 5'-GAAGCTTCTC GAGGAAGGGGCAGGTGGAGCG-3' (*Hind*III sites un-

Fig. 1. Construction of a phylogenetic tree based on 16S/18S (Prokaryotes and Eukaryotes, respectively) rRNA of various cutinase-producing organisms reported in the literature.

T. fusca (circle) used for designing primers and *P. cepacia* (or *B. cepacia*) (circle) used as the source of the organism for PCR amplification of cutinase genes.

derlined) and the second set of primer consisted of forward primer 5'-GGAATTCGGATCCAATGGCTGTGATGACCCCCCG-3' (*Bam*HI sites underlined) and reverse primer 5'-GAAGCTTCTCGAGGAACGGGCAGGTGGAGC-3' (*Hind*III sites underlined). Genomic DNA of *P. cepacia* NRRL B 2320 was prepared using the Sigma genomic DNA prep kit following the manufacturer's instructions. High fidelity *taq* polymerase (Phusion *taq* from NEB, US) was used for amplification of genes. The PCR amplicon was purified with a PCR purification kit (HiMedia, India) following the manufacturer's instructions and double digested with *Bam*HI and *Xho*I, directionally cloned into pET22b (+) and transformed in to *E. coli* DH5 α . Gene sequences of the clones were confirmed by sequencing and transformed into expression host *E. coli* BL21 (DE3) (Stratagene). Both the recombinant genes were expressed in TB medium at 37°C with 250 rpm shaking by inducing the culture at cell density $\sim A_{600nm}$ 0.75 with 0.6 mM IPTG. After 6 h incubation, 5 ml culture was pelleted and suspended in 500 μ l of potassium phosphate buffer (50 mM, pH 8), sonicated for 3 min, and centrifuged and the supernatant was used to measure cutinase activity and SDS-PAGE analysis.

Results and Discussion

Purification of cutinase

The production of cutinase was carried out according to the method described in MATERIALS AND METHODS. The activity of cutinase was found to be 336 U ml⁻¹ towards *p*-NPB and 3.12 U ml⁻¹ towards the cutinase-specific substrate *p*-NMSH. The crude extracellular protein was precipitated by the addition of ammonium sulphate (80% saturation). The precipitated enzyme was dissolved in minimal volume of Tris HCl buffer (20 mM, pH 8.0) followed by dialysis. The result of purification of cutinase from *P. cepacia* NRRL B 2320 is summarized in Table 1. The purification of cutinase in-

volved using ammonium sulphate precipitation followed by cation exchange chromatography using a CM cellulose column and followed by two gel filtration steps (Sephadex G-100 and Sephacryl S-300) (Fig. 2a).

The retention of cutinase on a CM cellulose column is dependent on the pH of the buffer used. The adsorbed cutinase from the CM cellulose column was eluted with a gradient of 0–500 mM NaCl. The enzyme was purified approximately 89.84-fold with an overall yield and specific activity of 17.56% and 5,359.15 U mg⁻¹, respectively. The purified cutinase was found to be homogeneous as evident from SDS-PAGE (Fig. 2a). The molecular mass of the enzyme was observed to be 26.5 kDa from SDS-PAGE and 26.25 kDa from MALDI-TOF-MS (Fig. 2b). It was also observed that cutinase was a monomeric protein from the MALDI-TOF-MS. Cutinase from *P. cepacia* NRRL B 2320 was successfully purified to homogeneity using Sephacryl S-300 chromatography with a 17.56% yield, which is the first purification report on cutinase from *P. cepacia*. Sebastian and Kolattukudy (1988) reported that the molecular mass of bacterial cutinase lies between the molecular mass of fungal cutinase (22 kDa) and pollen cutinase (40 kDa).

Characterization of cutinase

Effect of different effectors. To determine the influence of metal cofactors on the activity, cutinase was incubated with metal chelator EDTA or metal ions and then assayed for activity against *p*-NPB. One millimolar of divalent metal ions, Mn, Ni, or Ca did not exhibit significant impact on the enzyme activity, whereas, Zn, Fe, or Cu have shown an inhibitory effect. Some of the divalent metal ions (Mg²⁺, Ca²⁺ and Co²⁺) and monovalent metal ions (K⁺ and Na⁺) were found to enhance the enzyme activity at 1 mM concentration. The inhibitory effect of Zn²⁺ and Hg²⁺ suggests that a sulphhydryl group may be present in the vicinity of active

Table 1. Summary of steps employed in purification of cutinase from *P. cepacia* NRRL B 2320.

Purification step	Enzyme activity (U)	Protein (mg)	Specific activity (U mg ⁻¹)	Fold	%yield
Crude	191,300	3,207.58	59.64	—	100.00
(NH ₄) ₂ SO ₄ precipitation	121,833.34	219.35	555.42	9.31	63.69
CM-cellulose column	75,454.55	57.90	1,303.14	21.85	39.44
Sephadex G-100	55,274.24	17.75	3,114.50	52.21	28.89
Sephacryl S-300	33,592.28	6.27	5,359.15	89.84	17.56

Fig. 2. (a) SDS-PAGE of cutinase from *P. cepacia* NRRL B 2320 at different stages of purification. Lane M: Protein molecular marker; Lane 1: Ammonium sulphate precipitated fraction; Lane 2: CM cellulose fraction; Lane 3: Sephadex G-100 fraction; Lane 4 and 5: Sephacryl S-300 fraction. (b) MALDI-TOF-MS analysis of purified cutinase.

site of the enzyme. EDTA (1 mM or 10 mM) did not affect the activity of cutinase (Table 2), suggesting that divalent ions are not essential for cutinase activity. The activity of the cutinase was tested in the presence of the nonionic surfactants Triton X-100 and Tween 80 and the anionic surfactants SDS and sodium deoxycholate (Table 2). Triton X-100 and Tween 80 did not affect the enzyme activity at a concentration of 1 mM or 10 mM in the reaction mixture, whereas sodium deoxycholate slightly enhanced the cutinase activity at both 1 and 10 mM. In the presence of 1 mM or 10 mM of SDS, the activity of cutinase was decreased. A similar observation was also reported by Chen et al. (2010) for cutinase from *Thermobifida fusca*. The high stability in surfactants reveals that the enzyme is more suitable for any kind of detergent formulations. The stability of the enzyme in organic solvents makes the enzyme more attractive for synthetic (esterification and transesterification) reactions. Generally, enzyme catalyzed esterification reaction is used between fatty acid and alcohol to synthesize alkyl esters, but this reaction is reversible. So, at higher water concentration, the equilibrium for the enzyme-catalyzed reaction shifts to the reverse direction forming again the initial reactants. To use the enzyme for the synthesis of esters, it must have stability at minimum water concentration and with organic solvents (de Barros et al., 2009; Dutta and Dasu, 2011). The cutinase from *P. cepacia* has shown

better stability in the solvents, viz. butanol, isooctane, chloroform, *n*-hexane and isoamyl alcohol (Table 2), than any previously reported cutinase (Chen et al., 2010). Thus the enzyme purified from *P. cepacia* has potential to be used in several industries. The inhibition of the cutinase by PMSF confirms that cutinase has a serine group in its active site and thereby supports the fact that cutinase from *P. cepacia* also under the same serine hydrolase group.

Substrate specificity. The specificity of the enzyme towards various chain length esters was investigated using *p*-nitrophenyl-fatty acyl esters (Table 3). The enzyme showed maximum specificity towards short-chain fatty acid esters. The hydrolytic activity was the maximum in the case of esters of C₄–C₆ fatty acid. Further increase in the chain length of fatty acid causes a decrease in activity. The enzyme showed significantly higher activity towards *p*-nitrophenyl butyrate than *p*-nitrophenyl palmitate.

Kinetic analysis. The kinetic parameters for the cutinase were determined for the commonly used substrate, *p*-NPB. The values of K_m and V_{max} of cutinase were determined by steady state kinetic analysis (Fig. 3). Plots of the reaction velocities versus substrate concentration (0–2.0 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 cutin-

Table 2. Effect of different metal ions and effectors on cutinase activity.

Metal ions or effectors	Concentration (mM)	Relative activity (%)
Control	—	100 ± 0.50
KCl	1	106.56 ± 1.67
	10	105.92 ± 1.49
NaCl	1	138.01 ± 1.09
	10	104.79 ± 0.50
MgCl ₂	1	120.50 ± 1.88
	10	108.51 ± .86
CaCl ₂	1	122.71 ± 2.76
	10	66.44 ± 0.52
CoCl ₂	1	133.44 ± 1.53
	10	41.98 ± 2.45
MnCl ₂	1	97.08 ± 1.15
	10	44.67 ± 2.03
NiCl ₂	1	99.63 ± 3.28
	10	25.03 ± 1.39
HgCl ₂	1	N.D.
	10	N.D.
ZnSO ₄	1	82.15 ± 3.56
	10	23.57 ± 2.36
FeSO ₄	1	80.22 ± 0.98
	10	20.97 ± 0.53
CuSO ₄	1	15.41 ± 0.71
	10	4.46 ± 1.60
EDTA	1	108.07 ± 1.76
	10	105.93 ± 1.09
β-Mercaptoethanol	1	94 ± 0.25
	10	91.72 ± 0.54
PMSF	1	5.03 ± 1.84
SDS	1	89.76 ± 0.88
	10	71.06 ± 1.88
Tween 80	1	99.14 ± 0.21
	10	97.80 ± 0.33
Triton X-100	1	96.11 ± 0.71
	10	93.47 ± 0.16
Sodium deoxycholate	1	107.50 ± 0.08
	10	106.67 ± 0.67
Methanol	75%	9.65 ± 0.025
Ethanol	75%	2.66 ± 0.068
Isopropanol	75%	N.D.
Butanol	75%	103.70 ± 1.36
Dioxan	75%	77.44 ± 0.83
Isoamyl alcohol	75%	96.42 ± 2.38
DMSO	75%	50.90 ± 1.92
n-Hexane	75%	81.55 ± 2.29
Acetone	75%	8.10 ± 0.05
DCM	75%	22.19 ± 0.911
Chloroform	75%	100 ± 1.9
Isooctane	75%	96.5 ± 1.41

Table 3. Specificity of the cutinases towards the acyl chain length of different esters.

Substrate	% Activity
p-Nitrophenyl butyrate (C4)	100 ± 2
p-Nitrophenyl valerate (C5)	96.25 ± 1.5
p-Nitrophenyl caprylate (C8)	85.36 ± 2.1
p-Nitrophenyl laurate (C12)	81.41 ± 1.56
p-Nitrophenyl palmitate (C16)	50.13 ± 1.88

Fig. 3. 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 the cutinase-catalyzed reaction.

ase were found to be 0.23 mM, $7.955 \text{ U } \mu\text{g}^{-1}$ and $5 \times 10^3 \text{ s}^{-1}$, respectively. The K_m for other cutinases from *T. fusca* and *F. solani pisi* was reported to be 0.673 and 0.272 (Chen et al., 2010). Previously, Sebastian and Kolattukudy (1988) also reported a K_m of 0.27 mM for *P. putida* cutinase. Substrate specificity of the cutinase from *P. cepacia* was similar to that of *P. putida* and higher than that of *T. fusca*.

Effect of pH and temperature on activity and pH stability of purified enzyme. 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. 4a). Maximum cutinase activity was obtained at pH 8.0 and 40°C, and decreased significantly (50%) when the pH was decreased to 6.5 (Fig. 4a and b). The enzyme showed stability at alkaline pH (pH 8.0–10.0) as it retained 90% of its original activity after incubation for 24 h (Fig. 4). The purified cutinase exhibited maximum activity at a temperature of 37°C. The activity de-

Fig. 4. Effect of (a) pH on enzyme activity (●), residual activity (○) and (b) temperature on enzyme activity of cutinase purified from *P. cepacia* NRRL B 2320.

creased sharply above and below the optimum temperature range (35–45°C) with almost 65% loss of its original activity at 60°C (Fig. 4b). No significant enzyme activity was lost when the purified enzyme was pre-incubated at 40°C for 60 min; beyond this temperature the enzyme became increasingly unstable.

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

The experimental design matrix and the corresponding results of CCD experiments to determine the effects of enzyme and substrate volume are shown in Table 4, along with the mean predicted values. Using the experimental results of CCD, a regression model equation was developed for predicting the maximum cutinase activity given by Eq. (7).

$$Y_{\text{EnzymeActivity}} = -70786.1 + 144.2X_1 + 605.1X_2 - 0.1X_1^2 - 5.2X_2^2 - 0.4X_1X_2 \quad (7)$$

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

According to the analysis of variance (ANOVA) of the quadratic regression model (data not shown) the model is highly significant, as is manifested from the Fisher F test (mean square regression: mean square residual is 79.72) with a very low probability value ($P_{\text{model}} > F$ is 0.0001). The goodness of the model as checked by the coefficient of determination, R^2 , implies that the sample variation of 98.28% for cutinase activity is attributed to the chemical parameters and

that only 1.72% of the total variation is not explained by the model.

The significant interaction between the two variables was observed from the contour plot in Fig. 5a. The optimum levels of substrate and enzyme volumes were found to be 971.4 μl and 24.2 μl (1.39 μg), respectively. These conditions have shown higher enzyme activity (6,680.56 U ml^{-1}) as compared to that used presently for the analysis of enzyme activity. After optimization, cutinase activity was increased by 500 U ml^{-1} in the purified sample.

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

Each enzyme has a characteristic pH and temperature optima beyond which the reduction in activity is observed and, therefore, the effect of these parameters on the performance of cutinase was studied using a central composite design. The design matrix and corresponding results of observed and predicted enzyme activity at each combination are given in Table 5. Using the experimental results of CCD, a regression model equation was developed to predict the maximum cutinase activity given by Eq. (8).

$$Y_{\text{EnzymeActivity}} = -329957 + 70800X_1 + 3231X_2 - 389X_1^2 - 17X_2^2 - 250X_1X_2 \quad (8)$$

where X_1 = pH and X_2 = temperature.

The results were analyzed using ANOVA as appropriate to the experimental design used (data not shown). According to the ANOVA of the quadratic re-

Table 4. 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 volume.

Run order	Experimental values						Enzyme activity (U ml ⁻¹)	
	Amount of substrate			Enzyme used			Observed ^a	Predicted
	X ₁		X ₂					
	Coded value	Actual value		Coded value	Actual value			
		μl	μg		μl	μg		
1	−1	860	67.0	−1	12	0.691	4,393.03 ± 10	4,463.67
2	+1	1,100	85.69	−1	12	0.69	5,433.63 ± 4	5,277.11
3	−1	860	67.0	+1	30	1.72	5,589.59 ± 15	5,810.23
4	+1	1,100	85.69	+1	30	1.72	5,063.13 ± 16	5,056.60
5	0	980	76.34	0	21	1.21	6,623.33 ± 21	6,575.27
6	0	980	76.34	0	21	1.21	6,620.11 ± 8	6,575.27
7	0	980	76.34	0	21	1.216	6,610.55 ± 6	6,575.27
8	−1.414	810.3	63.11	0	21	1.21	4,836.07 ± 18	4,643.39
9	+1.414	1,149.7	89.56	0	21	1.216	4,557.11 ± 12	4,685.69
10	0	980	76.34	+1.414	8.3	0.47	5,355.26 ± 9	5,429.28
11	0	980	76.34	−1.414	33.7	1.94	6,363.62 ± 14	6,225.50
12	0	980	76.34	0	21	1.21	6,630.33 ± 17	6,669.33
13	0	980	76.34	0	21	1.21	6,615 ± 8	6,669.33
14	0	980	76.34	0	21	1.21	6,634.44 ± 5	6,669.33

^aThe observed values of cutinase activity were the mean values of duplicates with standard deviation (Mean $\pm$ S.D.).

Fig. 5. 2D contour plot of cutinase activity at different levels of (a) substrate and enzyme volume (b) pH and temperature under assay condition.

gression model, the model is highly significant, as is evident from the Fisher F test (65.42) with a very low probability value (0.0001). This indicates that the combined effects of pH and temperature significantly contributed to maximizing the cutinase activity and the R^2 (97.95%) value confirmed the good fit of the model.

The elliptical shape of the 2D contour plot in Fig. 5b suggests the high significance of the interaction among the two parameters. Cutinase activity of

8,835.50 U ml^{-1} was observed at optimum levels of pH (7.9) and temperature (36.4°C). After optimization of process parameters, cutinase activity was increased by 1.3-fold in the purified sample. An overall 1.42-fold increase in activity was observed after optimizing both the assay and process conditions.

Enzyme deactivation studies

The deactivation rate is proportional to the active en-

Table 5. 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	6,680.46 ± 18	6,121.40
2	+1	8.7	-1	37	7,150 ± 15	6,723.10
3	-1	7	+1	48	6,318.18 ± 20	6,254.19
4	+1	8.7	+1	48	2,104.17 ± 8	2,118.90
5	0	7.85	0	42.5	8,309.68 ± 14	8,633.35
6	0	7.85	0	42.5	8,225.81 ± 25	8,633.35
7	0	7.85	0	42.5	8,382.80 ± 10	8,633.35
8	-1.414	6.64	0	42.5	3,151.51 ± 5.6	3,452.62
9	+1.414	9.05	0	42.5	850.89 ± 8.6	1,040.66
10	0	7.85	+1.414	34.7	7,813.98 ± 28	8,371.7
11	0	7.85	-1.414	50.2	5,363.44 ± 26	5,296.60
12	0	7.85	0	42.5	8,225.81 ± 31	7,899.99
13	0	7.85	0	42.5	8,227.96 ± 15	7,899.99
14	0	7.85	0	42.5	8,227.96 ± 18.5	7,899.99

^aThe observed values of cutinase activity were the mean values of duplicates with standard deviation (Mean ± S.D.).

Table 6. Effect of temperature at different pH on the deactivation constant (k_d) and half life time ($t_{1/2}$) of the purified enzyme and estimation of thermodynamic parameters during cutinase deactivation.

pH	Temp. (°C)	k_d (h ⁻¹)	$t_{1/2}$ (h)	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	40	0.024	28.63	183.26	244.04	185.95	3.65×10^{29}	106.88-102.00
	45	0.112	6.18					
	50	0.5871	1.18					
	55	0.8401	0.824					
	60	1.85	0.372					
7.0	40	0.024	28.87	298.18	610.65	300.86	5.34×10^{48}	107.04-94.83
	45	0.282	2.45					
	50	1.22	0.547					
	55	7.704	0.089					
	60	26.63	0.026					
8.0	40	0.03	23.1	278.66	558.62	281.34	1×10^{46}	103.82-92.64
	45	1.74	0.398					
	50	8.59	0.080					
	55	18.61	0.037					
	60	28.91	0.024					
9.0	40	0.6026	1.15	199.87	321.05	202.53	3.85×10^{33}	99.38-94.56
	45	1.79	0.385					
	50	9.32	0.074					
	55	18.35	0.037					

R^2 of plot of $\ln(E_d/E)$ versus t is 0.909.

zyme concentration. The deactivation process was modeled as first-order kinetics and the deactivation rate constant was evaluated. The minimum value of k_d observed for cutinase was 0.024 h^{-1} (Table 6). The above-mentioned minimum deactivation constant was found at 40°C with pH 6 as well as pH 7. The deactivation process was found to be faster at higher pH (pH 8, pH 9) than lower pH (pH 6, pH 7) for cutinase. These results demonstrate that the enzyme is more stable at pH 6 and pH 7 at higher temperatures ($>40^\circ\text{C}$) although at 40°C the enzyme is stable for more than 20 h at pH 8 and 1 h at pH 9.

Estimation of thermodynamic parameters

The change in enthalpy and entropy was calculated by transition state theory (Eq. 4). The results are shown in Table 6 for purified cutinase from *P. cepacia* NRRL B 2320. Solvent and structural effects are the two major factors which influence the numerical values of ΔH^* and ΔS^* . An increase in entropy and enthalpy values was observed when pH increased from 6 to 7. Beyond pH 7, there is a decrease in both the entropy and enthalpy. The values of ΔG^* (calculated from Eq. 5) are given in Table 6 for the enzyme. The decrease in entropy and enthalpy values was observed with an increase in pH. Probably, at higher pH, the stable three-dimensional structure of the active site of the enzyme gets altered, resulting in a decrease in residual activity. Positive values of ΔH^* would be expected 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. The positive value of ΔS^* indicates that the protein solution became more disordered as it was 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 temperature dependency of the first-order deactivation rate constant was studied by the Arrhenius equation (Eq. 6). The activation energy (E_A) and frequency factor (k_0) were estimated from that equation and they are shown in Table 6. It was found that the maximum activation energy at optimum pH and further increase in pH, results in a decrease of activation energy.

The deactivation energy increased at higher temperatures suggesting that cutinase requires more energy to deactivate. This is in agreement with the result

that the cutinase is more stable at pH 6 and pH 7 at 40°C rather than pH 8 and pH 9.

Conformational change of cutinase during thermal deactivation

Tryptophan proved to be an important intrinsic fluorescent probe (amino acid), which is used to estimate the nature of the microenvironment of the residue. From the fluorescence spectra (Fig. 6) a red shift of $\lambda_{\text{max}} \approx 10 \text{ nm}$, and decrease in intensity was observed with an increase in temperature for all combinations of pH levels. At room temperature and 40°C there was no change in spectra, which suggests that the protein retains its native form at these temperatures, whereas the further increase in temperature results in a gradual decrease in intensity and red shift in wavelength.

The disorderedness of deactivated protein at higher temperatures was further confirmed by CD spectral analysis (Fig. 7). From the CD spectra, it was observed that there was an increase in ellipticity θ (mdeg) from 208 to 220 nm as the temperature increased from room temperature to 70°C . This increase in ellipticity value shows that there was loss of α -helices (208 nm and 220 nm) and β -sheet structures (218 nm) at higher temperatures. The minimum ellipticity was observed at room temperature and the change in ellipticity was much less when the temperature increased up to 50°C . However, at 60°C and 70°C the prominent increase in ellipticity was observed. This observation suggests that the enzyme retains its secondary structure at 40°C and 50°C but loses the same when incubated at 60°C and above. Moreover, there was a decrease in ellipticity with increasing temperature near 200 nm, which reveals that the rise in temperature results in disorderedness of the protein. This trend is similar for all four pH levels. Previously, Melo et al. (2001) had also observed the loss of α -helix structure upon unfolding of cutinase at higher temperatures and in the presence of guanidine hydrochloride.

Identification, cloning and expression of cutinase encoding genes

PCR with both Set 1 and Set 2 of primers showed $\sim 1 \text{ kb}$ amplification (Fig. 8) and the BLASTn result of the sequence of the genes with Set 1 (corresponding to 960 nucleotides) and Set 2 (corresponding to 906 nucleotides) primers showed complete homology with Gene YP_288943 and YP_288944 of *Thermobifida fusca* cutinase, respectively. The similarity in the cutin-

Fig. 6. The fluorescence spectra of the 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 temperatures (— RT, ... 40°C , --- 50°C , — · — 60°C , — — 70°C) for 15 min. The excitation was done at 295 nm and emission was measured from 315 nm to 450 nm.

Fig. 7. The CD spectra of the cutinase enzyme at different pH and temperature.

0.07 mg ml^{-1} protein was heated at different pH values (6.0 to 9.0) at different temperatures (— RT, ... 40°C , --- 50°C , — · — 60°C , — — 70°C) for 15 min. The CD spectrum was obtained from 190 to 250 nm.

Fig. 8. PCR amplification of genomic DNA of *P. cepacia* with Set1 primers (Lane2) and Set 2 primers (Lane1) M- 1 kb marker, 1- *cut_2*, 2- *cut_1*.

Fig. 9. SDS-PAGE (12%) showing recombinant cutinase expressed at the 6th h.

(a) Lane M: marker, 1: *E. coli* BL21 (DE3) without vector, 2: *E. coli* BL21 (DE3) with pET22b (+), 3: un-induced *E. coli* BL21 (DE3) with *cut_1* constructs 4: *E. coli* BL21 (DE3) expressing *cut_1* protein. (b) Lane M: marker, 1: *E. coli* BL21 (DE3) without vector, 2: *E. coli* BL21 (DE3) with pET22b (+), 3: un-induced *E. coli* BL21 (DE3) with *cut_2* constructs, 4: *E. coli* BL21 (DE3) expressing *cut_2* protein.

ase genes in two different bacteria may be due to horizontal gene transfer (Furushita et al., 2003). Previously Furushita et al. (2003) also reported 100% sequence similarity of a tetracyclin resistance gene between one fish farm B strain and *Aeromonas salmonicida*, *Salmonella enterica* serovar Typhimurium. SDS-PAGE showed the induced cutinase band near 29 kDa (Fig. 9a and b). The difference in the size of wild-type cutinase (26.2 kDa) and the recombinant cutinase (~29 kDa) would be due to the presence of a native signal peptide (59 amino acids and 41 amino acids for *cut_1* and *cut_2*, respectively) in the recombinant cutinase. As depicted in Fig. 10, the activity (towards *p*-NPB) in wild *P. cepacia* was 190 U ml⁻¹ which is similar to the cutin-

Fig. 10. Comparison of wild-type cutinase production in *P. cepacia* with the expression of recombinant cutinases in *E. coli* BL21 (DE3).

ase expression by each of the two genes individually, and the expression of both recombinant cutinases (*cut_1* and *cut_2*) (387.46 U ml⁻¹) was increased 2-fold to that of the cutinase from wild *P. cepacia* (190 U ml⁻¹).

Conclusion

A novel cutinase from *P. cepacia* with high solvent and surfactant tolerance was purified to homogeneity with 89.84-fold purification and 17.56% of yield. The molecular size of the purified protein was found to be 26.25 kDa by MALDI-TOF-MS analysis. The cutinase was found to be stable in a broad range of pH (6.5–9.5) and temperature (35–60°C). The activity of cutinase was increased by 1.42-fold under optimal assay and process conditions. The estimated thermodynamic parameters, the red shift observed in fluorescence spectra, and the change in ellipticity in CD spectra suggest that the deactivation of the enzyme is due to a change in the secondary structure of the enzyme. Cutinase-encoding genes were identified, cloned and expressed in *E. coli* BL21 (DE3). The identified genes showed similarity to bacterial cutinase.

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