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A thermoalkaliphilic halotolerant esterase from *Rhodococcus* sp. LKE-028 (MTCC 5562): Enzyme purification and characterization

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ABSTRACT

A newly isolated *Rhodococcus* sp. LKE-028 (MTCC 5562) from soil samples of *Gangotri* region of Uttarakhand Himalayan produced a thermostable esterase. The enzyme was purified to homogeneity with purification fold 62.8 and specific activity 861.2 U mg^{-1} proteins along with 26.7% recovery. Molecular mass of the purified enzyme was 38 kDa and values of K_m and V_{max} were 525 nM and 1666.7 U mg^{-1} proteins, respectively. The esterase was active over a broad range of temperature (40–100 °C) and pH (7.0–12.0). The esterase was most active at pH 11.0. The optimum temperature of enzyme activity was 70 °C and the enzyme was completely stable after 3 h pre-incubation at 60 °C. Metal ions like Ca^{2+}, Mg^{2+} and Co^{2+} stimulated enzyme activities. Purified esterase remarkably retained its activity with 10 M NaCl. Enzyme activity was slightly increased in presence of non-polar detergents (Tween 20, Tween 80 and Triton X 100), and compatible with oxidizing agents (H_2O_2) and reducing agents (β-mercaptoethanol). Activities of the enzyme was stimulated in presence of organic solvents like DMSO, benzene, toluene, methanol, ethyl alcohol, acetone, isoamyl alcohol after 10 days long incubation. The enzyme retained over 75% activity in presence of proteinase K. Besides hyperthermostability and halotolerancy the novelty of this enzyme is its resistance against protease.

1. Introduction

Enzymes expressed by extremophiles offer new opportunities where biocatalysis and biotransformations play a pivotal role. Major road block of industrial usage of known esterases is their limited thermal and pH stability in various industrial processes [1].

Microbial esterases have attracted considerable attention because of its current applications in food, pharmaceutical, and chemical areas [2]. Useful reactions performed by esterases include resolution of racemic mixtures by transesterification, initiation and regioselective hydrolysis, synthesis of natural and non-natural pro-drugs, non-polar detergents, polyesters, and additives [3]. Generally, most of these reactions occur in non-aqueous environments. Esterolytic enzymes have been reported in various extremophiles including members of the order *Thermoplasmatales* and *Sulfolobales* [4–6]. In recent years, scientific and industrial significance of these extremophiles has increased intensely due to functional and structural stability of their proteins [7]. Esterases are most widely used biocatalysts in fine chemical applications, mainly because they could be applied efficiently in production of optically pure compounds. Compared to other enzymes, industrial application of esterases is relatively mature and there is an increasing interest in high throughput tools for discovery and characterization of these enzymes [8]. Despite growing interest in thermophiles and their biocatalysts, only a limited number of esterases have been characterized from thermophilic Archaea and Bacteria [5,6].

Therefore, hunt for new microbial esterase is important for the development of thermostable enzymes and its applications. Enzyme resistance to denaturation in organic solvents is correlated with their extremophilic characteristics such as high temperature and salt tolerance (high level of toxic compounds tolerance) as well as resistance to protease [2]. Esterases have a wide range of applications of which few employ in combination with amylase and protease. Nevertheless, there are few documents on proteolysis-resistant lipases. Zhang et al. [9] and Dutta and Ray [10] have produced lipases from *Streptomyces fradiae* and *Bacillus cereus* respectively which were resistant to commercial neutral and alkaline proteases. Lipases produced by *Pseudomonas aeruginosa*, *Bacillus pumilus* and *Bacillus licheniformis* were found to be resistant to co-produced native proteases [11–13]. Rees et al. [14] detected esterase enzyme activities encoded by novel genes present in

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environmental DNA libraries. Still not many reports on protease resistance esterases have been found.

In this paper, we are reporting purification and characterization of an organic solvent and protease stable thermoalkaliphilic halotolerant esterase from thermophilic *Rhodococcus* sp. LKE-028 isolated from Gangotri region of *Uttarakhand Himalaya*.

2. Experimental methodology

2.1. Microorganism: culture conditions and identification

Bacterial pure cultures were isolated from treated soil collected around *Gangotri* region (average height 10,000 ft) of *Uttarakhand Himalaya* (India). Soil sample (physical–chemical properties during collection; temperature 6 °C, pH 8.5, moisture content 25%, w/v) were dried at 80 °C for 72 h before inoculating in sterile distilled water (5%, w/v). Soil free aqueous phase was inoculated into nutrient broth (NB) supplied with glucose containing (composition in g L^{-1}): peptone, 5.0; yeast extract, 3.0; NaCl, 5.0; glucose, 10.0; pH 7.0 after proper serial dilution, and incubated at 60 °C for 5 days. Randomly selected colonies were screened for esterolytic activity on tributyrin agar plate containing 1.5% tributyrin in solidified nutrient agar medium at 60 °C for 3 days. Further 4 days incubation at 40 °C was continued to achieve bright clear zone on plate [9]. On the basis of plate assay, one isolated strain designated as LKE-028 was selected for further experiments. Master culture was preserved in 40% (v/v) glycerol at −80 °C.

Isolated strain LKE-028 was identified by partial 16S rDNA gene sequencing. Extraction and purification of DNA were carried out by GenElute™ bacterial genomic DNA kit (Sigma) following the manufacturer's instructions. Primers selected for PCR amplification experiments were, 5′-CAGGCCTAACACATGCAAGTC (forward primer) and 5′-GGGCGGWGTGTACAAGGC (reverse primer). Amplified DNA was purified by GenElute™ bacterial genomic DNA kit (Sigma). 16S rDNA sequence was aligned with submitted sequences available in NCBI database using clustal W software, and phylogenetic position of the isolate was depicted using TREEVIEW program (3.0).

2.2. Enzyme production and quantification

Isolate LKE-028 was cultured in shake flasks containing modified NB (composition same as Section 2.1) at 60 °C with constant shaking at 140 rpm. Culture supernatant was collected after 24 h culture by centrifugation at 10×10^3 r.c.f. for 5 min to recover extracellular enzyme. Esterase activity was determined spectrophotometrically using *p*-nitrophenyl acetate (*p*NP acetate) as substrate as described by Sana et al. [15] with some modification. 250 μL culture supernatant containing enzyme was incubated with 50 μL of 2 mM *p*NP acetate and 50 mM 200 μL McIlvaine buffer (pH 7.2) at 60 °C for 30 min. Reaction was terminated immediately by adding 500 μL of chilled buffer and suspended particles were removed by centrifuging at 10×10^3 r.c.f. for 5 min. Amount of *p*NP acetate released by esterase catalyzed hydrolysis was measured at λ_{410} nm against enzyme free blank. One unit of esterase activity was defined as the amount of enzyme needed to liberate 1 μM *p*NP acetate/min under this condition [15]. Total protein was measured by method described by Lowry et al. [16] with bovine serum albumin as standard. During chromatographic purification steps, protein concentration was measured as a function of its absorbance at λ_{280} nm.

2.3. Purification of esterase

LKE-028 esterase was purified to homogeneity in four steps. Crude enzyme solution (cell free supernatant) was cooled and ammonium sulfate crystals were slowly added with constant stirring up to 30% saturation. The solution was allowed to stand overnight at 4 °C. Precipitate was collected after centrifugation at 12×10^3 r.c.f. at 4 °C for 15 min and dissolved in 20 mM Tris–Cl buffer (pH 8.0) to achieve a volume of 1/10th of the original crude sample. Undissolved substance was removed by centrifuging at 12×10^3 r.c.f. at 4 °C for 15 min, precipitated protein were estimated and dialyzed against 20 mM Tris–Cl buffer (pH 8.0) to remove excess salts. Concentrated protein solution was loaded in a DEAE cellulose (Himedia, India) column (2.2 cm × 20 cm) pre-equilibrated with 20 mM Tris–Cl buffer (pH 8.0). Column was washed with same buffer until λ_{280} nm of effluent become zero and bound proteins were eluted by NaCl gradient (0–0.5 M, flow rate 0.2 mL min^{-1}). Each fraction of 1 mL volume was collected and those with high specific activity were pooled and lyophilized. Lyophilized powder was dissolved in minimum volume of 20 mM Tris–Cl buffer (pH 8.0) and was passed through pre-equilibrated carboxy methyl cellulose (CM-cellulose) (Sigma–Aldrich, USA) column (2.2 cm × 25 cm). Column was then washed until λ_{280} nm of effluent reached zero. Bound proteins were eluted by NaCl gradient (0–0.5 M) in same buffer. Active fractions were pooled and loaded to a sephadex-G-100 (Fluka chemicals, Switzerland) column (2.25 cm × 35 cm) and fractions of 1 mL each were collected at a flow rate of 5 mL h^{-1}. Each purification step was monitored by SDS-PAGE. High specific activity fractions of purified enzyme were pooled and lyophilized for further characterization.

2.4. Electrophoresis, molecular mass determination and activity staining

SDS-PAGE was carried out according to Laemmli [17] with 7.5% polyacrylamide. Molecular weight markers (14–97 kD; purchased from Sigma) were run along with samples to determine molecular mass of the target protein. Protein bands were visualized by sensitive silver staining method.

Purified enzyme was evaluated in native PAGE for activity staining with the same gel composition, pH and voltage like SDS-PAGE; only difference being SDS replaced by equivalent amounts of suitable buffer in gel preparation, electrode buffer and sample buffer. The reducing agent (β-mercaptoethanol) was excluded in the sample buffer and the sample was not boiled prior to application. After electrophoresis, gel was washed with 100 mM Tris–Cl buffer (pH 7.5), followed by treatment with 3 mM *p*NP acetate solution in McIlvein buffer (pH 7.5) for 30 min. Gel was finally incubated in developing solution with 2 mM Fast Red TR (Sigma–Aldrich, USA) in 100 mM sodium phosphate buffer (pH 7.5). Esterase activity was detected by appearance of a deep purple colored band in gel. All PAGEs were immediately photographed with GelDoc™ XR (Bio-Rad, USA) [18].

3. Characterization of the enzyme

3.1. Effect of pH and temperature on enzyme activity and stability

Effect of pH on esterase activity was determined at 70 °C within pH ranges of 5.0–12.0 by various buffer solutions, viz. sodium acetate (50 mM; pH 5.0–6.0), potassium phosphate (50 mM; pH 6.0–8.0), Tris–Cl buffer (50 mM; pH 7.0–9.0), glycine–NaOH (50 mM; pH 8.0–11.0) and Na_2HPO_4–NaOH (50 mM; pH 12.0) buffer. Enzyme activity was quantified using *p*NP acetate as substrate as mentioned earlier. To investigate pH stability, lyophilized enzymes were dissolved in buffers of different pH mentioned above and pre-incubated at 70 °C for 2 h. Reaction pH was adjusted with same buffer which has been used for pre-incubation and finally residual activity was determined.

Effect of temperature on enzyme activity was determined between 20 °C and 120 °C at pH 11.0 following standard assay methods using *p*NP acetate as substrate. Thermostability was investigated by pre-incubating enzyme solutions at 60–90 °C at pH 11.0 for 150 min. After 30 min interval, residual activities were quantified and compared with non pre-incubated sample.

On the basis of these findings all characterization studies were conducted in triplicate sets at 70 °C and pH 11.0.

3.2. Substrate specificity of the purified enzyme

Relative enzyme activity was measured on a broad range of substrates (procured from Sigma–Aldrich, USA) including different fatty acid esters viz. a series of *p*NP esters, triglycerides and naphthyl esters.

3.2.1. p-Nitrophenyl esters

Assay with different *p*NP ester substrates was performed with standard assay technique. *p*NP acetate was replaced by corresponding substrates [19].

3.2.1.1. α-Naphthyl esters. Reaction mixture of 250 μL enzyme solution, 50 μL α-naphthyl acetate and 200 μL glycine–NaOH buffer was incubated with 50 μL 10 mM Fast Red TR. Activity was quantified at λ_{560} nm by measuring the degree of substrate hydrolysis. Spontaneous substrate hydrolysis without enzyme was considered as blank. One unit of enzyme activity was defined as amount of enzyme essential to release 1 μM α-naphthol in 1 h. Methods were adopted from the work done by Sana et al. [15] with slight modifications.

3.2.1.2. β-Naphthyl esters. Assay mixture containing 50 μL β-naphthyl acetate, 200 μL of glycine–NaOH buffer, and 250 μL enzyme was incubated for 30 min and reaction was terminated by chilling on ice. Esterolytic activity was measured at λ_{320} nm. One

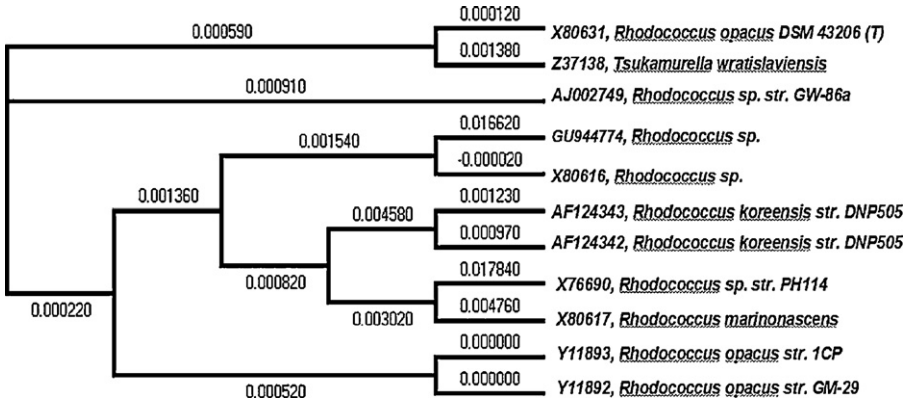

Fig. 1. Phylogenetic dendrogram, indicating the position and distance matrix based comparison of the strain *Rhodococcus* sp. LKE-028 (GenBank accession number: GU944774).

unit of enzyme activity was defined as amount of enzyme preparation capable of releasing 1 μM β-naphthol in 1 h [20].

3.2.2. Fatty acid esters

Enzymatic hydrolysis of fatty acid esters was determined by titration of liberated fatty acid. Reaction mixture containing 400 μL of 10 mM fatty acid ester, 300 μL glycine–NaOH buffer and 300 μL enzyme solution was incubated for 30 min and then stopped by addition of terminating agent (10 μL 1:1 (v/v) mixture of acetone and ethanol and 10 μL of 1% (w/v) phenolphthalein in a 9:1 (v/v) mixture of ethanol and H_2O). Final mixture was titrated with 0.05 M NaOH until mixture turned red. Amount of liberated fatty acid was estimated from the volume of NaOH required to reach end point. One unit of enzyme activity was defined as the amount of enzyme required to produce 1 μM fatty acid in 1 h [21].

3.3. Kinetic determinations

Initial rate of reaction for *p*NP acetate hydrolysis was calculated by estimating esterase activity with different substrate concentrations from 25 to 2500 nM Michaelis–Menten constant (K_m) and rate of reaction (V_{max}) were determined according to Lineweaver–Burk plot [22].

3.4. Effect of metal ions, inhibitors and denaturing chemicals

Enzyme was incubated for 1 h in 50 mM glycine–NaOH buffer at 70 °C with different metal ions (Ba^{2+}, Ca^{2+}, Co^{2+}, Cu^{2+}, Fe^{3+}, Hg^{2+}, K^{+}, Mg^{2+}, Mn^{2+} and Zn^{2+}); inhibitors (EDTA, iodoacetic acid, PMSF), reducing agent (β-mercaptoethanol) and oxidizing agent (hydrogen peroxide) with final concentration of 5 mM and surface active agents (SDS, Triton X 100, Tween 20 and Tween 80) with final concentration of 1 and 2% (v/v). Residual activity was measured following standard assay method with *p*NP acetate as substrate. Enzyme activity treated in same way but without any additive was considered 100%.

3.5. Effect of sodium chloride on enzyme activity

Effect of salinity on enzyme activity was studied in presence of different NaCl concentrations ranging from 0 to 10 M. Purified enzyme was preincubated in 50 mM glycine–NaOH buffer containing different NaCl concentrations at 70 °C for 3 h and then quantified. Enzyme activity treated in same way without NaCl was considered 100%.

3.6. Effect of organic solvents on enzyme activity and stability

Stability of purified esterase was determined against various organic solvents. In different screw cap test tubes, enzyme solution in 50 mM glycine–NaOH buffer was mixed with different organic solvents (enzyme:solvent::2:1) and incubated at 70 °C with constant moderate shaking for 10 days. Residual activity of each aliquot was measured by standard assay procedure at different interval of days. Loss of solvents due to evaporation was replenished with fresh solvent. Relative activities were calculated on the basis of enzyme activity, considering solvent at 0 day without incubation is 100%.

3.7. Stability of enzyme against protease

Enzyme stability was also checked against proteinase K (Sigma–Aldrich, USA). Purified esterase was incubated with different conc. (5–100 U μg^{-1} proteinase K) at 70 °C for 30 min. Esterase activity was measured with *p*NP acetate as substrate.

4. Results

4.1. Microbial and phylogenetic characterization and enzyme production of Rhodococcus sp. LKE-028

Isolated LKE-028 was found to possess potent esterolytic activity when qualitatively screened with tributyrin in nutrient agar medium at 60 °C for bright clear zone on the plate. A more visible clear zone was found with further incubation at 40 °C for 4 days. Morphological and physiological characteristics of isolate 'LKE-028' showed a *Coccus* morphotype. Based on nucleotide of 16S rRNA gene sequence of the isolate LKE-028 was found to be a member of *Rhodococcus* family (GenBank accession no. GU944774). Taxonomic position is shown in phylogenetic tree (Fig. 1). Strain has been deposited in 'MTCC', Chandigarh (CSIR-IMT, India) with code name MTCC 5562.

4.2. Enzyme purification and molecular weight

Enzyme was purified up to 62-fold with 26.7% recovery, specific activity of finally purified enzyme being 861.2 U mg^{-1} proteins (Table 1). Pure enzyme showed a single band on SDS-PAGE to prove its homogeneity. On the basis of molecular weight marker ran parallel with proteins during each purification steps in SDS-PAGE, target protein was found to have molecular weight of 38 kDa.

Table 1
Summary of the results of the purification of esterase from *Rhodococcus* sp. LKE-028.

Purification step	Total activity (U)	Protein (mg)	Specific activity (U mg^{-1})	Purification fold	Recovery (%)
Crude extraction	17,480	1280	13.7	1.0	100
30% $(NH_4)_2SO_4$ fraction	14,690	196.7	74	5.4	84
DEAE ion-exchange	9470	20.3	465.5	33.9	54.1
CM-ion-exchange	6450	10.9	590.2	43	36.8
Sephadex gel-filtration	4670	5.4	861.2	62.8	26.7

Table 2
Effects of different substrates on the activity of the esterase purified from *Rhodococcus* sp. LKE-028.

Substrate	Specific activity (U mg^{-1} protein)	% Relative activity	Substrate	Specific activity (U mg^{-1} protein)	% Relative activity
*p*NP acetate	861.2	100	Ethyl laurate	0.0	0
*p*NP butyrate	856.8	99.5	Ethyl lactate	86.1	10
*p*NP carprylate	491.7	57.1	Ethyl linolate	0.0	0
*p*NP palmitate	368.5	42.8	Ethyl oleate	55.6	6.5
α-Napthyl acetate	577.8	67.1	Ethyl octanate	356.5	41.4
β-Napthyl acetate	109.5	12.7	Ethyl palmitate	58.2	6.8
Ethyl butyrate	438.3	50.9	Isopropyl-myristate	0.0	0
Ethyl caprylate	294.5	34.2	Triacetin	384.9	44.7
Ethyl decanoate	72.49	8.4	Tributyrin	298.8	34.7
Ethyl formate	0.0	0	Trilaurin	0.0	0
Ethyl isolaurate	0.0	0	Tripalmitin	0.0	0

Esterase activity was detected by appearance of a deep purple colored band in gel with activity staining (Fig. 2).

4.3. Effect of pH and temperature on enzyme activity and stability

Optimum pH for enzyme activity was 11.0 and it remained active over a broad range of pH (8.0–12.0) (Fig. 3a) where it retained almost 80% of its residual activity (activity at pH 11.0 was considered to be 100%). This enzyme was stable in the pH range between 5.0 and 11.0 for a time span of 120 min. Fig. 3b illustrates residual activity at different pH values after 30, 60, 90 and 120 min of pre-incubation. LKE-028 esterase retained 50% of its residual activity even after 120 min pre-incubation up to pH 11.0. When pre-incubated at pH 12.0, a gradual decrease in activity was observed with increased pre-incubation time and at 120 min it almost lost 75% of its residual activity.

Optimum activity of the purified enzyme was found at 70 °C (Fig. 4a) and it remained active over a temperature range of 40–90 °C, retaining almost 70% of its residual activity (activity at optimum temperature was considered to be 100%). Thermal stability profile of LKE-028 esterase in form of relative activity has been presented in Fig. 4b. Enzyme was completely stable at 60 °C up to 3 h and retained more than 80% activity up to 80 °C for 3 h incubation. At 90 °C a sharp fall in residual activity was observed with increase in pre-incubation time.

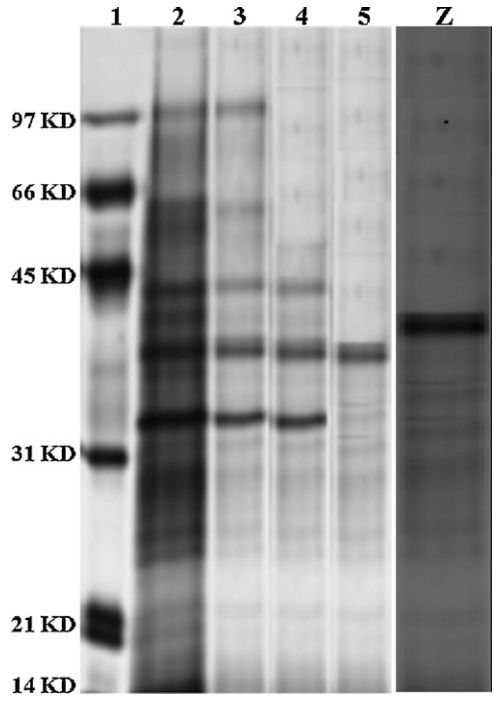

Fig. 2. SDS-PAGE and zymography of the purified esterase from *Rhodococcus* sp. LKE-028. Molecular weight marker (lane 1); crude enzyme after precipitation (lane 2); active fractions after anionic exchange chromatography (lane 3); active fractions after cationic exchange chromatography (lane 4); purified esterase after molecular sieving chromatography (lane 5); zymogram of the purified esterase (lane Z).

4.4. Substrate specificity of the LKE-028 esterase

Enzyme activity was studied on different type of substrates as shown in Table 2. Enzyme was found to be most active with *p*NP acetate and *p*NP butyrate. Increasing length of fatty acid side chain hindered residual activity of enzyme. α-napthyl acetate was a better substrate than its β isomer. Enzyme showed relatively lesser activity toward 'fatty acids from triglycerides' (Table 2).

4.5. Kinetic determinations

Michaelis–Menten constant (K_m) and rate of reaction (V_{max}) were determined according to Lineweaver–Burk plot [22]. Apparent K_m and V_{max} values, determined for LKE-028 esterase by using *p*NP acetate as substrate (Fig. 5) were 525 nM and 1666.7 U mg^{-1} protein respectively.

4.6. Effect of metal ions, inhibitors and denaturing chemicals

Enzyme activity was studied on different metal ions as shown in Table 3. Almost 60% of activity was found with Ba^{2+} and Zn^{2+}, where as Ca^{2+}, Mg^{2+} and Co^{2+} enhanced enzyme activity (Table 3). Enzyme was completely inhibited by well-known serine inhibitor (PMSF) indicating that this enzyme belonged to serine hydrolyses family (Table 4). LKE-028 esterase has also been found stable in

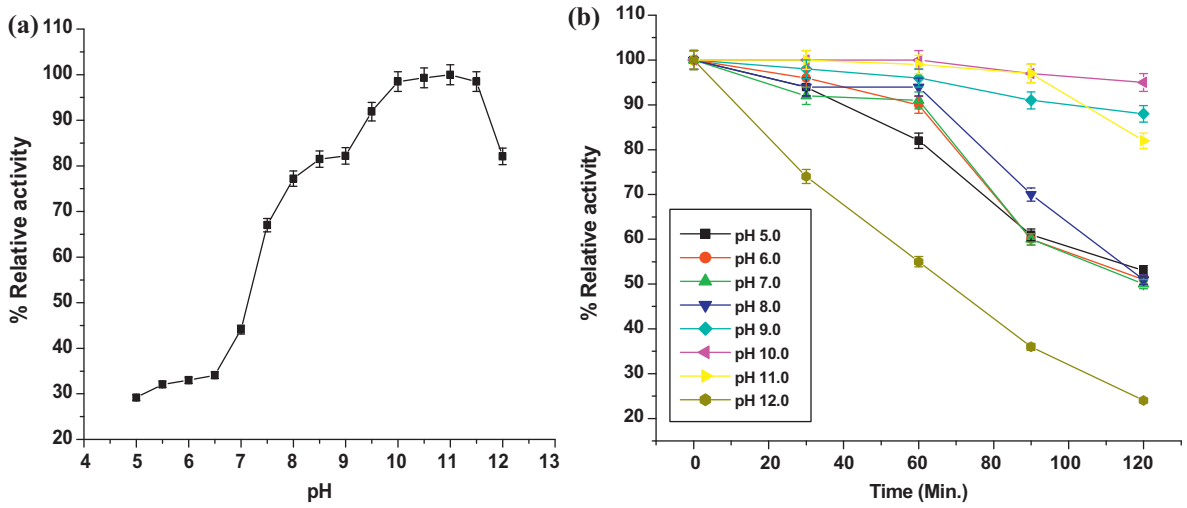

Fig. 3. Effect of pH (a) and stability (b) on the activity of esterase from *Rhodococcus* sp. LKE-028.

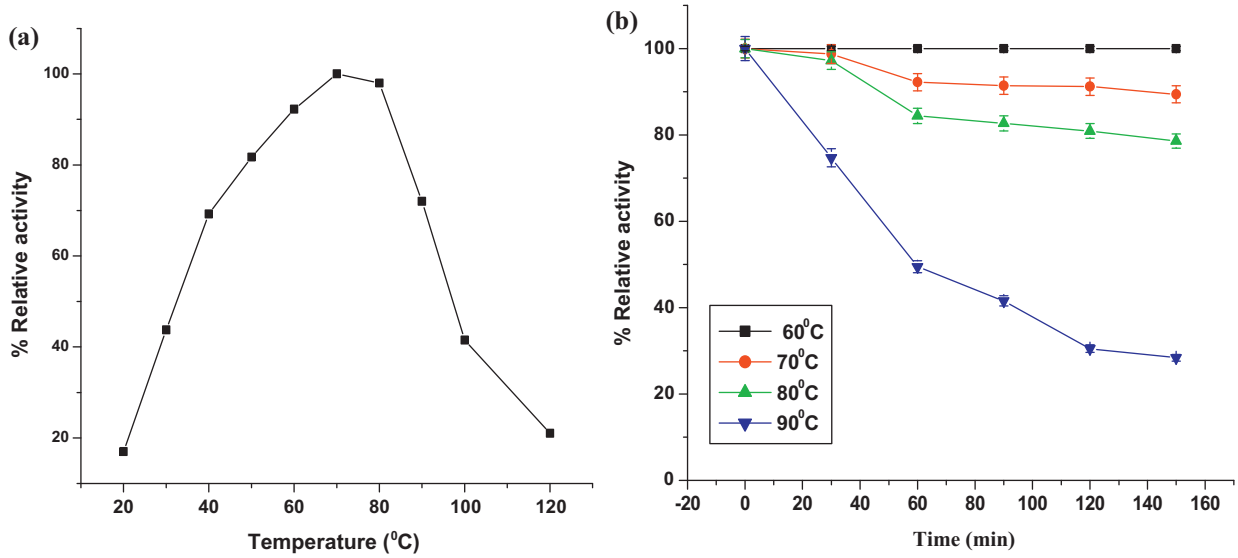

Fig. 4. Effect of temperature on activity (a) and stability (b) of esterase from *Rhodococcus* sp. LKE-028.

presence of both oxidizing and reducing agents and retained more than 60% activity in presence of β-mercaptoethanol and hydrogen peroxide. In presence of surfactants like SDS, Tween 20, Tween 80 and Triton X 100 (1% and 2%) enhancement of enzyme activity has been observed.

Table 3
Effects of metal ions on the activity of the esterase purified from *Rhodococcus* sp. LKE-028.

Metal ions	Specific activity (U mg^{-1} protein)	% Relative activity
Control	861.2	100
Ca^{2+}	1444.2	167.7
Hg^{2+}	769	89.3
Fe^{3+}	726.8	84.4
K^{+}	769	89.1
Cu^{2+}	810.3	94.1
Co^{2+}	1073.9	124.7
Mg^{2+}	1492.4	173.3
Fe^{2+}	688.9	80
Zn^{2+}	375.4	43.6
Ba^{2+}	291.9	33.9

4.7. Effect of sodium chloride on enzyme activity

Purified esterase remarkably retained 100% activity when incubated with 10 M NaCl. Activity enhanced about 50% from that

Table 4
Effects of inhibitors and surfactant on the activity of the esterase purified from *Rhodococcus* sp. LKE-028.

Inhibitors/reducing/oxidizing agent/surfactant	Specific activity (U mg^{-1} protein)	% Relative activity
Control	861.2	100
EDTA	769.0	89.1
Iodoacetic acid	726.8	84.4
PMSF	15	1.7
β-Mercaptoethanol	571.1	66.3
H_2O_2	677.5	78.6
SDS	951	110.43
Triton X 100 (1%)	1162.6	135
Triton X 100 (2%)	1095.4	127.2
Tween 20 (1%)	1179.8	137
Tween 20 (2%)	1162.6	135
Tween 80 (1%)	1205.6	140
Tween 80 (2%)	1194.4	138.7

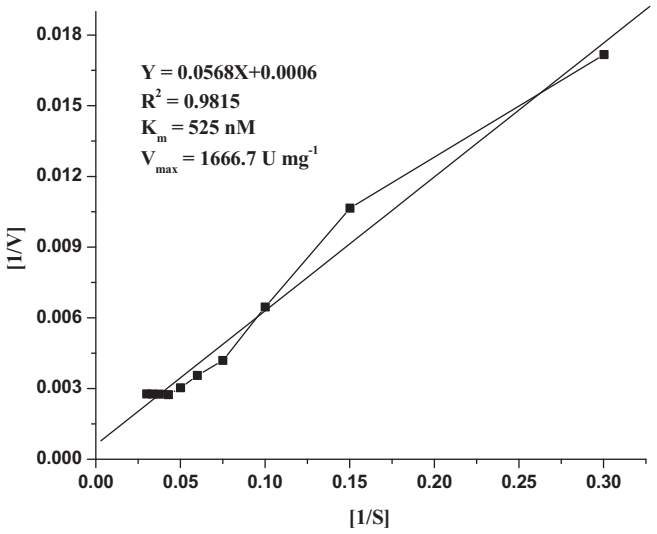

Fig. 5. Lineweaver–Burk plot of esterase from *Rhodococcus* sp. LKE-028 for the kinetic analysis with *p*NP acetate.

Table 5
Effects of NaCl on the activity of the esterase purified from *Rhodococcus* sp. LKE-028.

NaCl (M)	Specific activity (U mg^{-1} protein)	% Relative activity
Control	861.2	100
1 M	1731	201
2 M	1301.2	151.1
3 M	1216.8	141.3
4 M	1165.2	135.3
5 M	1080.8	125.5
10 M	869.8	101

without NaCl when incubated with 2.0 M sodium chloride (Table 5).

4.8. Effect of organic solvents on enzyme activity and stability

Purified LKE-028 esterase showed stability in presence of DMSO, benzene, toluene, methanol, ethyl alcohol, acetone, isoamyl alcohol. Enzyme retained considerable activity after 10 days of incubation in presence of isoamyl alcohol, DMSO and benzene compared to other solvents (Fig. 6). In case of DMSO and benzene a

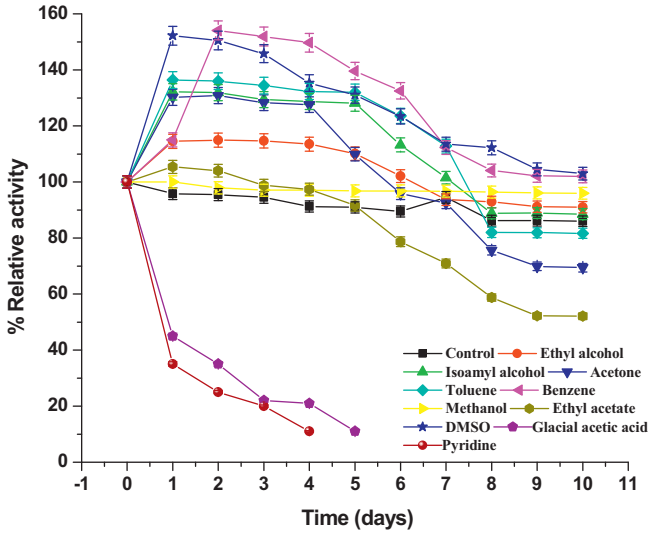

Fig. 6. Effect of different organic solvents on the stability of esterase from *Rhodococcus* sp. LKE-028.

Table 6
Effects of proteinase K on the stability of the esterase purified from *Rhodococcus* sp. LKE-028.

Proteinase K (U μg^{-1})	Specific activity (U mg^{-1} protein)	% Relative activity
Control	861.2	100
5	861	99.9
10	774.2	89.9
20	715.6	83.1
50	659.6	76.6
100	218.7	25.4

1.1–1.7-fold increase in specific activity (with respect to control) was observed over a period of 10 days.

4.9. Stability of enzyme against protease

Enzyme was found to be active after treatment with proteinase K. LKE-028 esterase retained over 75% of its residual activity up to 50 U μg^{-1} proteinase K (Table 6). A sharp loss in activity was observed with further increase in proteinase-K concentration up to 100 U μg^{-1}.

5. Discussion

Not many extremophilic esterases from thermophiles have been reported, except there are few reports of esterase activity in some *Rhodococcus* sp. [23–25]. This paper describes purification and characterization of a salt, organic solvent and protease stable thermoalkaliphilic esterase from a thermophilic *Rhodococcus* sp. LKE-028, a soil bacterium, isolated from *Gangotri* region of *Uttarakhand Himalaya*. LKE-028 esterase was purified from culture supernatant in four steps and achieved approximately 4 times recovery in comparison with reported esterase BSE01 [10] and higher specific activity (861.2 U mg^{-1} protein) (Table 1) with 2 times recovery in comparison with *Thermobifida fusca* esterase [26].

LKE-028 esterase displayed high activity under neutral to alkaline condition and no activity below pH 5.0. Highest level of activity was measured at pH 11.0. Interestingly, enzyme was extremely stable, with little loss in activity after incubation up to 2 h in alkaline conditions up to pH 11.0. pH stability profile of *Rhodococcus* sp. LKE-028 esterase showed that enzyme retained over 50% activity at broad range between pH 6.0 and 11.0 (Fig. 3) up to 2 h at room temperature compared to thermophile *Anoxybacillus gonensis A4* esterase [27]. LKE-028 displayed alkaliphilic properties that have been previously detected only in enzymes isolated from extreme environments [14]. Industrial processes often require different pH as well as high temperatures and majority of known enzymes need to be stabilized under these conditions; therefore, there is a great interest in enzymes that are derived from extremophiles and stable without pretreatment [6,28,29,30]. LKE-028 esterase was optimally active at 70 °C (Fig. 4). At 90 °C LKE-028 displayed a half-life ($t_{1/2}$) of 50 min and no significant reduction in activity was observed at 80 °C. Enzyme was active in broad range of temperature between 40 and 120 °C. Manco et al. investigated thermostability of esterase Est1 and Est2 from *Bacillus acidocaldarius* [31,32]. Est2 had a $t_{1/2}$ of 10 min at 90 °C and 30% of the initial activity was recovered after Est1 had been incubated for 90 min at 75 °C. Sobek and Gorisch [33] reported that residual activity of *Sulfolobus acidocaldarius* esterase declined to 92% after purified enzyme had been incubated for 1 h at 90 °C. These results provide strong evidence that esterase from *Rhodococcus* sp. LKE-028 possesses unique properties for large-scale applications at extreme pH and temperature.

Substrate specificity of purified esterase from *Rhodococcus* sp. LKE-028 was studied by using various *p*NP and ethyl esters of

Table 7
Comparative feature regarding various characteristics of esterase from *Rhodococcus* sp. LKE-028.

Strain/source	Optimum		NaCl conc. limit (%, M)	Reference
	Temperature (°C)	pH		
Rhodococcus sp. LKE-028	70	11	10.0 M	Present work
Thalassobacillus sp. strain DF-E4	40	8.5	0.5 M	Xiao-Yan, et al. [53]
Penicillum notatum NRRL-1249	30	5.5	-	Atta et al. [54]
Thermus scotoductus SA-01	80	7.0	–	Du Plessis et al. [56]
Haloarcula marismortui (ATCC 43049).	45	9.5	3.4 M	Rao et al. [55]
Bacillus species BSE-01	45	8.0	1.0 M	Sana et al. [15]
Anoxybacillus gonensis A4	60-80	5.5	-	Ozlem et al. [27]
Esterase EstA3 from metagenome (drinking water)	50	9.0	–	Elend et al. [52]
Esterase EstCE1 From metagenome (soil)	47	10.0	–	Elend et al. [52]
Rhodococcus sp. strain MB1	–	–	–	Bresler et al. [24]

straight chain fatty acids ranging in chain length from C_2 (acetate) to C_{14} (myristate). Our enzyme showed substrate specificity on a set of substrates typical for lipases and esterases, including *p*NP and ethyl esters. *p*NP and ethyl esters with acyl chain lengths bigger than C_8 were not best substrates for LKE-028. Maximum enzyme activity was observed toward *p*NP esters and ethyl esters of chain length ranging from C_2 and C_6 (Table 2), found to be typical substrates for esterases. *p*NP esters were hydrolyzed by LKE-028 at higher rates compared to ethyl esters. Further titration tests using triglycerides as substrates confirmed that only short chain substrates were converted by LKE-028 esterase. Specific activities of LKE-028 esterase with triacetin, tributyrin and tripalmitin were similar to most of the tested substrates, indicating LKE-028 esterase was also able to hydrolyze long chain fatty acids. These results indicated that enzyme belonged to esterase class rather than lipases. Broad substrate specificity of this robust LKE-028 enzyme was remarkable and they could be of use to produce a diverse range of high-value products.

It is known that metal ions play important role in maintaining structural stability and protein integrity through binding to amino acid residues with negative charges in specific sites [34]. In presence of Ca^{2+} and Mg^{2+} our esterase activity was enhanced up to 167.7 and 173.3% (Table 3). Stimulatory effect of Ca^{2+} might be attributed to better alignment of enzyme on substrate molecule and neutralization of fatty acids liberated from substrate [35]. The same stimulatory effect of Ca^{2+} was recorded for *Avena fatua* esterase [36]. Other metals such as Co^{2+} and Mg^{2+} had significant effect on enzyme activity in our study (Table 3). These observations reflect similarities those described by Mohamed et al. [36], Baigori et al. [37] and Nourse et al. [38]. Interestingly, LKE-028 was inhibited by addition of Ba^{2+} or Zn^{2+} ions and slightly inhibited with Cu^{2+} in hydrolysis medium (Table 3). These results are comparable with esterases from turkey pharyngeal tissue and *Cucurbita pepo* which were fully inhibited by the presence of Cu^{2+} or Zn^{2+} using *p*NP esters as substrate [39,40]. From literature data it is evident that many lipases and esterases are activated or inhibited by metal ions. Intracellular esterase from *Serratia marcescens* 345 was activated by Mg^{2+} ions [41], acetylcholinesterase is activated by monovalent Na^+, K^+ and divalent Ca^{2+} and Mg^{2+} cations [42] and in case of pancreatic lipase both alkali and alkali earth metal ions, especially Na^+, Ca^{2+} and Mg^{2+} stabilized the enzyme [43]. Several esterases have been reported to be Ca^{2+} dependent [44]. Zn^{2+} ions strongly inhibited EstB1 and EstB2 esterase activity, whereas addition of Ca^{2+} and Mg^{2+} ions slowly deactivated both esterases [18]. Catalytic triad conserved in esterases contains an active serine residue and therefore serine inhibitor such as PMSF inhibits activity of these serine hydrolases. This was substantiated by the inhibition of LKE-028 in the presence of 5 mM PMSF (Table 4). Inhibition in the presence of PMSF was also observed for the thermostable esterase EstA and EstB from thermoacidophilic euryarchaeon *Picrophilus torridus* [45], Est1 and Est2 from *B. acidocaldarius* [31,32], thermostable esterase from *S. acidocaldarius* [33] and thermostable SsoP1 [8]. In contrast, lipase from *Acinetobacter calcoaceticus* was not inhibited by PMSF, possibly caused by an active serine buried deeply within the molecule [46]. There are increasing evidences that a disulfide bridge is essential for functionality of some esterases. Most of the esterases and lipases have a serine residue at active site of the enzyme [47]. But lipases can be distinguished from esterases by having a hydrophobic domain (lead) covering active site [48–50]. Therefore, lipases are either slightly or not inhibited by PMSF because the "lead" structure makes active site of enzyme inaccessible to the reagent. So as to determine the presence of a thiol group in esterase molecule, enzyme was incubated with β-mercaptoethanol at a concentration of 5 mM (Table 4) and was found to be non-influencive. From this result, it could be inferred that thiol group was present or critical for catalytic function.

Organic solvents can be advantageous in various industrial enzymatic processes. Reaction media used in biocatalytic esterification and trans-esterification contains less than 1% water. Use of organic solvents could increase solubility of non-polar substrates and thermal stability of enzymes, decrease water-dependent side reactions, or eliminate microbial contamination [51]. Organic solvents affected performance of enzymes in different ways. In this study, LKE-028 esterase depicted high stability in presence of many water miscible organic solvents. In presence of DMSO and benzene, LKE-028 esterase retained over 100% relative activities even after 10 days (Fig. 6). Remarkable stability of EstB1 and EstB2 esterase activity toward organic solvents was reported by Karpushova et al. [18]. LKE-028 esterase performed even better as activity significantly increased with 33.3% (v/v) DMSO. Methanol and ethyl alcohol slightly enhanced activity of our enzyme while alcohols inhibited EstB1 and EstB2. Metagenome derived esterase reported by Elend et al. [52] expressed maximum activity after 1 h incubation with 15% and 30% (v/v) DMSO, while LKE-028 esterase retained over 100% relative activity after incubation for 10 days as similar to BSE01 esterase from *Bacillus* sp. reported by Sana et al. [15].

Remarkable stability of EstB1 and EstB2 esterase and BSE01 esterase activity toward NaCl was reported by Karpushova et al. [18] and Sana et al. [15] due to their marine origin. LKE-028 esterase activity was increased significantly with 1 M NaCl and retained 100% activity up to 10 M NaCl (Table 5), and displayed 10 times more halotolerancy than previously reported esterases [15,18]. Comparative features regarding various characteristics of esterase LKE-028 has been given in Table 7.

High salt tolerance of LKE-028 indicates extremophilic nature of this halophilic enzyme. Thermostable, salt and organic solvent tolerant enzymes might be applied to treat industrial effluents

treatments, to produce inter-esterification substances in food industry, or to synthesize useful chemical compounds and non-aqueous biocatalysis for non-natural hydrolysis of substrate as well as modification of molecules. Stability of LKE-028 activity in presence of protease, organic solvents and salt, as well as in alkaline pH at high temperatures makes it a potential candidate for its application as a non-aqueous biocatalyst.

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