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A novel esterase from *Ralstonia* sp. M1: Gene cloning, sequencing, high-level expression and characterization

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Abstract

A newly isolated gene from *Ralstonia* sp. M1, encoding an esterase, was cloned in *Escherichia coli* and its nucleotide sequence determined. The 1.6 kb insert revealed one complete open reading frame, predicted to encode an esterase (320 aa, 34.1 kDa) with a p*I* of 9.86. *Est*R contained a putative oxyanion hole $H^{36}G^{37}$, a conserved pentapeptide $G^{103}HSLG^{107}$ and a conserved catalytic His^{265} and Asp^{237}. The *Est*R sequence shared 64–70 and 44–48% identity with the hydrolases/acyltransferases from *Burkholderia* strains and from *Ralstonia* strains, respectively, 44 and 38% identity with the lactone-specific esterase from *Pseudomonas fluorescens* and *Mesorhizobium loti*, respectively. The esterase *Est*R was expressed with a high level of 41 mg/g wet cells. The Ni–NTA-purified esterase *Est*R showed an optimal activity in the temperature range 60–65 °C and pH range 7.5–9.0 towards *p*-nitrophenyl caproate. The enzyme was found to be highly resistant to many organic solvents especially induced by ethanolamine. Metal ions showed slight effect on esterase activity. The inhibitor phenylmethanesulfonyl fluoride inhibited strongly the esterase. Triton X-45 induced the activation of *Est*R, but other detergents slightly to strongly decreased or completely inhibited. Among tested *p*-NP esters, caproate was the most preferential substrate of this esterase.

Keywords: *Ralstonia* sp. M1; Esterase gene; Cloning; Expression; Characterization

The α/β hydroxylase superfamily consists of esterases and lipases primarily characterized by their common fold (α/β hydroxylase fold), which is central, predominantly parallel β sheet flanked by α-helical connections. These enzymes also share a characteristic sequence motif, GXSXG, for most esterases and lipases, called the "nucleophilic elbow" [1,2]. The serine is embedded in this motif, and ester hydrolysis is mediated by a nucleophilic attack of the active serine on the carbonyl of the substrate in a charge-relay system with the two other amino acid residues: aspartic acid and histidine [1]. These amino acid residues constitute a catalytic triad in the specific order (serine–aspartic acid–histidine) in the polypeptide chain [3–6]. So most of the esterases and lipases resemble lipases and serine proteases in hydrolytic mechanism.

Esterases and lipases are carboxylic ester hydrolases (EC 3.1.1). Whereas lipases (EC 3.1.1.3) display high activity towards water-insoluble or emulsified substrates with long-chain fatty acids, the carboxylesterases (EC 3.1.1.1) show highest activity towards water-soluble or emulsified esters with relatively short fatty acid chains. Structural studies revealed that this difference is caused by the absence or presence of a "lid" structure, which is responsible for the interfacial activation of lipase [4,6,7].

Esterases and lipases are widely distributed in all kingdoms of life. They have been identified in a wide range of organisms and several of these have been cloned. Besides lipases, different esterases have been identified and cloned from various microbial strains including *Pseudomonas* [8,9], *Bacillus* [10,11] and *Acinetobacter* [12,13].

Microbial esterases and lipases are found to be relatively resistant to organic solvents [14], and thus they are important catalysts for region- and enantioselective reactions in organic synthesis, among the most important

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groups of biocatalysts in biotechnology. Esterases and lipases have been used successfully in organic synthesis of optically pure substances. A *Pseudomonas* esterase displayed high activity and enantioselectivity to (*S*)-ketoprofen ethyl ester [15]. An esterase from *Arthrobacter globiformis* was used in the resolution of ethyl chrysanthemate derivatives [16,17], which are key compounds during the synthesis of pyrethrin insecticides. A *Bacillus* carboxylesterase has been used for stereospecific resolution of *R*,*S*-naproxen esters to *S*-naproxen [18], which is an important anti-inflammatory drug, and a *p*-nitrobenzyl esterase was genetically engineered to synthesize cephalosporin-derived antibiotics [19].

Several *Ralstonia* species were already reported to produce lipolytic enzymes [20–23]. In previous studies, we isolated a novel lipase/chaperone gene pair from *Ralstonia* sp. M1, analyzed genes and characterized physicochemical properties of the recombinant proteins [22]. In this study, we characterized another gene from *Ralstonia* sp. M1 encoding a lipolytic enzyme, expressed the ORF in *Escherichia coli* and characterized the recombinant lipolytic enzyme.

Materials and methods

Chemicals and agents

p-Nitrophenyl esters were purchased from Sigma, and Bacto-tryptone and yeast extract were from Difco. Restriction enzymes, calf intestinal alkaline phosphatase and T4 ligase were supplied by Roche. The DNA Gel-Extraction Kit and Ni–NTA-matrix were from Qiagen. PCR mix and miniprep solutions were purchased from Bioneer. All other reagents were of analytical grade unless otherwise stated.

Plasmids, bacterial strains, and culture conditions

The clone M1-81 from the genomic DNA library constructed from the strain *Ralstonia* sp. M1 [23] was used for sequencing and isolation of the esterase gene *est*R. *Escherichia coli* XL1 blue containing the pUC19-derived plasmid pUM1-81 was grown routinely in LB medium with ampicillin (100 μg/ml) at 37 °C. The vector pET22b$^+$ (Invitrogen) and *E. coli* BL21 cells were used to overexpress the esterase gene *est*R under the control of the T7 promoter, which is inducible by IPTG.

DNA manipulations

Plasmid DNA isolation was carried out according to the instruction of Bioneer. DNA fragments and PCR products were excised from a 0.8% agarose gel and purified as described by Qiagen. DNA sequencing was performed by Genotech. *Escherichia coli* BL21 was transformed using electroporation method as described previously [23].

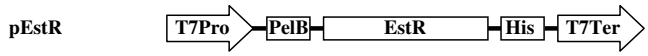

Fig. 1. Expression vector p*Est*R derived from the expression vector pET22b$^+$ for *E. coli* BL21 system. The inserts and restriction sites used for cloning are given. T7Pro, T7 promoter; PelB, PelB signal peptide; *Est*R, mature esterase; His, 6-histidine tag.

Plasmid construction and subcloning

To overexpress the esterase in *E. coli*, pET22b$^+$ vector was used. The 963-bp ORF encoding the esterase from *Ralstonia* sp. M1 was amplified from the plasmid pUM1-81 by PCR with the forward primer *Est*RF 5′-GGCCATGGTC CAGACCGTCCTGAT-3′, which introduces an *Nco*I site containing the start codon (ATG instead of the original GTG) and the reverse primer *Est*RR 5′-GGAAGCTTGG CTGACGCGGCGGCTTC-3′, which provides a *Hin*dIII site immediately upstream of the stop codon (TAG). The PCR product was digested with *Nco*I$^+$ *Hin*dIII and ligated to pET22b$^+$ linearized with the same enzymes, resulting in p*Est*R (Fig. 1), in which the *est*R gene is under the control of the T7 promoter and is inducible with IPTG; the plasmid also carries the ampicillin resistance marker, and provides a signal peptide at the 5′ end and a 6×His tag fused to the 3′ end of the ORF. The esterase *Est*Rhis encoded by the plasmid p*Est*R consists of the *pel*B leader, the ORF for the esterase and a sequence encoding the 6×His tag. Other standard recombinant DNA techniques were carried out as described by Sambrook et al. [24].

Gene expression

The recombinant esterase *Est*R was expressed in *E. coli* BL21 transformants containing p*Est*R as described previously [23].

Enzyme purification and estimation of protein concentrations

The fusion form *Est*Rhis carrying a C-terminal 6xHis tag was expressed in *E. coli*. To purify the esterase *Est*R, the cells (~200 mg wet weight) from a 50-ml culture in LB medium was harvested by centrifugation, and suspended in 1 ml of 5 mM imidazole buffer (pH 8.0). After ultrasonic cell disintegration (three bursts of 1 min each at 1 min intervals), the cell suspension was centrifuged at 10,000*g* at 4 °C for 15 min. The cell supernatant was applied to Ni–NTA column (Qiagen) according to the manufacturer's recommendation. The esterase *Est*R solution was used for characterization study. Protein concentrations were determined by Bradford method with Bio-Rad protein assay kit.

Esterase activity estimation

Esterase activity towards *p*-NP esters (acetate, propionate, caproate, caprylate, myristate and palmitate) was carried out as described previously [22].

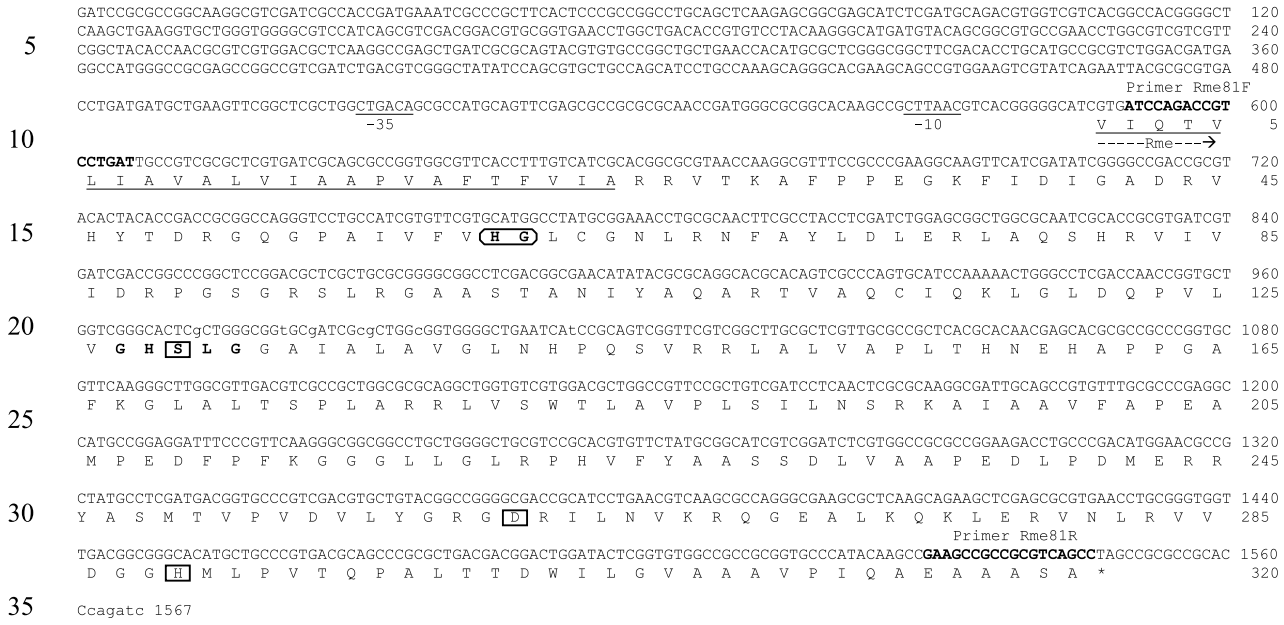

Fig. 2. Nucleotide sequence of the M1-81 insert and deduced amino acid sequence of *Est*R from *Ralstonia* sp. M1. Amino acids are aligned with the first nucleotide of each codon. Potential promoter regions (−10, −35) are labeled and underlined. The putative *Est*R signal sequence is underlined beneath the amino acid sequence. The conserved Ser, His and Asp are boxed. Putative oxyanion hole residues are depicted by a rhombus. Stop codons are marked with an asterisk. The sequence has been deposited in GenBank Accession No. AY320282. Bold marked letters, primer sequences for expression.

Effect of pH, temperature, organic solvents, detergents and metal ions on esterase activity

To determine the effect of various factors, 10 mM *p*-NP caproate was used as a substrate.

The optimum temperature for the enzyme activity was measured at pH 8.0 and at various temperatures from 25 to 90 °C. The optimum pH for the enzyme activity was measured at 55 °C and in 0.1 M (acetate, phosphate or Tris–HCl) buffers at different pH values 4.0–11.0.

The effect of detergents, organic solvents and metal ions or inhibitors on the esterase activity was determined by incubating the enzyme for 30 min at 30 °C in 0.1 M Tris–HCl buffer (pH 8.0) containing 0.2, 1 and 5% (w/v) of detergents; 10 and 30% (v/v) of organic solvents; and 0.1, 1 and 10 mM of metal ions or inhibitors, respectively. After treatment, the activity of the esterase mixture was determined at pH 8.0 and 55 °C using *p*-NP caproate as a substrate.

All measurements were carried out three times and from these values the average value was taken.

Gel electrophoresis

SDS–polyacrylamide gel electrophoresis (SDS–PAGE)[1] was carried out as described by Laemmli [25] with Bio-Rad equipment. SDS–PAGE was usually performed on gels containing 12.5% (w/v) acrylamide according to the manufacturer's recommendations.

DNA and amino acid sequence alignments

Sequence alignments were constructed and analyzed using the program Megalign DNAStar.

GenBank Accession numbers

The GenBank Accession No. for M1-81 sequence described in this paper is AY320282.

Results

Analysis of the esterase-encoding gene estR from Ralstonia sp. M1

The halo-forming colony M1-81 on 1% tributyrin agar plate was detected from the genomic DNA library constructed from the strain *Ralstonia* sp. M1 [23] and further analyzed on 1% tricaprylin agar plates to distinguish esterases from lipases. The colony M1-81 did not show any activity on 1% tricaprylin after 2 days incubation at 37 °C. The insert M1-81 in the plasmid pUM1-81 (pUC19$^+$ insert M1-81) was sequenced (Fig. 2). Analysis of the M1-81 insert (1.6 kb) revealed a single open reading frame (ORF) of 963 bp (positions 587–1549) (Fig. 2). Based on the homologies of the deduced amino acid sequences, this ORF encoded a putative esterase (320 aa) with the deduced molecular weight of 34 kDa and used GTG as a start codon. The G$^+$C content of *est*R was 67.6%. Upstream of *est*R is a putative transcription initiation site with a -10 region CTTAAC (position 564–568) and a -35 region CTGACA (position 510–515).

[1] *Abbreviations used:* SDS–PAGE, SDS–polyacrylamide gel electrophoresis; ORF, open reading frame; PMSF, phenylmethanesulfonyl fluoride.

*Analysis of the sequence of **Est**R*

The deduced amino acid sequence for *Est*R (34.1 kDa) is shown in Fig. 2. The presence of the semi-conserved active-site pentapeptide, Gly-His-Ser-Leu-Gly (positions 103–107), in the N-terminal region of the protein provided support for the identification of *Est*R as a member of esterases/lipases. On the basis of sequence comparisons with the lactone-specific esterase protein from *Pseudomonas fluorescens*, it can be concluded that Ser^{105}, His^{265} and Asp^{237} comprise the catalytic triad. $His^{36}Gly^{37}$ forms a putative oxyanion hole (Fig. 2).

Sequence comparison of EstR with other esterases

An alignment of the *Est*R sequence with corresponding sequences from other bacterial esterases (DNAStar) was used to construct the phylogenetic tree depicted in Fig. 3. The results revealed that *Est*R from *Ralstonia* M1 displays highest identity (64–70%) to corresponding ones from *Burkholderia* strains (Table 1), 38–44% identity to the lactone-specific esterases from *P. fluorescens* and *Mesorhizobium loti*, and 45–48% identity to esterases from *Ralstonia* strains. The active site pentapeptide GHSLG is entirely conserved throughout these esterases.

Expression and purification of the esterase

To overexpress the recombinant esterase in *E. coli*, the expression plasmid p*Est*R containing the esterase gene under the control of T7 promoter was constructed (Fig. 1). The esterase *Est*R was expressed from this plasmid in *E. coli* BL21 at a level of 41 g protein per gram (wet weight) of cells (Fig. 4, lane 2). *Est*R was purified by Ni–NTA chromatography. SDS–PAGE analysis showed only a single band (Fig. 4, lane 3). The molecular mass determined by SDS–PAGE (Fig. 4, lanes 2 and 3) was in good agreement with that calculated (34.1 kDa).

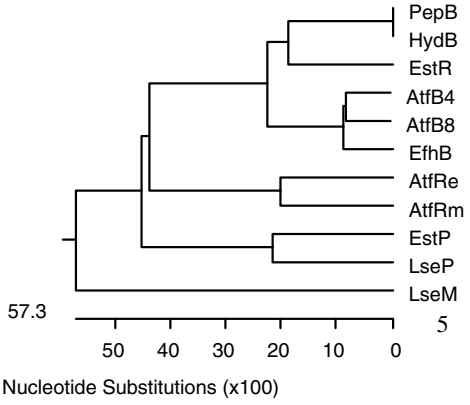

Fig. 3. Phylogenetic analysis of the esterases, based on the comparison of deduced amino acid sequences of the mature proteins. The phylogenetic tree was constructed using the DNAStar program. The following sequences were obtained from GenBank: PepB (peptidase from *Burkholderia pseudomallei* K96243, Accession No. YP_111976); HydB (hydrolase from *B. mallei* ATCC 23344, YP_104940); AtfB4 (hydrolase/acyltransferase from *B. cepacia* R18194, ZP_00217547); AtfB8 (hydrolase/acyltransferase from *B. cepacia* R1808, ZP_00222665); EfhB (ethyl ferulate hydrolase from *B. cepacia*, AAV97951); LseP (lactone-specific esterase from *P. fluorescens*, AAC36352); *Est*P (esterase from *P. fluorescens*, CAC34851); AtfRm (hydrolase/acyltransferase from *Ralstonia metallidurans* CH34, ZP_00023622); *Est*R (esterase from *Ralstonia* sp. M1, this study, AY320282); LseM (lactone-specific esterase from *M. loti*, NP_102005); AtfRe (hydrolase/acyltransferase from *Ralstonia eutropha* JMP134, ZP_00202627).

Table 1
Comparison of the sequences of the M1 esterase *Est*R with corresponding sequences from GenBank

Esterase-producing strain	Protein	%	Accession No.
B. pseudomallei K96243	Pepidase	69.6	YP_111976
B. mallei ATCC 23344	Hydrolase	69.6	YP_104940
B. cepacia R18194	Hydrolase/acyltransferase	66.2	ZP_00217547
B. cepacia R1808	Hydrolase/acyltransferase	65.5	ZP_00222665
B. cepacia	Ethyl ferulate-hydrolase	63.9	AAV97951
P. fluorescens	Lactone-specific esterase	44.3	AAC36352
P. fluorescens	Putative esterase	41.6	CAC34851
R. metallidurans CH34	Hydrolase/acyltransferase	44.6	ZP_00023622
Ralstonia sp. M1	Esterase	—	AY320282
Ralstonia eutropha JMP134	Hydrolase/acyltransferase	48.1	ZP_00202627
Mesorhizobium loti	Lactone-specific esterase	37.8	NP_102005

Substrate specificity

To determine the substrate specificity, the esterase *Est*R activity towards *p*-NP esters of different carbon chain length was measured spectrometrically at 405 nm (for C2–C8 acyl group) and 420 nm (for C14–C16 acyl group) at pH 8.0 and 55 °C. Among *p*-NP esters, the esterase *Est*R displayed the highest activity towards *p*-NP caproate (C6 acyl group) and fixed as 100%. The typical profile of chain length specificity of this esterase towards *p*-NP esters is

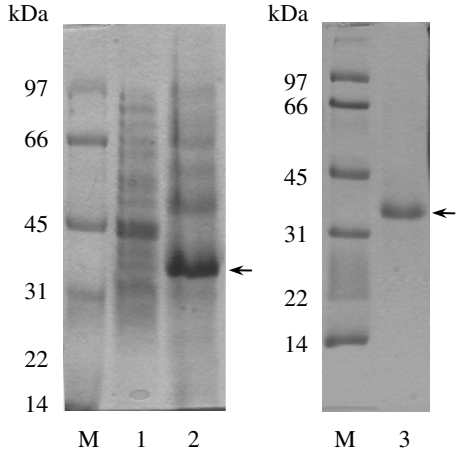

Fig. 4. SDS–PAGE of the overexpressed and Ni–NTA purified, tagged esterase *Est*R in *E. coli* BL21. Proteins were stained with Coomassie Brilliant Blue. Lane M, size standards (molecular weights indicated in kDa); lane 1, lysate of *E. coli* p*Est*R cells before IPTG induction; lane 2, lysate of *E. coli* p*Est*R cells before IPTG induction; lane 3, Ni–NTA-purified esterase *Est*Rhis.

Table 2
Activity[a] and substrate specificity of esterase from *Ralstonia* sp. M1

Substrate	Specific activity (units/mg protein)	Relative activity (%)
p-Nitrophenyl acetate	18.3	18
p-Nitrophenyl propionate	53.1	51
p-Nitrophenyl caproate	104.5	100
p-Nitrophenyl caprylate	67.5	65
p-Nitrophenyl myristate	ND[b]	—
p-Nitrophenyl palmitate	ND[b]	—

[a] The esterase activity was measured by spectrophotometric assay with 10 mM of *p*-nitrophenyl ester as a substrate and 20 μg of the purified esterase *Est*R at pH 8.0 and 55 °C.
[b] ND, not detected under specified conditions.

shown in Table 2. The activity towards *p*-NP myristate (C14 acyl group) and palmitate (C16 acyl group) was not detected under the specified conditions. The activity of the esterase *Est*R towards *p*-NP acetate (C2 acyl group), propionate (C3 acyl group) and caprylate (C8 acyl group) was 18, 51 and 65% of that towards *p*-NP caproate, respectively.

Effect of temperature on esterase activity

The optimum temperature of the esterase *Est*R was investigated using *p*-nitrophenyl caproate (C6 acyl) as a substrate at pH 9.0. The maximum temperature was 60 °C for hydrolysis of *p*-nitrophenyl caproate (Fig. 5). The esterase activity increased very gradually from 51% at 25 °C to the maximum of 100% at 60 °C and then decreased also gradually to 41% at 85 °C. The esterase is most active from 45 to 65 °C (86–100%). This esterase belongs to thermoactive enzymes.

Effect of pH on esterase activity

The optimum activity of the esterase *Est*R was investigated at 55 °C using *p*-nitrophenyl caproate as a substrate with a pH range from 4.0 to 11.0 (Fig. 6). The esterase showed maximum activity at pH 9.0. The relative activity increased very gradually from 8% at pH 4.0 to 58% at pH 7.0, then it remained relatively constant at 86–100% in a pH

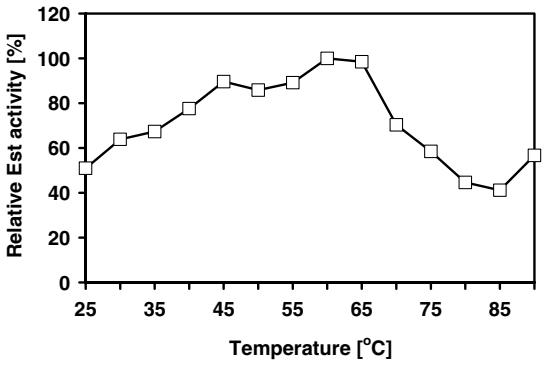

Fig. 5. Effect of temperature on the esterase activity. The activity of 20 μg of the purified esterase *Est*R was measured by spectrophotometer with 10 mM *p*-nitrophenyl caproate as a substrate at pH 8.0 and various temperatures 25–90 °C.

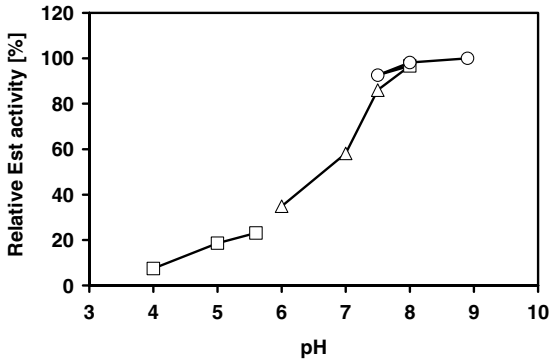

Fig. 6. Effect of pH on the esterase activity. The activity of 20 μg of the purified esterase *Est*R was measured by spectrophotometer with 10 mM *p*-nitrophenyl caproate as a substrate at 55 °C and various pH 4.0–11.0.

range from 7.5 to 9.0. When pH exceeded 9.0, the autohydrolysis was higher than the values measured at pH 9.0 and values of samples added with the enzyme. So, the residual activity could not be evaluated.

Effect of organic solvents on esterase activity

The effect of 10 and 30% (v/v) of organic solvents (Table 3) on esterase activity was investigated by incubating the esterase *Est*R for 30 min at 30 °C in 0.1 M Tris–HCl buffer (pH 8.0). In general, the esterase *Est*R from *Ralstonia* sp. M1 was very resistant to most organic solvents tested, except for 1- and *tert*-butanol. No significant effect on esterase *Est*R was detected after incubation with 10 and 30% of organic solvents for 30 min with these organic solvents, the esterase activity decreased or increased by up to 32% and up to 16% in comparison with the control sample without treatment, respectively. However, ethanolamine stimulated the esterase *Est*R strongly. The addition of 10 and 30% of ethanolamine increased the activity 6- to 15-fold, respectively. In contrast to other organic solvents, the addition of

Table 3
Effect of organic solvents on esterase activity[a]

Solvent	Remaining activity (%) at concentration (%) of	
	10	30
Methanol	98	110
Ethanol	100	104
Isopropanol	90	90
1-Butanol	19	14
tert-Butanol	86	22
Dichloromethane	116	78
Ethyl acetate	86	69
Acetonitrile	90	99
Acetone	88	68
Ethanolamine	569	1482
Hexane	99	88
DMSO	87	96
DMFA	96	97

[a] Twenty micrograms of the purified esterase *Est*R was incubated in 0.1 M Tris buffer, pH 8.0 at 30 °C for 30 min with 10 or 30% (v/v) of different solvents. The esterase activity was measured by spectrophotometric assay with 10 mM of *p*-nitrophenyl caproate as a substrate at pH 8.0 and 55 °C.

Table 4
Effect of metal ions and inhibitors on esterase activity[a]

Compound	Remaining activity (%) at concentration (mM) of		
	0.1	1	10
$CaCl_2$	88	87	96
$CuCl_2$	91	93	85
$FeCl_3$	88	73	83
$MgCl_2$	101	87	90
$MnCl_2$	94	95	92
$SnCl_2$	92	100	110
$ZnCl_2$	85	87	80
DEPC	96	88	90
EDTA	105	101	85
PMSF	77	80	17

[a] Twenty micrograms of the purified esterase *Est*R was incubated in 0.1 M Tris buffer, pH 8.0, at 30 °C for 30 min with 0.1, 1, or 10 mM of different metal ions or inhibitors. The esterase activity was measured by spectrophotometric assay with 10 mM of *p*-nitrophenyl caproate as a substrate at pH 8.0 and 55 °C.

10 and 30% of 1-butanol and 30% of *tert*-butanol inhibited the esterase strongly. The residual activity remained around 20% in comparison to the control sample.

Effect of ions and inhibitors on esterase activity

The effect of 0.1, 1 and 10 mM of various metal ions was determined by incubating the esterase *Est*R for 30 min in 0.1 M Tris–HCl buffer, pH 8.0. In general, all assayed metal ions showed no significant effect on the esterase *Est*R activity (Table 4). The addition of 0.1, 1 and 10 mM of the metal ions increased and decreased the esterase activity slightly by up to 27 and 10%, respectively (Table 4). No significant effect of the typical lipase/esterase inhibitors on the esterase *Est*R activity was detected except for 10 mM of PMSF (phenylmethanesulfonyl fluoride) with a decrease of 83%.

Effect of detergents on esterase activity

The effect of 0.2, 1 and 5% (w/v) of detergents including Tween 80, Tween 60, Tween 40, Triton X-100, Triton X-45 and SDS was measured by incubating the esterase *Est*R for 30 min at 30 °C in 0.1 M Tris–HCl buffer, pH 8.0. The addition of 0.2% (w/v) of Tween 80, Tween 60 and Tween 40 induced the esterase activation slightly (Table 5). The esterase activity increased by 1–10%. The addition of 1% of these detergents decreased the esterase activity slightly by up to 13%. However, the addition of 5% of these detergents decreased the activity by up to 48%. Triton X-100 showed an inhibitory effect on the esterase activity. The esterase activity decreased by 3–58% when incubating with 0.2–5% of Triton X-100. However, Triton X-45 stimulated the esterase strongly. The activity increased to 2.6–4-fold in comparison with the control sample. In contrast to other detergents, SDS showed a complete inhibition on the esterase activity.

Table 5
Effect of detergents on esterase activity[a]

Detergent	Remaining activity (%) at concentration (%) of		
	0.2	1	5
Tween 80	107	94	55
Tween 60	110	87	75
Tween 40	101	90	52
Triton X-100	97	71	42
Triton X-45	260	396	
SDS	0	0	0

[a] Twenty micrograms of the purified esterase *Est*R was incubated in 0.1 M Tris buffer, pH 8.0 at 30 °C for 30 min with 0.2, 1 or 5% (w/v) of different metal ions or inhibitors. The esterase activity was measured by spectrophotometric assay with 10 mM of *p*-nitrophenyl caproate as a substrate at pH 8.0 and 55 °C.

Discussion

Previously we reported the isolation of a lipase/chaperone gene pair from *Ralstonia* sp. M1, sequencing, analysis and expression in *E. coli*, in vitro refolding and characterization of physicochemical properties [22,23]. In this study, we further described another lipolytic enzyme from *Ralstonia* sp. M1: gene cloning, sequence analysis and expression in *E. coli* and characterization of some biophysical properties.

Sequencing of the 1.6 kb insert in the clone M1-81 revealed a single complete ORF, predicted to encode a lipolytic enzyme *Est*R. Its deduced amino acid sequences (320 aa) exhibited a significant level of homology to the reported sequences of a subset family of esterases from various sources, mainly the genera *Burkholderia* (64–70% identities), *Pseudomonas* (42–45% identities) and *Ralstonia* (48% identities). Similar to other reports on related gene coding for esterases, the ORF of *Ralstonia* sp. M1 esterase utilized GTG as a start codon rather than ATG [8,26]. A relatively high G^+C content (67.6%) was found in the predicted ORF, a typical feature of *Pseudomonas* and *Burkholderia* chromosomes [27].

The *Est*R preprotein is predicted to contain a putative 24-residue leader peptide, which is cleaved to form a 296-amino acid (31.7 kDa) mature protein with a p*I* of 9.86 [28].

The ORF of *Ralstonia* sp. M1 esterase had a consensus motif P-V-L-V-G-H-S-L-G-G. According the ProSite database [29], the lipase family also containing acetylcholine esterases, has a consensus motif of [LIV]-x-[LIVFY]-[LIVMST]-G-[HYWV]-S-x-G-[GSTAC] (S is the active site residue) [30]. Whereas the active site motif for the esterases is F-[GR]-G-x(4)-[LIVM]-x-[LIV]-x-G-x-S-[STAG]-G (S is the active site residue) [5]. Comparison of these motifs yielded that the motif for esterase *Est*R is contained in the lipase motif, not in esterase motif. This confirmed the motif for lipases is also contained in the esterase motif and vice versa [31].

Both lipolytic enzymes, esterases/lipases, show activity towards *p*-nitrophenyl esters, but different profiles. The lipase LipA from *Raltonia* sp. M1 showed the highest activity towards *p*-NP caprylate (C8 acyl group), but also activity towards *p*-NP myristate (C14 acyl group) and

palmitate (C16 acyl group) [22]. However, the esterase *Est*R also from this strain exhibited the highest activity towards *p*-NP caproate (C6 acyl group), but no activity towards *p*-NP esters of C^{14} or C^{16} acyl group was detected. Further, the esterase *Est*R did not show any activity towards triglycerides (data not shown). Other esterases from *Pseudmonas* sp. KCTC 10122BP, *Acinetobacter* sp. strain No. 6, showed the highest activity towards *p*-NP acetate among tested *p*-NP esters [8,12]. The esterase from *Aeropyrum pernix* K1 had activity also against *p*-NP esters of C^{12}, C^{16} and C^{18} acyl group and highest activity against *p*-NP caprylate [32].

One of the important characteristics required for applications in detergent formulations and biotransformations is that the enzyme should be active at high temperature and alkaline pH range. The esterase *Est*R from *Ralstonia* sp. M1 had a temperature optimum at 60–65 °C and pH ranging from 7.5 to 9.0. The residual activity could not be evaluated at pH 10.0–11.0 due to higher autohydrolysis than maximum activity at pH 9.0 and addition of *Est*R. The lipase LipA from the same strain *Ralstonia* sp. M1 showed a maximum activity also at high temperature 55–60 °C and alkaline pH 10.75 [22]. The optimal reaction temperature reported here is lower than that of the esterases from *A. pernix* K1 (90 °C) [32] and from *Archaeoglobus fulgidus* (80 °C) [33], and higher than that of the esterases from *Aspergillus ficuum* (40–45 °C) [34] and from *Fusarium oxysporum* (45 °C) [35]. However, the esterase *Est*R from *Raltonias* sp. M1 exhibited a pH optimum higher than these esterases from *A. pernix* K1 (pH 8.0) [32] and from *F. oxysporum* (pH 7.0) [35].

Similar to the lipase LipA from the same strain *Ralstonia* sp. M1 [22], *Est*R showed a very high resistance against organic solvents. In general, most organic solvents stimulated the activation of the lipase LipA as well as the wild-type enzyme from *Ralstonia* sp. M1 and had no effect on the esterase *Est*R. Ethanolamine induced both the lipase and esterase activation and increased the activity strongly by threefold [22] and 6–15-fold, respectively. 1-Butanol showed an obvious inhibitory effect on the esterase *Est*R and lipase LipA activity, the residual activity decreased to one-half and one-fifth in comparison to the control sample.

Tested ions showed the same effect on *Ralstonia* sp. M1 lipase and esterase activity, the residual activity increased or decreased slightly in both cases. The addition of the chelating agent EDTA decreased the lipase activity, indicating that EDTA accesses the putative Ca^{2+} binding site and removes ions [22], whereas no effect on the esterase activity was found. The addition of DEPC inhibited both the lipase and esterase slightly. The inhibition by histidine residue modifier DEPC suggests the possibility of histidine residues being involved in catalysis as well. PMSF indicated also an inhibitory effect on both lipase LipA [22] and esterase *Est*R activity, and is expected to inhibit lipase/esterase as they contain a serine as part of the catalytic triad.

Detergents induced the lipase activation, and the remaining activity increased up to three times. Whereas only Triton X-45 increased the esterase residual activity up to four times and other detergents showed a slight increase or moderately inhibitory effect. The addition of 1% (w/v) of SDS increased the lipase LipA activity strongly by one-half [22], whereas inhibited the esterase *Est*R completely.

The recombinant *Est*R from *Ralstonia* sp. M1 is an alkaline, thermo-active and highly organic-solvent-resistant and detergent-inducible esterase. These important features render the recombinant esterase *Est*R very attractive for biotechnological applications.

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