

Screening, Cloning and Biochemical Characterisation of Novel Esterases from *Bacillus* sp. associated with the Marine Sponge *Aplysina aerophoba*

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Keywords: marine *Bacillus*, sponge, *Aplysina aerophoba*, esterase

Abstract

Two novel esterases (EstB1 and EstB2) were isolated from a genomic library of *Bacillus* sp. associated with the marine sponge *Aplysina aerophoba*. EstB1 shows low identity (26-44 %) with the published hydrolases of the genus *Bacillus*, whereas EstB2 shows high identity (73-74 %) with the carboxylesterases from *B. cereus* and *B. anthracis*. Both esterases were efficiently expressed in *Escherichia coli* under the control of T7 promoter using the vector pET-22b(+). Recombinant EstB1 was purified in a single step to electrophoretic homogeneity by IMAC. A method for the refolding of inclusion bodies formed by the recombinant EstB2 was established to obtain active enzyme. Substrate specificity of the two enzymes towards *p*-nitrophenyl and methyl esters and the respective kinetic parameters K_m and V_{max} were determined. The temperature optima of EstB1 and EstB2 were determined to be in the range of 30-50°C and 20-35°C, respectively. The pH optima were found to be in the range of 6.5-7.5 and 6.5-8.0, respectively. Both enzymes showed the highest stability in up to 50 % (v/v) DMSO followed by methanol, ethanol and 2-propanol. The influence of high NaCl and KCl concentrations was tested. The inhibition effect of 10-50 mM Zn^{2+} and 50 mM Mg^{2+} and Ca^{2+} ions was observed for both esterases. 1-5 mM PMSF deactivated the enzymes, whereas β -mercaptoethanol, DTT and EDTA had no effect on the enzymes activity.

Introduction

Until nowadays terrestrial micro-organisms have been a great source for novel enzymes and diverse biologically active compounds which are employed directly or modified for biotechnological exploitation. In contrast, marine micro-organisms are hardly used for industrial purposes and even not yet investigated strongly concerning their biotechnological potential. As many of them are adapted to survive in ecological niches at extreme conditions (temperature, pH, high pressure, high salt concentrations), they might be a promising repository of novel biocatalysts and other bioactive compounds.

Sponges (Phylum Porifera) are one of the oldest living organisms whose origin dates back to the Precambrian more than 600 millions years ago (Brusca and Brusea 1999). Sponges are associated with a large amount of different micro-organisms including cyanobacteria (Vacelet 1971), diverse heterotrophic bacteria (Hentschel et al. 2002), unicellular algae (Wilkinson 1992) and zoochlorellae (Gilbert and Allen 1973). Although in some of the sponges (e.g. the Mediterranean *Aplysina aerophoba*) the associated micro-organisms constitute up to 40 % of

the host biomass (Vacelet 1971), they are hardly characterized and nearly nothing is known about their physiological role in the symbiosis or associations with their hosts.

Recently we have isolated and characterized a novel *Bacillus* strain associated with the Mediterranean sponge *Aplysina aerophoba* (will be published elsewhere). This paper describes the cloning, sequencing and expression of two novel esterases originally produced from this marine *Bacillus* strain.

Hydrolytic enzymes such as lipases (EC 3.1.1.3) and esterases (EC 3.1.1.1) still belong to the most important biocatalysts because they accept a broad range of non-natural substrates, they are usually very stable in organic solvents and some of them exhibit high stereoselectivity, for example in the kinetic resolution of racemates or the desymmetrization of prostereogenic compounds (Bornscheuer and Kazlauskas 1999). The three-dimensional structures of both enzymes show the characteristic α/β fold (Ollis et al. 1992) composed of α -helices and β -sheets, which is also found in haloperoxidases and epoxide hydrolases. Most of these hydrolases contain the consensus sequence Gly-X-Ser-X-Gly around the active site serine and function via a Ser-Asp-His catalytic triad (Glu instead of Asp in some lipases, e.g. *Geotrichum candidum* lipase).

The difference between lipases and esterases is their ability to act on surface display. Esterases act on water soluble carboxyl ester molecules composed of short-chain fatty acids. Lipases catalyse the hydrolysis of fats and oils at water-lipid interface and reversing the reaction in non-aqueous media, due to a hydrophobic domain (lid) covering the active site of lipases. Every year novel biotechnological applications are established using lipases and esterases for synthesis of biopolymers and biodiesel, for the production of pharmaceuticals, agrochemicals and flavour compounds. Therefore, identification and isolation of novel lipases and esterases genes are of special interest of industry.

The objective of this study was isolation and preliminary biochemical characterisation of novel lipases or esterases from the lipolytic active *Bacillus* species associated with the marine sponge *Aplysina aerophoba*.

Materials and Methods

Chemicals and reagents

Artificial salt water (ASW) was obtained from the Zoological and Botanical Garden Wilhelma, Stuttgart, Germany. Other chemicals used in this work were purchased from the commercially available sources.

Bacterial strains, plasmids and culture conditions

Bacillus sp. was isolated from the mesohyl of the homogenised marine sponge *Aplysina aerophoba* under aerobic conditions on ASW agar plates (Brümmer et. al., manuscript in preparation) and grown in ASW based media containing 75% (v/v) sterile filtrated ASW, 0.5 % (w/v) peptone and 0.01 % (w/v) yeast extract at 30°C. *Escherichia coli* DH5 α (Clontech, Heidelberg, Germany) and the plasmid pUC18 were used for the construction of genomic DNA libraries of *Bacillus* sp.. *E. coli* BL21(DE3) (Novagen, Madison, USA) was used as host cells for the transformation of expression vector pET-22b(+) (Novagen). *E. coli* strains were grown in Luria-Bertani (LB) liquid medium or on LB agar plates at 30°C or 37°C (Luria et al. 1960). The media was supplemented with 100 μ g/ml ampicillin for the selection of plasmids. For experiments utilizing α -complementation, isopropyl-thio- β -galactoside (IPTG) and 5-bromo-4-chloro-3-indoyl- β -D-galactoside (X-Gal) were added to LB agar media at concentrations of 119 mg/l and 40 mg/l, respectively.

DNA isolation and manipulation

Restriction analysis and DNA modifications were performed using enzymes purchased from MBI Fermentas (MBI Fermentas, St. Leon-Rot, Germany) according to the recommendations of the manufacturer. Plasmid DNA was isolated using Plasmid Midi Kit (Qiagen, Hilden, Germany). QIAquick Gel extraction Kit (Qiagen) was used to recover DNA fragments from agarose gels. Chromosomal DNA of *Bacillus* sp. was prepared according to the method of Lebaron (Lebaron et al., 1998). Recombinant DNA manipulations were carried out using standard protocols (Sambrook et al., 1989). A method of Chung (Chung et al. 1989) was used to transform *E. coli* with plasmid DNA.

Construction of a *Bacillus* sp. genomic DNA library and screening for lipolytic enzymes

Chromosomal DNA from *Bacillus* sp. was isolated and digested with *EcoRI*, *PstI*, *HindIII* or *NdeI*. 1.5 to 6.0 kilobase pair (kbp) DNA fragments were excised from 1% agarose gels and DNA was recovered. The pUC18 vector was digested with *EcoRI*, *PstI*, *HindIII* or *NdeI*, dephosphorylated with calf intestinal alkaline phosphatase and ligated with the chromosomal fragments, which were cut with the respective nuclease. Ligation was done using T4 DNA ligase from MBI for 10-12 h at 16°C. Chemically competent *E. coli* cells were transformed with the ligation products individually and plated onto LB agar plates containing X-Gal and IPTG such that 50 to 100 colonies were visible after overnight growth. The colonies were transferred onto sterile flax filters and replicated onto LB agar plates supplemented with 1 %

tributylin. The identity of putative positive clones was confirmed by restriction analysis and DNA sequencing.

DNA Sequencing

DNA cycle sequencing reactions (Sanger et al., 1997) were carried out on both strands of double-stranded templates, using the big dye terminator kit (Perkin Elmer, Wellesley, Mass., USA). The sequencing products were analysed on ABI 373A DNA sequencer (Applied Biosystems, Foster City, CA, USA). Computer analysis of the DNA sequences and the deduced amino acid sequences was performed with the Software SeqMan™ II of the DNASTar packet. Search for nucleotide and amino acid sequence similarities was done on the web server of the National Centre for Biotechnology Information (<http://www.ncbi.nlm.nih.gov>) in the non-redundant nucleotide (nr-nt) or amino acid (nr-aa) databases using BLASTX, BLASTP and BLASTN programmes (Atschul et al. 1990).

Protein sequencing

N-terminal amino acid sequencing of the purified EstB2 was based on the degradation method developed by Edmann (Edmann 1950) and carried out with a protein sequencer 470A (Applied Biosystems).

Cloning of the *estB1* and *estB2*

The *estB1* and *estB2* genes were amplified from the inserts (*insBS1* and *insBS2*) of two clones of the *Bacillus* sp. genomic DNA library with lipolytic activity. The *estB1* gene was amplified using the oligonucleotides 5'-GGAATTCCATATGATGGGGAGCAACAACG-3' and 5'-ATAAGAATGCGGCCGCTTT CTCTAAAAAGTCAG-3'. The *NdeI* site was introduced at the 5'-end of the gene, whereby the *NotI* site was introduced at the 3'-end, thus generating a fusion protein with six His of the vector. The PCR fragments were cleaved with *NdeI* and *NotI* and inserted into the respective sites of pET-22b(+) resulting in pET-*estB1*. The *estB2* gene was amplified with two primers (5'-CGCGGATCCATGAAAGTTGTTGCACCAAAG-3' and 5'-ATAAGAATGCGGCCGCTT ATTCTTCCAGTCAAGTCC-3'), which introduced a *BamHI* site before the start codon and a *NotI* site just after the stop codon. The PCR fragments were cleaved with *BamHI* and *NotI* and ligated into the respective sites of pET-22b(+) to give pET-*estB2*.

Expression of the *estB1* and *estB2*

E. coli BL21(DE3) were freshly transformed with the expression vectors pET-*estB1* and pET-*estB2* and grown at 37°C in 400 ml LB media until the early exponential phase (OD₆₀₀ 0.4-0.8). The EstB1 and EstB2 production was induced with 0.01-1 mM IPTG and cultivation was continued at 30°C for 4 h. *E. coli* cells were then harvested by centrifugation (4000 g) and washed twice with 50 mM potassium phosphate buffer, pH 7.5, 4°C. *E. coli* cell extracts were prepared by sonication (3 times for 2 min, the power level set between 4 and 5, 50 % output, Branson Sonifier 250 (Branson, Dietzenbach, Germany)) and used for purification or to assay esterase activity.

Purification of the EstB1

Purification of the His₆-tagged EstB1 esterase was performed under native conditions by IMAC using the cobalt-based TALON™ Superflow Resin (Clontech, Palo, Alto, USA) according to the manufacturers recommendations.

Refolding of the EstB2

Isolation of inclusion bodies was performed according to the method of Rudolph and Lilie (Rudolph and Lilie 1996). The pure inclusion bodies were dissolved in extraction buffer (50 mM potassium phosphate buffer, pH 7.5, 5 M urea, 1 mM DTT and 1 mM EDTA) to a final protein concentration of 1 mg/ml and incubated for 3 h at RT with slight agitation. The solubilized inclusion bodies were dialyzed at 4°C for 10-12 h with 50 mM potassium phosphate buffer, pH 7.5, 1 mM DTT and 1 mM EDTA.

SDS-PAGE

Proteins from the crude extracts and from various purification steps were analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (Laemmli 1970). A low molecular weight (LMW) standard (BioRad Laboratories, Richmond, USA) was used as a reference.

Measurement of hydrolase activity

Esterase activity was assayed and visualized on zymograms using 12.5 % SDS-polyacrylamide gels. The protein samples containing esterases were separated by SDS-PAGE and renatured in 100 mM Tris-HCl buffer, pH 7.5 containing 0.5 % Triton X-100 for 4 h at 4°C. The gels were finally incubated for 5 min at RT in developing solution consisting of 3

mM α -naphthyl acetate, 1 mM Fast Red TR (Sigma), and 100 mM sodium phosphate buffer, pH 7.5. Esterase activity was detected by the appearance of purple coloured bands in the gels. The esterase activity of the crude extracts and purified enzymes was measured spectrophotometrically towards *p*-nitrophenyl (*p*NP) esters (Bornscheuer and Kazlauskas 1999) and by a pH-Stat assay towards methyl esters (Peled and Krenz 1981). One unit of esterase activity was defined as the amount of enzyme that released 1 μ mol of *p*-nitrophenol per min or 1 μ mol of acetic acid per min under assay conditions.

Chloroperoxidase activity of the EstB1 was measured spectrophotometrically towards monochlorodimedone as described by Wiesner and co-workers (Wiesner et al. 1976).

Biochemical Characterisations

The temperature and pH optimum of the EstB1 and EstB2 were determined by a pH-Stat assay towards 200 mM methyl acetate, which ensured a large excess of substrate. The temperature optimum was performed at pH 7.5 after 5 min incubation of the esterase at a given temperature. The pH optimum was measured after 5 min incubation of the enzyme at 35°C. 50 mM acetate buffer, sodium phosphate buffer and Tris-HCl were used for the measurements in pH ranges of 2-5, 5-7.5 and 7.5-10, respectively. The influence of 10-50 % (v/v) organic solvents, 0.1-2.0 M NaCl and KCl salts, metal ions at the 1-10 mM concentrations and 1-10 mM PMSF and EDTA on the EstB1 and EstB2 activity was measured by standard spectrometric assay towards *p*NP acetate. The effect of β -mercaptoethanol and DTT (1-10 mM) was measured by a pH-stat assay using methyl acetate as a substrate. In each experiment 0.2 mg of the purified EstB1 and EstB2 were used. Kinetic constants K_m and V_{max} for the hydrolysis of *p*NP acetate and *p*NP butyrate were measured spectrophotometrically at pH 7.5 using 0.05 mg of the purified EstB1 or EstB2. *p*NP esters were used at concentrations ranging from 10 mM to 150 mM. All values were determined in triplicate and were corrected for the autohydrolysis of the substrates. The deviations for all data were between 0.2 % and 5.3 %.

Nucleotide sequence accession number

The nucleotide sequences of the *estB1* and *estB2* have been deposited in the National Centre for Biotechnological Information (Bethesda, Md.) and assigned the accession numbers AY640622 and AY640623, respectively.

Results

Isolation of esterase-encoding fragments from a genomic library of *Bacillus* sp. nucleotide sequence analysis

In the DNA library pool consisting of 93000 clones one *Nde*I and one *Eco*RI clone exhibiting lipolytic activity were detected. The plasmids of these clones were isolated and analysed by restriction analysis. The DNA inserts of these plasmids were designated *insBS1* (2.2 kb) and *insBS2* (1.9 kb) and sequenced by primer walking on both strands. The lipolytic activity encoded in the *insBS1* and *insBS2* could not be induced with IPTG, indicating that the genes were transcribed from their own promoters.

Sequence analysis of the 2283 bp *insBS1* fragment revealed three open reading frames (ORFs). The deduced amino acid sequence revealed an 942-bp ORF1 extending from an ATG start codon at position 192 to the TAG stop codon at position 1134. A putative ribosomal binding site was identified 15 bps upstream of the ATG codon, which most probably represents the translation initiation site. Two possible -10 and -35 promoter regions were found at positions 120-125 and 97-102; 89-93 and 58-63 respectively. ORF1, designated *estB1*, encodes a putative 314-amino acid protein called EstB1 with a predicted molecular weight of 35.30 kDa. It exhibited similarity to the amino acid sequences of the 3-oxoadipate enol-lactonase from *Bacillus cereus* ATCC 14579 (44 % identity), abhydrolase α/β hydrolase fold from *Bacillus anthracis* A2012 (45 % identity) and the putative chloroperoxidase from *Clostridium acetobutylicum* (26 % identity). The amino acid sequence Gly-Trp-Ser-Thr-Gly, (residues 114 to 117) found in the EstB1, fits the Gly-X1-Ser-X2-Gly motif found in most bacterial lipases and esterases. Two other ORFs (ORF2 and ORF3) found in the *insBS1* were located downstream from the *estB1* in the same orientation like *estB1*. The ORF2 encodes a putative protein of 213 amino acid with a predicted molecular weight of 22.69 kDa. It shows similarity to the 3-oxyacyl reductase from *Thermotoga maritime* (51 % identity) and the adh_short chain dehydrogenase from *Bacillus anthracis* A2012 (47 % identity).

A 744-bp ORF designated *estB2* with an ATG start codon at position 1085 and a TAA stop codon at position 1829 was detected in the 1977 bp *insBS2*. This ORF encodes a putative protein called EstB2 composed of 248 amino acids with a deduced molecular weight of 28.32 kDa. A putative ribosomal binding site is located 15 bp upstream of the ATG start codon. Putative -10 and -35 promoter consensus regions were detected at 1117-1021 and 986-990 positions, correspondently. The EstB2 shows high similarity to the carboxylesterase from *Bacillus cereus* ATCC 14579 (74 % identity) and proteins of the dienelactone hydrolase

family from *Bacillus anthracis* A2012 (73 % identity). The consensus sequence Gly-Leu-Ser-Leu-Gly was found at positions 91 to 94 in the EstB2. The upstream and downstream flanking sequences of the *estB2* were analysed for putative ORFs, but no significant similarity to published sequences was found.

Cloning and expression of the *estB1* and *estB2*

The *estB1* and *estB2* were PCR amplified from a genomic DNA library of *Bacillus* sp. and inserted into the expression vector pET-22b(+) resulting pET-*estB1* and pET-*estB2* vectors, correspondently. The designed medium-copy vectors allowed the expression of the cloned sequences in *E. coli* BL21(DE3) under the control of T7 promoter.

Recombinant expression of *estB1* and *estB2* was monitored by SDS-PAGE, zymogram and activity measurements with *pNP* acetate. The SDS-PAGE analysis of *E. coli* BL21(DE3)/pET-*estB1* lysates indicated the presence of a 35 kDa protein, which corresponds to the theoretically calculated mass of the EstB1 (**fig. 1 A**). The SDS-PAGE revealed that the induction time does not play a crucial role in the recombinant EstB1 production. The amount of the recombinant protein increased rapidly within the first hour of induction and was found to be nearly constant during the following induction time. When measuring the enzymatic activity (U/mg total protein) of the *estB1*, a tendency to slightly increased values with longer induction times was recognized (data not shown). The highest activity (10 U/mg total protein) was observed in the cell lysate obtained from a 500-ml culture after 4 h induction as measured spectrophotometrically towards *pNP* acetate.

The SDS-PAGE analysis of *E. coli* BL21(DE3)/pET-*estB2* revealed two proteins of 28 kDa and 32 kDa, respectively (**fig. 2 A**). Based on the N-terminal sequences of two proteins (MKYLLPTAAAGLLLLAAQPAMAMDIGINSDPMKV for the protein at 32 kDa, MDIGINSDPMKVVAPKPFTFE for the protein at 28 kDa) the bands could be referred to the EstB2 with and without the *pelB* leader sequence. The EstB2 with *pelB* leader sequence was first expressed to the cytoplasm and then it was exported to the periplasm where the leader sequence was deleted by the signal peptidase. Analogue to EstB1 also the amount of EstB2 increased rapidly within the first hour of induction and remained constant until the cell harvesting. 1 U/ml of the esterase activity was obtained from a 500-ml culture after 4 h induction. Although induction of the gene expression was done at various conditions (temperature, concentration of IPTG), the major part of the EstB2 was consistently produced as inclusion bodies.

Kommentar: Seite: 10
Besser auf Proteinmenge beziehen
als auf volumen

FIGURE 1

FIGURE 2

Purification of the EstB1

The EstB1 was purified from a crude extract of *E. coli* BL21(DE3)/pET-*estB1* in a single step by immobilized cobalt ion affinity chromatography, which yielded an almost homogeneous esterase. The purity of the EstB1 was confirmed by SDS-PAGE (**fig. 1 B**). The specific activity of the purified EstB1 towards *p*NP acetate consisted of 96 U/mg which corresponds to a purification factor of 34. 50 mg active EstB1 was obtained from 1 l of cultural broth. Based on the catalytic activity of the purified esterase, the expression level of the active enzyme in recombinant *E. coli* was estimated to be 20 % of the total soluble protein.

Refolding of the EstB2

Refolding was performed to convert the EstB2 inclusion bodies into soluble, active protein. Different urea concentrations were tested to denature the inclusion bodies. Full solubilisation was observed in 5 M urea, therefore this concentration was used for further refolding processes. The homogeneity of the refolded protein is demonstrated by SDS-PAGE (**fig. 2 B**). The specific activity of the refolded EstB2 towards *p*NP acetate was 31 U/mg. This corresponded to 40 mg of the active EstB2 obtained after refolding from 1 l of cultural broth.

Biochemical Characterisation of the EstB1 and EstB2

Substrate Specificity

The substrate specificity of the purified from *E. coli* EstB1 and EstB2 was studied by using various *p*NP esters and methyl esters of straight-chain fatty acids ranging in chain length from C₂ (acetate) to C₁₄ (myristate). The substrate specificity of the EstB1 and EstB2 is represented in **Table 1**. In the case of *p*NP esters EstB1 showed the highest activity towards *p*NP acetate (96 U/mg), *p*NP butyrate (147 U/mg) and *p*NP caproate (23 U/mg). K_m and V_{max} of the EstB1 were determined with *p*NP acetate and *p*NP butyrate as substrate and calculated from Lineweaver-Burk plots using a least-squares best fit of the Michaelis-Menten equation (Table 1). The highest activity of the EstB1 towards acyclic aliphatic esters was observed with methyl acetate (111 U/mg), methyl butyrate (109 U/mg) and methyl caproate (76 U/mg) as substrates. Since the EstB1 shows similarity to the 3-oxoadipate enol-lactonase and a putative chloroperoxidase, the enzyme was additionally tested for lactonase and chloroperoxidase

activity. No detectable lactonase or chloroperoxidase activity could be detected. The highest activity of the EstB2 with *p*NP esters was measured towards *p*NP acetate (31 U/mg), *p*NP butyrate (52 U/mg) and *p*NP caproate (25 U/mg). K_m and V_{max} values of the EstB2 were calculated from Lineweaver-Burk plots derived from the initial measurements of the rates of *p*NP acetate and *p*NP butyrate hydrolysis (Table 1). The maximum specific activity of the EstB2 towards methyl esters was observed with methyl acetate (48 U/mg), methyl butyrate (44 U/mg) and methyl caproate (22 U/mg). The activity of the EstB2 decreased with the acyl chain length, whereby the EstB2 preferentially hydrolyses *p*NP acetate and methyl butyrate. Since the EstB2 shows similarity on amino acid level to proteins of the lactonase family, lactonase activity towards γ -butyrolactone and δ -hexanolactone was also measured. The specific activity of the EstB2 purified from *E. coli* was 2 U/mg towards γ -butyrolactone and δ -hexanolactone, correspondently.

TABLE1

Temperature and pH Optimum

Different temperature and pH optima were observed in comparative studies of the EstB1 and EstB2 esterases (**fig. 3a, 3b**). The EstB1 exhibited its temperature optimum in a broad temperature range between 30°C (84 %) and 50°C (62 %), that indicates a slight thermophilic character of the enzyme. The temperature optimum of the EstB2 esterase was observed in a temperature range between 20°C and 35°C. The temperature profile of the EstB2 showed a steadily increasing activity up to 35°C. 33 %, 64 % and 98 % of EstB2 activity was measured at 10°C, 20°C and 30°C, respectively. Further increasing of the temperature resulted in a strong inactivation of the enzyme. The complete inactivation of the EstB2 was observed at 40°C.

The EstB1 showed the highest activity under neutral conditions between pH 6.5 and pH 7.5. The activity of the EstB1 was 20 % and 75 % at pH 6.0 and pH 6.5, correspondently with its maximum at pH 7.5. The activity of the EstB2 was 54 % and 42 % at pH 6.5 and pH 8.0, respectively.

FIGURE 3

Influence of organic solvents

The influence of DMSO, methanol, ethanol and 2-propanol on the EstB1 and EstB2 activities is represented on **fig. 4a** and **fig. 4b**, respectively. The EstB2 activity was less influenced by DMSO in comparison with the EstB1. The activities of the EstB1 measured with 20 % (v/v) and 50 % (v/v) DMSO consisted 71 % and 7 %, respectively. 75 % and 41 % of the EstB2 activity was measured with 20 % (v/v) and 50 % (v/v) DMSO. Additionally, the stability of both esterases in 10 % (v/v) DMSO was determined during 1 h at 35°C. No loss of activity neither of EstB1 nor of EstB2 was observed during incubation. Methanol stronger inhibited the EstB1 and EstB2 activity in comparison with DMSO. 47 % and 73 % of the EstB1 and EstB2 activity was measured with 10 % (v/v) of methanol, correspondently. 30 % (v/v) methanol led to the complete deactivation of the EstB1 and EstB2 esterases. The strongest inhibition effect on the EstB1 and EstB2 activity was observed with ethanol and 2-propanol. 25 % and 54 % of the EstB1 and EstB2 activity was measured with 10 % (v/v) ethanol, correspondently. In the presence of 10 % (v/v) 2-propanol 15 % and 2 % of the EstB1 and EstB2 activity was detected.

FIGURE 4

Influence of salt concentrations

Since the EstB1 and EstB2 esterases were isolated from marine *Bacillus*, the influence of 0.1-2 M NaCl and KCl salts on the enzymes activity was determined (**fig. 5a** and **fig. 5b**). The EstB1 was found to be more stable in NaCl and KCl salts in comparison with the EstB2. NaCl and KCl similar inhibited the EstB1 activity. Up to a concentration of 0.5 M KCl had less influence on the EstB2 activity compared with NaCl. The activity of the EstB1 in the presence of 1 M of NaCl and KCl was 94 % and 88 %, correspondently. The increase of NaCl and KCl concentrations to 2 M resulted in 69 % and 80 % activity of the EstB1, respectively. 52 % and 68 % of the EstB2 activity was observed with 1 M of NaCl and KCl, whereby 2 M NaCl and KCl led to the decrease of activity to 29 % and 53 %, correspondently.

FIGURE 5

Influence of metal ions

The EstB1 and EstB2 activity was strongly inhibited by Zn^{2+} ions. The inhibition of the EstB1 was observed with 1 mM Zn^{2+} and consisted 26 % activity. 10 mM Zn^{2+} ions decreased the EstB1 activity to 12 % and 50 mM Zn^{2+} ions complete deactivated the EstB1 esterase. The strong inhibition effect of 1 mM Zn^{2+} ions was observed for the EstB2 (11 % activity), whereby 10 mM Zn^{2+} ions completely inactivated the protein. Mg^{2+} and Ca^{2+} ions have less influence on the EstB1 and EstB2 activity in comparison with Zn^{2+} ions. The EstB1 was less influenced by Mg^{2+} ions then by Ca^{2+} ions. No influence on the EstB1 activity was measured in the presence of 1-10 mM Mg^{2+} and Ca^{2+} ions, whereas 50 mM Mg^{2+} and Ca^{2+} ions lowered the EstB1 activity to 89 % and 51 %, correspondently. 79 % and 28 % activity was measured for the EstB1 with 100 mM Mg^{2+} and Ca^{2+} ions, respectively. Contrary to the EstB1, Mg^{2+} and Ca^{2+} ions influenced the EstB2 activity in a similar way. The decrease of the EstB2 activity to 31 % and 35 % was measured with 50 mM Mg^{2+} and Ca^{2+} ions, respectively. The increasing the Mg^{2+} and Ca^{2+} concentrations to 100 mM resulted in 12 % and 9 % of the EstB2 activity.

Influence of the inhibitors PMSF, β -mercapthoethanol, DTT and EDTA

Of the inhibitors analysed, PMSF was the most effective in the inhibition of the EstB1 and EstB2 esterases. 57 % of the EstB1 activity was observed in the presence of 1 mM PMSF. 5 mM PMSF completely deactivated the enzyme. The EstB2 was fully deactivated already by 1 mM PMSF. No effect on the EstB1 and EstB2 activity was observed in the presence of 1-10 mM β -mercapthoethanol, DTT and EDTA.

Discussion

Two novel esterase genes *estB1* and *estB2* were isolated from a genomic DNA library of *Bacillus* sp. associated with the Mediterranean sponge *Aplysina aerophoba*. The presence of putative transcription regions detected upstream of the *estB1* and *estB2*, suggests that they are transcribed from the original *Bacillus* promoters. Due to the lack of other ORFs upstream or downstream of the *estB2* it was assumed that it is transcribed monocistronically. The protein sequence alignment of the first esterase EstB1 revealed low similarity to the 3-oxadipate enol-lactonase from *B. cereus*, α/β hydrolase fold from *B. anthracis* and the putative chloroperoxidase from *Clostridium acetobutylicum*. The alignment of the second esterase EstB2 showed high similarity to the carboxylesterase from *B. cereus* and to the proteins of the

dienelactone hydrolase family from *B. anthracis*. The physiological roles of these esterases and chloroperoxidases are still unknown. Many peroxidases are able to oxidise halide ions, except fluoride, to an electrophilic species which then reacts with an electron-rich acceptor forming carbon-halogen bonds (Morrison and Schonbaum 1976). This in combination with the ever increasing number of halogenated organic compounds isolated from marine algae (van Pee 2001) suggests that peroxidase catalysed halogenation plays a crucial role in the biogenesis of the halogenated compounds in the marine environment. These enzymes contain the catalytic triad and the α/β -hydrolase fold which are typical for the proteins of the serine hydrolase family. Activity of lipases and esterases is dependent on a charge relay system involving an active-site Ser-Asp/Glu-His triad, whereby the active site serine is commonly conserved in a Gly-X-Ser-X-Gly motif with X as a variable residue (Bornscheuer and Kazlauskas 1999). The putative active site serine of the EstB1 and EstB2 is believed to reside in the deduced amino acid sequences Gly-Trp-Ser-Thr-Gly and Gly-Leu-Ser-Leu-Gly, correspondently. PMSF is a potential inhibitor of serine hydrolases. By a nucleophilic reaction the hydroxyl group of serine attacks the sulfonyl group of PMSF which leads to the irreversible sulfonylation of the active serine. The inhibition of the EstB1 and EstB2 with PMSF implies that Ser residues are important for their activity and therefore the EstB1 and EstB2 could belong to the proteins of the serine hydrolase family.

The EstB1 could be efficiently purified from *E. coli* to homogeneity in a single step by IMAC yielding 96 U/mg specific activity as determined by hydrolysis of *p*NP acetate. The major part of the EstB2 expressed in *E. coli* under various temperatures and IPTG concentrations was accumulated in inclusion bodies. The mechanism of the formation of inclusion bodies is still not well understood. It seems that they are formed from intermediates of the folding pathway (Mitraki and King 1998). Chaperons and proteases are major components of the cellular protein quality control system devoted to prevent the occurrence of misfolded polypeptides (Carrio 2000). Traub and co-workers demonstrated that the correct folding of lipases from *Pseudomonas* sp. KWI 56 and *Chromobacterium viscisum* in *E. coli* depends on the presence of three specific chaperons (Traub et al. 2001). In this work a method for the refolding of EstB2 was established which gave 31 U/mg specific activity as determined by hydrolysis of *p*NP acetate. The refolded protein was more than 90 % homogeneous; further purification was not required.

The EstB1 and EstB2 showed a substrate specificity on a set of typical for lipases and esterases substrates including *p*NP esters and methyl esters. The *p*NP and methyl esters with acyl chain length bigger then C₈ were poor substrates for the EstB1 and EstB2. The highest

activity of both enzymes was observed towards *p*NP esters and methyl esters of C2-C6 being typical substrates for esterases. *p*NP esters were hydrolysed by the EstB1 at lower rates compared to methyl esters, whereby no preference on *p*NP- or methyl esters was detected for the EstB2. In case of the EstB1 an increase of fatty acid chain length from C2 to C4 resulted in a decrease of the respective K_m , whereas V_{max} was not influenced. In case of EstB2 increasing the fatty acid chain length from C2 to C4 resulted in a decrease of the K_m and V_{max} values. These trends indicate a preference of both esterases for *p*NP esters with a chain length of C4 and the ability of EstB1 to stabilise the transition state of C2 and C4 substrates in a similar manner.

Due to similarities between the cloned *Bacillus* esterases and chloroperoxidases and lactonases respectively, chloroperoxidase and lactonase activities were additionally tested on both enzymes. Haloperoxidases are widespread in marine environment and have been found in a variety of seaweeds (Tromp 1991). The presence of especially high levels of lipid-extractable halometabolites in some species of sponges and red algae was also detected (Hewson and Hager 1998). Anyway, no chloroperoxidase and lactonase activity was detected for the EstB1; also EstB2 displays very low activity towards lactones compared to *p*NP esters and methyl esters.

The temperature optima of EstB1 and EstB2 was found to be different. EstB1 is highly active in a wide temperature range between 30°C and 50°C that indicates the slight thermophilic property of the enzyme. The temperature optimum of the EstB2 was found to be in a narrow range between 20°C and 35°C. The pH optima of EstB1 and EstB2 were found to be in a narrow neutral range between pH 6.5 and pH 7.5 and pH 6.5 to pH 8.0, respectively.

From literature data it is known that many lipases and esterases are activated (or inhibited) by metal ions. For example, the intracellular esterase from *Serratia marcescens* 345 is activated by Mg^{2+} ions (Bachkatova and Severina 1980), the acetylcholinesterase is activated by monovalent Na^+ , K^+ and divalent Ca^{2+} and Mg^{2+} cations (Hofer 1984), and in the case of pancreatic lipase both alkali and alkali earth metal ions, especially Na^+ , Ca^{2+} and Mg^{2+} stabilized the enzyme (Schandl and Pittner 1984). Several lipases have been reported to be calcium-dependent (Simons 1999). The influence of Zn^{2+} , Ca^{2+} and Mg^{2+} ions on the EstB1 and EstB2 activity was tested in this work: in both cases even small concentrations of Zn^{2+} ions strongly inhibited the EstB1 and EstB2 activity, whereas the addition of Ca^{2+} and Mg^{2+} ions slowly decreased the activity of both esterases.

The influence of different organic solvents was tested on the activity of EstB1 and EstB2 because nearly all lipase- or esterase-catalysed esterification reactions used in

biotechnological applications are performed in non-aqueous medium. Among the tested organic solvents a remarkable stability in up to 50 % (v/v) of DMSO was shown for EstB1 and EstB2. Both esterases were inhibited in a similar way by methanol, ethanol and 2-propanol. The strongest inhibition effect on the EstB1 and EstB2 esterases was measured for 2-propanol followed by ethanol and methanol.

Due to the marine origin of EstB1 and EstB2 the influence of different NaCl and KCl concentrations on the enzymes activity was tested. Both enzymes display a high stability in high NaCl and KCl concentrations, whereby the EstB1 was found to be more stable in comparison with EstB2. Nearly 80 % and 50 % of the EstB1 and EstB2 activity remained in the presence of 2 M KCl.

The influence of different chemicals including EDTA, β -mercaptoethanol and DTT on the EstB1 and EstB2 esterases was determined. It was shown that EDTA, β -mercaptoethanol and DTT had no influence on the EstB1 and EstB2 activity.

In conclusion, the particular microbial ecology of the sponge mesohyl with respect to the high numbers of taxonomically diverse bacteria provides potential for biotechnology in terms of novel enzymes. Two novel esterases from the novel marine *Bacillus* sp. have been cloned, expressed, purified and preliminary biochemical characterised.

Acknowledgements

Part of this work (FB) was supported by the German Federal Ministry of Education and Research (BMBF) through the project Centre of Excellence *BIOTECmarin* (FO345D) and by the Ministry of Science, Research and Arts of the State of Baden-Württemberg and the University of Stuttgart.

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Legends

Figure 1 Expression **A** and purification **B** of the esterase EstB1 from *Bacillus* sp. in *E. coli* BL21(DE3). Protein samples were separated in 12.5% SDS-polyacrylamide gels and stained with Coomassie blue (*lanes A 1-8; B 1-3*) and α -naphthyl acetate and Fast Red (zymogram) (*lanes A 8-10*). **A** Cell lysat of *E. coli* BL21(DE3) bearing the empty vector (*lane 2*); and of *E. coli* BL21(DE3) transformed with pET-*estB1* without induction (*lane 3*); after 1, 2, 3 and 4 h induction (*lanes 5-8*, respectively); **B** Soluble fraction of *E. coli* BL21(DE3) transformed with pET-*estB1* after 4 h induction (*lane 2*); fraction after IMAC purification (*lane 3*). **A, B** *Lanes 1* – low molecular weight protein standard (BioRad) with bands corresponding to molecular masses of 97.4, 66.2, 45.0, 31.0, 21.5 and 14.4 kDa; *arrow* indicates the EstB1 esterase.

Figure 2 Expression **A** and refolding **B** of the esterase EstB2 from *Bacillus* sp. in *E. coli* BL21(DE3). Protein samples were separated in 12.5% SDS-polyacrylamide gels and stained with Coomassie blue (*lanes A 1-7; B 1-4*) and α -naphthyl acetate and Fast Red (zymogram) (*lanes A 8-10*). **A** Cell lysat of *E. coli* BL21(DE3) bearing the empty vector (*lane 2*); and of *E. coli* BL21(DE3) transformed with pET-*estB2* without induction (*lane 3*); after 1, 2, 3 and 4 h induction (*lanes 4-7*, respectively); **B** Insoluble fraction of *E. coli* BL21(DE3) transformed with pET-*estB2* after 4 h induction (*lane 2*); soluble fraction of *E. coli* BL21(DE3) transformed with pET-*estB2* after 4 h induction (*lane 3*); fraction after refolding (*lane 4*). **A, B** *Lanes 1* - low molecular weight protein standard (BioRad) with bands corresponding to molecular masses of 97.4, 66.2, 45.0, 31.0, 21.5 and 14.4 kDa; *arrow* indicates the EstB2 esterase.

Figure 3 Temperature optimum of the EstB1 and EstB2 esterases (a). The enzymes activity was measured towards methyl acetate at various temperatures at pH 7.5. pH optimum of the EstB1 and EstB2 esterases (b). The enzymes activity was measured towards methyl acetate at various pH values at 35°C.

Figure 4 Influence of DMSO, methanol, ethanol and 2-propanol on the EstB1 (a) and EstB2 (b) activity. The enzyme activity was measured towards *p*NP acetate at 35°C and pH 7.5

Figure 5 Influence of NaCl and KCl on the EstB1 (a) and EstB2 (b) activity. The enzyme activity was measured towards *p*NP acetate at 35°C and pH 7.5

Table 1 Substrate specificity and kinetic data of the purified EstB1 and EstB2 measured by standard spectrometric assay towards *p*NP esters and pH-start assay towards methyl esters

FIGURE 1

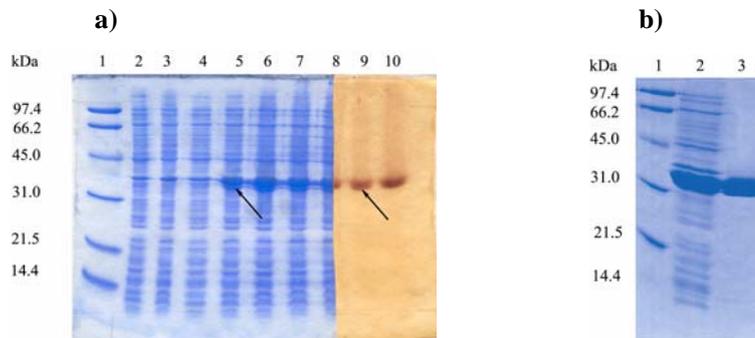


FIGURE 2

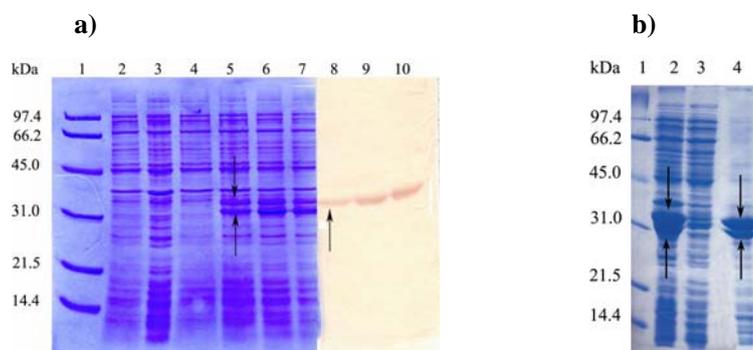


FIGURE 3

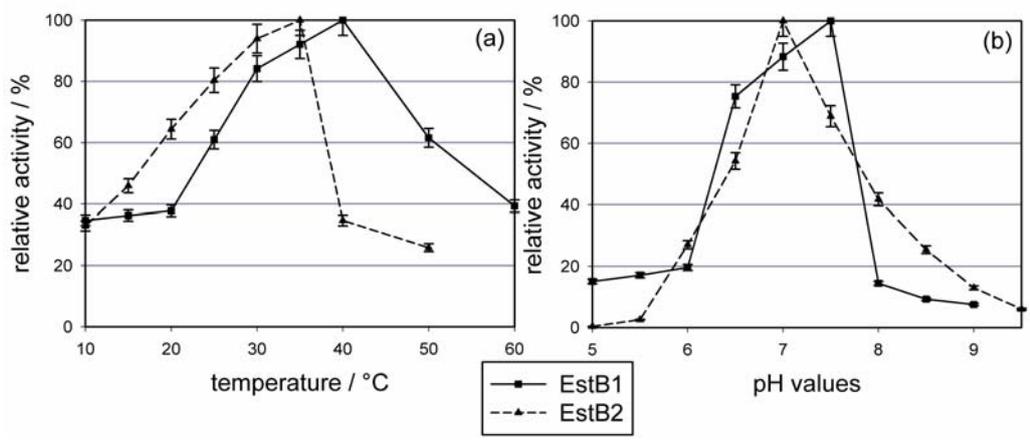


FIGURE 4

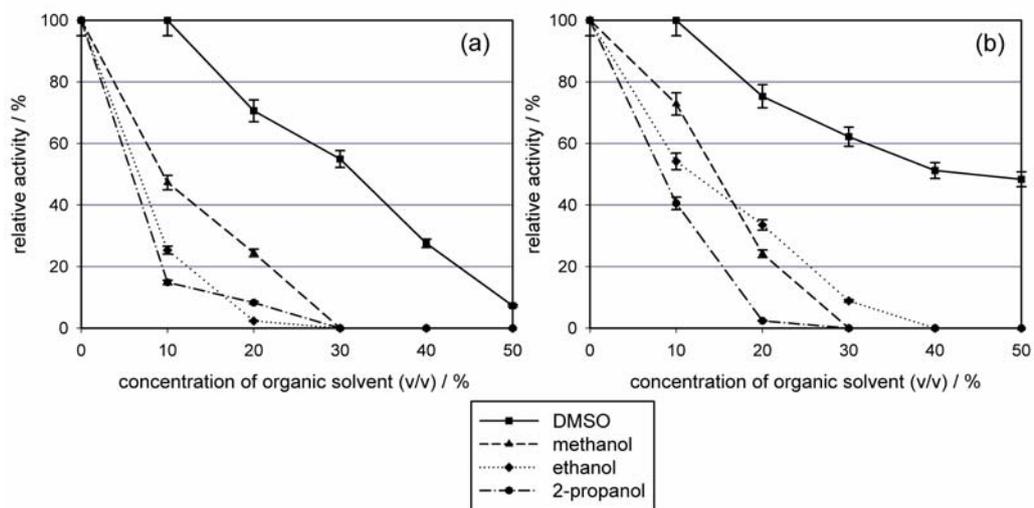
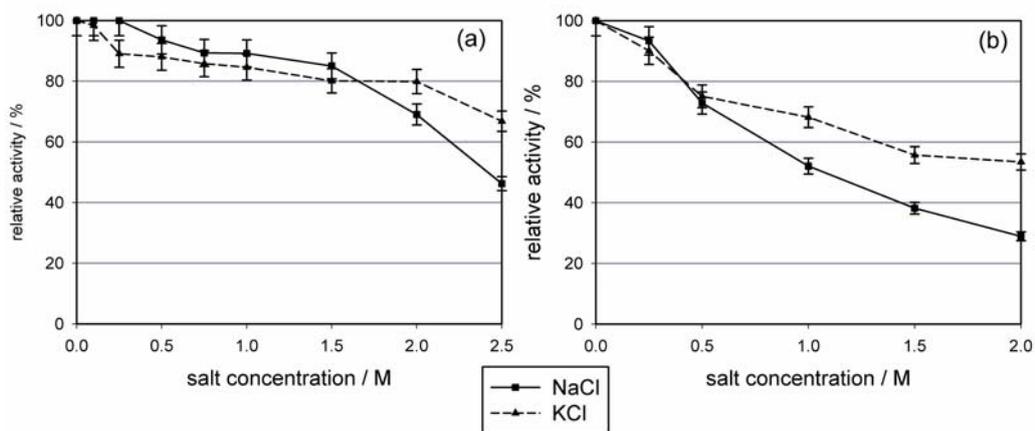


FIGURE 5



TABLES

Table 1 Substrate specificity and kinetic data of the purified EstB1 and EstB2 measured by standard spectrometric assay towards *p*NP esters and pH-start assay towards methyl esters

Substrate	Specific activity of EstB1 (U/mg)	K_m (M)	V_{max} (M min ⁻¹ mg of protein ⁻¹)	Specific activity of EstB2 (U/mg)	K_m (M)	V_{max} (M min ⁻¹ mg of protein ⁻¹)
<i>p</i> NP acetate	96	4·10 ⁻³	8·10 ⁻⁵	31	1·10 ⁻³	2·10 ⁻⁴
<i>p</i> NP butyrate	147	2·10 ⁻⁴	3·10 ⁻⁵	52	2·10 ⁻⁴	4·10 ⁻⁵
<i>p</i> NP caproate	23			25		
<i>p</i> NP caprylate	14			10		
<i>p</i> NP caprate	12			9		
<i>p</i> NP laurate	3			3		
methyl acetate	111			48		
methyl butyrate	109			35		
methyl caproate	76			22		
methyl caprylate	30			15		
methyl caprate	17			6		
methyl laurate	4			2		
methyl myristate	-			-		

Supplementary Information

Screening, Cloning and Biochemical Characterisation of Novel Esterases from *Bacillus* sp. associated with the Marine Sponge *Aplysina aerophoba*

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1 catatgaaacgatctgcacacgattatgtcctaagtgagcttggcctcac
-35

61 cgaagaacattcgatttacttggaggaatatggacacatcggacaaatcg
-10 -35

111 atcagattatcgctgaagctagctgaagcacaaggcaagattcaagca
-10

161 ggtgatgtagtcgttctagtagtctgcaggaattggctatgcatgggggagc
rbs M G S

211 aacaacgataatatggggaaacgaggaggaaatttaatgattacaatacc
N N D N M G K R G G N L M I T I

261 aactgttcataaagtcagccttctaatggagaagtgatgggatatcgaa
P T V H K V S L P N G E V M G Y R

311 aacgagatggaggagaaaaacaatcttactcgttcatggtaatatgacg
K R D G G E K T I L L V H G N M T

361 tcttcaaaacattgggaccttttcttcgaaacatttctgcttcttatac
S S K H W D L F F E T F P A S Y

411 gctgggtgcatcgatcgatgatgagaggggttggtgaatcatcttataacaaaa
T L V A I D M R G F G E S S Y N K

461 gagttgagggaaatagaggattttgctcaagatcttaaattctttgtagat
R V E G I E D F A Q D L K F F V D

511 cagctaggattaaacgattttacgatgatcggctggctccactggaggagc
Q L G L N D F T M I G W S T G G

561 cgtttgcagcagtttgaagcgcagtaccctggatattgagataagatcg
A V C M Q F E A Q Y P G Y C D K I

611 tcctcatttcttctgcttcaacaaggggttatccttcttcttgggacgcat
V L I S S A S T R G Y P F F G T H

661 tctgacgggacgccagatcttaaccaaaggctgaaaacagttgacgatat
S D G T P D L N Q R L K T V D D

711 agaaaaagatccgatgagaacgatcccgattcaacaggcctatgatacgg
 I E K D P M R T I P I Q Q A Y D T

761 gcaatcgagcgccttctaaaaacgatttggaactctcttatctacaccac
 G N R A L L K T I W N S L I Y T H

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 N Q P E E K R Y E A Y V D D M M

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 S S V T N G L T E G T N Q A N L I

961 cgtatccctgttctcgttttgcggtggagaacgagatcttgttatctcaaa
 R I P V L V L R G E R D L V I S

1011 agaaatgacagaagaaatcgtcgaagaccttggtacgaattcaacgtaca
 K E M T E E I V E D L G T N S T Y

1061 aggagttgtctgcatccggtcactctccattcatcgatgattgtgatcag
 K E L S A S G H S P F I D D C D Q

1101 cttacgaatattattactgacttttttagagaaa**tagg**aggaatattatga
 L T N I I T D F L E K

1161 ggttgcaaga

Figure 1 Nucleotide sequence and the deduced amino acid sequence of the esterase gene *estB1* from *Bacillus* sp. and flanking regions (numbering according to sequence deposited at GenBank, accession number AY640622) The ribosomal binding site (RBS) and two putative -10 and -35 regions found in the 5' non-coding region are bolded and underlined. Start and stop codons are shown in bold letters. The deduced amino acid sequence is given below in the one-letter code according to the codon usage of *E. coli*. The esterase/lipase consensus sequence Gly-X1-Ser-X2-Gly is bolded and underlined.

I Y A P T F V V Q A R H D E M I

1671 tacggatagtgcgaaatgtcattcatgacaatatcgaatctgatgagaaaa
N T D S A N V I H D N I E S D E K

1721 gcttaaaatgggtatgaaaattcaactcatgtcatcacgtaggaaaagaa
S L K W Y E N S T H V I T L G K E

1771 aaagaagtccttcatgaggatgtgctcgaatctttaaaccggacttgactg
K E V L H E D V L E F L N G L D

1821 gaaagaat**ta**atcgttaatttggttgaaggaaggtgaaacgatatggcag
W K E

1871 aagaacatgtacaaaaaatattgagctacatgaaagacgaggcatataaa

1921 ccattaacgggttaaagaacttgaagaagtgttcggcattcaagattcgag

1971 tgaattc

Figure 2 Nucleotide sequence and the deduced amino acid sequence of the esterase gene *estB2* from *Bacillus* sp. and flanking regions (numbering according to sequence deposited at GenBank, accession number AY640623). The ribosomal binding site (RBS) and putative -10 and -35 regions found in the 5' non-coding region are bolded and underlined. Start and stop codons are shown in bold letters. The deduced amino acid sequence is given below in the one-letter code according to the codon usage of *E. coli*. The esterase/lipase consensus sequence Gly-X1-Ser-X2-Gly is bolded and underlined.

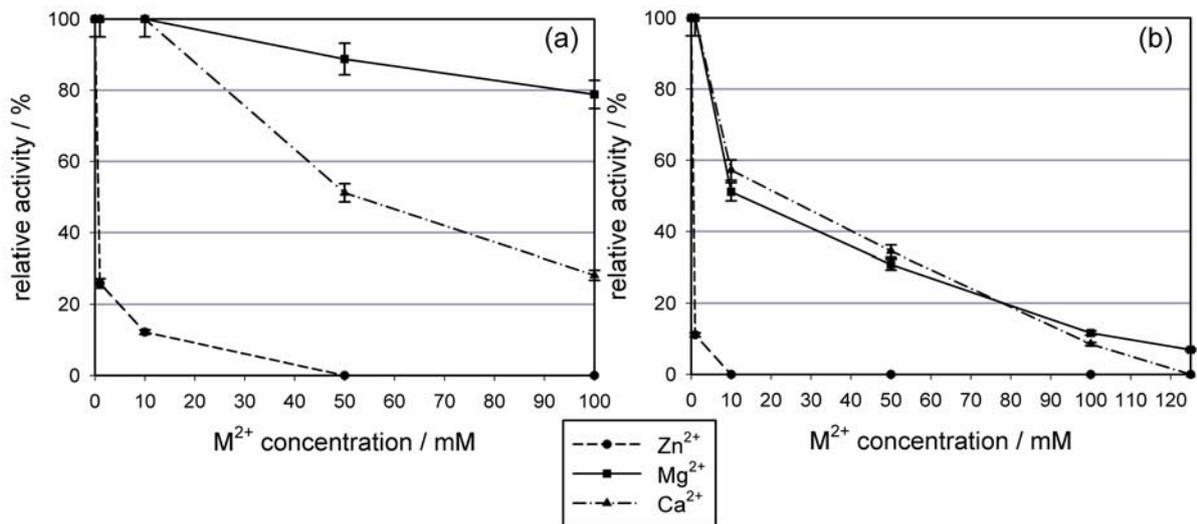


Figure 3 Influence of Zn²⁺, Mg²⁺ and Ca²⁺ ions on (a) the EstB1 and (b) the EstB2 activity. The enzyme activity was measured toward *p*NP acetate at 35°C and pH 7.5